รายงานวิจัยฉบับสมบูรณ์

ชื่อโครงการวิจัย

ปฏิสัมพันธ์ระหว่างแอคติโนมัยสีทเอนโดไฟต์กับพืชไร่ในแง่การเจริญและความเครียดโดยการวิเคราะห์ทรานสคริปชัน

Investigation of interaction between endophytic actinomycetes and crop plants on growth and stress by transcriptional analysis

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Abstract

In this study, plant growth promoting (PGP)-endophytic bacteria including actinomycetes were intensively investigated for their beneficial roles towards crop plants including rice, sugarcane, mungbean, and maize. The results demonstrated that co-inoculation of diazotrophic bacteria and actinomycetes significantly enhance the growth parameters of sugarcane greater than that of individual and un-inoculated plants. Genome analysis of those actinomycetes revealed genes involved in PGP traits such as phosphate solubilization, IAA production, siderphore production, ACC deaminase, fungal cell wall degradation family 18 and 19 chitinases, and host plant colonization. To verify the role of ACC deaminase trait towards stress tolerance, endophytic *Enterobacter* sp. EN-21, *Streptomyces* sp. GMKU 336, *Streptomyces* sp. GKU 895, and *Streptomyces venezuelae* ATCC 10712 were comprehensively investigated *in vivo*. The results showed that *Enterobacter* sp. EN-21 promoted growth and salt tolerance in sugarcane; while, *Streptomyces* sp. GMKU 336, *Streptomyces* sp. GKU 895, and *S. venezuelae* ATCC 10712 enhanced growth and salt tolerance in rice. Moreover, *Streptomyces* sp. GMKU 336 helped mungbean to tolerate flooding. Those endophytes helped plants to tolerate salt stress by significantly increasing plant growth, chlorophyll and proline contents, osmotic balance, K^+ and Ca^+ contents, and relative water content (RWC); but decreasing stress ethylene, reactive oxygen species (ROS), malondialdehyde (MDA), Na^+ content, and Na^+/K^+ ratio. Expression profiles of stress responsive genes in some crop plants exhibited up- and down-regulation corresponding to the physiological parameters. The ACC deaminase mutation and overproduction strains were also manipulated and applied to clearify the role of such trait *in vivo*. Transcriptomic data obtained from salt-susceptible rice associated with *Streptomyces* sp. GKU 895 indicated up-regulation of genes involved in enhancement of growth, antioxidant enzymes, compatible solutes, phytohormones and photosynthesis. Further analysis of RNA-seq data is required to understand the molecular interaction of *Streptomyces* and salt tolerance of rice. Moreover, chitinase-producing endophytic *Streptomyces* sp. GKU 322 helped protect maize from foot rot and wilting disease of maize plants caused by *Fusarium moniliforme* DOAC 1224. In addition, a novel species, *Actinomadura barringtoniae* sp. nov. GKU 128, was proposed.

Keywords

Actinomycete, Endophyte, Plant Growth Promotion, Salt Stress, Transcription, Crop

บทคัดย่อ

งานวิจัยนี้ได้ศึกษาบทบาทหน้าที่ของแบคทีเรียเอนโดไฟต์ รวมทั้งแอคติโนมัยสีท ที่มีสมบัติส่งเสริมการเจริญพืช (plant growth promotion; PGP) ต่อพืชไร่ ได้แก่ ข้าว อ้อย ถั่วเขียว และข้าวโพด ผลการทดลองแสดงให้เห็นว่าการปลูกเชื้อแบบผสมของเชื้อกลุ่ม diazotroph กับแอคติโนมัยสีท สามารถเพิ่มการเจริญของอ้อยได้ดีกว่าการปลูกเชื้อเดี่ยวหรือไม่ใส่เชื้อ เมื่อวิเคราะห์ลำดับเบสจีโนมของเชื้อแอคติโนมัยสีท พบว่าประกอบไปด้วยยีนที่เกี่ยวข้องกับ PGP ได้แก่ การละลายฟอสเฟต การผลิตฮอร์โมนพืช IAA การสร้างสารไซเดอร์โรฟอร์ การสร้างเอนไซม์ ACC deaminase และ chitinase ที่ย่อยผนังเซลล์เชื้อรา รวมทั้งยีนที่เกี่ยวข้องกับการเข้าอาศัยในพืช การทดลองนี้ได้ศึกษาบทบาทหน้าที่ของ ACC deaminase ที่ช่วยให้พืชทนความเครียดอย่างละเอียด โดยเลือกใช้ *Enterobacter* sp. EN-21, *Streptomyces* sp. GMKU 336, *Streptomyces* sp. GKU 895 และ *Streptomyces venezuelae* ATCC 10712 ปลูกเข้าสู่พืช ผลการทดลองพบว่า *Enterobacter* sp. EN-21 ช่วยส่งเสริมการเจริญของอ้อยในสภาวะเค็ม ขณะที่ *Streptomyces* sp. GMKU 336, *Streptomyces* sp. GKU 895, และ *S. venezuelae* ATCC 10712 ช่วยส่งเสริมการเจริญและทนเค็มในข้าว นอกจากนี้ *Streptomyces* sp. GMKU 336 ยังช่วยให้ถั่วเขียวทนน้ำท่วมได้อีกด้วย แอคติโนมัยสีทเอนโดไฟต์เหล่านี้ช่วยพืชทนเค็มโดยเพิ่มการเจริญ เพิ่มปริมาณคลอโรฟิล โปรลีน โปแตสเซียมและแคลเซียม ปรับสมดุลออสโมซิส และเพิ่มปริมาณน้ำสัมพัทธ์ (relative water content, RWC) แต่ลดเอธิลีน reactive oxygen species (ROS) และ malondialdehyde (MDA) ลดปริมาณโซเดียม และสัดส่วนโซเดียมต่อโปแตสเซียม เมื่อตรวจสอบการแสดงออกของยีนที่เกี่ยวข้องกับการตอบสนองความเครียดในพืช พบว่ามียีนที่แสดงออกเพิ่มขึ้นและลดลงสอดคล้องกับผลทางสรีรวิทยาของพืช งานวิจัยนี้ยังได้สร้างสายพันธุ์กลายของ *Streptomyces* ที่ไม่สร้าง ACC deaminase และสายพันธุ์กลายที่สร้างแบบ over-expression เพื่อใช้ทดสอบหน้าที่ของยีนนี้ในพืชด้วย จากข้อมูลทรานสคริปโตมของข้าวไม่ทนเค็มที่ปลูกเชื้อ *Streptomyces* sp. GKU 895 ในสภาวะเค็มแสดงให้เห็นว่า กลุ่มยีนที่เกี่ยวข้องกับการเจริญ เอนไซม์ที่เกี่ยวข้องกับแอนติออกซิแดนท์ สารให้ความสมดุล ฮอร์โมนพืช และการสังเคราะห์แสง มีการแสดงออกที่เพิ่มมากขึ้น อย่างไรก็ตาม การข้อมูลทรานสคริปโตมระหว่าง *Streptomyces* และข้าว ยังคงต้องวิเคราะห์อย่างต่อเนื่องเพื่อให้เข้าใจภาพรวมของการทนเค็มของข้าว อันเนื่องมาจากแอคติโนมัยสีทเอนโดไฟต์ นอกจากนี้ยังพบว่า *Streptomyces* sp. GKU 322 ที่สร้างเอนไซม์ chitinase ยังสามารถช่วยป้องกันข้าวโพดจากโรคเหี่ยวและโคนเน่าที่เกิดจากเชื้อรา *Fusarium moniliforme* DOAC 1224 ได้ด้วย ในงานวิจัยนี้ยังได้รายงานเชื้อแอคติโนมัยสีทเอนโดไฟต์สปีชีส์ใหม่ด้วย คือ *Actinomadura barringtoniae* sp. nov. GKU 128

คำสำคัญ

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Executive Summary

Environmental friendly endophytic bacteria, particularly actinomycetes, have been intensively investigated to enhance growth of crop plants such as rice, sugarcaneand increase abiotic stress tolerance. In this study, endophytic bacteria including actinomycetes were isolated from roots of sugarcane and were characterized for plant growth promoting (PGP) traits including 1-aminocyclopropane-1-decarboxylate deaminase (ACC deaminase), indole-3-acetic acid (IAA), nitrogen fixation, phosphate solubilization and siderophore production. Based on morphological and 16S rRNA sequence analysis, the endophytes distributed into 14 genera of which the most dominant species belong to *Bacillus*, *Enterobacter*, *Microbispora* and *Streptomyces*. After co-inoculation of two diazotrophs, *Bacillus* sp. EN-24 and *Enterobacter* sp. EN-21; and two strains of actinomycetes, *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895, the growth parameters of sugarcane were significantly greater than that of individual and un-inoculated plants.

Genomes of *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895 were then sequenced and analyzed. The draft genome of *Microbispora* sp. GKU 823 is 9.43 Mbp with a G+C content of 71.3%. The genome contains genes related to PGP traits and secondary metabolite gene clusters including phosphate solubilization, IAA production, fungal cell wall degradation family 18 chitinase, nonribosomal peptide synthetase (NRPS), Type I polyketide synthase (T1PKS), terpene, bacteriocins, siderophores, and lanthipeptide. The draft genome of *Streptomyces* sp. GKU 895 showed 8.29 Mbp with a G+C content of 70.7%. The genome possesses genes associated with PGP traits, host plant colonization, and secondary metabolite gene clusters including ACC deaminase, IAA synthesis, phosphate solubilization, fungal cell wall degradation family 18 and 19

chitinases, salicylate hydroxylase, butyrolactones, siderophores, terpenes, bacteriocins, melanins, ectoine, Type II polyketide synthases (T2PKS), NRPS, NRPS-bacteriocin, Type III polyketide synthase (T3PKS), T3PKS-NRPS, T3PKS-terpene-butyrolactone, and terpene-butyrolactone-NRPS.

In this work, an action of ACC deaminase trait of endophytic bacteria including actinomycetes was intensively investigated *in vivo*. ACC deaminase converts a precursor of ethylene, ACC, in plants into ammonia and α-ketobutyrate and consequently reduced stress ethylene while plants are under environmental stress. The results showed that ACC deaminase-producing *Enterobacter* sp. EN-21 colonized in the intercellular space of sugarcane roots and helped sugarcane tolerate to salinity by significantly reducing proline, malondialdehyde (MDA), ethylene and Na^+ content; but markedly increasing chlorophyll and K^+ contents. Similarly, ACC deaminase-producing endophytic *Streptomyces* sp. GMKU 336 associated with Thai jasmine rice Khao Dok Mali 105 cultivar (*Oryza sativa* L. cv. KDML105) under salt stress condition significantly increased rice growth, chlorophyll, proline, K^+ and Ca^+ contents and relative water content (RWC); but decreased ethylene, reactive oxygen species (ROS), Na^+content, and Na^+/K^+ ratio when compared to rice not inoculated and those inoculated with the *acdS*-deficient mutant. Expression profiles of stress responsive genes involved in the ethylene pathway, *ACO1* and *EREBP1*, were significantly down-regulated; while *acdS* encoding ACC deaminase of strain GMKU 336 was up-regulated *in vivo*. Furthermore, genes involved in osmotic balance (*BADH1*), Na^+ transporters (*NHX1* and *SOS1*), calmodulin (*Cam1-1*), and antioxidant enzymes (*CuZn-SOD1* and *CATb*) were up-regulated; whereas, a gene implicated in a signaling cascade, *MAPK5*, was down-regulated. Furthermore, *Streptomyces* sp. GMKU 336 associated with mung bean CN72 [Vigna radiata (L.) Wilczekcv. CN72] significantly increased plant elongation and biomass, chlorophyll, leaf area, leaf color, adventitious roots, but reduced ethylene level under flooding conditions when compared to un-inoculated plants and those inoculated with the *acdS*-deficient mutant.

The ACC deaminase gene, *acdS*, of *S. venezuelae* ATCC 10712 was cloned into an expression plasmid to generate *S. venezuelae*/pIJ86-*acdS*. Expression of *acdS* and production of ACC deaminase of *S. venezuelae*/pIJ86-*acdS* were significantly higher than the unmodified strain. *S. venezuelae* on its own augmented rice growth and significantly increased more tolerance to salinity by reduction of ethylene, ROS and Na^+ content, while accumulating more proline, chlorophyll, RWC, MDA, and K^+ contents than those of uninoculated controls. The overproducer did not alter chlorophyll, RWC, or MDA further – while it did boost more shoot weight and elongation, and significantly regulated salt tolerance of rice by increasing proline and reducing

ethylene and Na^+ further than that of the wild type. Study of transcriptomic profiling of ACC-deaminase producing endophytic *Streptomyces* sp. GKU 895-inoculated and uninoculated rice plants grown under non-salt and salt stresses indicated enhancement of growth and salt tolerance in salt-susceptible indica rice cultivar IR29 by reduction of ethylene, ROS, but induction of antioxidant enzymes, compatible solutes, phytohormones and photosynthesis. Further RNA-seq data analysis is required to understand the molecular interaction of endophytic actinomycetes and salt tolerance of rice.

Moreover, chitinase-producing endophytic actinomycetes were studied to inhibit fingal pathogen. *Streptomyces* sp. GMKU 301 and GMKU 322 decomposed *F. moniliforme* DOAC 1224 hyphae during dual culture on colloidal chitin agar (CCA). Under pot culture conditions, *Streptomyces* sp. GMKU 322 controlled foot rot and wilting disease of maize plants caused by *F. moniliforme* DOAC 1224 better than *Streptomyces* sp. GMKU 301. The results indicated that *Streptomyces* sp. GMKU 322 has the capacity of strong antagonistic mechanism towards *F. moniliforme* DOAC 1224 by exhibiting high chitinase activity, high fungal cell-wall degrading activity, but weak anti-fungal activity.

In addition, a novel species, *Actinomadura barringtoniae* sp. nov. GKU 128, was validly proposed using polyphasic characteristics.

In conclusion, this work have demonstrated that PGP-endophytic actinomycetes help enhance growth, salt tolerance, and fungal pathogenic resistance of crop plants including rice, mungbean, sugarcane and maize. Plant transcriptional profiles together with plant physiology evidently supported the beneficial role of endiphytic actinmycetes towards crop plants. It is very promising that endophytic actinomycetes will become the important agents in term of environmental friendly application in sustainable agriculture in the near future.

เนื้อหางานวิจัยและ output ที่ได้จากโครงการ

Section 1

Diversity of plant growth-promoting bacterial endophytes associated with sugarcane roots and their effect of growth by co-inoculation

Introduction

Sugarcane (*Saccharum officinarum* L.) is a tropical plant belonging to the grass family, Gramineae, as well as maize, rice, sorghum and wheat. Sugarcane is the world's largest crop and is economically important in Thailand, which ranks fourth among sugarcane producers on the world market, after Brazil, India and China (Food and Agriculture Organization of the United Nations 2015). Although sugar is the primary product from sugarcane, ethanol has become a new high-value product because of the worldwide interest in replacing gasoline with biofuel.

For several decades, boosting crop yields by chemical fertilizers and various nutrients and pesticides has dominated agronomy (Tilman 1998). Now, the use of environmentally friendly strategies for reducing the use of chemicals to enhance growth of sugarcane plants is an ultimate goal of sustainable agriculture. One of the alternative ways to promote plant growth and protect plants from diseases is the application of bacterial endophytes. These are free-living bacteria that colonize the inside of the plants and can affect plant growth by direct and indirect mechanisms (Gupta and others 2000; Glick 2012). They are termed plant growth-promoting endophytes (PGPE) (Taulé and others 2011) with various PGP traits including solubilization of rock phosphates, production of siderophores to scavenge iron, production of phytohormones, fixation of nitrogen, secretion of 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce ethylene levels in plants and production of specialized metabolites to protect plants from phytopathogens (Gupta and others 2000; Mendes and others 2007; Glick 2012). A range of PGPE including endophyic actinomycetes significantly increase vegetative growth and grain yield of several crops, including *jatropha*, maize, rice, sorghum, sugarcane and wheat (Olivares and others 1997; Yanni and others 2001; Govindarajan and others 2007; Luna and others 2010; Rungin and others 2012; de Jesus Santos and others 2014; Alves and others 2015; Qin and others 2015) by increasing shoot and root lengths as well as biomass through the secretion of plant growth-promoting substances, increasing the availability of nutrients, or promoting growth by combined modes of action (Vessey 2003). Nevertheless, the application of single bacterial strains resulted in inconsistencies in the field (Bashan and Holguin 1997). Therefore, co-inoculation of compatible bacteria has been found to be more significantly enhanced the growth of plants than individual inoculation. For example,

co-inoculation of mixtures of diazotrophic bacteria gave better growth of rice and sugarcane (Govindarajan and others 2007); and co-culture of diazotrophs and fungal endophytes revealed better growth and yield of chickpea (Verma and others 2014).

This study aimed to select powerful endophytic PGP-diazotrophs and actinomycetes isolated from sugarcane plants in Thailand. The diversity of culturable root-associated endophytic bacteria and actinomycetes was systematically analyzed and their PGP-traits and plant growth-promoting effects on sugarcane plants were thoroughly evaluated after individual and co-culture inoculation.

Materials and Methods

Isolation of culturable endophytic bacteria and actinomycetes from sugarcane

Five-month old healthy sugarcane plants cultivars LK 92-11 and KK 3, commercial canes mainly planted in Thailand for sugar industry, were obtained from Kud Chock and Nongkonthai sugarcane plantations, Phu Khiao, Chaiyaphum, Thailand through Mitr Phol Sugarcane Research Center. The plant samples were vigorously shaken to loosen attached soil from the roots and air-dried at room temperature (RT) overnight, then kept at 4 ºC till use.

To isolate root-associated endophytic bacteria, sugarcane roots were surface sterilized using the method of Rachniyom and others (2015). The roots were first rinsed under running tap water for 2–3 min to remove soil particles, and then immersed with shaking in sterilized 0.1 % (v/v) Tween 20 for 5 min, followed by 70 % ethanol for 5 min, 1% sodium hypochlorite solution for 10 min, 10 % (w/v) sodium hydrogen carbonate solution for 10 min and finally washed three times with sterile distilled water.

Endophytic bacteria were isolated according to the method for isolation of nitrogen- fixing bacteria described by Cavalcante and Dobereiner (1988). The roots were crushed in 5 % sucrose solution and ten-fold serial dilutions were made. A 100 µl sample of each dilution was dropped into semisolid LGI medium (Baldani and others 2014) and incubated at 28 ºC for 7–10 days. A veil-like pellicle near the surface of the medium appeared and was streaked onto LGI agar and nutrient agar (NA) supplemented with 50 µg ml^{-1} nystatin and 50 µg ml^{-1} cycloheximide and incubated at 28 ºC for 3–7 days. The final rinsing water was also spread on both media in confirm surface sterilization. Colonies of bacteria were picked and purified on LGI agar and NA. The pure cultures were classified by Gram staining and morphological observation under the light microscope. Bacteria were stored in 40 % glycerol solution at –80 ºC.

For isolation of endophytic actinomycetes, the surface-sterilized roots were macerated in ¼ Ringer's solution (0.9 % NaCl, 0.042 % KCl, 0.048 % $CaCl_2$, 0.02 % $NaHCO_3$). The root

materials and the liquid solution were respectively placed and spread onto starch casein agar (SCA) (Küster and Williams 1964) and water agar (WA) supplemented with 50 µg ml^{-1} nalidixic acid, 100 µg ml^{-1} ampicillin, 50 µg ml^{-1} nystatin and 50 µg ml^{-1} cycloheximide and incubated at 28 ºC for 2–4 weeks. The final rinsing water was also spread on both media to confirm surface sterilization. Colonies of endophytic actinomycetes were picked and purified on mannitol soya (MS) agar (Hobbs and others 1989). The pure cultures were stored in 20 % glycerol solution at –80 ºC.

Identification of endophytic bacteria

All of bacterial endophytes were grouped based on their colony morphology on agar plates. Endophytic bacteria were grown on NA and Tryptic Soy Agar (TSA) media at 28 ºC for 2 days. Endophytic actinomycetes were grown on International *Streptomyces* Project (Shirling and Gottlieb 1966) media no. 2, 3 and 4 at 28 ºC for 14 days. The representatives of endophytic bacteria and actinomycetes were selected based on morphological characteristics for 16S rRNA gene sequencing and analysis. Genomic DNA of bacteria was prepared by a standard protocol (Green and Sambrook 2012). The 16S rRNA gene was amplified using universal primers and the PCR protocol of (Lane 1991). For actinomycetes, genomic DNA was prepared according to Kieser and others (2000). 16S rRNA genes were amplified using primers and the PCR protocol of Rachniyom and others (2015). PCR products were purified using Gel/PCR DNA fragment extraction kit (Geneaid, Taiwan) according to the manufacturer's protocol and subjected to DNA sequencing at Macrogen Inc. (Korea). All sequences obtained from the 16S rRNA genes were edited manually using BioEdit (Hall, 1999), then compared with type strains using the EzTaxon–e server (Kim and others 2012a). The sequences of the isolates and the most similar species were aligned using CLUSTAL X program version 2.0 (Larkin and others 2007). Phylogenetic tree was constructed using neighbour-joining algorithm (Saitou and Nei 1987) in MEGA software version 6.0 (Tamura and others 2013). The topology of tree was evaluated with bootstrap analysis based on 1000 resamplings (Felsenstein 1985). A distance matrix was generated using Kimura's two-parameter model (Kimura 1980).

Characterization of plant growth-promoting traits

Indole-3-acetic acid (IAA) production

IAA production was determined by a colorimetic method (Pilet and Chollet 1970). Endophytes were inoculated into glucose-beef extract broth supplemented with 10 mM L-tryptophan and incubated at 28 ºC for 7 days in the dark. The culture was then centrifuged and 1

ml of the supernatant was transferred into a vial containing 2 ml of Salkowski's reagent (Pilet and Chollet 1970). The mixture was left at RT for 30 min in the dark. Development of a pink-red color indicated IAA production.

Siderophore production

An overnight-NB culture of bacteria (10^7 CFU ml^{-1}) or an agar plug of the 14-day growth of actinomycetes on MS agar was respectively dropped or placed on chrom azurol S medium (Schwyn and Neilands 1987) and incubated at 28 ºC for 7 days for non-actinomycete bacteria and 14 days for actinomycetes. An orange halo indicated siderophore production.

Phosphate solubilization

Phosphate solubilization was detected by the modified method of Rodríguez and Fraga (1999). An overnight-NB culture of bacteria (10^7 CFU ml^{-1}) or an agar plug of 14-day growth of actinomycetes on MS agar was respectively dropped or placed on Pikovskaya's agar (Pikovskaya 1948) containing 0.5 % (w/v) $Ca_3(PO_4)_2$ and incubated at 28 ºC for 7 days for non-actinomycete bacteria and 14 days for actinomycetes. Formation of a clear zone indicated solubilizing ability.

1-Aminocyclopropane-1-carboxylate (ACC) deaminase activity

ACC deaminase activity was determined by the method of Glick and others (1995) using N-free medium (Nfb) (Dobereiner 1976) for bacteria and minimal medium (MM) (Kieser and others 2000) for actinomycetes containing 0.3 m mol l^{-1} ACC (Sigma, USA) as a sole nitrogen source. MM with 0.1 % (w/v) $NH_4(SO_4)_2$ was used as a positive control and cultivation without ACC was used as a negative control. After incubation at 28 ºC for 7 days for non-actinomycete bacteria and 14 days for actinomycetes, colony growth on Nfb or MM with addition of ACC indicated ACC deaminase activity.

Nitrogen fixation and nifH gene amplification

Nitrogenase activity was determined by the acetylene reduction assay (ARA) according to Hardy and others (1973). An overnight-NB culture of bacteria (10^7 CFU ml^{-1}) was washed three times with distilled water to remove NB before transferring to 5 ml of semi-solid NFb medium and incubated at 28 ºC for 2 days. Next, 10 % (v/v) of gas in the headspace of the test tube was replaced by acetylene and left at RT for 1 h. Gas was collected from the headspace and analyzed for ethylene by gas chromatography (Hewlett Packard HP Series II, Japan). The nitrogen-fixing *Azospirillum brasilense* TS13 (Meunchang and others 2004) was used as a positive control and an

un-inoculated tube served as a negative control. All of the bacterial isolates and *Azospirillum brasilense* TS13 as positive control were subjected to *nifH* gene amplification using primers nifH-F (5'-AAAGGNGGNATCGGNAANTCCACCAC-3') and nifH-R (5'-TTGTTNGCNGCNTAC ATNGCCATCAT-3') and the PCR protocol described by (Rösch and others 2002).

Evaluation for antimicrobial activity

To evaluate antimicrobial activity, seven test microbes, *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 259233, *Aspergillus niger* ATCC 6275, *Colletotrichum falcatum* DOAC 1655 and *Fusarium moniliforme* DOAC 1224, were used. Antibacterial activity was evaluated by an overlay method (Anand and others 2006). An overnight-NB culture of bacteria (10^7 CFU ml^{-1}) or a 14 day-old MS agar plug of actinomycetes was respectively dropped or placed on NA plates and incubated for 24 h. The assay plates were overlaid with test bacteria (10^7 CFU ml^{-1}). Appearance of an inhibition zone indicated positive activity.

Antifungal activity was evaluated by dual-culture assay (Fokkema 1976). An overnight-NB culture of bacteria (10^7 CFU ml^{-1}) was spotted on potato dextrose agar and incubated for 24 h. Actinomycetes were streaked on one third of the area of PDA and allowed to grow for 7 days. An agar plug of the test fungus was prepared using a sterile cock borer (diameter 8 mm), placed opposite the bacterial colony and incubated at RT for 7 days. Inhibition of fungal growth was compared to the growth of the fungal control.

Evaluation of plant growth promotion by individual and co-inoculation

Endophytic bacteria and actinomycetes were selected based upon their PGP traits to evaluate sugarcane growth enhancement by individual and co-culture inoculation. To abnegate the effect of antagonistic activity among them, the selected isolates were tested for antibacterial activity against each other; and only the strains showing no activity were used to inoculate sugarcane. Tissue cultured sugarcane plants of cultivar LK 92-11 were obtained from the Plant Tissue Culture Unit at Central Laboratory and Greenhouse Complex, Kasetsart University (Kamphaengsaen Campus). Sugarcane plantlets were rinsed with sterile distilled water to remove remaining agar from the roots. The height of the plants was normalized to 6.5 cm by truncating shoots and roots. Plants were then inoculated with bacteria either individual or co-culture (10^7 CFU ml^{-1} of each strain) by the root dip method (Musson and others 1995) in a tissue culture bottle containing *Murashige and Skoog (MS0) medium (Murashige and Skoog 1962)* and immersed at RT for 24 h. Inoculated sugarcane plants were transferred to cleaned mini-pot (6 × 5.5 cm)

containing sterilized hydroponic materials (perlite:vermiculite, 3:1). The experiment was set up in completely randomized design (CRD) using five replicate mini-pots per treatment and each treatment was replicated twice. Plants were maintained in the greenhouse and supplied with sterilized sucrose-free MS0 broth twice a day for 60 days. The plants were then harvested and examined for root and shoot lengths, and root and shoot fresh/dry weights. Plants were surface sterilized and the inoculated endophytes were re-isolated according to the procedure described above. Plant growth parameters were statistically analyzed by one-way ANOVA and Tukeys multiple range tests (TMRT) using SPSS (version 16.0) at $p = 0.05$ to determine the efficacy of un-inoculated, individual and co-culture inoculation to promote growth of sugarcane plants.

Results

Distribution of sugarcane root-associated bacterial endophytes

One hundred and thirty five culturable root-associated bacterial isolates were obtained from sugarcane. They comprised 52 isolates of endophytic bacteria and 83 isolates of actinomycetes. According to the preliminary dereplication by morphological criteria and cultural characteristics, 19 and 11 isolates of endophytic bacteria and actinomycetes were assigned to the genus level using 16S rRNA sequence analysis, respectively. Sequence similarity of 16S rRNA genes of these endophytes (ranging from 1,282-1,513 bp) to the closest type strains from the EzTaxon–e server was 98.4–100 % (Table 1-1). All of the representative strains displayed considerable diversity which distributed under 11 families of *Bacillaceae*, *Enterobacteriaceae*, *Micrococcaceae*, *Micromonosporaceae*, *Moraxellaceae*, *Paenibacillaceae*, *Pseudomonadaceae*, *Staphylococcaceae*, *Streptomycetaceae*, *Streptosporangiaceae* and *Thermomonosporaceae* comprised of 10 genera of bacteria and 4 genera of actinomycetes (Fig. 1-1). *Streptomyces* was the most frequently isolated genus (n = 47, 34.81 %) followed by *Microbispora* (n = 29, 21.48 %), *Enterobacter* (n = 23, 17.04 %), *Bacillus* (n = 14, 10.37 %), *Actinomadura* (n = 5, 3.70 %), *Pantoea* (n = 4, 2.96 %), *Acinetobacter* (n = 2, 1·48 %), *Kosakonia* (n = 2, 1.48 %), *Micromonospora* (n = 2, 1.48 %), *Paenibacillus* (n = 2, 1.48 %), *Staphylococcus* (n = 2, 1.48 %), *Lysinibacillus* (n = 1, 0.74 %), *Micrococcus* (n = 1, 0.74 %) and *Pseudomonas* (n = 1, 0.74 %). 16S rRNA gene sequence similarity values of four strains, *Actinomadura* sp. GKU 822, *Microbispora* sp. GKU 823, GKU 898 and *Pantoea* sp. EN-29 with closest type strain were lower than 99 % (Table 1-1) and therefore probably represent new species.

Plant growth-promoting traits of sugarcane endophytes

The plant growth-promoting (PGP) traits, including antagonistic activity, were examined from 135 strains of sugarcane endophytes (Table 1-2). Amongst them, three strains of actinomycetes, *Streptomyces* sp. GKU 833, GKU 879 and GKU 895, and two strains of bacteria, *Bacillus* sp. EN-27 and *Enterobacter* sp. EN-21, showed the highest activity of PGP traits. The highest frequency of PGP-trait possessed by both endophytic bacteria and actinomycetes in this study was siderophore production, followed by IAA production and phosphate solubilization (Table 1-2). Most of the siderophore producing species belong to the genera *Streptomyces*, *Microbispora* and *Bacillus*; while IAA producing dominant species were *Streptomyces*, *Microbispora* and *Enterobactor*. Members of the genera *Streptomyces* and *Microbispora* were also the majority species capable of phosphate solubilizaton. However, the highest activity of phosphate solubilzation was found in *Pantoea* sp. EN-29 and EN-39 (data not shown). Besides, ACC deaminase-production was mainly detected in species belong to *Streptomyces* and *Bacillus* (Table 1-2).

To evaluate nitrogenase activity, endophytic bacteria were screened using the ARA method. Only six out of the 52 strains belonging to *Bacillus*, *Enterobacter*, *Lysinibacillus* and *Pantoea* could reduce acetylene gas, ranging between 14.59–25.39 nmol C_2H_4 h^{-1} $(10^7$ cells$)^{-1}$. The highest level of nitrogenase activity was found in *Enterobacter* sp. EN-21 (25.39) and *Bacillus* sp. EN-24 (20.11). To evaluate nitrogen fixation ability, *nifH* gene amplification was performed. Ten *nifH*-positive strains (19.23 % of the total) were obtained. There were five strains, namely *Bacillus* sp. EN-27, *Enterobacter* sp. EN-21, *Enterobacter* sp. EN-30, *Lysinobacillus* sp. EN-9 and *Pantoea* sp. EN-39 that exhibited both nitrogenase activity and a *nifH* gene (Fig. 1-2). However, the *nifH* gene was absent in an ARA-positive endophyte, *Bacillus* sp. EN-24.

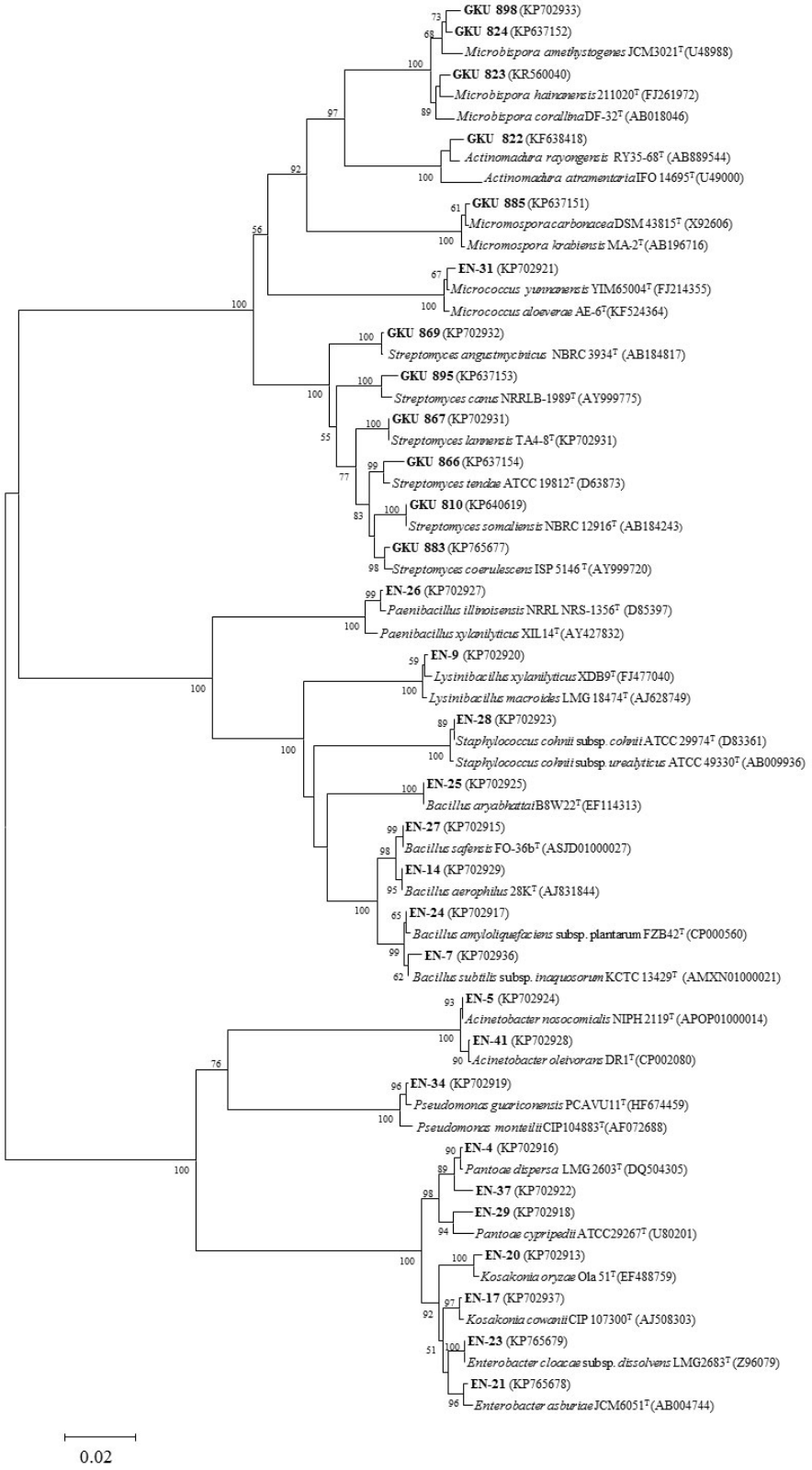

Fig. 1-1 Neighbour-joining tree based on 16S rRNA gene sequences showing relationship between the representative endophytic bacteria and actinomycetes isolated from sugarcane roots and the nearest type strains. Number of branch points indicate bootstrap value based on 1000 replications; only value above 50 % are shown. Bar, 0.01 substitutions per nucleotide position.

Table 1-1 Colony morphology and 16S rRNA sequence similarity of plant growth promoting bacterial endophytes from sugarcane to the closest type strains of valid described species

Isolates (n)	Colony morphology[a]	Closest type strains		Accession no.
		Species	Similarity (%)	
Actinomycetes				
GKU 822 (5)	Dv, Dv	*Actinomadura rayongensis* RY35-68^T	98.7	KF638418
GKU 824 (9)	Syb, Syb	*Microbispora amethystogenes* JCM3021^T	99.1	KP637152
GKU 898 (8)	So, Mo	*Microbispora amethystogenes* JCM3021^T	98.6	KP702933
GKU 823 (12)	Myp, Myp	*Microbispora hainanensis* 211020^T	98.8	KR560040
GKU 885 (2)	Gy, Gy	*Micromonospora carbonacea* DSM 43815^T	99.6	KP637151
GKU 869 (3)	Lgo, Lgo	*Streptomyces angustmycinicus* NBRC 3934^T	99.9	KP702932
GKU 895 (23)	Lgrb, Mr	*Streptomyces canus* NRRLB-1989^T	99.4	KP637153
GKU 867 (2)	Gg, Ly	*Streptomyces lannensis* TA4-8^T	99.9	KP702931
GKU 866 (5)	Lgg, Dgy	*Streptomyces tendae* ATCC 19812^T	99.1	KP637154
GKU 810 (2)	Pg, Lgyb	*Streptomyces somaliensis* NBRC 12916^T	99.9	KP640619
GKU 883 (12)	Pg, Bg	*Streptomyces coerulescens* ISP 5146 ^{T}	99.5	KP765677
Bacteria				
EN-5 (1)	C, E, Co, Cr, –ve	*Acinetobacter nosocomialis* NIPH 2119^T	100.0	KP702924
EN-41 (1)	C, E, P, W, –ve	*Acinetobacter oleivorans* DR1^T	100.0	KP702928
EN-24 (2)	I, L, Um, Cr, +ve	*Bacillus amyloliquefaciens* subsp. *plantarum* FZB42^T	100.0	KP702917
EN-25 (7)	C, E, Co, Cr, +ve	*Bacillus aryabhattai* B8W22^T	99.9	KP702925
EN-27 (2)	I, Cu, Co, Cr, +ve	*Bacillus safensis* FO-36b^T	100.0	KP702915
EN-14 (1)	C, U, Co, Cr, +ve	*Bacillus aerophilus* 28K^T	100.0	KP702929
EN-7 (2)	C, U, R, Cr, +ve	*Bacillus subtilis* subsp. *inaquosorum* KCTC 13429^T	99.7	KP702936
EN-21 (20)	C, E, Co, O, –ve	*Enterobacter asburiae* JCM6051^T	99.0	KP765678
EN-23 (3)	C, E, Co, W, –ve	*Enterobacter cloacae* subsp. *dissolvens* LMG2683^T	100.0	KP765679
EN-17 (1)	C, U, Co, Cr, –ve	*Kosakonia cowanii* CIP 107300^T	99.8	KP702937
EN-20 (1)	C, E, Co, W, –ve	*Kosakonia oryzae* Ola 51^T	99.7	KP702913
EN-9 (1)	I, E, Co, Cr, +ve	*Lysinibacillus macroides* LMG 18474^T	100.0	KP702920
EN-31 (1)	C, E, Co, Y, +ve	*Micrococcus yunnanensis* YIM65004^T	99.9	KP702921
EN-26 (2)	C, E, Co, O, +ve	*Paenibacillus illinoisensis* NRRL NRS-1356^T	99.8	KP702927
EN-29 (2)	I, E, R, Cr, –ve	*Pantoae cypripedii* ATCC29267^T	98.4	KP702918
EN-4 (1)	I, E, R, Cr, –ve	*Pantoae dispersa* LMG 2603^T	99.8	KP702916
EN-37 (1)	I, E, R, W, –ve	*Pantoae dispersa* LMG 2603^T	99.3	KP702922
EN-34 (1)	C, E, Co, Cr, –ve	*Pseudomonas guariconensis* PCAVU11^T	99.6	KP702919
EN-28 (2)	C, E, Co, Y, +ve	*Staphylococcus cohnii* subsp. *cohnii* ATCC 29974^T	100.0	KP702923

[a] Color of aerial and substrate mycelia of endophytic actinomycetes on ISP2 medium. Bg, blackish green; Dgy, dark greyish yellow; Dv, dark violet; Gg, greenish grey; Gy, greyish yellow; Lgg, light greenish grey, Lgo, light greyish olive; Lgrb, light greyish reddish brown; Lgyb, light greyish yellowish brown; Ly, light yellow; Mo, moderate orange; Mr, moderate reddish; Myp, moderate yellowish pink; Pg, paul green; So, strong orange; Syb, strong yellowish brown. Colony morphology of endophytic bacteria on NA medium. Form of colony (C/I; circular/irregular) , margin of colony (E/Cu/U/L; entire/curled/undulate/lobate) , elevation (Co/Um/R/P; convex/umbonate/raised/pulvinate) , color (Cr/Y/W/O; cream/yellow/white/off-white), Gram's straining (–ve/+ve; Gram negative/Gram positive). n, number of isolates.

Table 1-2 Number of positive isolates of sugarcane endophytic bacteria and actinomycetes with plant growth promoting traits

Genus (n)	**Plant growth promoting traits**[a] (%)					**Antagonistic activity** (%)						
	IAA	SD	P	ACC	ARA	BC	EC	PA	SA	AN	CF	FM
Actinomycetes												
Actinomadura (5)	2 (40)	4 (80)	–	–	ND	–	–	–	–	2 (40)	2 (40)	3 (60)
Microbispora (29)	14 (48.3)	20 (68.9)	19 (65.5)	–	ND	1 (3.4)	–	–	–	4 (13.8)	2 (6.9)	17 (58.6)
Micromonospora (2)	–	2 (100)	–	–	ND	–	–	–	–	2 (100)	1 (50)	2 (100)
Streptomyces (47)	26 (55.3)	33 (70.2)	18 (38.3)	11 (23.4)	ND	12 (25.5)	1 (2.1)	–	4 (8.5)	21 (44.6)	22 (46.8)	23 (48.9)
Total (83)	**42 (50.6)**	**59 (71.0)**	**37 (44.6)**	**11 (13.2)**	–	**13 (15.7)**	**1 (1.2)**	–	**4 (4.8)**	**29 (34.9)**	**27 (32.5)**	**45 (54.2)**
Bacteria												
Acinetobacter (2)	–	–	–	1 (50)	–	–	–	–	–	–	–	–
Bacillus (14)	3 (21.4)	10 (71.4)	3 (21.4)	7 (50)	2 (14.3)	5 (35.7)	6 (42.8)	–	1 (7.1)	4 (28.6)	8 (57.1)	8 (57.1)
Enterobacter (23)	11 (47.8)	9 (39.1)	2 (8.7)	3 (13)	2 (8.7)	–	–	–	–	–	4 (17.4)	5 (21.7)
Kosakonia (2)	1 (50)	–	–	–	–	–	–	–	–	–	–	–
Lysinibacillus (1)	–	–	–	1 (100)	1 (100)	–	–	–	–	–	–	–
Micrococcus (1)	1 (100)	–	–	–	–	–	–	–	–	–	–	–
Paenibacillus (2)	1 (50)	2 (100)	1 (50)	–	–	1 (50)	–	–	–	–	2 (100)	2 (100)
Pantoea (4)	2 (50)	–	3 (75)	–	1 (100)	–	–	–	–	–	–	3 (75)
Pseudomonas (1)	1 (100)	–	–	–	–	–	–	–	–	–	–	–
Staphylococcus (2)	–	–	–	1 (50)	–	–	–	–	–	–	1 (50)	1 (50)
Total (52)	**20 (38.5)**	**21 (40.4)**	**9 (17.3)**	**13 (25)**	**6 (11.53)**	**6 (11.5)**	**6 (11.5)**	–	**1 (1.9)**	**4 (7.7)**	**15 (28.8)**	**19 (36.5)**

[a] IAA, indole-3-acetic acid; SD, siderophore; P, phosphate solubilization; ACC, 1-aminocyclopropane-1-carboxylate deaminase; ARA, acetylene reduction assay; BC, *B. cereus* ATCC 11778; EC, *E. coli* ATCC 8739; PA, *Ps. aeruginosa* ATCC 15442; SA, *Staph. aureus* ATCC 259233; AN, *A. niger* ATCC 6275; CF, *C. falcatum* DOAC 1655; FM, *F. moniliforme* DOAC 1224; n, number of strains; ND, not determined; –, none

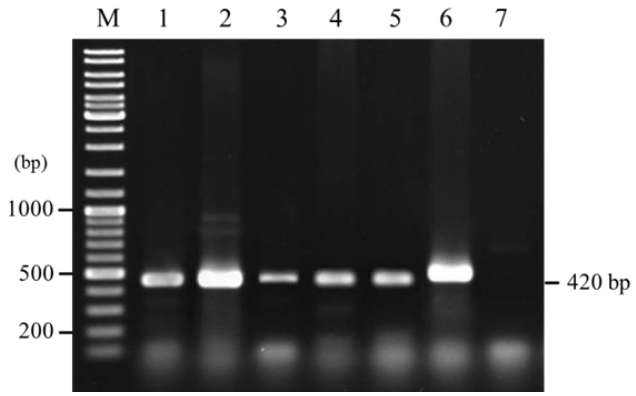

Fig. 1-2 Dinitrogenase reductase gene (*nifH*) amplification of endophytic diazotrophs. M, 1 kb DNA ladder; 1, *Azospirillum brasilense* TS13 (positive control); 2, *Enterobacter* sp. EN-21**;** 3, *Pantoea* sp. EN-39**;** 4, *Bacillus* sp. EN-27**;** 5, *Lysinibacillus* sp. EN-9; 6, *Enterobacter* sp. EN-30; 7, *Bacillus* sp. EN-15 (negative control)**.**

Antagonistic potential tests conducted against seven test strains of bacteria and fungi revealed that the most susceptible fungi that interacted with the endophytes were *Fusarium moniliforme*, followed by *Aspergillus niger* and *Colletotrichum falcatum*, while the most susceptible bacterium was *Bacillus cereus* (Table 1-2). Surprisingly, none of the endophytes inhibited *Pseudomonas aeruginosa*. Six strains of endophytes, *Bacillus* sp. EN-8, *Bacillus* sp. EN-15, *Bacillus* sp. EN-24, *Actinomadura* sp. GKU 870, *Streptomyces* sp. GKU 833 and *Streptomyces* sp. GKU 878, effectively inhibited growth of *A. niger* and the pathogenic fungi, *F. moniliforme* and *C. falcatum*, causing red rot disease in sugarcane (Alexander and Viswanathan 2002). *B. cereus* and *Escherichia coli* were the test bacteria most frequently inhibited by the endopytic bacteria as well as actinomycetes (Table 1-2).

Sugarcane plant growth enhancement

Based on PGP-traits, two strains of endophytic diazotrophs, *Bacillus* sp. EN-24 and *Enterobacter* sp. EN-21, and two strains of actinomycetes, *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895, were selected to evaluate growth enhancement of sugarcane in pot experiments (Table 1-3). Antagonistic test between each strain was performed and reviewed no effect to each other; therefore, they were suitable for co-culture inoculation. Nine treatments included (T1) *Enterobacter* sp. EN-21, (T2) *Bacillus* sp. EN-24, (T3) *Microbispora* sp. GKU 823, (T4) *Streptomyces* sp. GKU 895, (T5) EN-21+EN-24, (T6) GKU 823+GKU 895, (T7) GKU 823+EN-21+EN-24, (T8) GKU 895+EN-21+EN-24, (T9) GKU 823+GKU 895+EN-21+EN-24

and un-inoculated control were conducted to investigate individual and co-culture inoculation. Two-month old sugarcane plants treated by either individual or co-inoculation gave statistically significantly ($p \leq 0.05$) greater root and shoot lengths, and root and shoot biomass than un-inoculated plants (Table 1-4, Fig. 1-3). Either individual endophytic diazotroph or actinomycete inoculated plants (T1, T2, T3 and T4) gave measurable increments in root length (51–89 %), shoot fresh weight (67–106 %) and shoot dry weight (76–146 %) but remarkably increased in root fresh weight (147–278 %) (Table 1-4). The increase of root fresh weight was due to increased lateral root production (data not shown). These parameters were not significantly different when 2 strains of diazotrophs (T5) and actinomycetes (T6) were inoculated except shoot fresh (126 %) and dry weight (180 %) that T6 gave notably increased. The percentage increase in shoot length of individual and co-inoculated sugarcane were higher than that of the un-inoculated controls, especially T4, T5, T7 and T9, which were significantly (44–59 %) greater; while significant increase in root length was noticed in T7, T8 and T9 (125-128 %) (Table 1-4). The most significant enhancement of growth appeared in T7 and T8 in which each endophytic actinomycete was co-inoculated with both *Enterobacter* sp. EN-21 and *Bacillus* sp. EN-24. T7 and T8 caused drastic increases of 128–129 % in root length, 316–352 % in root fresh weight, 177–208 % in root dry weight, 37–58 % in shoot length, 116–126 % in shoot fresh weight and 146–160 % in shoot dry weight over those of un-inoculated treatments. The results suggested that the enhancement of growth in sugarcane was due to multiple growth-promoting traits from the mixture of the bacterial endophytes (Table 1-3). Our results indicated that co-inoculation of either endophytic *Microbispora* or *Streptomyces* combined with the mixture of *Enterobacter* and *Bacillus* gave higher growth of sugarcane than individual inoculation.

Remarkably, when four strains of endophytes were co-inoculated onto sugarcane plants (T9), some growth parameters such as root and shoot fresh/dried weights were significantly lower than those of T7 and T8 and some (root fresh/dried weight) were even lower than single strain inoculation (Table 1-4). Re-isolation experiments of the endophytes from two-month old sugarcane plants were then performed and the isolates were proved their identity by examination of colony morphology and 16S rRNA sequencing. Inoculated endophytic diazotrophs and actinomycetes abundantly appeared in roots but less in stems in every treatment (data not shown). About 10^4 CFU g fresh weight^{-1} were found in sugarcane roots inoculated with individual (T1, T2, T3, T4) and combinations of two bacterial strains (T5 and T6) (Table 1-4). However, the viable cell numbers of the endophytes decreased 10 times when more than two strains of bacteria were co-inoculated (T7, T8 and T9); particularly T9 gave the lowest. This might be explained by a reduction of plant biomass in T9 (Table 1-4) due to the low population of the endophytes. In

addition, this re-isolation experiment confirmed that *Enterobacter* sp. EN-21, *Bacillus* sp. EN-24, *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895 are true endophytes of sugarcane.

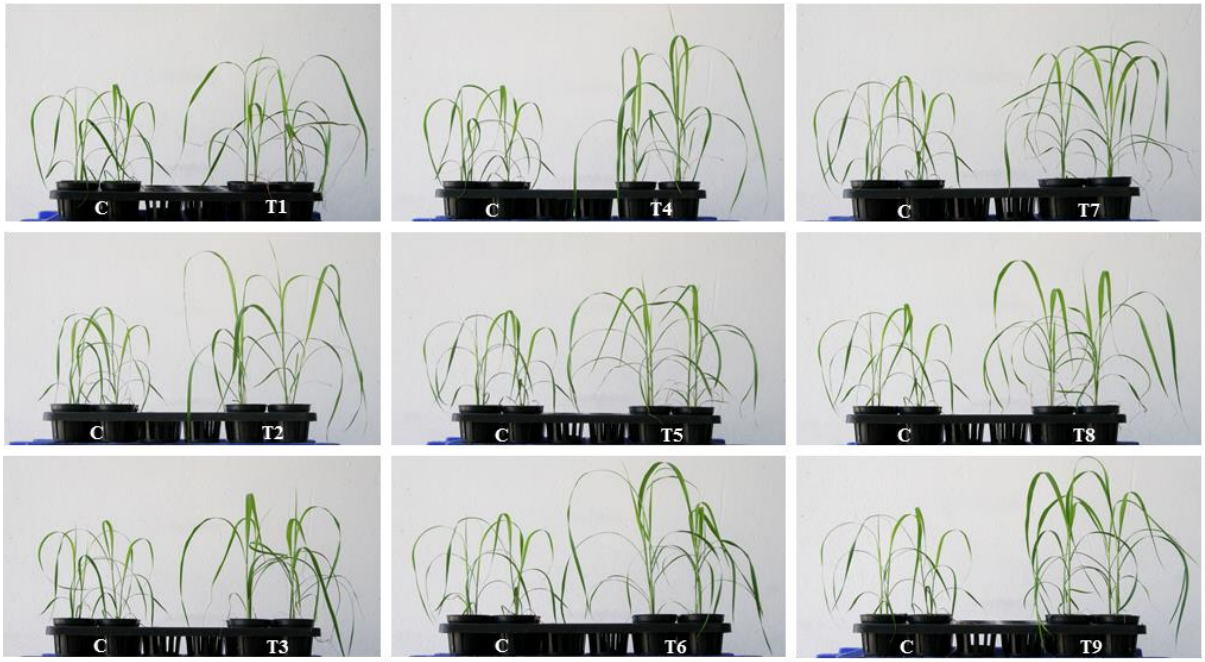

Fig. 1-3 Effects of individual and co-inoculation of endophytic bacteria and actinomycetes on growth promotion of sugarcane plants at 60 days after inoculation. T1, *Enterobacter* sp. EN-21; T2, *Bacillus* sp. EN-24; T3, *Microbispora* sp. GKU 823; T4, *Streptomyces* sp. GKU 895; T5, EN-21+EN-24; T6, GKU 823+GKU 895; T7, GKU 823+EN-21+EN-24; T8, GKU 895+EN-21+EN-24; T9, GKU 823+GKU 895+EN-21+EN-24 and C, un-inoculated control.

Table 1-3 Identification and PGP-traits of selected sugarcane diazotrophic bacteria and endophytic actinomycetes

Strains	Accessi	Closest type strains	Similarity (%)	Plant growth promoting traits[a]						Antagonistic activities			
				IAA	SD	P	ACC	ARA[b]	*nifH*	BC	AN	CF	FM
EN-21	KP765678	*Enterobacter asburiae* JCM6051^T	99.00	+	+	–	+	25.39	+[c]	–	–	–	–
EN-24	KP702917	*Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 ^{T}	99.52	–	+	–	–	20.11	–	+	+	+	+
GKU 823	KR560040	*Microbispora hainanensis* 211020 ^{T}	98.77	+	+	+	–	ND	ND	–	+	–	–
GKU 895	KP637153	*Streptomyces canus* NRRL B-1989 ^{T}	99.36	+	+	+	+	ND	ND	+	–	+	+

[a] IAA, indole-3-acetic acid; SD, siderophore; P, phosphate solubilization; ACC, 1-aminocyclopropane-1-carboxylatedeaminase; ARA, acetylene reduction assay; *nifH*, dinitrogenase reductase encoding gene; BC, *B. cereus* ATCC 11778; AN, *A. niger* ATCC 6275; CF, *C. falcatum* DOAC 1655; FM, *F. moniliforme* DOAC 1224; +, positive activity; –, negative activity; ND, not determined

[b] ARA values represent activity expressed as nmol C_2H_4 h^{-1} $(10^{-7}$ cells$)^{-1}$

[c] Accession No. KT275189

Table 1-4 Effect of individual and co-inoculation of endophytic diazotrophs and actinomycetes on growth of sugarcane plants at 60 days after inoculation

Treatments	Root			Shoot			Root re-isolation (CFU g fresh weight^{-1})	
	Length (cm)	FW[a] (g)	DW (g)	Length (cm)	FW (g)	DW (g)	Bacteria	Actinomycetes
Un-inoculated control	6.25 ± 0.96^{b}	1.04 ± 0.02^{f}	0.13 ± 0.02^{e}	30.58 ± 4.66^{b}	2.65 ± 0.08^{d}	0.30 ± 0.01^{e}	-	-
T1: *Enterobacter* sp. EN-21	9.45 ± 1.54^{ab}	3.24 ± 0.10^{de}	0.25 ± 0.03^{cd}	42.62 ± 6.64^{ab}	4.88 ± 0.16^{abc}	0.70 ± 0.03^{bc}	$5.27 \pm 0.35 \times 10^{4}$	-
T2: *Bacillus* sp. EN-24	11.43 ± 1.31^{ab}	3.93 ± 0.13^{abcd}	0.37 ± 0.01^{ab}	42.66 ± 3.48^{ab}	5.07 ± 0.22^{abc}	0.74 ± 0.04^{abc}	$8.22 \pm 0.17 \times 10^{4}$	-
T3: *Microbispora* sp. GKU 823	9.82 ± 1.53^{ab}	2.57 ± 0.05^{e}	0.16 ± 0.02^{e}	39.82 ± 2.22^{ab}	4.43 ± 0.10^{c}	0.53 ± 0.03^{d}	-	$9.47 \pm 0.07 \times 10^{3}$
T4: *Streptomyces* sp. GKU 895	11.85 ± 3.83^{ab}	3.34 ± 0.05^{cde}	0.26 ± 0.03^{cd}	43.98 ± 7.82^{a}	5.48 ± 0.12^{abc}	0.72 ± 0.05^{bc}	-	$2.05 \pm 0.50 \times 10^{4}$
T5: EN-21+EN-24	12.05 ± 1.87^{ab}	3.79 ± 0.14^{abcd}	0.22 ± 0.05^{de}	45.74 ± 6.30^{a}	4.62 ± 0.11^{bc}	0.63 ± 0.03^{cd}	$8.27 \pm 0.70 \times 10^{4}$	-
T6: GKU 823+GKU 895	11.49 ± 3.48^{ab}	4.24 ± 0.08^{abc}	0.33 ± 0.05^{abc}	40.64 ± 5.84^{ab}	5.99 ± 0.08^{a}	0.84 ± 0.09^{a}	-	$9.22 \pm 0.10 \times 10^{3}$
T7: GKU 823+EN-21+EN-24	14.25 ± 3.71^{a}	4.70 ± 0.10^{a}	0.36 ± 0.09^{ab}	48.22 ± 6.56^{a}	5.73 ± 0.07^{ab}	0.74 ± 0.07^{ab}	$6.67 \pm 0.81 \times 10^{3}$	$5.31 \pm 0.15 \times 10^{3}$
T8: GKU 895+EN-21+EN-24	14.30 ± 5.24^{a}	4.33 ± 0.05^{ab}	0.40 ± 0.04^{a}	41.92 ± 4.60^{ab}	5.98 ± 0.05^{a}	0.78 ± 0.06^{ab}	$4.72 \pm 0.65 \times 10^{3}$	$4.35 \pm 0.73 \times 10^{3}$
T9: GKU 823+GKU 895+EN-21+EN-24	14.12 ± 2.95^{a}	3.46 ± 0.10^{bcde}	0.28 ± 0.03^{bcd}	48.64 ± 2.21^{a}	5.29 ± 0.07^{abc}	0.73 ± 0.02^{bc}	$1.49 \pm 0.11 \times 10^{3}$	$3.01 \pm 0.85 \times 10^{3}$

Data are means ± standard deviations (SD) of five replicates. Means designated with same letters are not significantly different ($p \leq 0.05$) according to Turkey's multiple range test

[a] FW, fresh weight; DW, dried weight

Discussion

Plant growth-promoting endophytes (PGPE) are heterogeneous groups of bacteria that beneficial to host plants (Gaiero and others 2013). Several PGPE can encourage plant growth through the release of phytohormones, increment of nutrients and protection from phytopathogens. In this work, culture-dependent approach was used to isolate culturable root-associated bacteria including actinomycetes from sugarcane cultivated in Thailand. The sugarcane roots used for isolation in this study were successfully surface disinfested in which no colonies were detected from the final rinses on any medium agar used; thus, the isolated bacteria were true endophytes. From the isolation procedures used in this work, 38.52 % of endophytic bacteria and 61.58 % of actinomycetes were obtained. Fourteen genera were systematically identified and the most dominant species were *Bacillus*, *Enterobacter*, *Microbispora* and *Streptomyces*.

Diazotrophs appeared to be the most numerous bacteria so far isolated from sugarcane including *Azospirillum* spp., *Burkhoderia* spp., *Enterobacter* spp., *Gluconacetobacter diazotrophicus*, *Herbaspirillum* spp., *Klebsiella* spp., *Pantoea* spp. and *Pseudomonas* spp. (Asis and others 2000; Suman and others 2001; Perin and others 2006; Govindarajan and others 2007; Mendes and others 2007; Castro-González and others 2011; Taulé and others 2011; Lin and others 2012). Surprisingly, the well-known sugarcane diazotrophic species, including *Azospirillum* spp., *Burkholderia* spp., *Gluconacetobacter diazotrophicus*, and *Herbaspillum* spp. (Govindarajan and others 2007; Taulé and others 2011), were not found in this work. A plausible explanation for their notable absence could be that the composition of the endophytic bacterial community depends to some degree on a specificity of particular bacterial genotypes for particular sugarcane cultivars, different geographic origins and level of fertilizers used (Reis-Junior and others 2000; Taulé and others 2011). Moreover, the sugarcane root associated nitrogen-fixing bacteria in this work were isolated without using the enrichment method of reseeding in the N-free medium (Castro-González and others 2011), the amount of diazotrophic bacteria could, therefore, be affected and resulted in less population. The majority of endophytic bacteria isolated in this study were mainly found as members of genera *Bacillus* and *Enterobacter*. The results were in agreement with several reports of which *Bacillus* and *Enterobacter* were dominant sugarcane endophytes (Velazquez and others 2008; Magnani and others 2010; Taulé and others 2011).

Although endophytic actinomycetes have been isolated from several different kinds of plants including a variety of medicinal and crop plants (Coombs and Franco 2003; Tian and others 2007; Zhao and others 2011), this is the first report to describe the diversity of endophytic actinomycetes from sugarcane. The dominance of *Streptomyces* spp. in the culturable diversity of

root-associated endophytes in this report has been noticed in several previous studies from various plant species, i.e. wheat, rice, and herbaceous and medicinal plants (Coombs and Franco 2003; Tian and others 2007; Zhao and others 2011; Kim and others 2012; Li and others 2012). However, we found different genera of non-streptomycetes (*Actinomadura*, *Microbispora* and *Micromonospora*) associated with roots of sugarcane (Fig. 1). The presence of *Actinomadura*, *Microbispora* and *Micromonospora* was in agreement with previous reports that they are common genera found in root tissues of plants (Qin and others 2011; Mingma and others 2014). Although *Streptomyces* spp. was predominantly associated with sugarcane roots, we observed a large number of colonies of *Microbispora* spp. (21.48 %). The results suggested that *Microbispora* spp. also largely occupied in root tissues of sugarcanes. This work indicated that sugarcanes are rich bio-resources for diversity of endophytic bacteria including diazotrophs and actinomycetes.

PGP traits were examined in sugarcane endophytes and revealed that strains belong to genera *Streptomyces*, *Bacillus* and *Enterobacter* showed the highest activity of PGP traits. *Bacillus* and *Enterobacter* have been previously reported to promote the growth of maize, sugarcane and tobacco (Kloepper and others 2004; Lin and others 2012; Naveed and others 2014). The highest frequency of PGP-trait possessed by sugarcane endophytes in this study was siderophore production, followed by IAA production and phosphate solubilization. It has been suggested that siderophores are involved in both plant growth and health protection by chelating iron (Jaber and others 2002; Rungin and others 2012; Radzki and others 2013). Recent work of Rungin and others (2012) also confirmed that a siderophore-producing endophytic *Streptomyces* increased root/shoot lengths and biomass of rice and mung bean compared to the deficient mutant. It was demonstrated that bacterial endophytes carrying IAA trait could increase root elongation and could affect the development of lateral roots, which improves the plant's nutrient uptake from the rhizosphere (Idris and others 2007; Goudjal and others 2013). A recent report showed that *Microbispora* spp. and *Streptomyces* spp. exhibited a high variability in IAA production and resulted in significant promotion of shoot length of mandarin seedlings (Shutsrirung and others 2013). Members of PGPE in the genera *Bacillus*, *Enterobacter*, *Pseudomonas*, *Micromonospora* and *Streptomyces* are known as phosphate solubilizers (Hamdali and others 2008; Bashan and others 2013) with the ability to convert insoluble compounds of phosphorus into available phosphates that enhance nutrient availability to plants (Son and others 2006). However, the highest activity of phosphate solubilzation in this work was found in *Pantoea* spp. Recently, phosphate-solubilizing *Pantoea* has been reported as a plant growth promoter which increased height and dry weight of *Lotus tenuis* cv. Pampa INTA (Castagno and others 2011). PGPE harboring ACC deaminase in

sugarcane could facilitate plant growth by conversion of ACC to ammonia and α-ketobuterate, which bacteria can consume and consequently lower the ethylene level in plants (Glick 2005). Most of the endophytic bacteria isolated from sugarcane have been reported as diazotrophs, with effects on plant growth promotion (Govindarajan and others 2007; Lin and others 2012). In this work, only 11.53 % out of sugarcane endophytic bacteria showed nitrogenase activity belonging to *Bacillus*, *Enterobacter*, *Lysinibacillus* and *Pantoea* (Table 1-2). Although the standard protocol for isolation of diazotrophic bacteria was employed (Cavalcante and Dobereiner 1988), fewer ARA-positives were often obtained from plants such as rye grass (Habibi and others 2014) and rice (Rangjaroen and others 2015). Our ranges of ARA activity were in accordance with previous reports of *Enterobacter* spp. and *Bacillus* spp. (Habibi and others 2014). When *nifH* gene amplification was investigated to evaluate nitrogen fixation ability of ARA-positive endophytes, 19.23 % of the total was detected (Table 1-2). There were only five strains exhibited both nitrogenase activity and a *nifH* gene (Fig. 1-2). Our results were in agreement with Yim and others (2009) in which *nifH* gene could not be amplified from all ARA positives. Although, ARA is a common method to detect nitrogenase activity of microbial cultures and has been widely used to consequently identify nitrogen-fixing bacteria (Hardy and others 1973), it was suggested that such activity varies with growth stage, culture condition, and media composition. Hence, amplification of the *nifH* gene, encoding dinitrogenase reductase, was additionally used to perform the possible nitrogen-fixing ability. Although the *nifH* gene is the most widely used marker gene for identification of nitrogen-fixing bacteria, nucleotide sequences of *nifH* genes were diverse in many microorganisms (Waugh and others 1995; Zehr and others 2003). In this experiment, degenerate primers were used for amplification of *nifH* gene (Rösch and others 2002), nevertheless, it was yet absence in an ARA-positive *Bacillus* sp. EN-24 (Fig. 1-2). The absence of the *nifH* gene could therefore be explained by the variability of this gene (Waugh and others 1995; Zehr and others 2003). It is suggested that the other nitrogenase genes, *nifD* and *nifK*, could be used to confirm the nitrogen-fixing ability since *nifHDK* genes are known to encode the components of the nitrogenase enzyme complex (Howard and Rees 1996).

Some recent reports have indicated that root nodule-associated *Micromonospora* has the possible ability to fix atmospheric nitrogen (Trujillo and others 2010; Lorena and others 2012), but endophytic *Micromonospora* from medicago plants apparently had no functional nitrogenase and consequently no nitrogen fixation occurred (Martunez and others 2014). Because nitrogen fixation in endophytic actinomycetes has not yet been definitively explained and there is very little information about it, ARA assays were not performed on actinomycetes in this work.

Antagonistic tests revealed susceptible bacteria and fungi including *F. moniliforme* and *C. falcatum* causing red rot disease in sugarcane (Alexander and Viswanathan 2002) that interacted with the endophytes. *B. cereus* and *E. coli* were most frequently inhibited (Table 1-2), which was consistent with a previous report (Hassan and others 2010). The results of this study indicated that endophytic bacteria and actinomycetes from sugarcane carried multiple traits of PGP and are therefore considered to be safe agents to apply for plant growth enhancement and to control phytopathogens in sugarcane plantations.

Based on PGP-traits, two endophytic diazotrophs, *Bacillus* sp. EN-24 and *Enterobacter* sp. EN-21, and two strains of actinomycetes, *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895, were selected to evaluate growth enhancement of sugarcane plants by individual and co-inoculation. Two-month old sugarcane plants treated by either individual or co-inoculation gave significantly greater biomass and lengths of root and shoot than un-inoculated control (Table 1-4, Fig. 1-3). The increase of root fresh weight was due to increased lateral root production, which might be the effect of plant hormone production by the endophytes (Idris and others 2007; Goudjal and others 2013). The most significant enhancement of growth appeared in the treatment that each endophytic actinomycete was co-inoculated with both *Enterobacter* sp. EN-21 and *Bacillus* sp. EN-24. It has been demonstrated that several diazotrophic bacteria, including *Bacillus* and *Enterobacter*, could promote growth of sugarcane (Lin and others 2012) as well as *jatropha*, maize, rice, sorghum, sugarcane and wheat (Olivares and others 1997; Yanni and others 2001; Govindarajan and others 2007; Luna and others 2010; Rungin and others 2012; de Jesus Santos and others 2014; Alves and others 2015; Qin and others 2015). Furthermore, co-inoculation of five diazotrophs (*Azospirillum*, *Burkholderia*, *Gluconacetobacter diazotrophicus*, *Klebsiella* and *Pseudomonas*) was reported to give better growth of rice (Govindarajan and others 2007); while, co-culture of diazotrophs and a fungal endophyte, *Trichoderma*, revealed better growth and yield of chickpea (Verma and others 2014). It was suggested that the combination of phosphate solubilizing, nitrogen-fixing and phytohormone producing bacteria could provide a more balanced nutrition for plants and stimulate more growth of plants. For examples, combination of a phosphate-solubilizing *Bacillus megaterium*, nitrogen-fixing *Bacillus subtilis* and *Rhizobium leguminosarum bv. phaseoli* caused a significant enhancement of seed yield and an uptake of macronutrients and micronutrients elements in common bean (Elkoca and others 2010). Mixed inoculation of *Rhizobium*, a phosphate-solubilizing *B. megaterium* sub sp. *phospaticum* strain-PB and a biocontrol fungus *Trichoderma* sp. was reported to increase seed germination, nutrient uptake, plant height, number of branches, nodulation, pea yield, and total biomass of chickpea

compared to either individual inoculation or an un-inoculated control (Rudresh and others 2005). Therefore, understanding of plant growth-promoting properties of endophytic bacteria and actinomycetes suggest that these bacteria merit further investigation for potentially safe and environmentally friendly biofertilizers which can help us limit the use of chemical fertilizers in agriculture.

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Section 2

Genome sequencing and analysis of endophytic actinomycetes isolated from sugarcane

Introduction

Sugarcane is an economically important crop for the production of sugar and biofuels. Plant growth-promoting endophytes are heterogeneous groups of bacteria that reside mutually within plant tissues and appear to provide many benefits to the plant host. *Streptomyces* sp. GKU 895 and *Microbispora* sp. GKU 823 are root-associated bacterium isolated from sugarcane plants and has been reported to enhance growth of sugarcane by harboring plant growth promoting (PGP) traits (Kruasuwan and Thamchaipenet, 2016). The 16S rRNA sequences analysis showed that strain GKU 823 and GKU 895 closely related to *Microbispora hainanensis* 211020^T (98.8% similarity) and *Streptomyces canus* NRRLB-1989^T (99.4% similarity), respectively.

Materials and Methods

Endophytic actinomycetes

Two strains of sugarcane growth-promoting bacterial endophytic actinomycetes, *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895, were used in this study.

Genomic DNA extraction

Strains GKU 823 and GKU 895 were cultured in NB medium at 30°C, 200 rpm for 7 days. Then, 1 mL of NB-culture were centrifuged at 8,000 × g for 5 min and supernatants were removed. Total genomic DNA was extracted using ISOLATE II genomic DNA extraction kit (BIOLINE, UK) according to manufacture instruction. The cell pellets were resuspended with 180 µL nuclease-free water supplemented with 200 µL of 10 mg mL^{-1} lysozyme and incubated at 37°C for 1 h. After that, 25 µL proteinase K was added and further incubated at 56°C for 3 h. The suspension was briefly vortexed and 200 µL lysis buffer G3 was added and incubated at 70°C for 10 min. 210 µL ethanol was then added to the sample and vigorously vortexed. All samples were added to the column and then centrifuge for 1 min at 11,000 ×g. Flow-through was discarded and the silica membrane column was washed with 500 µL wash buffer GW1 followed by 600 µL wash buffer GW2 and centrifuged at 11,000 × g for 1 min. The column was transferred to a new microcentrifuge tube, 50 µL preheated elution buffer G was added and incubated at RT for 3 min, and centrifuged at 11,000 × g for 1 min. The quality and quantity of genomic DNA were determined using Nanodrop® 2000 (Thermo Fisher Scientific, Wilmington, USA).

Ion Torrent PGM sequencing

Library preparation

Genomic DNA of both strains, GKU 823 and GKU 895, were used for preparation of genomic library. The library was prepared using NEBNext® fast DNA fragmentation & library prep set for Ion Torrent™ kit (New England®, Biolab, USA).

Fragmentation and end repair of DNA

Genomic DNA (10 ng-1 µg) was mixed with 2 µL NEBNext DNA fragmentation reaction buffer and adjusted to 18.5 µL of total volume. The sample was vortexed for 3 sec, pulse spin and placed on ice. Then, 1.5 µl of NEBNext DNA fragmentation master mix was added to the microfuge tube. The mixture was vortexed for 3 sec, pulse spinned and then incubated in a thermal cycler at 25°C for 20 min, followed by at 70°C for 10 min, and hold at 4°C (BioRad, USA).

Preparation of adaptor ligated DNA

The fragmented DNA samples were ligated with adapter using the protocol according to NEBNext® fast DNA fragmentation & library prep set for Ion Torrent™ kit. Briefly, 1 µL sterile nuclease-free water, 4 µL T4 DNA ligase buffer, 10 µL NEBNext DNA library adaptors (5 µL of barcodes and 5 µL of adapters), 1 µL *Bst* 2.0 WarmStart DNA polymerase, and 4 µL T4 DNA ligase were added to the microfuge and mixed by pipetting up and down several times. Incubated the contents in a thermal cycler at 25°C for 15 min, followed by 65°C, 5 min, and hold at 4°C. Finally, 5 µL stop buffer was added, vortexed and pulse-spin.

Cleanup of adapter ligated DNA

The adapter ligated DNA was cleaned by adding 72 µL of AMPure XP Beads to the sample and mixed by pipetting up and down. The solution was incubated at RT for 5 min followed by pulse-spin and placed in a magnetic rack for 2-3 min. Then, the supernatant was carefully discarded without disturbing the beads. 500 µL freshly prepared 80% ethanol was added and incubated at RT for 30 sec. The beads were dried at RT for 5 min and resuspended with 25 µL nuclease-free water. Pulse-spin the beads and placed the tube in the magnetic rack until the beads have collected to the side of the tube and the solution is clear. Afterward, 20 µL of the supernatant was transferred to a new clean tube.

E-gel size selection

To select the target size fragment, approximately 400 bp of amplified library was selected by E-Gel® SizeSelect™ agarose gels (Thermo Fisher Scientific, Wilmington, USA) according to manufacture protocol. Firstly, 2% agarose gel cassette was inserted into the E-Gel® until right edge. Before loading, 20 µL of nuclease-free water was added to the purified ligated DNA (40 µL of total volume) and 20 µL of ligated DNA was loaded to the loading well. Next, 10 µL of diluted DNA ladder (1:40 dilution) was added to ladder well and 25 µL of nuclease-free water was added to all empty wells in the large top and bottom rows (collection wells). Then, 10 µL was added to the center well (lane M) of the bottom row and run SizeSelect 2% program for 16-20 min. During the run, refilled the collection wells to 25 µL with ~10 µL of nuclease-free water and run time set to 0.5-2.5 min. Monitoring the middle marker well for the desired fragment length, and stop the run when the maker number 7 and 8 located between the M well. Finally, the solution from the collection wells was collected using pipette and washed the well with 10 µL nuclease-free water, collected the solution, and pooled the solution together. The total recovered volume is ~30 µL from each well.

PCR amplification of adapter ligated DNA

To enrich the number of adapter ligated DNA, the cleaned adapter ligated DNA was amplified using thermal cycler PCR (Biorad, USA) with 46 µL leaned adapter ligated DNA, 4 µL primer and 50 µL NEBNext high-fidelity 2× PCR master mix. The mixture was subsequently subjected to thermo cycler at 98°C for 30 sec, 8 cycles at 98°C for 10 sec, 58°C for 30 sec, 72°C for 30 sec, and 72°C for 5 min and hold at 4°C.

Assess library quantity and quality by Qubit® fluorometer and Bioanalyzer

The amplified library was cleaned by adding 100 µL of AMPure XP Beads to the sample and mixed by pipetting up and down and followed the protocol described in 2.3.4 above. 20 µL of the supernatant was transferred to a clean tube. The concentration of amplified library was quantified by Qubit® fluorometer (Thermo Fisher Scientific, Wilmington, USA) and library size was analysed by Agilent high sensitivity DNA kit (Agilent Technologies, USA) according to manufacture instruction.

Ion OneTouch™ 2 amplified library preparation

Prepare template-positive Ion PGM™ Hi-Q™ Ion Sphere™ particles

To sequence the genome of actinomycetes by Ion Torrent™, amplified library was prepared template-positive using Ion OneTouch™ 2 amplified library preparation kit (Thermo Fisher Scientific, Wilmington, USA) according to the manufacture protocol. The amplified library was diluted to adjust the concentration to 100 pM (25 µL of total volume) and mixed with 25 µL nuclease-free water, 50 µL Ion PGM™ Hi-Q™ enzyme mix, and 100 µL Ion PGM™ Hi-Q™ ISPs (total volume as 1000 µL). Then, the complete amplification solution was mixed using a vortex mixer at maximum speed for 5 sec and proceeded immediately to fill to Ion OneTouch™ reaction filter. The reaction filter was inserted into the three holes on the top stage of the Ion OneTouch™ 2 instrument and carefully recovered both Ion OneTouch™ recovery tubes from the instrument after finished.

Recover the template-positive Ion PGM™ Hi-Q™ ISPs

To remove excess Ion PGM™ OT2 recovery solution from the ISPs, used a pipette but 100 µL of the recovery solution from each recovery tube remained. The ISPs was suspended in the remaining Ion PGM™ OT2 recovery solution by pipetting up and down until each pellet disperses in the solution. 500 µL of Ion OneTouch™ wash solution was added to each recovery tube and pipetted up and down to disperse the ISPs. The suspension from both tubes was transferred to a new 1.5-mL tube. Stored the ISPs at 2-8°C for up to 3 days until used.

Ion Sphere assay

To measure the Ion Sphere prior to perform sequencing, 2 µL of the ISPs solution was taken from Ion PGM™ OT2 recovery solution and analyzed by Qubit® fluorometer (Invitrogen, USA). The percentage of template-positive ISPs was calculated from the ratio of the Alexa Fluor 647 dye signal to the Alexa Fluor 488 dye signal.

Enrich the template-positive Ion PGM™ Hi-Q™ Ion Sphere™ Particles

The template-positive Ion PGM™ Hi-Q™ ISPs was centrifuged at 15,500 × g for 2.5 min and the supernatants were discarded but 100 µL of the recovery solution remained. Pipetted the ISPs up and down in remaining solution for 10 times, then the suspension was transferred into well 1 of the 8-well strip collected from the Ion OneTouch™ ES Supplies Kit (Thermo Fisher Scientific, Wilmington, USA) according the manufacture protocol. After that filled 130 µL of resuspended

Dynabeads® MyOne™ Streptavidin C1 Beads in MyOne™ beads wash solution into well 2, 300 µL of Ion OneTouch™ wash solution into well 3 to 5, and 300 µL of freshly-prepared melt-off solution into well 7. Pipetted the contents of well 2 up and down to resuspend the beads before starting the run. Finally, collected the finished sample and kept at 2-8°C until used.

Ion PGM™ Sequencing

Enriched template-positive ISPs preparation

The Ion Sphere™ particles obtained from ES was vortexed and centrifuged for 2 sec before aliquot. The content was added with 5 µL of control Ion Sphere™ particles into 0.2 mL non-polystyrene PCR tube and mixed by thoroughly pipetting up and down and centrifuged at 15,500 × g for 2 min. Then, the supernatant was carefully removed without disturbing the pellet and remained 15 µL in the tube. 12 µL of the sequencing primer was added and the tube was then placed in the thermal cycler and the program was run following 95°C for 2 min, and 37°C for 2 min using the heated lid option. After annealing the sequencing primer, 3 µL of Ion PGM™ sequencing polymerase was added, the sample was then pipetted up and down and incubated at RT for 5 min.

Ion 316™ chip checking

To ensure the chip is properly functioned prior to loading the sample, the chip was checked tests. Firstly, a new chip was removed from its packaging and labeled. The chip was placed on the Ion PGM™ sequencer grounding plate and run on the main menu to test a new Ion 316™ Chip. Then, the new chip was removed and placed it on the grounding plate while inserted a used chip in the socket and close the clamp.

Chip loading

To transfer the ISPs solution to the chips, the liquid inside the chip was discarded by tilted the chip 45 degrees and inserted the pipette tip firmly into the loading port and removed as much liquid as possible from the loading port. To completely empty the chip, the chip was placed upside-down in the centrifuge adapter bucket and centrifuged for 5 sec followed by loading the sample on the chip. The ISPs sample was collected (~30 µL) and loaded directly into the chip by slowly dial down the pipette at a rate of ~1 µL per sec. Subsequently, the chip was transferred to the Mini Fuge with the chip tab pointing in and centrifuged for 30 sec. The sample inside the chip was mixed by tilted the chip 45 degrees and slowly pipetted the sample up and down for ten times

followed by transferring the chip to the Mini Fuge with the chip tab pointing out, then centrifuged for 30 sec. Repeat the steps of mixing for four times and centrifuged for 30 sec with the previously opposite chip tab pointing. Finally, discarded the liquid by tilted the chip at a 45-degree angle and slowly removed by dialing the pipette.

Chip sequencing

Placed the ISPs sample filled chip on the Ion PGM™ sequencer grounding plate and selected the planned run that previously created in the Torrent Browser (http://130.159.63.93). The chip was removed and placed on the grounding plate while inserted a used chip in the socket and close the clamp. Then, run the planned program.

MinION sequencing

Total genomic DNA of endophytes were sequenced using MinION sequencer following ligation sequencing kit 2D (SQK-LSK208, R9.4) protocol.

DNA fragmentation and DNA repairing

Total genomic DNA of strains GKU 823 and GKU 895 were fragmented using the standard Covaris g-TUBE protocol. Briefly, the g-TUBE containing 1-1.5 µg genomic DNA in 45 µL was spun for 1 min. Then, inverted the g-TUBE, spun again for 1 min to collect the fragmented DNA and transferred the 45 µL fragmented DNA to a clean 1.5 mL Eppendorf DNA LoBind tube. Added 8.5 µL nuclease-free water, 6.5 µL FFPE repair buffer, and 2 µL FFPE repair mix to the 45 µL fragmented DNA tube, mixed gently and incubated at 20 °C for 15 min. After that 62 µL of the resuspended beads was added to the FFPE-repair reaction and mixed gently by flicking the tube and incubated on a rotator mixer at RT for 5 min. The tube was then placed on a magnet to pellet and pipetted off the supernatant. Keep on magnet, beads were washed twice with 200 µL of freshly prepared 70% ethanol without disturbing the pellet. 70% ethanol was subsequently discarded using a pipette, and allowed to dry briefly. Finally, pellet was resuspended in 46 µL nuclease-free water, incubated at RT for 2 min. 1 µL of fragmented and repaired DNA was quantified using a Qubit fluorometer (recovery aim > 1 µg).

End-repair and dA-tail of double-stranded DNA fragments

To perform end repair and dA-tailing of fragmented DNA, 7 µL Ultra II End-prep reaction buffer, 3 µL Ultra II End-prep enzyme mix, and 5 µL nuclease-free water were added to 45 µL

fragmented DNA. The sample was then transferred to a 0.2 ml PCR tube and incubated at 20 °C for 5 min, and 65 °C for 5 min using the thermal cycler. Next, the sample was transferred back to a new 1.5 mL DNA LoBind Eppendorf tube and 60 µL of resuspended AMPure XP beads was added to the end-prep reaction and mixed by pipetting. The tube was placed on a magnet to pellet and pipette off the supernatant. Keep on magnet, beads were washed twice with 200 µL of freshly prepared 70% ethanol without disturbing the pellet. 70% ethanol was then discarded using a pipette, and allowed to dry briefly. Finally, pellet was resuspended in 31 µL nuclease-free water, incubated at RT for 2 min. 1 µL of end-prepped DNA was quantified using a Qubit fluorometer (recovery aim > 700 ng).

Adaptor ligation and AMPure XP bead binding

End-prepped DNA was ligated by adding 30 µL end-prepped DNA with 20 µL Adapter Mix and 50 µL Blunt/TA Ligation Master Mix. Mixed gently by flicking the tube, and incubated the reaction at RT for 10 min. Then, 40 µL of resuspended AMPure XP beads was added to the adapter ligation reaction from the previous step and mixed by pipetting and incubated on a rotator mixer at RT for 5 min. The tube was placed on magnetic rack, allowed beads to pellet and pipetted off supernatant. 140 µL of the Adapter Bead Binding buffer was added to the beads. Close the tube lid, and resuspended the beads by flicking the tube. The tube was again placed to the magnetic rack, allowed beads to pellet and pipetted off the supernatant. Next, pellet was resuspended in 15 µL Elution Buffer and incubated at RT for 10 min. Finally, beads were pelleted on magnet until elute is clear and colourless. 15 µL of elute was removed and retained into a clean 1.5 Eppendorf DNA LoBind tube.

Priming and loading the SpotON Flow Cell

The adaptor ligated DNA was sequenced using spot-on flow cell Mk1 (R9.4). Firstly, flipped back the MinION lid and slide the priming port cover clockwise so till it is visible. Removed a few volumes of the buffer and small bubble from the flow cell using pipette. Then, 800 µL of the priming mix prepared by mixing 520 µL nuclease-free water with 480 µL RBF was loaded into the Flow Cell via the priming port. After that, prepared library was loaded by mixing of 35 µL RBF, 2.5 µL nuclease-free water, 25.5 µL LLB and 12 µL DNA library. Complete the flow cell priming by additionally loading 200 µL of the priming mix into the Flow Cell via the priming port. Next, 75 µL of DNA library was added to the flow cell via the SpotON sample port in a dropwise fashion. Close the priming port and replaced the MinION lid and started the run.

Genome sequences analysis

Ion PGM reads (.fastq) and MinION reads (.fast5) of GKU 823 and GKU 895 were respectively collected from Torrent server (http://130.159.63.93) and MinKNOW. The MinION reads were extracted to .fastq files using pore tool (Loman and Quinlan, 2014), then uploaded to Galaxy (http://localhost:8080/galaxy/) for further analysis. The genome sequences were assembled into contigs by SPAdes genome assembler (version 1.4) with default parameters and nanopore option. Low coverage and short contigs or scaffolds were removed by Filter SPAdes output (version 0.1) (Bankevich et al., 2012). The contiqs or scaffolds of assembled genome were evaluated the quality by QUAST (Quality Assessment Tool for Genome Assemblies, version 1.0.0) (Gurevich et al., 2013). To identify genomic features (i.e., protein-encoding genes and RNA) and annotate their functions in filtered assembly genome, Rapid Annotation using Subsystem Technology (RAST, http://rast.nmpdr.org/rast.cgi) (Aziz et al., 2008) was used. Total number of rRNA was predicted by RNAmmer version 1.2 (Lagesen et al., 2007). The biosynthetic gene cluster for secondary metabolites was predicted by antiSMASH (http://antismash. secondarymetabolites.org/) (Weber et al., 2015).

Result and Discussion

Genomic DNA extraction

Total genomic DNA of *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895 was extracted and measured the concentration by using Nanodrop. The concentration of genomic DNA was calculated as ng mL^{-1} that shown in the Table 2-1

Table 2-1 Genomic DNA concentration extracted from *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895

Sample	Conc. (ng μL^{-1})	Total conc. (µg)	$A_{260/280}$	$A_{260/230}$
GKU 823	191.9	9.2112	1.85	1.89
GKU 895	124.7	5.2374	1.87	2.65

Total genomic DNA of both endophytic actinomycetes was replicatively extracted. The highest concentration of GKU 823 and GKU 895 were respectively calculated to 191.9 ng μL^{-1} and 124.7 ng μL^{-1}. Therefore, both of these concentrations were used for library construction in the further experiments.

DNA fragmentation and size selection

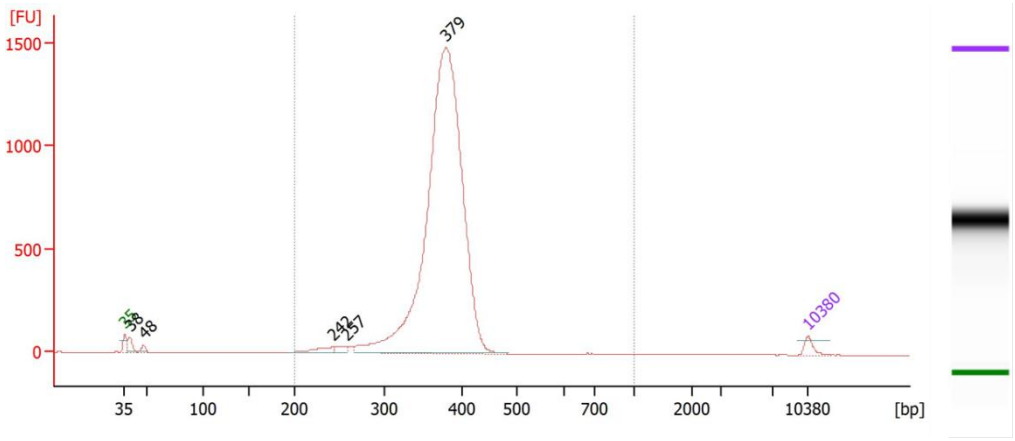
To construct the DNA library, fragmentation of DNA and selection of DNA size were performed. In this work, the estimated size was approximately 400-bp (Fig. 2-1) that selected by E-Gel® SizeSelect™ Agarose Gel to prepare as a template for further sequencing. The collected DNA was determined by Qubit® fluorometer for assess library concentration and Bioanalyzer for sizing, quantitation and quality of selected DNA. Concentration of DNA library of *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895 showed 4.82×10^3 ng mL^{-1} and 5.98×10^3 ng mL^{-1}, respectively.

Bioanalyzer analysis found that the target size of selected DNA of *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895 were respectively 379-bp and 421-bp (Fig. 2-1A and 2-1B). Both of selected size shown clearly single peak on the bioanalyzer analysis and gave highly amount of DNA concentration and molarity (Table 2-2). GKU 823 and GKU 895 showed 20,923.59 pmol L^{-1} and 23,881.15 pmol L^{-1}, respectively of selected DNA concentration after calculated from bioanalyzer system. Hence, this content will be used for diluted library calculation in the step of Ion OneTouch™ 2 amplified library preparation.

Enrichment of template-positive Ion Sphere™ Particles

Library fragments were clonally amplified onto Ion Sphere Particles (ISPs) through emulsion PCR and then enriched for template-positive ISPs. More specifically, PGM emulsion PCR reactions utilized the Ion OneTouch™ 2 amplified library preparation kit, and as specified in the accompanying protocol, emulsions and amplification were generated using the Ion OneTouch™ 2 System. Following recovery, enrichment was completed by selectively binding the ISPs containing amplified library fragments to streptavidin coated magnetic beads, removing empty ISPs through washing steps, and denaturing the library strands to allow for collection of the template-positive ISPs. Percentage of template-positive ISPs was calculated from the ratio of the Alexa Fluor 647 dye signal to the Alexa Fluor 488 dye signal. The template-positive ISPs showed 14% (suitable as 10-30%) absorbance ratio when compared to Alexa Fluor 488 and 647 dye signal that suggested the templated ISPs was suitable for sequencing.

A



B

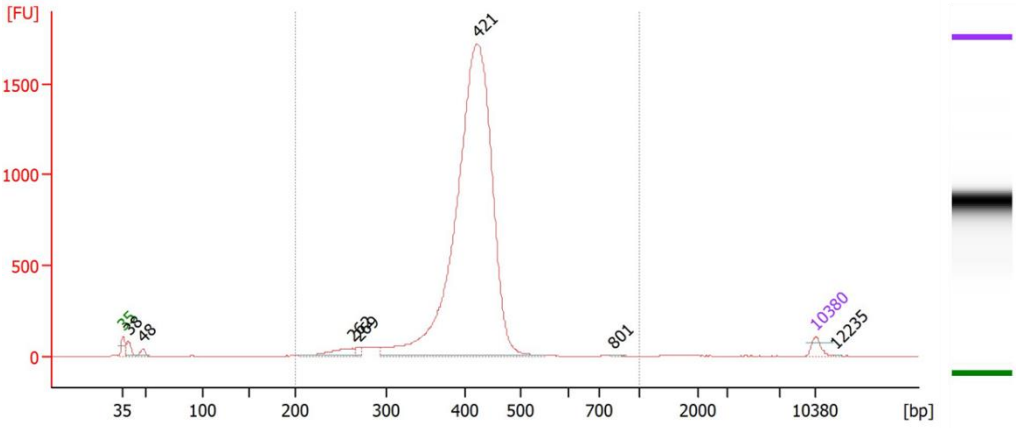


Fig. 2-1 Electropherograms of high-quality bioanalyzer runs of sequencing libraries showing a sharp peak at 379-bp fragment of *Microbispora* sp. GKU 823 (A), and 421-bp fragment of *Streptomyces* sp. GKU 895 (B).

Table 2-2 Selected size and DNA concentration of *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895 derived from bioanalyzer analysis

Bacterial strain	**Size** (bp)	**Concentration** (pg μ^{-1})	**Molarity** (pmol L^{-1})
GKU 823	379	10,512.56	42,010.70
GKU 895	421	9,016.90	32,418.30

Sequence reads analysis

The templated ISPs were sequenced by Ion 316™ Chip v2 with 1200 analysis flows for approximately 7 hrs. After that, reads summary was interpreted by Torrent server. The results demonstrated that the ISP was filled in the well by 71% (Fig. 2-2). The GKU 823 genome was sequenced using the platform Ion PGM system generating 1,230,781 reads (with approximately 30× coverage) with an average read length of 225 bp. The GKU 895 genome produced 1,019,643 reads (with approximately 30× coverage) with an average read length of 240 bp of Ion PGM and generated 21,275 2D MinION reads with an average length of 2,979 bp.

Genome analysis

Genome description of *Microbispora* sp. GKU 823

The genome character of *Microbispora* sp. GKU 823 was summarized in Table 2-3. QUAST analysis found that the GKU 823 genome consisted of 9,430,099 bp, assembled into 262 contigs (30× coverage) with an N_{50} of 69,483 bp. The largest contig obtained is 321,219 bp in length.

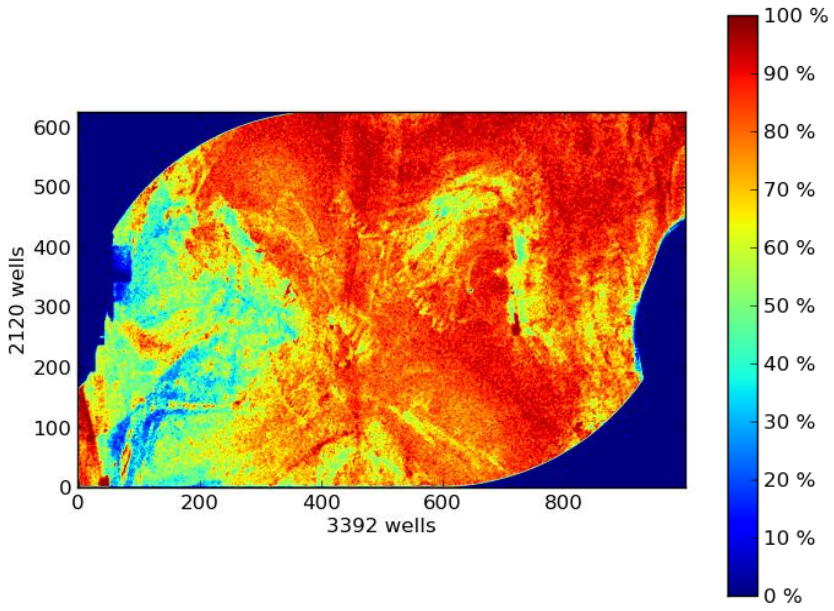


Fig. 2-2 ISPs loading density of the templated ISPs of *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895.

Table 2-3 Summary of genome sequencing results of *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895

Strain	Genome size (bp)	G+C content (%)	No. contigs	N_{50} (bp)	No. of CDSs[a]	No. of tRNAs
GKU 823	9,430,099	71.3	262	69,483	9,248	58
GKU 895	8,296,413	70.7	190	61,010	8,897	64

The draft genome of *Microbispora* sp. GKU 823 is estimated to be 9,430,099 bp with a G+C content of 71.3% (Fig. 2-3). The annotation predicted a total 9,248 coding sequences, 58 tRNA and 3 rRNA genes. The average nucleotide identity values of the genome were calculated using BlastN (ANIb) in JSpeciesWS (Richter et al., 2015). The genome comparison revealed that *Microbispora* sp. GKU 823 had an ANIb value of 92.47% similar to *Microbispora rosea* NRRLB-2630, 90.70% to *M. rosea* NRRL B-2631 and 81.20% *Microbispora* sp. ATCC PTA-5024. Genes related to PGP traits (Table 2-4) including phosphate solubilization (alkaline phosphatase, and isocitrate dehydrogenase; (Sola-Landa et al., 2005); IAA production [tryptophan 2-monooxygenase; (Spaepen and Vanderleyden, 2011; Jog et al., 2014)] and a gene involved in fungal cell wall degradation family 18 chitinase (Kawase et al., 2004) were detected in the genome of *Microbispora* sp. GKU 823. Moreover, genes involved in stress tolerance (betaine aldehyde dehydrogenase, proline dehydrogenase, superoxide dismutase and trehalose synthase (Liu et al., 2016) were also present. These genes sustain the capability of *Microbispora* sp. GKU 823 to promote growth of sugarcane. AntiSMASH predicted 23 secondary metabolite gene clusters in the genome of *Microbispora* sp. GKU 823 (Table 2-5) including seven gene clusters of nonribosomal peptide synthetase (NRPS), four gene clusters of Type I polyketide synthase (T1PKS) and terpene, three gene clusters of bacteriocin, two gene clusters of siderophore (including desferrioxamine E), and a single gene cluster encoding a lanthipeptide. These secondary metabolite gene clusters indicate that endophytic *Microbispora* species are potential sources of novel specialized metabolites.

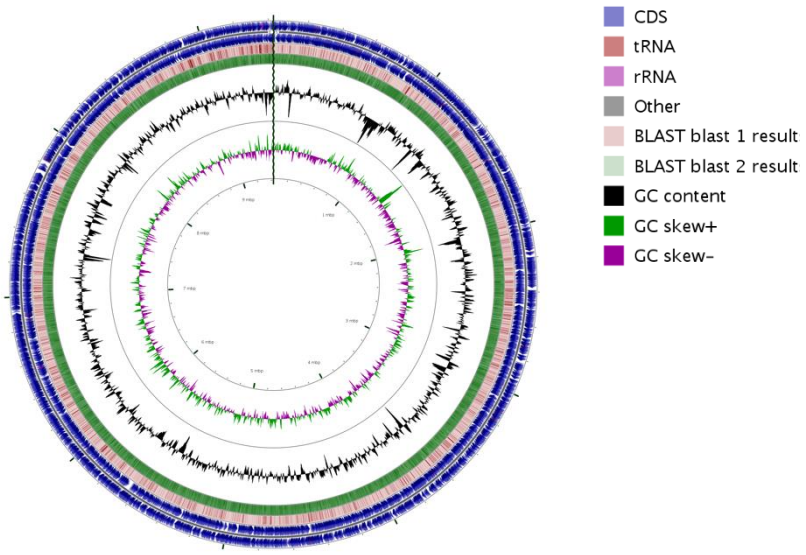


Fig. 2-3 Linear genome map of *Microbispora* sp. GKU 823. The genome-map was drawn using CGView Server.

Table 2-1 Plant growth related genes of *Microbispora* sp. GKU 823 annotated by RAST

Contig ID	Start	Stop	Strand	Gene function
Chitinase gene				
NODE_191_length_8153_cov_15.7941	3837	2359	-	Chitinase
NODE_191_length_8153_cov_15.7941	5485	3872	-	Chitinase
NODE_191_length_8153_cov_15.7941	6849	5491	-	Chitinase
NODE_191_length_8153_cov_15.7941	8111	7221	-	Chitinase
NODE_1_length_321219_cov_13.67	130858	129743	-	Chitinase
NODE_24_length_97179_cov_15.2805	49939	51651	+	Chitinase
NODE_36_length_81640_cov_15.0213	17718	19556	+	Chitinase
NODE_36_length_81640_cov_15.0213	45474	46658	+	Chitinase
NODE_42_length_69483_cov_15.0619	58352	55755	-	Chitinase
NODE_48_length_65177_cov_12.9238	53301	51100	-	Chitinase
NODE_51_length_61246_cov_13.6422	30019	28361	-	Chitinase
NODE_55_length_58359_cov_15.2684	40802	40248	-	Chitinase
NODE_55_length_58359_cov_15.2684	49229	50956	+	Chitinase
NODE_58_length_54599_cov_13.128	10965	10660	-	Chitinase
NODE_74_length_45164_cov_14.2634	45090	44821	-	Chitinase
NODE_75_length_44645_cov_18.7495	8410	9528	+	Chitinase
NODE_83_length_40414_cov_14.2508	4339	9372	+	Chitinase

NODE_88_length_36731_cov_13.3983	1048	3603	+	Chitinase
NODE_93_length_35936_cov_12.679	30513	29404	-	Chitinase
NODE_96_length_34747_cov_15.2612	12534	10762	-	Chitinase
NODE_97_length_34645_cov_11.6739	13579	12179	-	Chitinase
NODE_169_length_13762_cov_18.1344	3099	1315	-	Chitinase A precursor
IAA producing gene				
NODE_40_length_71054_cov_15.0107	31010	32911	+	Tryptophan 2-monooxygenase
Phosphate solubilizing gene				
NODE_35_length_81646_cov_13.4205	745	14	-	Alkaline phosphatase
NODE_157_length_15632_cov_14.6091	12440	11073	-	Alkaline phosphatase precursor
NODE_37_length_81310_cov_13.3767	64052	62835	-	Isocitrate dehydrogenase [NADP]
Plant colonizing gene				
NODE_11_length_124666_cov_15.4887	85464	86921	+	Salicylate hydroxylase
NODE_132_length_23610_cov_11.5779	21994	20813	-	Salicylate hydroxylase
NODE_17_length_109018_cov_11.7436	14809	13577	-	Salicylate hydroxylase
NODE_25_length_93394_cov_15.5553	42926	44599	+	Salicylate hydroxylase
NODE_2_length_277066_cov_14.3341	43157	41556	-	Salicylate hydroxylase
NODE_2_length_277066_cov_14.3341	147141	148319	+	Salicylate hydroxylase
NODE_3_length_157938_cov_13.1331	57005	55497	-	Salicylate hydroxylase
NODE_40_length_71054_cov_15.0107	19777	21315	+	Salicylate hydroxylase
NODE_61_length_53331_cov_13.5523	12482	13771	+	Salicylate hydroxylase
NODE_90_length_36411_cov_14.6388	15704	16219	+	Salicylate hydroxylase
NODE_90_length_36411_cov_14.6388	16329	16874	+	Salicylate hydroxylase
Siderophore producing gene				
NODE_101_length_33233_cov_14.3692	16927	15425	-	Desferrioxamine E biosynthesis protein DesA, Siderophore biosynthesis L-2,4-diaminobutyrate decarboxylase
NODE_101_length_33233_cov_14.3692	15438	14143	-	Desferrioxamine E biosynthesis protein DesB, Siderophore biosynthesis protein, monooxygenase
NODE_101_length_33233_cov_14.3692	14134	13574	-	Desferrioxamine E biosynthesis protein DesC, Siderophore synthetase small component
NODE_101_length_33233_cov_14.3692	13577	11751	-	Desferrioxamine E biosynthesis protein DesD, Siderophore synthetase superfamily
Stress responsive genes				
NODE_36_length_81640_cov_15.0213	57720	56260	-	Betaine aldehyde dehydrogenase

NODE_143_length_19756_cov_14.5264	522	1433	+	Proline dehydrogenase (Proline oxidase)
NODE_2_length_277066_cov_14.3341	148439	149086	+	Superoxide dismutase [Cu-Zn] precursor
NODE_59_length_54406_cov_16.2243	31686	31081	-	Superoxide dismutase [Fe]
NODE_36_length_81640_cov_15.0213	11371	13041	+	Trehalose synthase
NODE_51_length_61246_cov_13.6422	44792	43041	-	Trehalose synthase
NODE_56_length_55701_cov_13.7322	18800	17061	-	Trehalose synthase

Table 2-2 Secondary metabolites gene clusters of *Microbispora* sp. GKU 823 predicted by Anti-SMASH

Type	**Cluster**	**Most similar known** (% of genes similarity)	**MIBiG BGC-ID**
Bacteriocin	1		
Bacteriocin	13		
Bacteriocin	18		
Lantipeptide	2		
NRPS	4	Mannopeptimycin (33%)	BGC0000388 c1
NRPS	5	Tallysomycin (12%)	BGC0001048 c1
NRPS	6	Taromycin (10%)	BGC0000439 c1
NRPS	8	Coelibactin (100%)	BGC0000324 c1
NRPS	14		
NRPS	15	C-1027 (13%)	BGC0000965 c1
NRPS	21		
Siderophore	11		
Siderophore	17	Desferrioxamine B (83%)	BGC0000940 c1
T1PKS	7	Maklamicin (21%)	BGC0001288 c1
T1PKS	19		
T1PKS	20	Epothilone (20%)	BGC0000990 c1
TIPKS	23		
Terpene	3	K-252a (5%)	BGC0000814 c1
Terpene	12		
Terpene	16	Frankiamicin (14%)	BGC0001197 c1
Terpene	22	Geosmin (100%)	BGC0000661 c1
Other	9	Auricin deoxysugar moieties (13%)	BGC0000727 c1
Other	10	Meilingmycin (2%)	BGC0000093 c1

Genome description of *Streptomyces* sp. GKU 895

Streptomyces sp. GKU 895 showed 8,296,413 bp of genome size, assembled into 190 contigs (30× coverage), with a G+C content of 70.7% and 8,897 putative coding sequences (Table 2-3). The genome was annotated using RAST server, which identified 8,208 coding sequences. RNAmmer and tRNAscan-SE revealed 6 rRNA genes and 64 tRNA genes (Fig. 2-4). RAST annotation revealed that the genome of *Streptomyces* sp. GKU 895 possesses genes associated with plant growth promotion (Table 2-6) including *acdS* gene encoding ACC deaminase; genes involved in IAA synthesis [indoleacetamide hydrolase and nitrilase (Manulis et al., 1994; Spaepen and Vanderleyden, 2011)]; genes which assist in mineral phosphate solubilization including isocitrate dehydrogenase, citrate synthase, and purple acid phosphatase (Sharma et al., 2013; Jog et al., 2014); genes for the degradation of fungal cell walls family 18 and 19 chitinases (Gherbawy et al., 2012); and genes involved in host plant colonization such as salicylate hydroxylase (Ambrose et al., 2015). AntiSMASH analysis predicts 28 gene clusters involved in specialized metabolite production in the *Streptomyces* sp. GKU 895 (Table 2-7). These consist of six gene clusters encoding putative butyrolactones, three gene clusters of siderophores (including desferrioxamine E) and terpenes, two gene clusters for bacteriocins, melanins, Type II polyketide synthases (T2PKS), and a single gene cluster encoding ectoine, nonribosomal peptide synthetase (NRPS), NRPS-bacteriocin, Type III polyketide synthase (T3PKS), T3PKS-NRPS, T3PKS-terpene-butyrolactone, and terpene-butyrolactone-NRPS.

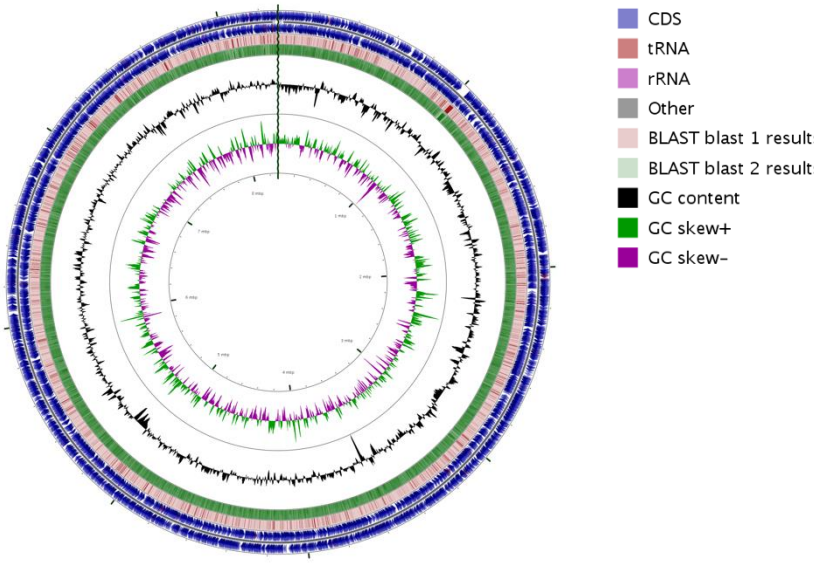


Fig. 2-4 Linear genome map of *Streptomyces* sp. GKU 895. The genome-map was drawn using CGView Server.

Table 2-3 Plant growth related genes of *Streptomyces* sp. GKU 895 annotated by RAST

Contig ID	Start	Stop	Strand	Gene function
ACC deaminase producing gene				
NODE_122_length_28852_cov_12.0526	13127	12111	-	1-aminocyclopropane-1-carboxylate deaminase
NODE_39_length_66281_cov_16.9454	62411	61509	-	1-aminocyclopropane-1-carboxylate deaminase
Chitinase gene				
NODE_118_length_30645_cov_13.4124	9045	10760	+	Chitinase
NODE_12_length_126731_cov_12.8486	64820	66505	+	Chitinase
NODE_14_length_120079_cov_16.8604	13227	11533	-	Chitinase
NODE_23_length_95968_cov_11.2574	65178	63943	-	Chitinase
NODE_25_length_90086_cov_12.2972	61412	60327	-	Chitinase
NODE_31_length_79858_cov_11.56	25814	24087	-	Chitinase
NODE_31_length_79858_cov_11.56	29596	30726	+	Chitinase
NODE_59_length_54037_cov_13.4319	33298	32333	-	Chitinase
NODE_59_length_54037_cov_13.4319	34593	33352	-	Chitinase
NODE_59_length_54037_cov_13.4319	37441	34712	-	Chitinase
NODE_86_length_41997_cov_17.4605	13784	15613	+	Chitinase
NODE_10_length_140474_cov_11.164	72673	73788	+	Chitinase 19-1
IAA producing gene				
NODE_31_length_79858_cov_11.56	47675	49042	+	Indoleacetamide hydrolase
NODE_31_length_79858_cov_11.56	49063	50073	+	Nitrilase
NODE_14_length_120079_cov_16.8604	64916	64326	-	nitroreductase
NODE_8_length_145384_cov_28.3855	89174	88584	-	nitroreductase
Phosphate solubilising gene				
NODE_10_length_140474_cov_11.164	45922	48144	+	Isocitrate dehydrogenase
NODE_101_length_35684_cov_16.4872	4034	5323	+	Citrate synthase (si)
NODE_32_length_79367_cov_13.7918	52926	51760	-	Citrate synthase (si)
NODE_32_length_79367_cov_13.7918	53025	54284	+	Citrate synthase (si)
NODE_66_length_48370_cov_15.0399	29179	27998	-	Citrate synthase (si)
NODE_152_length_21071_cov_11.8311	12554	11955	-	Endonuclease/exonuclease/ phosphatase
NODE_152_length_21071_cov_11.8311	12832	12566	-	Endonuclease/exonuclease/ phosphatase
NODE_24_length_92104_cov_13.9498	2037	1084	-	Endonuclease/exonuclease/ phosphatase

Plant-colonizing gene				
NODE_13_length_123875_cov_10.7579	50790	49222	-	Salicylate hydroxylase
NODE_1_length_213532_cov_11.9484	39690	37888	-	Salicylate hydroxylase
NODE_87_length_41498_cov_11.689	19632	18817	-	Salicylate hydroxylase
Siderophore producing gene				
NODE_3_length_175223_cov_13.0625	153208	151766	-	Desferrioxamine E biosynthesis protein DesA, siderophore biosynthesis
NODE_3_length_175223_cov_13.0625	151782	150493	-	Desferrioxamine E biosynthesis protein DesB, siderophore biosynthesis protein, monooxygenase
NODE_3_length_175223_cov_13.0625	150496	149957	-	Desferrioxamine E biosynthesis protein DesC, siderophore synthetase small component
NODE_3_length_175223_cov_13.0625	149960	148188	-	Desferrioxamine E biosynthesis protein DesD, siderophore synthetase superfamily
NODE_3_length_175223_cov_13.0625	151782	150493	-	Desferrioxamine E biosynthesis protein DesB, siderophore biosynthesis protein, monooxygenase
NODE_3_length_175223_cov_13.0625	150496	149957	-	Desferrioxamine E biosynthesis protein DesC, siderophore synthetase small component
NODE_3_length_175223_cov_13.0625	149960	148188	-	Desferrioxamine E biosynthesis protein DesD, siderophore synthetase superfamily, group C, Siderophore synthetase component, ligase
Stress responsive genes				
NODE_131_length_26606_cov_13.2922	16728	17288	+	Betaine aldehyde dehydrogenase
NODE_131_length_26606_cov_13.2922	17315	17626	+	Betaine aldehyde dehydrogenase

NODE_131_length_26606_cov_13.2922	17641	18225	+	Betaine aldehyde dehydrogenase
NODE_182_length_13169_cov_11.0962	1126	2595	+	Betaine aldehyde dehydrogenase
NODE_77_length_45423_cov_14.0625	44030	43146	-	Proline dehydrogenase (Proline oxidase)
NODE_77_length_45423_cov_14.0625	44030	43146	-	Proline dehydrogenase (Proline oxidase)
NODE_18_length_103295_cov_14.2372	97854	97207	-	superoxide dismutase [Fe-Zn]
NODE_18_length_103295_cov_14.2372	97854	97207	-	superoxide dismutase [Fe-Zn]
NODE_2_length_185022_cov_17.4582	125281	123581	-	Trehalose synthase

Table 2-4 Secondary metabolites gene clusters of *Streptomyces* sp. GKU 895 predicted by Anti-SMASH

Type	**Cluster**	**Most similar known** (% of genes similarity)	**MIBiG BGC-ID**
Bacteriocin	12	Informatipeptin (57%)	BGC0000518 c1
Bacteriocin	28		
Bacteriocin-NRPS	13	Teicoplanin (9%)	BGC0000440 c1
Butyrolactone	1		
Butyrolactone	4	Lactonamycin (8%)	BGC0000238 c1
Butyrolactone	19		
Butyrolactone	22	Rabelomycin (6%)	BGC0000262 c1
Butyrolactone	25		
Butyrolactone	26	Marineosin (9%)	BGC0000091 c1
Butyrolactone-T3PKS-Terpene	5	Merochlorin (43%)	BGC0001083 c1
Butyrolactone-Terpene-NRPS	14	Carotenoid (45%)	BGC0000633 c1
Ectoine	7	Ectoine (100%)	BGC0000853 c1
Ladderane-OtherKS	23	A54145 (3%)	BGC0000291 c1
Melanin	9	Melanin (80%)	BGC0000909 c1
Melanin	27	Melanin (71%)	BGC0000908 c1
NRPS	24	Meilingmycin (2%)	BGC0000093 c1
Other	10	Pristinamycin (2%)	BGC0000952 c3
Other	15		
Siderophore	2	Desferrioxamine (100%)	BGC0000940 c1
Siderophore	11	Scabichelin (20%)	BGC0000423 c1

Siderophore	20		
T2PKS	6	Auricin (27%)	BGC0000201 c1
T2PKS	21	Spore pigment (75%)	BGC0000271 c1

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Section 3

Sugarcane growth enhancement and salt tolerance affected by ACC-producing endophytic diazotrophic *Enterobacter* sp. EN-21

Introduction

Salinity stress is a major factor limiting the productivity of crop plants by high concentrations of salts in the soil. Over 20% of total irrigated land has been injured by high salinity and nearly 800 million hectares (more than 6%) of the world's total land throughout the world are salt affected (Pitman and Läuchli, 2002; Rana and Mark, 2008). In Thailand, there are 2.3 million hectares of total area were salt-affected soils that influences to lowing yield of commercial crop plantation such as maize, rice and sugarcane plants (Arunin and Pongwichian, 2015). Sugarcane (*Saccharum* sp.) is a major sugar producing crop in the tropical and subtropical regions and is economically important in Thailand as a sugar and bioethanol production. Sugarcane plants have been classified to glycophytes which are susceptible to high salt concentration that exerts plants to reduce growth, leading to low productivity, particularly sugar content (Gomathi and Thandapani, 2005; Hussain et al., 2004).

Plants display a severally responses to biotic and abiotic stress that enable them to tolerate severe conditions involving physiological, and biochemical processes (Mahajan and Tuteja, 2005). In salt stress, two main physiological mechanisms were responded by plants. Firstly, osmotic, which drastically alters water balance of the plants when salt concentration around the root plants and second, ionic, which causes toxic effects by excess ion unbalance in plant cells (Rana and Mark, 2008). Moreover, high salinity causes both physiological and metabolic changes in plants. For examples, tomato and Arabidopsis treated with 200 mM NaCl displayed decreased plant biomass (Kangmin et al., 2014), sorghum plants (Singh and Jha, 2016) and white clover (Han et al., 2014) significantly increased osmoprotectant molecule (proline) and lipid peroxidation (malondialdehyde, MDA) under salinity treatment.

The salt stress on plants can be mitigated by decreased accumulation of proline, lowering oxidative stress (lipid peroxidation), sodium (Na) contents and ethylene emission. Decreased plant metabolic changes and enhanced plants growth have been documented in plant inoculated with plant growth-promoting bacterial endophytes (PGPE). Arrange of ACC deaminase-producing bacterial endophytes such as *Bacillus*, *Enterobacter* and *Pseudomonas* have showed beneficial interaction with plants under severe stresses (Ali et al., 2014; Kangmin et al., 2014; Nadeem et al., 2009; Yaish et al., 2015). Hence, treated plants with ACC-producing bacteria could be promoted

growth and tolerated to stressed environments by conversion of ACC to ammonia and α-ketobutyrate, which bacteria can consume and consequently lower the ethylene level in plants (Glick, 2005) that mentioned in previously reports in tomato (Ali et al., 2014; Kangmin et al., 2014), Arabidopsis (Kangmin et al., 2014) and red pepper (Siddikee et al., 2011).

PGPE are bacteria that inhabit inside plant tissues and promote growth of plants. The capability to colonize root plants and has been considered the major characteristic of endophytes that exert a beneficial effect for crop yield enhancement (Santoyo et al., 2016). PGPE can encourage plant growth through the release of phytohormones, increment of nutrients, and protected from the critical stress environments (Madhaiyan et al., 2013; Mayak et al., 2004). Previously report showed that sugarcane plants treated with plant growth-promoting endophytic diazotrophic *Enterobacter* sp. EN-21 promotes growth under greenhouse experiment plants but have not yet been determined the colonized patterns to host plants (Kruasuwan and Thamchaipenet, 2016). Therefore, a better understanding of the endophytic diazotrophic EN-21 colonization patterns is a crucial prerequisite for the development of effective ways to distribute the biofertilizer the field.

In the present study, ACC deaminase-producing endophytic diazotrophic *Enterobacter* sp. EN-21 was inoculated to sugarcane plants under salinity condition to study their efficiency in enhancing growth and salt stress tolerance. Moreover, colonized of *gfp*-tagged endophytic diazotrophic *Enterobacter* sp. EN-21 on roots of sugarcane plants were determined.

Materials and Methods

Bacterial growth conditions

Endophytic diazotrophic *Enterobacter* sp. EN-21 previously isolated from root of sugarcane plants was streaked on NA and incubated at 37 °C for 24 h. Strain EN-21 was collected from NA, transferred into Tryptic Soybean Broth (TSB) and incubated at 37 °C with 7,500 × g for 10 min to yield 10^8 CFU mL^{-1} determined by plate counting.

Qualification of ACC deaminase activity

Enterobacter sp. EN-21 was quantified the ACC deaminase activity by monitoring the amount of α-ketobutyric acid generated from the cleavage of ACC as described by Penrose and Glick (2003). The EN-21 was cultured first in TSB (rich medium) and then twice washed with DF salt minimal medium and transferred the same medium supplemented with 3 mM ACC (Sigma–Aldrich, USA) as a sole source of nitrogen to induce the ACC deaminase activity. The amount of

α-ketobutyrate was determined by comparing the absorbance at 540 nm of the sample to a standard curve of α-ketobutyrate ranging between 0.02 and 0.2 μmol. Protein content was performed as described by Bradford (1976). The ACC deaminase activity was estimated as the amount of α-ketobutyrate produced in nmol mg^{-1} protein h^{-1}.

Sugarcane growth promotion under salt stress condition

One-month of sugarcane tissue cultures (*Saccharum officinarum* cv. LK92-11) were obtained from the Plant Tissue Culture Unit at Central Laboratory and Greenhouse Complex, Kasetsart University (Kamphaengsaen Campus). Sugarcane plantlets were prepared and cultivated according to Kruasuwan and Thamchaipenet (2016). Briefly, sugarcane plants were inoculated with cell suspensions of EN-21 (10^8 CFU mL^{-1}) by the root dip method in a tissue culture bottle containing *Murashige and Skoog (MS0) medium (Murashige and Skoog, 1962)* and immersed at room temperature (RT) for 24 h. Inoculated sugarcane plants were transferred to cleaned mini-pot (6 × 5.5 cm) containing sterilized sand and hydroponic materials (sand:perlite:vermiculite, 2:2:1). The experiment was set up in completely randomized design (CRD) using five replicate mini-pots per treatment and each treatment was replicated twice. Plants were maintained in the greenhouse and supplied with sterilized sucrose-free MS0 broth twice a day for 30 days. At 30 day after inoculation, salts stress treatments were irrigated using sterilized distilled water containing 200 mM NaCl for 7 days in the same condition as previously described.

Plant growth parameters evaluation

Sugarcane plants at 7 days after irrigation (DAI) were measured plant growth parameters. Plant biomass production was determined as total plant lengths, dry and fresh weights. Salt tolereant index (STI) was calculated as formula described by Baha and Bekki (2015).

Proline accumulation analysis

Sugarcane leaf samples (50 mg) were snap-frozen and homogenized immediately with liquid nitrogen. The sample powder was mixed with 3% (v/v) sulfosalicylic acid followed by centrifuged at 14,000 × g for 10 min. The supernatant was reacted with ninhydrin solution (1.25 g ninhydrin powder in 30 mL glacial acetic acid and 20 mL 6 M phospholic acid) and glacial acetic acid for 1 h at 95 °C and cool on ice. The reaction mixture was extracted and mixed vigorously with toluene for 15-20 sec. Red-color organic layer was separated and added to a cuvette for absorbance measurement of the ninhydrin-proline complex at 520 nm by spectrophotometry. The

proline concentration was determined from a standard curve of commercial proline and calculated using the formula as described by Bates et al. (1973).

Lipid peroxidase (MDA content) determination

Lipid peroxidation of plant membrane lipids was estimated by measuring the malondialdehyde (MDA) content obtained by the thiobarbituric acid (TBA) reaction. Fresh leaf samples (50 mg) were homogenized immediately with liquid nitrogen and mixed with 80% (v/v) ethanol followed by centrifuged at 14,000 × g for 10 min. The supernatant was reacted with either (i) with TBA solution (20% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene), or (ii) without TBA solution (containing the above plus 0.65% TBA). Samples were then mixed vigorously and heated for 1 h at 95 °C, cooled on ice and centrifuged at 14,000 × g for 5 min. Absorbance at 400, 523 and 600 nm were used for measuring the TBA-MDA complex in plants. The MDA level was calculated by the formula as described by Hodges et al. (1999).

Estimation of total chlorophyll contents

Total chlorophyll was extracted according to the method of Porra et al. (1989). Sugarcane leaf samples (50 mg) were homogenized immediately with liquid nitrogen and crushed in 80% (v/v) acetone, subsequently centrifuge at 9000 × g for 10 min at 4 °C. The absorbance at 645 and 663 nm were measured by spectrophotometry. Total chlorophyll content of collected supernatants was estimated based on chlorophyll equations of Arnon (1949).

Ethylene emission analysis

Ethylene emission was analyzed by the method of Madhaiyan et al. (2007). Sugarcane plants collected from the greenhouse were placed in 250 mL bottom with a rubber septum and sealed with a plastic paraffin film followed by 4 h inoculation. One milliliter of headspace air was sampled and analyzed for ethylene by gas chromatography (GC 7890A, Agilent Technologies, USA) packed with Poropak-N column at 60 °C, equipped with a flame ionization detector. The amount of ethylene emission was calculated as nmol of ethylene g^{-1} FW h^{-1} by compared to a standard curve generated with pure ethylene.

Total-K and -Na measurements

Total-K and -Na contents were measured from root and shoot of 5 DAI sugarcane plants. Plant samples were dried at 60 °C and ground for nutrient analysis. The ground samples were

analyzed by Soil-Fertilizer-Environment Scientific Development Project, Department of Soil Science, Faculty of Agriculture, Kasetsart University. The concentration of Na and K were respectively quantified and calculated as mg g^{-1} DW.

Re-colonization of sugarcane roots by endophytic diazotroph EN-21

A competent cell of endophytic diazotrophic *Enterobacter* sp. EN-21 was prepared followed by the method of Chung et al. (1989). pBZ1 plasmid (pRK404 containing *Hin*dIII/*Eco*RI fragment (*gfp*) from pEGFP) (Noisangiam et al., 2012) was transformed into competent cells by electroporation technique in a 100 µL cuvette using 1.8 kV, a capacitance of 25 µF and a resistance of 200 µΩ. To select the *gfp*-transformed cells, cellular suspension was plated in low-salt LA medium supplemented with 30 mg mL^{-1} tetracycline. Plasmid extracted from *gfp*-transformed cells was digested with *Hin*dIII/*Eco*RI to confirm the presence of *gfp* fragment (750 bp). Positive strains of *gfp*-transformed cells were confirmed by observing under fluorescent microscopy to visualize the expression of green fluorescent protein.

One-month tissue culture of sugarcane plants (cv. LK92-11) were inoculated with cell suspension of *gfp*-tagged *Enterobacter* sp. EN-21 (10^8 CFU mL^{-1}) by root-dip method for overnight. The inoculated plants were cultivated as previously described for 7 days. At successive time (24 h and 7 days after inoculation), inoculated and uninoculated sugarcane roots were carefully washed with sterile distilled water, cut into small pieces by hand and embedded on a bridge slide with 0.25% (w/v) agarose gel as describe by Lin et al. (2012) and analyzed by confocal laser scanning microscope (CLSM, Nikon C2, USA). The excitation at 470-495 nm and emission at 515-565 nm were used to visualize bacterial cells and to reveal structure of the plant tissues, respectively.

Results

ACC-producing endophytic diazotrophic *Enterobacter* sp. EN-21

ACC deaminase activity was measured by growing *Enterobacter* sp. EN-21 in DF salt minimal medium and monitoring the amount of α-ketobutyric acid generated from the cleavage of ACC. Strain EN-21 was better able to grown in DF salt minimal medium supplemented with 3 mM ACC than DF medium (data not shown). The ACC deaminase activity of strain EN-21 was quantified by measuring the amount of α-ketobutyrate that exhibited as 102.47 nmol α-ketobutyrate mg^{-1} protein h^{-1}.

Sugarcane growth promotion affected by *Enterobacter sp.* EN-21 under salt stress condition

Alleviation of the salt stress of sugarcane plants by inoculation of ACC-producing *Enterobacter* sp. EN-21 was investigated under greenhouse condition. Growth parameters of inoculated and uninoculated sugarcane plants were evaluated at 7 DAI with and without addition 200 mM NaCl. Compared to uninoculated plants, treated plants with strain EN-21 gave significantly enhancement of total plant lengths and fresh weights in both non-salt stressed and salt stressed conditions (Fig. 3-1A and C). In salt stressed condition, uninoculated plants significantly reduced total plant lengths (9.31%), dry weights (23.30%) and fresh weights (22.25%), respectively. Nevertheless, all of plant parameters were statistically recovered when plants treated with *Enterobacter* sp. EN-21 (Fig. 3-1). Additionally, the calculation of STI revealed that plant inoculated with strain EN-21 (81.10%) appear to be more tolerant to salt than uninoculated plants (68.70%).

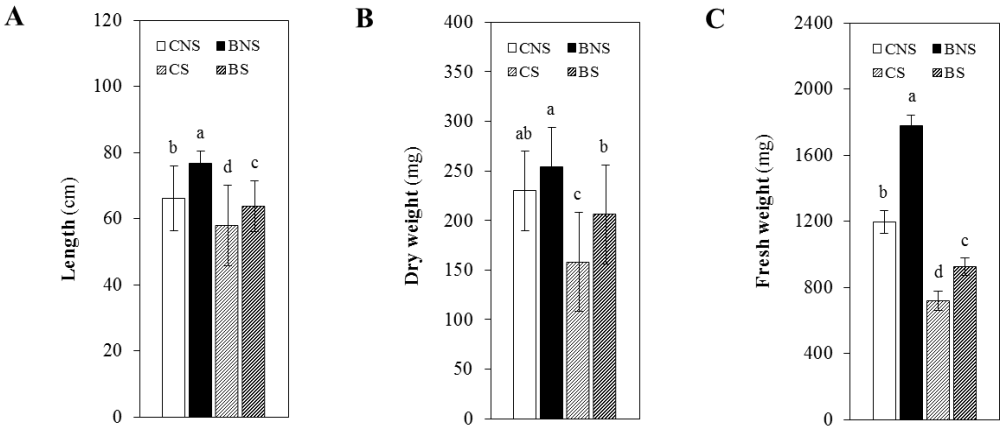

Fig. 3-1 Effect of *Enterobacter* sp. EN-21 on length (A), dry weight (B) and fresh weight (C) of sugarcane plants. Data are mean ± standard deviation of five plants per treatment with two different experiments. The same letter does not differ significantly at $p \leq 0.05$. CNS, non-salt stressed control plants; BNS, non-salt stressed inoculated plants; CS, salt stressed control plants and BS, salt stressed inoculated plants

Effect of *Enterobacter* sp. EN-21 on biochemical markers

At 7 DAI, proline and MDA accumulations were not significantly changed in *Enterobacter* sp. EN-21 inoculated plants compared to uninoculated plants under non-salt stressed conditions (Fig. 3-2). Salt stress was induced proline and MDA contents in uninoculated plants which were highly recorded at 769.74 ng g^{-1} FW and 26.44 nmol g^{-1} FW. However, treated plants with EN-21 were decreased the level of proline by 27.27% and significantly declined the MDA level by

32.45% (Fig. 3-2A and B). Total chlorophyll content of inoculated plants was not significantly altered in both non-salt stressed and salt stressed condition. Conversely, compared to corresponding to *Enterobacter* sp. EN-21 treated plants, uninoculated plants was reduced total chlorophyll content by 15.20% under salt stressed condition (Fig. 3-2C). Treated plants with strain EN-21 induce ethylene level in non-salt stressed plants and significantly augmented in salt-stressed uninoculated plants (89.24±1.70 nmol g^{-1} FW h^{-1}). Nevertheless, the emission of ethylene by plants was declined by 26.61% in salt stress inoculated sugarcane plants (Fig. 3-2D).

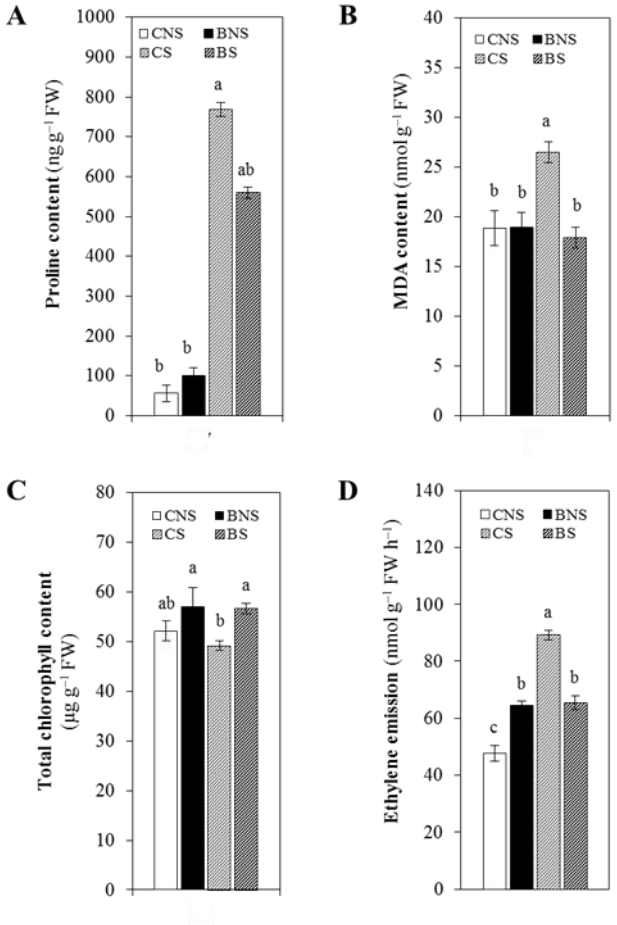

Fig. 3-2 Effect of *Enterobacter* sp. EN-21 on proline (A), MDA (B), total chlorophyll contents (C) and ethylene emission (D) of sugarcane plants. Data are mean ± standard deviation of five plants per treatment with two different experiments. The same letter does not differ significantly at $p \leq 0.05$. CNS, non-salt stressed control plants; BNS, non-salt stressed inoculated plants; CS, salt stressed control plants and BS, salt stressed inoculated plants

Na^+ content was significantly enhanced in salt stressed sugarcane plants by 13-fold when compared to the non-salt treated control (Fig. 3-3A). The results demonstrated that *Enterobacter* sp. EN-21 inoculated plants showed about 3-fold of Na^+ content lower than that of salt stressed uninoculated sugarcane (Fig. 3-3A). On the contrary, K^+ content drastically declined by 1.6-fold in salt stressed plants when compared to the non-salt treated control (Fig. 3-3B). Markedly, plants inoculated with *Enterobacter* sp. EN-21 revealed highly significant increment of K^+ content by 2.1-fold when compared to the salt stressed uninoculated plants (Fig. 3-3B).

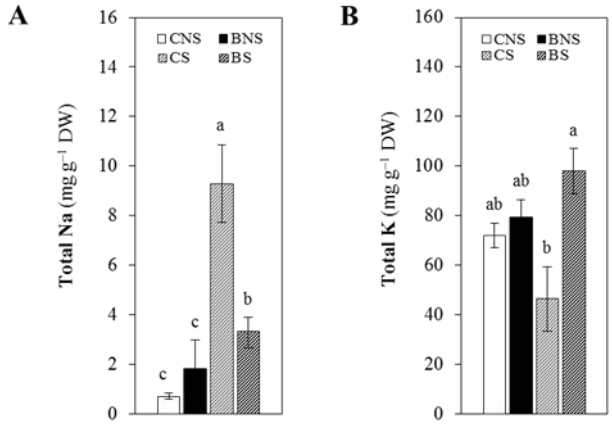

Fig. 3-3 Effect of *Enterobacter* sp. EN-21 on total-Na (A) and total-K (B) contents of sugarcane plants. Data are mean ± standard deviation of five plants per treatment with two different experiments. The same letter does not differ significantly at $p \leq 0.05$. CNS, non-salt stressed control plants; BNS, non-salt stressed inoculated plants; CS, salt stressed control plants and BS, salt stressed inoculated plants

Sugarcane root colonisation of gfp-tagged *Enterobacter* sp. EN-21

ACC-producing *Enterobacter* sp. EN-21 was tagged with *gfp* gene in pBZ1. The EN-21 harboring *gfp* gene in pBZ1 presented green-color colony on low-salt LA medium supplemented with 30 mg mL^{-1} tetracycline. Moreover, the *gfp*-tagged EN-21 showed 750 bp fragment of *gfp* gene after digested with *Hin*dIII/*Eco*RI which confirmed successfully tagged with pBZ1 (data not shown). Then, its expression was visualized by florescence microscopy that observed a strongly green fluorescence in the transformants following exposition to fluorescent light while the wild-type strain did not fluoresce (Fig. 3-4A). At 24 h after inoculation, green fluorescent *gfp*-tagged *Enterobacter* sp. EN-21 cells individually or in groups were primarily found to attach to root border cells surrounding root cap and gathered at the root junctions between root epidermal cells and lateral root junction zones (Fig. 3-4B-D). CLSM analysis found that individual bacterial cells

colonized in intercellular spaces of root sugarcane plants at 7 DAI (Fig. 3-4E-F). In control materials, non-fluorescent wild-type cells were also observed to colonize on root surfaces and in intercellular spaces of sugarcane roots (data not shown).

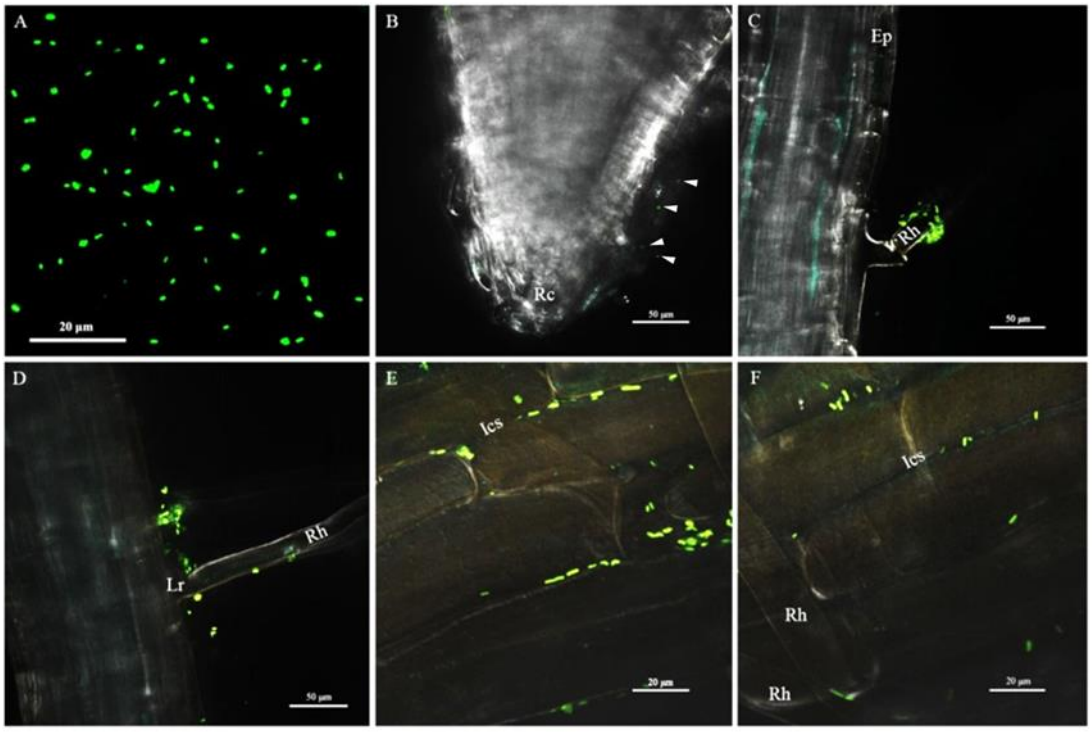

Fig. 3-4 Microscopic observation of *gfp*-tagged *Enterobacter* sp. EN-21 under fluorescence microscopy, scale bar, 20 µm (A), colonization of sugarcane roots by *gfp*-tagged *Enterobacter* sp. EN-21 cells at 24 h (B-D) and 7 days (E-F). CLSM images present bacterial GFP fluorescence. (B) individual bacterial cells colonized at an emergence site by a root border cells surrounding root cap (Rc). (C) group of bacterial cells colonized at the root hairs (Rh) (D) and at the lateral root junctions (Lr). (E-F) longitudinal hand-sections of sugarcane roots colonized by *gfp*-tagged EN-21 at the intercellular spaces (Ics), scale bar of A-C, 50 µm and E-F, 20 µm.

Discussion

Salinity is a significant problem affecting agriculture worldwide which reduces crop yields and agricultural productivity and is predicted to become a larger problem in the coming decades. PGPE can encourage plant growth through the direct and indirect mechanisms such as nitrogen fixation, production of plant hormones and reduction of biotic and abiotic stresses by ACC deaminase producing. To investigate the effect of endophytic diazotroph on sugarcane growth promotion and salt stress tolerance. In this study, we decided to test the plant growth-promoting

endophytic diazotrophic *Enterobacter* sp. EN-21, previously successfully employed to facilitate sugarcane growth under individual and co-inoculation with endophytic actinomycetes, for its ability to promote plant growth in the presence of salt. Endophytic diazotrophic *Enterobacter* sp. EN-21 was originally isolated from root of sugarcane plants that closely related to *Enterobacter asburiae* JCM6051^{T} (99% similarity) and severally produced PGP agents such as indole-3-acetic (IAA) production, siderophore production, nitrogen fixation, in addition to ACC deaminase activity (Kruasuwan and Thamchaipenet, 2016). A preliminary screening of salt stress tolerant in sugarcane plants found that the plants were tolerated to salt stress (200 mM NaCl) up to 7 DAI (data not shown). Therefore, in this study, sugarcane plants were measured in term of plant growth parameters and biochemical markers at 7 DAI.

Although *Enterobacter asburiae* firstly isolated from clinical specimens (Brenner et al., 1986), however, the species have also been reported to be associated with plants as endophytes (Asis and Adachi, 2004; Kruasuwan and Thamchaipenet, 2016; Yaish, 2016). In this study, treated sugarcane plants with *Enterobacter* sp. EN-21 significantly increase plant lengths, dry and fresh weights after salt affected plants at 7 DAI (Fig. 3-1) and increase the STI when compared to uninoculated plants. Sugarcane growth promotion and salt stress tolerance driven by strain *Enterobacter* sp. EN-21 might be due to ACC deaminase activity of the bacterium. Our results are in agreement with the previous report for salt tolerance in Arabidopsis, maize and tomato plants induced by PGPE (Ali et al., 2014; Kangmin et al., 2014; Nadeem et al., 2009). These results demonstrate that ACC deaminase-producing *Enterobacter* sp. EN-21 could be protected sugarcane plants and facilitated their growth under salt stress condition.

In response to salt stress, plant activates various kinds of physiological and biochemical processes. In this study, we study the effect of bacterial ACC deaminase to improve plant growth inhibition caused by salt stress through decreased metabolic changes in plants. Proline and MDA are important molecules for osmotic regulation and membrane lipid peroxidation under biotic and abiotic stresses. High-salinity stress inhibits plant water uptake, causes ionic toxicity, osmotic stress and oxidative damage (Rana and Mark, 2008). Therefore, accumulation of osmoprotectants such as proline and MDA, which decreases osmotic stress and enable plants to tolerate to salt stress condition (Pottosin et al., 2014). Wheat plants inoculated with ACC-producing *Enterobacter* sp. SBP-6 and white clove treated with *Bacillus subtilis* GB03 caused a significant reduction in the proline and MDA contents as compared to control plant treated with salt stress (Singh and Jha, 2016; Han et al., 2014). Similarity, inoculated with strain ACC-producing *Enterobacter* sp. EN-

21 resulted in decreased proline and MDA levels as a consequence of salt stress in sugarcane plants which are a reflection of a lower severity of salt-induced stress in these plants.

Salinity also adversely affects plant growth by high internal Na accumulation which is common plant response to salinity condition. Salt stress increased total-Na content in uninoculated plants, however, inoculation with *Enterobacter* sp. EN-21 showed 64.59% reduction (Fig. 3-3A). The high salt load in the uninoculated plants may be due to changes in gene expression (such as the sodium transporters HKT1 and SOS1) (Shabala and Cuin, 2008; Shi et al., 2002) and trigger salt toxicity in the plants (Rana and Mark, 2008) that resulted in the death of leaves and reduce the total photosynthetic leaf area (data not shown). Besides the positive effects of *Enterobacter* sp. EN-21 on the Na balance, the bacterium also enhanced the uptake of K in plants. Similarity to previously reports, maize plant inoculated with ACC deaminase-producing *Pseudomonas fluorescens* and pea plants treated with ACC deaminase-containing rhizobacterium *Variovorax paradoxus* 5C-2 noticed to regulate of nutritional balance, ion homeostasis and photosynthesis which was more uptake K and gave high K^+/Na^+ ratio when compared to salt stressed uninoculated plants (Nadeem et al., 2009; Wang et al., 2016). Therefore, enhancing the uptake of K and decreasing of Na in treated plant tissues may be one of the mechanisms for ion homoeostasis and photosynthesis.

Reduction of crop yield was observed in various plant species exposed to salinity stresses which was linked to the decline in photosynthesis such as tomato (Ali et al., 2014), cucumber (Kang et al., 2015) and rice (Turan and Tripathy, 2015). Our results indicated significantly decreases in total chlorophyll contents under salt stress in uninoculated plants. Reduction of total chlorophyll contents in sugarcane plants was affected by the inhibition of chlorophyll synthesis, together with the activation of its degradation by the enzyme chlorophyllase (Santos, 2004). On the other hand, ACC-producing *Enterobacter* sp. EN-21 treated sugarcane plants grown in a saline environment were facilitated by the activation of total chlorophyll content (Fig. 3-2C).

Ethylene is an important plant growth regulator that functions in the stress signaling and also quickly stimulated by salinity and some other stresses (Tao et al., 2015). ACC-producing bacteria could be promoted growth and tolerated to stressed environments by conversion of ACC to ammonia and α-ketobutyrate, which bacteria can consume and consequently lower the ethylene level in plants (Glick, 2005) that mentioned in previously reports in tomato (Ali et al., 2014; Kangmin et al., 2014), Arabidopsis (Kangmin et al., 2014) and red pepper (Siddikee et al., 2011). Similarity, the present study revealed that plant ethylene level was increased in salt stress uninoculated plants and inoculated plants with ACC deaminase-producing bacteria reduced the

ethylene emission by 26.61%. It was suggested that ACC deamiase-producing *Enterobacter* sp. EN-21 promote growth and alleviate ethylene stress from salt toxicity by bacterial ACC deaminase activity.

Sugarcane roots colonized by ACC deaminase-producing endophytic diazotrophic *Enterobacter* sp. EN-21 was similar to that of other endophytes described so far. For examples, *Pseudomonas* spp. was predominantly detected along the whole root hairs that involves invasion of root hair cells (Prieto et al., 2011). *gfp*-tagged endophytic nitrogen-fixing *Klebsiella variicola* DX120E colonized at elongation zones of root maturation and entered roots by cracks at the emergence site of lateral roots and at disrupted epidermis (Wei et al., 2014) which was similar to sugarcane plants inoculated with *Microbacterium* sp.16SH that entered roots via cracks at the lateral root junctions and penetration of epidermal cells (Lin et al., 2012). Sugarcane root-associated *Enterobacter* sp. strain 35-1was observed to colonize the intercellular spaces and the junctions of the lateral roots with the parent root of *Brassica oleracea* (Zakria et al., 2008a) and rice plants (Zakria et al., 2008b). In this study found that *Enterobacter* sp. EN-21was found in the root caps (Rc) and forms microcolony in an extracellular matrix cemented to the root hairs (Rh) at early interaction (24 h) (Fig 3-4B-C). The most significant colonization feature of the *gfp*-tagged *Enterobacter* sp. EN-21 cells is locally restricted aggregates on the root hair surfaces and lateral root junctions (Fig 3-4C-D). At 7 DAI found that the *Enterobacter* sp. EN-21 cells were penetrated into intercellular spaces (Ics) of epidermal cells by cracked at lateral root junction (Fig 3-4E-F). Invasion of root tissues might be affected of *in vitro* endoglucanase secretion (Compant et al., 2005; Madhaiyan et al., 2013). Our study clearly showed that strain EN-21 colonized the roots of sugarcane plants and its modes of action must be truly endophytes.

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Section 4

Molecular interaction of ACC deaminase-producing endophytic *Streptomyces* sp. GMKU 336 towards salt-stress resistance of *Oryza sativa* L. cv. KDML105

Introduction

Salinity is one of the major environment stress factors that reduces plant cell division, growth and productivity. Recently, plant growth-promoting (PGP) bacteria have been identified that enhance tolerance to salinity by plants, particularly bacteria associated with the plants[1]. Endophytic actinomycetes are of special interest because they not only produce various bioactive secondary metabolites to protect plants from infectious diseases[2], but they also show ability to enhance plant growth by carrying several PGP traits including production of siderophores to capture iron, production of plant hormones such as auxins and cytokinins, solubilization of phosphate and other minerals to supply nutrients[3,4]. Moreover, they facilitate plant growth under stress caused by drought, heavy metals, flooding and high salt by reducing stress associated with ethylene via production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase[5,6].

Ethylene has long been recognized as a hormone that controls plant responses to growth limiting conditions. Under stress conditions, the ethylene level is increased via the ethylene pathway that transforms the precursor ACC into the final product, ethylene[1]. A diverse group of endophytic PGP bacteria is able to reduce plant ethylene levels by the action of ACC deaminase (ACCD). ACCD (encoded by the *acdS* gene) converts ACC in plants to ammonia and α-ketobutyrate, which the bacteria consume as nitrogen sources[7]. Therefore, ACCD-producing bacteria stimulate plant ACC efflux and subsequently decrease ACC concentration and ethylene production[1]. The consequence of this interaction is an increase in root/shoot elongation and protection of the plant from the inhibitory effects of ethylene[8]. Thus, plants associated with endophytic ACCD-producing bacteria become more resistant to stress.

So far, the role and interaction of ACCD-producing endophytic actinomycetes to promote plant growth under salt stress has been less studied. This research therefore focused on ACCD-producing endophytic *Streptomyces* sp. GMKU 336 and its ability to enhance the growth of rice under salt stress conditions. An ACCD-deficient mutant of *Streptomyces* sp. GMKU 336 was constructed and compared with the effect of growth promotion of rice with the wild type under salt stress *in situ*. This work demonstrated that strain GMKU 336 increases salt tolerance of salt-sensitive Thai jasmine rice Khao Dok Mali 105 cultivar (KDML105). Expression profiles of stress responsive genes of rice associated with strain GMKU 336 are demonstrated and the impact of the

interaction is discussed. Understanding of such interaction will lead to sustainably utilize ACCD-producing endophytic actinomycetes to enhance growth and salt tolerance in rice growing in saline soil.

Materials and Methods

Bacterial strain, identification and NaCl tolerance

Streptomyces sp. GMKU 336 was isolated from roots of a medicinal plant, *Clerodendrum serratum* (L.) Moon, collected from Khaohinson Royal Development Study Center, Chachoengsao Province, Thailand on starch casein agar (SCA)[9]. Its 16S rRNA gene was amplified and sequenced using primers listed in Table 1 (Genbank accession number KR870352). The sequence was analyzed and verified using EzTaxon-e database[56]. The NaCl tolerance of strain GMKU 336 was determined by growing on inorganic salt-starch agar (ISP-4) with addition of 1–12% (w/v) NaCl and incubated at 28±2 °C for 14–21 days.

Determination of phosphate solubilization, indole-3-acetic acid and siderophore production

Streptomyces sp. GMKU 336 was grown in tryptic soy broth (TSB) at 28±2 °C, 200 rpm for 5 days. The cell culture was dropped onto Pikovskaya agar[57] containing tricalcium phosphate and further incubated for 5 days. Presence of a clear zone indicates solubilization of phosphate.

Strain GMKU 336 was inoculated into TSB supplemented with tryptophan (500 μg mL^{-1}) at 28±2 °C, 200 rpm in the dark for 7 days. 1 mL supernatant was mixed with 2 mL Salkowski's reagent[58] and incubated for 30 min at room temperature. Development of a pink color indicates indole-3-acetic acid (IAA) production[58].

A YM agar plug of 5-day growth of strain GMKU 336 was placed on a chrome azurol S (CAS) agar[59] and incubated at 28±2 °C for 3 days. An orange halo zone indicates siderophore production.

Determination of ACC deaminase activity

Streptomyces sp. GMKU 336 was grown on mannitol soybean agar (MS) for 5 days and streaked on nitrogen-free minimal medium agar (MM), and MM agar supplemented with either 2 gL^{-1} $(NH_4)_2SO_4$ or 3 mM ACC (Sigma-Aldrich) and incubated at 28±2 °C in the dark for 7 days. Growth and sporulation on MM agar supplemented with ACC (MM-ACC) indicates ACC deaminase (ACCD) activity[4].

For quantitative determination of ACCD activity, strain GMKU 336 was grown in TSB at 28±2 °C, 200 rpm for 3 days. Cells were washed twice with 0.1 M Tris-HCl (pH 8.5) and resuspended in MM-ACC broth followed by incubation at 28±2 °C, 200 rpm for 3 days. Cells were collected, washed twice and resuspended in 0.1 M Tris-HCl (pH 8.5). Cells were lysed by sonication and ACCD activity in the supernatant was assayed[7]. An aliquot of 200 mL of supernatant was incubated with 50 mM ACC at 30 °C for 1 h. The enzyme reaction was then stopped by adding 1.8 mL of 0.56 M HCl and 0.3 mL of 0.1% (w/v) 2,4-dinitrophenylhydrazine (prepared in 2 M HCl solution) and incubated at 30 °C for 15 min. The colorimetric reaction was then stopped by adding 2 mL of 2 M NaOH and the absorbance at 540 nm was determined by comparing to a standard curve of α-ketobutyrate.

Construction of an ACCD-deficient mutant

Since *Streptomyces* sp. GMKU 336 was newly isolated and its genome has not yet been sequenced, partial ACCD gene (*acdS*) was obtained by PCR amplification using specific primers[9] and annealing temperature listed in Table 4-1. The primers were designed based on conserved amino acid regions[60] with minimal degeneracy[9]. The PCR product was cloned into a non-replicative vector, pIJ8671[61] to obtain pIJ8671/*acdS*. Next, pIJ8671/*acdS* was transformed into *E. coli* ET12567(pUZ8002)[62] to perform an intergeneric conjugation[63] with 24-h mycelium of strain GMKU 336. The mutants were selected by thiostrepton resistance (50 μg mL^{-1}) and screened for deficiency of ACCD activity. Insertional inactivation of *acdS* in mutant was verified by PCR amplification using specific primers listed in Table 1 of (i) thiostrepton resistance gene, (ii) partial *acdS* gene, and (iii) the absence of a 5.4-kb long PCR product (presence in pIJ8671/*acdS*) (Fig. 4-1).

Table 4-1 List of primers used in this study

Primers	Sequence (5'–3')	Description (gene)	T_a (°C)	Reference
926F	AAACTCAAAGGAATTGACGG	16S rDNA for sequencing		54
ATT026	TGGACTACCAGGGTATCTAATC			
1530R	AAGGAGATCCAGCCGCA	16S rDNA	55	55
STR1F	TCACGGAGAGTTTGATCCTG			
aco-F	GATAGCGTGTGTACCACAGCGACC	*ACO1*	64	71
aco-R	CACGGTACAGCACGCCGCAC			
acs-F	GCCGAATTCGATGGTGAGCCAAGTGGTCG	*ACS1*	57	72
acs-R	GAGCGCGTGGGGGTTCTTCT			

act-F	AGCTATCGTCCACAGGAA	*act1*	49	73
act-R	ACCGGAGCTAATCAGAGT			
Apr N-2	CCCCGGCGGTGTGCT	5.4 kb long amplicon	69	3
ATT012	TGACTGAGTTGGACACCATCGC			
ATT012	TGACTGAGTTGGACACCATCGC	*thio*^{r}	58	63
ATT013	CCTGTCGATCCTCTCGTGCAG			
ATT082F	CAGTCCAACCACACSCGSCAG	*acdS*	65	This work
ATT082R	GCCATSGACTTSCCCTCGTASAC			
BADH-For	TAGCTTCACATCCCCATGTG	*BADH1*	57	12
BADH_R	TAGACGAGAAGTAGCACTGC			This work
CATb_FW	ATGGATCCCTACAAGCATCG	*CATb*	57	12
CATb_R	GGCTCCCCTTGCATGAACGAC			This work
CuZn-SOD1F	TTAACAATGGTGAAGGCTG	*CuZn- SOD*	57	This work
CuZn-SOD1R	TGTAGTGTGGCCCAGTTGA			
EREBP-For	GGGCCCTCTCCAGAGATAAC	*EREBP1*	57	12
EREBP-Rev	TGCAGCTTCTTCAGCACTGT			
hrdBF	CCGAGTCTGTGATGGCGCTC	*hrdB*	62	70
hrdBR	TTGGTGGCGGTGCGCTTGAC			
MAPK-For	CGACATGATGACGGAGTACG	*MAPK5*	57	12
MAPK5_R	CGTCCTCGTTCCGTATG			This work
NHX-For	GCTAGATTTGAGCGGCATTC	*NHX1*	57	12
NHX1-R	TCAATATCCAATGCATCCATC			This work
OsCam1-1F	ACCGTGCATTGCCGTATTAG	*Cam1-1*	57	74
OsCam1-1R	GCAAGCCTTAACAGATTCAC			
salT-F	GGAATATGCCATTGGTCCAT	*salT*	55	11
salT-R	GTCTTGCAGTGGAATGCTGA			
SOS-For	TCTAGTCGTTGCCAGGCTTT	*SOS1*	55	12
SOS-Rev	TCATTGATCATGCTCCCGTA			This work

Inoculation of rice plants with *Streptomyces* sp. GMKU 336 and its deficient mutant

Healthy seeds of Thai jasmine rice Khao Dok Mali 105 cultivar (KDML105), *Oryza sativa* L. cv. KDML105 were surface sterilized by immersion in 70% (v/v) ethanol for 1 min, 1% (w/v) sodium hypochlorite for 10 min and washed six times with sterile distilled water before transferring to a sterile moist chamber and incubation at room temperature in the dark for 3 days. Rice seedlings were grown under artificial light with light intensity at 8,000 lux for 16 h daily at room temperature for 7 days. The roots of seedlings was cut to the same length and were then immersed in individual sterile glass beakers containing 10^8 spores mL^{-1} of *Streptomyces* sp. GMKU 336 or the mutant for 4 h. The seedlings were re-located to a moist sponge support for 1 day before transferring to a 20-L container filled with ½ Yoshida solution[64] for 7 days and replaced with Yoshida solution for 7 days. Next, the nutrient solution was changed to Yoshida solution supplemented with 150 mM NaCl for 7 days. The pH of the nutrient solution was maintained

between 5.0–5.5 throughout the growth period. A positive control of non-salt stressed rice was also grown under the same conditions without NaCl treatment.

Symptoms of salt toxicity were evaluated according to the standard evaluation system used at the International Rice Research Institute (IRRI)[10].

Endophytic streptomycetes was re-isolated by surface-sterilized protocol as previously described[9]. The final washed solution was examined to ensure that the surface plant materials were actually sterilized. Re-isolated colonies of endophytic streptomycetes were randomly selected for analysis by 16S rDNA gene sequencing. In addition, re-isolated ACCD-deficient mutants were further verified by (i) growth on MS supplemented with thiostrepton (50 μg mL^{-1}); (ii) deficiency of ACCD activity; and (iii) amplification of thiostrepton resistant gene.

Determination of chlorophyll and ethylene contents

Approximately 100 mg of leaf fresh weight was ground in liquid nitrogen and chlorophyll was extracted twice by adding 1.0 mL DMSO and then sonicated[65]. The chlorophyll content of the supernatants were measured at 645 and 663 nm within 20 min after the extraction.

Ethylene production was measured by enclosing the whole rice plants in a 250-mL sealed glass container containing 50 mL acetylene for 1 h. A 1 mL gas sample was withdrawn and quantified by gas chromatography at PGPR Biofertlizer and Aerated Compost Soil Microbiology Research Group, Soil Science Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

Determination of proline, ion, and relative water contents

250 mg fresh weight of the whole plantlets were ground in liquid nitrogen and mixed with 5 mL aqueous sulfosalicylic acid (3% w/v) and filtered through Whatman® No. 1. 1 mL of filtrate was mixed with an equal volume of glacial acetic acid and ninhydrin reagent (1.25 mg ninhydrin, 30 mL glacial acetic acid, and 20 mL 6 M H_3PO_4) and incubated for 1 h at 100 °C in boiling water. The reaction was terminated by placing the test tube in an ice bath. Next, the reaction mixture was vigorously mixed with 2 mL toluene. After warming at 25 °C, proline was measured at 520 nm[66].

Rice shoots were assayed for Na^+, K^+ and Ca^{2+} contents at the Department of Soil Science, Faculty Agriculture, Kasetsart University, Bangkok, Thailand.

The relative water content (RWC) of plant leaves was examined[67]. ~10 cm of leaf was cut off from the middle part of the youngest fully expanded leaf, weighed and placed in a tube. The tube was kept on ice and was filled with distilled water and kept in the dark at 4 °C overnight. The

leaf was blotted dry and weighed. The samples were then dried at 70 °C for 3 days and weighed. The RWC was calculated from each weigh[66].

ROS staining and estimation of lipid peroxidation

For detection of superoxide[68], rice leaves were immersed in 25 mL of nitrobluetrazolium (NBT) solution (0.5 μg mL^{-1} NBT in 10 mM phosphate buffer, pH 7.6) for 3 h in the dark. For detection of hydrogen peroxide[69], leaves were immersed in 25 mL 3,3'-diaminobenzidine (DAB) solution (1 μg mL^{-1} DAB in 50 mM Tris-acetate buffer, pH 5.0) for 8 h. After staining, both treatments were boiled in 95% (v/v) ethanol for 30 min. The leaves were then immersed in 40% glycerol for 16 h before color detection.

The amount of lipid peroxidation was determined by estimating malondialdehyde (MDA)[69]. Rice shoots were ground in 80% (v/v) ethanol (1 g fresh weight $25mL^{-1}$). 1 mL aliquots of samples were added with 1 mL of either (i) –TBA solution [20% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene], or (ii) +TBA solution containing –TBA solution plus 0.65% thiobarbituricacid (TBA) and mixed vigorously before heating at 95 °C for 25 min. Absorbance was determined at 440 nm, 532 nm, and 600 nm and MDA equivalents were calculated[69].

Streptomycete RNA extraction and semi qRT-PCR analysis

Streptomyces sp. GMKU 336 was grown in TSB at 28±2°C, 200 rpm for 3 days. Cells were washed twice with 0.1 M Tris-HCl (pH 8.5) and resuspended in MM-ACC broth followed by incubation at 28±2°C, 200 rpm for 3 days. Total RNA was extracted following the manufacturer's protocol of Trizol® Reagent (Invitrogen). cDNA was synthesized using the Thermo Scientific RevertAid First strand cDNA synthesis Kit (Thermo Scientific). Semi-quantitative RT-PCR analysis was performed using cDNA products, the corresponding primers listed in Supplementary Table S3, and Phusion® Hot Start II-High Fidelity DNA polymerase (Thermo Scientific). The expression level of each product was quantified by Gel Doc™ XR+ with Image Lab™ Software (Biorad) and normalized against the expression of a housekeeping gene, *hrdB*[70].

Rice RNA extraction and transcription analysis of genes involved in salt stress response by real-time PCR

Total RNA was extracted from shoots following the manufacturer's protocol for Trizol® Reagent (Invitrogen) and treated with DNase I (Thermo Scientific). cDNA was synthesized using the Thermo Scientific RevertAid First strand cDNA synthesis Kit (Thermo Scientific). KAPA

SYBR® FAST qPCR Master Mix (2X) (KAPA BIOSYSTEMS) was used for quantification in Master Cycler Realplex 4 (Eppendorf). The primers for real-time PCR are listed in Table 1. The mean value was calculated and normalized with actin (*act1*) as internal control.

Statistical analysis

All data from the experiments were calculated and statistically evaluated from biological and technical triplicates. The data were analyzed with one way analysis of variance (ANOVA) and Duncan's test to determine any significant differences between groups at $P<0.05$. All statistical analyses were performed using the SPSS 20.0 for Windows software (SPSS Inc.).

Results

Characterization of plant growth promoting traits of *Streptomyces* sp. GMKU 336

As part of a program to discover PGP endophytic actinomycetes from medicinal plants, *Streptomyces* sp. GMKU 336 was recovered from the roots of *Clerodendrum serratum* (L.) Moon[9]. The strain is most closely related to *Streptomyces hydrogenans* NBRC 13475^T, with 99.86% identity based on 16S rDNA gene sequence analysis (GenBank accession number KR870352). Screening of plant growth promoting (PGP) traits of strain GMKU 336 revealed characteristics of phosphate solubilization, siderophore production, 1-aminocyclopropane-1-carboxylate deaminase (ACCD) activity but no indole-3-acetic acid (IAA) production (data not shown). Strain GMKU 336 shows moderate halophilic type as it tolerates NaCl up to 6% (w/v).

Construction of ACCD-deficient mutant

Streptomyces sp. GMKU 336 displayed ACCD activity at 2.85±0.15 μmol α-ketobutyrate mg protein^{-1} h^{-1}. Furthermore, the expression profile of the ACCD gene (*acdS*) by semi-quantitative RT-PCR revealed high expression when bacteria were consuming ACC as a sole nitrogen source (data not shown). An ACCD-deficient mutant of strain GMKU 336 was then constructed by insertional inactivation of *acdS* (GenBank accession number KT000002). The mutant constructed showed no ACCD activity and the disruption was verified by PCR analysis (Fig. 4-1). The mutant was stable up to five generation of growth without thiostrepton selection. The mutant was reverted to wild type by further selection without the antibiotic up to ten generations. The revertant showed the same ACCD activity and all other properties as wild type (data not shown).

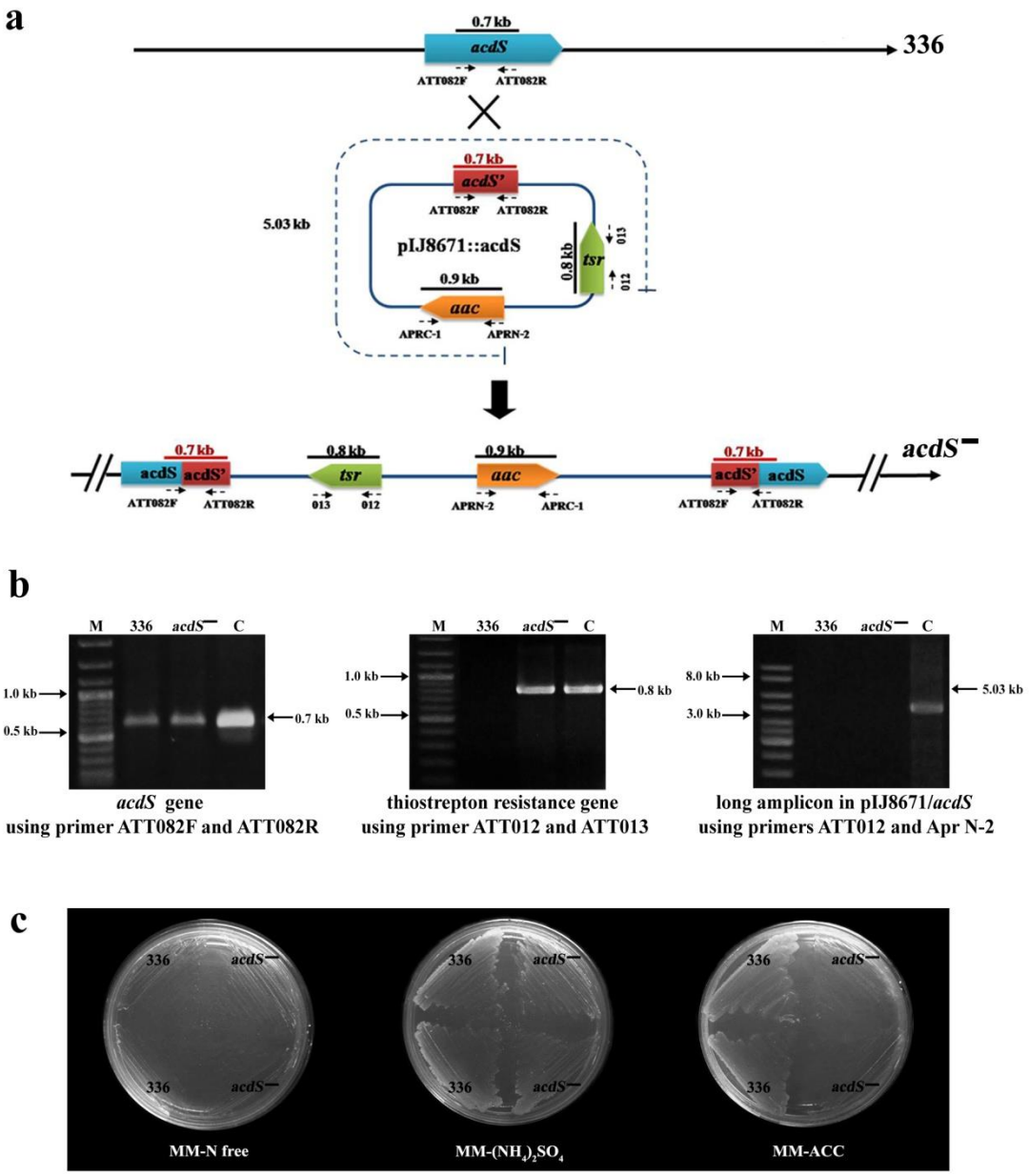

Figure 4-1 Characterization of *Streptomyces* sp. GMKU 336 and the ACCD-deficient mutant. (a) Illustration of the insertion inactivation of the *acdS* gene. Small dashed arrows indicate primers used for PCR amplification tests; (b) PCR amplification tests for *acdS* gene, thiostrepton resistant gene and long amplicon in pIJ8671/*acdS*; (c) ACC deaminase activity on MM-N free, MM-$(NH_4)_2SO_4$, and MM-ACC media. M, 1-kb ladder; C, pIJ8671/*acdS*; 336, *Streptomyces* sp. GMKU 336; *acdS*$^-$, ACCD-deficient mutant.

Effect of ACCD-producing *Streptomyces* sp. GMKU 336 inoculated rice plants under salt stress

Streptomyces sp. GMKU 336 and its ACCD-deficient mutant were inoculated into KDML105. The growth parameters were observed after 7 days of treatment with 150 mM NaCl under hydroponic conditions and compared with non-salt treatment (Fig. 4-2 and Table 4-2). Re-isolation of wild type and mutant from both salt and non-salt treatments was about 10^4 CFU g root fresh weight^{-1} (Table 4-2). Both strains were confirmed by 16S rRNA gene sequencing and PCR analysis (data not shown). In addition, un-inoculated plants were shown not to harbor *Streptomyces* sp. GMKU 336 as well as other endophytic actinomycetes. The results indicated that the surface sterilized protocol of rice seeds was successful and the hydroponic condition used in this study are free from contamination.

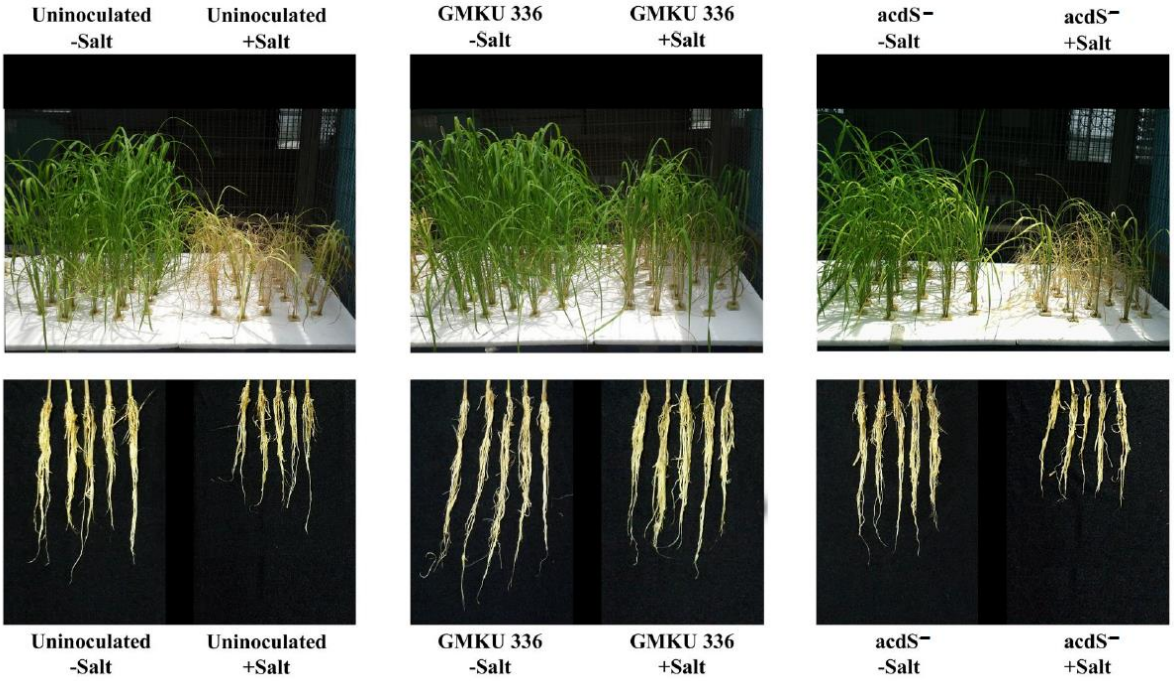

Figure 4-2 Effect of ACCD-producing endophytic *Streptomyces* sp. GMKU 336 on *Oryza sativa* L. cv. KDML105 after 7 days of salt stress under hydroponic conditions. Uninoculated, plants without bacteria inoculation; GMKU 336, plants inoculated with *Streptomyces* sp. GMKU 336; acdS$^-$, plants inoculated with ACCD-deficient mutant; -Salt, non-salt treatment; +Salt, salt treatment (150 mM NaCl).

Table 4-2 Plant growth and physiological parameters of *Oryza sativa* L. cv. KDML105 with and without ACCD-producing *Streptomyces* sp. GMKU 336 and ACCD-deficient mutant under salt (150 mM NaCl) and non-salt treatments.

Parameters	Treatments					
	Uninoculated -Salt	Uninoculated +Salt	GMKU 336 -Salt	GMKU 336 +Salt	acdS⁻ -Salt	acdS⁻ +Salt
Shoot length (cm)	78.70±1.61^{ab}	60.40±1.05^{d}	81.8±1.53^{a}	70.60±1.87^{c}	76.40±1.28^{b}	58.23±0.91^{d}
Root length (cm)	20.87±0.49^{ab}	15.60±1.06^{c}	23.17±0.90^{a}	20.43±0.78^{b}	19.00±0.82^{ab}	16.20±1.05^{c}
Shoot fresh weight (g)	0.90±0.01^{b}	0.54±0.05^{d}	1.50±0.03^{a}	0.66±0.031^{c}	0.89±0.08^{b}	0.52±0.05^{d}
Root fresh weight (g)	0.19±0.01^{b}	0.12±0.01^{d}	0.27±0.03^{a}	0.16±0.01^{c}	0.19±0.01^{b}	0.16±0.01^{d}
Shoot dry weight (g)	0.18±0.01^{b}	0.08±0.01^{d}	0.28±0.01^{a}	0.12±0.01^{c}	0.16±0.01^{b}	0.07±0.02^{d}
Root dry weight (g)	0.05±0.01^{bc}	0.02±0.01^{d}	0.06±0.01^{a}	0.03±0.01^{cd}	0.05±0.01^{ab}	0.02±0.01^{d}
Total chlorophyll (mg g FW^{-1})	0.66±0.02^{a}	0.27±0.01^{c}	0.66±0.02^{a}	0.46±0.01^{b}	0.65±0.01^{a}	0.30±0.01^{c}
Ethylene production (µmol g DW^{-1} h^{-1})	0.98±0.13^{b}	2.15±0.11^{a}	0.78±0.06^{b}	1.01±0.10^{b}	0.97±0.13^{b}	1.93±0.06^{a}
RWC (%)	98.44±0.44^{a}	80.56±0.97^{c}	98.54±0.47^{a}	91.91±0.32^{b}	98.23±0.53^{a}	81.26±1.16^{c}
Proline content (µg g FW^{-1})	8.13±0.76^{c}	23.83±0.18^{b}	8.73±1.15^{c}	28.97±0.48^{a}	8.63±0.41^{c}	24.80±1.73^{b}
Na^{+} content (mg g DW^{-1})	3.33±0.68^{c}	30.60±3.40^{a}	3.10±0.46^{c}	18.63±1.12^{b}	2.77±0.31^{c}	27.83±2.14^{a}
K^{+} content (mg g DW^{-1})	44.90±3.97^{a}	31.93±1.63^{c}	44.83±4.68^{a}	38.37±2.75^{b}	45.27±2.32^{a}	29.27±1.60^{c}
Na^{+}/K^{+}	0.08±0.02^{c}	0.96 ±0.07^{a}	0.07±0.02^{c}	0.49±0.04^{b}	0.06±0.01^{c}	0.95±0.09^{a}
Ca^{2+} content (mg g DW^{-1})	8.10±0.20^{a}	6.13±0.40^{c}	7.90±0.46^{a}	6.90±0.30^{b}	8.10±2.32^{a}	6.23±0.31^{c}
MDA content (uM g FW^{-1})	29.40±0.10^{c}	55.63±1.19^{a}	28.93±0.72^{c}	33.07±1.72^{b}	28.93±1.05^{c}	55.67±0.64^{a}
Salt injury score	ND	7.40±0.40^{a}	ND	3.20±0.36^{b}	ND	7.60±0.52^{a}
Salt tolerant type	ND	Susceptible	ND	Tolerant	ND	Susceptible
Colonization (10^{4} CFU g root FW^{-1})	0.00	0.00	8.50±1.40	4.66 ±3.05	5.48 ±3.47	4.84 ±3.19

Values are mean of three replicates ± standard error of mean. Different letters indicated statistical differences between treatments (Duncan's test, $P<0.05$). Uninoculated, plants without bacteria inoculation; GMKU 336, plants inoculated

with *Streptomyces* sp. GMKU 336; acdS⁻, plants inoculated with ACCD-deficient mutant; -Salt, non-salt treatment; +Salt, salt treatment (150 mM NaCl); DW, dry weight; FW, fresh weight; ND, not determined.

Under non-salt conditions, ACCD-producing *Streptomyces* sp. GMKU 336 slightly enhanced plant elongation compared to un-inoculated controls (Fig 3a and b), but significantly increased plant biomass (1.2–1.6 fold) including shoot/root fresh and dry weights (Fig 4-3c–f). Rice inoculated with the ACCD-deficient mutant showed similar plant growth parameters to those of un-inoculated controls (Fig. 4-3). Under salt-stress conditions, all growth parameters were significantly reduced when compared to the non-salt treatments (Fig. 4-3). Therefore, strain GMKU 336 was able to promote growth of KDML105 with or without salt treatment.

A symptom of salt toxicity in the KDML105 was evaluated using the standard scoring protocol[10]. Under salt treatment, complete cessation of growth was scored as 'susceptible' in un-inoculated plants and those inoculated with the ACCD-deficient mutant, whereas nearly normal growth was scored as 'tolerant' in plants inoculated with strain GMKU 336 (Fig. 4-2 and Table 4-2).

Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on plant physiology

Indices of plant physiology including chlorophyll content, ethylene production, relative water content, and proline content revealed constant level of each parameters of all treatments of non-salt stressed plants (Fig. 4-4). Under salt-stress conditions, a significant decrease in chlorophyll content of rice was observed in all treatments, compared to those grown in non-salt conditions. However, the KDML105 inoculated with *Streptomyces* sp. GMKU 336 had a significantly higher (1.7-fold) chlorophyll content compared to that of an un-inoculated control (Fig. 4-4a and Table 4-2). Significant induction of ethylene production (2-fold) was observed in un-inoculated plants and those inoculated with the ACCD-deficient mutant, compared to those of non-salt treatment (Fig. 4-4b, Table 4-2). Remarkably and in contrast to that of non-salt treatment, KDML105 inoculated with strain GMKU 336 had no increment in ethylene level (Fig. 4-4b). The water content of all treatments was drastically reduced after exposure to salt stress. Nevertheless, plants inoculated with strain GMKU 336 accumulated water significantly more than the un-inoculated control (Fig.4- 4c). Furthermore, proline content was increased in all salt-stressed plants, but those inoculated with strain GMKU 336 had significantly higher proline content than un-inoculated controls (Fig.4- 4d). The results suggested that strain GMKU 336 has a positive effect on the physiology of rice to tolerate salinity.

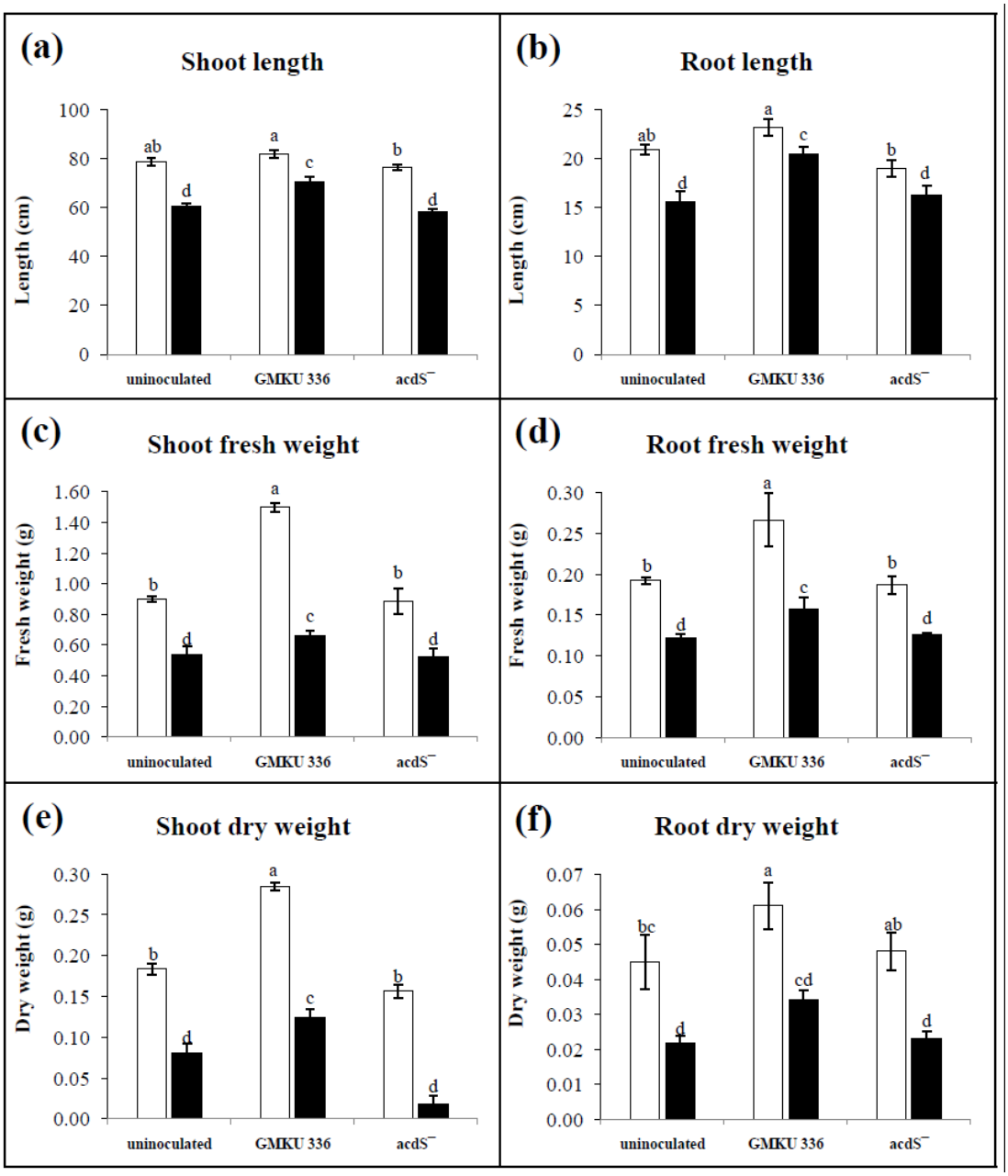

Figure 4-3 Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on growth of *Oryza sativa* L. cv. KDML105 after 7 days of salt stress under hydroponic conditions. (a), shoot length; (b), root length, (c), shoot fresh weight; (d), root fresh weight; (e), shoot dry weight; (f), root dry weight. Values are mean of three replicates ± standard error of mean. Different letters indicated statistical differences between treatments (Duncan's test, $P<0.05$). Uninoculated, plants without bacteria inoculation; GMKU 336, plants inoculated with *Streptomyces* sp. GMKU336; acdS⁻, plants inoculated with ACCD-deficient mutant; white bar, non-salt treatment; black bar, salt treatment (150 mM NaCl).

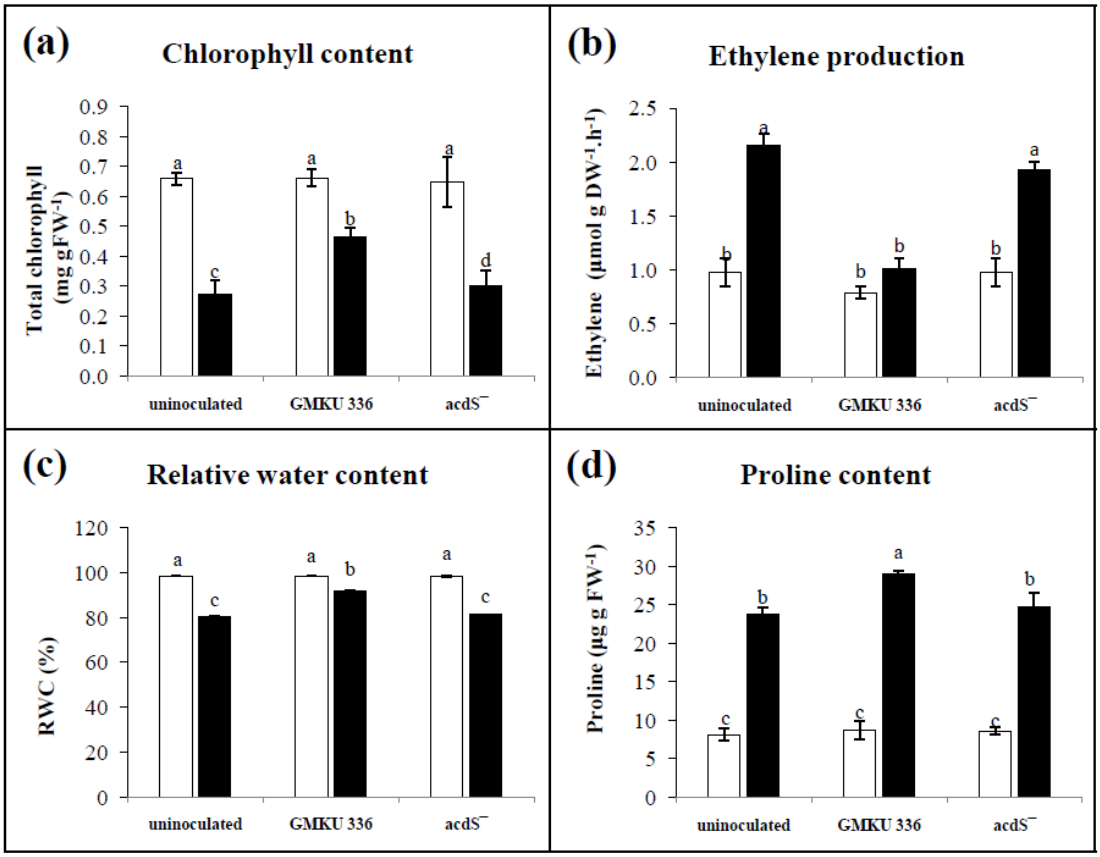

Figure 4-4 Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on plant physiology of *Oryza sativa* L. cv. KDML105. (a), chlorophyll content; (b), ethylene production; (c), relative water content; (d), proline content. Values are mean of three replicates ± standard error of mean. Different letters indicated statistically differences between treatments (Duncan's test, $P<0.05$). Uninoculated, plants without bacteria inoculation; GMKU 336, plants inoculated with *Streptomyces* sp. GMKU 336; acdS^{-}, plants inoculated with ACCD-deficient mutant; white bar, non-salt treatment; black bar, salt treatment (150 mM NaCl).

Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on plant ion content

Under normal conditions, the ion content of KDML105, specifically Na^+, K^+, and Ca^{2+}, was at the same level in all treatments (Fig. 4-5). Under salt-stress conditions, the Na^+ content of all plant treatments was significantly increased (Fig. 4-5a). Un-inoculated plants and those inoculated with the ACCD-deficient mutant had a Na^+ content nearly 10-fold higher than plants with the corresponding non-salt treatment, whereas plants inoculated with *Streptomyces* sp. GMKU 336 had a Na^+ content that was only 6-fold higher than the non-salt control (Fig. 4-5a and Table 4-2). By contrast, the K^+ content of all plant treatments was significantly decreased under

salt-stress conditions (Fig. 4-5b). However, plants inoculated with strain GMKU 336 had a smaller decrease in K^+ content compared to un-inoculated plants and those inoculated with the ACCD-deficient mutant (Fig. 4-5b). Taken together, there was a 13-fold increment in Na^+/K^+ ratio for un-inoculated plants and those inoculated with the ACCD-deficient mutant when compared to the non-salt treatment. On the other hand, the increment in Na^+/K^+ ratio was only 6-fold in plants inoculated with strain GMKU 336 (Fig. 4-5c and Table 4-2). Salt stress also caused a decrease in Ca^{2+} content in all plant treatments (Fig. 4-5d). However, plants inoculated with strain GMKU 336 had significantly less reduction in Ca^{2+} content than un-inoculated plants or those inoculated with the ACCD-deficient mutant (Fig. 4-5d). The results implied that strain GMKU 336 helps maintain ion balance and, thus, increases salt tolerance in KDML105.

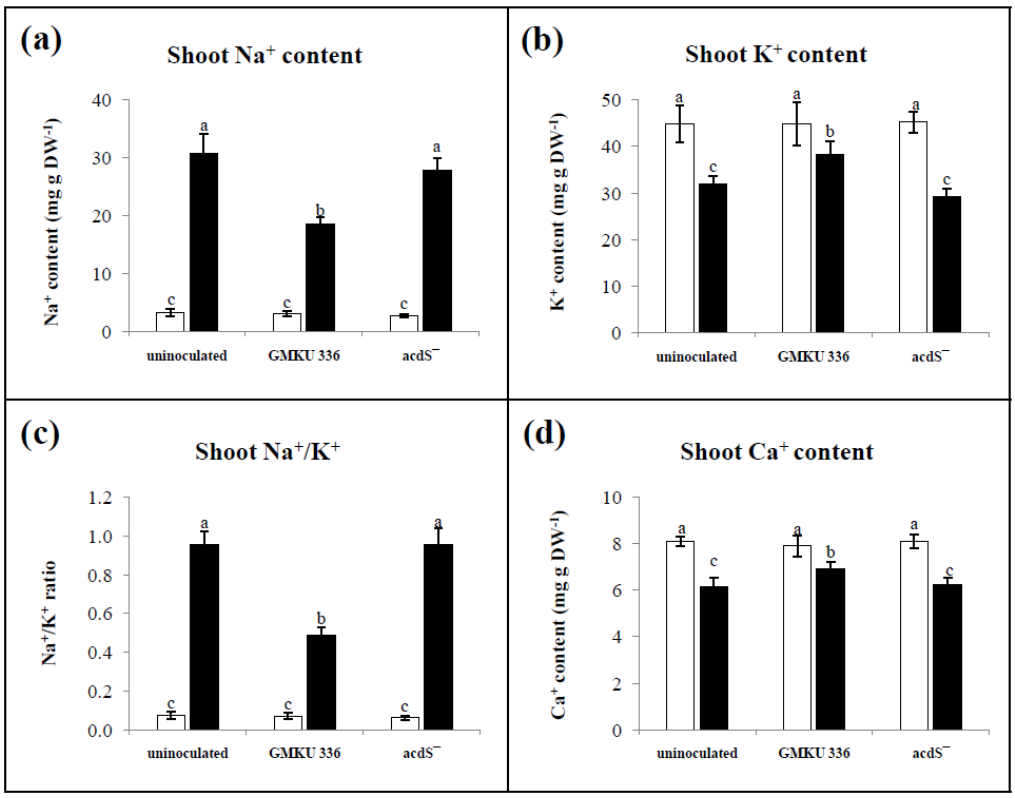

Figure 4-5 Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on ion content of shoots of *Oryza sativa* L. cv. KDML105. (a), Na^+ content; (b), K^+ content; (c), Na^+/K^+ ratio; (d), Ca^{2+} content. Values are mean of three replicates ± standard error of mean. Different letters indicated statistically significant differences between treatments (Duncan's test, $P<0.05$). Uninoculated, plants without bacteria inoculation; GMKU 336, plants inoculated with *Streptomyces* sp. GMKU 336; acdS$^-$, plants inoculated with ACCD-deficient mutant; white bar, non-salt treatment; black bar, salt treatment (150 mM NaCl).

Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on reactive oxygen species (ROS)

A significant increase of lipid peroxidation determined by estimating production of malondialdehyde (MDA) content was observed in all plant treatments exposed to salt (Fig. 4-6a). Un-inoculated plants and those inoculated with the ACCD-deficient mutant accumulated MDA nearly 2-fold higher than the non-salt treatments. By contrast, plants inoculated with *Streptomyces* sp. GMKU 336 had a MDA content less than half of both treatments (Fig. 4-6a and Table 4-2). ROS were detected in leaves by staining with nitrobluetrazolium (NBT) (Fig. 4-6b) and 3,3'-diaminobenzidine (DAB) (Fig. 4-6c), which indicate the presence of superoxide and hydrogen peroxide, respectively. In the presence of salt, leaves had higher staining indicative of both ROS species; however, plants inoculated with strain GMKU 336 showed less staining than the other treatments (Fig. 4-6b and c).The results suggested that strain GMKU336 reduces ROS in salt-stressed rice.

Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on expression profile of genes involved in the ethylene pathway

As KDML105 inoculated with *Streptomyces* sp. GMKU 336 maintained the same level of ethylene either with or without salt treatment similar to those un-inoculated plants and those inoculated with the ACCD-deficient mutant under non-salt condition (Fig. 4-4b), gene expression patterns of ACC synthase (*ACS1*), ACC oxidase (*ACO1*) and the ethylene responsive element binding protein (*EREBP1*) were investigated by real-time PCR. All three genes were expressed at the same basal level in all plant treatments under non-salt conditions (Fig. 4-7a–c). The expression level of *ACS1* was about 5-fold up-regulated in all salt-stressed plants compared to the corresponding non-salt controls (Fig. 4-7a). The expression profiles of *ACO1* and *EREBP1* in un-inoculated plants and those inoculated with the ACCD-deficient mutant were about 3–4 fold higher than the non-salt treatments, whereas plants inoculated with strain GMKU 336 had 1-fold lower expression (Fig. 4-7b and Table 4-3). Moreover, expression of the *acdS* gene encoding ACCD in strain GMKU 336 was only detected *in vivo* within salt-stressed rice (Fig. 4-7d). The results indicated that strain GMKU 336 reduces ethylene content through the action of ACCD and consequently down-regulated *ACO1* and *EREBP1* genes in rice.

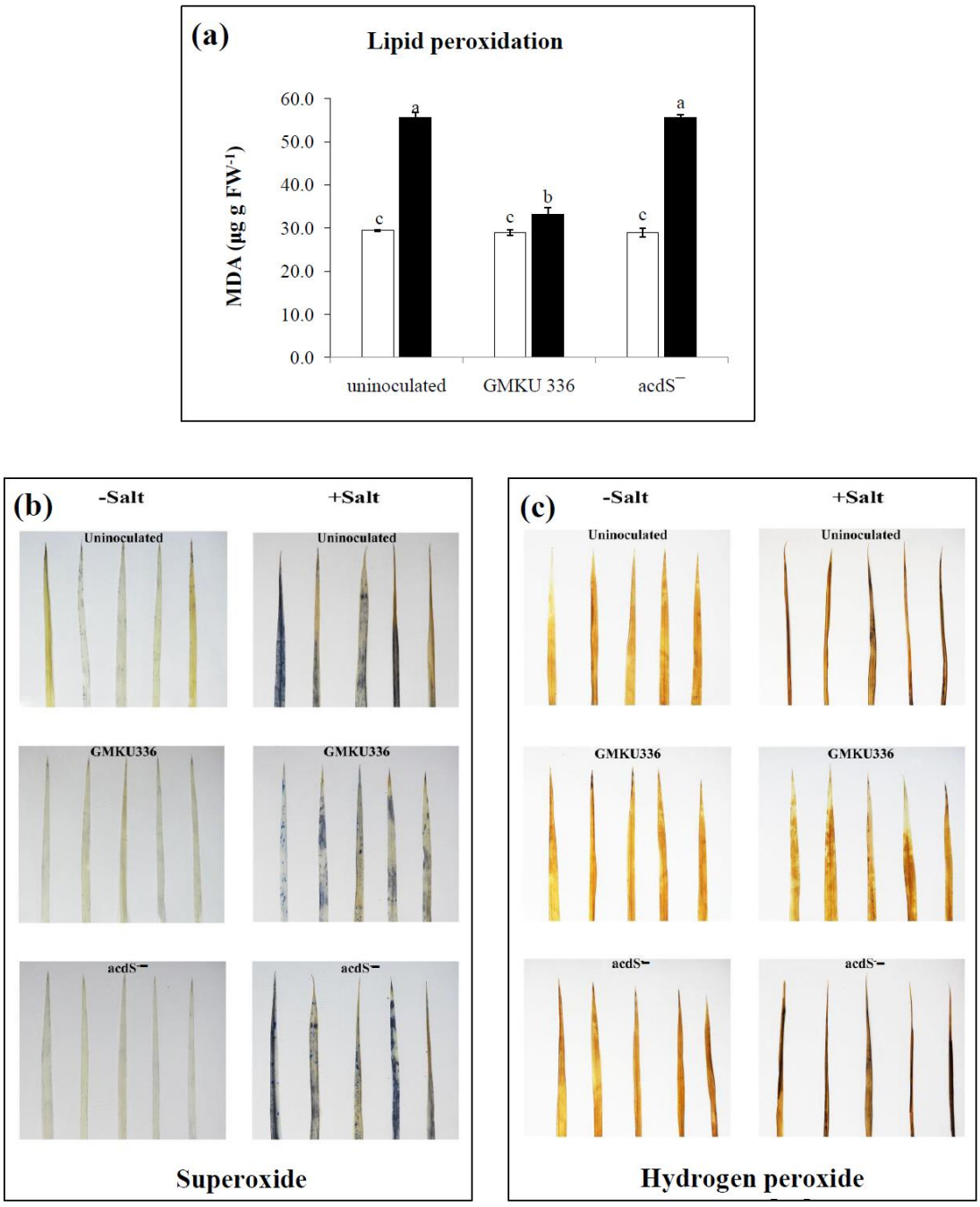

Figure 4-6 Effect of ACCD-producing *Streptomyces* sp. GMKU 336 in reactive oxygen species (ROS) in *Oryza sativa* L. cv. KDML105. (a), lipid peroxidation; (b), superoxide by NBT staining; (c), hydrogen peroxide by DAB staining. Values are mean of three replicates ± standard error of mean. Different letters indicated statistical differences between treatments (Duncan's test, $P<0.05$). Uninoculated, plants without bacteria inoculation; GMKU 336, plants inoculated with *Streptomyces* sp. GMKU 336; acdS⁻, plants inoculated with ACCD-deficient mutant; white bar, non-salt treatment; black bar, salt treatment (150 mM NaCl).

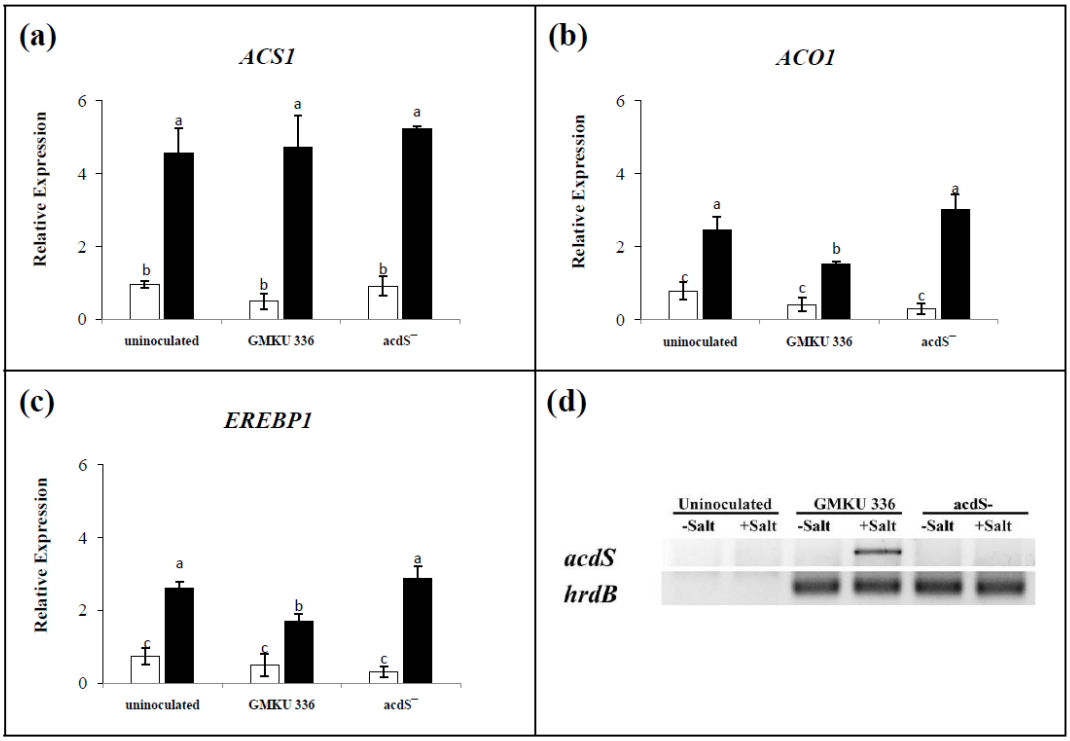

Figure 4-7 Transcriptional analysis of genes involved in ethylene production in *Oryza sativa* L. cv. KDML105 associated with *Streptomyces* sp. GMKU 336. (a), ACC synthase (*ACS1*); (b), ACC oxidase (*ACO1*); (c), ethylene responsive element binding proteins (*EREBP1*); (d), ACCD (*acdS*) of *Streptomyces* sp. GMKU 336. The two gels cropped from different gels. The histogram represents mean of the expression ratio, relative to the actin gene (*act1*). Values are mean of three replicates ± standard error of mean. Different letters indicated statistically-significant differences between treatments (Duncan's test, $P<0.05$). Uninoculated, plants without bacteria inoculation; GMKU 336, plants inoculated with *Streptomyces* sp. GMKU 336; acdS⁻, plants inoculated with ACCD-deficient mutant; *hrdB*, RNA polymerase principal sigma factor gene of *Streptomyces* sp. GMKU 336; white bar/-Salt, non-salt treatment; black bar/+Salt, salt treatment (150 mM NaCl).

Table 4-3 Transcriptional levels of genes involved in salt stress response in *Oryza sativa* L. cv. KDML105 with and without ACCD-producing *Streptomyces* sp. GMKU 336 and ACCD-deficient mutant under salt (150 mM NaCl) and non-salt treatments.

Relative expression	Treatments					
	Uninoculated -Salt	Uninoculated +Salt	GMKU 336 -Salt	GMKU 336 +Salt	acdS⁻ -Salt	acdS⁻ +Salt
ACS1	0.96±0.20^{b}	4.56±0.66^{a}	0.48±0.21^{b}	4.72±0.87^{a}	0.90±0.27^{b}	5.21±0.08^{a}
ACO1	0.78±0.24^{c}	2.46±0.35^{a}	0.41±0.17^{c}	1.51±0.07^{b}	0.29±0.16^{c}	3.02±0.41^{a}
EREBP1	0.74±0.23^{c}	2.60±0.17^{a}	0.49±0.31^{c}	1.69±0.21^{b}	0.31±0.02^{c}	2.86±0.33^{a}
salT	0.98±0.09^{b}	1.27±0.06^{a}	0.93±0.11^{b}	1.44±0.30^{a}	0.70±0.16^{b}	1.37±0.05^{a}
BADH1	0.87±0.14^{c}	1.65±0.04^{b}	0.90±0.10^{c}	4.42±0.32^{a}	0.93±0.15^{c}	1.68±0.15^{b}
NHX1	0.87±0.13^{c}	1.42±0.14^{b}	1.04±0.09^{c}	2.53±0.30^{a}	0.88±0.10^{c}	1.60±0.10^{b}
SOS1	0.87±0.13^{c}	2.02±0.42^{b}	0.65±0.07^{c}	3.09±0.13^{a}	0.70±0.10^{c}	0.13±0.13^{b}
Cam1-1	0.95±0.11^{c}	1.69±0.15^{b}	1.10±0.11^{c}	3.23±0.57^{a}	0.84±0.08^{c}	1.75±0.07^{b}
MAPK5	0.95±0.18^{c}	3.23±0.17^{a}	0.85±0.14^{c}	1.83±0.12^{b}	0.91±0.11^{c}	3.21±0.21^{a}
CuZn-SOD1	0.99±0.04^{c}	2.27±0.12^{b}	0.73±0.24^{c}	3.67±0.31^{a}	0.97±0.06^{c}	2.34±0.13^{b}
CATb	0.93±0.08^{c}	2.10±0.10^{b}	0.97±0.09^{c}	3.00±0.05^{a}	0.94±0.37^{c}	1.99±0.21^{b}

Values are mean of three replicates ± standard error of mean. Different letters indicated statistical differences between treatments (Duncan's test, $P<0.05$). Uninoculated, plants without bacteria inoculation; GMKU 336, plants inoculated with *Streptomyces* sp. GMKU 336; acdS⁻, plants inoculated with ACCD-deficient mutant; -Salt, non-salt treatment; +Salt, salt treatment (150 mM NaCl).

Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on expression of salt-stress responsive genes

Eight candidate genes of rice encoded proteins involved in the salt stress response were transcriptionally analyzed in un-inoculated plants and those inoculated with wild type or mutant bacteria, under salt and non-salt conditions. The genes were: *salT* (salt stress responsive protein)[11], *BADH1* (betaine aldehyde dehydrogenase)[12], *NHX1* (Na^+/H^+ antiporter)[13], *SOS1* (salt overlay sensitive 1 protein)[13], *Cam1-1* (calmodulin)[14], *MAPK5* (mitogen activated protein kinase 5)[12],

CuZn-SOD1 (superoxide dismutase)[15], and *CATb* (catalase)[12]. Expression profiles of all genes were observed at similar basal level in plants grown under non-salt conditions (Fig. 4-8). When KDML105 were exposed to salt, the salt-induced positive control gene, *salT*, was up-regulated to a similar level in all treatments (Fig. 4-8a). The expression profile of *BADH1*, involved in competition of solute production, was up-regulated in all salt-stressed plants. However and significantly, it was 4-fold higher in plants inoculated with *Streptomyces* sp. GMKU 336 compared to un-inoculated plants and those inoculated with the ACCD-deficient mutant (Fig. 4-8b and Table 4-3).

Genes involved in Na^+ transport, specifically a member of vacuole Na^+/H^+ transporters (*NHX1*) and salt overlay sensitive 1 protein (*SOS1*), were significantly (1.6–2.3 fold) up-regulated in salt-stressed un-inoculated plants and those inoculated with the ACCD-deficient mutant, when compared to the non-salt controls. Rice inoculated with strain GMKU 336 had expression levels that were 3–4 fold higher than control (Fig. 4-8c and d, Table 4-3). Up-regulation of the *Cam1-1* gene, a Ca^+ sensor involved in plant signaling by calmodulin, was 1.8-fold higher than the non-salt treatment in salt-stressed un-inoculated plants and those inoculated with the ACCD-deficient mutant. However, plants inoculated with strain GMKU 336 had expression levels that were 1-fold higher than both treatments (Fig. 4-8e and Table 4-3). High expression of the *MAPK5* gene, encoding a kinase protein, was detected in un-inoculated plants and those inoculated with the ACCD-deficient mutant when compared to those of non-salt treatments. By contrast, plants inoculated with strain GMKU 336 had nearly 2-fold expression when compared to its control (Fig. 4-8f and Table 4-3).

Further analysis of gene expression encoding antioxidant enzymes, superoxide dismutase (*CuZn-SOD1*) and catalase (*CATb*), revealed 2.1–2.3 fold up-regulation in salt-stressed un-inoculated plants and those inoculated with the ACCD-deficient mutant when compared to the non-salt treatments. Significantly, expression levels of plants inoculated with strain GMKU 336 were 3.2–3.7 times higher than the non-salt controls (Fig. 4-8g and h, Table 4-3). All of these results indicate that strain GMKU 336 has a positive influence to salt stress response gene expression in KDML105 by up-regulation of *BADH1*, *NHX1*, *SOS1*, *Cam1-1*, *CuZn-SOD1*, and *CATb*, and down-regulation of *MAPK5*, and increases salt tolerant in rice as a consequence.

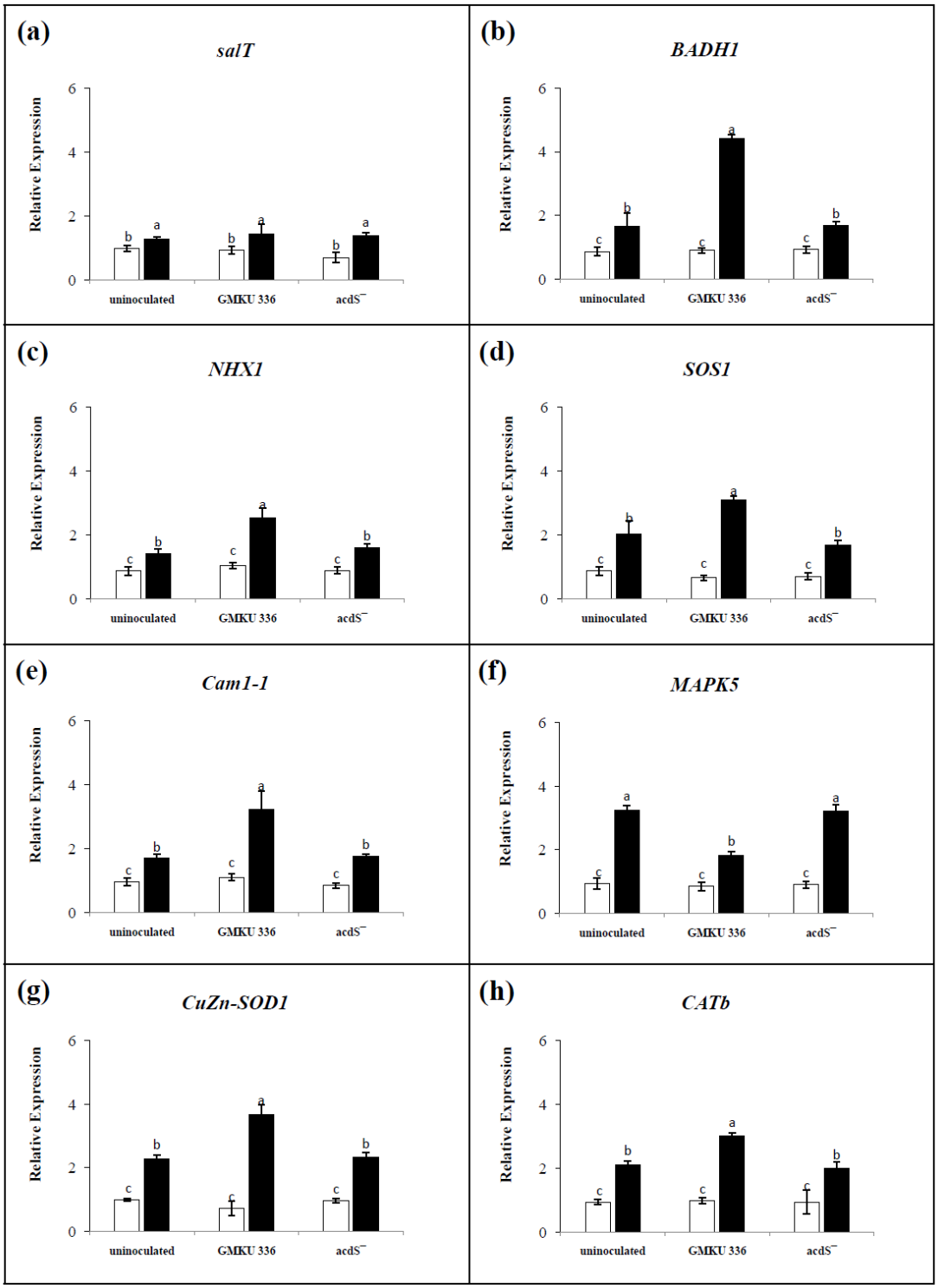

Figure 4-8 Transcriptional analysis of genes involved in salt stress response in *Oryza sativa* L. cv. KDML105 associated with *Streptomyces* sp. GMKU 336. (a), salt stress responsive protein (*salT*); (b), betaine aldehyde dehydrogenase (*BADH1*); (c), Na^+/H^+ antiporter (*NHX1*); (d), salt overlay sensitive 1 protein (*SOS1*); (e), calmodulin (*Cam1-1*); (f), mitogen activated protein kinase (*MAPK5*); (g), superoxide dismutase (*CuZn-SOD1*); (h), catalase (*CATb*). The histogram

represents mean of expression ratio, relative to the actin gene (*act1*). Values are mean of three replicates ± standard error of mean. Different letters indicated statistically-significant differences between treatments (Duncan's test, $P<0.05$). Uninoculated, plants without bacteria inoculation; GMKU 336, plants inoculated with *Streptomyces* sp. GMKU 336; acdS⁻, plants inoculated with ACCD-deficient mutant; white bar, non-salt treatment; black bar, salt treatment (150 mM NaCl).

Discussion

Recently, actinomycetes have been reported to promote plant growth as well as alleviate various abiotic stresses including salinity and osmotic stress via the action of 1-aminocyclopropane-1-carboxylate deaminase (ACCD)[5,6]. However, the role of ACCD-producing endophytic actinomycetes in promoting plant growth under stress conditions has not yet been investigated systematically. *Streptomyces* sp. GMKU336 was used in this study as it displayed highest ACCD activity amongst other strains in the screening program[9] and showed halophilic property which is suitable to investigate the *in vivo* molecular interactions of this strain in partnership with the salt-sensitive Thai jasmine rice Khao Dok Mali 105 cultivar (KDML105) under salt-stress conditions. In addition, strain GMKU 336 revealed endophytic ability in rice although it was isolated from medicinal plant. The result was in agreement with previous report that endophytic *Streptomyces* isolated from one plant species could mutually resided in the other different plant species by *in vitro* inoculation[3]. In this work, an ACCD-deficient mutant of strain GMKU 336 was constructed by insertional inactivation of the *acdS* gene to define definitively the role of ACCD on plant growth and salt tolerance.

Under non-salt conditions, KDML105 inoculated with ACCD-producing *Streptomyces* sp. GMKU 336 had significantly enhanced shoot and root biomass but not obviously extended shoot and root lengths. This might be due to the lack of IAA production of this strain that would not encourage the elongation of plants. However, the results for plant growth were consistent with previous work showing that ACCD-producing *Streptomyces* have an ability to enhance growth of tomato[5], *Arabidopsis*[16], halophytic *Limonium sinense*[17] and sugarcane[4]. The growth effect has also been found in other bacteria such as ACCD-producing *Pseudomonas*[18], *Enterobacter*[19], and *Bacillus*[20] that enhanced growth of canola.

Addition of salt had a negative effect on plant growth parameters in all plant treatments. However, KDML105 inoculated with *Streptomyces* sp. GMKU 336 maintained high shoot and root elongation when compared to un-inoculated plants and those inoculated with the ACCD-deficient mutant. The results were in agreement with previous work that an ACCD-producing

Streptomyces increased shoot and root growth of halophytic *Ligustrum sinense* under salt-stress treatment[6]. ACCD-producing *Bacillus* significantly increased seed germination and promoted growth of rice KDML105[21] and indica rice[12]. Likewise, an ACCD-producing *Pseudomonas* increased yield and enhanced salt resistance in various plants including canola[22] and tomato[23] which was not observed with its *acdS* mutant strain. Since KDML105 is a salt-sensitive cultivar[24], symptoms of salt toxicity were observed in un-inoculated plants and those inoculated with the ACCD-deficient mutant. By contrast, inoculation of KMDL105 with strain GMKU 336 resulted in the rice exhibiting better resistance to salinity stress.

Salt stress reduced chlorophyll content in all KDML105 treatments. However, plants inoculated with *Streptomyces* sp. GMKU 336 had less reduction of chlorophyll content. The result was similar to that with PGP *Azospirillum*[25] and ACCD-producing *Bacillus*[12] which significantly increased chlorophyll content in respective maize and rice grown in high salt conditions. Furthermore, salt stress significantly accelerated ethylene synthesis in all rice treatments. By contrast, rice inoculated with strain GMKU 336 maintained ethylene content at the same level as that of non-salt treatment. The result is supported by other reports that ACCD-producing *Pseudomonas* suppressed ethylene synthesis and decelerated chlorophyll decay in wheat under salt-stress conditions[26]. Besides, lower amount of ACC, a precursor of ethylene, was observed in canola inoculated with ACCD-producing *Pseudomonas*[18] and *Enterobacter*[19]. It was also reported that *Streptomyces* enhanced plant growth by lowering the plant ACC and ethylene levels in tomato[5].

Salt stress induced high activities of ACC synthase and ACC oxidase and subsequently produced high levels of ethylene[1]. In this work, expression profiles of rice genes involved in the ethylene pathway including ACC synthase (*ACS1*) and ACC oxidase (*ACO1*) were up-regulated in all plants treated with salt. Here, we report the remarkable reduction of ethylene in KDML105 inoculated with *Streptomyces* sp. GMKU 336, which is correlated with low expression of the *ACO1* gene. Expression of the *acdS* gene encoding ACCD of strain GMKU 336 was only observed *in vivo* with salt-treated rice. The results indicated that lower expression of the *ACO1* gene of salt-treated rice inoculated with strain GMKU 336 was due to the expression of *acdS* gene of the bacteria which converted the ACC to ammonia and α-ketobutyrate and subsequently reduced the level of the stress molecule, ethylene. The results were in agreement with the expression of *acdS* of *Mesorhizobium* spp.[28] and *Sinorhizobium* sp. BL3[29] in nodules of chickpea and mungbean, respectively under salinity condition. However, the expression was not detected in mungbean inoculated with an *acdS*-deficient mutant of strain BL3[29]. The gene encoding an ethylene

responsive element binding protein (*EREBP1*) was up-regulated in all plants treated with salt. EREBP is a member of the ethylene-response factor (ERF) family[30], which plays an important role in abiotic stress response[31]. Up-regulation of *EREBP1* was observed in tobacco during drought and salt stress[32]. Here, we report that KDML105 inoculated with *Streptomyces* sp. GMKU 336 expressed *EREBP1* at significantly lower levels. Canola inoculated with ACCD-producing *Pseudomonas putida* UW4 had reduced expression of *ERF*, while plants inoculated with an ACCD-deficient mutant had increased the expression[33].

Under salt-stress conditions, plants adapt by producing competition solutes such as proline and glycine betaine that help to stabilize proteins and cell structures, osmotic balance, scavenge reactive oxygen species (ROS)[34], and increases chlorophyll content[35]. Here we report that the water and proline contents of all salt-treated rice were significantly increased, but highly accumulated in plants inoculated with *Streptomyces* sp. GMKU 336. The results correlated with previous reports that PGP *Dietzia*[36] and ACCD-producing *Bacillus*[12] improved salt tolerance in respective wheat and rice by enhancement of proline content. Furthermore, the transcription profile of the betaine aldehyde dehydrogenase gene (*BADH1*) that converts choline to glycine betaine was up-regulated in all salt-stressed rice and expressed at the highest level in plants associated with strain GMKU 336. The result was in agreement with ACCD-producing *Bacillus* that maintained osmotic adjustment in rice under salinity condition by accumulation of glycine betaine and up-regulation of the *BADH1* gene[12].

It is generally known that the maintenance of low cytosolic Na^+ concentrations and Na^+/K^+ homeostasis are important for tolerance to salinity. ACC could promote the production of ethylene and improve the response to salinity-induced injury by homeostasis of Na^+/K^{+12}. High accumulation of Na^+ inside the cells inhibits K^+ uptake and results in an increase in Na^+/K^+ ratio that is inversely related to the level of salt tolerance[37]. Here we report that decreased Na^+ content and increased K^+ resulted in a reduction of the Na^+/K^+ ratio in salt-stressed rice inoculated with *Streptomyces* sp. GMKU 336. It was reported that PGPB may regulate the uptake of Na^+/K^+ and maintain a nutritional balance in plants[38]. The results were in agreement with previous reports that cotton inoculated with ACCD-producing *Klebsiella* showed high K^+ concentrations that resulted in enhancement of salt tolerance[39].

Increases in the uptake of Na^+ in shoot vacuoles could enhance salt tolerance in plants. Therefore, the most direct way to manage excess cytoplasmic Na^+, which is toxic to plant cells, is to pump the excess Na^+ to a vacuole catalyzed by a Na^+/H^+ antiporter[13]. We observed up-regulation of the Na^+/H^+ antiporter gene (*NHX1*) in all salt-stressed rice, which was significantly highest in

plants inoculated with *Streptomyces* sp. GMKU 336. Similarly, high expression level of *NHX* was observed in wheat inoculated with PGP *Dietzia* which correlated with enhancement of salt tolerance[40]. Na^+ efflux is one of the mechanisms that maintains the level of Na^+ in the cytoplasm. Salt overlay sensitive 1 (SOS1) is the only Na^+ efflux protein located at the plant plasma membrane[13]. We report here that expression of the *SOS1* gene was increased in all salt stressed rice and significantly highest in plants inoculated with strain GMKU 336. The results were in agreement with other reports of overexpression of the *SOS1* gene in salt tolerant rice[41] and *Arabidopsis*[42]. The results indicated that excess Na^+ was reduced by the up-regulation of *NHX1* and *SOS1* genes which was an effect of strain GMKU 336 to enhance growth and salt tolerance of rice.

The calcium signaling network is one of the signal cascades involved in transient changes in cytosolic Ca^{2+} concentration, which was reported to be a key messenger in the salt stress response[43]. A decrease in Ca^{2+} content under stress condition was previously reported in KDML105[24] and other rice salt-sensitive lines[44]. Here we report a decrease in Ca^{2+} content as a result of salt stress in all salt treated rice. However, plants inoculated with *Streptomyces* sp. GMKU 336 maintained a significantly higher Ca^{2+} content. Increase of Ca^{2+} content was also observed in eggplant[45] and cotton[46] inoculated with PGP *Pseudomona*s. Furthermore, the expression profile of *Cam 1-1*, involved in calmodulin, was significantly up-regulated in all salt-stressed rice, but highest in plants inoculated with strain GMKU 336. The binding of Ca^{2+} to the calmodulin complex is able to regulate a variety of cellular processes implicated in salt and other stresses[14], therefore, *Cam1-1* gene is a significant player in the Ca^{2+} signal transduction network. The responses of high Ca^{2+} content and *Cam1-1* gene expression on rice KDML105 to salt stress suggested that strain GMKU 336 plays a positive role to induce calmodulin and Ca^{2+} content to help rice tolerate salinity.

The response of plant cells to salt stress is controlled by multiple mechanisms linked to stress and other developmental responses. Ethylene was additionally reported to mediate crosstalk between mitogen activated protein kinase (MAPK) signaling pathways[47]. Plant MAPK cascades are thought to play a key role in biotic and abiotic stress responses, hormone response, cell division and development in rice[48]. We report here that the expression profile of *MAPK5* was significantly up-regulated in all salt-stressed rice, but had lower expression in plants inoculated with *Streptomyces* sp. GMKU 336. Similarly, a low expression level of *MAPK5* was observed in rice inoculated with *Bacillus amyloliquefaciens* NBRISN13 that increased salt tolerance of rice in soil[12]. However, *MAPK* gene expression profiles of rice[49], pea[50], and *Arabidopsis*[51], were up-

regulated during ethylene induction. The results suggested that lower ethylene production in rice inoculated with strain GMKU 336 under salt-stress treatment might reflect the low expression level of the *MAPK5* gene.

In rice, salinity triggers MAPK cascades to stabilize ACC synthase activity that enhances ethylene production and ethylene signaling, which then promotes ROS accumulation leading to lipid peroxidation (high accumulation of MDA content) and growth inhibition[52]. In this experiment, salinity significantly induced high accumulation of MDA content and ROS including superoxide and hydrogen peroxide in all salt-stressed rice. Remarkably, *Streptomyces* sp. GMKU 336 reduced ROS leading to a reduction in lipid peroxidation in rice. Earlier studies suggested that induction of ROS scavenging anti-oxidative enzymes such as superoxide dismutase (SOD) and catalase (CAT) were associated with salt tolerance in sugar beet[53] and pea[13]. Here we report that the transcription levels of superoxide dismutase (*CuZn-SOD1*) and catalase (*CATb*) genes were significantly up-regulated in all salt-stressed rice and particularly highest in plants inoculated with strain GMKU 336. The results correlated with previous reports that PGPB reduced salt-induced lipid peroxidation through modulation of expression of ROS-scavenging enzymes[54]. The results indicated that strain GMKU 336 helps salt-stressed rice plants by reduction of lipid peroxidation and ROS levels and consequently promotes plant growth by induction of antioxidant enzymes.

In addition, up-regulation of *salT* was observed in all salt-stressed rice at the same expression level. *SalT* is one of the key genes for salt stress response that has been used as a marker for a salt-induced positive control gene[12] whose transcript is not induced by ethylene[55]. We have shown up-regulation of *salT* in all salt stressed KDML105, supporting the view that this salt-sensitive cultivar changed in level of transcripts of salt stress responsive genes when exposed to salt.

In conclusion, all of the experimental data indicate that ACCD-producing endophytic *Streptomyces* sp. GMKU 336 promoted growth and protected salt-sensitive *Oryza sativa* L. cv. KDML105 from salt stress damage. This endophytic streptomycete enhanced salt tolerance in rice by lowering stress-induced ethylene via the action of ACCD; reduction of lipid peroxidation and Na^+/K^+ ratio but increasing Ca^{2+} content; chlorophyll content, accumulation of osmoprotectants: proline and glycine betaine. The plant physiology also correlated with expression profiles of stress responsive genes in rice associated with strain GMKU 336.

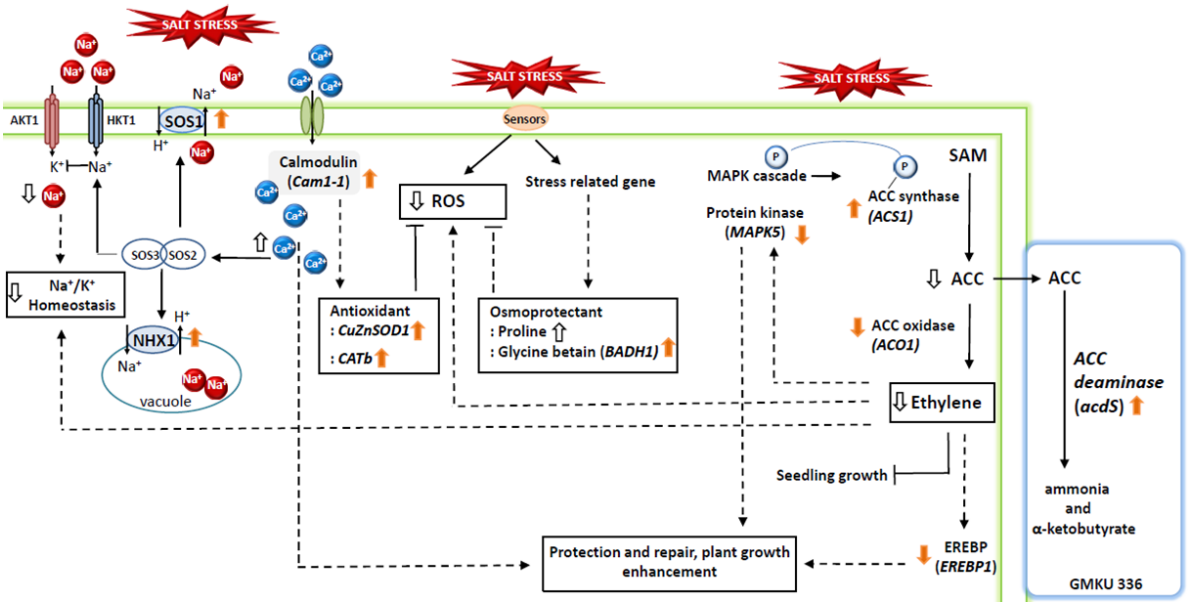

Figure 4-9 Molecular interaction scheme of ACCD-producing endophytic *Streptomyces* sp. GKU 336 associated with *Oryza sativa* L. cv. KDML105 under salt stress. Salt stress induces the ethylene biosynthesis pathway by up-regulation of *ACS1*. However, ACC is consumed by the *acdS* gene encoding ACCD of *Streptomyces* sp. GMKU 336, whereas *ACO1* and *EREBP1* are down-regulated and ethylene is reduced as a consequence. Depletion of ethylene induced less expression of *MAPK5* as well as lowering ROS accumulation. Salt tolerance in rice is enhanced by increases in proline and glycine betaine by up-regulation of the *BADH1* gene. Ca^{2+}content is increased and Na^{+}/K^{+} ratio is decreased which are correlated with up-regulation of *Cam1-1*, *SOS1* and *NHX1* genes. Ca^{2+} signal activates the SOS3/SOS2 protein kinase complex which negatively regulates the activity of Na^{+} ion channel. Association of Ca^{2+} and calmodulin activates antioxidant enzymes (*CuZn-SOD1* and *CATb*) which subsequently inhibits ROS. Bold orange arrow indicates gene regulation, bold white arrow indicates plant physiological regulation, black arrow indicates positive regulation, dashed arrow indicates indirect positive regulation, black line with bar end indicates inhibition, and dashed line with bar end indicates indirect inhibition.

The molecular interaction scheme of rice and *Streptomyces* sp. GMKU336 under salt stress is summarized in Fig. 4-9. *AcdS* encoding ACCD of strain GMKU 336 was up-regulated *in vivo*, while *ACO1* and *EREBP1* were down-regulated and implicated in reduction of ethylene production in rice. Depleted ethylene induced less expression of *MAPK5*, that plays a role in salt tolerance as well as lowering ROS accumulation and consequently enhances plant growth. The presence of *Streptomyces* sp. GMKU 336 also enhances salt tolerance in rice by increasing proline and glycine

betaine by up-regulation of *BADH1* gene expression to balance the osmotic pressure between rice tissues and salt-stress environment. Furthermore, an increase in Ca^{2+} and decrease in Na^+/K^+ homeostasis were correlated with up-regulation of the *Cam1-1* gene of calmodulin and *SOS1* and *NHX1* genes related to SOS pathway. The Ca^{2+} signal activates the SOS3/SOS2 protein kinase complex which negatively regulates the activity of the Na^+ ion channel[13]. Consequently, the plasma membrane Na^+/H^+ antiporter (*SOS1*) was phosphorylated and drives the cytoplasmic Na^+ into a vacuole, thus maintaining cellular ion homeostasis under salt stress. In addition, association of Ca^{2+} with calmodulin contributes to the antioxidant defense system by up-regulation of *CuZn-SOD1* and *CATb* gene expression to increase the production of antioxidant enzymes which subsequently inhibits ROS. Noticeably, plants inoculated with the ACCD-deficient mutant exhibited plant growth parameters, physiology and expression of all plant stress responsive genes in the same manner as those of un-inoculated controls. This supports the positive role of ACCD of *Streptomyces* sp. GMKU 336 in growth promotion and salt tolerance of rice.

It is clearly demonstrate for the first time that ACCD-producing *Streptomyces* sp. GMKU 336 enhances growth and salt tolerance by regulation of stress responsive genes of plants *in vivo* under salt-stress condition. Knowledge of the interaction is crucial to understand the relationship between rice plants and endophytic actinomycetes that is essential for further applications of endophytes as potential environmental friendly biofertilizers in saline soil.

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Section 5

Positive role of ACC deaminase-producing endophytic *Streptomyces* sp. GMKU 336 on flooding resistance of mung bean

Introduction

Mung bean [*Vigna radiata* (L.) Wilczek] is one of the most important crops for human nutritional needs. In tropical and subtropical region such as Thailand, heavy rainfall in the rainy season frequently induces short-term flooding in crop fields. Soil flooding has long been identified as a major abiotic stress that resulted in decreasing of growth and crop production (Ahmed *et al*., 2002; Kumar *et al*., 2013; Parent *et al.*, 2008). In recent years, a new approach has been developed to improve stress in plants by treating crop seeds and seedlings with plant growth-promoting bacteria (PGPB) harboring 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD) (Farwell *et al*., 2007). PGPB convert ACC, a precursor of ethylene in plants, to ammonia and α-ketobutyrate which the bacteria can consume by an action of ACCD (Honma and Shinomura, 1978), thereby lower the level of stressed ethylene in plants (Glick, 2004). Plants inoculated with ACCD-producing PGPB were markedly more resistant to the deleterious effects of stress ethylene under flooding condition including *Ocimum sanctum* and tomato (Barnawal *et al*., 2012; Grichko and Glick, 2001; Saleem *et al*., 2007).

Recently, endophytic actinomycetes have been recognized as new members of PGPB due to their properties to protect plants from infectious diseases (Misk and Franco 2011) and ability to enhance plant growth by carrying several PGP-traits including siderophore production (Rungin *et al*., 2012), plant hormone production (Kruasuwan and Thamchaipenet, 2016), phosphate solubilization (El-Tarabily *et al*., 2008) and ACCD production (El-Tarabily, 2008; Qin *et al*., 2014). However, the role of ACCD-producing endophytic actinomycetes to promote plant growth under flooding stress has been less studied. Newly, an endophytic *Streptomyces* sp. GMKU 336 was employed to facilitate growth of rice under salt-stress conditions by reducing stress ethylene via the action of ACCD by converting a precursor of ethylene, ACC, in plants into ammonia and α-ketobutyrate and consequently reduced ethylene, reactive oxygen species (ROS), Na^+, and Na^+/K^+ ratio (Jaemsaeng *et al*., 2018). In this work, *Streptomyces* sp. GMKU 336 was further investigated on its ability to enhance growth of mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72] under flooding conditions.

Materials and Methods

Endophytic streptomycete strains

Endophytic *Streptomyces* sp. GMKU 336 was isolated from medicinal plant, *Clerodendrum serratum* (L.) Moon (Ak-kee-ta-wan) (Indananda, 2013). Strain GMKU 336 displays ACCD activity at 2.85±0.15 μmol α-ketobutyrate/mg protein/h and harbors PGP-traits including phosphate solubilization and siderophore production but no indole-3-acetic acid (IAA) production (Jaemsaeng *et al.*, 2018). The ACCD-deficient mutant was constructed by insertional inactivation of *acdS* gene (encoding for ACCD) of the wildtype strain and showed no ACCD activity (Jaemsaeng *et al*., 2018).

Inoculation of mung bean with endophytic streptomycetes

Healthy seeds of mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72] were obtained from the Chai Nat Field Crops Research Center, Field Crops Research Institute, Department of Agriculture and Cooperatives, Chai Nat, Thailand. The seeds were surface sterilized by immersion in 70% (v/v) ethanol for 1 min, 1% (w/v) sodium hypochlorite for 5 min and washed six times with sterile distilled water. Then, sterilized seeds were soaked in sterile distilled water for 4 h in dark, transferred to a sterile moist chamber and incubation at room temperature in the dark for 2 days for seed germination. Fifteen seedlings were used for each experiment and performed in triplicate. Pruned-root dip method (Musson *et al.*, 1995) was used to inoculate the seedlings by cutting 0.5 cm from the root tip using a sterile scalpel. Then, the seedlings were immersed in individual sterile glass beakers containing 10^8 spores/mL of *Streptomyces* sp. GMKU 336 or its ACCD-deficient mutant for 4 h. The seedlings were re-located to a pot containing sterile soil for 7 days. Next, mung bean plants were flooded with 5 inch of standing water above the soil surface for 21 days. For positive control, mung bean was grown under the same condition without flooding treatment. The plants were harvested and examined for shoot/root elongation, biomass, and plant physiology. The experiment was conducted in green house during August to October 2017.

Re-isolation of endophytic streptomycetes

Re-isolation of endophytic streptomycetes from mung bean plants was performed by modifying the method of Rachniyom *et al*. (2015). Roots and shoots of mung bean were cut separately into small pieces and rinsed with 0.1% (v/v) Tween 20 for 5 min. Then, plant materials were surface sterilized by soaking in 1% (w/v) sodium hypochlorite for 10 min and then immersing in 70% (v/v) ethanol for 1 min and soaked in 10% (w/v) $NaHCO_3$ solution for 5 min. Next, surface-

sterilized plant materials were washed in sterile water three times and crushed in ¼ Ringer's solution (0.9% NaCl, 0.042% KCl, 0.048% $CaCl_2$, 0.02% $NaHCO_3$). The resulting solution was spread onto starch casein agar (SCA; Küster and Williams, 1964) supplemented with 100 mg/mL ampicillin, 2.5 U/mL penicillin G, 50 mg/mL amphotericin B and 50 mg/mL cycloheximide. The final washed solution was spread on the medium to ensure that there was no epiphyte contamination. Colonies of endophytic streptomycetes were observed after incubation at 28±2 °C for 7 days and were randomly selected for analysis by 16S rDNA gene sequencing using primers and PCR condition described by Rachniyom *et al.* (2015).

Determination of survival rate

Survival rate of mung bean plants under flooding condition was calculated in percentage compared with those under non-flooding condition. Plants exhibited rot, wilt, and died were recorded as dead, whereas surviving plants were recorded as alive.

Determination of leaf color, leaf area, and chlorophyll content

Leaf color was compared with the standardized four panel IRRI leaf color chart (LCC) ranging from yellowish green (No. 2) to dark green (No. 5) (International Rice Research Institute, IRRI). Leaf area was measured and calculated on grid paper.

Chlorophyll was extracted by DMSO as described by Hiscox and Israelstam (1979). Approximately 100 mg of leaf fresh weight from each treatment was ground in liquid nitrogen. The macerated leaves were extracted twice by adding 1.0 mL DMSO and sonicated at 30 hz in a waterbath sonicator for 2 min. The extracts were centrifuged and the chlorophyll content of the supernatants were measured at 645 and 663 nm within 20 min after the extraction. Chlorophyll content was calculated by Arnon's equations (Arnon, 1949).

Determination of ethylene

Ethylene production was measured by enclosing the whole mung bean plants in a 250-mL sealed glass container containing 50 mL acetylene for 1 h. 1 mL gas sample was withdrawn and quantified by gas chromatography at PGPR Biofertlizer and Aerated Compost Soil Microbiology Research Group, Soil Science Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

Statistical analyses

All data from the experiments were calculated and statistically evaluated on fifteen seedlings for each condition from biological and technical triplicates. The data were analyzed with one way analysis of variance (ANOVA) and Duncan's test to determine any significant differences between groups at $p<0.05$. All statistical analyses were performed using the SPSS 20.0 for Windows software (SPSS Inc., Chicago, IL, USA).

Results

Effect of ACCD-producing *Streptomyces* sp. GMKU 336 inoculated mung bean under flooding conditions

Streptomyces sp. GMKU 336 and its ACCD-deficient mutant were inoculated into mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72]. The growth parameters of mung bean were observed after 21 days of flooding treatment in comparison to the non-flooding treatment (Figure 5-1A and 1B, Table 5-1). Under non-flooding condition, *Streptomyces* sp. GMKU 336 significantly enhanced plant elongation in shoot/root lengths, and plant biomass in shoot/root fresh and dry weights when compared to the un-inoculated controls (Table 5-1). Mung bean inoculated with ACCD-deficient mutant showed similar plant growth parameters to those of un-inoculated controls plants (Figure 5-1A and 1B, Table 5-1).

Under flooding condition, plants elongation and biomass significantly reduced in all plant treatments (Table 5-1). However, ACCD-producing *Streptomyces* sp. GMKU 336 inoculated mung bean showed significantly increment of shoot/root elongation and biomass (shoot/root fresh and dry weight) when compared to those of un-inoculated controls (Table 5-1). Moreover, the development of new adventitious roots were greatly accelerated by flooding stress, in particularly, plants associated with strain GMKU 336 produced more adventitious roots than other treatments (Figure 5-1B). In contrast, no development of adventitious roots were observed in non-flooding controls (Figure 15-B). Furthermore, the survival rate of un-inoculated plants was reduced 20% when compared to *Streptomyces* sp. GMKU 336 inoculated plants and those non-flooding treatments (Table 5-2). ACCD-deficient mutant inoculated plants exhibited similar trend in plant elongation and biomass to un-inoculated plants in all treatments (Figure 5-1B; Table 5-1). Therefore, ACCD-producing *Streptomyces* sp. GMKU 336 is able to promote growth of mung bean CN72 with or without flooding stress.

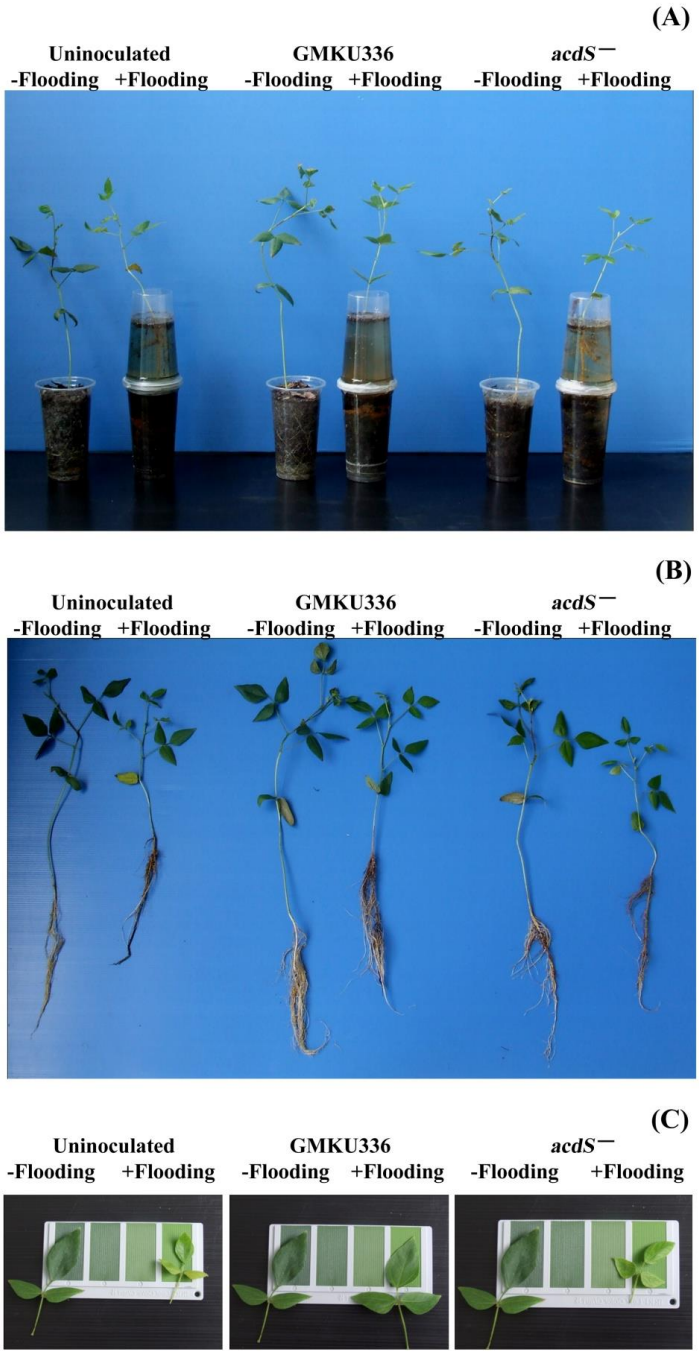

Fig. 5-1 Effect of ACCD-producing endophytic *Streptomyces* sp. GMKU 336 on mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72] after 21 days of flooding stress: (A) flooding conditions; (B) whole plants, (C) leaf size and color. Uninoculated = without bacteria treatment; GMKU336 = plant inoculated with *Streptomyces* sp. GMKU 336; acdS⁻ = plant inoculated with ACCD-deficient mutant; –Flooding = control condition; + Flooding =flooding condition (5 inch flooding).

Table 5-1 Plant growth and biomass of mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72] with and without ACCD-producing *Streptomyces* sp. GMKU 336 and ACCD-deficient mutant under flooding and non-flooding treatments

Parameters	Treatments					
	Uninoculated –Flooding	Uninoculated +Flooding	GMKU336 –Flooding	GMKU336 +Flooding	acdS⁻ –Flooding	acdS⁻ +Flooding
Shoot length (cm/plant)	44.3±0.18^{b}	25.11±0.17^{d}	46.4±0.23^{a}	27.19±0.27^{c}	43.84±0.17^{b}	23.25±0.25^{e}
Root length (cm/plant)	18.53±0.16^{b}	17.07±0.16^{d}	24.41±0.15^{a}	18.37±0.15^{b}	17.87±0.21^{c}	16.45±0.11^{e}
Shoot fresh weight (g/plant)	1.82±0.02^{b}	0.74±0.02^{d}	1.93±0.02^{a}	0.82±0.02^{c}	1.80±0.02^{b}	0.62±0.02^{e}
Root fresh weight (g/plant)	0.71±0.01^{b}	0.44±0.01^{d}	0.81±0.01^{a}	0.61±0.01^{c}	0.69±0.01^{b}	0.34±0.01^{e}
Shoot dry weight (g/plant)	0.45±0.01^{b}	0.17±0.01^{d}	0.54±0.01^{a}	0.28±0.01^{c}	0.43±0.02^{b}	0.12±0.01^{e}
Root dry weight (g/plant)	0.17±0.01^{b}	0.12±0.01^{d}	0.22±0.01^{a}	0.14±0.01^{c}	0.16±0.01^{b}	0.12±0.01^{d}

Values are mean of tree replicates ± standard error of means (n = 15). Different letters indicated statistically differences between treatments (Duncan's test $p < 0.05$). Uninoculated = without bacteria treatment; GMKU336 = plant inoculated with *Streptomyces* sp. GMKU 336; acdS⁻ = plant inoculated with ACCD-deficient mutant; –Flooding = control condition; +Flooding = flooding condition (5 inch flooding)

Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on leaf and chlorophyll

Leaf area was markedly reduced under flooding stress in all treatments (Table 5-2). A significant reduction of leaf area of un-inoculated plants and those inoculated with the ACCD-deficient mutant was about 2.5-fold when compared to the corresponding non-flooding treatments (Figure 5-1C, Table 5-2). However, leaf area reduction of mung bean associated with *Streptomyces* sp. GMKU 336 was about 1.2-fold lower than the non-flooding treatment (Figure 5-1C, Table 5-2). Furthermore, leaf color of mung bean under flooding stress was altered from dark green to yellowish green compared to non-flooding treatments (Figure 5-1C). Conversely, leaf color of mung bean associated with *Streptomyces* sp. GMKU 336 was greener than un-inoculated plants and those inoculated with the ACCD-deficient mutant (Figure 5-1C).

Table 5-2 Plant physiological parameters of mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72] with and without ACCD-producing *Streptomyces* sp. GMKU 336 and ACCD-deficient mutant under flooding and non-flooding treatments

Parameters	Treatments					
	Uninoculated –Flooding	Uninoculated +Flooding	GMKU336 –Flooding	GMKU336 +Flooding	acdS⁻ –Flooding	acdS⁻ +Flooding
Total chlorophyll (mg/g FW)	12.23±0.22[a]	6.16±0.27[c]	12.19±0.18[a]	9.34±0.27[b]	12.03±0.17[a]	5.39±0.19[c]
Leaf area (cm^2/leaf)	13.84±0.90[a]	5.77±0.63[c]	14.29±0.72[a]	11.27±1.03[b]	13.57±1.01[a]	5.16±0.34[c]
Ethylene level (pmol/h/g FW)	1.62±0.12[c]	8.58±0.52[a]	1.45±0.03[c]	2.81±0.11[b]	1.50±0.03[c]	8.03±0.38[a]
Survival rate (%)	100±0.00[a]	80±0.00[b]	100±0.00[a]	100±0.00[a]	100±0.00[a]	75.5±2.23[c]
Colonization (10^4 CFU/g root FW)	ND	ND	4.96±0.08	4.56±0.42	4.65±0.38	4.56±0.43

Values are mean of tree replicates ± standard error of means (n = 15). Different letters indicated statistically differences between treatments (Duncan's test $p < 0.05$). Uninoculated = without bacteria treatment; GMKU336 = plant inoclated with *Streptomyces* sp. GMKU 336; acdS⁻ = plant inoculated with ACCD-deficient mutant; –Flooding = control condition; +Flooding = flooding condition (5 inch flooding); FW = fresh weight; ND = not detected.

Chlorophyll content in mung bean was equally determined in all treatments under normal condition, but was significantly decreased under flooding treatments (Table 5-2). A significant reduction of 2-2.3 folds of chlorophyll content was observed in un-inoculated mung bean and those inoculated with the ACCD-deficient mutant when compared to non-flooding controls (Table 5-2). However, *Streptomyces* sp. GMKU 336 inoculated plants maintained 1.5-1.7 folds higher chlorophyll content than those of un-inoculated and ACCD-deficient mutant inoculated plants (Table 5-2). The results suggested that ACCD-producing *Streptomyces* sp. GMKU 336 enhances leaf area and leaf color as well as chlorophyll content of mung bean under flooding stress.

Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on ethylene production

Under non-flooding condition, low ethylene levels were determined in all plant treatments (Table 5-2). The ethylene level was drastically increased 5.3-fold after flooding stress exposure in un-inoculated mung bean and those inoculated with the ACCD-deficient mutant when compared to the corresponding non-flooding controls. However, *Streptomyces* sp. GMKU 336 inoculated

plants significantly maintain ethylene level 3-fold lower than the un-inoculated control (Table 5-2).

Re-isolation of the endophytic streptomycetes from inoculated mung bean

Re-isolation of wild type and mutant in both flooding and non-flooding treatments was about 10^4 CFU/g root fresh weight (Table 5-2). Both strains were validated by 16S rRNA gene sequencing (data not shown). In addition, un-inoculated plants did not harbor endophytic *Streptomyces* sp. GMKU 336.

Discussion

ACCD-producing bacteria enhance the growth of a wide range of plants in the presence of various biotic and abiotic stresses, including pathogen damage, flooding, drought, salt and organic and inorganic contaminants (Glick, 2004). Recently, *Streptomyces* sp. GMKU 336 has been reported to promote plant growth under salinity stress via the action of ACCD (Jaemsaeng *et al*., 2018). Here we report further work to define the role of ACCD on plant growth and flooding tolerance of mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72] by strain GMKU 336 and its ACCD-deficient mutant.

Streptomyces sp. GMKU 336 has been proved as a true endophyte since it was able to localize and multiply within rice (*Oryza sativa* L. cv. KDML105) without causing any obviously deleterious effects (Jaemsaeng *et al*., 2018). In this work, both *Streptomyces* sp. GMKU 336 and its ACCD-deficient mutant were inoculated into mung bean CN72 and were able to re-isolate from the plants in every conditions. Although, *Streptomyces* sp. GMKU 336 was originally isolated from medicinal plant, *Clerodendrum serratum* (L.) Moon (Indananda, 2013), the results indicated that it has endophytic ability with wide range host both monocot and dicot plants. The results are in agreement with previous report that an endophytic *Streptomyces* sp. GMKU 3100 that originally isolated from rice was able to neutrally colonize in mung bean (Rungin *et al*., 2012). It is clearly indicated that endophytic actinomycetes are none host specific and behave as free-living bacteria. It was suggested that endophytic bacteria may migrate from rhizosphere to rhizoplane of their plant hosts and then move to other organs such as roots, stems, leaves, flowers as well as fruits and seeds (Misk and Franco, 2011).

The effect of ACCD-producing *Streptomyces* sp. GMKU 336 and its ACCD-deficient mutant on plant growth were investigated in mung bean CN72 after flooding treatment for 21 days. Under non-flooding conditions, mung bean inoculated with strain GMKU 336 significantly

enhanced shoot/root length and biomass when compared to un-inoculated plants and those inoculated with the ACCD-deficient mutant. The results for plant growth were consistent with previous work showing that ACCD-producing *Streptomyces* sp. GMKU 336 have an ability to enhance growth of rice KDML105 (Jaemsaeng *et al*., 2018). The growth effect has also been found in other ACCD-producing *Streptomyces* that enhanced growth of *Arabidopsis* (Palaniyandi *et al*., 2013), halophytic *Limonium sinense* (Qin *et al*., 2014), sugarcane (Kruasuwan and Thamchaipenet, 2016), and tomato (El-Tarabily, 2008).

ACCD-producing *Streptomyces* sp. GMKU 336 inoculated mung bean substantially tolerated to flooding stress, it maintained high shoot and root elongation when compared to un-inoculated plants and those inoculated with the ACCD-deficient mutant. The results were in agreement with previous work that an ACCD-producing *Pseudomonas* increased shoot and root growth of tomato (Grichko and Glick, 2001) and canola (Farwell *et al*., 2007) under flooding conditions.

The decline in photosynthesis under flooding has been reported in mung bean (Ahmed *et al*., 2002). Here, leaf chlorophyll content drastically decreased in flooding plants compared to non-flooding ones. However, ACCD-producing *Streptomyces* sp. GMKU 336 reduced this negative effect, and maintained the greener leaf color than the un-inoculated flooding controls. The results are in agreement with previous reports that treatment of ACCD-producing bacteria such as *Achromobacter*, *Herbaspirillum*, *Serratia*, and *Ochrobactrum* on *Ocimum sanctum* (Barnawal *et al*., 2012) and *Pseudomonas* on tomato (Grichko and Glick, 2001) increased photosynthetic rates which leading to some protection against flooding stress.

Morphological adaptation by reducing leaf area and induced formation of adventitious roots improved flooding tolerance in mung bean (Ahmed *et al*., 2002). Here we report that flooding treatment also resulted in decreasing of leaf area and affected the plant to root rot that was observed in un-inoculated plants and those inoculated with the ACCD-deficient mutant. By contrast, mung bean inoculated with ACCD-producing *Streptomyces* sp. GMKU 336 maintained leaf area and increased adventitious root development. Likewise, ACCD-producing *Pseudomonas* clearly stimulated the development of both adventitious roots and stem in tomato under flooding stress (Grichko and Glick, 2001).

Flooding causes an increase in accumulation of stress ethylene in plants. Plants inoculated with ACCD-producing PGPB have been documented to show substantial tolerance to flooding stress by lowering stress ethylene (Glick, 2004; Saleem *et al*., 2007). In this work, significant induction of ethylene production was observed in all plant treatments under flooding stress.

Remarkably, mung bean CN72 inoculated with *Streptomyces* sp. GMKU 336 showed lower increment in ethylene level. The results suggested that ACCD-producing *Streptomyces* sp. GMKU 336 decreased ethylene production of mung bean via an action of ACCD. We have recently reported that *Streptomyces* sp. GMKU 336 associated with rice KDML105 that exposed to salt stress significantly expressed *acdS* gene *in vivo* which converted a precursor of ethylene, ACC, in plants into ammonia and α-ketobutyrate, and consequently reduced ethylene, reactive oxygen species (ROS), Na^+ content and Na^+/K^+ ratio (Jaemsaeng *et al*., 2018). Therefore, it is implied that *Streptomyces* sp. GMKU 336 enhances flooding tolerance in mung bean in the same manner as salt tolerance in rice.

In conclusion, all of the experimental data indicated that ACCD-producing endophytic *Streptomyces* sp. GMKU 336 promoted growth of mung bean CN72 both under normal and flooding conditions. The presence of *Streptomyces* sp. GMKU 336 also enhances flooding tolerance in mung bean CN72 by decreasing ethylene production and increasing plant growth and biomass, chlorophyll content, leaf area, leaf color, formation of adventitious roots. Noticeably, plants inoculated with the ACCD-deficient mutant exhibited plant growth parameters and physiology in the same manner as those of un-inoculated controls. These results clearly indicated the beneficial effects of ACCD-producing endophytic *Streptomyces* sp. GMKU 336 on plant growth promotion in various stress tolerance including salinity and flooding.

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Section 6

Modulation of salt tolerance in Thai jasmine rice (*Oryza sativa* L. cv. KDML105) by *Streptomyces venezuelae* ATCC 10712 expressing ACC deaminase

Introduction

Soil salinity in arid regions is often an important limiting factor for cultivation of agricultural crops such as maize, rice, and sugarcane. Excess of salt affects plant growth by increasing stress factors, such as ethylene production, Na^+ accumulation, and reactive oxygen species (ROS) which is detrimental to the plant's physiology, leading to growth impairment[1-3].

Streptomycetes have been recognized recently as plant growth promoting (PGP) bacteria that can protect plants from infectious diseases and enhance plant growth through several PGP-traits, such as siderophore production, plant hormone production, and phosphate solubilization[4-6]. Furthermore, PGP-bacteria assist plants to grow under severe condition caused by drought, flooding, salinity, and phytopathogens by the action of 1-aminocyclopropane-1-carboxylate (ACC) deaminase[7-11]. ACC deaminase, encoded by the *acdS* gene, is responsible for the breakdown of ACC, which is the direct precursor of ethylene in all higher plants, into ammonia and α-ketobutyrate - which bacteria consume as nitrogen and carbon sources[12]. Overexpression of *acdS* in endophytic bacteria remarkably improved plant growth and alleviated stresses in plants, when compared to uninoculated plants and those of wild type inoculation. For example, ACC deaminase-overproducing strains of *Pseudomonas putida* ameliorated flooding stress in tomato[13], *Sinorhizobium meliloti* improved growth and copper tolerance in *Medicago lupulina*[14], and *Serratia grimesii* enhanced growth and the level of plant protection against seed-borne pathogens in the common bean[15].

Streptomyces venezuelae was discovered from soil and, thus far, has been known as a cell factory for the production of diverse natural products including chloramphenicol, watasemycin, and venemycin[16-18]. Although the genome sequence of *S. venezuelae* was determined and characterized[19], the information was used mainly for investigation of gene clusters involved in antibiotic biosynthesis. The genome sequence has never been inspected for a role of plant-beneficial functions; likewise, *S. venezuelae* has never been documented as a PGP-endophytic bacterium. Recently, genes contributing to PGP-traits including *acdS* were not only present in genome sequences of PGP-rhizobacteria (PGPR) but also found in those of non-PGPR[20,21].

On this basis, we examined genes related to PGP-function in all genome sequences of members of a genus *Streptomyces* available in the GenBank database, in particular *acdS*.

Surprisingly, *acdS* was present in many genomes of non-PGP-endophytic *Streptomyces*, including *S. venezuelae*. To address the possible beneficial role of *S. venezuelae* interacting beneficially with plants and modulating salt stress, *S. venezuelae* was inoculated into the salt-sensitive Thai jasmine rice KDML105 cultivar. Furthermore, the effects of overexpression of *acdS* within *S. venezuelae* towards rice growth and salt tolerance were investigated. The physiology of rice associated with *S. venezuelae* and its overexpressed mutant under salt stress condition are discussed.

Materials and Methods

Bacterial salt tolerance and plant growth promoting (PGP) traits

Streptomyces venezuelae ATCC 10712 was grown and maintained on mannitol soybean agar (MS)[45]. Salt tolerance was determined by growth of colonies on ISP 2 (Difco™) supplemented with 1–4% NaCl (w/v) at 28 °C for 7 days.

Proline accumulation was determined by growing *S. venezuelae* in 10 mL tryptic soy broth (TSB) supplemented with 1–3% NaCl at 28 °C for 3 days. Cells were treated with 2 mL 20% trichloroacetic acid, mixed and centrifuged. The aqueous solution was mixed with 2 mL ninhydrin solution (1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid) and 2 mL glacial acetic acid, and incubated at 95 °C for 1 h, then cooled on ice. The reaction mixture was extracted and mixed vigorously with 4 mL toluene for 15–20 sec. The absorbance of the red-colored organic layer of the ninhydrin-proline complex was measured at 520 nm by spectrophotometry. Proline concentration was determined from a standard curve of commercial proline and calculated as described by Bates, et al.[46].

Indole-3-acetic acid (IAA) was determined by a colorimetric method[47]. *S. venezuelae* was grown in the dark in glucose-beef extract broth supplemented with 10 mM L-tryptophan at 28 °C for 7 days. The culture was then centrifuged and 2 mL of supernatant was mixed with 1 mL of Salkowski's reagent[48]. The mixture was left at room temperature for 30 min in the dark. IAA production was indicated by development of a pink-red color.

ACC deaminase activity was monitored by the amount of α-ketobutyrate generated from ACC cleavage as described by Penrose and Glick[49]. *S. venezuelae* was cultured in TSB and washed twice before transferring onto minimal medium (MM) containing 3 mM ACC as a sole source of nitrogen and incubated on a rotary shaker in the dark for 0, 24, 48, 72 and 98 h. The amount of α-ketobutyrate was determined by measuring absorbance at 540 nm and comparing to a standard curve of α-ketobutyrate. Protein content was performed according to Bradford[50]. ACC deaminase activity was expressed as α-ketobutyrate production in nmol mg^{-1} protein h^{-1}.

Construction of ACC deaminase-overexpressing mutant

The ACC deaminase gene (*acdS*) (SVEN_RS07535) was retrieved from the genome sequence of *S. venezuelae* ATCC 10712 (Accession no. NC_018750). Specific primers for amplification of *acdS* were designed as ATT151F (5′-TTTTTTAAGCTTGAGATGACGGCGATGGGCGAGTT-3′) and ATT151R (5′-TTTTTTCATATGCCGACCAGCAGCCGTCACTCAAC-3′) including respectively *Hind*III and *Nde*I sites (underlined). PCR conditions were initially 98 °C, 30 sec; and 30 cycles of 98 °C, 10 sec; 69 °C, 30 sec; 72 °C, 1 min; and finally at 72 °C, 10 min. The PCR product was then cloned into the pJET cloning vector (Fermentas, USA) and subcloned into constitutive multi-copy expression plasmid pIJ86 under *ermE** promoter[51] to obtain pIJ86-*acdS*. Next, pIJ86-*acdS* was transformed into *E. coli* ET12567/pUZ8002[52] and intergeneric conjugation was performed using 24-h mycelium of *S. venezuelae* as described Vitayakritsirikul, et al.[23]. Exconjugants (*S. venezuelae*/pIJ86-*acdS*) were selected by apramycin (100 μg mL^{-1}) and thiostrepton (50 μg mL^{-1}) resistance, and verified by (i) PCR amplification of the thiostrepton resistance gene using primers and conditions as described previously Rungin, et al. [5] and (ii) ACC deaminase activity. *S. venezuelae*/pIJ86 was also constructed as a control.

RNA purification and semi-quantitative RT-PCR

S. venezuelae/pIJ86 and *S. venezuelae*/pIJ86-*acdS* were grown in TSB for 24 h, then harvested by centrifugation, washed twice with 0.1 M Tris-HCl (pH 8.5) and inoculated onto MM medium containing 3 mM ACC and incubated for 72 h. Total RNA was isolated using TRIzol (Ambion, USA) and treated with RNase-free DNase I according to the manufacturer's protocol (Thermo Fisher Scientific, USA). cDNA was synthesized using a RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Semi-quantitative RT-PCR analysis of *acdS* gene was performed using cDNA and primers, ATT165F (5′- CGGGTGATCTGCTCGTGGGTCGGTA-3′) and ATT165R (5′-GCGGGCTTCGGCATCGGCTT-3′), using Phusion Hot Start II-High Fidelity DNA polymerase (Thermo Fisher Scientific, USA). PCR conditions started with 98 °C, 30 sec; and 30 cycles of 98 °C, 10 sec; 58 °C, 30 sec; 72 °C, 1 min; and finally at 72 °C, 10 min. The expression level of *acdS* was quantified by Gel Doc™ XR + with Image Lab™ Software (Biorad, USA) and normalized against the expression of a housekeeping gene, *hrdB*[53].

Analysis of rice growth parameters

Thai jasmine rice seeds (*Oryza sativa* L. cv. KDML105) were surface sterilized by 70% (v/v) ethanol for 1 min followed by 15 min in 5% (w/v) sodium hypochlorite and thoroughly rinsed

with sterile distilled water before transferring into a sterile moist chamber and incubated at room temperature in the dark for 7 days. Roots of seedlings were cut into the same length and individually immersed into sterile glass beakers containing 10^8 spores mL^{-1} of either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* and incubated for 24 h. Seedlings were then re-located to a moist sponge support for 1 day before transferring to a 20-L container filled with half-Yoshida solution (YS)[54] for 14 days. Next, salt stress was introduced by replacing the nutrient solution with YS supplemented with 150 mM NaCl and further incubated for 7 days. The pH of nutrient solution was maintained between 5.0–5.5 throughout the growth period. A positive control of non-salt stressed rice was grown under the same conditions without NaCl treatment. Growth parameters of non-salt and salt-stressed rice plants at 7 days were determined for root and shoot lengths, fresh (FW) and dry (DW) weights.

Analysis of ethylene level and proline accumulation

Ethylene emission was analyzed by the method of Cristescu, et al.[55]. 7-day rice plants were placed in a 550 mL bottle tightly sealed with a rubber septum and left for 1 h. Fifty millilitres of headspace air was sampled and analyzed for ethylene by gas chromatography (GC 7890A, Agilent Technologies, USA) packed with a Poropak-N column at 60 °C, equipped with a flame ionization detector. The amount of ethylene emission was calculated as nmol of ethylene g^{-1} FW h^{-1} by comparison to a standard curve generated with pure ethylene.

For proline content, fresh leaf samples (50 mg) were immediately homogenized with liquid nitrogen. The powder was mixed with 3% (v/v) sulfosalicylic acid and centrifuged. The aqueous solution was mixed with ninhydrin solution and glacial acetic acid following the protocol described above.

Analysis of total chlorophyll and relative water content (RWC)

Total chlorophyll was measured according to the method of Porra, et al.[56]. Fresh leaf samples (50 mg) were immediately homogenized with liquid nitrogen. The powder was dissolved in DMSO and centrifuged at 4 °C for 10 min. Absorbance was measured at 645 and 663 nm by spectrophotometry. Total chlorophyll content was calculated based on chlorophyll equations of Arnon[57].

RWC was determined according to the method of Mostofa and Fujita[58]. Leaf fresh weight was measured and soaked in distilled water for 6 h to determine a turgid weight. The leaves were

then dried at 60 °C for 72 h to determine a dry weight. RWC was calculated from each weigh according to Smart and Bingham [59].

Determination of Na^+ and K^+ contents

Na^+ and K^+ contents were analyzed using an atomic absorption spectrophotometer according to the method of Johnson and Ulrich[60] at The Soil-Fertilizer-Environment Scientific Development Project, Department of Soil Science, Faculty of Agriculture, Kasetsart University. The concentrations of Na^+ and K^+ were quantified and calculated as mg g^{-1} DW.

Analysis of lipid peroxidation and reactive oxygen species (ROS) staining

Lipid peroxidation of leaf samples was estimated by measuring the amount of malondialdehyde (MDA) by a colorimetric method[61]. Fresh leaf samples (50 mg) were immediately homogenized with liquid nitrogen and mixed with 80% ethanol followed by centrifugation. The aqueous solution was mixed with either (i) –TBA solution [20% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene], or (ii) +TBA solution (0.65% TBA in –TBA solution). Samples were mixed vigorously and heated at 95 °C for 1 h, cooled on ice and centrifuged. The TBA-MDA complex absorbance was measured at 400, 523 and 600 nm by spectrophotometry. The MDA level was calculated as described by Hodges, et al.[61].

ROS staining of leaf samples was detected using nitrotetrazolium blue chloride (NBT) and 3,3′-diaminobenzidine (DAB) for superoxide and hydrogen peroxide, respectively following the protocol described by Kumar, et al.[62]. Leaf samples were separately immersed in 25 mL 2.5 mM NBT staining solution (pH 7.5) and 5 mM DAB staining solution (pH 3.8) for 24 h at room temperature in the dark. The leaves were then decolorized by boiling in 95% (v/v) ethanol for 30 min and further immersed in 60% glycerol for 16 h before color detection.

Statistical analysis

Data were subjected to statistical analysis using standard ANOVA and Tukey's multiple range tests of SPSS (version 18.0). Data were presented as mean ± S.E. calculated from four plants per treatment in three different replicates, with a different letter indicating statistical significance at $p<0.05$. ACC deaminase activity and gene expression ratio data were analysed statistically using a t test at $p<0.05$. The values represented the mean ± S.E. of three replicates and an asterisk represents a statistically-significant change in expression.

Results

Salt tolerance and PGP-traits of *S. venezuelae*

Analysis of salt tolerance of *S. venezuelae* ATCC 10712 revealed that it had tolerated NaCl up to 3% (w/v). During growth in 3% NaCl, proline was accumulated significantly, at 36.66 ± 0.24 µM in cells (Table 6-1). Moreover, *S. venezuelae* had ACC deaminase activity of 364.21 ± 19.28 nmol α-ketobutyrate mg protein^{-1} h^{-1} and produced IAA at 21 ± 1.02 µg mL^{-1} (Table 6-1).

Table 6-1 Plant growth promoting (PGP) traits of *Streptomyces venezuelae*

PGP-traits	
ACC deaminase	364.21 ± 19.28 nmol α-keto.mg protein^{-1}.h^{-1}
IAA production	21 ± 1.02 µg.mL^{-1}
Proline accumulation	
0% NaCl	21.12 ± 0.30 µM
1% NaCl	25.71 ± 0.39 µM
2% NaCl	30.33 ± 0.59 µM
3% NaCl	36.66 ± 0.24 µM

Characterization of ACC deaminase-overexpressing *S. venezuelae*

An ACC deaminase-overexpressing mutant, *S. venezuelae*/pIJ86-*acdS*, was constructed and verified by resistance to apramycin and thiostrepton. The wild type with empty plasmid, *S. venezuelae*/pIJ86, was also constructed as a control. In comparison with *S. venezuelae*/pIJ86, ACC deaminase activity of *S. venezuelae*/pIJ86-*acdS* was enhanced 5-fold from 72–96 h of incubation in MM containing 3 mM ACC (Fig. 6-1a, Table 6-2). This result correlated with the high expression profile of *acdS* by *S. venezuelae*/pIJ86-*acdS* at 72 h (2.7-fold) when compared to that of wild type control (Fig. 6-1b, Table 6-3). The ACC deaminase activity of *S. venezuelae*/pIJ86-*acdS* were relatively stable when re-streaked for up to 5 generations without antibiotic selection (data not shown).

Plant colonization and growth promotion by *S. venezuelae*

S. venezuelae/pIJ86 was successfully inoculated into Thai jasmine rice cv. KDML105 grown under hydroponic condition with and without salt treatment. *S. venezuelae*/pIJ86 was re-isolated from rice under both treatments at about 10^4 CFU g root fresh weight^{-1} (Table 6-4)

indicating that *S. venezuelae* had the ability to colonize inside plants. In addition, un-inoculated plants did not harbor any streptomycete (data not shown) showing that rice seeds were surface sterilized effectively, and the hydroponic conditions used in this study were free from contamination. Growth parameters of inoculated and uninoculated rice KDML105 were evaluated at 7 days after being treated with and without 150 mM NaCl. In comparison to uninoculated plants, rice associated with *S. venezuelae*/pIJ86 had significant increases of shoot/root lengths and shoot/root fresh/dry weights in both non-salt and salt treatments (Figs. 6-2a-f).

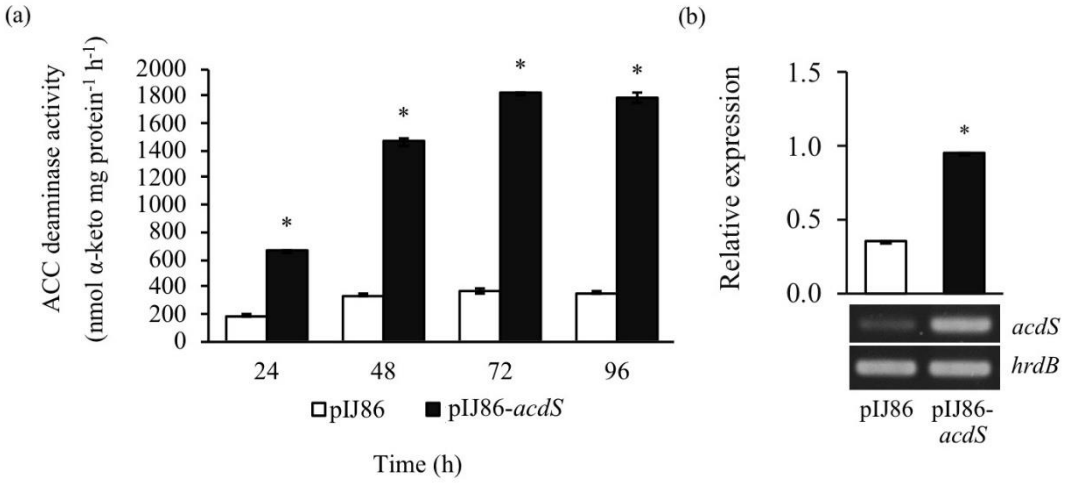

Fig. 6-1 ACC deaminase activity (a) and semi-quantitative RT-PCR analysis of expression of *acdS* (b) of *S. venezuelae*/pIJ86 (pIJ86) and *S. venezuelae*/pIJ86-*acdS* (pIJ86-*acdS*). The values represent the mean ± S.E. of three replicates and an asterisk (*) indicate statistically significant changes in expression (t test, $p<0.05$).

Table 6-2 ACC deaminase activity of *Streptomyces venezuelae* and its overexpressing mutant grown in MM supplemented with 0.3 mM ACC at 24, 48, 72 and 96 h. The values show the mean ± S.E. of three replicates and an asterisk (*) represents statistically significant different activity (t test, $p<0.05$). pIJ86, *S. venezuelae*/pIJ86; pIJ86-*acdS*, *S. venezuelae*/pIJ86-*acdS*.

Strain	ACC deaminase activity (nmol α-keto mg protein^{-1} h^{-1})			
	24 h	48 h	72 h	96 h
pIJ86	189.18 ± 10.98	334.70 ± 14.02	364.21 ± 19.28	355.21 ± 12.28
pIJ86-*acdS*	659.68 ± 11.48*	1,465.68 ± 25.34*	1,820.10 ± 13.05*	1,783.10 ± 40.05*

Table 6-3 Semi-quantitative RT-PCR expression profile of *acdS* in *S. venezuelae*/pIJ86 (pIJ86) and *S. venezuelae*/pIJ86 (pIJ86-*acdS*). The expression ratio of *acdS* gene was normalized against the expression of a housekeeping gene, *hrdB*. The values show the mean ± S.E. of three replicates and an asterisk (*) represents statistically-significant different activity (t test, $p<0.05$).

Strain	Expression ratio
pIJ86	0.351 ± 0.001
pIJ86-*acdS*	0.950 ± 0.006*

Effect of overexpression of ACC deaminase on plant growth parameters

Growth parameters including shoot/root length and shoot/root fresh/dry weights of rice KDML105 inoculated with *S. venezuelae*/pIJ86 were enhanced significantly, when compared with uninoculated plants in both non-salt and salt treatments (Figs. 6-2a-f, Supplementary Fig. 6-3). Similar to the original strain, its overexpressing mutant, *S. venezuelae*/pIJ86-*acdS*, greatly promoted growth of rice in both non-salt and salt stress conditions (Figs. 6-2a-f, Fig. 6-3), but highly increased shoot length and biomass in particular more than those inoculated with wild type control under salt stress conditions (Figs. 6-2a-c).

Effect of overexpression of ACC deaminase on plant ethylene

At 7 days after irrigation with 150 mM NaCl, the ethylene level of uninoculated plants was increased about 2-fold when compared with those grown without salt (Fig. 6-4a, Table 6-4). When rice was associated with *S. venezuelae*/pIJ86, the ethylene level was reduced 1.6-fold when compared to uninoculated plants (Fig. 6-4a, Table 6-4). When rice was inoculated with *S. venezuelae*/pIJ86-*acdS*, the ethylene level was decreased 2-fold when compared to uninoculated plants (Fig. 6-4a, Table 6-4). The results indicated that overexpression of ACC deaminase facilitated salt tolerance in plants by reduction of ethylene to the same level as that of the non-salt treatment control.

Effect of overexpression of ACC deaminase on proline content

Under non-salt conditions, proline content was unaffected in rice KDML105 inoculated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* (Fig. 6-4b). Under salt-stress conditions, the proline content was 3.4-fold higher in uninoculated plants compared to those grown in non-salt conditions (Fig. 6-4b, Table 6-4). Nonetheless, the proline content of plants associated

with *S. venezuelae*/ pIJ86 was increased significantly compared to the uninoculated control and even higher in plants inoculated with *S. venezuelae*/pIJ86-*acdS* (Fig. 6-4b). The results demonstrated that overexpression of ACC deaminase facilitated salt tolerance in rice by escalation of proline content.

Table 6-4 Plant growth parameters of rice KDML105 associated with and without ACC deaminase-producing *Streptomyces venezuelae* and the ACC deaminase-overexpressing mutant under non-salt and salt stress treatments. The values show the mean ± S.E. of twelve replicates and different letters are significantly different (Tukey's test, $p<0.05$). C, uninoculated rice control; pIJ86, rice inoculated with *S. venezuelae/*pIJ86; pIJ86-*acdS*, rice inoculated with *S. venezuelae*/pIJ86-*acdS*; ND, not detected.

Plant growth parameters	0 mM NaCl			150 mM NaCl		
	C	pIJ86	pIJ86-*acdS*	C	pIJ86	pIJ86-*acdS*
Length (cm)						
Shoot	67.17 ± 1.06^{bc}	78.75 ± 2.54^{a}	80.75 ± 1.78^{a}	59.92 ± 1.29^{d}	66.25 ± 0.39^{c}	72.58 ± 0.50^{b}
Root	19.08 ± 0.66^{ab}	20.75 ± 0.60^{a}	20.50 ± 0.44^{a}	15.08 ± 0.29^{d}	16.92 ± 0.23^{c}	18.00 ± 0.17^{bc}
Fresh weight (g plant^{-1})						
Shoot	1.09 ± 0.04^{b}	1.62 ± 0.06^{a}	1.56 ± 0.04^{a}	0.52 ± 0.03^{d}	0.84 ± 0.02^{c}	1.07 ± 0.02^{b}
Root	0.18 ± 0.00^{b}	0.22 ± 0.01^{a}	0.22 ± 0.01^{a}	0.13 ± 0.01^{c}	0.21 ± 0.01^{a}	0.02 ± 0.01^{a}
Dry weight (g plant^{-1})						
Shoot	0.16 ± 0.01^{b}	0.23 ± 0.01^{a}	0.23 ± 0.01^{a}	0.11 ± 0.01^{c}	0.17 ± 0.005^{b}	0.20 ± 0.00^{a}
Root	0.03 ± 0.00^{c}	0.04 ± 0.00^{a}	0.04 ± 0.00^{a}	0.02 ± 0.00^{d}	0.03 ± 0.00^{bc}	0.04 ± 0.00^{ab}
Relative water content (%)	96.06 ± 1.14^{a}	96.87 ± 1.01^{a}	96.79 ± 1.08^{a}	64.11 ± 2.39^{c}	89.08 ± 1.81^{b}	92.93 ± 0.66^{b}
Ethylene emission (µmoles g^{-1} FW h^{-1})	0.92 ± 0.04^{c}	0.69 ± 0.02^{d}	0.68 ± 0.03^{d}	1.98 ± 0.10^{a}	1.24 ± 0.07^{b}	0.93 ± 0.04^{c}
Proline content (µmole g^{-1} FW)	35.50 ± 1.58^{d}	34.66 ± 1.28^{d}	39.33 ± 3.34^{d}	119.85 ± 4.25^{c}	144.68 ± 7.58^{b}	168.29 ± 9.07^{a}
Total chlorophyll (mg g^{-1} FW)	0.10 ± 0.02^{d}	0.17 ± 0.03^{a}	0.17 ± 0.00^{a}	0.13 ± 0.00^{c}	0.15 ± 0.01^{b}	0.15 ± 0.00^{b}
MDA (nmol g^{-1} FW)	28.79 ± 1.50^{c}	27.48 ± 1.23^{c}	28.21 ± 1.26^{c}	42.63 ± 1.10^{a}	33.45 ± 1.38^{b}	32.88 ± 1.77^{b}
Na^{+}content (mg g^{-1} DW)	0.63 ± 0.09^{c}	0.70 ± 0.03^{c}	0.77 ± 0.03^{c}	35.53 ± 3.94^{a}	27.83 ± 5.76^{ab}	24.83 ± 2.18^{b}
K^{+}content (mg g^{-1} DW)	29.00 ± 6.14^{b}	38.83 ± 3.99^{a}	40.40 ± 4.30^{a}	12.50 ± 2.15^{c}	27.47 ± 1.83^{b}	33.00 ± 2.96^{ab}
CFU g root FW^{-1}	ND	2.45×10^{4}	6.03×10^{3}	ND	7.19×10^{4}	2.74×10^{4}

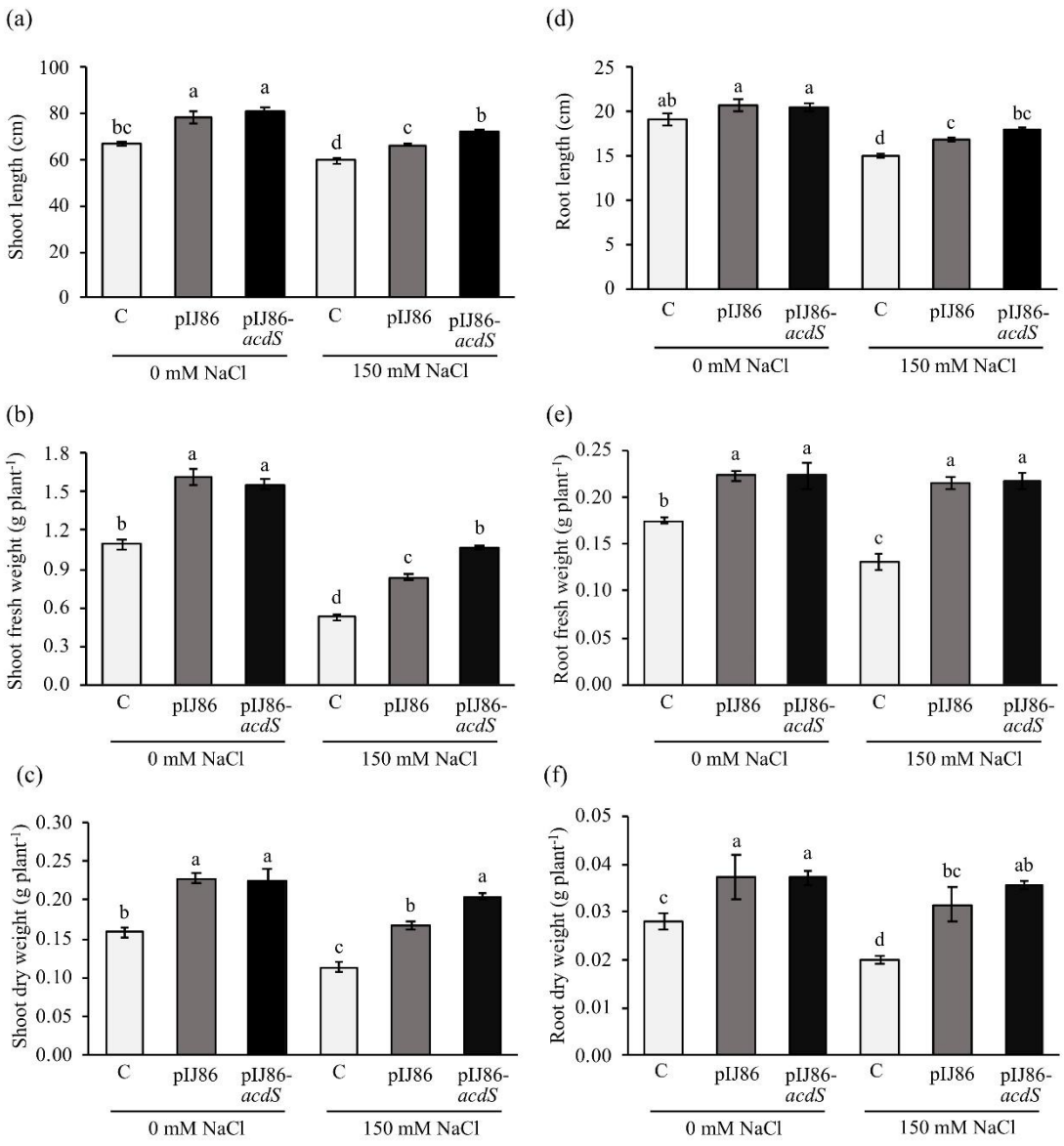

Fig. 6-2 Effect of ACC deaminase-producing *Streptomyces venezuelae* on shoot length (a), shoot fresh weight (b), shoot fresh weight (c), root length (d), root fresh weight (e), and root dry weight (f) of rice plants under non-salt (0 mM NaCl) and salt stress (150 mM NaCl) conditions. The values show the mean ± S.E. of twelve replicates and bars carrying different letters are significantly different (Tukey's test, $p<0.05$). C, uninoculated rice control; pIJ86, rice inoculated with *S. venezuelae*/pIJ86; pIJ86-*acdS*, rice inoculated with *S. venezuelae*/pIJ86-*acdS*.

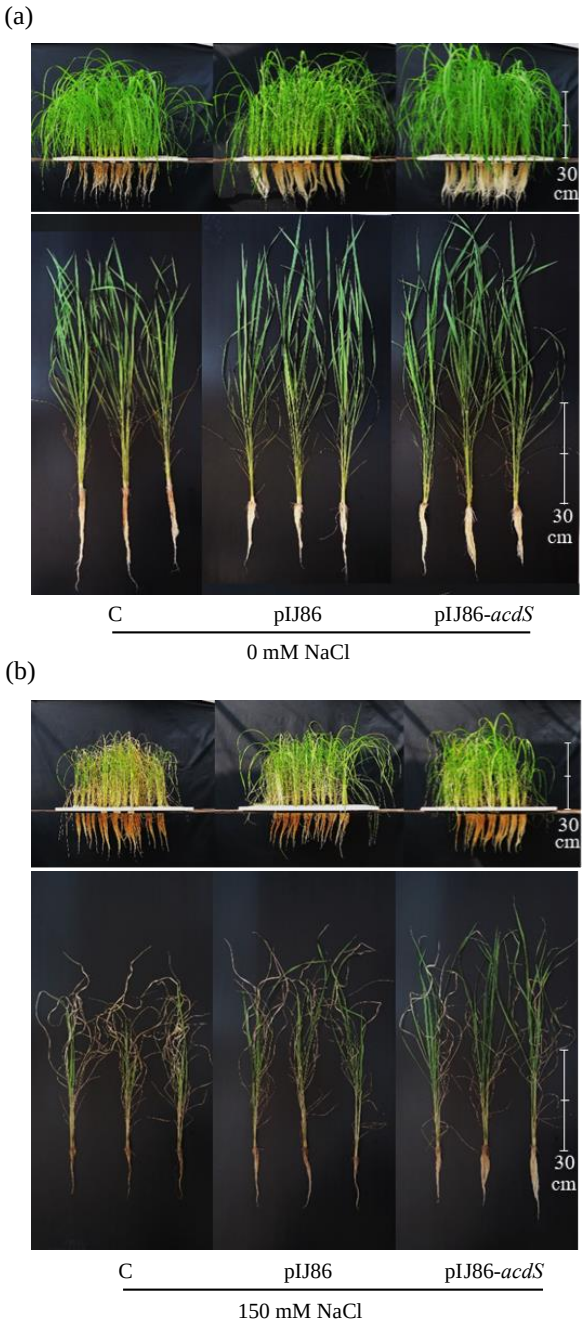

Fig. 6-3 Effect of ACC deaminase-producing *Streptomyces venezuelae* and its overexpressing mutant on the physiology of rice KDML105 after 7 days of non-salt (a) and salt stress (b) treatments under hydroponic conditions. C, uninoculated rice control; pIJ86, rice inoculated with *S. venezulae*/pIJ86; pIJ86-*acdS*, rice inoculated with *S. venezulae*/pIJ86-*acdS*.

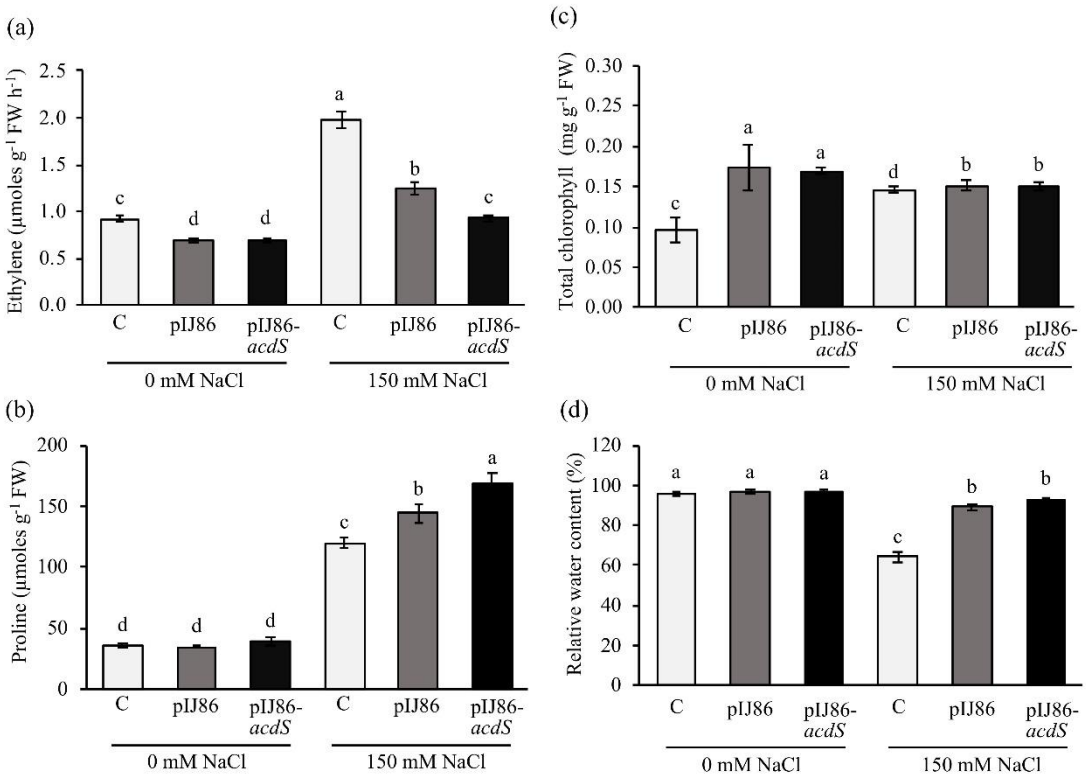

Fig. 6-4 Effect of ACC deaminase-producing *Streptomyces venezuelae* on ethylene (a), proline (b), total chlorophyll (c), and relative water contents (RWC) (d) of rice plants under non-salt (0 mM NaCl) and salt stress (150 mM NaCl) conditions. The values show the mean ± S.E. of twelve replicates and bars carrying different letters are significantly different (Tukey's test, $p<0.05$). C, uninoculated rice control; pIJ86, rice inoculated with *S. venezuelae*/pIJ86; pIJ86-*acdS*, rice inoculated with *S. venezuelae*/pIJ86-*acdS*.

Effect of overexpression of ACC deaminase on total chlorophyll and RWC

The total chlorophyll content of rice KDML105 inoculated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* (1.8-fold) was augmented significantly when compared to uninoculated plants under non-salt conditions (Fig. 6-4c, Table 6-4). Under salt stress conditions, rice associated with either the wild-type control or the ACC deaminase-overexpressing mutant maintained a higher chlorophyll content compared to that of uninoculated rice (Fig. 6-4c). RWC of uninoculated plants under salt treatment was 1.5-fold decreased when compared to untreated controls (Fig. 6-4d, Table 6-4). Significantly, RWC in rice associated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* was 1.4-fold higher when compared to the uninoculated control. The results suggested that *S. venezuelae* induced salt tolerance in rice by elevation of chlorophyll content and RWC.

Effect of overexpression of ACC deaminase on Na^+ and K^+ contents

When rice was grown under salt stress conditions, Na^+ was accumulated up to 56-fold compared to that of non-salt treatment (Fig. 6-5a, Table 6-4). Significantly, the Na^+ content in rice inoculated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* decreased about 1.4-fold when compared to uninoculated rice (Fig. 6-5a, Table 6-4). On the contrary, the K^+ content decreased (2.3-fold) when rice was grown under salt stress conditions (Fig. 6-5b). However, rice inoculated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* had significantly increased K^+ content under both non-salt and salt treatments (Fig. 6-5b, Table 6-4). Markedly, rice inoculated with the ACC deaminase-overexpressing mutant had the highest significant increase in K^+ content by 2.6-fold when compared to the uninoculated control under salt stress conditions (Fig. 6-5b, Table 6-4). The results demonstrated that overexpression of ACC deaminase helped salt tolerance in rice by reduction of Na^+ content, and increase in K^+ content.

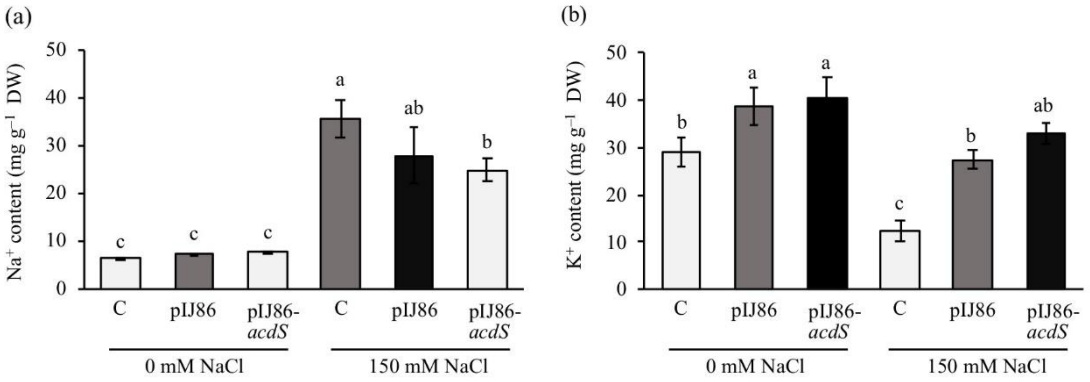

Fig. 6-5 Effect of ACC deaminase-producing *Streptomyces venezuelae* on Na^+ (a) and K^+ (b) contents of rice plants under non-salt (0 mM NaCl) and salt stress (150 mM NaCl) conditions. The values show the mean ± S.E. of twelve replicates and bars carrying different letters are significantly different (Tukey's test, $p<0.05$). C, uninoculated rice control; pIJ86, rice inoculated with *S. venezuelae/*pIJ86; pIJ86-*acdS*, rice inoculated with *S. venezuelae*/pIJ86-*acdS*.

Effect of overexpression of ACC deaminase on ROS

Salt stress drastically induced lipid peroxidation. The MDA content was increased up to 1.5-fold in rice grown under salt stress conditions (Fig. 6-6a, Table 6-4). However, rice KDML105 inoculated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* had a significant reduction in MDA content - about 1.3-fold when compared to the uninoculated control (Fig. 6-6a, Table 6-4). ROS in leaves were detected by the presence of superoxide and hydrogen peroxide by staining

with nitrobluetrazolium (NBT) (Fig. 6-6b) and 3,3′-diaminobenzidine (DAB) (Fig. 6-6c), respectively. In the presence of salt, both ROS species were present, shown by the intense staining of leaves; however rice inoculated with *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* showed fainter staining than those of the uninoculated control (Figs. 6-6b,c). The results indicated that *S. venezuelae* helped salt tolerance in rice by reduction of MDA content and ROS species. However, under salt stress conditions, the overexpression of ACC deaminase did not induce those characteristics more than those of the wild type control.

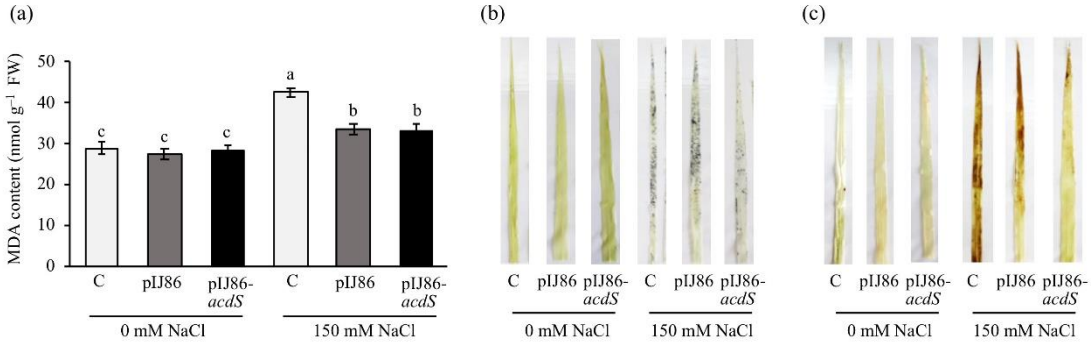

Fig. 6-6 Effect of ACC deaminase-producing *Streptomyces venezuelae* on MDA content (a), histochemical NBT staining (b), and DAB staining (c) of rice plants under non-salt (0 mM NaCl) and salt stress (150 mM NaCl) conditions. The values indicate the mean ± S.E. of twelve replicates and bars carrying different letters are significantly different (Tukey's test, $p<0.05$). C, uninoculated rice control; pIJ86, rice inoculated with *S. venezuelae*/pIJ86; pIJ86-*acdS*, rice inoculated with *S. venezuelae*/pIJ86-*acdS*.

Discussion

ACC deaminase is a bacterial enzyme found in several PGP-bacteria including *Bacillus*, *Enterobacter*, *Pseudomonas* and *Streptomyces*. Its improved stress tolerance of plants to drought, flooding, salinity and phytopathogens[7-11]. Interestingly, increasing the ACC deaminase activity by overexpression of the corresponding gene in PGP-bacteria remarkably facilitated growth and alleviated environmental stresses of host plants more than those of wild type strains[13-15].

In this work *acdS*, encoding ACC deaminase located in the genome of *S. venezuelae* ATCC 10712, was cloned and expressed in this strain. The overexpressing mutant, *S. venezuelae*/pIJ86-*acdS*, had higher ACC deaminase activity, compared to *S. venezuelae*/pIJ86. The results were in agreement with previous reports that overexpression of ACC deaminase in *Mesorhizobium cicero*, *Serratia grimesii* and *Sinorhizobium meliloti* resulted in higher ACC deaminase activity, compared

to the corresponding wild type strains[14,15,22]. In addition, *acdS* expression under the *ermE* promoter of multi-copy plasmid pIJ86 in *S. venezuelae* without antibiotic selection was maintained up to 5 generations, consistent with the previous report[23]. Interestingly, *S. venezuelae* showed endophytic ability in rice plants; which was proven by re-isolation of the bacterium responsible for promotion of rice growth from plant tissues; a trait that is herein shown for the first time for this bacterium. Soil actinomycetes, therefore, potentially act as endophytes, supporting the hypothesis that bacterial communities in the rhizosphere, rhizoplane, and endosphere of rice root microbiomes were overlapping[24]. In this work, it was demonstrated for the first time that *S. venezuelae* behaves as a PGP-endophytic bacterium.

Under normal conditions, rice inoculated with *S. venezuelae*/pIJ86 significantly increased biomass of shoot and root, and elongation. This might be due to an action of IAA produced by this strain that would encourage plant growth and elongation. Moreover, the results were in agreement with previous work showing that ACC deaminase-producing *Streptomyces* have the ability to enhance growth of *Jatropha curcas*, mung bean, sugarcane, and rice[6,8,10,25]. Apart from *Streptomyces*, IAA and ACC deaminase-producing species from the genera *Agromyces*, *Bacillus*, *Enterobacter*, *Methylophaga*, *Microbacterium*, *Paenibacillus*, and *Pseudomonas* were also reported to promote growth of canola, rice, sugarcane, and tomato[26,27].

Under salt stress conditions, rice plants inoculated with either *S. venezuelae*/pIJ86 or its overexpressing mutant, *S. venezuelae*/pIJ86-*acdS*, showed enhanced growth parameters compared to those of uninoculated controls. However, shoot length and biomass of rice associated with the ACC deaminase-overexpressing mutant were significantly greater than plants inoculated with the unmodified strain. Our results were in congruence with other studies in which ACC deaminase overexpressing strains of *Pseudomonas putida* and *Serratia grimesii* promoted growth of tomato and common bean respectively, compared to wild type strains[13,15]. Therefore, the results unambiguously demonstrated that ACC deaminase-overexpressing *S. venezuelae* facilitated rice growth better than the original strain under salt stress conditions.

It is generally known that ethylene production is a main response in plants exposed to environmental stress. Salinity induced a high level of ethylene via the actions of ACC synthase and ACC oxidase towards ACC, an ethylene precursor. Whereas, ACC deaminase of bacteria assists plants in responding by conversion of ACC into ammonia and α-ketobutyrate and, thus, reducing ethylene as a consequence[28]. In this work, the ethylene levels were significantly lower when rice was associated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* compared to that of the uninoculated control. Our results were similar to previous reports that ethylene levels

in rice and sugarcane were reduced by ACC deaminase-producing *Streptomyces* sp. GMKU 336[10] and *Enterobacter* sp. EN-21[9] respectively, under salt stress conditions. The ethylene level was lowest in rice inoculated with the ACC deaminase-overexpressing mutant, correlating with the high ACC deaminase activity of this strain. The results were consistent with another report that overexpression of ACC deaminase in endophytic *Pseudomonas* spp. enhanced salt tolerance in tomato by reducing ethylene production[29]. In addition, a lower amount of ACC was observed in tomatoes inoculated with ACC deaminase-overexpressing psychrotolerant bacteria under chilling stress[30].

Proline accumulation is one of the adaptation mechanisms of plants under salt stress. At 7 days after irrigation with salt, the proline content of rice associated with *S. venezuelae* was high and particularly higher in rice inoculated with the ACC deaminase-overexpressing mutant. The results agreed with data on ACC-deaminase producing *Dietzia natronolimnaea* and *Streptomyces* sp. GMKU 336, associated with wheat and rice respectively – which induced elevated proline content[31,32]. Accumulation of higher levels of proline stabilized proteins, cell structures, and osmotic balance[33] in rice associated with *S. venezuelae*/pIJ86-*acdS* and, thus, accelerated salt tolerance.

Reduction of total chlorophyll and RWC of plants are generally the first notable effects of salt stress such as those reported in black gram and rice[10,34,35]. In this work, the total chlorophyll and RWC of rice plants were increased significantly in plants under salt treatment, when inoculated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS*. The results were in congruence with other studies in which ACC deaminase-producing *Enterobacter* sp. SBP-6 in wheat[32], *Enterobacter* cloacae HSNJ4 in canola[36], *Bacillus subtilis* RJ46, *Ochrobactrum pseudogrignonense* RJ12, and *Pseudomonas* sp. RJ15 in black gram and pea[37], and bacterial consortia in avocado[38] increased chlorophyll level more than those of non-inoculated plants, when under salt stress. Moreover, the results were in agreement with other for the ACC deaminase-overexpressing endophytic *Pseudomonas* spp., which improved photosynthetic performance and water content in tomato[29]. The results suggested that *S. venezuelae* facilitates rice growth in saline environments by increasing total chlorophyll and RWC. However, as the ACC deaminase-overproducing *S. venezuelae* enhanced chlorophyll content and RWC equally to those of the wild type control, it can be concluded that the overexpression of ACC deaminase did not influence those characters.

Excess accumulation of Na^+ and inhibition of K^+ uptake under salt stress are very harmful for plant cells, leading to growth impairment[3]. Several reports have indicated that increasing the

K^+/Na^+ ratio is crucial for salt tolerance in plants[39-41]. In this work, the Na^+ content was significantly enhanced, while the K^+ content was decreased drastically in salt-stressed uninoculated rice. On the contrary, rice inoculated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* had markedly reduced Na^+ content and enhanced K^+ content. The results were similar to recent reports that ACC deaminase-producing *Dietzia natronolimnaea* and *Streptomyces* sp. GMKU 336 enhanced salt tolerance in plants by increasing the K^+/Na^+ ratio via up-regulation of the Na^+/H^+ antiporter gene (*NHX1*) involved in maintenance of the Na^+ level in the cytoplasm[10,42]. Besides, the increment in K^+/Na^+ ratio was observed in maize, pea, and sugarcane associated respectively with ACC deaminase-producing *Pseudomonas fluorescens*, *Variovorax paradoxus* 5C-2, and *Enterobacter* sp. EN-21, under salinity stress[9,43,44].

ROS production plays a crucial role as signalling molecules involved in stress conditions including attack by pathogens, drought, and salinity which leads to high accumulation of MDA, a product of membrane lipid peroxidation[1,2]. In this study, rice KDML105 inoculated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* had significantly decreased MDA content under salt stress conditions. Moreover, histochemical staining with NBT and DAB indicated that levels of H_2O_2 and O^{2-} were reduced in the corresponding leaves. The results were in agreement with previous reports that ACC deaminase-producing endophytes caused a reduction in MDA content, including *Bacillus subtilis* GB03 in white clover[31], *Enterobacter* sp. EN-21 in sugarcane[9], *Streptomyces* sp. GMKU 336 in Thai jasmine rice[10], and *Dietzia natronolimnaea* in wheat[42]. Moreover, higher accumulation of proline in rice associated with *S. venezuelae* might help stabilize ROS[33] and, thus, alleviate salt stress by modulation of the antioxidant system.

This work is the first demonstration that *S. venezuelae* carries PGP-traits and promotes growth of rice KDML105 endophytically under normal and salinity conditions. Moreover, the ACC deaminase-overexpressing mutant, *S. venezuelae*/pIJ86-*acdS*, enhanced rice growth and salt tolerance more than the original strain. The physiology of the rice benefitted remarkably from the ACC-deaminase trait. Overproduction of ACC deaminase of *S. venezuelae* is an important model to investigate how excessive ACC deaminase-producing inocula can be effective for crop health improvement under severe conditions.

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Section 7

Transcriptomic analysis of salt-susceptible indica rice cultivar IR29 associated with ACC deaminase-producing endophytic actinomycete

Introduction

There are several ways that plants response to salt stress including change in various physiological and metabolic processes and inhibition of crop production (Gupta and Huang, 2014). Many reports have been described that inoculation of PGP bacterial endophytes such as *Achromobacter*, *Pseudomonas* and *Streptomyces* ultimately helped plants on growth enhancement and modulated salt stress (Karthikeyan et al., 2012; Ali et al., 2014; Yaish et al., 2015; Jaemsaeng et al., 2018). However, there are only few studies with the application of high-throughput techniques (e.g. RNA-seq) in plant-PGPE interactions under severe conditions. Therefore, transcriptomic profiling of salt-sensitive indica rice cultivar IR29 inoculated with ACC-deaminase producing *Streptomyces* sp. GKU 895 under non-salt and salt stress conditions was investigated using Ion PGM sequencing.

Materials and Method

Bacterial inoculation and plant cultivation

An ACC deaminase-producing endophytic actinomycete was prepared for inoculation to rice plants according to the method previously described. Salt-sensitive indica rice variety IR29 (*Oryza sativa* cv. IR29) was kindly provided by Nakhon Ratchasima Rice Research Center (Nakhon Ratchasima, Thailand). Rice seeds were surface sterilised with 70% ethanol for 5 min followed by 2% sodium hypochlorite for 45 min and grown on sterilised water saturated tissue papers in plastic trays for 7 days in the dark. Seven-day-old rice seedlings were inoculated with spore suspension actinomycetes for 4 h. Yoshida culture solution was used as a control treatment. Seedlings were cultured hydroponically in a half-strength Yoshida culture solution (½YS) for 7 days, then, transferred to full-strength YS for 7 days at green house condition. After 14 days, rice seedlings were subjected to control (YS) and salt stress treatment (150 mM NaCl). After 7 days post irrigation (DPI), seedlings were collected and immediately snap-frozen with liquid nitrogen and keep at –80°C for until use. The experiment was set up in completely randomised design (CRD) using ten replicate plants per treatment and each treatment was replicated twice.

Plant growth parameter determination

Plant growth parameters at 7 DPI were determined for root and shoot dry weights. Salt tolerant index (STI) was calculated as previously described. Data were statistically analysed by one-way ANOVA and Tukey's multiple range tests (TMRT) using SPSS (version 18.0) to determine the significant difference among means of the treatment at $P \leq 0.05$. Data were showed in mean ± standard deviation calculated from five plants per treatment in three different replicates. Different letters indicate statistical significance at $P \leq 0.05$.

RNA isolation

Total RNA from ten individual plants was extracted by CTAB method according to Yu and Goh (2000) (two biological replications). Ten plant samples were immediately ground in liquid nitrogen and suspended in 5 mL Hao buffer [2% (v/v) β-mercaptoethanol, 2% (w/v) hexadecyltrimethyl-ammonium bromide, 100 mm Tris [tris(hydroxymethyl)aminomethane]-HCl (pH 7.5), 20 mm EDTA, 2 m NaCl, and 1% (w/v) polyvinylpyrrolidone], then incubated at 60°C for 1 h. The supernatant was collected by centrifugation at 7,500 ×*g*, 4°C, 15 min and subsequently three-time extracted with chloroform: isoamyl (24:1). The supernatant was transferred to new 50 mL sterilised Falcon tube and precipitated with 0.9 volume isopropanol at –20°C for 10 min followed by centrifugation at 7,500 ×*g*, 4°C, 15 min. The RNA pellet was washed with cold 75% EtOH and resuspended with 900 µL cold-DEPC water and precipitated with one-third volume of 10 M LiCl at –20°C for overnight. The pellet was washed with 200 µL 2.5 M LiCl followed by cold 70% EtOH, dried, and dissolved with 40 µL sterilised DEPC water. RNA purity and concentration were estimated quantity and quality using NanoDrop ND-1000 spectrophotometer (NanoDrop technologies Inc., USA) and gel electrophoresis. RNA was treated with DNaseI to remove contaminant DNA and preserved at –80°C.

Ribosomal RNA depletion

Ribosomal RNA of plants and endophyte were removed from total RNA samples using Ribo-Zero Magnetic Kit (plant seeds/roots, Epicenter, USA) and Ribo-Zero Magnetic Kit (Gram-positive bacteria, Epicenter, USA) respectively, following the manufacture's instruction. Twenty-six microliters of total RNA (1-5 µg) was mixed with 4 µL Ribo-zero reaction buffer and 10 µL Ribo-zero rRNA removal solution and incubated at 68°C for 10 min followed by at RT for 5 min. Next, the treated RNA was added to 1.5 mL microcentrifudge tube containing the washed magnetic beads, pipetting at least 10 times and then incubated at RT for 5 min and 50°C for 5 min. The

microcentrifuge tubes were immediately placed on a magnetic stand for at least 1 min or until the solution was clear. The supernatant (90 µL) was carefully collected and transferred to new 1.5-mL RNase-free microcentrifuge tube and immediately placed on ice. The rRNA-depleted samples were purified by adding 90 µL RNase-free water, 18 µL of 3 M sodium acetate, 2 µL of glycogen (10 mg mL^{-1}) and 3 volumes (600 µL) of ice-cold 100% ethanol. The reaction was left at –20°C for 1 h, and then centrifuged at ≥10,000 ×*g* for 30 min. The RNA pellet was washed twice with 200 µL of ice-cold 70% ethanol and centrifuged at ≥10,000 ×*g* for 5 min and air dried at RT for 5 min. Finally, the pellet was dissolved in 10 µL of RNase-free water and immediately stored at –80°C until used.

Whole transcriptome library preparation

Library was prepared as described in the Ion Total RNA-Seq Kit v2 library preparation guide (Ion Torrent manual reversion B.0).

RNA fragmentation

The rRNA-depleted total RNA was fragmented using RNase III by adding 1 µL of RNase III, 1 µL RNase III reaction buffer to 10 µL rRNA-depleted total RNA and incubated at 37°C for 3 min in thermal cycle. Immediately after the incubation, 20 µL of nuclease-free water was added and the tube was placed the fragmented RNA on ice. The rRNA-depleted total RNA was purified according the manufacturing's protocol. The fragmented RNA was eluted with 12 µL nuclease-free water and quantified using Nanodrop® spectrophotometer.

RNA hybridisation and ligation

The rRNA-depleted total RNA was hybridised by adding 5 µL of hybridisation master mix (2 µL Ion Adaptor Mix v2 and 3 µL Hybridisation solution) and incubated at 65ºC, 10 min and 30ºC, 5 min. Then, 12 µL of ligation master mix (10 µL 2× ligation buffer and 2 µL ligation enzyme mix) was added and incubated at 30ºC for 1 h.

Reverse transcription (RT) and cDNA purification

The ligation reaction (20 µL) was mixed with 16 µL of RT master mix (2 µL nuclease-free water, 4 µL 10× RT buffer, 2 µL 2.5 mM dNTP mix and 8 µL Ion RT primer v2) and incubated at 70ºC for 10 min. After that, 4 µL 10× SuperScript® III enzyme mix was added and incubated at

42ºC for 30 min. The cDNA samples were purified according to the manufacturing's protocol and eluted with 12 µL nuclease-free water.

cDNA amplification

The cDNA samples were barcoded by mixed with 46 µL barcoded master mix (45 µL Platinum® PCR superMix High Fidelity and 1 µL Ion Xpress™ RNA 3' Barcode primers) and 1 µL Ion Xpress™ RNA-seq Barcode BC primer. The barcoded library was incubated in a thermal cycler at 94ºC, 2 min, 2 cycles at 94ºC, 30 sec, 50ºC, 30 sec, 68ºC, 30 sec, 16 cycles at 94ºC, 30 sec, 62ºC, 30 sec, 68ºC, 30 sec and hold at 68ºC, 5 min. The cDNA was purified according to the manufacturing's protocol and eluted with 15 µL nuclease-free water. The cDNA samples were qualified by Agilent® High Sensitivity DNA kit with Agilent® 2100 Bioanalyser® instrument.

Pool barcoded whole trancriptome library

The barcoded cDNA library was diluted to 50 pM and then mixed an equal volume of each dilution library to prepare a pool of the barcoded libraries.

Ion Sphere particle preparation and sequencing

The barcoded cDNA library was used to prepare template-positive Ion PGM™ Hi-Q™ Ion Sphere™ particles using Ion PGM™ Hi-Q™ OT2 kit according to the protocol. The Ion particles were enriched using Ion OneTouch™ ES Supplies Kit according to the protocol. Then, the particles were sequencing using Ion Torrent platform following the protocol.

Sequencing data processing

All read sequences of RNA-seq of plants were analysed using The Discovery Environment (DE, https://de.cyverse.org/de/) in the CyVerse server. Firstly, the reads were qualified by FastQC followed by aligned against the rice genome (*Oryza sativa* IRGSP 1.0) using HISAT2 (Kim *et al.*, 2015). The rice-mapped reads were respectively assembled and merged to transcripts using Cufflinks and Cuffmerge. Finally, Cuffdiff was used to calculated differential gene expression in the treatments relative to the respective control treatments (Trapnell *et al.*, 2010, Trapnell *et al.*, 2012).

Gene ontology (GO) analysis

Functional categorisation of the differential expressed genes (DEG) were performed using singular enrichment analysis (SEA) in a agriGO against the rice genome (*Oryza sativa*) (Du *et al.*, 2010). A GO category was considered significant if it had a hypergeometric test $P<0.05$, a Benjamini-Yekutieli FDR<0.05, and at least five mapped entries. Gene functional description of DEGs was determined using UniplotKB (https://www.uniprot.org/). Venn diagrams of pair-wise overlap between the up- and down-regulated DEGs were analysed by Venny 2.0.2 (Oliveros, 2007). Heatmaps of log2 FC (fold change) of DEGs were generated using the R package (version 3.4.0).

Raw reads accession number

All raw read sequences retrieved from Ion Torrent server were deposited to NCBI database as a sequence read archive (SRA) accession number SRP126983 in the BioProject number PRJNA422335.

Results

Mapping of RNA-seq read sequences

The RNA-seq analysis was carried out in two independent samples (biological replicates) of each treatment, yielding a total 8 sequencing libraries with were designed to non-salt control plants (NSC), non-salt inoculated plants (NSB), salt stressed control plants (SC), and salt stressed inoculated plants (SB). Ion Torrent PGM was used to sequence all of 8 libraries which generated approximately 1.9 million bp with mean of 96 bp read lengths of sequenced data. The sequenced reads were firstly quantified and qualified by FastQC and mapped to the *Oryza sativa* IRGSP-1.0 in the DE database using HISAT2. The reads were mapped to rice genome by 66-83% (Fig. 7-1).

Analysis of differentially expressed genes (DEGs)

The two independent biological replicates of RNA-seq reads from SC, NSC, SB and NSB samples were mapped to rice genome using HISAT2. The fragment per kilobase of transcript per million mapped reads (FPKM) was counted using Cuffdiff program with false discovery rate (FDR) cutoff at 0.05 in order to find significant changes in transcript expression between control and treatments. Number of up- and down-regulated DEGs of each treatment was showed in the Figure 7-2. In total 1,154 DEGs of SC, 859 (74.4%) and 259 DEGs (25.6%) were up- and down-regulated, resprctively. In total 10,306 DEGs of NSC, 10,233 (99.2%) and 73 DEGs (0.8%) were

up- and down-regulated, respectively. In total 866 DEGs of SB, 853 (98.5%) and 13 DEGs (1.5%) were respectively up- and down-regulated. In total 478 DEGs of NSB, the number of down-regulated DEGs (332 DEGs, 69.5%) were, however, much highly than up-regulated DEGs (146 DEGs, 30.5%) which were in contrast to SC, NSC and SB.

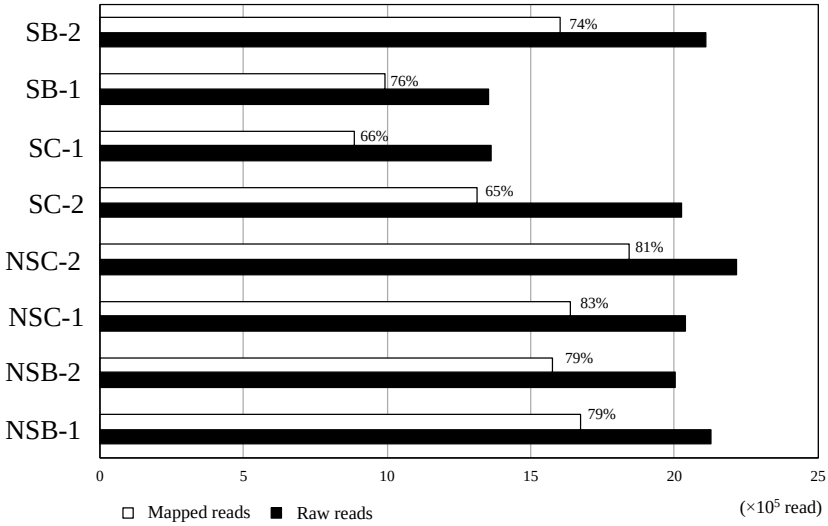

Figure 7-1 HISAT2 mapping percentage of 8 sequenced reads with reference rice genome (*Oryza sativa* IRGSP-1.0)

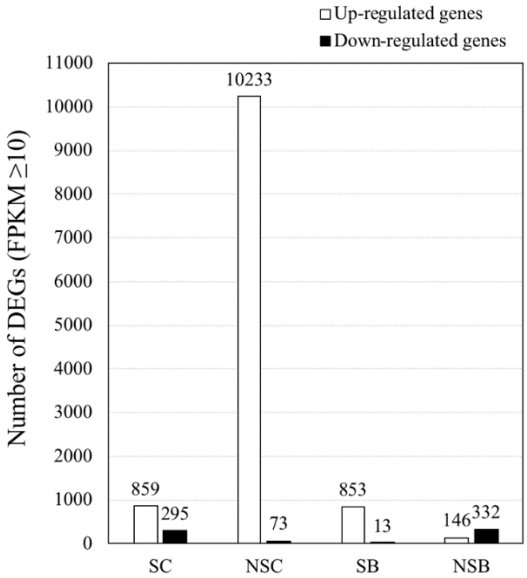

Figure 7-2 Number of differentially expressed genes (DEGs) of up- and down-regulated genes of SC, NSC, SB and NSB conditions.

Gene Ontology (GO) terms of DEGs analysis

An agriGO analysis of DEGs in each treatment identified 111, 15, 100 and 34 GO terms (hypergeometric test, $P<0.05$; Benjamini-Yekutieli, FDR<0.05) in SC, NSC, SB and NSB conditions, respectively. GO terms classification of DEGs found that most of up-regulated DEGs were possessed to Biological Process category (21-66%) followed by Cellular Component (13-79%) and Molecular Function (14-37%) (Fig. 7-3). Moreover, all of down-regulated DEGs were assigned to Biological Process category.

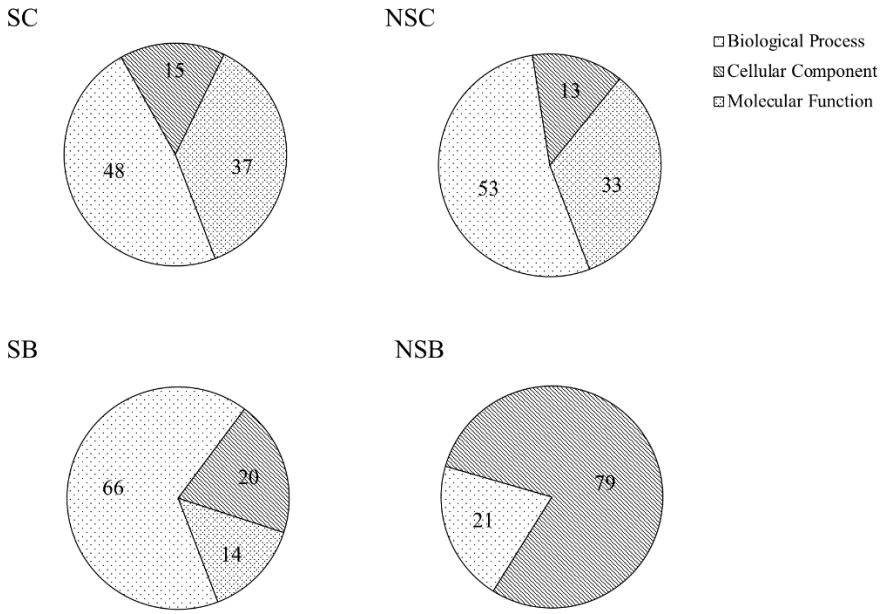

Figure 7-3 An agriGO classification of GO category of up-regulated DEGs gene in SC, NSC, SB and NSB conditions

To classify the function of the DEGs, GO term assignment was carried out by agriGO. The top three GO terms in the "Biological Process" category of DEGs in SC, NSC, SB and NSB conditions were showed in the Table 10. For up-regulated DEGS, 590 of 859 DEGs in SC mapped onto agriGO database of *Oryza sativa* and the top three GO terms were translation, photosynthesis and metabolic process. In NSC, 7819 of 10,233 DEGs mapped to database and the GO terms were cellular nitrogen compound metabolic process, translation and macromolecule localization. In SB, 722 of 853 DEGs were mapped and the GO terms were response to stimulus, abiotic stimulus and stress. Moreover, 79 of 146 mapped DEGs belonged to photosynthesis GO terms in the NSB. For down-regulated DEGs, 201 of 295 mapped DEGs in the SC were classified to response to stimulus, abiotic stimulus and stress GO terms. In NSC, 22 of 73 DEGs mapped to database and the GO

terms were regulation of primary metabolic process, regulation of cellular biosynthetic process and cellular metabolic process. In SB, 10 of 13 mapped DEGs was only assigned to metabolic process. In addition, 235 of 332 mapped DEGs of NSB belonged to cellular nitrogen compound metabolic process, aromatic amino acid family metabolic process and carbohydrate metabolic process GO terms (Table 7-1).

Table 7-1 The top three GO terms in the Biological Process category of up- and down-regulated DEGs

Treatment	GO term	Description	p-value	FDR
Up-regulated DEGs				
SC	GO:0006412	translation	2.00E-43	2.00E-40
	GO:0015979	photosynthesis	3.20E-22	1.50E-19
	GO:0008152	metabolic process	4.60E-16	1.50E-13
NSC	GO:0034641	cellular nitrogen compound metabolic process	2.60E-14	1.20E-10
	GO:0006412	translation	6.60E-08	1.50E-04
	GO:0033036	macromolecule localization	4.40E-06	6.50E-03
SB	GO:0050896	response to stimulus	7.40E-46	8.30E-43
	GO:0009628	response to abiotic stimulus	1.00E-30	5.70E-28
	GO:0006950	response to stress	3.60E-28	1.40E-25
NSB	GO:0015979	photosynthesis	1.50E-37	8.30E-36
	GO:0019684	photosynthesis, light reaction	1.20E-21	3.20E-20
	GO:0009765	photosynthesis, light harvesting	7.00E-21	1.30E-19
Down-regulated DEGs				
SC	GO:0050896	response to stimulus	2.10E-05	7.20E-03
	GO:0006950	response to stress	1.10E-04	1.80E-02
	GO:0042221	response to chemical stimulus	2.10E-04	1.80E-02
NSC	GO:0080090	regulation of primary metabolic process	1.20E-03	1.30E-02
	GO:0031326	regulation of cellular biosynthetic process	9.20E-04	1.30E-02
	GO:0031323	regulation of cellular metabolic process	1.10E-03	1.30E-02
SB	GO:0008152	metabolic process	4.60E-03	3.80E-02
NSB	GO:0034641	cellular nitrogen compound metabolic process	3.40E-04	4.10E-02
	GO:0009072	aromatic amino acid family metabolic process	3.20E-04	4.10E-02
	GO:0005975	carbohydrate metabolic process	1.60E-04	4.10E-02

Core set of up- and down-regulated DEGs of rice plants responses to salt stress

Venn diagram was performed to investigate overlapping of up- and down-regulated DEGs of four treatments (Fig. 7-4). From 10,647 up-regulated DEGs, a core set of 5 genes were up-regulated in response to all treatments but was not found in down-regulated DEGs (Figs. 7-4a and 7-4b). Among the core sets of shared up-regulated DEGs, genes related to metabolic process GO term and response to stress GO term were expressed. A similar pattern terms can be observed in the significance analysis of the GO terms that metabolic process and respond to stress were enriched in each treatment. Remarkably, GO term in response to abiotic stimulus was highest up-regulated in SB condition (Fig. 7-4c).

Exclusive set of up- and down-regulated DEGs of rice plants responses to salt stress

From 10,647 up-regulated DEGs, 8,988 DEGs (84.41%), 85 (0.79%), 304 (2.85%) and 16 (0.15%) were classified into excusive DEGs of NSC, SC, SB and NSB, respectively (Fig. 7-4a). In total of 617 down-regulated DEGs, 60 (9.72%), 203 (32.90%), 248 (40.19%) and 12 (1.94%) DEGs were classified into exclusively set of NSC, SC, SB and NSB, respectively (Fig. 7-4b).

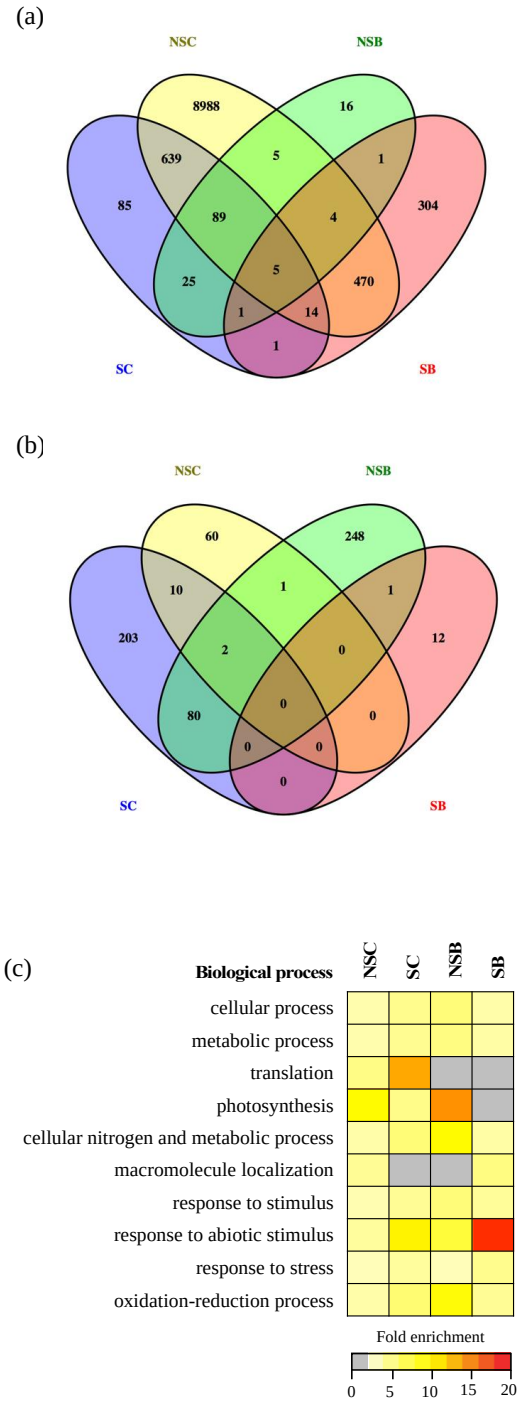

Figure 7-4 Venn diagrams and enriched GO terms of DEGs of four treatments. (a) up-regulated DEGs and (b) down-regulated DEGs. (c) Enriched GO terms associated with the core sets of up-regulated DEGs in all treatments. The fold enrichment was plotted in yellow-red colour scale which grey indicates no enriched GO terms and red indicates high enriched GO terms.

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Section 8

Characterization of chitinase-producing endophytic actinomycetes antagonistic to *Fusarium moniliforme* and their potential for biocontrol of foot rot and wilting disease of maize plants

Introduction

Endophytic actinomycetes as known as Gram-positive filamentous bacteria that reside in the tissue of living plants and do not visibly harm the host plants (Stone *et al.*, 2000). They have been isolated from a variety of healthy plant species ranging from crop plants (El-Tarabily *et al.*, 2009; Lee *et al.*, 2008; Shanmugam *et al.*, 2013; Tan *et al.*, 2006; Tian *et al.*, 2007), halophytic plants (Bian *et al.*, 2012; Qin *et al.*, 2014) and medicinal plants (Qin *et al.*, 2009; Zhao *et al.*, 2011). These microbes inhabit various organ of the host plants such as roots, stems and leaves. In general, *Streptomyces* were the most predominant species follow by *Microbispora*, *Micromonospora*, *Nocardia*, *Nocardioides* and *Streptosporangium* as common genera (Qin *et al.*, 2011). The usefulness of endophytic actinomycetes as biocontrol agents against phytopathogenic fungi has increased over the past years. They have been demonstrated the potential against various soil-borne fungal pathogens (Kim *et al.*, 2012a; Misk & Franco, 2011; Shimizu *et al.*, 2009; Verma *et al.*, 2011).

Actinomycetes, particulary *Streptomyces* species, are known to produce fungal cell-wall degrading enzymes such as cellulases, chitinases, β-1,3, β-1,4, β-1,6 glucanases and proteases (El-Tarabily, 2006; Fróes *et al.*, 2012; Macagnan *et al.*; 2008, Quecine *et al.*; 2008, Xue *et al.*; 2013). Remarkably, chitinase plays an important role to dissolve cell wall structure of fungi. Because of the chitin polymer have an enormous tensile strength and significantly contribute to the overall integrity of the fungal cell wall (Bago *et al.*, 1996; Bowman & Free, 2006; Specht *et al.*, 1996). Chitinases are able to randomly hydrolyze the β-1,4 glycosidic bonds of *N*-acetylglucosamine residues in chitin. Based on amino acid sequence similarities within the catalytic domain, chitinases are classified into glycosyl hydrolase families 18 and 19 (Funkhouser & Aronson, 2007; Kawase *et al.*, 2004). Moreover, family 18 chitinases can be further classified into subfamilies A, B and C (Watanabe *et al.*, 1993). Bacteria produce chitinases mainly to utilize chitin as a carbon and nitrogen sources for their growth (Huang *et al.*, 2012). Many chitinase producing bacteria could produce only family 18 chitinases, while some bacteria, especially *Streptomyces* species could produce both family 18 and 19 chitinases (Watanabe *et al.*, 2003). Multiple chitinase genes of each family are commonly found in the genomes of many *Streptomyces* (Kawase *et al.*, 2006;

Williamson *et al.*, 2000). To date, *Streptomyces* chitinases of the family 18 subfamily A and family 19 have been correlated to antifungal activity and added advantage in biocontrol (Itoh *et al.*, 2003; Xiayun *et al.*, 2012).

Fusarium moniliforme is an important soil-borne fungal pathogens, which worldwide distributed and attacks a wide range of vegetables and field crops (Bacon & Nelson, 1994). It causes foot rots and wilting disease of maize plants (*Zea mays* L.), adversely affecting the yield loss and substantial damage in several area (MacDonald & Chapman, 1997; Pal *et al.*, 2001). There are no maize cultivars with complete resistance to fungal pathogens and fungicides are not potent enough to protect the plants from infection by these pathogens (Pal *et al.*, 2001). Therefore, application of biocontrol agents could be the best alternative to minimize the incidence of diseases. Chitinase-producing endophytic actinomycetes have been investigated as biocontrol agents of phytopathogenic fungi since they advantage of safety and environmental-friendly. For instance, endophytic *Streptomyces* are known as major producers of chitinases and exhibited antifungal activity against various phytopathogenic fungi under laboratory (Haggag & Abdallh, 2012). Biocontrol strategies for suppression of pathogenic fungi by chitinase producing endophytic actinomycetes *in planta* were still under discovering.

In this study, endophytic actinomycetes were identified and screened for their chitinase production, fungal cell-wall degrading activity, family 18 subfamily A and family 19 chitinase genes, as well as antagonistic activity against *Fusarium moniliforme* DOAC 1224 *in vitro*. The promising candidate strains were investigated the decomposition of fungal hyphae under light and scanning electron microscope. Importantly, these strains were demonstrated for their effectiveness in controlling foot rot and wilting disease in maize plants under pot culture conditions.

Material and Methods

Fungus strain and endophytic actinomycetes isolates

Plant pathogenic fungus *F. moniliforme* DOAC 1224 was obtained from the Plant Protection Research and Development Office, Department of Agriculture, Thailand. It was grown on potato dextrose agar (PDA; Difco) at 25 °C and stored in PDA slant at 4 °C. All of the 133 endophytic actinomycetes were recovered from the culture collections of Genetics-Microbiology Kasetsart University (GMKU) and Genetics Kasetsart University (GKU) of Actinobacteria Research Unit at Department of Genetics, Faculty of Science, Kasetsart University, Thailand. They were grown on mannitol-soya (MS) agar (Hobbs *et al.*, 1989) at 28 °C and stored in 20% (v/v) glycerol suspension at –20 °C.

Molecular characterization of the endophytic actinomycetes

Endophytic actinomycetes were identified by partial sequencing of 16S rRNA gene. Total genomic DNA was extracted according to Kieser *et al.* (2000). PCR amplification of 6S rRNA gene was carried out with specific primers and conditions described by Indananda *et al.* (2010) and Rachniyom *et al.* (2015b). PCR products were purified using a Gel/PCR DNA Fragment Extraction Kit (Geneaid, Taiwan) and sequenced commercially by Macrogen (Korea). The obtained gene sequences were compared with corresponding sequences of type strains retrieved from the GenBank database using the EzTaxon-e server (Kim *et al.*, 2012b).

Screening of chitinase-producing endophytic actinomycetes

Chitinase production was determined by growing endophytic actinomycetes in semi-solid starch-casein medium (Küster & Williams, 1964) with the addition of 0.1% agar and incubated in a rotary shaker at 200 rpm for 7 days at 28 °C. Aliquot of 25 µl of culture suspension was dropped onto colloidal chitin agar (CCA) containing the basal medium (Nawani *et al.*, 2002) supplemented with 0.5% colloidal chitin. The colloidal chitin was prepared from shrimp shell chitin powder (Sigma) according to the method of Ahmadi *et al.* (2008) with some modifications. Five grams of chitin powder was added slowly into 90 ml of concentrated HCl under vigorous stirring for 2 h. The mixture was added to 500 ml of ice-cold 95% ethanol with rapid stirring and kept overnight at 4 °C. The precipitate was collected by centrifugation at 6000 g for 10 min at 4 °C and washed with 0.1 M sodium phosphate buffer (pH 7.0) until the colloidal chitin become natural (pH 7.0). Development of clear zones were recorded after 14 days of incubation at 28 °C in the dark. The experiment was performed in triplicate.

Determination of fungal cell wall-degrading activity

Fungal cell wall-degrading ability was determined on mycelial fragments agar (MFA) by modified method of Valois *et al.* (1996). The fungus, *F. moniliforme* DOAC 1224 was grown in 30 ml potato dextrose broth (PDB; Difco) and incubated on a rotary shaker at 110 rpm for 7 days at 25 °C. The mycelial mats were collected by filtration through Whatman no. 1 filter paper, washed with sterile deionized water (DW), resuspended in 30 ml sterile DW, and then homogenized using blender for 1 min. The suspended mycelial fragments were centrifuged in a refrigerator at 7500 g for 20 min at 4 °C and washed with sterile DW. The mycelial fragments were then resuspended in 30 mL sterile DW and subjected to ultrasonic disintegration in an ice

bath for 20 min of 30 sec power on and 15 sec power off in a ultrasonicator. The mycelial pellets were centrifuged, washed 3 times with sterile DW and then added 3 volumes of sterile DW. The suspended mycelial pellets were added to the basal medium containing methyl blue (0.005, w/v). The culture suspension of 7-day growth of chitinase-producing endophytic actinomycetes in semi-solid starch-casein medium were dropped onto MFA plate. Development of clear zones were recorded after 10 days of incubation at 28 °C in the dark. The experiment was performed in triplicate.

Molecular detection of chitinase genes

Family 18 subfamily A and family 19 chitinase genes from chitinase-producing endophytic actinomycetes were amplified by using specific primers, namely chi18AF (5'-GACACCTGGGACCAGCCGCTG-3'), chi18AR (5'-TAGAAGCCGAYGCCGAKSAGCA-3'), chi19F (5'-CAGTTCRACCARATGTTCCCG-3') and chi19R (5'-CGTTGATSGASCGGATSG TCT-3'). The PCR reaction was performed in a total volume of 20 µl containing; 0.2 mM of each dNTP, 10 pmol of each primer, 5% DMSO, 1.25 mM $MgCl_2$, 5 ng of DNA, 2 µl of 10X *Taq* polymerase and 0.5 U of *Taq* DNA polymerase. The PCR conditions were adjusted to 3 min for initial denaturation at 95 °C and then 30 cycles of 1 min at 95 °C, 1 min at 56 °C (*chi18A*) or 64 °C (*chi19*), 1 min at 72 °C and finally 7 min at 72 °C. The PCR products were purified and confirmed by DNA sequencing. The nucleotide sequences were manually assessed for similarities against the NCBI non-redundant protein database using BLASTX algorithm.

***In vitro* evaluation of fungal antagonism**

The evaluation of fungal antagonism was performed by dual culture technique (Tahtamouni *et al.*, 2006) with slight modification. Each chitinase-producing endophytic actinomycetes was streak on one side of starch casein agar (SCA; Küster & Williams, 1964) plates and incubated at 28 °C for 7 days. The mycelial plugs (6 mm) of *F. moniliforme* DOAC 1224 from 7-day growth were placed at the opposite side of the actinomycetes at 4-cm distance. The fungal plug was also placed on SCA plate separately as a control treatment. The diameter of the inhibition zone was recorded after 8 days of incubation at room temperature. The experiment was performed in duplicate.

Investigation of chitinase-producing endophytic *Streptomyces* on *F. moniliforme* hyphae

The best performing strains of chitinase-producing endophytic *Streptomyces* sp. GMKU 301 and GMKU 322, were investigated for their effect on *F. moniliforme* DOAC 1224 hyphae using dual culture assay. An agar block (4x2 cm) was taken from CCA plate and transferred to the center of slide. The culture suspension of 7-day growth of individual *Streptomyces* in semi-solid starch-casein medium was dropped on one side of agar block and incubated at 28 °C for 5 days in the dark. The mycelial of *F. moniliforme* DOAC 1224 was inoculated at the opposite side at 2-cm distance. The inoculated slides were incubated at 25 °C for 3 days in moist chambers. The fungal hyphae from the interaction zones were observed under light and scanning electron microscope (JEOL model: JSM-5410).

Evaluation of biocontrol efficacy of chitinase-producing endophytic *Streptomyces* under pot culture conditions

Chitinase-producing endophytic *Streptomyces* sp. GMKU 301 and GMKU 322 were evaluated for their biocontrol efficacy against *F. moniliforme* DOAC 1224 causing foot rots and wilting disease of maize plants (cv. Nakhonsawan 3). Maize seeds were surface sterilized by soaking in 1% sodium hypochlorite for 1 min and subsequently immersing in 70% ethanol for 5 min. Surface-sterilized seeds were then washed in sterile water 3 times. The seeds were pre-germinated on moist paper towels in a sterile plastic box at room temperature in the dark for 3–4 days. The seedlings were inoculated with individual *Streptomycetes* by pruned-root dip method (Musson *et al.*, 1995) and soaked in spore suspension (10^8 cfu/ml) for 4 hours on a rotary shaker at 110 rpm. Excess spore suspensions were drained off and seedlings were then planted into the mini-pot (4 cm in diameter) containing autoclaved soil. The mini-pots were placed in the greenhouse and supplied with water once a day. Thirteen-day-old seedlings were transferred into fresh pot (24 cm in diameter) with 5 plants per pot. One day after transferring the seedlings, each seedling was inoculated with 2 mycelial plugs (6 mm in diameter) of *F. moniliforme* DOAC 1224 on the collar region and then covered with parafilm to avoid dryness. Three days after pathogen inoculation, the parafilm was removed from seedling. Un-inoculated plants were treated with agar plug that without mycelial of fungi. There were 6 treatments as follows; (1) GMKU 301, (2) GMKU 322, (3) GMKU 301 with *F. moniliforme* DOAC 1224, (4) GMKU 322 with *F. moniliforme* DOAC 1224, (5) *F. moniliforme* DOAC 1224, and (6) un-inoculated control. Fourteen-day after pathogen inoculation, the plants were recorded disease index and calculating biocontrol efficacy. The experiment was performed in triplicate.

The disease index was evaluated as previously described (Pal *et al*., 2001) with some modification based on the disease scores 0–5, as follow; 0-no symptom, 1-appearance of lesions at the collar region, 2-large lesions at the collar region, 3-moderate rotting at the collar region, loss of turgor at the top with slight drooping, 4-extensive rotting at the collar region, wilting and drooping of the shoot, and 5-plant completely wilted, dead and dry. Disease scores were converted to disease incidence (Xue *et al.*, 2009) by using the following formula; disease incidence (%) = [Σ (the number of plants in this index x disease index) / (total number of plants investigated x highest disease index)] x 100. All treatments were arranged in a randomized complete block design. Data were subjected to analysis by one-way ANOVA (SPSS; version 16.0) and significant differences between means were determined using Duncan's multiple range test at $P \leq 0.05$. Biocontrol efficiency was calculated as described by Xue *et al*. (2009) using the following formula; biocontrol efficiency = [(disease incidence of control - disease incidence of bacteria treated plants) / disease incidence of control] x 100%. Finally, the maize stems were cut into longitudinal-cross section and then observed the symptom of disease under a stereomicroscope.

Results

Identification of endophytic actinomycetes

One hundred and thirty-three unique endophytic actinomycetes were originally isolated from the interior tissues of Thai medicinal plants. 16S rRNA gene sequence compared to the GenBank database suggested that these endophytes belonged to the genera *Actinoallomurus* (*n*=2, 1.5%), *Actinomadura* (*n*=16, 12.0%), *Dactylosporangium* (*n*=1, 0.8%), *Microbispora* (*n*=15, 11.2%), *Micromonospora* (*n*=6, 4.5%), *Nocardia* (*n*=3, 2.2%), *Nocardiopsis* (*n*=1, 0.8%), *Nonomuraea* (*n*=3, 2.2%), *Promicromonospora* (*n*=1, 0.8%), *Saccharopolyspora* (*n*=1, 0.8%), *Streptomyces* (*n*=83, 62.4%) and *Streptosporangium* (*n*=1, 0.8%). It was suggested that medicinal plants are good sources for diversity of endophytic actinomycetes.

Chitinase-producing endophytic actinomycetes

Endophytic actinomycetes were screened for their chitinase production ability on CCA plates. Eighty strains (60.2%) out of one hundred and thirty-three strains could produce chitinases. They belong to the members of genera *Actinoallomurus* (n=1, 1.25%), *Actinomadura* (n=8, 10%), *Dactylosporangium* (n=1, 1.25%), *Microbispora* (n=14, 17.5%), *Micromonospora* (n=5, 6.25%), *Nonomuraea* (n=1, 1.25%), *Promicromonospora* (n=1, 1.25%), *Saccharopolyspora* (n=1, 1.25%) and *Streptomycetes* (n=48, 60%). In this study, the strains were grouped according to chitinase

activity (diameter of clear zone) into 4 categories; 30 strains showed high activity (clear zone ≥5.0 mm), 22 strains showed moderate activity (3.0–4.9 mm), 28 strains showed low activity (1.1–2.9 mm) and 53 strains showed no activity (≤1.1 mm). According to these categories, *Micromonospora* sp. GMKU 358 (clear zone 19.5±1.3 mm), *Actinomadura* sp. GMKU 331 (17.0±1.0 mm), *Streptomyces* sp. GMKU 342 (17.7±3.2 mm) and *Microbispora* sp. GMKU 340 (15.7±1.9) showed the widest clear zone of chitinase production on CCA plates.

Fungal cell wall-degrading activity

Chitinase-producing endophytic actinomycetes were determined for their fungal cell wall-degrading activity on MFA plates. Seventy-seven strains (96.3%) out of eighty strains could exhibited fungal cell wall-degrading activity. The strains were grouped according to fungal cell wall-degrading activity (diameter of clear zone) into 4 categories; 49 strains showed high activity (clear zone≥5.0 mm), 16 strains showed moderate activity (3.0–4.9 mm), 12 strains showed low activity (1.1–2.9 mm) and 3 strains showed no activity. According to these categories, *Streptomyces* sp. GKU 148 (clear zone 15.3±0.6 mm), *Streptomyces* sp. GKU 112 (15.0±1.0 mm), *Microbispora* sp. GMKU 350 (14.7±0.6 mm) and *Actinomadura* sp. GMKU 356 (14.0±1.7) showed the widest clear zone of fungal cell wall-degrading activity on MFA plates. The results showed that 20 strains (26.0%) of the positive strains were able to produce chitinases and degraded fungal cell-wall with high activity. They belong to the members of genera *Actinomadura* (n=2, 10%), *Dactylosporangium* (n=1, 5%), *Microbispora* (n=8, 40%), *Micromonospora* (n=2, 10%), *Promicromonospora* (n=1, 5%), and *Streptomyces* (n=6, 30%).

Detection of chitinase genes

Genes encoding family 18 subfamily A and family 19 chitinases were detected from 77 chitinase-producing endophytic actinomycetes. Based on the published sequences of the actinomycete chitinases in database, specific primers for chitinase genes were designed. The amplified fragments of *chi18A* and *chi19* genes were approximately 580 and 460 bp, respectively. Some amplified fragments were purified and sequenced. The nucleotide and deduced amino acid sequences were perfectly matched to the family 18 subfamily A and family 19 chitinases of actinomycetes. As the result, the strains were grouped according to present/absent of chitinase genes into 4 categories: 39 strains (50.65%) presented both *chi18A* and *chi19* genes, 6 strain (7.79%) presented only *chi18A* gene, 2 strains (2.60%) presented only *chi19* gene and 30 strains (38.96%) absented both genes. According to these categories, 26 strains of *Streptomyces*, 4 strains

of *Microbispora* (GMKU 340, GMKU 362, GMKU 363 and GMKU 372), 4 strains of *Actinomadura* (GKU 126, GKU 127, GKU 154 and GKU 157), 2 strains of *Micromonospora* (GMKU 326 and GMKU 330) and *Actinoallomurus* sp. GMKU 370, *Dactylosporangium* sp. GKU 133, and *Nonomuraea* sp. GKU 150 were presented both *chi18A* and *chi19* genes. Whereas, 2 strains of *Microbispora* (GMKU 364 and GMKU 378), 2 strains of *Micromonospora* (GMKU 353 and GMKU 358), *Actinomadura* sp. GKU 125 and *Streptomyces* sp. GKU 105 were presented only *chi18A* gene. Although, *Streptomyces* sp. GKU 171 and *Promicromonospora* sp. GMKU 303 were presented only *chi19* gene.

Antagonism against *F. moniliforme* DOAC 1224

Chitinase-producing endophytic actinomycetes were evaluated for their potential antagonism against phytopathogenic fungi on SCA plates. Thirty-three strains (84.62%) out of thirty-nine strains could exhibited the antagonism against mycelial growth of *F. moniliforme* DOAC 1224 *in vitro*. The strains were grouped according to antagonistic activity into 4 categories; 4 strains showed strong inhibition (inhibition zone≥10 mm), 11 strains showed moderate inhibition (1–9 mm.), 18 strains showed weak inhibition (no inhibition zone, but abnormal growth of fungus was observed at the interaction zone) and 6 strains showed no inhibition (fungus overgrew the colony of actinomycete). According to these categories, 4 strains of *Streptomyces* (12.12%) showed strong inhibition against *F. moniliforme* DOAC 1224. *Streptomyces* sp. GMKU 301 (inhibition zone 20.5±0.7 mm) was showed the widest inhibition zone of antagonistic activity on SCA plates, followed by GMKU 354 (19.0±1.4 mm), GMKU 355 (14.0±1.4 mm) and GKU 101 (11.5±0.7 mm). The other genera like *Actinoallomurus* (GMKU 370), *Actinomadura* (GKU 126, GKU 128 and GKU 157), *Microbispora* (GMKU 362 and GMKU 363) and *Micromonospora* (GMKU 330) were also showed antagonistic activity against *F. moniliforme* DOAC 1224. All strains that showed no inhibition were members of non-streptomycete strains (18.18%). They belong to the genera *Microbispora* (GMKU 340 and GMKU 372), *Actinomadura* (GKU 154), *Dactylosporangium* (GKU 133), *Micromonospora* (GMKU 326) and *Nonomuraea* (GKU 150).

Selection and characterization of chitinase-producing endophytic actinomycetes

On the basis of results that obtained from chitinase and fungal cell-wall degrading activities, chitinase genes and antagonistic activity. The best performing strains, namely *Streptomyces* sp. GMKU 301 and GMKU 322, were selected for further studies, these strains showed high chitinase activity (clear zone 5.7±1.0 and 14.0±2.0 mm) and presented both *chi18A*

and *chi19* genes. Conceptual translation of *chi18A* gene sequence of *Streptomyces* sp. GMKU 301 (496 nt) revealed 93% identity to family 18 subfamily A chitinase of *Streptomyces griseoflavus* (accession number WP 004925477) and *Streptomyces hirsutus* (WP 055591765), and *chi19* gene sequence (410 nt) revealed 100% identity to family 19 chitinase of *Streptomyces aureofaciens* (WP 052837276) and *Streptomyces* sp. CCM_MD2014 (WP 061446277), *chi18A* gene sequence of *Streptomyces* sp. GMKU 322 (590 nt) revealed 99% identity to family 18 subfamily A chitinase of *Streptomyces griseorubens* (WP 037641602) and *chi19* (407 nt) revealed 100% identity to family 19 chitinase of *S. griseorubens* (WP 037638087). Phylogenetic tree of chitinase was constructed and family of Chi18A and Chi19 was distinguish separated into 2 clades (Fig. 8-1). Additionally, *Streptomyces* sp. GMKU 301 were showed moderate fungal cell-wall degrading activity (clear zone 3.3±0.6 mm) and highest strong inhibition (inhibition zone 20.5±0.7 mm) against *F. moniliforme* DOAC 1224 *in vitro*, whereas *Streptomyces* sp. GMKU 322 showed high fungal cell-wall degrading activity (9.3±1.5 mm) and weak inhibition against *F. moniliforme* DOAC 1224.

Effect of chitinase-producing endophytic *Streptomyces* on *F. moniliforme* hyphae

Chitinase-producing endophytic *Streptomycetes* sp. GMKU 301 and GMKU 322 were investigated for their effect on *F. moniliforme* DOAC 1224 hyphae during dual culture on CCA medium. Observations of the interaction zone between *Streptomyces* sp. GMKU 301 and *F. moniliforme* DOAC 1224 under light microscope revealed lysis of the fungal hyphae, while the interaction zone between *Streptomyces* sp. GMKU 322 and *F. moniliforme* DOAC 1224 revealed distortions of the fungal hyphae. These interaction zone were confirmed under scanning electron microscope. *Streptomyces* sp. GMKU 301 and *F. moniliforme* DOAC 1224 interaction appeared distinct holes on the fungal hyphae, while *Streptomyces* GMKU 322 and *F. moniliforme* DOAC 1224 interaction appeared decomposition of the fungal hyphae.

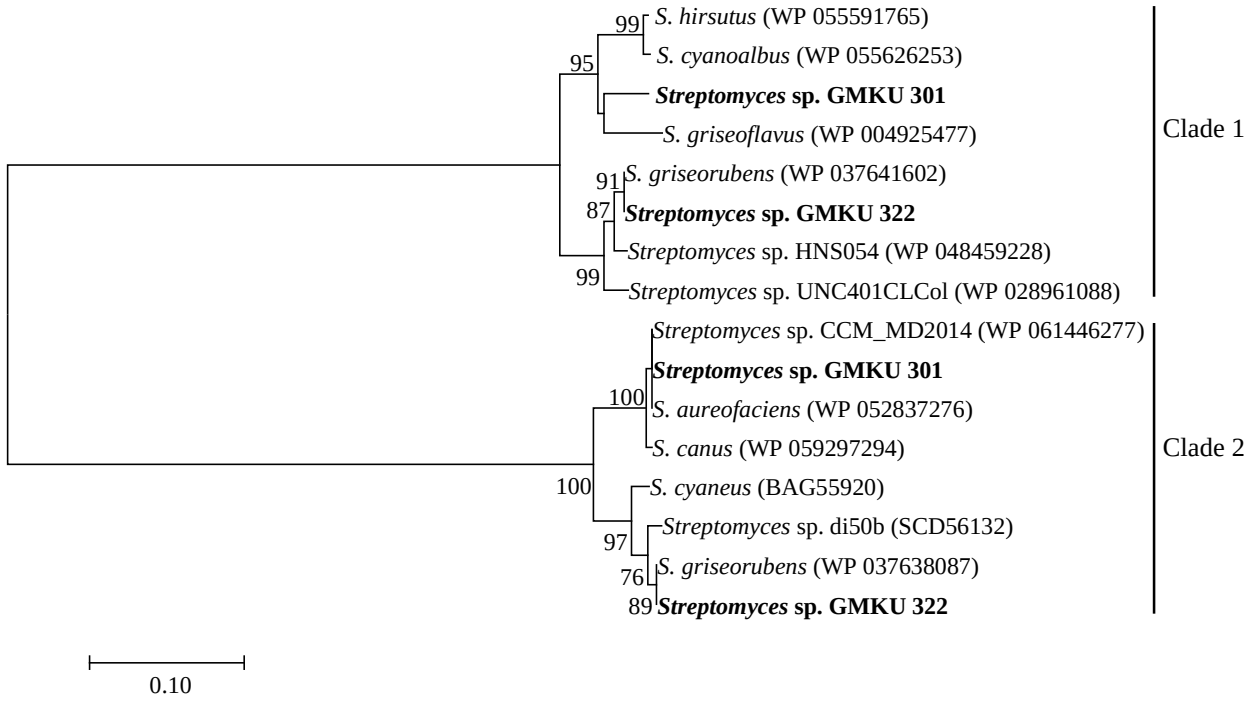

Fig. 8-1 Phylogenetic tree of chitinases based on protein sequence alignment of the conserved regions. The tree was constructed by neighbor-joining method. Bootstrap values based on 1,000 replications shown at branch point and the bar represents 10% sequence divergence. Only values of 70% or greater are included. Sequences derived from the database are shown with their accession numbers. The sequences in clade 1 belongs to family 18 subfamily A chitinases and clade 2 belongs to family 19 chitinases.

Biocontrol efficacy of chitinase-producing endophytic *Streptomyces* under pot culture conditions

Chitinase-producing endophytic *Streptomyces* sp. GMKU 301 and 322 were evaluated for their potential for biocontrol of foot rot and wilting disease of maize plants under pot culture conditions. Fourteen-day after *F. moniliforme* DOAC 1224 inoculation, control plants showed high disease incidence (82.67%) with the majority of plants completely wilted and dead (Table 8-1). However, plants were treated with individual *Streptomyces* sp. GMKU 301 and GMKU 322 exhibited the significant ($P \leq 0.05$) reduction in disease incidence (Table 8-1). The greatest incidence of disease reduction was achieved in plants treated with *Streptomyces* sp. GMKU 322 (Table 8-1). The biocontrol efficacy of individual *Streptomyces* sp. GMKU 301 and GMKU 322 in controlling foot rot and wilting disease of maize plants caused by *F. moniliforme* DOAC 1224 up reached 43.33–65.91% (Table 8-1). Longitudinal-cross sections of stems, control plants showed completely rotten symptom. Whereas, plant treated with individual *Streptomyces* sp. GMKU 301

and GMKU 322 rendered some infection. Additionally, plant treated with individual *Streptomyces* sp. GMKU 301 and 322 without pathogen inoculation did not produce any harmful effects on the plant growth.

Table 8-1 Effect of chitinase-producing endophytic *Streptomyce*s antagonistic to *F. moniliforme* DOAC 1224 of maize plants under pot culture conditions. All results were made 14-days after *F. moniliforme* DOAC 1224 inoculation.

Treatment	**Disease incidence (%) ***	**Biocontrol efficacy (%) ‡**
GMKU 301	0.00 [a †]	–
GMKU 322	0.00 [a]	–
GMKU 301 + *F. moniliform* DOAC 1224	46.67 ± 1.33 [c]	43.33 ± 3.33
GMKU 322 + *F. moniliform* DOAC 1224	28.00 ± 2.31 [b]	65.91 ± 3.70
F. moniliform DOAC 1224	82.67 ± 2.67 [d]	–
Un-inoculated control	0.00 [a]	–

* Percentage of disease incidence was determined as [Σ (the number of plants in this index x disease index) / (total number of plants investigated x highest disease index)] x 100.

† Values are mean ± standard error of 3 independent experiment. Each experiment was conducted in 5 replicates. Values followed by the same letter are not significantly (P≤0.05) different according to Duncan's multiple range test.

‡ Percentage of biocontrol efficacy was determined as [(disease incidence of control - disease incidence of bacteria treated plants) / disease incidence of control] x 100%.

Discussion

Among actinomycetes, streptomycetes are well known as decomposers of chitin in soil (Metcalfe *et al.*, 2002). Streptomycete chitinases play important roles cooperatively in the biodegradation of chitin in soil ecosystem as well as in protection of plants against pathogenic fungi (Gomes *et al.*, 2000; Watanabe *et al.*, 1999). There were records use of endophytic actinomycetes for chitinase production (El-Tarabily, 2003; Haggag & Abdallh, 2012; Quecine *et al*., 2008; Taechowisan *et al*., 2003). In most cases, colloidal chitin is used as substrate for isolation of chitinase-producing organisms (Felse & Panda, 2000), indicated that these microbes could degraded and utilized chitin as substrate for growth and multiplication (Shanmugam *et al*., 2013). In this study, we found that different strains of streptomycetes and non-streptomycetes (*Actinomadura*, *Microbispora* and *Micromonospora*) could produce chitinases. It was suggested that chitinase production are widely distributed in actinomycetes. However, none of the members

of genus *Nocardia* (GMKU 327, GKU 152 and GKU 156) could produce chitinases but little work have been reported in *Nocardia orientalis* (Nanjo *et al.*, 1990; Usui *et al.*, 1987).

The fungal cell wall is a complex structure composed of chitin, glucans, mannans and glycoproteins, there is evidence of extensive cross-linking between these components (Adams 2004; Bowman & Free, 2006). Most of chitin is considered to be located near to the plasma membrane and responsible to provide the fungal cell (Bowman & Free, 2006). Therefore, several attempts have been made to use of chitinase-producing microorganisms as fungal disease protectants (Hariprasad *et al.*, 2011). In this study, the use of basal medium supplemented with mycelial fragments of *F. moniliforme* DOAC 1224 helped to rapidly select chitinase-producing endophytic actinomycetes that capable of degrading fungal cell walls. Mycelial fragments agar used previously to select glucanase-producing actinomycetes for biocontrol of *Phytophthora fragariae* (Valois *et al*., 1996) and *Pythium aphanidermatum* (El-Tarabily, 2006). However, we found that most of chitinase-producing endophytic actinomycetes (96.3%) could degraded fungal cell walls, indicating that they may play an important role in fungal disease suppression.

Family 18 chitinase appear to encompass most bacterial chitinase genes sequenced so far, however, recently family 19 chitinase genes were discovered in streptomycetes (Metcalfe *et al*., 2002). Studies of chitin utilization system of streptomycetes revealed that existence of multiple chitinase genes in their genomes. They suggested the high multiplicity of chitinase genes of streptomycetes that act synergistically to degrade chitin and potentially enabling them to hydrolyze the natural diversity of chitin type (Chater *et al*., 2010; Xiayun *et al*., 2012). However, detailed studies of enzymes that degrade chitin from fungal cell walls is scarce (Chater *et al*., 2010). The previous comparison of enzymatic and antifungal properties between family 18 and 19 chitinases from *Streptomyces coelicolor* A3(2) reported only family 19 chitinase (*chiF*) that showed significant antifungal activity (Kawase *et al*., 2006). Several studies of molecular cloning and expression of genes encoding family 19 chitinase from *Streptomyces* sp. indicated that purified chitinases exhibited remarkable antifungal activity (Hoster *et al.*, 2005,;Okazaki *et al.*, 2004; Tsujibo *et al.*, 2000; Yamashita & Okazaki, 2004). The success of disruption of gene encoding family 19 chitinase (*chiB*) in *Nocardiopsis prasina* OPC-131 resulted in a mutant that reduced antifungal activity (Tsujibo *et al.*, 2003). Nowadays, antifungal activity of family 18 chitinases have been reported only in *Bacillus* sp. (Xiao *et al.*, 2009; Yang *et al.*, 2009; Zhang *et al.*, 2012), *Stenotrophomonas maltophilia* (Jankiewicz *et al.*, 2012), *Streptomyces rimosus* (Brzezinska *et al.*, 2013) and *Streptomyces roseolus* (Xiayun *et al*., 2012) and some reports of them speculated that these chitinases belong to family 18 subfamily A (Jankiewicz *et al*., 2012; Xiayun *et al*., 2012).

These data agreed well with bacterial chitinases in family 18 subfamily A and family 19 are involved in antifungal activity. In our study, we confirmed chitinase-producing endophytic actinomycetes harboring chitinase genes that involved in antifungal activity. Specific primers of family 18 subfamily A and family 19 chitinase genes were designed and used to identify the chitinase genes in streptomycetes and non-streptomycetes. Chitinase-producing endophytic actinomycetes that presented both chitinase genes were found in genera *Actinoallomurus*, *Actinomadura*, *Dactylosporangium*, *Microbispora*, *Micromonospora*, *Nonomuraea* and *Streptomyces*. The results demonstrated that family 18 subfamily A and family 19 chitinase genes are broadly dispersed in actinomycetes. There were reports on molecular screening for chitinase production from streptomycetes (Gherbawy *et al*., 2012; Williamson *et al*., 2000) but little work has been done from non-streptomycetes (Kawase *et al*., 2004). Some strains of chitinase-producing endophytic actinomycetes were presented either family 18 subfamily A or family 19 chitinase genes, this results are also agreed with PCR amplification of chitinase genes from streptomycetes by using specific primers (Williamson *et al*., 2000). However, some strains of chitinase-producing endophytic actinomycetes were unable to detect neither family 18 subfamily A family 19 chitinase genes. Previous studies indicated cellulose-degrading microorganisms such as species of *Cellulomonas*, *Clostridium* and *Streptomyces* apparently have the ability to degrade and utilize chitin as a nitrogen source (Korn-Wendisch *et al.*, 1992; Reguera & Leschine, 2001), because cellulases can hydrolyze the glycosidic linkages of chitin (Simpson & Barras, 1999).

Chitinase-producing microorganisms have been postulated to play an important role in antagonism and biocontrol of phytopathogenic fungi (Brzezinska *et al.*, 2014). The SCA media appears to be suitable for screening of antagonism activity since it supported actinobacteria growth (Qin *et al*., 2011) and stimulate maximum antifungal activity (Augustine *et al.*, 2004). Among chitinase-producing endophytic actinomycetes tested, *Streptomyces* (66.67%) were found to be an effective antagonism against *F. moniliforme* DOAC 1224 *in vitro*. It has been reported the role of streptomycetes as potential agents in the biocontrol of various soil-borne pathogenic fungi such as *Alternaria brassicicola*, *Colletotrichum gleosporioides*, *Fusarium oxysporum*, *Pennicillium digitatum*, *Pyricularia oryzae*, *Rhizoctonia solani* and *Sclerotium rolfsii* (Joo, 2005; Khamna *et al.*, 2009; Prabavathy *et al.*, 2006; Prapagdee *et al.*, 2008). In addition, some genera of non-streptomycetes were presented here, including *Actinoallomurus*, *Actinomadura*, *Microbispora* and *Micromonospora* also exhibited antagonism against *F. moniliforme* DOAC 1224. Other genera like *Actinoplanes*, *Amorphosporangium*, *Nocardia*, *Nocardioides*, *Streptosporangium* and *Streptoverticillium* have the potential suppressive to several soil-borne pathogenic fungi (El-

Tarabily, 2006). We found that 46% of chitinase-producing endophytic non-streptomycetes did not inhibited the mycelial growth of *F. moniliforme* DOAC 1224, which indicated that their chitinase producing ability on SCA plates could not overcome the fungal growth. However, most bacteria are capable of producing other metabolites and antibiotics that could also be involved in antagonism activity (Martínez-Absalón *et al.*, 2014).

Previous reports proved that *Fusarium oxysporum* treated with purified chitinase from endophytic *Streptomyces aureofaciens* CMUAc130 showed the inhibition of spore germination and fungal cell-wall lysis under light microscope (Taechowisan *et al*., 2003). Likewise, *Aspergillus parasiticus* treated with purified endochitinase from *Streptomyces* sp. RC1071 showed the inhibition of spore germination and considerable reduction in hyphal width (Gomes *et al.*, 2001). *Fusarium oxysporum* f. sp. *lycopersici* treated with culture filtrate *Streptomyces griseorubens* E44G showed relatively thicker with granulated surfaces of hyphae under scanning electron microscope (Al-Askar *et al.*, 2015). The best performing strains of chitinase-producing endophytic actinomycetes *Streptomyces* sp. GMKU 301 and GMKU 322 were demonstrated the decomposition of *F. moniliforme* DOAC 1224 hyphae during dual culture on CCA media, observed by light and scanning electron microscope. However, *Streptomyces* sp. GMKU 301 gave the widest inhibition zone of antagonistic activity on SCA medium but gave weak inhibition on CCA medium. These suggested that SCA medium has high nutrient concentration that supported the growth of actinobacteria (Qin *et al*., 2011) and stimulated maximum antifungal activity in some strain of *Streptomyces* (Augustine *et al*., 2004). Whereas, CCA medium stimulated the production of chitinases (Nawani *et al*., 2002). It was suggested that the decomposition of *F. moniliforme* DOAC 1224 hyphae on CCA medium by *Streptomyces* sp. GMKU 301 and GMKU 322 may be primarily influenced by chitinases.

Chitinase-producing rhizobacteria have been used as fungal disease protestants under greenhouse conditions, such as use of *Bacillus atrophaeus* S2BC-2 antagonistic to *Fusarium oxysporum* f. sp. *zingiberi* for control of rhizome rot of ginger (Shanmugam *et al*., 2013) and *Bacillus subtitlis* CRB20 antagonistic to *Fusarium oxysporum* for management of fusarium wilt in tomato (Hariprasad *et al*., 2011). Slimene *et al.* (2015) isolated *Bacillus licheniformis* S213 from Tunisian soil that against *Phoma medicaginis* infection in *Medicago trancatula*. Meanwhile, the success of *Serratia marcescens*, *Streptomyces viridodiasticus* and *Micromonospora carbonaces* isolated from rhizospheric soil, individually or in combination, were exhibited the antagonistic to *Sclerotinia minor* and significantly reduce basal drop disease of lettuce (El-Tarabily *et al.*, 2000). The biocontrol efficacy of chitinase-producing endophytic *Streptomyces* sp. GMKU 322 in

controlling foot rot and wilting disease of maize plants caused by *F. moniliforme* DOAC 1224 was higher than *Streptomyces* sp. GMKU 301 under pot culture conditions. It is interesting that *in vitro* test showed they could exhibited high chitinase activity, while *Streptomyces* sp. GMKU 301 exhibited moderate fungal cell-wall degrading activity and highest strong inhibition against *F. moniliforme* DOAC 1224, *Streptomyces* sp. GMKU 322 exhibited high fungal cell-wall degrading activity and weak inhibition against *F. moniliforme* DOAC 1224. Therefore, it can be suggested that *Streptomyces* sp. GMKU 322 has the capacity of strong antagonistic mechanism towards *F. moniliforme* DOAC 1224 in plant and appeared to be relate the production of chitinases and fungal cell-wall degrading enzymes. This is the first report to describe the use of chitinase-producing endophytic *Streptomyces* for bioncontrol of *F. moniliforme* in maize plants.

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Section 9

Identify of novel species of endophytic actinomycetes *Actinomadura barringtoniae* sp. nov. GKU 128

Introduction

The genus *Actinomadura* was firstly established by Lechevalier and Lechevalier [1] and was proposed to belong to the family Thermomonosporaceae [2, 3]. At present, the genus *Actinomadura* comprises 61 recognized species with validly published names (http://www.bacterio.net/actinomadura.html). The most recent species, *Actinomadura alkaliterrae*, has just been described [4]. The genus *Actinomadura* represents aerobic, Gram-strain-positive, non-acid-alcohol-fast, non-motile actinomycete that generates abundantly branched, non-fragmenting substrate and aerial mycelia. The aerial mycelium differentiated into various spore chain morphologies. The spore chains are generally short (sometime long), straight, hooked or spiral with folded, irregular, rugose, smooth, spiny or warty surface ornament. Members of the genus *Actinomadura* are characterized by the presence of meso-diaminopimelic acid and N-acetylmuramic acid in the cell wall peptidoglycan. Mycolic acids are absent. The whole-cell hydrolysates contain galactose, glucose, madurose, mannose and ribose. The cell membranes contain diphosphatidylglycerol (DPG) and phosphatidylglycerol (PG) as major phospholipids. The predominant menaquinone is MK-9(H6). The cellular fatty acid is rich in branched saturated and unsaturated fatty acids, including tuberculostearic acid. The genomic DNA G + C content is 66–73 mol% [5].

Most *Actinomadura* species are recovered from soils [5]. Novel strains have been recently isolated from alkaline soil [4], geothermally heated soil [6], maddy soil [7], mountain soils [8, 9], peat swamp forest soil [10] and Saharan soils [11, 12]. Some strains of the species are pathogens of humans and animals [13-15]. A few strains are reported as endophytes, namely *Actinomadura flavalba* and *Actinomadura syzygii* isolated from medicinal plants [16, 17].

Materials and Methods

Isolation of endophytic actinomycetes

Endophytic actinomycetes were isolated from excised roots of indian oak tree [*Barringtonia acutangula* (L.) Gaertn.] collected at Khao Khitchakut district, Chantaburi province, Thailand. The roots were surface-sterilized as described by Rachniyom et al. [18]. Colonies were appeared on starch-casein agar [19] supplemented with ampicillin (100 µg ml-1), penicillin G (2.5

U ml-1), amphotericin B (50 µg ml-1) and cycloheximide (50 µg ml-1) after incubation at 28 °C for 3–4 weeks. A single colony was purified and cultured on mannitol-soybean (MS) agar [20]. The pure culture was maintained in 20% (v/v) glycerol suspension at –80 °C.

Morphological and physiological characteristics of strain GKU 128

Morphological and physiological characteristics of strain GKU 128 were determined in comparison with those of phylogenetically closely related type strains, *A. nitritigenes* NBRC 15918T and *A. fibrosa* JCM 9371T. Cultural characteristics were determined on nutrient agar (NA; Difco), Czapek's agar (CZA; ATCC medium 312) and various International Streptomyces Project (ISP) media [21], namely yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salt-starch-agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract-iron agar (ISP 6) and tyrosine agar (ISP 7) after incubation at 27 °C for 21 days. Mycelium and soluble pigment colour were determined by comparison with colour chips from the Colour Harmony Manual [22]. Spore morphology and ornamentation of strain GKU 128T were observed by light and scanning electron microscopy (model JSM-6610LV; JEOL) using culture grown on ISP 2 medium at 28 °C for 7 weeks. The motility of spores were observed after flooding the culture plate (MS agar at 28 °C for 7 weeks) with releasing buffer [23] and incubated at 30 °C for 30 min.

Growth at various temperatures (5–50 °C using a temperature gradient incubator), pH values (3.0–11.0 at intervals of 1.0 pH unit) and NaCl concentrations [0–7 % (w/v) at intervals of 1.0 %] were assessed using ISP 2 medium at 28 °C for 14–21 days. For pH values, medium was adjusted with biological buffers: citrate buffer for pH 3.0–5.0, phosphate buffer for pH 6.0–8.0 and bicarbonate-carbonate buffer for pH 9.0–11.0. Catalase and oxidase activities were observed with 3 % (v/v) hydrogen peroxide solution and 1 % (v/v) tetramethyl-p-phenylenediamine solution, respectively. Acid production from carbohydrates and decomposition of adenine, hypoxanthine, L-tyrosine, xanthine, casein and urea were determined using the method of Gordon et al. [24]. Reduction of nitrate was observed using nitrate broth (Difco). Starch hydrolysis was examined on ISP 4 medium. Gelatin liquefaction was evaluated on gelatin medium (2.0 % glucose, 0.5 % peptone, 20 % gelatin; pH 7.0). Coagulation and peptonization of milk were observed in 10 % (v/v) skimmed milk broth (Difco). Production of H2S and melanin pigments were determined on peptone iron agar (Difco) and ISP 7 medium. Citrate utilization was tested on Simmons' citrate agar (Difco). Degradation of Tweens 20 and 80 (1 %, w/v) were examined by using Sierra medium [25]. Utilization of carbon and nitrogen sources was examined on ISP 9 medium and basal medium supplemented with a final concentration of 1 % and 0.1 % (w/v) of the tested carbon and nitrogen

sources, respectively [21, 26]. Enzyme activities were tested by using the API ZYM system (bioMérieux) according to the manufacturer's instructions.

Chemotaxonomic analyses of strain GKU 128

Freeze-dried cells of strain GKU 128 were obtained from culture grown in ISP 2 broth on a rotary shaker at 27 °C for 7 days. The isomer of diaminopimelic acid in the cell wall and sugar in whole cell hydrolysate were determined by TLC according to the method of Hasegawa et al. [27]) and Stanck and Roberts et al. [28], respectively. The N-acyl type of muramic acid in the peptidoglycan was examined using the method of Uchida and Aida [29]. Mycolic acids was detected by TLC following the procedure of Tomiyasu [30]. Phospholipids in the cell were extracted and detected by two-dimensional TLC according to the method proposed by Minnikin et al. [31]. Menaquinones were extracted and purified by using the method of Collins et al. [32] and were analyzed by LC/MS (model JSM-T100LP; JEOL) with CAPCELL PAK C18 UG 120 column (Shiseido) at 210 nm. Methyl esters of cellular fatty acids were extracted and analyzed by GC (model 6850 series II; Agilent) with HP Ultra 2 column (Agilent) according to the instructions for the Sherlock Microbial Identification System (version 6.1; MIDI), using the RTSBA6 library for identification fatty acids [33]. The genomic DNA G+C content was determined by following the procedure of Tamaoka and Komagata [34], and was analyzed by HPLC (model L-2455, Hitachi) with CAPCELL PAK C18 UG 120 column (Shiseido) at 270 nm.

16S rRNA sequencing and phylogenetic analysis

Genomic DNA of strain GKU 128 was extracted from a culture grown on MS agar at 28 °C for 7 days according to the method of Kieser et al. [35]. Conditions of PCR amplification of the 16S rRNA gene, purification and sequencing were carried out as described previously [18]. The 16S rRNA gene sequence of strain GKU 128 was compared with corresponding sequences of reference type strains via the EzBioCloud server [36], and the value of pairwise sequence similarities were achieved. The program CLUSTAL X version 2.0 [37] was used to align the sequences of strain GKU 128 together with the related taxa retrieved from the GenBank/EMBL/ DDBI database. Phylogenetic trees were reconstructed using the neighbour-joining [38], maximum-likelihood [39] and maximum-parsimony [40] algorithms by MEGA software version 7.0 [41]. A distance matrix of the neighbour-joining and maximum-likelihood trees were generated using Kimura's two parameter model [42]. In addition, nearest-neighbour-interchange (NNI) was used for the heuristic method for a maximum-likelihood tree. Likewise, subtree pruning-regrafting

(SPR) method (number of initial tree=10; MP search level=1; maximum number trees to retain=100) was carried out for a maximum-parsimony tree. All positions containing gaps and missing data were eliminated from the dataset. The topology of the phylogenetic trees was evaluated by bootstrap analysis [43] based on 1000 resampled datasets.

DNA-DNA relatedness

The taxonomic position of strain GKU 128 was confirmed by DNA-DNA hybridization with the closely related species, *A. nitritigenes* NBRC 15918T and *A. fibrosa* JCM 9371T by fluorometrically determination using photobiotin-labelled DNA probes [50] by a microplate reader (model SH-9000, Corona Electric). Hybridization was performed at 50 °C with eight replications for each sample and the DNA-DNA relatedness values were expressed as the means of two independent determinations.

Results and Discussion

Isolation of endophytic actinomycetes

Thirty-one isolates were identified from the roots indian oak tree [*Barringtonia acutangula* (L.) Gaertn.]. Most of the isolates belonged to the genus *Streptomyces* (n=20) and some less common isolates were classified into the genera *Actinomadura* (n=7), *Dactylosporangium* (n=1), *Nocardia* (n=2), and *Nonomuraea* (n=1). In this study, an *Actinomadura* strain GKU 128 was described using a polyphasic approach.

Morphological and physiological characteristics of strain GKU 128

Strain GKU 128 showed good growth on NA, CZA, ISP 3, ISP 4, ISP 6 and ISP 7 media and moderate growth on ISP 2 and ISP 5. Aerial and substrate mycelia were well developed. The substrate mycelium was yellowish brown, grayish yellow brown to brownish gray (ISP 2, ISP 4, ISP 6 and NA), grayish olive to olive black (CZA and ISP 3), dusty yellow (ISP 5) and oyster white (ISP 7). The aerial mycelium was white (ISP 3, ISP 5, ISP 7 and NA), light brownish gray (ISP 2) and pale orange (ISP 4), except on ISP 6 and CZA in which it was not produced. Antique gold pigment could be observed on ISP 2 medium (Fig. 9-1, Table 9-1 and Table 9-2). Strain GKU 128 formed an extensively branched, non-fragmenting substrate and aerial mycelia. The aerial mycelium was differentiated into straight to flexuous chains of spores (six or more spores per chain). Each spore was oval to cylindrical (0.6 x 0.8 µm) with smooth surface, and not motile (Fig. 9-2). Phenotypic characteristic revealed that strain GKU 128 grew at 14–38 °C (optimal 24–32

°C), at pH range 7.0–8.0 (optimal pH 7.0) and at 0–5 % (w/v) NaCl (optimal 0–1 %). Other physiological and biochemical properties are showed in the species description. Furthermore, strain GKU 128 exhibited different phenotypic characteristics from the reference type strains, *A. nitritigenes* NBRC 15918T and *A. fibrosa* JCM 9371T (Table 9-1).

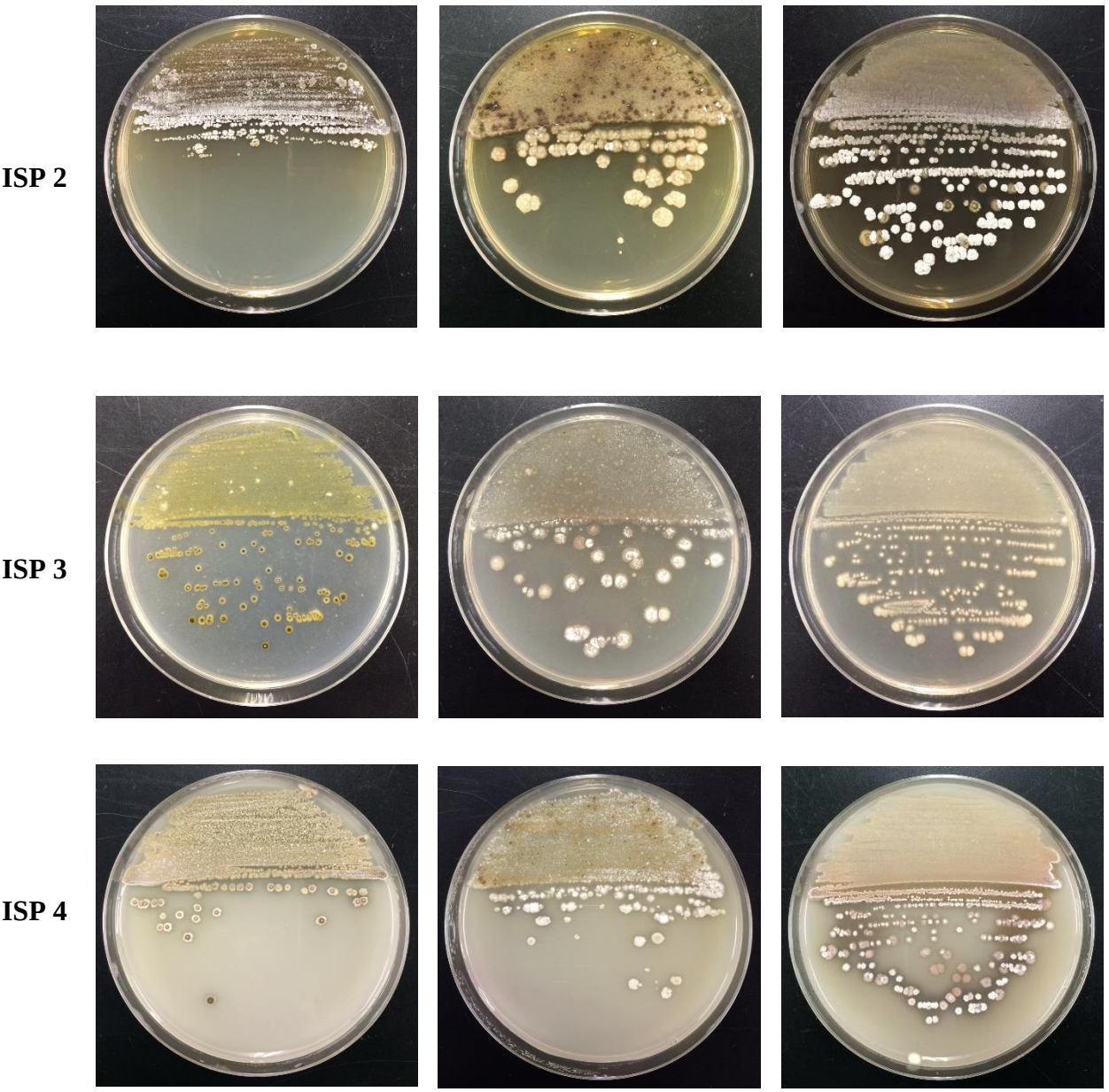

Fig. 9-1 Cultural characteristics of strain GKU 128 (left) and closely related type strains, *A. nitritigenes* NBRC 15918^{T} (middle), and *A. fibrosa* JCM 9371^{T} (right) after incubation on various medium at 27 °C for 21 days.

Table 9-1 Differential of the phenotypic characteristics of strain GKU 128 and the closely related species of the genus *Actinomadura*. Strains: 1, GKU 128; 2, *A. nitritigenes* NBRC 15918^{T}; 3, *A. fibrosa* JCM 9371^{T}. +, positive; –, negative; w, weekly positive. All data were determined in this study.

Characteristics	**1**	**2**	**3**
Colony characteristics on ISP 2 medium			
Growth	Moderate	Abundant	Abundant
Aerial mycelium	Light brownish gray	White	White
Substrate mycelium	Yellowish brown	Grayish yellow brown	Pale yellow brown
Soluble pigment	Antique gold	None	None
Growth at pH 8.0	+	+	–
Growth at 5 % (w/v) NaCl	+	–	–
Acid production from:			
Adonitol	–	+	–
L-Arabinose	+	–	–
D-Mannitol	+	–	–
Sucrose	w	–	–
Decomposition of:			
Adenine	+	–	–
Starch	+	–	+
L-Tyrosine	+	–	–
Urea	–	+	–
Nitrate reduction	+	+	–
Gelatin liquefaction	+	–	–
Carbon utilization of:			
Adonitol	+	–	–
L-Arabinose	+	+	–
D(-)-Fructose	+	+	–
Glycerol	+	+	–
myo-Inositol	+	+	–
D-Maltose	+	–	–
D-Mannitol	+	+	–
D-Mannose	+	+	–
D-Raffinose	+	+	–
L-Rhamnose	+	+	–
D-Xylose	–	+	+
Nitrogen utilization of:			
Potassium nitrate	+	–	–
L-Proline	+	–	+
L-Valine	+	–	–
Enzyme activities of:			
α-galactosidase	w	–	–
α-mannosidase	+	w	–

Table 9-2 Culture characteristics of strain GKU 128 and the closely related species of the genus *Actinomadura* grown at 27 °C for 21 days. Strains: 1, GKU 128; 2, *A. nitritigenes* NBRC 15918^T; 3, *A. fibrosa* JCM 9371^T. All data were determined in this study. No soluble pigments were produced on any of the tested media.

Medium	Growth and cultural characteristic	1	2	3
NA	Growth	Abundant	Abundant	Abundant
	Aerial mycelium	White	White	White
	Substrate mycelium	Grayish yellow brown	Light olive brown	Light gray
CZA	Growth	Abundant	Abundant	Abundant
	Aerial mycelium	None	White	None
	Substrate mycelium	Grayish olive	Light brownish gray	Light brownish gray
ISP 3	Growth	Abundant	Abundant	Abundant
	Aerial mycelium	White	White	None
	Substrate mycelium	Olive black	Light brown gray	Pale brown
ISP 4	Growth	Abundant	Abundant	Abundant
	Aerial mycelium	Pale orange	White	Pink
	Substrate mycelium	Light brownish gray	Pale yellow brown	Pale red
ISP 5	Growth	Moderate	Moderate	Abundant
	Aerial mycelium	White	White	Pink
	Substrate mycelium	Dusty yellow	Light brown gray	Gray reddish red
ISP 6	Growth	Abundant	Abundant	Moderate
	Aerial mycelium	None	None	None
	Substrate mycelium	Light brownish gray	Light brownish gray	Light brownish gray
ISP 7	Growth	Abundant	Abundant	Abundant
	Aerial mycelium	White	White	Pale orange
	Substrate mycelium	Oyster white	Pale yellow brown	Orange

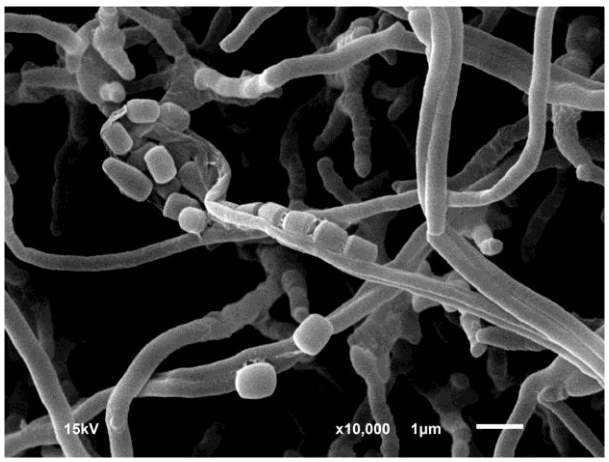

Fig. 9-2 Scanning electron micrographs of straight to flexuous chain of spores with smooth surface ornament of strain GKU 128 grown on ISP 2 medium at 28 °C for 7 weeks. Bar, 1 µm.

Chemotaxonomic analyses of strain GKU 128

Strain GKU 128 revealed chemical markers typical of members of the genus *Actinomadura*. The cell wall contained meso-diaminopimelic acid as the diagnostic peptidoglycan diamino acid and whole-cell hydrolysate was found to contain madurose as the diagnostic sugar, along with glucose, mannose, rhamnose and ribose, showing that it possessed cell-wall type III [44] and whole-cell sugar pattern B [45]. The N-acyl type of muramic acid in the peptidoglycan was acetyl. Mycolic acids were absent. The diagnostic phospholipids were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylinositolmannoside (PIM) and four unknown phospholipids (PLs) (Fig. 9-3), which corresponded to phospholipid type PI [46]. The predominant menaquinones were MK-9(H6) (67.6 %), with small amounts of MK-9(H8) (17.6 %), MK-9(H0) (12.8 %) and MK-9(H4) (2.0 %). The major fatty acids were C16:0 (44.8 %), C18:1 ω9c (21.5 %) and 10-methyl C18:0 (tuberculostearic acid; 9.5 %) with minor amounts of C18:0 (5.0 %), C14:0 (4.8 %), summed feature 3 (C16:1 ω6c and/or C16:1 ω7c; 4.7 %), C17:0 (2.3 %), C17:1 ω8c (1.2 %), C16:1 ω9c (1.1 %), which corresponding to fatty acid type 3a [47]. The major fatty acid profile of the strain was similar to that of *A. nitritigenes* NBRC 15918T and *A. fibrosa* JCM 9371T [10, 48, 49]. The genomic DNA G + C content was 70.5 mol%.

Phylogenetic analysis of strain GKU 128

The almost-complete 16S rRNA gene sequence (1497 nt) of strain GKU 128 were determined and compared with available sequences in public database. The results indicated that strain GKU 128 was a member of the genus *Actinomadura* [1]. It highly shared 16S rRNA gene sequence similarity with *Actinomadura nitritigenes* NBRC 15918T (99.2 %), *Actinomadura montaniterrae* CYP1-1BT (98.8 %), *Actinomadura fibrosa* JCM 9371T and *Actinomadura hibisca* NBRC 15177T (98.7 %). Lower levels of 16S rRNA gene sequence similarity (<98.5 %) were found with the type strains of all other recognized species of the genus *Actinomadura*. The neighbour-joining and maximum-likelihood trees indicated that strain GKU 128 was most closely related to *A. nitritigenes* NBRC 15918T and *A. fibrosa* JCM 9371T (Fig. 9-4 and Fig. 9-5). However, a maximum-parsimony tree showed that strain GKU 128 comprised a separate branch from *A. nitritigenes* NBRC 15918T and *A. fibrosa* JCM 9371T within the genus *Actinomadura* (Fig. 9-6).

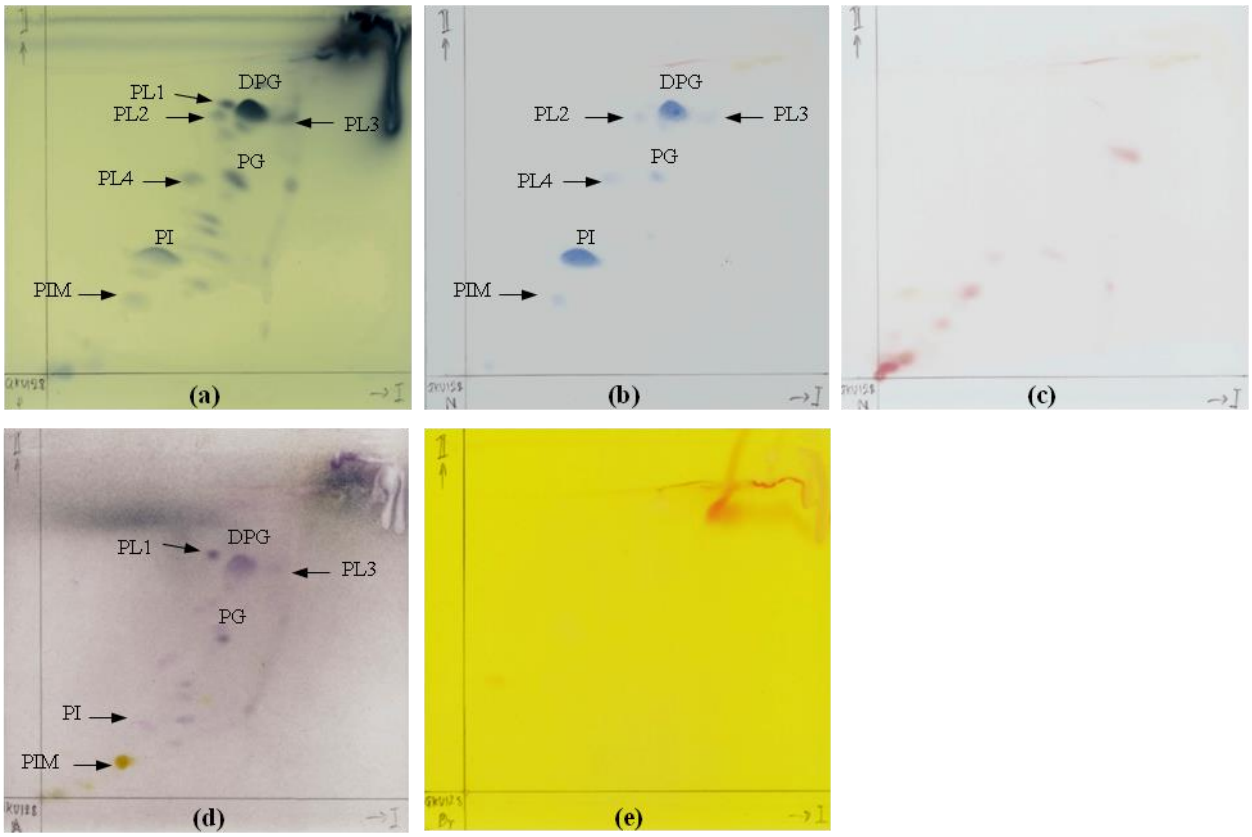

Fig. 9-3 Phospholipid profiles by two-dimensional TLC detected with phosphomolybdic acid (a), molybdenum blue (b), ninhydrin (c), anisaldehyde (d), and Dragendorff (e) spray reagents. Chloroform:methanol:water (65:25:4) was used in the first direction, followed by chloroform:acetic acid:methanol:water (80:15:12:4) in the second direction. Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidyl inositolmannoside; and PLs (PL1, PL2, PL3 and PL4), unidentified phospholipids.

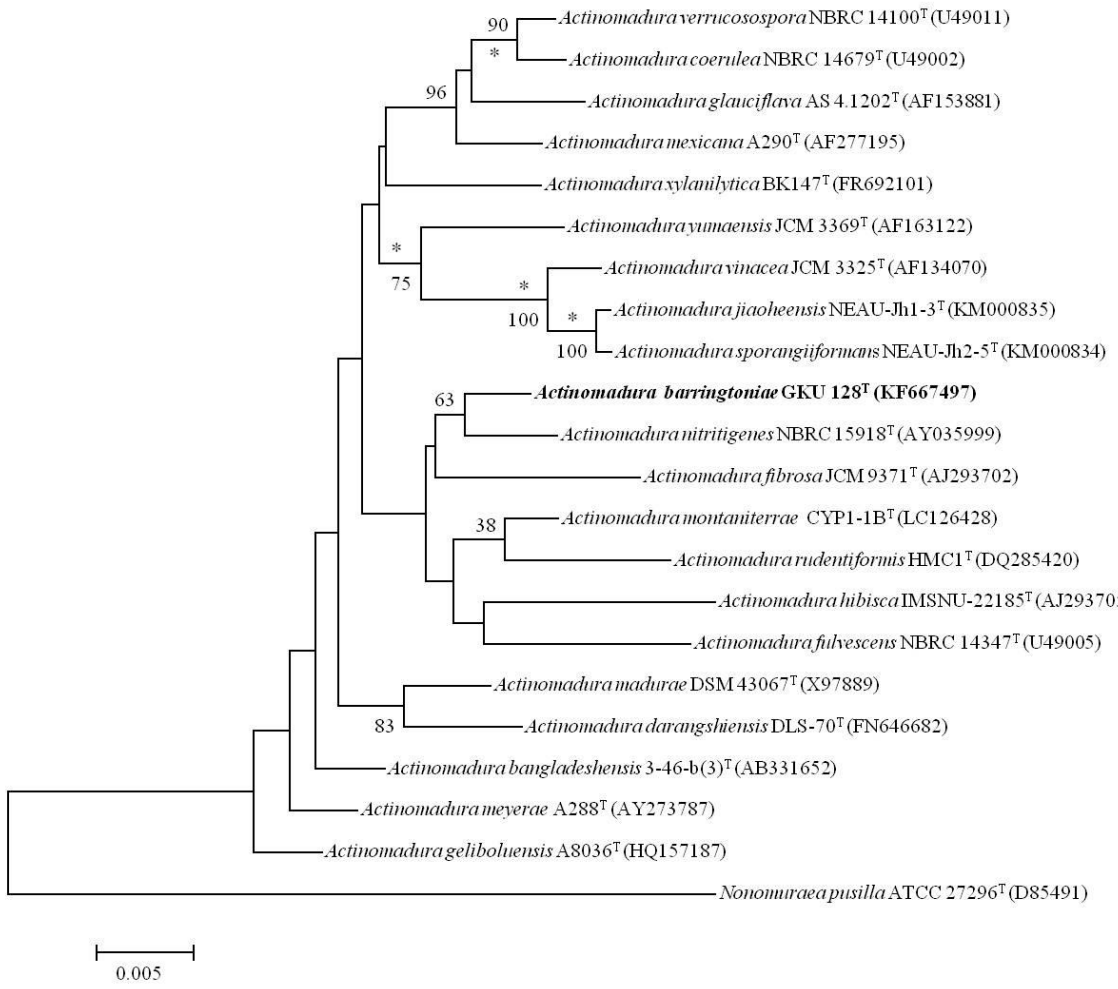

Fig. 9-4 Neighbour-joining tree based on almost-complete 16S rRNA gene sequences (1497 nt) showing the relationship between strain GKU 128 and some related species of the genus *Actinomadura*. *Nonomuraea pusilla* ATCC 27296 T (GenBank accession no. D85491) was used as an outgroup. Asterisks denote branches that were also recovered in the maximum-likelihood and maximum-parsimony trees. Numbers at branch points indicate bootstrap percentages (based on 1000 replications); only values >50 % are shown. Bar, 0.005 substitutions per nucleotide position.

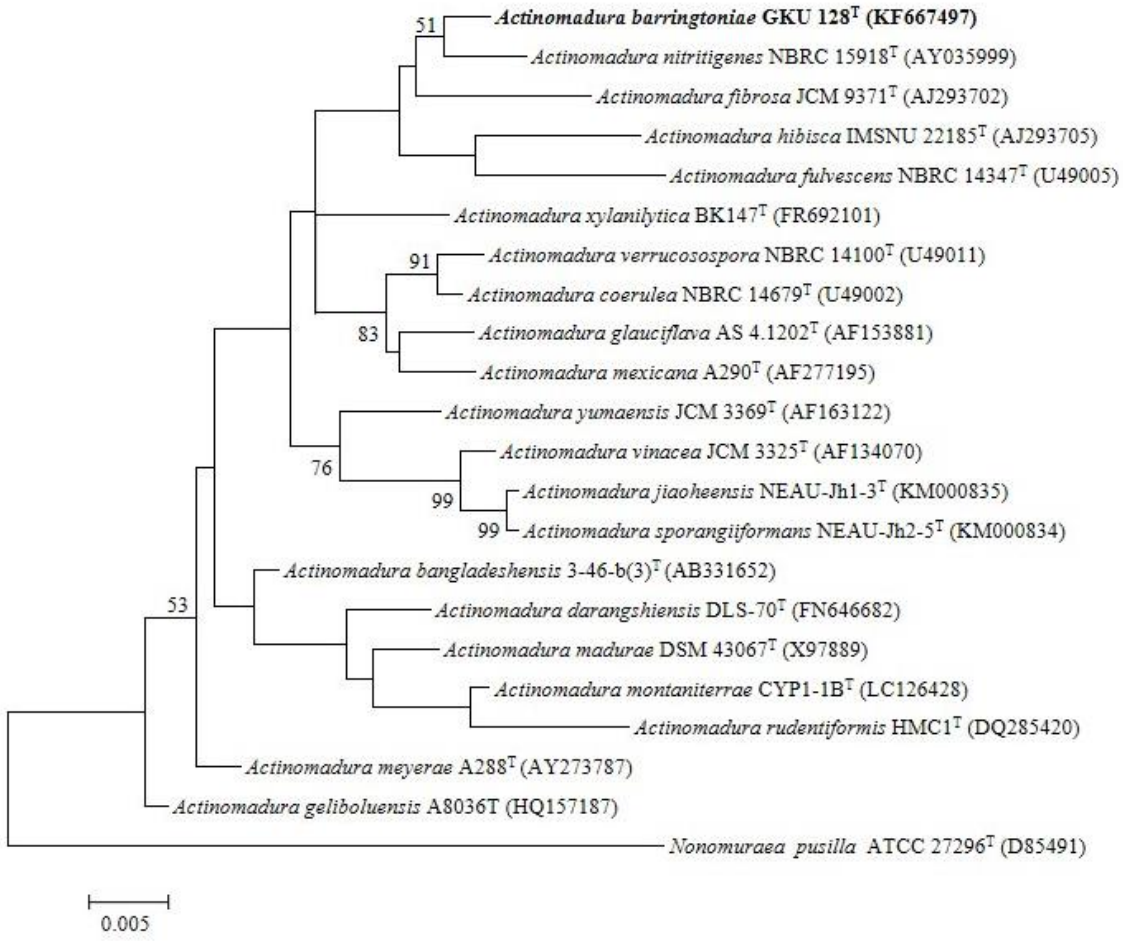

Fig. 9-5 Maximum-likelihood tree based on almost-complete 16S rRNA gene sequences (1497 nt) showing the relationship between strain GKU 128 and some related species of the genus *Actinomadura*. *Nonomuraea pusilla* ATCC 27296^{T} (GenBank accession no. D85491) was used as an outgroup. Numbers at branch points indicate bootstrap percentages (based on 1000 replications); only values >50 % are shown. Bar, 0.005 substitutions per nucleotide position.

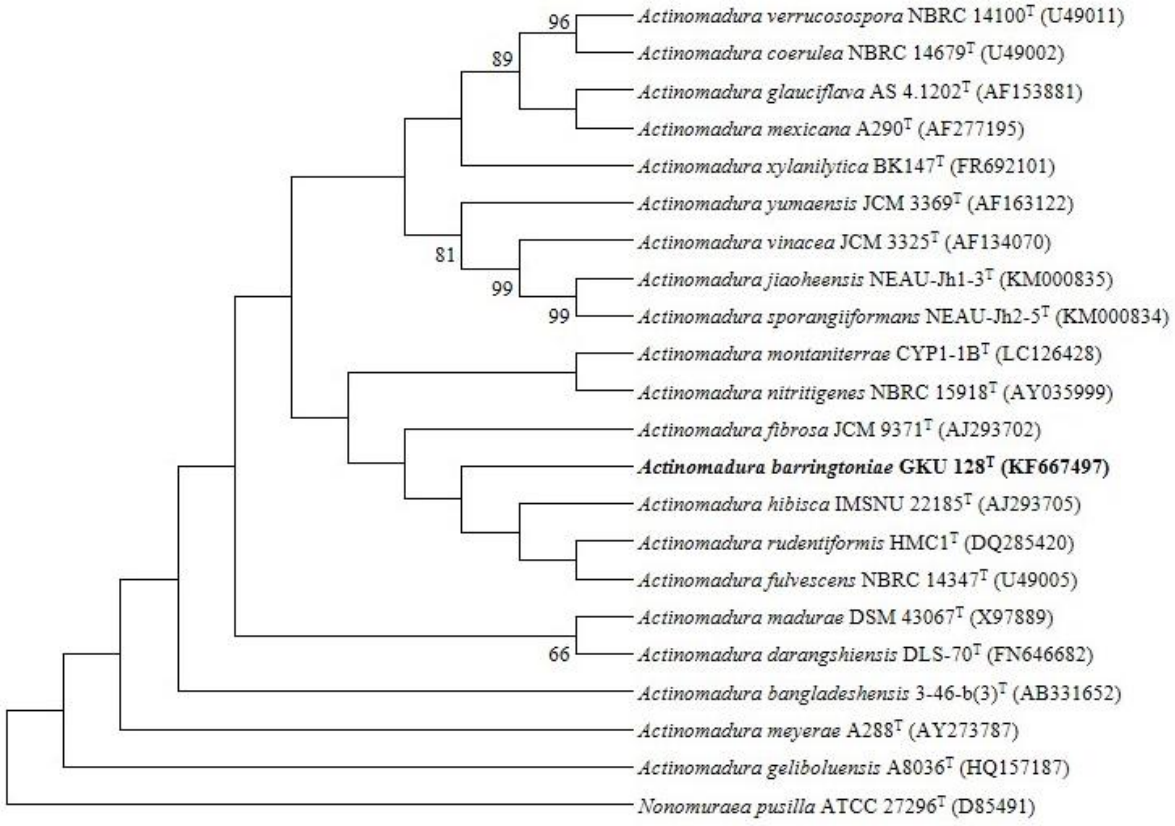

Fig. 9-6 Maximum-parsimony tree based on almost-complete 16S rRNA gene sequences (1497 nt) showing the relationship between strain GKU 128 and some related species of the genus *Actinomadura*. *Nonomuraea pusilla* ATCC 27296^T (GenBank accession no. D85491) was used as an outgroup. Numbers at branch points indicate bootstrap percentages (based on 1000 replications); only values >50 % are shown.

DNA-DNA hybridization studies

Level of DNA-DNA relatedness between strain GKU 128 and the closely related species, *A. nitritigenes* NBRC 15918T and *A. fibrosa* JCM 9371T were 18.4 + 4.1 % and 13.7 + 4.3 %, respectively. These values are well below the 70 % cut-off point recommended for the definition of bacterial species [51].

The phenotypic and genotypic characteristics of strain GKU 128 clearly distinguished from its closest phylogenetic neighbours (Table 8-1 and Table 8-2). Phylogenetic analyses indicated that strain GKU 128 was most closely related to *A. nitritigenes* NBRC 15918T supported with the neighbour-joining and maximum-likelihood trees. However, strain GKU 128 possesses straight to flexuous chain of spores with smooth surface ornament different from *A. nitritigenes* NBRC 15918 that possesses straight to hook-like chain of spores with warty surface ornament [48]. Based on

the results of the polyphasic taxonomic study, strain GKU 128 represents the novel species of the genus *Actinomadura* for which the name *Actinomadura barringtoniae* sp. nov. is proposed.

Description of *Actinomadura barringtoniae* sp. nov.

Actinomadura barringtoniae (bar.ring.to'ni.ae. N.L. gen. n. barringtoniae of *Barringtonia acutangula* (L.) Gaertn., the indian oak tree from which the type strain was isolated).

Cells are aerobic, Gram-stain-positive, non-motile actinomycete which form extensively branched substrate and aerial mycelia. Abundant aerial mycelium is produced on ISP 2, ISP 4, ISP 5 and ISP 7 media and differentiated into straight to flexuous chains of six or more spores. The spores are smooth and oval to cylindrical in shaped (0.6 x 0.8 µm in size). Antique gold pigment is produced on ISP 2 medium. The optimum temperature and pH for growth is 24–32 °C and 7.0, respectively. NaCl tolerance is up to 5 % (w/v). Catalase, gelatin liquefaction, nitrate reduction and oxidase are positive, but H_2S production, melanin pigment, milk coagulation and peptonization are negative. Acid is produced from L-arabinose, D-mannitol and sucrose, but not from adonitol, D-(-)-fructose, D-glucose, glycerol, D-maltose, D-mannose, D-raffinose, L-rhamnose, D-sorbitol and D-xylose. Adenine, casein, hypoxanthine, starch, Tween 20, Tween 80 and L-tyrosine are degraded, but citrate, urea and xanthine are not. Adonitol, L-arabinose, D-(-)-fructose, D-glucose, glycerol, D-maltose, D-mannitol, D-mannose, myo-inositol, D-raffinose, L-rhamnose, sucrose and D-trehalose are utilized as a sole carbon source, but D-sorbitol and D-xylose are not. L-arginine, L-asparagine, L-cysteine, L-histidine, potassium nitrate, L-proline and L-valine are utilized as a sole nitrogen source. In the API ZYM system, positive for N-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, α-chymotrypsin, cystine arylamidase, esterase (C4), esterase lipase (C8), β-glucosidase, leucine arylamidase, α-mannosidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase, while weakly positive for α-galactosidase, β-galactosidase, α-glucosidase and trypsin, but negative for α-fucosidase, β-glucuronidase and lipase (C 4). The cell wall peptidoglycan contains meso-diaminopimelic acid and whole-cell sugars are glucose, madurose, mannose, rhamnose and ribose. The N-acyl type of muramic acid is acetyl. Mycolic acids are absent. The major phospholipids are diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylinositolmannoside (PIM). The predominant menaquinone is MK-9(H6) with minor amounts of MK-9(H8), MK-9(H0) and MK-9(H4). The major fatty acids are C16:0, C18:1 ω9c, and 10-methyl C18:0 (tuberculostearic acid). The genomic DNA G + C content is 70.5 mol%.

The type strain GKU 128T was kept at public culture collection as TBRC 7225T and NBRC 113074T.

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Output ที่ได้จากโครงการวิจัย

1. International publications

There are 8 publications achieved under this research grant no. BRG5880004.

1.1 Yoolong, S., Kruasuwan, W., Phạm, H.T.T., Jaemsaeng, R., Jantasuriyarat, C. and Thamchaipenet, A. (2019). Modulation of salt tolerance in Thai jasmine rice (*Oryza sativa* L. cv. KDML105) by *Streptomyces venezuelae* ATCC 10712 expressing ACC deaminase. *Sci. Rep.* 9(1): 1275. doi: 10.1038/s41598-018-37987-5.

1.2 Kruasuwan, W. and Thamchaipenet, A. (2018). 1-Aminocyclopropane-1-carboxylate (ACC) deaminase-producing endophytic diazotrophic *Enterobacter* sp. EN-21 modulates salt-stress response in sugarcane. *J. Plant Growth Regul.* 37(3): 849-858.

1.3 Jaemsaeng, R., Jantasuriyarat, C., Thamchaipenet, A. (2018). Positive role of 1-aminocyclopropane-1-carboxylate deaminase-producing endophytic *Streptomyces* sp. GMKU 336 on flooding resistance of mung bean. *Agri. Nat. Resour*. 52(4): 330-334.

1.4 Rachniyom, H., Matsumoto, A., Inahashi, Y., Take, A., Takahashi, Y., Thamchaipenet, A. (2018). *Actinomadura barringtoniae* sp. nov., an endophytic actinomycete isolated from the roots of *Barringtonia acutangula* (L.) Gaertn. *Int J Syst Evol Microbiol*. 68(5): 1584-1590.

1.5 Jaemsaeng, R., Jantasuriyarat, C., Thamchaipenet, A. (2018). Molecular interaction of 1-aminocyclopropane-1-carboxylate deaminase (ACCD)-producing endophytic *Streptomyces* sp. GMKU 336 towards salt-stress resistance of *Oryza sativa* L. cv. KDML105. *Sci. Rep*. 8(1): 1950. doi: 10.1038/s41598-018-19799-9.

1.6 Kruasuwan W., Hoskisson, P.A., Thamchaipenet, A. (2017). Draft genome sequence of root-associated sugarcane growth promoting *Microbispora* sp. GKU 823. *Genome Announc*. 5 (29) doi: 10.1128/genomeA.00647-17.

1.7 Kruasuwan W., Salih, T.S., Brozio, S., Hoskisson, P.A., Thamchaipenet, A. (2017). Draft genome sequence of plant growth-promoting endophytic *Streptomyces* sp. GKU 895 isolated from the roots of sugarcane. *Genome Announc*. 5 (19) doi: 10.1128/genomeA.00358-17.

1.8 Kruasuwan, W. and Thamchaipenet, A. (2016). Diversity of culturable plant growth-promoting bacterial endophytes associated with sugarcane roots and their effect of growth by co-inoculation of diazotrophs and actinomycetes. *J. Plant Growth Regul.* 35 (4): 1074-1087.

2. Invitation speaker in national and international conferences (13 invitations)

1. Thamchaipenet, A. (2019). Molecular plant-actinomycete interaction towards salt stress of rice. The 21st National Genetics Conference, The Zign Hotel, Pataya, Chonburi, Thailand, 20-22 June 2019.

2. Thamchaipenet, A. (2018). Beneficial plant microbiome for abiotic stress tolerance: a case study by endophytic actinomycetes. International workshop on Empowering Agricultural Research through (Meta)Genomics. Faculty of Science, Kasetsart University, Bangkok, Thailand, 18-21 June 2018.

3. Thamchaipenet, A. (2018). ACC deaminase-producing endophytic actinomycetes modulate growth and salt tolerance of rice. Special seminar, Toyama Prefectural University, Toyama, Japan, 6 December 2018.

4. Thamchaipenet, A. (2018). Transcriptomic analysis of beneficial effect of ACC-deaminase producing endophytic *Streptomyces* associating with indica rice under salt stress. The 9th International Conference on Computational Systems-Biology and Bioinformatics (CSBio 2018), KMUTT, Bangkok, Thailand, 10-13 December 2018.

5. Thamchaipenet, A. (2017). Endophytic actinomycetes: diversity, new bioactive compounds and plant growth promotion traits. The 2nd International Conference on Biosciences (ICoBio) 2017, IPB International Convention Center (IICC), Bogor, Indonesia, 8-10 August 2017.

6. Thamchaipenet, A. (2017). Impact of ACC deaminase-producing endophytic streptomycetes on growth and salt tolerance of rice plants. The 18th International Symposium on the Biology of Actinomycetes (ISBA 18), Jeju, Korea, 23-27 May 2017.

7. Thamchaipenet, A. (2017). Effect of ACC-deaminase producing endophytic *Streptomyces* sp. GMKU 336 towards salt stress in rice plants. Plant Genomics & Gene Editing Asia Congress, Eaton Hotel, Hong Kong, 10-11 April 2017.

8. Thamchaipenet, A. (2016). Endophytic actinomycetes as biofertitizer and biocontrol agents. The Workshop on MicrobiaI Resources: Applications in Agriculture, Faculty of Pharmacy, Chulalongkorn University, Bnagkok, Thailand, 23 December 2016.

9. Thamchaipenet, A. (2016). Endophytic actinomycetes: new taxa, bioactive compounds and plant growth enhancers. The 4th Workshop on Science and Technology Cooperation, Biodiversity: secure our future, Xishuangbanna Tropical Botanical Garden, China, 21-24 November 2016.

10. Thamchaipenet, A. (2016). Molecular interaction of endophytic actinomycetes associated with Thai jasmine rice towards salt stress. Recent advances in genomics and genetics (RAGG2016), BITEC, Bangkok, Thailand, 21-22 September 2016.

11. Thamchaipenet, A. (2016). Endophytic actinomycetes: diversity, bioactive compounds and plant growth promotion. The 3rd Seminar of Priority Universities for Cooperation in Thailand. Kasetsart University, Bangkok, Thailand, 19 August 2016.

12. Thamchaipenet, A. (2016). Diversity and applications of endophytic actinomycetes from Thai plants. International Conference on Innovative Approaches in Applied Sciences and Technologies (iCiAst-2016). Kasetsart University, Bangkok, Thailand, 1-5 February 2016.

13. Thamchaipenet, A. (2015). Endophytic actinomycetes: diversity and applications. The 27th Annual Meeting of the Thai Society for Biotechnology and International Conference. Mandarin Hotel, Bangkok, Thailand, 17-20 November 2015.

3. Postdoctorate and PhD/MS students

Postdoctorate fellow:

- Dr. Hathairat Rachniyom

PhD students:

- Dr. Ratchaniwan Jaensaeng
- Dr. Worarat Kruasuwan

MS student:

- Mr. Suranan Yoolong
- Ms. Huyen Thi Thanh Pham

4. Awards

1. Scientific Educational Research Society (SERS) Fellow-2015, India
2. Science and Technology Research Grant, Thailand Toray Science Foundation (TTSF), 2017

ลงนาม

(รศ.ดร. อรินทิพย์ ธรรมชัยพิเนต)

วันที่ 20 มีนาคม พ.ศ. 2562

Appendix

1. International publications

1.1 Yoolong, S., Kruasuwan, W., Phạm, H.T.T., Jaemsaeng, R., Jantasuriyarat, C. and Thamchaipenet, A. (2019). Modulation of salt tolerance in Thai jasmine rice (*Oryza sativa* L. cv. KDML105) by *Streptomyces venezuelae* ATCC 10712 expressing ACC deaminase. *Sci. Rep.* 9(1): 1275. doi: 10.1038/s41598-018-37987-5.

1.2 Kruasuwan, W. and Thamchaipenet, A. (2018). 1-Aminocyclopropane-1-carboxylate (ACC) deaminase-producing endophytic diazotrophic *Enterobacter* sp. EN-21 modulates salt-stress response in sugarcane. *J. Plant Growth Regul.* 37(3): 849-858.

1.3 Jaemsaeng, R., Jantasuriyarat, C., Thamchaipenet, A. (2018). Positive role of 1-aminocyclopropane-1-carboxylate deaminase-producing endophytic *Streptomyces* sp. GMKU 336 on flooding resistance of mung bean. *Agri. Nat. Resour*. 52(4): 330-334.

1.4 Rachniyom, H., Matsumoto, A., Inahashi, Y., Take, A., Takahashi, Y., Thamchaipenet, A. (2018). *Actinomadura barringtoniae* sp. nov., an endophytic actinomycete isolated from the roots of *Barringtonia acutangula* (L.) Gaertn. *Int J Syst Evol Microbiol*. 68(5): 1584-1590.

1.5 Jaemsaeng, R., Jantasuriyarat, C., Thamchaipenet, A. (2018). Molecular interaction of 1-aminocyclopropane-1-carboxylate deaminase (ACCD)-producing endophytic *Streptomyces* sp. GMKU 336 towards salt-stress resistance of *Oryza sativa* L. cv. KDML105. *Sci. Rep*. 8(1): 1950. doi: 10.1038/s41598-018-19799-9.

1.6 Kruasuwan W., Hoskisson, P.A., Thamchaipenet, A. (2017). Draft genome sequence of root-associated sugarcane growth promoting *Microbispora* sp. GKU 823. *Genome Announc*. 5 (29) doi: 10.1128/genomeA.00647-17.

1.7 Kruasuwan W., Salih, T.S., Brozio, S., Hoskisson, P.A., Thamchaipenet, A. (2017). Draft genome sequence of plant growth-promoting endophytic *Streptomyces* sp. GKU 895 isolated from the roots of sugarcane. *Genome Announc*. 5 (19) doi: 10.1128/genomeA.00358-17.

1.8 Kruasuwan, W. and Thamchaipenet, A. (2016). Diversity of culturable plant growth-promoting bacterial endophytes associated with sugarcane roots and their effect of growth by co-inoculation of diazotrophs and actinomycetes. *J. Plant Growth Regul.* 35 (4): 1074-1087.

SCIENTIFIC REPORTS

OPEN

Modulation of salt tolerance in Thai jasmine rice (*Oryza sativa* L. cv. KDML105) by *Streptomyces venezuelae* ATCC 10712 expressing ACC deaminase

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1-Aminocyclopropane-1-carboxylate (ACC) deaminase is a plant growth promoting (PGP) trait found in beneficial bacteria including streptomycetes and responsible for stress modulation. The ACC deaminase gene, *acdS*, of *S. venezuelae* ATCC 10712 was cloned into an expression plasmid, pIJ86, to generate *S. venezuelae*/pIJ86-*acdS*. Expression of *acdS* and production of ACC deaminase of *S. venezuelae*/pIJ86-*acdS* were significantly higher than the unmodified strain. The ACC deaminase-overexpressing mutant and the wild type control were inoculated into Thai jasmine rice (*Oryza sativa* L. cv. KDML105) under salt stress conditions. *S. venezuelae* on its own augmented rice growth and significantly increased more tolerance to salinity by reduction of ethylene, reactive oxygen species (ROS) and Na^+ contents, while accumulating more proline, total chlorophyll, relative water content (RWC), malondialdehyde (MDA), and K^+ than those of uninoculated controls. The overproducer did not alter chlorophyll, RWC, or MDA further–while it did boost more shoot weight and elongation, and significantly regulated salt tolerance of rice by increasing proline and reducing ethylene and Na^+ contents further than that of the wild type. This work is the first illustration of the beneficial roles of *S. venezuelae* to enhance plant fitness endophytically by promotion of growth and salt tolerance of rice.

Soil salinity in arid regions is often an important limiting factor for cultivation of agricultural crops such as maize, rice, and sugarcane. Excess of salt affects plant growth by increasing stress factors, such as ethylene production, Na^+ accumulation, and reactive oxygen species (ROS) which is detrimental to the plant's physiology, leading to growth impairment[1–3].

Streptomycetes have been recognized recently as plant growth promoting (PGP) bacteria that can protect plants from infectious diseases and enhance plant growth through several PGP-traits, such as siderophore production, plant hormone production, and phosphate solubilization[4–6]. Furthermore, PGP-bacteria assist plants to grow under severe condition caused by drought, flooding, salinity, and phytopathogens by the action of 1-aminocyclopropane-1-carboxylate (ACC) deaminase[7–11]. ACC deaminase, encoded by the *acdS* gene, is responsible for the breakdown of ACC, which is the direct precursor of ethylene in all higher plants, into ammonia and α-ketobutyrate - which bacteria consume as nitrogen and carbon sources[12]. Overexpression of *acdS* in endophytic bacteria remarkably improved plant growth and alleviated stresses in plants, when compared to uninoculated plants and those of wild type inoculation. For example, ACC deaminase-overproducing strains of *Pseudomonas putida* ameliorated flooding stress in tomato[13], *Sinorhizobium meliloti* improved growth and copper tolerance in *Medicago lupulina*[14], and *Serratia grimesii* enhanced growth and the level of plant protection against seed-borne pathogens in the common bean[15].

Streptomyces venezuelae was discovered from soil and, thus far, has been known as a cell factory for the production of diverse natural products including chloramphenicol, watasemycin, and venemycin[16–18]. Although the

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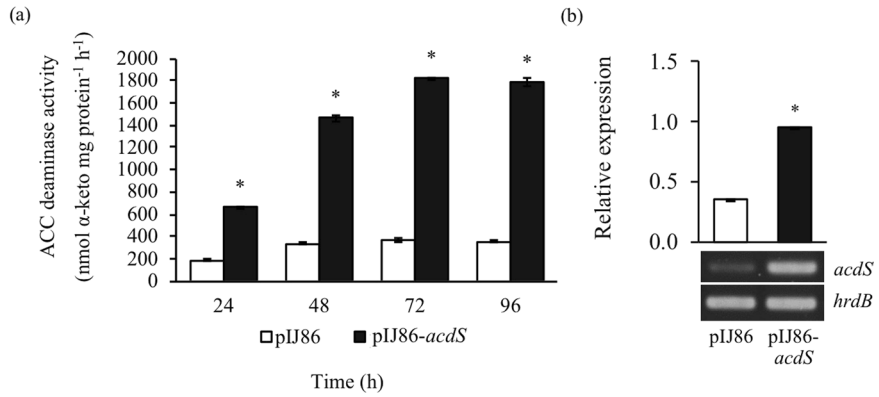

Figure 1. ACC deaminase activity (**a**) and semi-quantitative RT-PCR analysis of expression of *acdS* (**b**) of *S. venezuelae*/pIJ86 (pIJ86) and *S. venezuelae*/pIJ86-*acdS* (pIJ86-*acdS*). The values represent the mean ± S.E. of three replicates and an asterisk (*) indicate statistically significant changes in expression (t test, $p < 0.05$).

genome sequence of *S. venezuelae* was determined and characterized[19], the information was used mainly for investigation of gene clusters involved in antibiotic biosynthesis. The genome sequence has never been inspected for a role of plant-beneficial functions; likewise, *S. venezuelae* has never been documented as a PGP-endophytic bacterium. Recently, genes contributing to PGP-traits including *acdS* were not only present in genome sequences of PGP-rhizobacteria (PGPR) but also found in those of non-PGPR[20,21].

On this basis, we examined genes related to PGP-function in all genome sequences of members of a genus *Streptomyces* available in the GenBank database, in particular *acdS*. Surprisingly, *acdS* was present in many genomes of non-PGP-endophytic *Streptomyces*, including *S. venezuelae*. To address the possible beneficial role of *S. venezuelae* interacting beneficially with plants and modulating salt stress, *S. venezuelae* was inoculated into the salt-sensitive Thai jasmine rice KDML105 cultivar. Furthermore, the effects of overexpression of *acdS* within *S. venezuelae* towards rice growth and salt tolerance were investigated. The physiology of rice associated with *S. venezuelae* and its overexpressed mutant under salt stress condition are discussed.

Results

Salt tolerance and PGP-traits of *S. venezuelae*. Analysis of salt tolerance of *S. venezuelae* ATCC 10712 revealed that it had tolerated NaCl up to 3% (w/v). During growth in 3% NaCl, proline was accumulated significantly, at 36.66 ± 0.24 µM in cells (Supplementary Table S1). Moreover, *S. venezuelae* had ACC deaminase activity of 364.21 ± 19.28 nmol α-ketobutyrate mg protein^{-1} h^{-1} and produced IAA at 21 ± 1.02 µg mL^{-1} (Supplementary Table S1).

Characterization of ACC deaminase-overexpressing *S. venezuelae*. An ACC deaminase-overexpressing mutant, *S. venezuelae*/pIJ86-*acdS*, was constructed and verified by resistance to apramycin and thiostrepton. The wild type with empty plasmid, *S. venezuelae*/pIJ86, was also constructed as a control. In comparison with *S. venezuelae*/pIJ86, ACC deaminase activity of *S. venezuelae*/pIJ86-*acdS* was enhanced 5-fold from 72–96 h of incubation in MM containing 3 mM ACC (Fig. 1a, Supplementary Table S2). This result correlated with the high expression profile of *acdS* by *S. venezuelae*/pIJ86-*acdS* at 72 h (2.7-fold) when compared to that of wild type control (Figs 1b and S1 and Table S3). The ACC deaminase activity of *S. venezuelae*/pIJ86-*acdS* were relatively stable when re-streaked for up to 5 generations without antibiotic selection (data not shown).

Plant colonization and growth promotion by *S. venezuelae*. *S. venezuelae*/pIJ86 was successfully inoculated into Thai jasmine rice cv. KDML105 grown under hydroponic condition with and without salt treatment. *S. venezuelae*/pIJ86 was re-isolated from rice under both treatments at about 10^4 CFU g root fresh weight^{-1} (Supplementary Table S4) indicating that *S. venezuelae* had the ability to colonize inside plants. In addition, un-inoculated plants did not harbor any streptomycete (data not shown) showing that rice seeds were surface sterilized effectively, and the hydroponic conditions used in this study were free from contamination. Growth parameters of inoculated and uninoculated rice KDML105 were evaluated at 7 days after being treated with and without 150 mM NaCl. In comparison to uninoculated plants, rice associated with *S. venezuelae*/pIJ86 had significant increases of shoot/root lengths and shoot/root fresh/dry weights in both non-salt and salt treatments (Fig. 2a–f).

Effect of overexpression of ACC deaminase on plant growth parameters. Growth parameters including shoot/root length and shoot/root fresh/dry weights of rice KDML105 inoculated with *S. venezuelae*/pIJ86 were enhanced significantly, when compared with uninoculated plants in both non-salt and salt treatments (Figs 2a–f and S2). Similar to the original strain, its overexpressing mutant, *S. venezuelae*/pIJ86-*acdS*, greatly promoted growth of rice in both non-salt and salt stress conditions (Figs 2a–f and S2), but highly increased shoot length and biomass in particular more than those inoculated with wild type control under salt stress conditions (Fig. 2a–c).

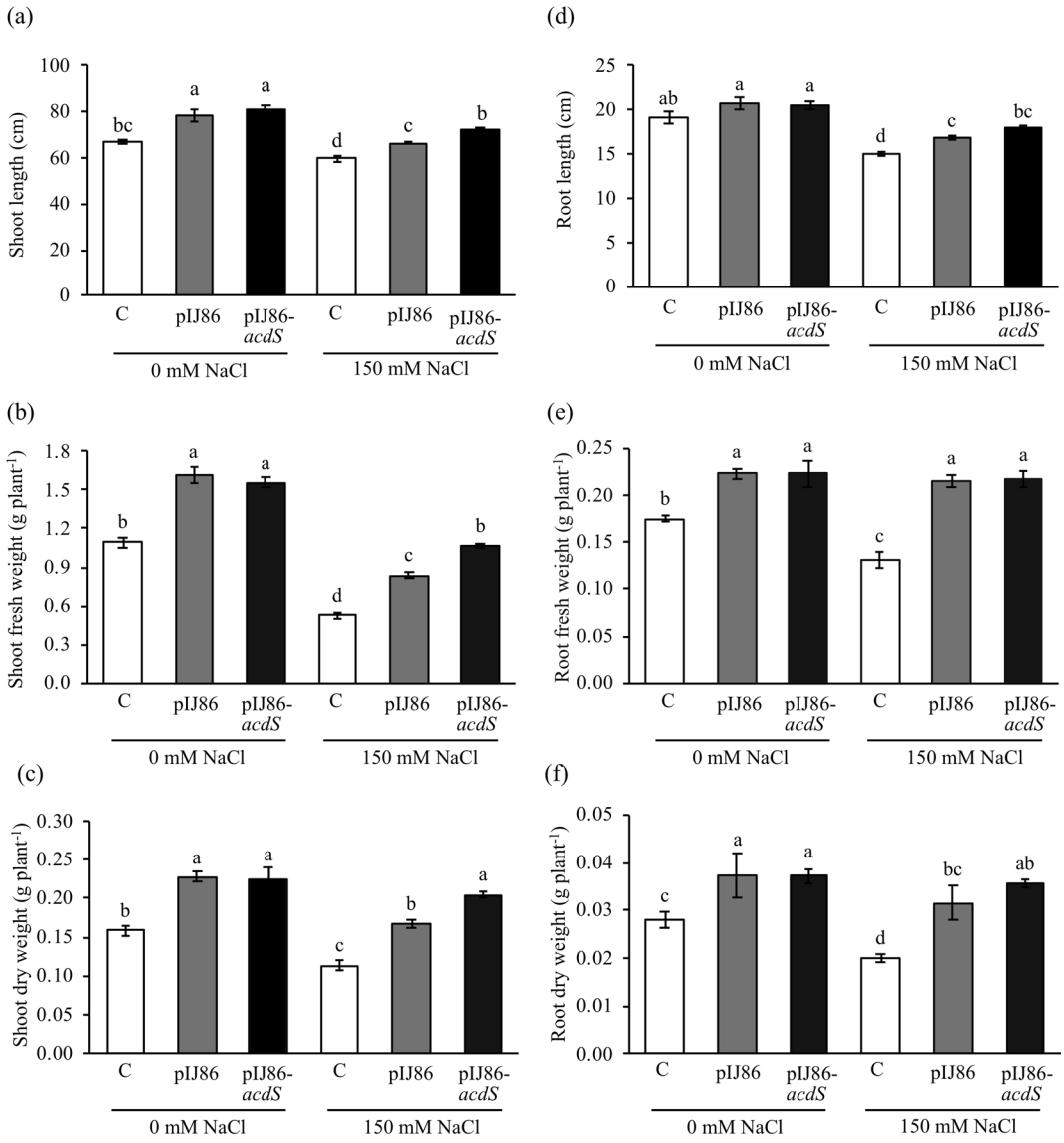

Figure 2. Effect of ACC deaminase-producing *Streptomyces venezuelae* on shoot length (**a**), shoot fresh weight (**b**), shoot dry weight (**c**), root length (**d**), root fresh weight (**e**), and root dry weight (**f**) of rice plants under non-salt (0 mM NaCl) and salt stress (150 mM NaCl) conditions. The values show the mean ± S.E. of twelve replicates and bars carrying different letters are significantly different (Tukey's test, $p < 0.05$). C, uninoculated rice control; pIJ86, rice inoculated with *S. venezuelae*/pIJ86; pIJ86-*acdS*, rice inoculated with *S. venezuelae*/pIJ86-*acdS*.

Effect of overexpression of ACC deaminase on plant ethylene. At 7 days after irrigation with 150 mM NaCl, the ethylene level of uninoculated plants was increased about 2-fold when compared with those grown without salt (Fig. 3a, Supplementary Table S4). When rice was associated with *S. venezuelae*/pIJ86, the ethylene level was reduced 1.6-fold when compared to uninoculated plants (Fig. 3a, Supplementary Table S4). When rice was inoculated with *S. venezuelae*/pIJ86-*acdS*, the ethylene level was decreased 2-fold when compared to uninoculated plants (Fig. 3a, Supplementary Table S4). The results indicated that overexpression of ACC deaminase facilitated salt tolerance in plants by reduction of ethylene to the same level as that of the non-salt treatment control.

Effect of overexpression of ACC deaminase on proline content. Under non-salt conditions, proline content was unaffected in rice KDML105 inoculated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* (Fig. 3b). Under salt-stress conditions, the proline content was 3.4-fold higher in uninoculated plants compared to those grown in non-salt conditions (Fig. 3b, Supplementary Table S4). Nonetheless, the proline content of plants associated with *S. venezuelae*/ pIJ86 was increased significantly compared to the uninoculated control and even higher in plants inoculated with *S. venezuelae*/pIJ86-*acdS* (Fig. 3b). The results demonstrated that overexpression of ACC deaminase facilitated salt tolerance in rice by escalation of proline content.

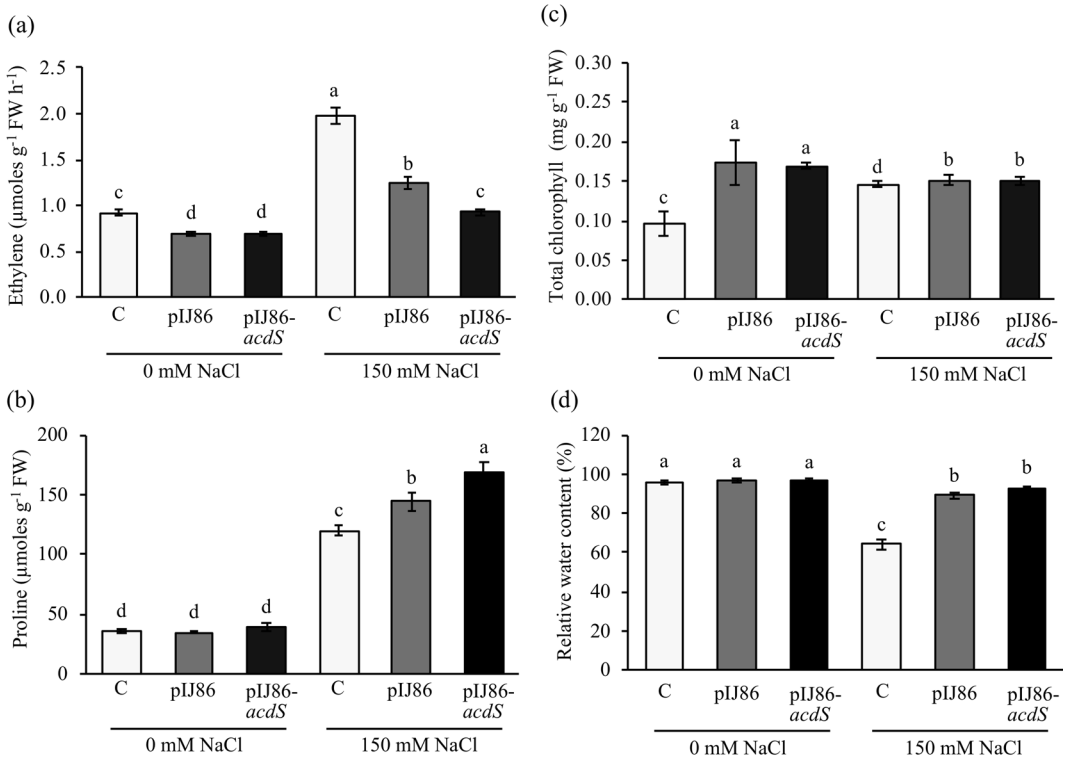

Figure 3. Effect of ACC deaminase-producing *Streptomyces venezuelae* on ethylene (**a**), proline (**b**), total chlorophyll (**c**), and relative water contents (RWC) (**d**) of rice plants under non-salt (0 mM NaCl) and salt stress (150 mM NaCl) conditions. The values show the mean ± S.E. of twelve replicates and bars carrying different letters are significantly different (Tukey's test, $p < 0.05$). C, uninoculated rice control; pIJ86, rice inoculated with *S. venezuelae*/pIJ86; pIJ86-*acdS*, rice inoculated with *S. venezuelae*/pIJ86-*acdS*.

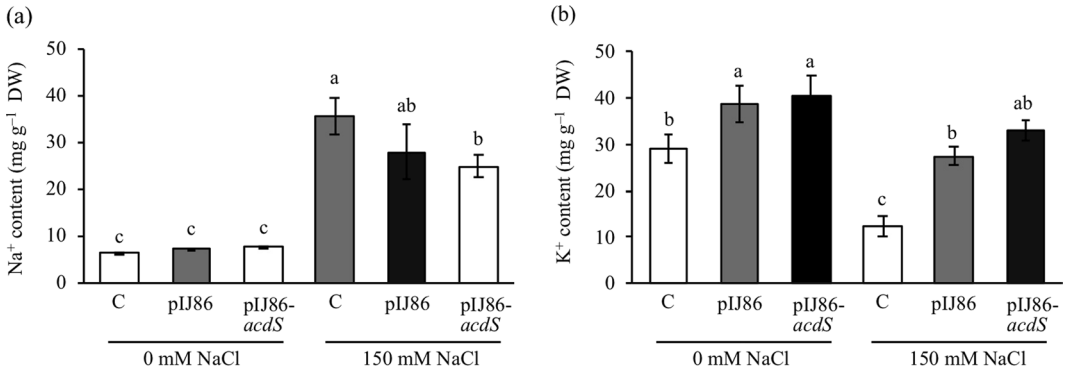

Figure 4. Effect of ACC deaminase-producing *Streptomyces venezuelae* on Na^+ (**a**) and K^+ (**b**) contents of rice plants under non-salt (0 mM NaCl) and salt stress (150 mM NaCl) conditions. The values show the mean ± S.E. of twelve replicates and bars carrying different letters are significantly different (Tukey's test, $p < 0.05$). C, uninoculated rice control; pIJ86, rice inoculated with *S. venezuelae*/pIJ86; pIJ86-*acdS*, rice inoculated with *S. venezuelae*/pIJ86-*acdS*.

Effect of overexpression of ACC deaminase on total chlorophyll and RWC. The total chlorophyll content of rice KDML105 inoculated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* (1.8-fold) was augmented significantly when compared to uninoculated plants under non-salt conditions (Fig. 3c, Supplementary Table S4). Under salt stress conditions, rice associated with either the wild-type control or the ACC deaminase-overexpressing mutant maintained a higher chlorophyll content compared to that of uninoculated rice (Fig. 3c). RWC of uninoculated plants under salt treatment was 1.5-fold decreased when compared to untreated controls (Fig. 3d, Supplementary Table S4). Significantly, RWC in rice associated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* was 1.4-fold higher when compared to the uninoculated control. The results suggested that *S. venezuelae* induced salt tolerance in rice by elevation of chlorophyll content and RWC.

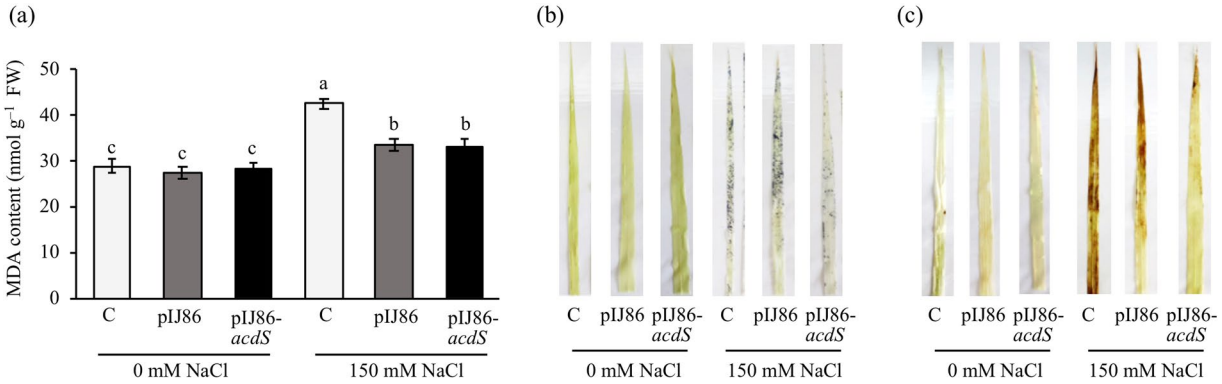

Figure 5. Effect of ACC deaminase-producing *Streptomyces venezuelae* on MDA content (**a**), histochemical NBT staining (**b**), and DAB staining (**c**) of rice plants under non-salt (0 mM NaCl) and salt stress (150 mM NaCl) conditions. The values indicate the mean ± S.E. of twelve replicates and bars carrying different letters are significantly different (Tukey's test, $p < 0.05$). C, uninoculated rice control; pIJ86, rice inoculated with *S. venezuelae*/pIJ86; pIJ86-*acdS*, rice inoculated with *S. venezuelae*/pIJ86-*acdS*.

Effect of overexpression of ACC deaminase on Na^+ and K^+ contents. When rice was grown under salt stress conditions, Na^+ was accumulated up to 56-fold compared to that of non-salt treatment (Fig. 4a, Supplementary Table S4). Significantly, the Na^+ content in rice inoculated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* decreased about 1.4-fold when compared to uninoculated rice (Fig. 4a, Supplementary Table S4). On the contrary, the K^+ content decreased (2.3-fold) when rice was grown under salt stress conditions (Fig. 4b). However, rice inoculated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* had significantly increased K^+ content under both non-salt and salt treatments (Fig. 4b, Supplementary Table S4). Markedly, rice inoculated with the ACC deaminase-overexpressing mutant had the highest significant increase in K^+ content by 2.6-fold when compared to the uninoculated control under salt stress conditions (Fig. 4b, Supplementary Table S4). The results demonstrated that overexpression of ACC deaminase helped salt tolerance in rice by reduction of Na^+ content, and increase in K^+ content.

Effect of overexpression of ACC deaminase on ROS. Salt stress drastically induced lipid peroxidation. The MDA content was increased up to 1.5-fold in rice grown under salt stress conditions (Fig. 5a, Supplementary Table S4). However, rice KDML105 inoculated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* had a significant reduction in MDA content - about 1.3-fold when compared to the uninoculated control (Fig. 5a, Supplementary Table S4). ROS in leaves were detected by the presence of superoxide and hydrogen peroxide by staining with nitrobluetrazolium (NBT) (Fig. 5b) and 3,3′-diaminobenzidine (DAB) (Fig. 5c), respectively. In the presence of salt, both ROS species were present, shown by the intense staining of leaves; however rice inoculated with *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* showed fainter staining than those of the uninoculated control (Fig. 5b,c). The results indicated that *S. venezuelae* helped salt tolerance in rice by reduction of MDA content and ROS species. However, under salt stress conditions, the overexpression of ACC deaminase did not induce those characteristics more than those of the wild type control.

Discussion

ACC deaminase is a bacterial enzyme found in several PGP-bacteria including *Bacillus*, *Enterobacter*, *Pseudomonas* and *Streptomyces*. Its improved stress tolerance of plants to drought, flooding, salinity and phytopathogens[7–11]. Interestingly, increasing the ACC deaminase activity by overexpression of the corresponding gene in PGP-bacteria remarkably facilitated growth and alleviated environmental stresses of host plants more than those of wild type strains[13–15].

In this work *acdS*, encoding ACC deaminase located in the genome of *S. venezuelae* ATCC 10712, was cloned and expressed in this strain. The overexpressing mutant, *S. venezuelae*/pIJ86-*acdS*, had higher ACC deaminase activity, compared to *S. venezuelae*/pIJ86. The results were in agreement with previous reports that overexpression of ACC deaminase in *Mesorhizobium cicero*, *Serratia grimesii* and *Sinorhizobium meliloti* resulted in higher ACC deaminase activity, compared to the corresponding wild type strains[14,15,22]. In addition, *acdS* expression under the *ermE* promoter of multi-copy plasmid pIJ86 in *S. venezuelae* without antibiotic selection was maintained up to 5 generations, consistent with the previous report[23]. Interestingly, *S. venezuelae* showed endophytic ability in rice plants; which was proven by re-isolation of the bacterium responsible for promotion of rice growth from plant tissues; a trait that is herein shown for the first time for this bacterium. Soil actinomycetes, therefore, potentially act as endophytes, supporting the hypothesis that bacterial communities in the rhizosphere, rhizoplane, and endosphere of rice root microbiomes were overlapping[24]. In this work, it was demonstrated for the first time that *S. venezuelae* behaves as a PGP-endophytic bacterium.

Under normal conditions, rice inoculated with *S. venezuelae*/pIJ86 significantly increased biomass of shoot and root, and elongation. This might be due to an action of IAA produced by this strain that would encourage plant growth and elongation. Moreover, the results were in agreement with previous work showing that ACC

deaminase-producing *Streptomyces* have the ability to enhance growth of *Jatropha curcas*, mung bean, sugarcane, and rice[6,8,10,25]. Apart from *Streptomyces*, IAA and ACC deaminase-producing species from the genera *Agromyces*, *Bacillus*, *Enterobacter*, *Methylophaga*, *Microbacterium*, *Paenibacillus*, and *Pseudomonas* were also reported to promote growth of canola, rice, sugarcane, and tomato[26,27].

Under salt stress conditions, rice plants inoculated with either *S. venezuelae*/pIJ86 or its overexpressing mutant, *S. venezuelae*/pIJ86-*acdS*, showed enhanced growth parameters compared to those of uninoculated controls. However, shoot length and biomass of rice associated with the ACC deaminase-overexpressing mutant were significantly greater than plants inoculated with the unmodified strain. Our results were in congruence with other studies in which ACC deaminase overexpressing strains of *Pseudomonas putida* and *Serratia grimesii* promoted growth of tomato and common bean, respectively compared to wild type strains[13,15]. Therefore, the results unambiguously demonstrated that ACC deaminase-overexpressing *S. venezuelae* facilitated rice growth better than the original strain under salt stress conditions.

It is generally known that ethylene production is a main response in plants exposed to environmental stress. Salinity induced a high level of ethylene via the actions of ACC synthase and ACC oxidase towards ACC, an ethylene precursor. Whereas, ACC deaminase of bacteria assists plants in responding by conversion of ACC into ammonia and α-ketobutyrate and, thus, reducing ethylene as a consequence[28]. In this work, the ethylene levels were significantly lower when rice was associated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* compared to that of the uninoculated control. Our results were similar to previous reports that ethylene levels in rice and sugarcane were reduced by ACC deaminase-producing *Streptomyces* sp. GMKU 336[10] and *Enterobacter* sp. EN-21[9] respectively, under salt stress conditions. The ethylene level was lowest in rice inoculated with the ACC deaminase-overexpressing mutant, correlating with the high ACC deaminase activity of this strain. The results were consistent with another report that overexpression of ACC deaminase in endophytic *Pseudomonas* spp. enhanced salt tolerance in tomato by reducing ethylene production[29]. In addition, a lower amount of ACC was observed in tomatoes inoculated with ACC deaminase-overexpressing psychrotolerant bacteria under chilling stress[30].

Proline accumulation is one of the adaptation mechanisms of plants under salt stress. At 7 days after irrigation with salt, the proline content of rice associated with *S. venezuelae* was high and particularly higher in rice inoculated with the ACC deaminase-overexpressing mutant. The results agreed with data on ACC-deaminase producing *Dietzia natronolimnaea* and *Streptomyces* sp. GMKU 336, associated with wheat and rice respectively – which induced elevated proline content[31,32]. Accumulation of higher levels of proline stabilized proteins, cell structures, and osmotic balance[33] in rice associated with *S. venezuelae*/pIJ86-*acdS* and, thus, accelerated salt tolerance.

Reduction of total chlorophyll and RWC of plants are generally the first notable effects of salt stress such as those reported in black gram and rice[10,34,35]. In this work, the total chlorophyll and RWC of rice plants were increased significantly in plants under salt treatment, when inoculated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS*. The results were in congruence with other studies in which ACC deaminase-producing *Enterobacter* sp. SBP-6 in wheat[32], *Enterobacter* cloacae HSNJ4 in canola[36], *Bacillus subtilis* RJ46, *Ochrobactrum pseudogrignonense* RJ12, and *Pseudomonas* sp. RJ15 in black gram and pea[37], and bacterial consortia in avocado[38] increased chlorophyll level more than those of non-inoculated plants, when under salt stress. Moreover, the results were in agreement with other for the ACC deaminase-overexpressing endophytic *Pseudomonas* spp., which improved photosynthetic performance and water content in tomato[29]. The results suggested that *S. venezuelae* facilitates rice growth in saline environments by increasing total chlorophyll and RWC. However, as the ACC deaminase-overproducing *S. venezuelae* enhanced chlorophyll content and RWC equally to those of the wild type control, it can be concluded that the overexpression of ACC deaminase did not influence those characters.

Excess accumulation of Na^+ and inhibition of K^+ uptake under salt stress are very harmful for plant cells, leading to growth impairment[3]. Several reports have indicated that increasing the K^+/Na^+ ratio is crucial for salt tolerance in plants[39–41]. In this work, the Na^+ content was significantly enhanced, while the K^+ content was decreased drastically in salt-stressed uninoculated rice. On the contrary, rice inoculated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* had markedly reduced Na^+ content and enhanced K^+ content. The results were similar to recent reports that ACC deaminase-producing *Dietzia natronolimnaea* and *Streptomyces* sp. GMKU 336 enhanced salt tolerance in plants by increasing the K^+/Na^+ ratio via up-regulation of the Na^+/H^+ antiporter gene (*NHX1*) involved in maintenance of the Na^+ level in the cytoplasm[10,42]. Besides, the increment in K^+/Na^+ ratio was observed in maize, pea, and sugarcane associated respectively with ACC deaminase-producing *Pseudomonas fluorescens*, *Variovorax paradoxus* 5C-2, and *Enterobacter* sp. EN-21, under salinity stress[9,43,44].

ROS production plays a crucial role as signalling molecules involved in stress conditions including attack by pathogens, drought, and salinity which leads to high accumulation of MDA, a product of membrane lipid peroxidation[1,2]. In this study, rice KDML105 inoculated with either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* had significantly decreased MDA content under salt stress conditions. Moreover, histochemical staining with NBT and DAB indicated that levels of H_2O_2 and O^{2-} were reduced in the corresponding leaves. The results were in agreement with previous reports that ACC deaminase-producing endophytes caused a reduction in MDA content, including *Bacillus subtilis* GB03 in white clover[31], *Enterobacter* sp. EN-21 in sugarcane[9], *Streptomyces* sp. GMKU 336 in Thai jasmine rice[10], and *Dietzia natronolimnaea* in wheat[42]. Moreover, higher accumulation of proline in rice associated with *S. venezuelae* might help stabilize ROS[33] and, thus, alleviate salt stress by modulation of the antioxidant system.

This work is the first demonstration that *S. venezuelae* carries PGP-traits and promotes growth of rice KDML105 endophytically under normal and salinity conditions. Moreover, the ACC deaminase-overexpressing mutant, *S. venezuelae*/pIJ86-*acdS*, enhanced rice growth and salt tolerance more than the original strain. The physiology of the rice benefitted remarkably from the ACC-deaminase trait. Overproduction of ACC deaminase of *S. venezuelae* is an important model to investigate how excessive ACC deaminase-producing inocula can be effective for crop health improvement under severe conditions.

Methods

Bacterial salt tolerance and plant growth promoting (PGP) traits. *Streptomyces venezuelae* ATCC 10712 was grown and maintained on mannitol soybean agar (MS)[45]. Salt tolerance was determined by growth of colonies on ISP 2 (Difco™) supplemented with 1–4% NaCl (w/v) at 28 °C for 7 days.

Proline accumulation was determined by growing *S. venezuelae* in 10 mL tryptic soy broth (TSB) supplemented with 1–3% NaCl at 28 °C for 3 days. Cells were treated with 2 mL 20% trichloroacetic acid, mixed and centrifuged. The aqueous solution was mixed with 2 mL ninhydrin solution (1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid) and 2 mL glacial acetic acid, and incubated at 95 °C for 1 h, then cooled on ice. The reaction mixture was extracted and mixed vigorously with 4 mL toluene for 15–20 sec. The absorbance of the red-colored organic layer of the ninhydrin-proline complex was measured at 520 nm by spectrophotometry. Proline concentration was determined from a standard curve of commercial proline and calculated as described by Bates, *et al.*[46].

Indole-3-acetic acid (IAA) was determined by a colorimetric method[47]. *S. venezuelae* was grown in the dark in glucose-beef extract broth supplemented with 10 mM L-tryptophan at 28 °C for 7 days. The culture was then centrifuged and 2 mL of supernatant was mixed with 1 mL of Salkowski's reagent[48]. The mixture was left at room temperature for 30 min in the dark. IAA production was indicated by development of a pink-red color.

ACC deaminase activity was monitored by the amount of α-ketobutyrate generated from ACC cleavage as described by Penrose and Glick[49]. *S. venezuelae* was cultured in TSB and washed twice before transferring onto minimal medium (MM) containing 3 mM ACC as a sole source of nitrogen and incubated on a rotary shaker in the dark for 0, 24, 48, 72 and 98 h. The amount of α-ketobutyrate was determined by measuring absorbance at 540 nm and comparing to a standard curve of α-ketobutyrate. Protein content was performed according to Bradford[50]. ACC deaminase activity was expressed as α-ketobutyrate production in nmol mg^{-1} protein h^{-1}.

Construction of ACC deaminase-overexpressing mutant. The ACC deaminase gene (*acdS*) (SVEN_RS07535) was retrieved from the genome sequence of *S. venezuelae* ATCC 10712 (Accession no. NC_018750). Specific primers for amplification of *acdS* were designed as ATT151F (5′-TTTTTTAAGCTTGAGATGACGGCGATGGGCGAGTT-3′) and ATT151R (5′-TTTTTTCATATGCCGACCAGCAGCCGTCACTCAAC-3′) including respectively *Hind*III and *Nde*I sites (underlined). PCR conditions were initially 98 °C, 30 sec; and 30 cycles of 98 °C, 10 sec; 69 °C, 30 sec; 72 °C, 1 min; and finally at 72 °C, 10 min. The PCR product was then cloned into the pJET cloning vector (Fermentas, USA) and subcloned into constitutive multi-copy expression plasmid pIJ86 under *ermE** promoter[51] to obtain pIJ86-*acdS*. Next, pIJ86-*acdS* was transformed into *E. coli* ET12567/pUZ8002[52] and intergeneric conjugation was performed using 24-h mycelium of *S. venezuelae* as described by Vitayakritsirikul, *et al.*[23]. Exconjugants (*S. venezuelae*/pIJ86-*acdS*) were selected by apramycin (100 μg mL^{-1}) and thiostrepton (50 μg mL^{-1}) resistance, and verified by (i) PCR amplification of the thiostrepton resistance gene using primers and conditions as described previously by Rungin, *et al.*[5] and (ii) ACC deaminase activity. *S. venezuelae*/pIJ86 was also constructed as a control.

RNA purification and semi-quantitative RT-PCR. *S. venezuelae*/pIJ86 and *S. venezuelae*/pIJ86-*acdS* were grown in TSB for 24 h, then harvested by centrifugation, washed twice with 0.1 M Tris-HCl (pH 8.5) and inoculated onto MM medium containing 3 mM ACC and incubated for 72 h. Total RNA was isolated using TRIzol (Ambion, USA) and treated with RNase-free DNase I according to the manufacturer's protocol (Thermo Fisher Scientific, USA). cDNA was synthesized using a RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Semi-quantitative RT-PCR analysis of *acdS* gene was performed using cDNA and primers, ATT165F (5′-CGGGTGATCTGCTCGTGGGTCGGTA-3′) and ATT165R (5′-GCGGGCTTCGGCATCGGCTT-3′), using Phusion Hot Start II-High Fidelity DNA polymerase (Thermo Fisher Scientific, USA). PCR conditions started with 98 °C, 30 sec; and 30 cycles of 98 °C, 10 sec; 58 °C, 30 sec; 72 °C, 1 min; and finally at 72 °C, 10 min. The expression level of *acdS* was quantified by Gel Doc™ XR + with Image Lab™ Software (Biorad, USA) and normalized against the expression of a housekeeping gene, *hrdB*[53].

Analysis of rice growth parameters. Thai jasmine rice seeds (*Oryza sativa* L. cv. KDML105) were surface sterilized by 70% (v/v) ethanol for 1 min followed by 15 min in 5% (w/v) sodium hypochlorite and thoroughly rinsed with sterile distilled water before transferring into a sterile moist chamber and incubated at room temperature in the dark for 7 days. Roots of seedlings were cut into the same length and individually immersed into sterile glass beakers containing 10^8 spores mL^{-1} of either *S. venezuelae*/pIJ86 or *S. venezuelae*/pIJ86-*acdS* and incubated for 24 h. Seedlings were then re-located to a moist sponge support for 1 day before transferring to a 20-L container filled with half-Yoshida solution (YS)[54] for 14 days. Next, salt stress was introduced by replacing the nutrient solution with YS supplemented with 150 mM NaCl and further incubated for 7 days. The pH of nutrient solution was maintained between 5.0–5.5 throughout the growth period. A positive control of non-salt stressed rice was grown under the same conditions without NaCl treatment. Growth parameters of non-salt and salt-stressed rice plants at 7 days were determined for root and shoot lengths, fresh (FW) and dry (DW) weights.

Analysis of ethylene level and proline accumulation. Ethylene emission was analyzed by the method of Cristescu, *et al.*[55] 7-day rice plants were placed in a 550 mL bottle tightly sealed with a rubber septum and left for 1 h. Fifty millilitres of headspace air was sampled and analyzed for ethylene by gas chromatography (GC 7890A, Agilent Technologies, USA) packed with a Poropak-N column at 60 °C, equipped with a flame ionization detector. The amount of ethylene emission was calculated as nmol of ethylene g^{-1} FW h^{-1} by comparison to a standard curve generated with pure ethylene.

For proline content, fresh leaf samples (50 mg) were immediately homogenized with liquid nitrogen. The powder was mixed with 3% (v/v) sulfosalicylic acid and centrifuged. The aqueous solution was mixed with ninhydrin solution and glacial acetic acid following the protocol described above.

Analysis of total chlorophyll and relative water content (RWC). Total chlorophyll was measured according to the method of Porra, *et al.*[56]. Fresh leaf samples (50 mg) were immediately homogenized with liquid nitrogen. The powder was dissolved in DMSO and centrifuged at 4 °C for 10 min. Absorbance was measured at 645 and 663 nm by spectrophotometry. Total chlorophyll content was calculated based on chlorophyll equations of Arnon[57].

RWC was determined according to the method of Mostofa and Fujita[58]. Leaf fresh weight was measured and soaked in distilled water for 6 h to determine a turgid weight. The leaves were then dried at 60 °C for 72 h to determine a dry weight. RWC was calculated from each weigh according to Smart and Bingham[59].

Determination of Na^+ and K^+ contents. Na^+ and K^+ contents were analyzed using an atomic absorption spectrophotometer according to the method of Johnson and Ulrich[60] at The Soil-Fertilizer-Environment Scientific Development Project, Department of Soil Science, Faculty of Agriculture, Kasetsart University. The concentrations of Na^+ and K^+ were quantified and calculated as mg g^{-1} DW.

Analysis of lipid peroxidation and reactive oxygen species (ROS) staining. Lipid peroxidation of leaf samples was estimated by measuring the amount of malondialdehyde (MDA) by a colorimetric method[61]. Fresh leaf samples (50 mg) were immediately homogenized with liquid nitrogen and mixed with 80% ethanol followed by centrifugation. The aqueous solution was mixed with either (i) −TBA solution [20% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene], or (ii) +TBA solution (0.65% TBA in −TBA solution). Samples were mixed vigorously and heated at 95 °C for 1 h, cooled on ice and centrifuged. The TBA-MDA complex absorbance was measured at 400, 523 and 600 nm by spectrophotometry. The MDA level was calculated as described by Hodges, *et al.*[61].

ROS staining of leaf samples was detected using nitrotetrazolium blue chloride (NBT) and 3,3′-diaminobenzidine (DAB) for superoxide and hydrogen peroxide, respectively following the protocol described by Kumar, *et al.*[62]. Leaf samples were separately immersed in 25 mL 2.5 mM NBT staining solution (pH 7.5) and 5 mM DAB staining solution (pH 3.8) for 24 h at room temperature in the dark. The leaves were then decolorized by boiling in 95% (v/v) ethanol for 30 min and further immersed in 60% glycerol for 16 h before color detection.

Statistical analysis. Data were subjected to statistical analysis using standard ANOVA and Tukey's multiple range tests of SPSS (version 18.0). Data were presented as mean ± S.E. calculated from four plants per treatment in three different replicates, with a different letter indicating statistical significance at $p < 0.05$. ACC deaminase activity and gene expression ratio data were analysed statistically using a t test at $p < 0.05$. The values represented the mean ± S.E. of three replicates and an asterisk represents a statistically-significant change in expression.

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Author Contributions

S.Y. and A.T. conceived and designed the experiments. S.Y., H.P. performed the experiments. S.Y., W.K., R.J., C.J. and A.T. analyzed the data. S.Y., W.K., R.J. and A.T. wrote the manuscript. All authors have reviewed the manuscript and have given approval to the final version.

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1-Aminocyclopropane-1-carboxylate (ACC) Deaminase-Producing Endophytic Diazotrophic *Enterobacter* sp. EN-21 Modulates Salt–Stress Response in Sugarcane

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Abstract
Soil salinity is a wide-reaching environmental problem that lowers the yield of commercial crops such as maize, rice, and sugarcane. In this study, we examined the effect of 1-aminocyclopropane-1-carboxylate (ACC) deaminase-producing endophytic diazotrophic *Enterobacter* sp. EN-21 on growth promotion, salt tolerance, and root colonization of sugarcane. *Enterobacter* sp. EN-21 inoculated and uninoculated sugarcane plants were grown in a greenhouse with and without 200 mM NaCl for 7 days. Sugarcane inoculated with *Enterobacter* sp. EN-21 substantially increased in total plant length, dry, and fresh weights in both non-salt and salt treatments. Under the salt–stress condition, *Enterobacter* sp. EN-21 significantly reduced proline, malondialdehyde, ethylene emission, and Na^+ accumulation in sugarcane but markedly increased total chlorophyll content and K^+ accumulation. The *gfp*-tagged *Enterobacter* sp. EN-21 was observed to colonize early at the root cap, root hairs, and lateral root junctions of sugarcane and later localized in intercellular spaces. Altogether the results of this study indicated that ACC deaminase-producing *Enterobacter* sp. EN-21 is a true endophyte and able to promote growth and enhance salt tolerance in sugarcane.

Keywords ACC deaminase · Salt stress · Colonization · Endophyte · *Enterobacter* · Sugarcane

Introduction

Salinity stress caused by high concentrations of salt in soil is a major factor limiting the growth and productivity of crop plants. Over 20% of total irrigated land has been damaged by high salinity and nearly 800 million hectares (more than 6%) of the world's total land is salt affected (Pitman and Läuchli 2002; Munns and Tester 2008). In Thailand, there are 2.3 million hectares where salt-affected soil influences the low yield of commercial crop plantations such as maize, rice, and sugarcane (Arunin and Pongwichian 2015). Sugarcane (*Saccharum* sp.) is a major sugar-producing crop in the tropical and subtropical regions and is economically important in Thailand for sugar and bioethanol production. Sugarcane is classified as a glycophyte which is susceptible to high salt concentrations that would cause reduction of growth and particularly low productivity of sugar content (Hussain and others 2004; Gomathi and Thandapani 2005).

Plants display several responses to biotic and abiotic stresses that enable them to tolerate severe conditions involving physiological and biochemical processes (Mahajan and Tuteja 2005). In salt stress, two main physiological mechanisms are affected: osmotic balance, which drastically alters the water balance of plant cells when the salt concentration rises around the rhizosphere; and ionic concentration, which causes toxic effects by excess of ion unbalance in plant cells (Munns and Tester 2008). Moreover, high salinity not only causes physiological changes but also metabolic alterations in plants. For examples, when red pepper (Siddikee and others 2011), rice (Sarkar and others 2017), sorghum (Singh and Jha 2016), white clover (Han and others 2014), tomato,

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and *Arabidopsis* (Kangmin and others 2014) were treated with 200 mM NaCl, they displayed decreased plant biomasses and significant increases of osmoprotectant proline, lipid peroxidation, and malondialdehyde (MDA).

Although rhizospheric bacteria have been known to ameliorate the effect of many deleterious conditions including salt stress (Forni and others 2017), plant growth-promoting bacterial endophytes (PGPEs) such as *Achromobacter, Enterobacter*, and *Pseudomonas* have also been noticed (Karthikeyan and others 2012; Ali and others 2014; Yaish and others 2015). PGPE are bacteria that live inside plant tissues and enhance the growth of plants. The capability to colonize plant roots has been considered as the major characteristic of endophytes that exert a beneficial effect for crop yield enhancement (Santoyo and others 2016). PGPEs encourage plant growth through the release of phytohormones, increment of nutrients, and protection of plants from critical environmental stresses (Mayak and others 2004; Madhaiyan and others 2013). In salt–stress conditions, PGPEs help reduce plant metabolic changes by decreasing the accumulation of proline and lowering oxidative stress (lipid peroxidation) and ethylene emission (Ali and others 2014; Yaish and others 2015; Barnawal and others 2016). Modification of the plant metabolic profile and increment of plant growth have been investigated in plants inoculated with PGPEs, particularly those carrying 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. ACC deaminase-producing bacterial endophytes such as *Paenibacillus, Brachybacterium, Enterobacter*, and *Pseudomonas* have showed beneficial interactions with plants under severe stresses (Ali and others 2014; Yaish and others 2015; Barnawal and others 2016). Hence, ACC deaminase-producing bacteria could promote plant growth and increase plant tolerance to environmental stresses by the conversion of ACC to ammonia and α-ketobutyrate for their consumption and consequently lower the ethylene level in plants (Glick 2005).

Previously, we reported sugarcane growth enhancement by root-associated diazotrophic *Enterobacter* sp. EN-21 (Kruasuwan and Thamchaipenet 2016). Here, we further report the ACC deaminase trait of *Enterobacter* sp. EN-21 to facilitate growth of sugarcane under salt–stress conditions. Moreover, the strain was *gfp*-tagged to demonstrate its location and colonization within roots of sugarcane.

Materials and Methods

Bacterial Growth Conditions

Endophytic diazotrophic *Enterobacter* sp. EN-21 previously isolated from roots of sugarcane (Kruasuwan and Thamchaipenet 2016) was streaked on nutrient agar and incubated at 37 °C for 24 h. Then strain EN-21 was transferred into tryptic soybean broth (TSB) and incubated at 37 °C for 24 h. The bacterial suspension was then harvested by centrifugation at 8000 rpm for 10 min and resuspended in TSB to yield 10^8 CFU mL^{-1} determined by plate counting. The strain was further used for inoculation into sugarcane plants.

Quantification of ACC Deaminase Activity

Enterobacter sp. EN-21 was quantified for its ACC deaminase activity by monitoring the amount of α-ketobutyrate generated from the cleavage of ACC as described by Penrose and Glick (2003). Strain EN-21 was cultured in TSB and then washed twice with DF salt minimal medium and transferred to the same medium supplemented with 3 mM ACC (Sigma-Aldrich, USA) as a sole nitrogen source to induce ACC deaminase activity. The amount of α-ketobutyrate was determined by comparing the absorbance at 540 nm of the sample to a standard curve of α-ketobutyrate ranging from 0.02 to 0.2 µmol. Protein content was performed as described by Bradford (1976). ACC deaminase activity was calculated as the amount of α-ketobutyrate produced in nmol mg^{-1} protein h^{-1}.

Sugarcane Growth Promotion Under Salt–Stress Conditions

One-month old sugarcane tissue cultures (*Saccharum officinarum* cv. LK92-11) were obtained from the Plant Tissue Culture Unit at Central Laboratory and Greenhouse Complex, Kasetsart University (Kamphaengsaen Campus). Sugarcane plantlets were prepared and cultivated according to Kruasuwan and Thamchaipenet (2016). Briefly, sugarcane plants were inoculated with a cell suspension of *Enterobacter* sp. EN-21 (10^8 CFU mL^{-1}) by the root dip method (Musson and others 1995) in a tissue culture bottle containing Murashige and Skoog (MS0) medium (Murashige and Skoog 1962) and immersed at room temperature (RT) for 24 h. Inoculated sugarcane plants were transferred to cleaned mini-pots (6×5.5 cm^2) containing sterilized sand:perlite:vermiculite (2:2:1). The experiment was set up in completely randomized design using five-replicate mini-pots per treatment and each treatment was replicated twice. Plants were maintained in the greenhouse and supplied with sterilized sucrose-free MS0 broth twice a day for 30 days. At 30-day after inoculation, salt stress was introduced by irrigation of sterilized distilled water containing 200 mM NaCl twice a day for 7 days in the same condition as above described. After that, plants were collected and growth parameters and physiological properties were measured as described below.

Plant Growth Parameters Evaluation

Plant growth parameters of sugarcane at 7 days after irrigation (DAI) were determined for total plant length, dry, and fresh weights. The salt tolerant index (STI) was calculated as STI = (DWSP/DWNSP) × 100, where DWSP is dry weight of the stressed plant and DWNSP is dry weight of the non-stressed plant (Baha and Bekki 2015).

Proline Accumulation Analysis

Sugarcane leaf samples (50 mg) were snap-frozen and immediately homogenized with liquid nitrogen. The sample powder was mixed with 3% (v/v) sulfosalicylic acid followed by centrifugation at 14,000×*g* for 10 min. The supernatant was mixed with ninhydrin solution (1.25 g ninhydrin in 30 mL glacial acetic acid and 20 mL 6 M phospholic acid) and glacial acetic acid and incubated at 95 °C for 1 h and cooled on ice. The reaction mixture was extracted and mixed vigorously with toluene for 15–20 s. The red-colored organic layer was separated and transferred to a cuvette for absorbance measurement of the ninhydrin-proline complex at 520 nm by spectrophotometry. The proline concentration was determined from a standard curve of commercial proline and calculated as described by Bates and others (1973).

Lipid Peroxidase (MDA Content) Determination

Lipid peroxidation of plant membrane lipids was detected by measuring the MDA content obtained by the thiobarbituric acid (TBA) reaction. Fresh leaf samples (50 mg) were immediately homogenized with liquid nitrogen and mixed with 80% ethanol followed by centrifugation at 14,000×*g* for 10 min. The supernatant was mixed with either (i) −TBA solution (20% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene), or (ii) +TBA solution (containing −TBA solution plus 0.65% TBA). Samples were then mixed vigorously and heated at 95 °C for 1 h, cooled on ice and centrifuged at 14,000×*g* for 5 min. The TBA–MDA complex was examined at absorbance 400, 523, and 600 nm by spectrophotometry. The MDA level was calculated as described by Hodges and others (1999).

Evaluation of Total Chlorophyll Contents

Total chlorophyll was extracted according to the method of Porra and others (1989). Sugarcane leaf samples (50 mg) were immediately homogenized with liquid nitrogen and crushed in 80% (v/v) acetone, subsequently centrifuged at 9000×*g* for 10 min at 4 °C. The aqueous samples were measured at absorbance 645 and 663 nm by spectrophotometry. Total chlorophyll content was calculated based on chlorophyll equations of Arnon (1949).

Ethylene Emission Analysis

Ethylene emission was analyzed by the method of Madhaiyan and others (2007). Ten sugarcane plant samples were placed in 250-mL bottles with a rubber septum and sealed with paraffin film followed by 4-h incubation. 1 mL of headspace air was sampled and analyzed for ethylene by a gas chromatograph (GC 7890A, Agilent Technologies, USA) packed with a Poropak-N column at 60 °C, equipped with a flame ionization detector. The amount of ethylene emission was calculated as nmol of ethylene g^{-1} FW h^{-1} by comparison to a standard curve generated with pure ethylene.

Measurement of Na^+ and K^+ Contents

Na^+ and K^+ contents were measured from roots and shoots of 7 DAI sugarcane plants. Plant samples were dried at 60 °C and ground to finely powder with pestle and mortar. The samples were digested with perchloric acid:nitric acid (5:2) at 180 °C. Ion analysis was analyzed using atomic absorption spectrophotometer according the method of Johnson and others (1959) by the Soil–Fertilizer–Environment Scientific Development Project, Department of Soil Science, Faculty of Agriculture, Kasetsart University. The concentration of Na^+ and K^+ were, respectively, quantified and calculated as mg g^{-1} DW.

Preparation of *gfp*-Tagged *Enterobacter* sp. EN-21

Competent cells of *Enterobacter* sp. EN-21 were prepared following the method of Chung and others (1989). The pBZ1 plasmid [pRK404 containing *gfp* (*Hin*dIII/*Eco*RI fragment) from pEGFP] (Noisangiam and others 2012) was introduced into the competent cells by electroporation in a 100-µL cuvette using 1.8 kV, a capacitance of 25 µF and a resistance of 200 µΩ. To select the *gfp*-transformants, the cell suspension was plated onto low-salt LA medium supplemented with 30 mg mL^{-1} tetracycline. Plasmid extracted from *gfp*-transformants was digested with *Hin*dIII/*Eco*RI to confirm the presence of the *gfp* fragment (750 bp). Positive transformants were confirmed by observing under fluorescent microscopy to visualize the expression of green fluorescent protein.

Re-inoculation of *gfp*-Tagged *Enterbacter* sp. EN-21 into Sugarcane

One-month tissue cultures of sugarcane plants (cv. LK92-11) were inoculated with a cell suspension of *gfp*-tagged *Enterobacter* sp. EN-21 (10^8 CFU mL^{-1}) by the root-dip method overnight. The inoculated plants were cultivated as described earlier. At successive times, 24 h and 7 days after inoculation, inoculated and uninoculated sugarcane roots

were carefully washed with sterile distilled water, cut into small pieces and embedded on a bridge slide with 0.25% (w/v) agarose gel as describe by Lin and others (2012) and analyzed by a confocal laser scanning microscope (CLSM, Nikon C2, USA). The excitation at 470–495 nm and emission at 515–565 nm were used to visualize bacterial cells and to reveal the structure of the plant tissues, respectively.

Statistical Analysis

Data were statistically analyzed by one-way ANOVA and Tukey's multiple range tests using SPSS (version 18.0) to determine the significant difference among means of the treatment at $P \leq 0.05$. Data are shown as mean ± standard deviation calculated from five plants per treatment in three different replicates. Different letters indicate statistical significance at $P \leq 0.05$.

Results

ACC-Deaminase Activity from *Enterobacter* sp. EN-21

ACC-deaminase activity of the endophytic diazotroph *Enterobacter* sp. EN-21 was measured by growing the endophyte in DF salt minimal medium and monitoring the amount of α-ketobutyrate generated from the cleavage of ACC. *Enterobacter* sp. EN-21 was able to grow better in DF salt minimal medium supplemented with 3 mM ACC than DF medium alone (data not shown). ACC deaminase activity of strain EN-21 was quantified by measuring the amount of α-ketobutyrate at 102.47 nmol α-ketobutyrate mg^{-1} protein h^{-1}.

Effect of *Enterobacter* sp. EN-21 on Sugarcane Growth Under Salt Stress

A preliminary screening of salt–stress tolerance in sugarcane plants demonstrated that *S. officinarum* cv. LK92-11 tolerated salt stress at 200 mM NaCl up to 7 DAI (data not shown). Alleviation of sugarcane plants under salt stress by inoculation of ACC deaminase-producing *Enterobacter* sp. EN-21 was investigated in pot experiments in greenhouse conditions. Growth parameters and biochemical characteristics of inoculated and uninoculated sugarcane plants were evaluated at 7 DAI with and without the addition of 200 mM NaCl. Compared to uninoculated plants, *Enterobacter* sp. EN-21-inoculated plants had significant enhancements of total lengths, total fresh, and dry weights of sugarcane plants in either salt or non-salt treatments (Fig. 1a–c, respectively). Under salt–stress conditions, inoculated plants had significantly increased total plant length, total fresh, and dry weights about 1.1–1.3-fold when compared to uninoculated plants. In addition, calculation of STI revealed that sugarcane plants inoculated with *Enterobacter* sp. EN-21 appeared to be more tolerant to salt (81.10%) than uninoculated plants (68.70%) (Table S1).

Effect of *Enterobacter* sp. EN-21 on Proline, MDA, Total Chlorophyll, and Ethylene Accumulation Under Salt Stress

At 7 DAI, salt stress drastically induced proline and MDA contents at 769.74 ng g^{-1} FW and 26.44 nmol g^{-1} FW, respectively, in uninoculated plants. Although the level of proline was increased in *Enterobacter* sp. EN-21-inoculated sugarcane plants under salt–stress conditions when compared to the non-salt control, it was lower by onefold when compared to the uninoculated control under salt stress

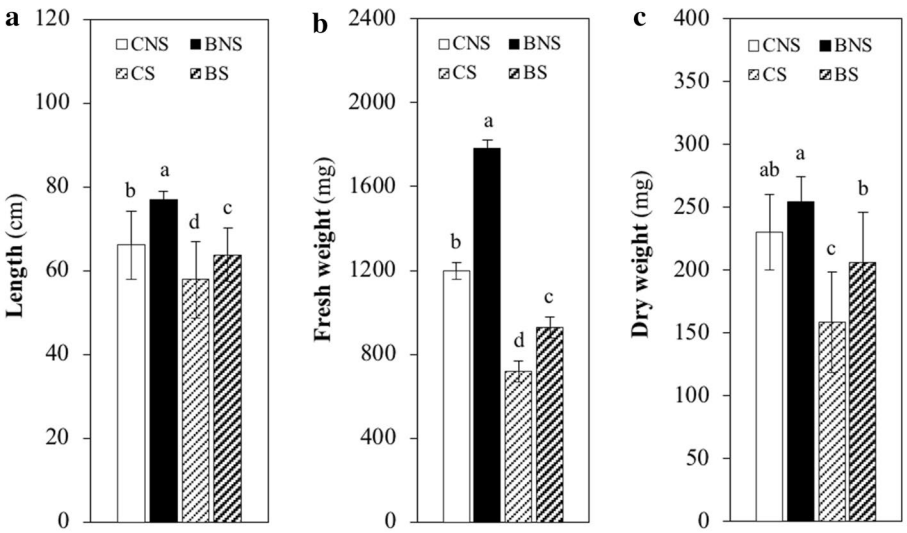

Fig. 1 Effect of *Enterobacter* sp. EN-21 on length (**a**), fresh weight (**b**), and dry weight (**c**) of sugarcane. *CNS* non-salt control plants, *BNS* non-salt inoculated plants, *CS* salt stressed control plants, and *BS* salt stressed inoculated plants

(Fig. 2a). Noticeably, the MDA level of inoculated plants under salt stress was sustained at the same level as the non-salt inoculated plants (Fig. 2b). Total chlorophyll content was constant in inoculated plants either with or without salt treatment, but was significantly reduced by onefold in salt stressed uninoculated plants when compared to the non-salt treated plants (Fig. 2c). Significantly, the ethylene level was augmented in uninoculated plants under salt stress by 1.4-fold when compared with non-salt control plants (Fig. 2d). Although the ethylene level was increased in inoculated sugarcane plants, it was maintained at the same level when plants were treated with salt (Fig. 2d).

Effect of *Enterobacter* sp. EN-21 on Na^+ and K^+ Contents

Na^+ content was significantly enhanced in salt stressed sugarcane plants by 13-fold when compared to the non-salt treated control (Fig. 3a). The results demonstrated that *Enterobacter* sp. EN-21-inoculated plants showed about a threefold lower Na^+ content than that of salt stressed uninoculated sugarcane (Fig. 3a). On the contrary, K^+ content drastically declined by 1.6-fold in salt stressed plants when compared to the non-salt treated control (Fig. 3b). Markedly, plants inoculated with *Enterobacter* sp. EN-21 revealed highly significant increments of K^+ content by 2.1-fold when compared to the salt stressed uninoculated plants (Fig. 3b).

Localization of *gfp*-Tagged *Enterobacter* sp. EN-21 in Sugarcane Roots

The ACC deaminase-producing endophytic diazotroph *Enterobacter* sp. EN-21 was tagged with *gfp* (pBZ1-*gfp*) and presented green-colored colonies on low-salt LA medium supplemented with 30 mg mL^{-1} tetracycline (data not shown). Visualized by fluorescence microscopy, *gfp*-tagged *Enterobacter* sp. EN-21 showed strong green fluorescence (Fig. 4a), whereas the wild-type strain did not (data not shown). At 24 h after inoculation into sugarcane plants, *gfp*-tagged cells were primarily found individually or in groups attaching to the root border, surrounding the root cap, and gathering at the root junctions between root epidermal cells and lateral root junction zones (Fig. 4b–d). CLSM analysis demonstrated that individual bacterial cells localized in the intercellular spaces of roots at 7 DAI (Fig. 4e, f). In uninoculated control plant material, bacterial cells were not observed to colonize root surfaces nor in intercellular spaces of sugarcane roots (data not shown).

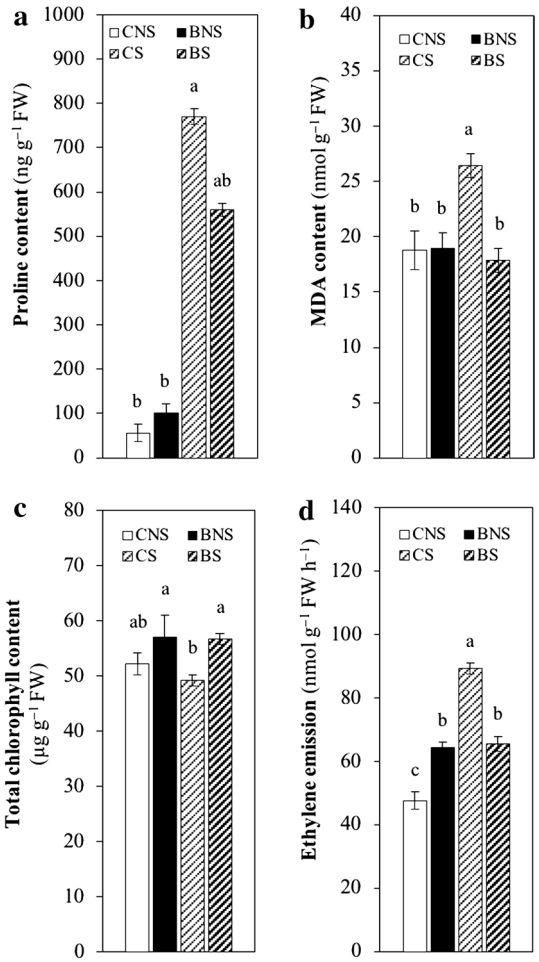

Fig. 2 Effect of *Enterobacter* sp. EN-21 on proline (**a**), MDA (**b**), total chlorophyll contents (**c**), and ethylene emission (**d**) of sugarcane. *CNS* non-salt control plants, *BNS* non-salt inoculated plants, *CS* salt stressed control plants, and *BS* salt stressed inoculated plants

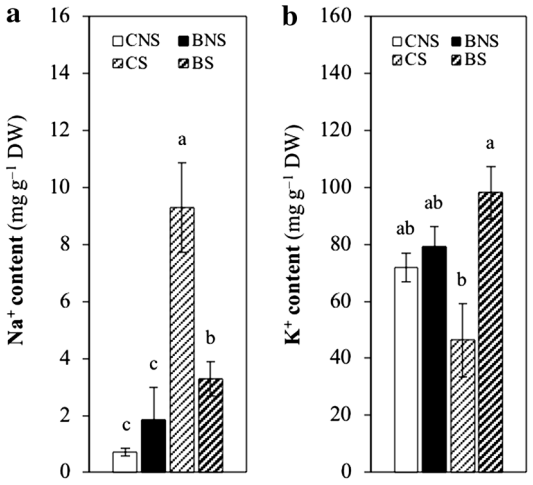

Fig. 3 Effect of *Enterobacter* sp. EN-21 on Na^+ (**a**) and K^+ (**b**) contents of sugarcane. *CNS* non-salt control plants, *BNS* non-salt inoculated plants, *CS* salt stressed control plants; and *BS* salt stressed inoculated plants

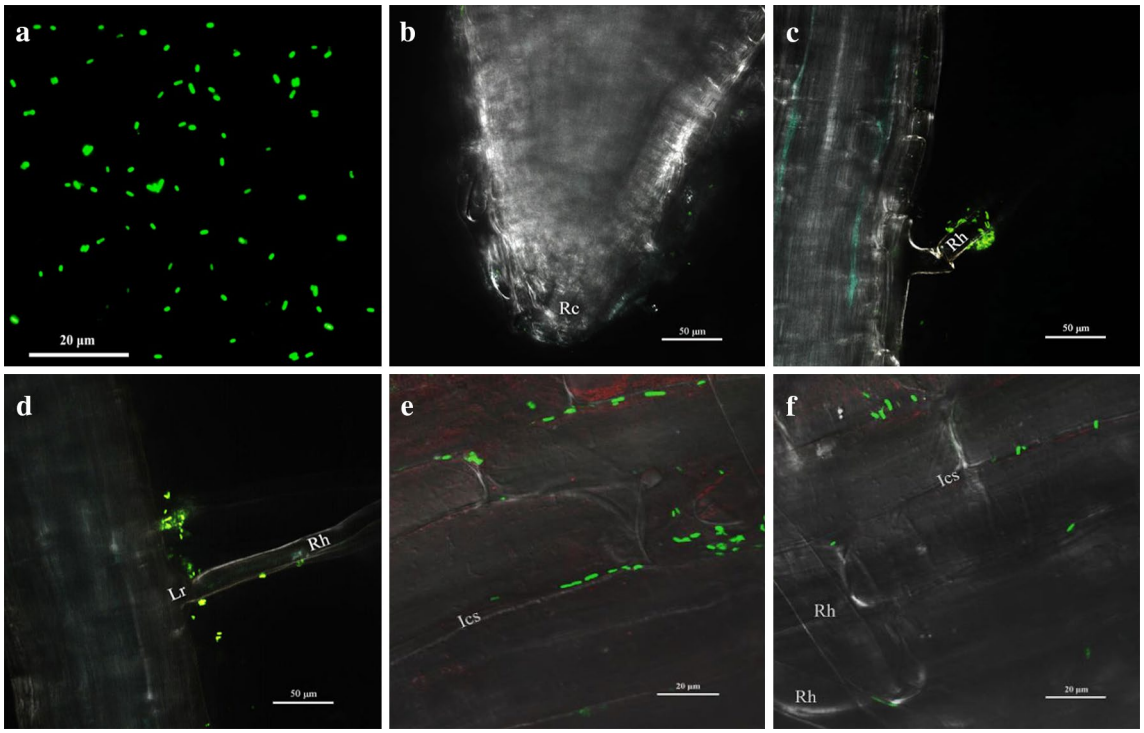

Fig. 4 Microscopic observation of *gfp*-tagged *Enterobacter* sp. EN-21 outside and inside sugarcane roots under fluorescence microscopy and CLSM images. *gfp*-tagged *Enterobacter* sp. EN-21 cells under fluorescence microscopy (**a**), colonization of *gfp*-tagged *Enterobacter* sp. EN-21 at sugarcane roots at 24 h (**b**–**d**) and 7 days (**e, f**). CLSM images present individual bacterial cells colonized at an emergence site by a root border cell surrounding root cap (Rc) (**b**), group of bacterial cells colonized at the root hairs (Rh) (**c**), and at the lateral root junctions (Lr) (**d**), bacterial cells colonized at intercellular spaces (Ics) (**e, f**), *Ep* epidermal cells. Scale bar of **a, e, f**, 20 µm, and **b**–**d**, 50 µm

Discussion

Salinity is a significant problem affecting agriculture worldwide that reduces agricultural productivity and is predicted to become a larger problem in the coming decades. PGPEs are known to encourage plant growth through direct and indirect mechanisms such as fixing of nitrogen, production of plant hormones, and reduction of biotic and abiotic stresses (Santoyo and others 2016). In this study, a sugarcane root-associated endophytic diazotrophic *Enterobacter* sp. EN-21 harboring PGP-traits including nitrogen fixation, production of indole-3-acetic (IAA), siderophores, and ACC deaminase, previously reported to facilitate sugarcane growth under individual and co-inoculation with endophytic actinomycetes (Kruasuwan and Thamchaipenet 2016), was further investigated for its ability to promote sugarcane growth in the presence of salt.

Salt stress reduced plant growth and increased ethylene levels in uninoculated sugarcane plants. However, sugarcane inoculated with ACC deaminase-producing *Enterobacter* sp. EN-21 had significantly increased plant lengths, fresh, and dry weights and reduced levels of ethylene after salt treatment at 7 DAI. Besides, the STI of inoculated sugarcane was increased when compared to uninoculated plants. The results suggested that the promotion of sugarcane growth and alleviation of salt stress are possibly driven by IAA and ACC deaminase produced by *Enterobacter* sp. EN-21. Our results are in agreement with the previous reports that IAA-overproducing *Sinorhizobium meliloti* RD64 inoculated *Medicago truncatula* (Bianco and Defez 2009), IAA- and ACC deaminase-producing *Klebsiella oxytoca* Rs-5 inoculated cotton (Liu and others 2013), and ACC deaminase-producing *Enterobacter* sp. associated with rice (Sarkar and others 2017) showed increased plant growth and salt tolerance. Therefore, the results unambiguously demonstrated that *Enterobacter* sp. EN-21 facilitated sugarcane growth under salt stress conditions.

In response to salt stress, plants generally activate various kinds of physiological and biochemical processes. *Enterobacter* sp. EN-21 not only ameliorated growth of sugarcane plants, but also facilitated metabolic changes under salt stress. At 7 DAI, salt stress drastically induced proline and MDA contents in uninoculated sugarcane, whereas lower amounts were detected in *Enterobacter* sp. EN-21-inoculated sugarcane plants. High salinity stress inhibits plant water uptake, causes ionic toxicity, osmotic stress, and

oxidative damage (Munns and Tester 2008). In response to it, plants exhibit leaf wilting and progressive senescence leading to increased MDA, a product of membrane lipid peroxidation, and proline contents (Yazici and others 2007; Barnawal and others 2016). Our results are in agreement with other plants inoculated with beneficial bacteria such as ACC deaminase-producing *Bacillus subtilis* GB03, *Enterobacter* sp. SBP-6, and *Streptomyces* sp. PGPA39 that caused reduction in proline and MDA contents in wheat, white clover, and tomato plants, respectively (Han and others 2014; Palaniyandi and others 2014; Singh and Jha 2016), but opposed to other reports that PGPB-inoculated mung bean, rice, and wheat increased proline content under salt stress conditions (Nautiyal and others 2013; Bharti and others 2016; Islam and others 2016). The reduction of proline content might be accompanied by an accumulation of other organic solutes that increased salt tolerance rather than proline (Hamdia and others 2004).

Furthermore, chlorophyll content of sugarcane under salt stress was significantly decreased when compared to non-salt stressed plants. Salinity stress was obviously linked to the decline in photosynthesis and crop yield such as those reports in tomato (Ali and others 2014), cucumber (Kang and others 2015), and rice (Turan and Tripathy 2015). However, when sugarcane was inoculated with *Enterobacter* sp. EN-21, the plants had significantly higher total chlorophyll content either with or without salt treatment. Our results are in congruence with other studies in which ACC deaminase-producing *Enterobacter* sp. SBP-6, *Enterobacter cloacae* HSNJ4, and IAA and ACC deaminase-producing bacterial consortia improved photosynthetic pigments and growth of wheat, canola, and avocado, respectively, better than those of non-inoculated plants under salt stress (Singh and Jha 2016; Barra and others 2017; Li and others 2017). The results suggested that ACC deaminase-producing *Enterobacter* sp. EN-21 facilitates sugarcane growth in saline environments by reduction of proline and MDA contents, and activation of total chlorophyll content.

Ethylene is an important plant growth regulator that functions in stress signaling which is quickly stimulated by biotic and abiotic stresses including salinity (Tao and others 2015). In this experiment, ethylene level was significantly increased in uninoculated sugarcane under salt stress when compared with non-salt treated plants. Under the non-salt condition, the ethylene level was, however, increased in *Enterobacter* sp. EN-21-inoculated sugarcane plants compared to the uninoculated control. This might be an effect of a plant hormone, IAA, produced by the strain EN-21 (Kruasuwan and Thamchaipenet 2016). IAA is known to facilitate plant growth and stimulates ethylene synthesis in plants by activation of the first enzyme in the pathway, ACC synthase, to convert ACC into ethylene. Therefore, bacterial IAA promotes plant growth and simultaneously stimulates ethylene synthesis that inhibits plant growth (Glick 2015; Olanrewaju and others 2017). Coincidentally, *Enterobacter* sp. EN-21 in this work maintained a low ethylene level by the action of ACC deaminase and at the same time enhanced sugarcane growth by IAA. Our results are in agreement with other reports that ACC deaminase-producing bacteria decreased ethylene levels in host plants, such as *Enterobacter* sp. EJ01 in tomato and *Arabidopsis* (Kangmin and others 2014); and *Brevibacterium iodinum* RS16, *Bacillus licheniformis* RS656 and *Zhihengliuela alba* RS111 in red pepper (Siddikee and others 2011). The results suggested that *Enterobacter* sp. EN-21 alleviates ethylene stress from salt toxicity by its ACC deaminase activity and consequently promoted growth of sugarcane.

Salinity also adversely affects plant growth by causing high internal Na^+ accumulation and disrupting plant potassium nutrition which is a common plant response to salinity conditions (Zhu 2001). In our work, Na^+ content was significantly enhanced in salt stressed sugarcane but K^+ content was drastically decreased when compared to the non-salt treated control (Fig. 3a, b). High Na^+ load in the uninoculated plants may be due to the increment of sodium transporters, *HKT1* and *SOS1* (Shi and others 2002; Shabala and Tracey 2008), and thus triggers salt toxicity in the plants resulting in the death of leaves and reduction in total photosynthesis (Munns and Tester 2008). Under salt–stress conditions, *Enterobacter* sp. EN-21-inoculated sugarcane showed significantly lower Na^+ content but higher K^+ content. *Enterobacter* sp. EN-21, therefore, not only positively affected Na^+ balance, but also enhanced the uptake of K^+ in salt stressed sugarcane. Similar to previous reports, ACC deaminase-producing *Pseudomonas fluorescens, Streptomyces* sp. KLBMP 5084 and *Variovorax paradoxus* 5C-2 inoculation of maize, halophyte *Limonium sinense*, and pea, respectively, increased uptake of K^+ and gave a high K^+/Na^+ ratio and consequently regulated nutritional balance, ion homeostasis and photosynthesis (Nadeem and others 2009; Wang and others 2016; Qin and others 2017).

In this study, *Enterobacter* sp. EN-21 was *gfp*-tagged and visualized near root caps, root hair surfaces, and lateral root junctions of sugarcane at 24 h of interaction, similarly to *Pseudomonas* spp. associated with olive roots (Prieto and others 2011). At 7 DAI, *Enterobacter* sp. EN-21 cells penetrated into intercellular spaces of epidermal cells, perhaps entering the roots by cracks at the emergence site of lateral roots and at disrupted epidermis similarly to those reported by *gfp*-tagged endophytic nitrogen-fixing *Klebsiella variicola* DX120E colonizing sugarcane roots (Wei and others 2014), *Microbacterium* sp. 16SH localized in sugarcane roots (Lin and others 2012), and sugarcane root-associated *Enterobacter* sp. 35-1 colonizing roots of *Brassica oleracea* (Zakria and others 2008a) and rice (Zakria and others 2008b). It was suggested that invasion

of endophytic bacteria into root tissues might be affected by in vitro endoglucanase secretion (Compant and others 2005; Madhaiyan and others 2013). Our study clearly showed that *Enterobacter* sp. EN-21 colonized the roots of sugarcane and, thus, its mode of action must be truly endophytic. Better understanding of the endophytic diazotrophic *Enterobacter* sp. EN-21 colonization patterns is a crucial prerequisite for the development of effective ways to distribute the endophyte as an added value biofertilizer in sugarcane plantations.

Based on the 16S rRNA sequence previously reported, *Enterobacter* sp. EN-21 is closely related to *Enterobacter asburiae* JCM6051^T (Kruasuwan and Thamchaipenet 2016). Although *E. asburiae* was first isolated from clinical specimens (Brenner and others 1986), this species has recently been reported to be associated with plants as endophytes (Asis and Adachi 2004; Kruasuwan and Thamchaipenet 2016; Yaish 2016). Therefore, it is encouraging to utilize this potential *Enterobacter* sp. EN-21 as a promising candidate for a safe and environmentally friendly bio-inoculant which may facilitate growth and yield enhancement of sugarcane plants under marginal saline lands.

Conclusion

This study demonstrated that the ACC deaminase-producing endophytic diazotrophic *Enterobacter* sp. EN-21 colonizes and facilitates growth of sugarcane under salt–stress condition. Our results provide evidence of a beneficial endophytic *Enterobacter* sp. EN-21 in plant growth promotion and a role of its ACC deaminase in salt–stress tolerance of sugarcane. *Enterobacter* sp. EN-21 mitigated salt stress of sugarcane plants by enhancement of plant biomass, chlorophyll content, and K^+ accumulation, with reduction of proline, MDA, ethylene emission, and Na^+ content. Monitoring of *gfp*-tagged *Enterobacter* sp. EN-21 in sugarcane roots displayed colonization in the rhizosphere region before entering the endosphere region as a true endophyte. These evaluations suggested a potential utilization of *Enterobacter* sp. EN-21 as a promising candidate for an environmentally friendly bio-inoculant to facilitate growth and salt tolerance for sugarcane plantations in the future.

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Compliance with Ethical Standards

Conflict of interest The authors declared that they have no conflict of interest.

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Original Article

Positive role of 1-aminocyclopropane-1-carboxylate deaminase-producing endophytic *Streptomyces* sp. GMKU 336 on flooding resistance of mung bean

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ABSTRACT

Previously, a plant growth-promoting endophytic *Streptomyces* sp., namely GMKU 336, has been shown to improve salt tolerance in rice through the action of 1-aminocyclopropane-1-carboxylate deaminase (ACCD) by converting a precursor of ethylene, (ACC) in plants into ammonia and α-ketobutyrate and consequently reducing the ethylene level. In this study, strain GMKU 336 and its ACCD-deficient mutant were further investigated regarding flooding tolerance in mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72]. The results clearly demonstrated that mung bean associated with *Streptomyces* sp. GMKU 336 significantly increased plant elongation and biomass, chlorophyll content, leaf area, leaf color and adventitious roots, and reduced the ethylene level under flooding conditions when compared to un-inoculated plants and those inoculated with the ACCD-deficient mutant. It was concluded that ACCD-producing *Streptomyces* sp. GMKU 336 that beneficially improved salt tolerance in rice also enhanced flooding tolerance in mung bean via the action of ACCD.

Introduction

Mung bean [*Vigna radiata* (L.) Wilczek] is one of the most important crops for human nutritional needs. In tropical and sub-tropical region such as Thailand, heavy rainfall in the rainy season frequently induces short-term flooding in crop fields and soil flooding has long been identified as a major abiotic stress that results in decreased growth and crop production (Ahmed et al., 2002; Kumar et al., 2013; Parent et al., 2008). In recent years, a new approach has been developed to improve stress in plants by treating crop seeds and seedlings with plant growth-promoting bacteria (PGPB) harboring 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD) (Farwell et al., 2007). PGPB convert ACC, a precursor of ethylene in plants, to ammonia and α-ketobutyrate which the bacteria can consume by the action of ACCD (Honma and Shimomura, 1978), thereby lowering the level of stressed ethylene in plants (Glick, 2004). Plants inoculated with ACCD-producing PGPB were markedly more resistant to the deleterious effects of stress ethylene under flooding conditions including *Ocimum sanctum* and tomato (Barnawal et al., 2012; Grichko and Glick, 2001; Saleem et al., 2007).

Recently, endophytic actinomycetes have been recognized as new members of PGPB due to their properties to protect plants from infectious diseases (Misk and Franco, 2011) and their ability to enhance plant growth by carrying several PGP-traits including siderophore production (Rungin et al., 2012), plant hormone production (Kruasuwan and Thamchaipenet, 2016), phosphate solubilization (El-Tarabily et al., 2008) and ACCD production (El-Tarabily, 2008; Qin et al., 2014). However, the role of ACCD-producing endophytic actinomycetes to promote plant growth under flooding stress has been less studied. Recently, the endophytic *Streptomyces* sp. GMKU 336 was employed to facilitate growth of rice under salt-stress conditions by reducing stress ethylene via the action of ACCD through converting a precursor of ethylene, ACC, in plants into ammonia and α-ketobutyrate and consequently reduced ethylene, reactive oxygen species (ROS), Na^+ and the Na^+/K^+ ratio (Jaemsaeng et al., 2018). In this work, *Streptomyces* sp. GMKU 336 was further investigated regarding its ability to enhance the growth of mung bean

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CN72 [*Vigna radiata* (L.) Wilczek cv. CN72] under flooding conditions.

Materials and methods

Endophytic streptomycete strains

Endophytic *Streptomyces* sp. GMKU 336 was isolated from the medicinal plant *Clerodendrum serratum* (L.) Moon (Ak-kee-ta-wan) (Indananda, 2013). Strain GMKU 336 displays ACCD activity at 2.85 ± 0.15 μmol α-ketobutyrate/mg protein/h and harbors PGP-traits including phosphate solubilization and siderophore production but no indole-3-acetic acid (IAA) production (Jaemsaeng et al., 2018). The ACCD-deficient mutant was constructed by insertional inactivation of *acdS* gene (encoding for ACCD) of the wildtype strain and showed no ACCD activity (Jaemsaeng et al., 2018).

Inoculation of mung bean with endophytic streptomycetes

Healthy seeds of mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72] were obtained from the Chai Nat Field Crops Research Center, Field Crops Research Institute, Department of Agriculture and Cooperatives, Chai Nat, Thailand. The seeds were surface sterilized by immersion in 70% volume per volume (v/v) ethanol for 1 min, 1% weight per volume (w/v) sodium hypochlorite for 5 min and washed six times with sterile distilled water. Then, sterilized seeds were soaked in sterile distilled water for 4 h in the dark, transferred to a sterile moist chamber and incubated at room temperature in the dark for 2 d for seed germination. Fifteen seedlings were used for each experiment, which was performed in triplicate. The pruned-root dip method (Musson et al., 1995) was used to inoculate the seedlings by cutting 0.5 cm from the root tip using a sterile scalpel. Then, the seedlings were immersed in individual sterile glass beakers containing 1×10^8 spores/mL of *Streptomyces* sp. GMKU 336 or its ACCD-deficient mutant for 4 h. The seedlings were re-located to a pot containing sterile soil for 7 d. Next, mung bean plants were flooded with 13 cm of standing water above the soil surface for 21 d. For the positive control, mung bean seedlings were grown under the same conditions without the flooding treatment. The plants were harvested and examined for shoot/root elongation, biomass and plant physiology. The experiment was conducted in a greenhouse during August to October 2017.

Re-isolation of endophytic streptomycetes

Re-isolation of endophytic streptomycetes from the mung bean plants was performed by modifying the method of Rachniyom et al. (2015). Roots and shoots of mung bean were cut separately into small pieces and rinsed with 0.1% (v/v) Tween 20 for 5 min. Then, plant materials were surface sterilized by soaking in 1% (w/v) sodium hypochlorite for 10 min and then immersing in 70% (v/v) ethanol for 1 min and soaking in 10% (w/v) $NaHCO_3$ solution for 5 min. Next, samples of the surface-sterilized plant material were washed in sterile water three times and crushed in ¼ Ringer's solution (0.9% NaCl, 0.042% KCl, 0.048% $CaCl_2$, 0.02% $NaHCO_3$). The resulting solution was spread onto starch casein agar (SCA; Küster and Williams, 1964) supplemented with 100 mg/mL ampicillin, 2.5 U/mL penicillin G, 50 mg/mL amphotericin B and 50 mg/mL cycloheximide. The final washed solution was spread on the medium to ensure that there was no epiphyte contamination. Colonies of endophytic streptomycetes were observed after incubation at 28 ± 2 °C for 7 d and were randomly selected for analysis using 16S rDNA gene sequencing with primers and PCR conditions as described by Rachniyom et al. (2015).

Determination of survival rate

The survival rate of the mung bean plants under flooding conditions was calculated as a percentage compared with those under the non-flooding conditions. Plants that exhibited rot, wilt and died were recorded as dead, whereas surviving plants were recorded as alive.

Determination of leaf color, leaf area, and chlorophyll content

The leaf color was compared with the standardized four panel International Rice Research Institute (IRRI) leaf color chart (LCC) ranging from yellowish green (No. 2) to dark green (No. 5). Leaf area was measured and calculated using grid paper.

Chlorophyll was extracted using dimethyl sulfoxide (DMSO) as described by Hiscox and Israelstam (1979). Approximately 100 mg of leaf fresh weight from each treatment was ground in liquid nitrogen. The macerated leaves were extracted twice by adding 1.0 mL DMSO and sonicated at 30 hz in a water bath sonicator for 2 min. The extracts were centrifuged and the chlorophyll content of the supernatants was measured at 645 and 663 nm within 20 min after the extraction. The chlorophyll content was calculated using Arnon's equations (Arnon, 1949).

Determination of ethylene

Ethylene production was measured by enclosing whole mung bean plants in a 250 mL sealed glass container containing 50 mL acetylene for 1 h. A gas sample (1 mL) was withdrawn and quantified using gas chromatography at the PGPR Biofertilizer and Aerated Compost Soil Microbiology Research Group, Soil Science Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

Statistical analyses

All data from the experiments were calculated and statistically evaluated on 15 seedlings for each condition from biological and technical triplicates. The data were analyzed using one-way analysis of variance and Duncan's test to determine any significant differences between groups at $p < 0.05$. All statistical analyses were performed using the SPSS 20.0 for Windows software (SPSS Inc.; Chicago, IL, USA).

Results

Effect of 1-aminocyclopropane-1-carboxylate deaminase-producing Streptomyces sp. GMKU 336 inoculated mung bean under flooding conditions

Streptomyces sp. GMKU 336 and its ACCD-deficient mutant were inoculated into mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72]. The growth parameters of mung bean were observed after 21 d of flooding treatment in comparison to the non-flooding treatment (Fig. 1A and B, Table 1). Under non-flooding conditions, *Streptomyces* sp. GMKU 336 significantly enhanced plant elongation in shoot/root lengths and the plant biomass in shoot/root fresh and dry weights when compared to the un-inoculated controls (Table 1). Mung bean inoculated with the ACCD-deficient mutant showed similar plant growth parameters to those of the un-inoculated controls plants (Fig. 1A and B, Table 1).

Under flooding conditions, plant elongation and biomass significantly reduced in all plant treatments (Table 1). However, ACCD-producing *Streptomyces* sp. GMKU 336 inoculated mung bean showed significantly more increment in shoot/root elongation

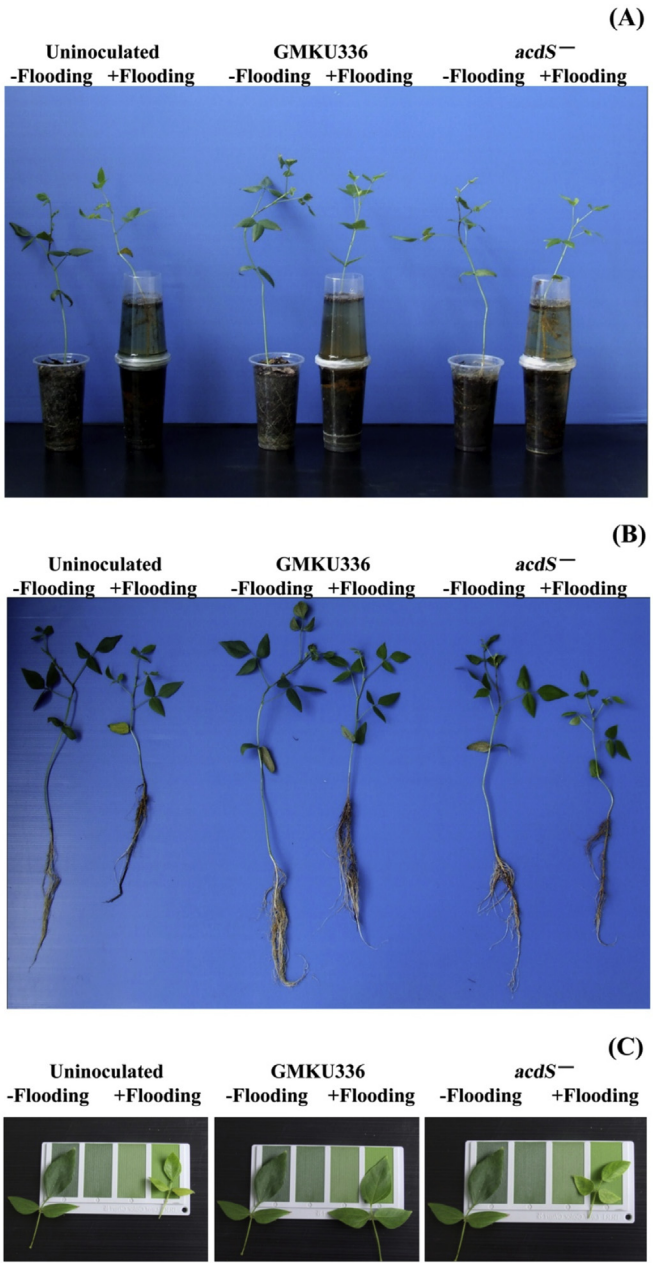

Fig. 1. Effect of 1-aminocyclopropane-1-carboxylate deaminase (ACCD)-producing endophytic *Streptomyces* sp. GMKU 336 on mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72] after 21 d of flooding stress: (A) flooding conditions; (B) whole plants, (C) leaf size and color, where Uninoculated = without bacteria treatment; GMKU336 = plant inoculated with *Streptomyces* sp. GMKU 336; acdS^{-} = plant inoculated with ACCD-deficient mutant; −Flooding = control condition; + Flooding = flooding conditions (13 cm flooding).

and biomass (shoot/root fresh and dry weight) compared to the un-inoculated controls (Table 1). Moreover, the development of new adventitious roots was greatly accelerated by flooding stress, particularly in plants associated with strain GMKU 336, which produced more adventitious roots than other treatments (Fig. 1B). In contrast, no development of adventitious roots was observed in the non-flooding controls (Fig. 1B). Furthermore, the survival rate of un-inoculated plants was reduced 20% compared to *Streptomyces* sp. GMKU 336-inoculated plants and those non-flooding treatments (Table 2). ACCD-deficient mutant inoculated plants exhibited similar trends in plant elongation and biomass to the un-inoculated plants in all treatments (Fig. 1B; Table 1). Therefore, ACCD-producing *Streptomyces* sp. GMKU 336 was able to promote the growth of mung bean CN72 with or without flooding stress.

Effect of 1-aminocyclopropane-1-carboxylate deaminase-producing Streptomyces sp. GMKU 336 on leaves and chlorophyll production

The leaf area was markedly reduced under flooding stress in all treatments (Table 2). A significant reduction in the leaf area of un-inoculated plants and those inoculated with the ACCD-deficient mutant was about 2.5-fold when compared to the corresponding non-flooding treatments (Fig. 1C, Table 2). However, leaf area reduction of mung bean associated with *Streptomyces* sp. GMKU 336 was about 1.2-fold lower than in the non-flooding treatment (Fig. 1C, Table 2). Furthermore, the leaf color of mung bean under flooding stress was altered from dark green to yellowish green compared to the non-flooding treatments (Fig. 1C). Conversely, the leaf color of mung bean associated with *Streptomyces* sp. GMKU 336 was greener than the un-inoculated plants and those inoculated with the ACCD-deficient mutant (Fig. 1C).

The chlorophyll content in mung bean was similar in all treatments under normal conditions but was significantly decreased under flooding treatments (Table 2). A significant reduction of 2–2.3 fold of the chlorophyll content was observed in un-inoculated mung bean and those inoculated with the ACCD-deficient mutant compared to the non-flooding controls (Table 2). However, *Streptomyces* sp. GMKU 336-inoculated plants maintained 1.5–1.7 fold higher chlorophyll content than un-inoculated and ACCD-deficient mutant inoculated plants (Table 2). These results suggested that ACCD-producing *Streptomyces* sp. GMKU 336 enhances the leaf area and leaf color as well as the chlorophyll content of mung bean under flooding stress.

Effect of 1-aminocyclopropane-1-carboxylate deaminase-producing Streptomyces sp. GMKU 336 on ethylene production

Under non-flooding conditions, low ethylene levels were determined in all plant treatments (Table 2). The ethylene level was substantially increased 5.3 fold after flooding stress exposure in the un-inoculated mung bean and in mung bean inoculated with the ACCD-deficient mutant compared to the corresponding non-flooding controls. However, *Streptomyces* sp. GMKU 336-inoculated plants had significantly 3-fold lower ethylene levels than the un-inoculated control (Table 2).

Re-isolation of the endophytic streptomycetes from inoculated mung bean

Re-isolation of the wild type and mutant in both flooding and non-flooding treatments was about 1×10^4 colony forming units (CFU)/g root fresh weight (Table 2). Both strains were validated using 16S rRNA gene sequencing (data not shown). In addition, un-inoculated plants did not harbor endophytic *Streptomyces* sp. GMKU 336.

Discussion

ACCD-producing bacteria enhance the growth of a wide range of plants in the presence of various biotic and abiotic stresses, including pathogen damage, flooding, drought, salt and organic and inorganic contaminants (Glick, 2004). Recently, *Streptomyces* sp. GMKU 336 has been reported to promote plant growth under salinity stress via the action of ACCD (Jaemsaeng et al., 2018). This study has contributed further work to define the role of ACCD in plant growth and flooding tolerance of mung bean CN72 [*Vigna*

Table 1
Plant growth and biomass of mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72] with and without 1-aminocyclopropane-1-carboxylate deaminase (ACCD)-producing *Streptomyces* sp. GMKU 336 and ACCD-deficient mutant under flooding and non-flooding treatments.

Parameters	Treatments					
	Uninoculated−Flooding	Uninoculated + Flooding	GMKU336−Flooding	GMKU336 + Flooding	acdS$^-$−Flooding	acdS$^-$+ Flooding
Shoot length (cm/plant)	44.3 ± 0.18^b	25.11 ± 0.17^d	46.4 ± 0.23^a	27.19 ± 0.27^c	43.84 ± 0.17^b	23.25 ± 0.25^e
Root length (cm/plant)	18.53 ± 0.16^b	17.07 ± 0.16^d	24.41 ± 0.15^a	18.37 ± 0.15^b	17.87 ± 0.21^c	16.45 ± 0.11^e
Shoot fresh weight (g/plant)	1.82 ± 0.02^b	0.74 ± 0.02^d	1.93 ± 0.02^a	0.82 ± 0.02^c	1.80 ± 0.02^b	0.62 ± 0.02^e
Root fresh weight (g/plant)	0.71 ± 0.01^b	0.44 ± 0.01^d	0.81 ± 0.01^a	0.61 ± 0.01^c	0.69 ± 0.01^b	0.34 ± 0.01^e
Shoot dry weight (g/plant)	0.45 ± 0.01^b	0.17 ± 0.01^d	0.54 ± 0.01^a	0.28 ± 0.01^c	0.43 ± 0.02^b	0.12 ± 0.01^e
Root dry weight (g/plant)	0.17 ± 0.01^b	0.12 ± 0.01^d	0.22 ± 0.01^a	0.14 ± 0.01^c	0.16 ± 0.01^b	0.12 ± 0.01^d

Uninoculated = without bacteria treatment; GMKU336 = plant inoculated with *Streptomyces* sp. GMKU 336; acdS$^-$ = plant inoculated with ACCD-deficient mutant; −Flooding = control conditions; +Flooding = flooding conditions (13 cm flooding).
Values are mean of tree replicates ± SE (n = 15). Different letters indicated statistical differences between treatments (Duncan's test $p < 0.05$).

Table 2
Plant physiological parameters of mung bean CN72 [*Vigna radiata* (L.) Wilczek cv. CN72] with and without 1-aminocyclopropane-1-carboxylate deaminase (ACCD)-producing *Streptomyces* sp. GMKU 336 and ACCD-deficient mutant under flooding and non-flooding treatments.

Parameters	Treatments					
	Uninoculated−Flooding	Uninoculated + Flooding	GMKU336−Flooding	GMKU336 + Flooding	acdS$^-$−Flooding	acdS$^-$+ Flooding
Total chlorophyll (mg/g FW)	12.23 ± 0.22^a	6.16 ± 0.27^c	12.19 ± 0.18^a	9.34 ± 0.27^b	12.03 ± 0.17^a	5.39 ± 0.19^c
Leaf area (cm^2/leaf)	13.84 ± 0.90^a	5.77 ± 0.63^c	14.29 ± 0.72^a	11.27 ± 1.03^b	13.57 ± 1.01^a	5.16 ± 0.34^c
Ethylene level (pmol/h/g FW)	1.62 ± 0.12^c	8.58 ± 0.52^a	1.45 ± 0.03^c	2.81 ± 0.11^b	1.50 ± 0.03^c	8.03 ± 0.38^a
Survival rate (%)	100 ± 0.00^a	80 ± 0.00^b	100 ± 0.00^a	100 ± 0.00^a	100 ± 0.00^a	75.5 ± 2.23^c
Endophytic colonization (1×10^4 CFU/g root FW)	ND	ND	4.96 ± 0.08	4.56 ± 0.42	4.65 ± 0.38	4.56 ± 0.43

Uninoculated = without bacteria treatment; GMKU336 = plant inoculated with *Streptomyces* sp. GMKU 336; acdS$^-$ = plant inoculated with ACCD-deficient mutant; −Flooding = control conditions; +Flooding = flooding conditions (13 cm flooding); FW = fresh weight; ND = not detected; CFU = colony forming units.
Values are mean of tree replicates ± SE (n = 15). Different letters indicated statistical differences between treatments (Duncan's test $p < 0.05$).

radiata (L.) Wilczek cv. CN72] by the strain GMKU 336 and its ACCD-deficient mutant.

Streptomyces sp. GMKU 336 has been proved as a true endophyte since it was able to localize and multiply within rice (*Oryza sativa* L. cv. KDML105) without causing any obvious, deleterious effects (Jaemsaeng et al., 2018). In the current work, both *Streptomyces* sp. GMKU 336 and its ACCD-deficient mutant were inoculated into mung bean CN72 and were able to re-isolate from the plants under all conditions. Although, *Streptomyces* sp. GMKU 336 was originally isolated from the medicinal plant *Clerodendrum serratum* (L.) Moon (Indananda, 2013), the results indicated that it has endophytic ability with a wide host range including both monocotyledonous and dicotyledonous plants. The results were in agreement with a previous report on endophytic *Streptomyces* sp. GMKU 3100 originally isolated from rice that was able to neutrally colonize mung bean (Rungin et al., 2012). It clearly indicated that endophytic actinomycetes are nonhost-specific and behave as free-living bacteria. It was suggested that endophytic bacteria may migrate from the rhizosphere to the rhizoplane of their plant hosts and then move to other organs such as roots, stems, leaves and flowers as well as fruits and seeds (Misk and Franco, 2011).

The effects of ACCD-producing *Streptomyces* sp. GMKU 336 and its ACCD-deficient mutant on plant growth were investigated in mung bean CN72 after flooding treatment for 21 d. Under non-flooding conditions, the mung bean plants inoculated with strain GMKU 336 significantly enhanced shoot/root length and biomass when compared to un-inoculated plants and those inoculated with the ACCD-deficient mutant. The results for plant growth were consistent with previous work showing that ACCD-producing *Streptomyces* sp. GMKU 336 has the ability to enhance growth of rice KDML105 (Jaemsaeng et al., 2018). The growth effect has also been found in other ACCD-producing *Streptomyces* for enhanced growth of *Arabidopsis* (Palaniyandi et al., 2013), halophytic *Limonium sinense* (Qin et al., 2014), sugarcane (Kruasuwan and Thamchaipenet, 2016) and tomato (El-Tarabily, 2008).

ACCD-producing *Streptomyces* sp. GMKU 336 inoculated mung bean plants substantially tolerated flooding stress and maintained high shoot and root elongation compared to un-inoculated plants and those inoculated with the ACCD-deficient mutant. The results were in agreement with previous work that an ACCD-producing *Pseudomonas* increased shoot and root growth of tomato (Grichko and Glick, 2001) and canola (Farwell et al., 2007) under flooding conditions.

A decline in photosynthesis under flooding has been reported in mung bean (Ahmed et al., 2002). Here, the leaf chlorophyll content substantially decreased in plants subjected to flooding compared to ones in the non-flooding treatment. However, ACCD-producing *Streptomyces* sp. GMKU 336 reduced this negative effect and maintained a greener leaf color than in the un-inoculated flooding controls. These results were in agreement with previous reports that treatment of ACCD-producing bacteria such as *Achromobacter*, *Herbaspirillum*, *Serratia* and *Ochrobactrum* on *Ocimum sanctum* (Barnawal et al., 2012) and of *Pseudomonas* on tomato (Grichko and Glick, 2001) increased photosynthetic rates which provided some protection against flooding stress.

Morphological adaptation by reducing the leaf area and the induced formation of adventitious roots improved flooding tolerance in mung bean (Ahmed et al., 2002). The current report found that flooding treatment also resulted in a decreased leaf area and affected plant-to-root rot that was observed in un-inoculated plants and those inoculated with the ACCD-deficient mutant. In contrast, mung bean inoculated with ACCD-producing *Streptomyces* sp. GMKU 336 maintained leaf area and increased adventitious root development. Likewise, ACCD-producing *Pseudomonas* clearly stimulated the development of both adventitious roots and the stem in tomato under flooding stress (Grichko and Glick, 2001).

Flooding causes an increase in the accumulation of stress ethylene in plants. Plants inoculated with ACCD-producing PGPB have been documented to show substantial tolerance to flooding stress by lowering stress ethylene (Glick, 2004; Saleem et al., 2007).

In the current work, a significant induction of ethylene production was observed in all plant treatments under flooding stress. Remarkably, mung bean CN72 inoculated with *Streptomyces* sp. GMKU 336 had a lower increment in the ethylene level. These results suggested that the ACCD-producing *Streptomyces* sp. GMKU 336 decreased ethylene production of mung bean via an action of ACCD. Jaemsaeng et al. (2018) recently reported that *Streptomyces* sp. GMKU 336 associated with rice KDML105 that had been exposed to salt stress significantly expressed the *acdS* gene *in vivo* which converted a precursor of ethylene, ACC, in plants into ammonia and α-ketobutyrate, and consequently reduced the levels of ethylene, reactive oxygen species (ROS) and the Na^+ content and Na^+/K^+ ratio. Therefore, it is implied that *Streptomyces* sp. GMKU 336 enhances flooding tolerance in mung bean in the same manner as salt tolerance in rice.

In conclusion, all of the experimental data indicated that ACCD-producing endophytic *Streptomyces* sp. GMKU 336 promoted growth of mung bean CN72 under both normal and flooding conditions. The presence of *Streptomyces* sp. GMKU 336 also enhanced flooding tolerance in mung bean CN72 by decreasing the ethylene production and by increasing the plant growth and biomass, chlorophyll content, leaf area, leaf color and formation of adventitious roots. Noticeably, plants inoculated with the ACCD-deficient mutant exhibited plant growth parameters and physiology in the same manner as the un-inoculated controls. These results clearly indicated the beneficial effects of ACCD-producing endophytic *Streptomyces* sp. GMKU 336 on plant growth promotion under various stress tolerance conditions (salinity and flooding).

Conflict of interest

There is no conflict of interest.

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Actinomadura barringtoniae sp. nov., an endophytic actinomycete isolated from the roots of *Barringtonia acutangula* (L.) Gaertn.

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Abstract

A novel actinomycete strain, designated GKU 128^T, isolated from the roots of an Indian oak tree [*Barringtonia acutangula* (L.) Gaertn.] at Khao Khitchakut district, Chantaburi province, Thailand, was characterized by using a polyphasic approach. The strain formed a branched substrate and aerial mycelia which differentiated into straight to flexuous chains of smooth-ornamented spores. Analysis of the cell wall revealed the presence of *meso*-diaminopimelic acid and *N*-acetylmuramic acid in the peptidoglycan. The whole-cell sugars were glucose, madurose, mannose, rhamnose and ribose. Mycolic acids were absent. The major phospholipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositolmannoside. The predominant menaquinones were MK-9(H_6), MK-9(H_8), MK-9(H_0) and MK-9(H_4). The major fatty acids were $C_{16:0}$, $C_{18:1}\omega 9c$ and 10-methyl $C_{18:0}$ (tuberculostearic acid). The genomic DNA G+C content was 70.5 mol%. Based on 16S rRNA gene sequence analysis, strain GKU 128^T was closely related to the type strains of *Actinomadura nitritigenes* NBRC 15918^T (99.2 % sequence similarity) and *Actinomadura fibrosa* JCM 9371^T (98.7 %). The levels of DNA–DNA relatedness between strain GKU 128^T and the closely related type species were less than 19 %. On the basis of phenotypic and genotypic characteristics, strain GKU 128^T could be distinguished from its closely related type strains and represents a novel species of the genus *Actinomadura*, for which the name *Actinomadura barringtoniae* sp. nov. (=TBRC 7225^T=NBRC 113074^T) is proposed.

The genus *Actinomadura* was first established by Lechevalier and Lechevalier [1] and was proposed to belong to the family *Thermomonosporaceae* [2, 3]. At present, the genus *Actinomadura* comprises 61 recognized species with validly published names (www.bacterio.net/actinomadura.html). The most recent species, *Actinomadura alkaliterrae*, has just been described [4]. The genus *Actinomadura* represents aerobic, Gram-strain-positive, non-acid-alcohol-fast, non-motile actinomycetes that generate abundantly branched, non-fragmenting substrates and aerial mycelia. The aerial mycelium differentiate into various spore chain morphologies. The spore chains are generally short (sometimes long), straight, hooked or spiral with folded, irregular, rugose, smooth, spiny or warty surface ornaments. Members of the genus *Actinomadura* are characterized by the presence of *meso*-diaminopimelic acid and *N*-acetylmuramic acid in the cell-wall peptidoglycan. Mycolic acids are absent. The whole-cell hydrolysates contain galactose, glucose, madurose, mannose and ribose. The cell membranes contain diphosphatidylglycerol and phosphatidylglycerol as major phospholipids. The predominant menaquinone is MK-9(H_6). The cellular fatty acid is rich in branched saturated and unsaturated fatty acids, including tuberculostearic acid. The genomic DNA G+C content is 66–73 mol% [5].

Most *Actinomadura* species are recovered from soils [5]. Novel strains have been recently isolated from alkaline soil [4], geothermally heated soil [6], muddy soil [7], mountain soils [8, 9], peat swamp forest soil [10] and Saharan soils [11, 12]. Some strains of the species are pathogens of humans and animals [13–15]. A few strains are reported as endophytes, namely *Actinomadura flavalba* and *Actinomadura syzygii* isolated from medicinal plants [16, 17]. During an investigation of endophytic actinomycetes from an Indian oak tree [*Barringtonia acutangula* (L.) Gaertn.] at

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Keywords: endophytic actinomycete; new species; *Actinomadura barringtoniae* sp. nov.; Indian oak tree.
Abbreviations: CZA, Czapek's agar; ISP, International *Streptomyces* Project; MS, mannitol-soybean; NA, nutrient agar; TLC, thin-layer chromatography.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GKU 128^T is KF667497.
One supplementary table and three supplementary figures are available with the online version of this article.

Khao Khitchakut district, Chantaburi province, Thailand, 31 isolates were identified from the roots. Most of the isolates belonged to the genus *Streptomyces* (*n*=20) and some less common isolates were classified into the genera *Actinomadura* (*n*=7), *Dactylosporangium* (*n*=1), *Nocardia* (*n*=2) and *Nonomuraea* (*n*=1). In this study, the taxonomic classification of an *Actinomadura* strain, designated GKU 128^T, is described by using a polyphasic approach.

Strain GKU 128^T was isolated from excised roots that had been surface-sterilized as described by Rachniyom *et al.* [18]. Colonies appeared on starch–casein agar [19] supplemented with ampicillin (100 μg ml^{-1}), penicillin G (2.5 U ml^{-1}), amphotericin B (50 μg ml^{-1}) and cycloheximide (50 μg ml^{-1}) after incubation at 28 °C for 3–4 weeks. A single colony was purified and cultured on mannitol–soybean (MS) agar [20]. The pure culture was maintained in 20 % (v/v) glycerol suspension at –80 °C.

Morphological and physiological characteristics of strain GKU 128^T were determined in comparison with those of phylogenetically closely related type strains, *Actinomadura nitritigenes* NBRC 15918^T and *Actinomadura fibrosa* JCM 9371^T. Cultural characteristics were determined on nutrient agar (NA; Difco), Czapek's agar (CZA; ATCC medium 312) and various International *Streptomyces* Project (ISP) media [21], namely yeast extract–malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salt–starch-agar (ISP 4), glycerol–asparagine agar (ISP 5), peptone–yeast extract–iron agar (ISP 6) and tyrosine agar (ISP 7) after incubation at 27 °C for 21 days. Mycelium and soluble pigment colour were determined by comparison with colour chips from the *Colour Harmony Manual* [22]. Spore morphology and ornamentation of strain GKU 128^T were observed by light and scanning electron microscopy (model JSM-6610LV; JEOL) using culture grown on ISP 2 medium at 28 °C for 7 weeks. The motility of spores were observed after flooding the culture plate (MS agar at 28 °C for 7 weeks) with releasing buffer [23] and incubated at 30 °C for 30 min. Growth at various temperatures (5–50 °C using a temperature gradient incubator), pH values (3.0–11.0 at intervals of 1.0 pH unit) and NaCl concentrations [0–7 % (w/v) at intervals of 1.0 %] were assessed using ISP 2 medium at 28 °C for 14–21 days. For pH values, medium was adjusted with biological buffers: citrate buffer for pH 3.0–5.0, phosphate buffer for pH 6.0–8.0 and bicarbonate–carbonate buffer for pH 9.0–11.0. Catalase and oxidase activities were observed with 3 % (v/v) hydrogen peroxide solution and 1 % (v/v) tetramethyl-*p*-phenylenediamine solution, respectively. Acid production from carbohydrates and decomposition of adenine, hypoxanthine, L-tyrosine, xanthine, casein and urea were determined using the method of Gordon *et al.* [24]. Reduction of nitrate was observed using nitrate broth (Difco). Starch hydrolysis was examined on ISP 4 medium. Gelatin liquefaction was evaluated on gelatin medium (2.0 % glucose, 0.5 % peptone, 20 % gelatin; pH 7.0). Coagulation and peptonization of milk were observed in 10 % (v/v) skimmed milk broth (Difco). Production of H_2S and melanin pigments were determined on peptone iron agar (Difco) and ISP 7 medium. Citrate utilization was tested on Simmons' citrate agar (Difco). Degradation of Tweens 20 and 80 (1 %, w/v) were examined by using Sierra medium [25]. Utilization of carbon and nitrogen sources was examined on ISP 9 medium and basal medium supplemented with a final concentration of 1 and 0.1 % (w/v) of the tested carbon and nitrogen sources, respectively [21, 26]. Enzyme activities were tested by using the API ZYM system (bioMérieux) according to the manufacturer's instructions.

For chemotaxonomic analyses, freeze-dried cells were obtained from culture grown in ISP 2 broth on a rotary shaker at 27 °C for 7 days. The isomer of diaminopimelic acid in the cell wall and sugar in whole-cell hydrolysate were determined by thin-layer chromatography (TLC) according to the method of Hasegawa *et al.* [27] and Stanck and Roberts *et al.* [28], respectively. The *N*-acyl type of muramic acid in the peptidoglycan was examined using the method of Uchida and Aida [29]. Mycolic acids was detected by TLC following the procedure of Tomiyasu [30]. Phospholipids in the cell were extracted and detected by two-dimensional TLC according to the method proposed by Minnikin *et al.* [31]. Menaquinones were extracted and purified by using the method of Collins *et al.* [32] and were analysed by liquid chromatography–mass spectrometry (model JSM-T100LP; JEOL) with a Capcell Pak C18 UG 120 column (Shiseido) at 210 nm. Methyl esters of cellular fatty acids were extracted and analysed by gas chromatography (model 6850 series II; Agilent) with an HP Ultra 2 column (Agilent) according to the instructions for the Sherlock Microbial Identification System (version 6.1; MIDI), using the RTSBA6 library for identification of fatty acids [33]. The genomic DNA G+C content was determined by following the procedure of Tamaoka and Komagata [34], and was analysed by high-performance liquid chromatography (model L-2455, Hitachi) with a Capcell Pak C18 UG 120 column (Shiseido) at 270 nm.

Genomic DNA of strain GKU 128^T was extracted from a culture grown on MS agar at 28 °C for 7 days according to the method of Kieser *et al.* [35]. Conditions of PCR amplification of the 16S rRNA gene, purification and sequencing were carried out as described previously [18]. The 16S rRNA gene sequence of strain GKU 128^T was compared with corresponding sequences of reference type strains via the EzBioCloud server [36], and the value of pairwise sequence similarities were achieved. The program CLUSTAL_X version 2.0 [37] was used to align the sequences of strain GKU 128^T together with the related taxa retrieved from the GenBank/EMBL/DDBI database. Phylogenetic trees were reconstructed using the neighbour-joining [38], maximum-likelihood [39] and maximum-parsimony [40] algorithms by using MEGA software version 7.0 [41]. A distance matrix of the neighbour-joining and maximum-likelihood trees were generated using Kimura's two parameter model [42]. In addition, nearest-neighbour-interchange was used for the heuristic method for a maximum-likelihood tree. Likewise,

subtree pruning-regrafting method (number of initial trees, 10; MP search level, 1; maximum of number trees to retain, 100) was carried out for the maximum-parsimony tree. All positions containing gaps and missing data were eliminated from the dataset. The topology of the phylogenetic trees was evaluated by bootstrap analysis [43] based on 1000 resampled datasets.

Strain GKU 128^T showed good growth on NA, CZA, ISP 3, ISP 4, ISP 6 and ISP 7 media and moderate growth on ISP 2 and ISP 5. Aerial and substrate mycelia were well developed. The substrate mycelium was yellowish brown, greyish yellow brown to brownish grey (ISP 2, ISP 4, ISP 6 and NA), greyish olive to olive black (CZA and ISP 3), dusty yellow (ISP 5) and oyster white (ISP 7). The aerial myceluim was white (ISP 3, ISP 5, ISP 7 and NA), light brownish grey (ISP 2) and pale orange (ISP 4), except on ISP 6 and CZA in which it was not produced. Antique gold pigment could be observed on ISP 2 medium (Tables 1 and S1, available in the online version of this article). Strain GKU 128^T formed an extensively branched, non-fragmenting substrate and aerial mycelia. The aerial mycelium was differentiated into straight to flexuous chains of spores (six or more spores per chain). Each spore was oval to cylindrical (0.6×0.8 µm) with a smooth surface, and non-motile (Fig. 1). Phenotypic characterization revealed that strain GKU 128^T grew at 14–38 °C (optimal 24–32 °C), at pH range 7.0–8.0 (optimal pH 7.0) and at 0–5 % (w/v) NaCl (optimal 0–1 %). Other physiological and biochemical properties are shown in the species description. Furthermore, strain GKU 128^T exhibited different phenotypic characteristics from the reference type strains, *A. nitritigenes* NBRC 15918^T and *A. fibrosa* JCM 9371^T (Table 1).

Strain GKU 128^T revealed chemical markers typical of members of the genus *Actinomadura*. The cell wall contained *meso*-diaminopimelic acid as the diagnostic peptidoglycan diamino acid and the whole-cell hydrolysate was found to contain madurose as the diagnostic sugar, along with glucose, mannose, rhamnose and ribose, showing that it possessed cell-wall type III [44] and whole-cell sugar pattern B [45]. The *N*-acyl type of muramic acid in the peptidoglycan was acetyl. Mycolic acids were absent. The diagnostic phospholipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositolmannoside and four unknown phospholipids (Fig. S1), which correspond to phospholipid type PI [46]. The predominant menaquinone was MK-9(H_6) (67.6 %), with small amounts of MK-9(H_8) (17.6 %), MK-9(H_0) (12.8 %) and MK-9(H_4) (2.0 %). The major fatty acids were $C_{16:0}$ (44.8 %), $C_{18:1}\omega9c$ (21.5 %) and 10-methyl $C_{18:0}$ (tuberculostearic acid; 9.5 %) with minor amounts of $C_{18:0}$ (5.0 %), $C_{14:0}$ (4.8 %), summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$; 4.7 %), $C_{17:0}$ (2.3 %), $C_{17:1}\omega8c$ (1.2 %), $C_{16:1}\omega9c$ (1.1 %), which corresponded to fatty acid type 3a [47]. The major fatty acid profile of the strain was similar to those of *A. nitritigenes* NBRC 15918^T and *A. fibrosa* JCM 9371^T [10, 48, 49]. The genomic DNA G+C content was 70.5 mol%.

Table 1. Differential of the phenotypic characteristics of strain GKU 128^T and the closely related species of the genus *Actinomadura*

Strains: 1, GKU 128^T; 2, *A. nitritigenes* NBRC 15918^T; 3, *A. fibrosa* JCM 9371^T. +, Positive; –, negative; w, weakly positive. All data were determined in this study.

Characteristic	1	2	3
Colony characteristics on ISP 2 medium:			
Growth	Moderate	Abundant	Abundant
Aerial mycelium	Light brownish grey	White	White
Substrate mycelium	Yellowish brown	Greyish yellow brown	Pale yellow brown
Soluble pigment	Antique gold	None	None
Growth at pH 8.0	+	+	–
Growth at 5 % (w/v) NaCl	+	–	–
Acid production from:			
Adonitol	–	+	–
L-Arabinose	+	–	–
D-Mannitol	+	–	–
Sucrose	w	–	–
Decomposition of:			
Adenine	+	–	–
Starch	+	–	+
L-Tyrosine	+	–	–
Urea	–	+	–
Nitrate reduction	+	+	–
Gelatin liquefaction	+	–	–
Carbon utilization of:			
Adonitol	+	–	–
L-Arabinose	+	+	–
D-(-)-Fructose	+	+	–
Glycerol	+	+	–
myo-Inositol	+	+	–
Maltose	+	–	–
D-Mannitol	+	+	–
D-Mannose	+	+	–
Raffinose	+	+	–
L-Rhamnose	+	+	–
D-Xylose	–	+	+
Nitrogen utilization of:			
Potassium nitrate	+	–	–
L-Proline	+	–	+
L-Valine	+	–	–
Enzyme activities of:			
α-Galactosidase	w	–	–
α-Mannosidase	+	w	–

The almost-complete 16S rRNA gene sequence (1497 nt) of strain GKU 128^T was determined and compared with available sequences in public database. The results indicated that strain GKU 128^T was a member of the genus *Actinomadura*

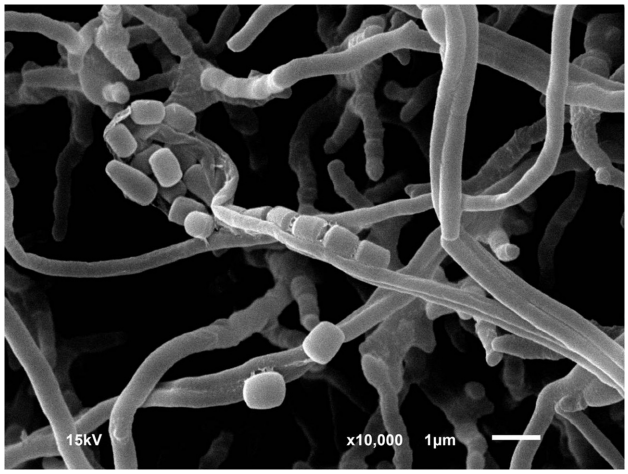

Fig. 1. Scanning electron micrographs of straight to flexuous chains of spores with a smooth surface ornament of strain GKU 128^T grown on ISP 2 medium at 28 °C for 7 weeks. Bar, 1 µm.

[1]. It shared high 16S rRNA gene sequence similarity with *A. nitritigenes* NBRC 15918^T (99.2 %), *Actinomadura montaniterrae* CYP1-1B^T (98.8 %), *A. fibrosa* JCM 9371^T and *Actinomadura hibisca* NBRC 15177^T (98.7 %). Lower levels of 16S rRNA gene sequence similarity (<98.5 %) were found to the type strains of all other recognized species of the genus *Actinomadura*. The neighbour-joining and maximum-likelihood trees indicated that strain GKU 128^T was most closely related to *A. nitritigenes* NBRC 15918^T and *A. fibrosa* JCM 9371^T (Figs 2 and S2). However, the maximum-parsimony tree showed that strain GKU 128^T comprised a separate branch from *A. nitritigenes* NBRC 15918^T and *A. fibrosa* JCM 9371^T within the genus *Actinomadura* (Fig. S3).

To confirm the taxonomic position of strain GKU 128^T, DNA–DNA hybridization studies were fluorometrically determined using photobiotin-labelled DNA probes [50] by a microplate reader (model SH-9000, Corona Electric). Hybridization was performed at 50 °C with eight replications for each sample and the DNA–DNA relatedness values were expressed as the means of two independent determinations. Level of DNA–DNA relatedness between strain GKU 128^T and the closely related species, *A. nitritigenes* NBRC 15918^T and *A. fibrosa* JCM 9371^T, were 18.4 ±4.1 and 13.7±4.3 %, respectively. These values are well below the 70 % cut-off point recommended for the definition of bacterial species [51].

The phenotypic and genotypic characteristics of strain GKU 128^T clearly distinguished it from its closest phylogenetic neighbours (Tables 1 and S1). Phylogenetic analyses indicated that strain GKU 128^T was most closely related to *A. nitritigenes* NBRC 15918^T, which was supported by the neighbour-joining and maximum-likelihood trees. However, strain GKU 128^T possesses straight to flexuous chain of spores with smooth a surface ornament, which is different to *A. nitritigenes* NBRC 15918^T that possesses straight to hook-like chain of spores with a warty surface ornament [48]. Based on the results of the polyphasic taxonomic study, strain GKU 128^T represents the novel species of the genus *Actinomadura*, for which the name *Actinomadura barringtoniae* sp. nov. is proposed.

DESCRIPTION OF *ACTINOMADURA BARRINGTONIAE* SP. NOV.

Actinomadura barringtoniae (bar.ring.to′ni.ae. N.L. gen. n. *barringtoniae* of *Barringtonia acutangula* (L.) Gaertn., the Indian oak tree from which the type strain was isolated).

Cells are aerobic, Gram-stain-positive, non-motile actinomycetes, which form extensively branched substrate and aerial mycelia. Abundant aerial mycelia are produced on ISP 2, ISP 4, ISP 5 and ISP 7 media and differentiate into straight to flexuous chains of six or more spores. The spores are smooth and oval to cylindrical in shaped (0.6×0.8 µm in size). Antique gold pigment is produced on ISP 2 medium. The optimum temperature and pH for growth is 24–32 °C and 7.0, respectively. NaCl tolerance is up to 5 % (w/v). Catalase activity, gelatin liquefaction, nitrate reduction and oxidase activity are positive, but H_2S production, melanin pigment, milk coagulation and peptonization are negative. Acid is produced from L-arabinose, D-mannitol and sucrose, but not from adonitol, D-(-)-fructose, D-glucose, glycerol, maltose, D-mannose, raffinose, L-rhamnose, D-sorbitol and D-xylose. Adenine, casein, hypoxanthine, starch, Tween 20, Tween 80 and L-tyrosine are degraded, but citrate, urea and xanthine are not. Adonitol, L-arabinose, D-(-)-fructose, D-glucose, glycerol, maltose, D-mannitol, D-mannose, *myo*-inositol, raffinose, L-rhamnose, sucrose and trehalose are utilized as a sole carbon source, but D-sorbitol and D-xylose are not. L-Arginine, L-asparagine, L-cysteine, L-histidine, potassium nitrate, L-proline and L-valine are utilized as a sole nitrogen source. In the API ZYM system, positive for *N*-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, α-chymotrypsin, cystine arylamidase, esterase (C 4), esterase lipase (C 8), β-glucosidase, leucine arylamidase, α-mannosidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase, while weakly positive for α-galactosidase, β-galactosidase, α-glucosidase and trypsin, but negative for α-fucosidase, β-glucuronidase and lipase (C 4). The cell-wall peptidoglycan contains *meso*-diaminopimelic acid and the whole-cell sugars are glucose, madurose, mannose, rhamnose and ribose. The *N*-acyl type of muramic acid is acetyl. Mycolic acids are absent. The major phospholipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylinositolmannoside. The predominant menaquinone is MK-9(H_6), with minor amounts of MK-9(H_8), MK-9(H_0) and MK-9(H_4). The major fatty acids are $C_{16:0}$, $C_{18:1}\omega 9c$ and 10-methyl $C_{18:0}$ (tuberculostearic acid). The genomic DNA G+C content is 70.5 mol%.

The type strain, GKU 128^T (=TBRC 7225^T=NBRC 113074^T), was isolated from the root of an Indian oak

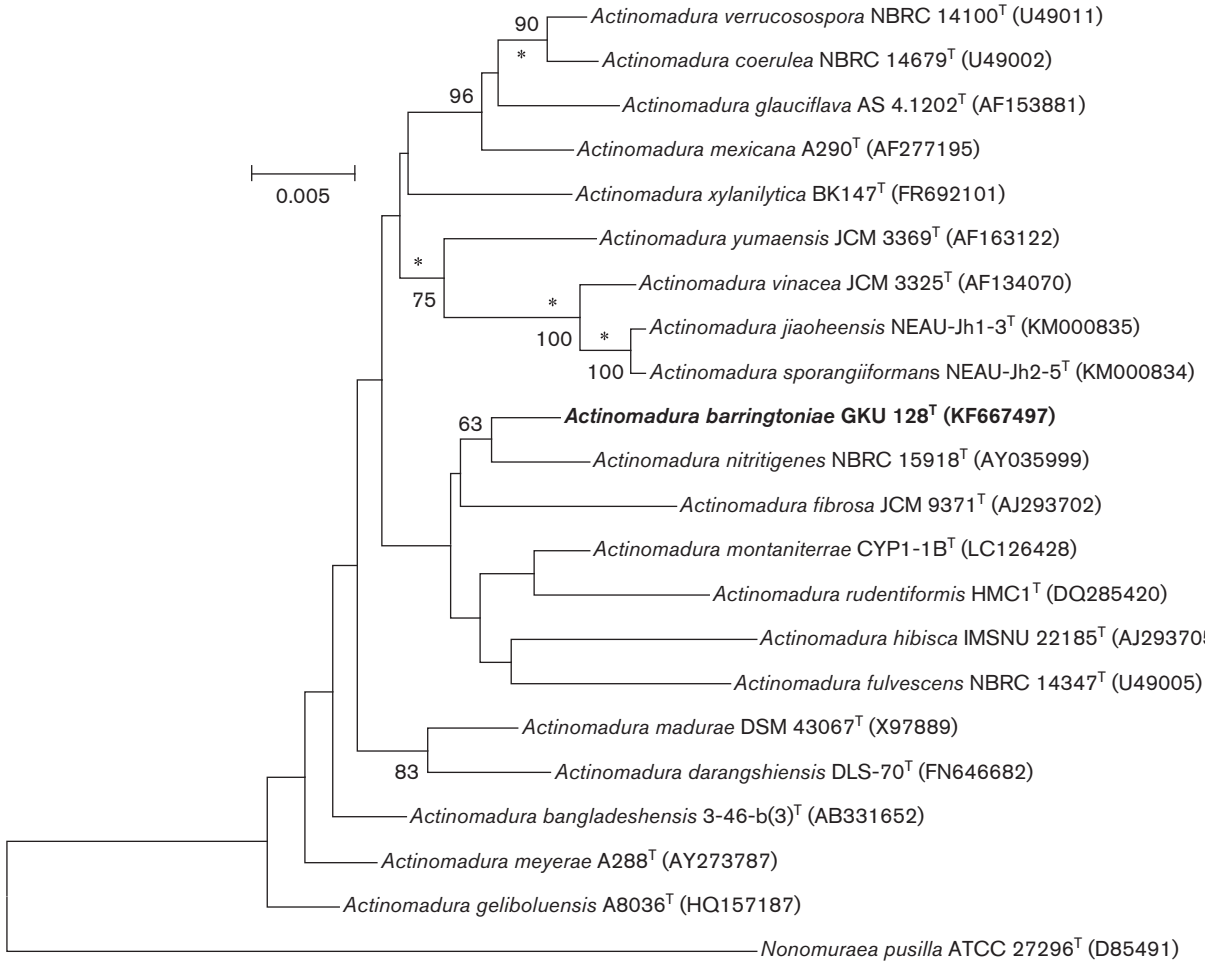

Fig. 2. Neighbour-joining tree based on almost-complete 16S rRNA gene sequences (1497 nt) showing the relationship between strain GKU 128^T and some related species of the genus *Actinomadura*. *Nonomuraea pusilla* ATCC 27296 ^{T} (GenBank accession no. D85491) was used as an outgroup. Asterisks denote branches that were also recovered in the maximum-likelihood and maximum-parsimony trees. Numbers at branch points indicate bootstrap percentages (based on 1000 replications); only values >50 % are shown. Bar, 0.005 substitutions per nucleotide position.

tree [*Barringtonia acutangula* (L.) Gaertn.] collected at Khao Khitchakut district, Chantaburi province, Thailand.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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Molecular interaction of 1-aminocyclopropane-1-carboxylate deaminase (ACCD)-producing endophytic *Streptomyces* sp. GMKU 336 towards salt-stress resistance of *Oryza sativa* L. cv. KDML105

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1-aminocyclopropane-1-carboxylate deaminase (ACCD)-producing endophytic *Streptomyces* sp. GMKU 336 and its ACCD-deficient mutant were inoculated into Thai jasmine rice Khao Dok Mali 105 cultivar (*Oryza sativa* L. cv. KDML105) under salt stress (150 mM NaCl) conditions. The results clearly indicated that *Streptomyces* sp. GMKU 336 significantly increased plant growth, chlorophyll, proline, K^+, Ca^+, and water contents; but decreased ethylene, reactive oxygen species (ROS), Na^+, and Na^+/K^+ ratio when compared to plants not inoculated and those inoculated with the ACCD-deficient mutant. Expression profiles of stress responsive genes in rice in association with strain GMKU 336 were correlated to plant physiological characteristics. Genes involved in the ethylene pathway, *ACO1* and *EREBP1*, were significantly down-regulated; while *acdS* encoding ACCD in *Streptomyces* sp. GMKU 336 was up-regulated *in vivo*. Furthermore, genes involved in osmotic balance (*BADH1*), Na^+ transporters (*NHX1* and *SOS1*), calmodulin (*Cam1-1*), and antioxidant enzymes (*CuZn-SOD1* and *CATb*) were up-regulated; whereas, a gene implicated in a signaling cascade, *MAPK5*, was down-regulated. This work demonstrates the first time that ACCD-producing *Streptomyces* sp. GMKU 336 enhances growth of rice and increases salt tolerance by reduction of ethylene via the action of ACCD and further assists plants to scavenge ROS, balance ion content and osmotic pressure.

Salinity is one of the major environment stress factors that reduces plant cell division, growth and productivity. Recently, plant growth-promoting (PGP) bacteria have been identified that enhance tolerance to salinity by plants, particularly bacteria associated with the plants[1]. Endophytic actinomycetes are of special interest because they not only produce various bioactive secondary metabolites to protect plants from infectious diseases[2], but they also show ability to enhance plant growth by carrying several PGP traits including production of siderophores to capture iron, production of plant hormones such as auxins and cytokinins, solubilization of phosphate and other minerals to supply nutrients[3,4]. Moreover, they facilitate plant growth under stress caused by drought, heavy metals, flooding and high salt by reducing stress associated with ethylene via production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase[5,6].

Ethylene has long been recognized as a hormone that controls plant responses to growth limiting conditions. Under stress conditions, the ethylene level is increased via the ethylene pathway that transforms the precursor

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ACC into the final product, ethylene[1]. A diverse group of endophytic PGP bacteria is able to reduce plant ethylene levels by the action of ACC deaminase (ACCD). ACCD (encoded by the *acdS* gene) converts ACC in plants to ammonia and α-ketobutyrate, which the bacteria consume as nitrogen sources[7]. Therefore, ACCD-producing bacteria stimulate plant ACC efflux and subsequently decrease ACC concentration and ethylene production[1]. The consequence of this interaction is an increase in root/shoot elongation and protection of the plant from the inhibitory effects of ethylene[8]. Thus, plants associated with endophytic ACCD-producing bacteria become more resistant to stress.

So far, the role and interaction of ACCD-producing endophytic actinomycetes to promote plant growth under salt stress has been less studied. This research therefore focused on ACCD-producing endophytic *Streptomyces* sp. GMKU 336 and its ability to enhance the growth of rice under salt stress conditions. An ACCD-deficient mutant of *Streptomyces* sp. GMKU 336 was constructed and compared with the effect of growth promotion of rice with the wild type under salt stress *in situ*. This work demonstrated that strain GMKU 336 increases salt tolerance of salt-sensitive Thai jasmine rice Khao Dok Mali 105 cultivar (KDML105). Expression profiles of stress responsive genes of rice associated with strain GMKU 336 are demonstrated and the impact of the interaction is discussed. Understanding of such interaction will lead to sustainably utilize ACCD-producing endophytic actinomycetes to enhance growth and salt tolerance in rice growing in saline soil.

Results

Characterization of plant growth promoting traits of *Streptomyces* sp. GMKU 336. As part of a program to discover PGP endophytic actinomycetes from medicinal plants, *Streptomyces* sp. GMKU 336 was recovered from the roots of *Clerodendrum serratum* (L.) Moon[9]. The strain is most closely related to *Streptomyces hydrogenans* NBRC 13475 ^{T}, with 99.86% identity based on 16S rDNA gene sequence analysis (GenBank accession number KR870352). Screening of plant growth promoting (PGP) traits of strain GMKU 336 revealed characteristics of phosphate solubilization, siderophore production, 1-aminocyclopropane-1-carboxylate deaminase (ACCD) activity but no indole-3-acetic acid (IAA) production (data not shown). Strain GMKU 336 shows moderate halophilic type as it tolerates NaCl up to 6% (w/v).

Construction of ACCD-deficient mutant. *Streptomyces* sp. GMKU 336 displayed ACCD activity at 2.85 ± 0.15 μmol α-ketobutyrate mg protein^{-1} h^{-1}. Furthermore, the expression profile of the ACCD gene (*acdS*) by semi-quantitative RT-PCR revealed high expression when bacteria were consuming ACC as a sole nitrogen source (data not shown). An ACCD-deficient mutant of strain GMKU 336 was then constructed by insertional inactivation of *acdS* (GenBank accession number KT000002). The mutant constructed showed no ACCD activity and the disruption was verified by PCR analysis (Supplementary Fig. S1). The mutant was stable up to five generation of growth without thiostrepton selection. The mutant was reverted to wild type by further selection without the antibiotic up to ten generations. The revertant showed the same ACCD activity and all other properties as wild type (data not shown).

Effect of ACCD-producing *Streptomyces* sp. GMKU 336 inoculated rice plants under salt stress. *Streptomyces* sp. GMKU 336 and its ACCD-deficient mutant were inoculated into KDML105. The growth parameters were observed after 7 days of treatment with 150 mM NaCl under hydroponic conditions and compared with non-salt treatment (Supplementary Fig. S2 and Supplementary Table S1). Re-isolation of wild type and mutant from both salt and non-salt treatments was about 10^4 CFU g root fresh weight^{-1} (Supplementary Table S1). Both strains were confirmed by 16S rRNA gene sequencing and PCR analysis (data not shown). In addition, un-inoculated plants were shown not to harbor *Streptomyces* sp. GMKU 336 as well as other endophytic actinomycetes. The results indicated that the surface sterilized protocol of rice seeds was successful and the hydroponic condition used in this study are free from contamination.

Under non-salt conditions, ACCD-producing *Streptomyces* sp. GMKU 336 slightly enhanced plant elongation compared to un-inoculated controls (Fig. 1a and b), but significantly increased plant biomass (1.2–1.6 fold) including shoot/root fresh and dry weights (Fig. 1c–f). Rice inoculated with the ACCD-deficient mutant showed similar plant growth parameters to those of un-inoculated controls (Fig. 1). Under salt-stress conditions, all growth parameters were significantly reduced when compared to the non-salt treatments (Fig. 1). Therefore, strain GMKU 336 was able to promote growth of KDML105 with or without salt treatment.

A symptom of salt toxicity in the KDML105 was evaluated using the standard scoring protocol[10]. Under salt treatment, complete cessation of growth was scored as 'susceptible' in un-inoculated plants and those inoculated with the ACCD-deficient mutant, whereas nearly normal growth was scored as 'tolerant' in plants inoculated with strain GMKU 336 (Supplementary Fig. S2 and Supplementary Table S1).

Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on plant physiology. Indices of plant physiology including chlorophyll content, ethylene production, relative water content, and proline content revealed constant level of each parameters of all treatments of non-salt stressed plants (Fig. 2). Under salt-stress conditions, a significant decrease in chlorophyll content of rice was observed in all treatments, compared to those grown in non-salt conditions. However, the KDML105 inoculated with *Streptomyces* sp. GMKU 336 had a significantly higher (1.7-fold) chlorophyll content compared to that of an un-inoculated control (Fig. 2a, Supplementary Table S1). Significant induction of ethylene production (2-fold) was observed in un-inoculated plants and those inoculated with the ACCD-deficient mutant, compared to those of non-salt treatment (Fig. 2b, Supplementary Table S1). Remarkably and in contrast to that of non-salt treatment, KDML105 inoculated with strain GMKU 336 had no increment in ethylene level (Fig. 2b). The water content of all treatments was drastically reduced after exposure to salt stress. Nevertheless, plants inoculated with strain GMKU 336 accumulated water significantly more than the un-inoculated control (Fig. 2c). Furthermore, proline content was increased in all salt-stressed

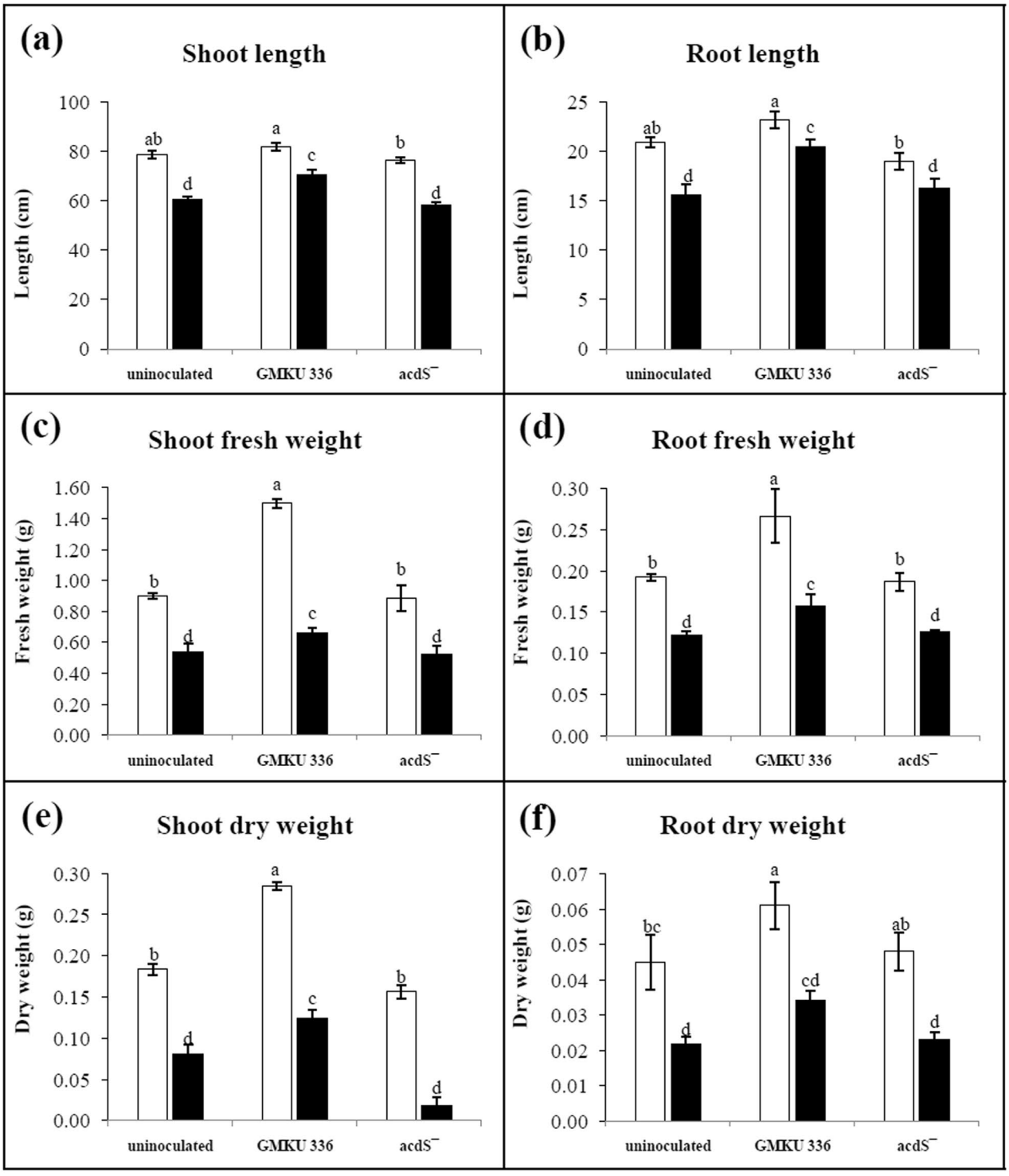

Figure 1. Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on growth of *Oryza sativa* L. cv. KDML105 after 7 days of salt stress under hydroponic conditions. (**a**) Shoot length; (**b**) root length, (**c**) shoot fresh weight; (**d**) root fresh weight; (**e**) shoot dry weight; (**f**) root dry weight. Values are mean of three replicates ± standard error of mean. Different letters indicated statistical differences between treatments (Duncan's test, $P < 0.05$). Uninoculated, plants without bacteria inoculation; GMKU 336, plants inoculated with *Streptomyces* sp. GMKU 336; acdS⁻, plants inoculated with ACCD-deficient mutant; white bar, non-salt treatment; black bar, salt treatment (150 mM NaCl).

plants, but those inoculated with strain GMKU 336 had significantly higher proline content than un-inoculated controls (Fig. 2d). The results suggested that strain GMKU 336 has a positive effect on the physiology of rice to tolerate salinity.

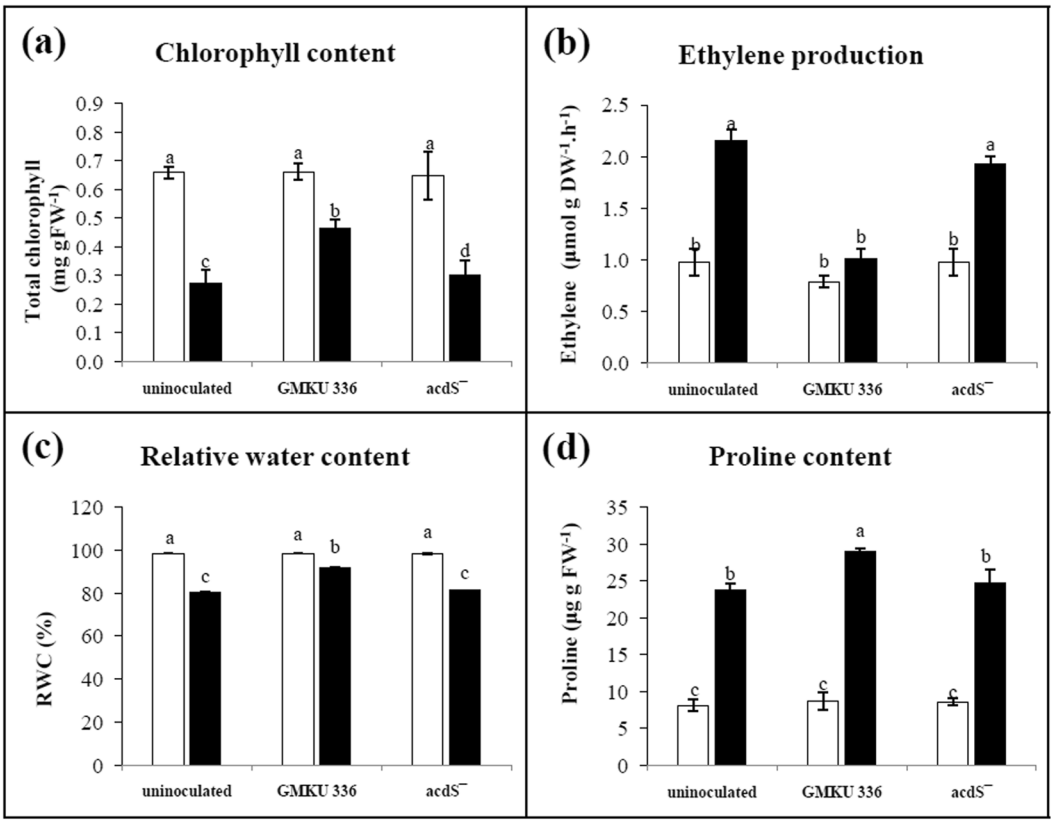

Figure 2. Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on plant physiology of *Oryza sativa* L. cv. KDML105. (**a**) Chlorophyll content; (**b**) ethylene production; (**c**) relative water content; (**d**) proline content. Values are mean of three replicates ± standard error of mean. Different letters indicated statistically differences between treatments (Duncan's test, $P < 0.05$). Uninoculated, plants without bacteria inoculation; GMKU 336, plants inoculated with *Streptomyces* sp. GMKU 336; acdS^{-}, plants inoculated with ACCD-deficient mutant; white bar, non-salt treatment; black bar, salt treatment (150 mM NaCl).

Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on plant ion content. Under normal conditions, the ion content of KDML105, specifically Na^+, K^+, and Ca^{2+}, was at the same level in all treatments (Fig. 3). Under salt-stress conditions, the Na^+ content of all plant treatments was significantly increased (Fig. 3a). Un-inoculated plants and those inoculated with the ACCD-deficient mutant had a Na^+ content nearly 10-fold higher than plants with the corresponding non-salt treatment, whereas plants inoculated with *Streptomyces* sp. GMKU 336 had a Na^+ content that was only 6-fold higher than the non-salt control (Fig. 3a, Supplementary Table S1). By contrast, the K^+ content of all plant treatments was significantly decreased under salt-stress conditions (Fig. 3b). However, plants inoculated with strain GMKU 336 had a smaller decrease in K^+ content compared to un-inoculated plants and those inoculated with the ACCD-deficient mutant (Fig. 3b). Taken together, there was a 13-fold increment in Na^+/K^+ ratio for un-inoculated plants and those inoculated with the ACCD-deficient mutant when compared to the non-salt treatment. On the other hand, the increment in Na^+/K^+ ratio was only 6-fold in plants inoculated with strain GMKU 336 (Fig. 3c, Supplementary Table S1). Salt stress also caused a decrease in Ca^{2+} content in all plant treatments (Fig. 3d). However, plants inoculated with strain GMKU 336 had significantly less reduction in Ca^{2+} content than un-inoculated plants or those inoculated with the ACCD-deficient mutant (Fig. 3d). The results implied that strain GMKU 336 helps maintain ion balance and, thus, increases salt tolerance in KDML105.

Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on reactive oxygen species (ROS). A significant increase of lipid peroxidation determined by estimating production of malondialdehyde (MDA) content was observed in all plant treatments exposed to salt (Fig. 4a). Un-inoculated plants and those inoculated with the ACCD-deficient mutant accumulated MDA nearly 2-fold higher than the non-salt treatments. By contrast, plants inoculated with *Streptomyces* sp. GMKU 336 had a MDA content less than half of both treatments (Fig. 4a, Supplementary Table S1). ROS were detected in leaves by staining with nitrobluetrazolium (NBT) (Fig. 4b) and 3,3′-diaminobenzidine (DAB) (Fig. 4c), which indicate the presence of superoxide and hydrogen peroxide, respectively. In the presence of salt, leaves had higher staining indicative of both ROS species; however, plants inoculated with strain GMKU 336 showed less staining than the other treatments (Fig. 4b and c).The results suggested that strain GMKU 336 reduces ROS in salt-stressed rice.

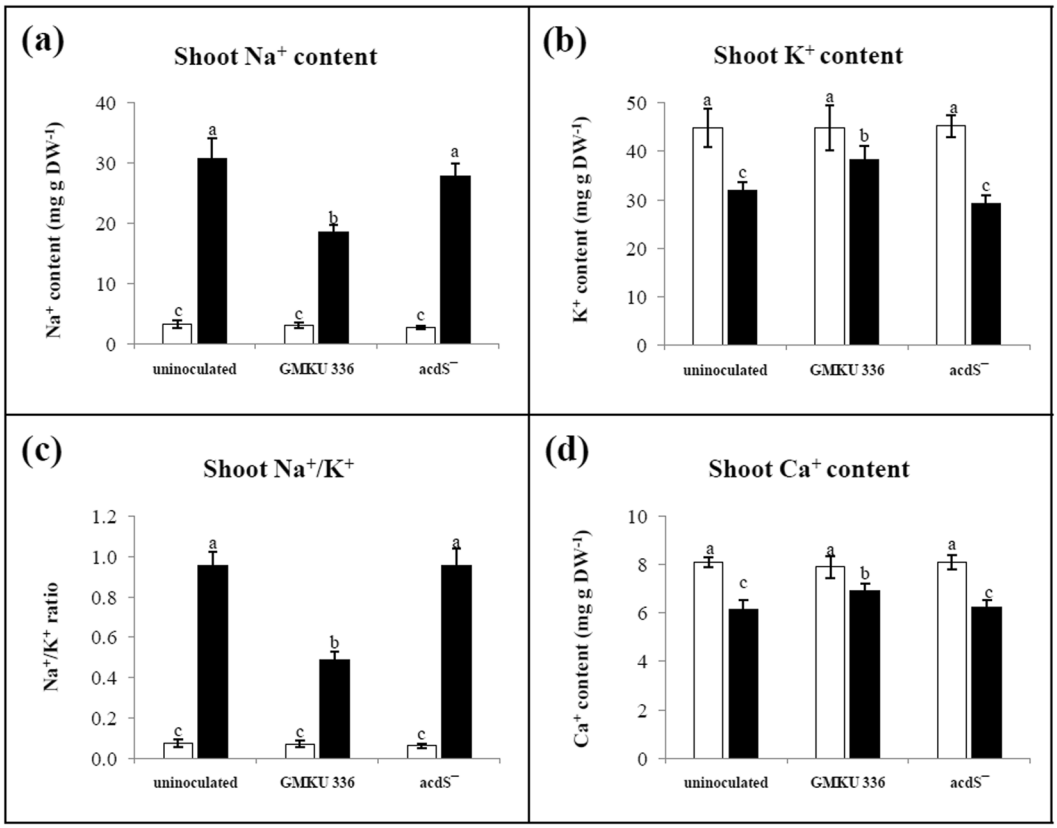

Figure 3. Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on ion content of shoots of *Oryza sativa* L. cv. KDML105. (**a**) Na^+ content; (**b**) K^+ content; (**c**) Na^+/K^+ ratio; (**d**) Ca^{2+} content. Values are mean of three replicates ± standard error of mean. Different letters indicated statistically significant differences between treatments (Duncan's test, $P < 0.05$). Uninoculated, plants without bacteria inoculation; GMKU 336, plants inoculated with *Streptomyces* sp. GMKU 336; acdS$^-$, plants inoculated with ACCD-deficient mutant; white bar, non-salt treatment; black bar, salt treatment (150 mM NaCl).

Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on expression profile of genes involved in the ethylene pathway. As KDML105 inoculated with *Streptomyces* sp. GMKU 336 maintained the same level of ethylene either with or without salt treatment similar to those un-inoculated plants and those inoculated with the ACCD-deficient mutant under non-salt condition (Fig. 2b), gene expression patterns of ACC synthase (*ACS1*), ACC oxidase (*ACO1*) and the ethylene responsive element binding protein (*EREBP1*) were investigated by real-time PCR. All three genes were expressed at the same basal level in all plant treatments under non-salt conditions (Fig. 5a–c). The expression level of *ACS1* was about 5-fold up-regulated in all salt-stressed plants compared to the corresponding non-salt controls (Fig. 5a). The expression profiles of *ACO1* and *EREBP1* in un-inoculated plants and those inoculated with the ACCD-deficient mutant were about 3–4 fold higher than the non-salt treatments, whereas plants inoculated with strain GMKU 336 had 1-fold lower expression (Fig. 5b, Supplementary Table S2). Moreover, expression of the *acdS* gene encoding ACCD in strain GMKU 336 was only detected *in vivo* within salt-stressed rice (Fig. 5d). The results indicated that strain GMKU 336 reduces ethylene content through the action of ACCD and consequently down-regulated *ACO1* and *EREBP1* genes in rice.

Effect of ACCD-producing *Streptomyces* sp. GMKU 336 on expression of salt-stress responsive genes. Eight candidate genes of rice encoded proteins involved in the salt stress response were transcriptionally analyzed in un-inoculated plants and those inoculated with wild type or mutant bacteria, under salt and non-salt conditions. The genes were: *salT* (salt stress responsive protein)[11], *BADH1* (betaine aldehyde dehydrogenase)[12], *NHX1* (Na^+/H^+ antiporter)[13], *SOS1* (salt overlay sensitive 1 protein)[13], *Cam1-1* (calmodulin)[14], *MAPK5* (mitogen activated protein kinase 5)[12], *CuZn-SOD1* (superoxide dismutase)[15], and *CATb* (catalase)[12]. Expression profiles of all genes were observed at similar basal level in plants grown under non-salt conditions (Fig. 6). When KDML105 were exposed to salt, the salt-induced positive control gene, *salT*, was up-regulated to a similar level in all treatments (Fig. 6a). The expression profile of *BADH1*, involved in competition of solute production, was up-regulated in all salt-stressed plants. However and significantly, it was 4-fold higher in plants inoculated with *Streptomyces* sp. GMKU 336 compared to un-inoculated plants and those inoculated with the ACCD-deficient mutant (Fig. 6b, Supplementary Table S2).

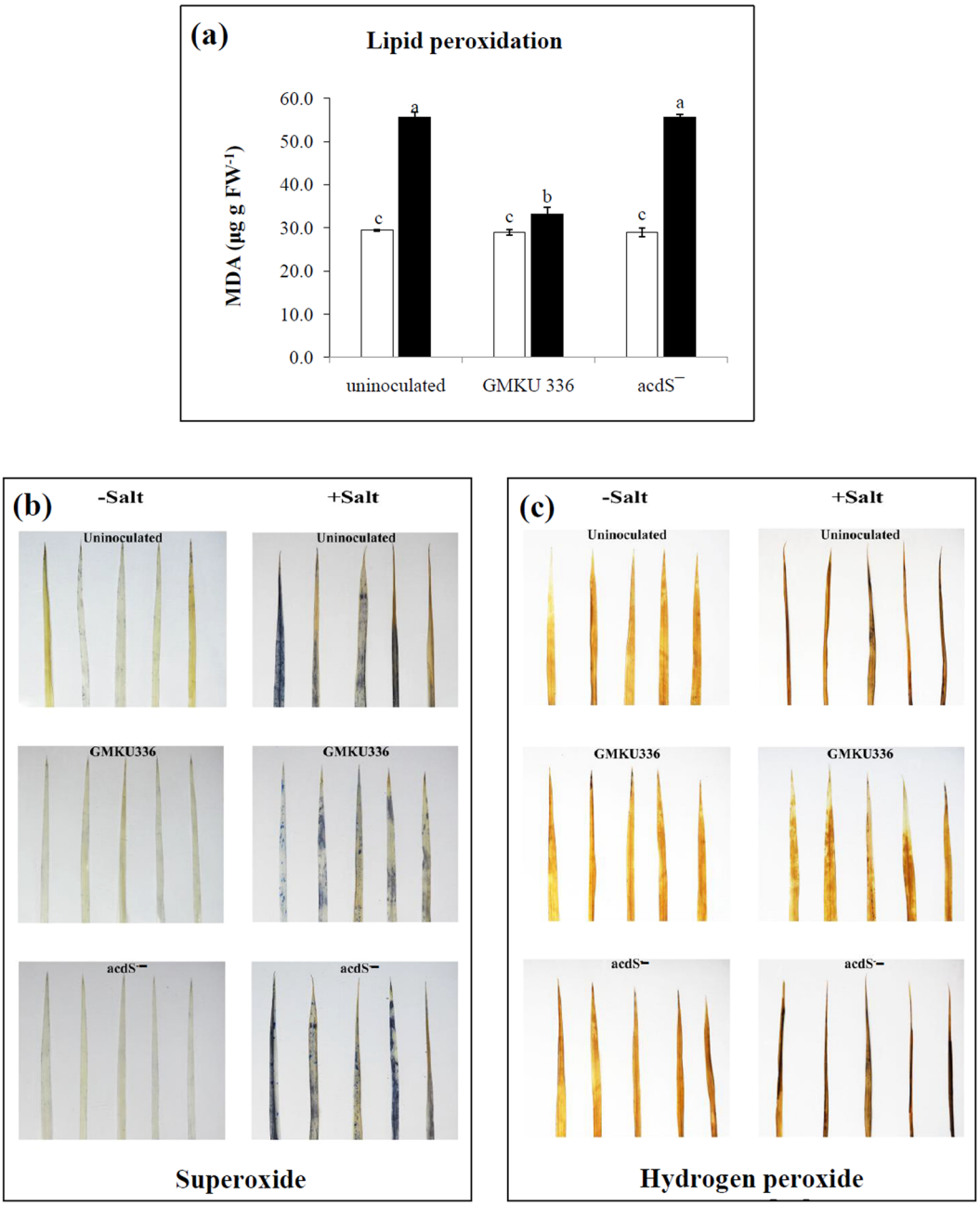

Figure 4. Effect of ACCD-producing *Streptomyces* sp. GMKU 336 in reactive oxygen species (ROS) in *Oryza sativa* L. cv. KDML105. (**a**) lipid peroxidation; (**b**) superoxide by NBT staining; (**c**) hydrogen peroxide by DAB staining. Values are mean of three replicates ± standard error of mean. Different letters indicated statistical differences between treatments (Duncan's test, $P < 0.05$). Uninoculated, plants without bacteria inoculation; GMKU 336, plants inoculated with *Streptomyces* sp. GMKU 336; acdS^{-}, plants inoculated with ACCD-deficient mutant; white bar, non-salt treatment; black bar, salt treatment (150 mM NaCl).

Genes involved in Na^+ transport, specifically a member of vacuole Na^+/H^+ transporters (*NHX1*) and salt overlay sensitive 1 protein (*SOS1*), were significantly (1.6–2.3 fold) up-regulated in salt-stressed un-inoculated plants and those inoculated with the ACCD-deficient mutant, when compared to the non-salt controls. Rice inoculated with strain GMKU 336 had expression levels that were 3–4 fold higher than control (Fig. 6c and d, Supplementary Table S2). Up-regulation of the *Cam1-1* gene, a Ca^+ sensor involved in plant signaling by calmodulin, was 1.8-fold higher than the non-salt treatment in salt-stressed un-inoculated plants and those inoculated with the ACCD-deficient mutant. However, plants inoculated with strain GMKU 336 had expression levels that were 1-fold higher than both treatments (Fig. 6e, Supplementary Table S2). High expression of the *MAPK5* gene, encoding a kinase protein, was detected in un-inoculated plants and those inoculated with the ACCD-deficient mutant when compared to those of non-salt treatments. By contrast, plants inoculated with strain GMKU 336 had nearly 2-fold expression when compared to its control (Fig. 6f, Supplementary Table S2).

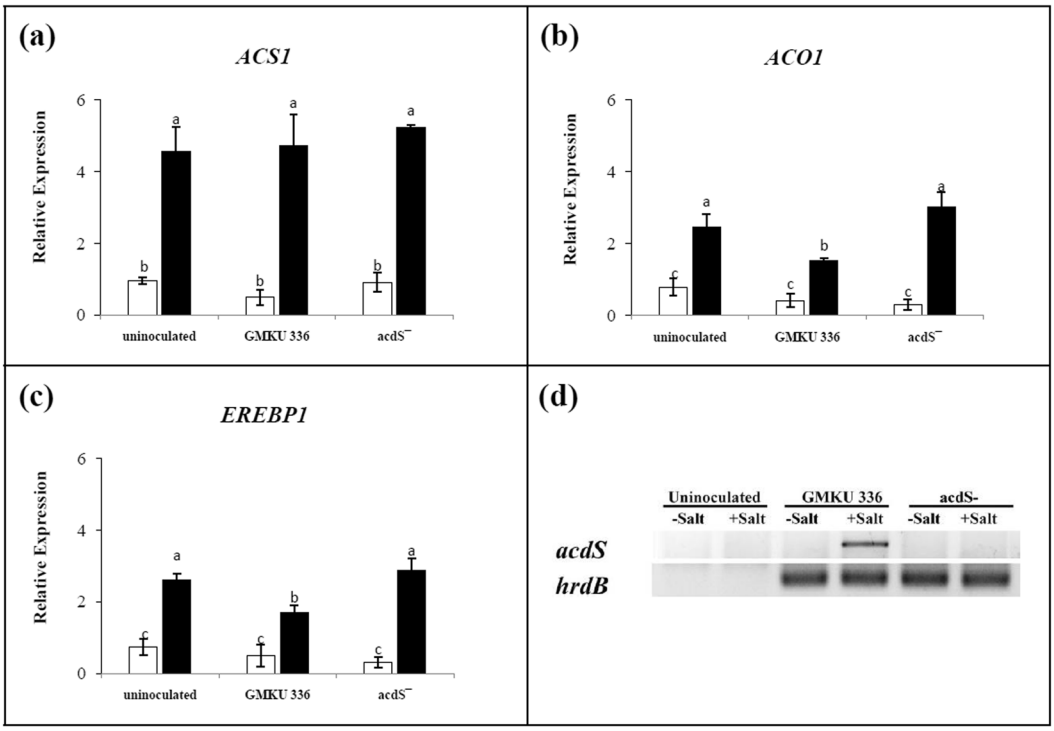

Figure 5. Transcriptional analysis of genes involved in ethylene production in *Oryza sativa* L. cv. KDML105 associated with *Streptomyces* sp. GMKU 336. (**a**) ACC synthase (*ACS1*); (**b**) ACC oxidase (*ACO1*); (**c**) ethylene responsive element binding proteins (*EREBP1*); (**d**) ACCD (*acdS*) of *Streptomyces* sp. GMKU 336. The two gels cropped from different gels (Supplementary Fig. S3). The histogram represents mean of the expression ratio, relative to the actin gene (*act1*). Values are mean of three replicates ± standard error of mean. Different letters indicated statistically-significant differences between treatments (Duncan's test, $P < 0.05$). Uninoculated, plants without bacteria inoculation; GMKU 336, plants inoculated with *Streptomyces* sp. GMKU 336; acdS⁻, plants inoculated with ACCD-deficient mutant; *hrdB*, RNA polymerase principal sigma factor gene of *Streptomyces* sp. GMKU 336; white bar/−Salt, non-salt treatment; black bar/+Salt, salt treatment (150 mM NaCl).

Further analysis of gene expression encoding antioxidant enzymes, superoxide dismutase (*CuZn-SOD1*) and catalase (*CATb*), revealed 2.1–2.3 fold up-regulation in salt-stressed un-inoculated plants and those inoculated with the ACCD-deficient mutant when compared to the non-salt treatments. Significantly, expression levels of plants inoculated with strain GMKU 336 were 3.2–3.7 times higher than the non-salt controls (Fig. 6g and h, Supplementary Table S2). All of these results indicate that strain GMKU 336 has a positive influence to salt stress response gene expression in KDML105 by up-regulation of *BADH1*, *NHX1*, *SOS1*, *Cam1-1*, *CuZn-SOD1*, and *CATb*, and down-regulation of *MAPK5*, and increases salt tolerant in rice as a consequence.

Discussion

Recently, actinomycetes have been reported to promote plant growth as well as alleviate various abiotic stresses including salinity and osmotic stress via the action of 1-aminocyclopropane-1-carboxylate deaminase (ACCD)[5,6]. However, the role of ACCD-producing endophytic actinomycetes in promoting plant growth under stress conditions has not yet been investigated systematically. *Streptomyces* sp. GMKU 336 was used in this study as it displayed highest ACCD activity amongst other strains in the screening program[9] and showed halophilic property which is suitable to investigate the *in vivo* molecular interactions of this strain in partnership with the salt-sensitive Thai jasmine rice Khao Dok Mali 105 cultivar (KDML105) under salt-stress conditions. In addition, strain GMKU 336 revealed endophytic ability in rice although it was isolated from medicinal plant. The result was in agreement with previous report that endophytic *Streptomyces* isolated from one plant species could mutually resided in the other different plant species by *in vitro* inoculation[3]. In this work, an ACCD-deficient mutant of strain GMKU 336 was constructed by insertional inactivation of the *acdS* gene to define definitively the role of ACCD on plant growth and salt tolerance.

Under non-salt conditions, KDML105 inoculated with ACCD-producing *Streptomyces* sp. GMKU 336 had significantly enhanced shoot and root biomass but not obviously extended shoot and root lengths. This might be due to the lack of IAA production of this strain that would not encourage the elongation of plants. However, the results for plant growth were consistent with previous work showing that ACCD-producing *Streptomyces* have an ability to enhance growth of tomato[5], *Arabidopsis*[16], halophytic *Limonium sinense*[17] and sugarcane[4]. The growth effect has also been found in other bacteria such as ACCD-producing *Pseudomonas*[18], *Enterobacter*[19], and *Bacillus*[20] that enhanced growth of canola.

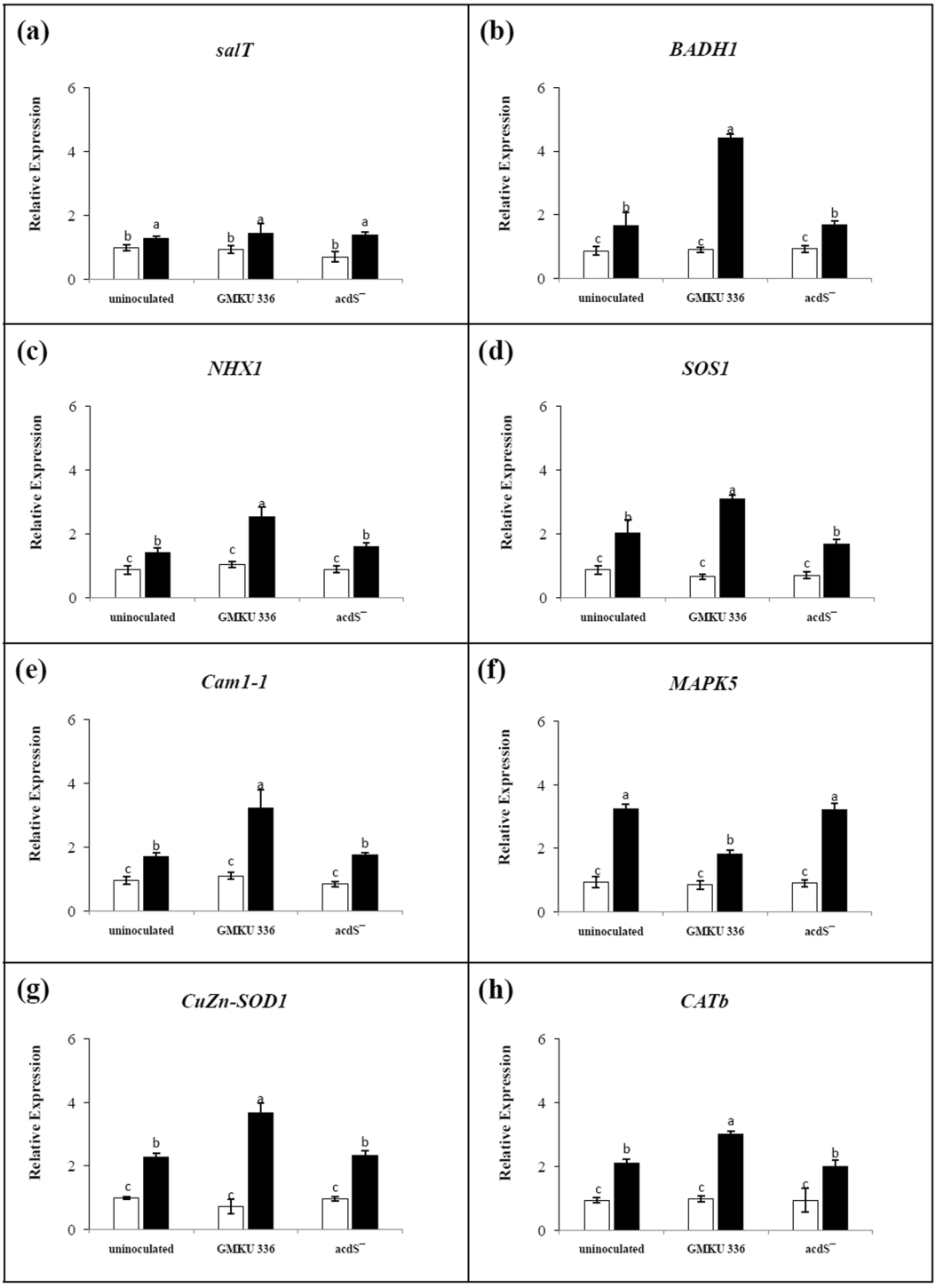

Figure 6. Transcriptional analysis of genes involved in salt stress response in *Oryza sativa* L. cv. KDML105 associated with *Streptomyces* sp. GMKU 336. (**a**) Salt stress responsive protein (*salT*); (**b**) betaine aldehyde dehydrogenase (*BADH1*); (**c**) Na^+/H^+ antiporter (*NHX1*); (**d**) salt overlay sensitive 1 protein (*SOS1*); (**e**) calmodulin (*Cam1-1*); (**f**) mitogen activated protein kinase (*MAPK5*); (**g**) superoxide dismutase (*CuZn-SOD1*); (**h**) catalase (*CATb*). The histogram represents mean of expression ratio, relative to the actin gene (*act1*). Values are mean of three replicates ± standard error of mean. Different letters indicated statistically-significant differences between treatments (Duncan's test, $P < 0.05$). Uninoculated, plants without bacteria inoculation; GMKU 336, plants inoculated with *Streptomyces* sp. GMKU 336; $acdS^-$, plants inoculated with ACCD-deficient mutant; white bar, non-salt treatment; black bar, salt treatment (150 mM NaCl).

Addition of salt had a negative effect on plant growth parameters in all plant treatments. However, KDML105 inoculated with *Streptomyces* sp. GMKU 336 maintained high shoot and root elongation when compared to un-inoculated plants and those inoculated with the ACCD-deficient mutant. The results were in agreement with previous work that an ACCD-producing *Streptomyces* increased shoot and root growth of halophytic *Ligustrum sinense* under salt-stress treatment[6]. ACCD-producing *Bacillus* significantly increased seed germination and promoted growth of rice KDML105[21] and indica rice[12]. Likewise, an ACCD-producing *Pseudomonas* increased yield and enhanced salt resistance in various plants including canola[22] and tomato[23] which was not observed with its *acdS* mutant strain. Since KDML105 is a salt-sensitive cultivar[24], symptoms of salt toxicity were observed in un-inoculated plants and those inoculated with the ACCD-deficient mutant. By contrast, inoculation of KMDL105 with strain GMKU 336 resulted in the rice exhibiting better resistance to salinity stress.

Salt stress reduced chlorophyll content in all KDML105 treatments. However, plants inoculated with *Streptomyces* sp. GMKU 336 had less reduction of chlorophyll content. The result was similar to that with PGP *Azospirillum*[25] and ACCD-producing *Bacillus*[12] which significantly increased chlorophyll content in respective maize and rice grown in high salt conditions. Furthermore, salt stress significantly accelerated ethylene synthesis in all rice treatments. By contrast, rice inoculated with strain GMKU 336 maintained ethylene content at the same level as that of non-salt treatment. The result is supported by other reports that ACCD-producing *Pseudomonas* suppressed ethylene synthesis and decelerated chlorophyll decay in wheat under salt-stress conditions[26]. Besides, lower amount of ACC, a precursor of ethylene, was observed in canola inoculated with ACCD-producing *Pseudomonas*[18] and *Enterobacter*[19]. It was also reported that *Streptomyces* enhanced plant growth by lowering the plant ACC and ethylene levels in tomato[5].

Salt stress induced high activities of ACC synthase and ACC oxidase and subsequently produced high levels of ethylene[1]. In this work, expression profiles of rice genes involved in the ethylene pathway including ACC synthase (*ACS1*) and ACC oxidase (*ACO1*) were up-regulated in all plants treated with salt. Here, we report the remarkable reduction of ethylene in KDML105 inoculated with *Streptomyces* sp. GMKU 336, which is correlated with low expression of the *ACO1* gene. Expression of the *acdS* gene encoding ACCD of strain GMKU 336 was only observed *in vivo* with salt-treated rice. The results indicated that lower expression of the *ACO1* gene of salt-treated rice inoculated with strain GMKU 336 was due to the expression of *acdS* gene of the bacteria which converted the ACC to ammonia and α-ketobutyrate and subsequently reduced the level of the stress molecule, ethylene. The results were in agreement with the expression of *acdS* of *Mesorhizobium* spp.[27] and *Sinorhizobium* sp. BL3[28] in nodules of chickpea and mungbean, respectively under salinity condition. However, the expression was not detected in mungbean inoculated with an *acdS*-deficient mutant of strain BL3[28]. The gene encoding an ethylene responsive element binding protein (*EREBP1*) was up-regulated in all plants treated with salt. EREBP is a member of the ethylene-response factor (ERF) family[29], which plays an important role in abiotic stress response[30]. Up-regulation of *EREBP1* was observed in tobacco during drought and salt stress[31]. Here, we report that KDML105 inoculated with *Streptomyces* sp. GMKU 336 expressed *EREBP1* at significantly lower levels. Canola inoculated with ACCD-producing *Pseudomonas putida* UW4 had reduced expression of *ERF*, while plants inoculated with an ACCD-deficient mutant had increased the expression[32].

Under salt-stress conditions, plants adapt by producing competition solutes such as proline and glycine betaine that help to stabilize proteins and cell structures, osmotic balance, scavenge reactive oxygen species (ROS)[33], and increases chlorophyll content[34]. Here we report that the water and proline contents of all salt-treated rice were significantly increased, but highly accumulated in plants inoculated with *Streptomyces* sp. GMKU 336. The results correlated with previous reports that PGP *Dietzia*[35] and ACCD-producing *Bacillus*[12] improved salt tolerance in respective wheat and rice by enhancement of proline content. Furthermore, the transcription profile of the betaine aldehyde dehydrogenase gene (*BADH1*) that converts choline to glycine betaine was up-regulated in all salt-stressed rice and expressed at the highest level in plants associated with strain GMKU 336. The result was in agreement with ACCD-producing *Bacillus* that maintained osmotic adjustment in rice under salinity condition by accumulation of glycine betaine and up-regulation of the *BADH1* gene[12].

It is generally known that the maintenance of low cytosolic Na^+ concentrations and Na^+/K^+ homeostasis are important for tolerance to salinity. ACC could promote the production of ethylene and improve the response to salinity-induced injury by homeostasis of Na^+/K^+ [12]. High accumulation of Na^+ inside the cells inhibits K^+ uptake and results in an increase in Na^+/K^+ ratio that is inversely related to the level of salt tolerance[36]. Here we report that decreased Na^+ content and increased K^+ resulted in a reduction of the Na^+/K^+ ratio in salt-stressed rice inoculated with *Streptomyces* sp. GMKU 336. It was reported that PGPB may regulate the uptake of Na^+/K^+ and maintain a nutritional balance in plants[37]. The results were in agreement with previous reports that cotton inoculated with ACCD-producing *Klebsiella* showed high K^+ concentrations that resulted in enhancement of salt tolerance[38].

Increases in the uptake of Na^+ in shoot vacuoles could enhance salt tolerance in plants. Therefore, the most direct way to manage excess cytoplasmic Na^+, which is toxic to plant cells, is to pump the excess Na^+ to a vacuole catalyzed by a Na^+/H^+ antiporter[13]. We observed up-regulation of the Na^+/H^+ antiporter gene (*NHX1*) in all salt-stressed rice, which was significantly highest in plants inoculated with *Streptomyces* sp. GMKU 336. Similarly, high expression level of *NHX* was observed in wheat inoculated with PGP *Dietzia* which correlated with enhancement of salt tolerance[35]. Na^+ efflux is one of the mechanisms that maintains the level of Na^+ in the cytoplasm. Salt overlay sensitive 1 (SOS1) is the only Na^+ efflux protein located at the plant plasma membrane[13]. We report here that expression of the *SOS1* gene was increased in all salt stressed rice and significantly highest in plants inoculated with strain GMKU 336. The results were in agreement with other reports of overexpression of the *SOS1* gene in salt tolerant rice[39] and *Arabidopsis*[40]. The results indicated that excess Na^+ was reduced by the up-regulation of *NHX1* and *SOS1* genes which was an effect of strain GMKU 336 to enhance growth and salt tolerance of rice.

The calcium signaling network is one of the signal cascades involved in transient changes in cytosolic Ca^{2+} concentration, which was reported to be a key messenger in the salt stress response[41]. A decrease in Ca^{2+} content under stress condition was previously reported in KDML105[24] and other rice salt-sensitive lines[42]. Here we

report a decrease in Ca^{2+} content as a result of salt stress in all salt treated rice. However, plants inoculated with *Streptomyces* sp. GMKU 336 maintained a significantly higher Ca^{2+} content. Increase of Ca^{2+} content was also observed in eggplant[43] and cotton[44] inoculated with PGP *Pseudomonas*. Furthermore, the expression profile of *Cam 1-1*, involved in calmodulin, was significantly up-regulated in all salt-stressed rice, but highest in plants inoculated with strain GMKU 336. The binding of Ca^{2+} to the calmodulin complex is able to regulate a variety of cellular processes implicated in salt and other stresses[14], therefore, *Cam1-1* gene is a significant player in the Ca^{2+} signal transduction network. The responses of high Ca^{2+} content and *Cam1-1* gene expression on rice KDML105 to salt stress suggested that strain GMKU 336 plays a positive role to induce calmodulin and Ca^{2+} content to help rice tolerate salinity.

The response of plant cells to salt stress is controlled by multiple mechanisms linked to stress and other developmental responses. Ethylene was additionally reported to mediate crosstalk between mitogen activated protein kinase (MAPK) signaling pathways[45]. Plant MAPK cascades are thought to play a key role in biotic and abiotic stress responses, hormone response, cell division and development in rice[46]. We report here that the expression profile of *MAPK5* was significantly up-regulated in all salt-stressed rice, but had lower expression in plants inoculated with *Streptomyces* sp. GMKU 336. Similarly, a low expression level of *MAPK5* was observed in rice inoculated with *Bacillus amyloliquefaciens* NBRISN13 that increased salt tolerance of rice in soil[12]. However, *MAPK* gene expression profiles of rice[47], pea[48], and *Arabidopsis*[49], were up-regulated during ethylene induction. The results suggested that lower ethylene production in rice inoculated with strain GMKU 336 under salt-stress treatment might reflect the low expression level of the *MAPK5* gene.

In rice, salinity triggers MAPK cascades to stabilize ACC synthase activity that enhances ethylene production and ethylene signaling, which then promotes ROS accumulation leading to lipid peroxidation (high accumulation of MDA content) and growth inhibition[50]. In this experiment, salinity significantly induced high accumulation of MDA content and ROS including superoxide and hydrogen peroxide in all salt-stressed rice. Remarkably, *Streptomyces* sp. GMKU 336 reduced ROS leading to a reduction in lipid peroxidation in rice. Earlier studies suggested that induction of ROS scavenging anti-oxidative enzymes such as superoxide dismutase (SOD) and catalase (CAT) were associated with salt tolerance in sugar beet[51] and pea[13]. Here we report that the transcription levels of superoxide dismutase (*CuZn-SOD1*) and catalase (*CATb*) genes were significantly up-regulated in all salt-stressed rice and particularly highest in plants inoculated with strain GMKU 336. The results correlated with previous reports that PGPB reduced salt-induced lipid peroxidation through modulation of expression of ROS-scavenging enzymes[52]. The results indicated that strain GMKU 336 helps salt-stressed rice plants by reduction of lipid peroxidation and ROS levels and consequently promotes plant growth by induction of antioxidant enzymes.

In addition, up-regulation of *salT* was observed in all salt-stressed rice at the same expression level. *SalT* is one of the key genes for salt stress response that has been used as a marker for a salt-induced positive control gene[12] whose transcript is not induced by ethylene[53]. We have shown up-regulation of *salT* in all salt stressed KDML105, supporting the view that this salt-sensitive cultivar changed in level of transcripts of salt stress responsive genes when exposed to salt.

In conclusion, all of the experimental data indicate that ACCD-producing endophytic *Streptomyces* sp. GMKU 336 promoted growth and protected salt-sensitive *Oryza sativa* L. cv. KDML105 from salt stress damage. This endophytic streptomycete enhanced salt tolerance in rice by lowering stress-induced ethylene via the action of ACCD; reduction of lipid peroxidation and Na^+/K^+ ratio but increasing Ca^{2+} content; chlorophyll content, accumulation of osmoprotectants: proline and glycine betaine. The plant physiology also correlated with expression profiles of stress responsive genes in rice associated with strain GMKU 336. The molecular interaction scheme of rice and *Streptomyces* sp. GMKU 336 under salt stress is summarized in Fig. 7. *AcdS* encoding ACCD of strain GMKU 336 was up-regulated *in vivo*, while *ACO1* and *EREBP1* were down-regulated and implicated in reduction of ethylene production in rice. Depleted ethylene induced less expression of *MAPK5*, that plays a role in salt tolerance as well as lowering ROS accumulation and consequently enhances plant growth. The presence of *Streptomyces* sp. GMKU 336 also enhances salt tolerance in rice by increasing proline and glycine betaine by up-regulation of *BADH1* gene expression to balance the osmotic pressure between rice tissues and salt-stress environment. Furthermore, an increase in Ca^{2+} and decrease in Na^+/K^+ homeostasis were correlated with up-regulation of the *Cam1-1* gene of calmodulin and *SOS1* and *NHX1* genes related to SOS pathway. The Ca^{2+} signal activates the SOS3/SOS2 protein kinase complex which negatively regulates the activity of the Na^+ ion channel[13]. Consequently, the plasma membrane Na^+/H^+ antiporter (*SOS1*) was phosphorylated and drives the cytoplasmic Na^+ into a vacuole, thus maintaining cellular ion homeostasis under salt stress. In addition, association of Ca^{2+} with calmodulin contributes to the antioxidant defense system by up-regulation of *CuZn-SOD1* and *CATb* gene expression to increase the production of antioxidant enzymes which subsequently inhibits ROS. Noticeably, plants inoculated with the ACCD-deficient mutant exhibited plant growth parameters, physiology and expression of all plant stress responsive genes in the same manner as those of un-inoculated controls. This supports the positive role of ACCD of *Streptomyces* sp. GMKU 336 in growth promotion and salt tolerance of rice.

It is clearly demonstrate for the first time that ACCD-producing *Streptomyces* sp. GMKU 336 enhances growth and salt tolerance by regulation of stress responsive genes of plants *in vivo* under salt-stress condition. Knowledge of the interaction is crucial to understand the relationship between rice plants and endophytic actinomycetes that is essential for further applications of endophytes as potential environmental friendly biofertilizers in saline soil.

Methods

Bacterial strain, identification and NaCl tolerance. *Streptomyces* sp. GMKU 336 was isolated from roots of a medicinal plant, *Clerodendrum serratum* (L.) Moon, collected from Khaohinson Royal Development Study Center, Chachoengsao Province, Thailand on starch casein agar (SCA)[9]. Its 16S rRNA gene was amplified and sequenced using primers listed in Supplementary Table S3[54,55] (Genbank accession number KR870352). The

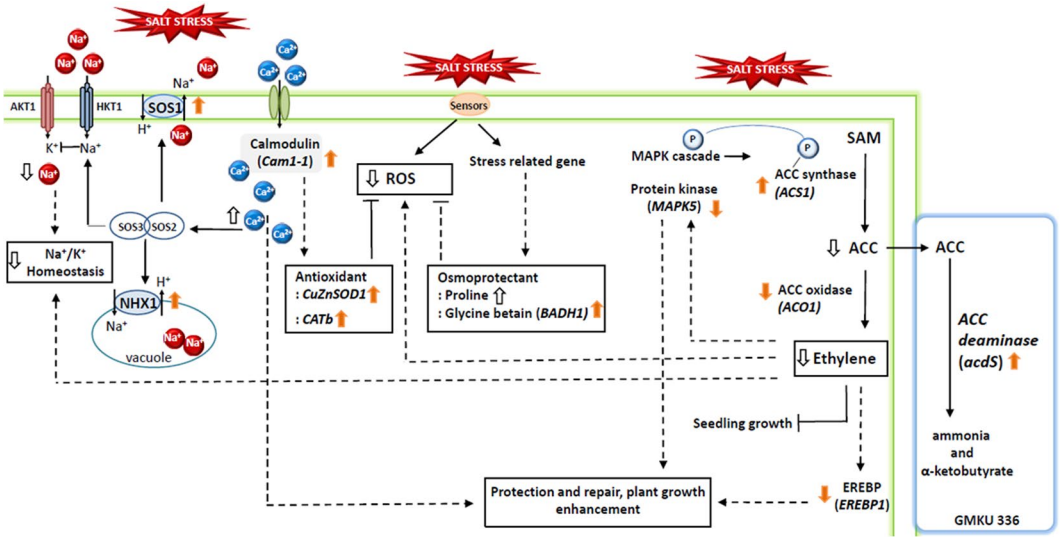

Figure 7. Molecular interaction scheme of ACCD-producing endophytic *Streptomyces* sp. GKU 336 associated with *Oryza sativa* L. cv. KDML105 under salt stress. Salt stress induces the ethylene biosynthesis pathway by up-regulation of *ACS1*. However, ACC is consumed by the *acdS* gene encoding ACCD of *Streptomyces* sp. GMKU 336, whereas *ACO1* and *EREBP1* are down-regulated and ethylene is reduced as a consequence. Depletion of ethylene induced less expression of *MAPK5* as well as lowering ROS accumulation. Salt tolerance in rice is enhanced by increases in proline and glycine betaine by up-regulation of the *BADH1* gene. Ca^{2+}content is increased and Na^{+}/K^{+} ratio is decreased which are correlated with up-regulation of *Cam1-1*, *SOS1* and *NHX1* genes. Ca^{2+} signal activates the SOS3/SOS2 protein kinase complex which negatively regulates the activity of Na^{+} ion channel. Association of Ca^{2+} and calmodulin activates antioxidant enzymes (*CuZn-SOD1* and *CATb*) which subsequently inhibits ROS. Bold orange arrow indicates gene regulation, bold white arrow indicates plant physiological regulation, black arrow indicates positive regulation, dashed arrow indicates indirect positive regulation, black line with bar end indicates inhibition, and dashed line with bar end indicates indirect inhibition.

sequence was analyzed and verified using EzTaxon-e database[56]. The NaCl tolerance of strain GMKU 336 was determined by growing on inorganic salt-starch agar (ISP-4) with addition of 1–12% (w/v) NaCl and incubated at 28 ± 2 °C for 14–21 days.

Determination of phosphate solubilization, indole-3-acetic acid and siderophore production. *Streptomyces* sp. GMKU 336 was grown in tryptic soy broth (TSB) at 28 ± 2 °C, 200 rpm for 5 days. The cell culture was dropped onto Pikovskaya agar[57] containing tricalcium phosphate and further incubated for 5 days. Presence of a clear zone indicates solubilization of phosphate.

Strain GMKU 336 was inoculated into TSB supplemented with tryptophan (500 μg mL^{-1}) at 28 ± 2 °C, 200 rpm in the dark for 7 days. 1 mL supernatant was mixed with 2 mL Salkowski's reagent[58] and incubated for 30 min at room temperature. Development of a pink color indicates indole-3-acetic acid (IAA) production[58].

A YM agar plug of 5-day growth of strain GMKU 336 was placed on a chrome azurol S (CAS) agar[59] and incubated at 28 ± 2 °C for 3 days. An orange halo zone indicates siderophore production.

Determination of ACC deaminase activity. *Streptomyces* sp. GMKU 336 was grown on mannitol soybean agar (MS) for 5 days and streaked on nitrogen-free minimal medium agar (MM), and MM agar supplemented with either 2 gL^{-1} $(NH_4)_2SO_4$ or 3 mM ACC (Sigma-Aldrich) and incubated at 28 ± 2 °C in the dark for 7 days. Growth and sporulation on MM agar supplemented with ACC (MM-ACC) indicates ACC deaminase (ACCD) activity[4].

For quantitative determination of ACCD activity, strain GMKU 336 was grown in TSB at 28 ± 2 °C, 200 rpm for 3 days. Cells were washed twice with 0.1 M Tris-HCl (pH 8.5) and resuspended in MM-ACC broth followed by incubation at 28 ± 2 °C, 200 rpm for 3 days. Cells were collected, washed twice and resuspended in 0.1 M Tris-HCl (pH 8.5). Cells were lysed by sonication and ACCD activity in the supernatant was assayed[7]. An aliquot of 200 mL of supernatant was incubated with 50 mM ACC at 30 °C for 1 h. The enzyme reaction was then stopped by adding 1.8 mL of 0.56 M HCl and 0.3 mL of 0.1% (w/v) 2,4-dinitrophenylhydrazine (prepared in 2 M HCl solution) and incubated at 30 °C for 15 min. The colorimetric reaction was then stopped by adding 2 mL of 2 M NaOH and the absorbance at 540 nm was determined by comparing to a standard curve of α-ketobutyrate.

Construction of an ACCD-deficient mutant. Since *Streptomyces* sp. GMKU 336 was newly isolated and its genome has not yet been sequenced, partial ACCD gene (*acdS*) was obtained by PCR amplification using specific primers[9] and annealing temperature listed in Supplementary Table S3. The primers were designed based on conserved amino acid regions[60] with minimal degeneracy[9]. The PCR product was cloned into a non-replicative

vector, pIJ8671[61] to obtain pIJ8671/*acdS*. Next, pIJ8671/*acdS* was transformed into *E. coli* ET12567(pUZ8002)[62] to perform an intergeneric conjugation[63] with 24-h mycelium of strain GMKU 336. The mutants were selected by thiostrepton resistance (50 μg mL^{-1}) and screened for deficiency of ACCD activity. Insertional inactivation of *acdS* in mutant was verified by PCR amplification using specific primers listed in Supplementary Table S3 of (i) thiostrepton resistance gene, (ii) partial *acdS* gene, and (iii) the absence of a 5.4-kb long PCR product (presence in pIJ8671/*acdS*) (Supplementary Fig. S1).

Inoculation of rice plants with *Streptomyces* sp. GMKU 336 and its deficient mutant. Healthy seeds of Thai jasmine rice Khao Dok Mali 105 cultivar (KDML105), *Oryza sativa* L. cv. KDML105 were surface sterilized by immersion in 70% (v/v) ethanol for 1 min, 1% (w/v) sodium hypochlorite for 10 min and washed six times with sterile distilled water before transferring to a sterile moist chamber and incubation at room temperature in the dark for 3 days. Rice seedlings were grown under artificial light with light intensity at 8,000 lux for 16 h daily at room temperature for 7 days. The roots of seedlings was cut to the same length and were then immersed in individual sterile glass beakers containing 10^8 spores mL^{-1} of *Streptomyces* sp. GMKU 336 or the mutant for 4 h. The seedlings were re-located to a moist sponge support for 1 day before transferring to a 20-L container filled with ½ Yoshida solution[64] for 7 days and replaced with Yoshida solution for 7 days. Next, the nutrient solution was changed to Yoshida solution supplemented with 150 mM NaCl for 7 days. The pH of the nutrient solution was maintained between 5.0–5.5 throughout the growth period. A positive control of non-salt stressed rice was also grown under the same conditions without NaCl treatment.

Symptoms of salt toxicity were evaluated according to the standard evaluation system used at the International Rice Research Institute (IRRI)[10].

Endophytic streptomycetes was re-isolated by surface-sterilized protocol as previously described[9]. The final washed solution was examined to ensure that the surface plant materials were actually sterilized. Re-isolated colonies of endophytic streptomycetes were randomly selected for analysis by 16S rDNA gene sequencing. In addition, re-isolated ACCD-deficient mutants were further verified by (i) growth on MS supplemented with thiostrepton (50 μg mL^{-1}); (ii) deficiency of ACCD activity; and (iii) amplification of thiostrepton resistant gene.

Determination of chlorophyll and ethylene contents. Approximately 100 mg of leaf fresh weight was ground in liquid nitrogen and chlorophyll was extracted twice by adding 1.0 mL DMSO and then sonicated[65]. The chlorophyll content of the supernatants were measured at 645 and 663 nm within 20 min after the extraction.

Ethylene production was measured by enclosing the whole rice plants in a 250-mL sealed glass container containing 50 mL acetylene for 1 h. A 1 mL gas sample was withdrawn and quantified by gas chromatography at PGPR Biofertlizer and Aerated Compost Soil Microbiology Research Group, Soil Science Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

Determination of proline, ion, and relative water contents. 250 mg fresh weight of the whole plantlets were ground in liquid nitrogen and mixed with 5 mL aqueous sulfosalicylic acid (3% w/v) and filtered through Whatman® No. 1. 1 mL of filtrate was mixed with an equal volume of glacial acetic acid and ninhydrin reagent (1.25 mg ninhydrin, 30 mL glacial acetic acid, and 20 mL 6 M H_3PO_4) and incubated for 1 h at 100 °C in boiling water. The reaction was terminated by placing the test tube in an ice bath. Next, the reaction mixture was vigorously mixed with 2 mL toluene. After warming at 25 °C, proline was measured at 520 nm[66].

Rice shoots were assayed for Na^+, K^+ and Ca^{2+} contents at the Department of Soil Science, Faculty Agriculture, Kasetsart University, Bangkok, Thailand.

The relative water content (RWC) of plant leaves was examined[67]. ~10 cm of leaf was cut off from the middle part of the youngest fully expanded leaf, weighed and placed in a tube. The tube was kept on ice and was filled with distilled water and kept in the dark at 4 °C overnight. The leaf was blotted dry and weighed. The samples were then dried at 70 °C for 3 days and weighed. The RWC was calculated from each weigh[66].

ROS staining and estimation of lipid peroxidation. For detection of superoxide[68], rice leaves were immersed in 25 mL of nitrobluetrazolium (NBT) solution (0.5 μg mL^{-1} NBT in 10 mM phosphate buffer, pH 7.6) for 3 h in the dark. For detection of hydrogen peroxide[69], leaves were immersed in 25 mL 3,3′-diaminobenzidine (DAB) solution (1 μg mL^{-1} DAB in 50 mM Tris-acetate buffer, pH 5.0) for 8 h. After staining, both treatments were boiled in 95% (v/v) ethanol for 30 min. The leaves were then immersed in 40% glycerol for 16 h before color detection.

The amount of lipid peroxidation was determined by estimating malondialdehyde (MDA)[69]. Rice shoots were ground in 80% (v/v) ethanol (1 g fresh weight 25 mL^{-1}). 1 mL aliquots of samples were added with 1 mL of either (i) −TBA solution [20% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene], or (ii) +TBA solution containing –TBA solution plus 0.65% thiobarbituricacid (TBA) and mixed vigorously before heating at 95 °C for 25 min. Absorbance was determined at 440 nm, 532 nm, and 600 nm and MDA equivalents were calculated[69].

Streptomycete RNA extraction and semi qRT-PCR analysis. *Streptomyces* sp. GMKU 336 was grown in TSB at 28 ± 2 °C, 200 rpm for 3 days. Cells were washed twice with 0.1 M Tris-HCl (pH 8.5) and resuspended in MM-ACC broth followed by incubation at 28 ± 2 °C, 200 rpm for 3 days. Total RNA was extracted following the manufacturer's protocol of Trizol® Reagent (Invitrogen). cDNA was synthesized using the Thermo Scientific RevertAid First strand cDNA synthesis Kit (Thermo Scientific). Semi-quantitative RT-PCR analysis was performed using cDNA products, the corresponding primers listed in Supplementary Table S3, and Phusion® Hot Start II-High Fidelity DNA polymerase (Thermo Scientific). The expression level of each product was quantified by Gel Doc™ XR + with Image Lab™ Software (Biorad) and normalized against the expression of a housekeeping gene, *hrdB*[70].

Rice RNA extraction and transcription analysis of genes involved in salt stress response by real-time PCR. Total RNA was extracted from shoots following the manufacturer's protocol for Trizol® Reagent (Invitrogen) and treated with DNase I (Thermo Scientific). cDNA was synthesized using the Thermo Scientific RevertAid First strand cDNA synthesis Kit (Thermo Scientific). KAPA SYBR® FAST qPCR Master Mix (2x) (KAPA BIOSYSTEMS) was used for quantification in Master Cycler Realplex 4 (Eppendorf). The primers for real-time PCR are listed in Supplementary Table 3[71–74]. The mean value was calculated and normalized with actin (*act1*) as internal control.

Statistical analysis. All data from the experiments were calculated and statistically evaluated from biological and technical triplicates. The data were analyzed with one way analysis of variance (ANOVA) and Duncan's test to determine any significant differences between groups at $P < 0.05$. All statistical analyses were performed using the SPSS 20.0 for Windows software (SPSS Inc).

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Author Contributions

R.J. and A.T. conceived and designed the work; R.J. performed the experiments and analyzed the data together with A.T. and C.J.; R.J. and A.T. wrote and prepared manuscript, all authors reviewed the manuscript.

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PROKARYOTES

Draft Genome Sequence of Root-Associated Sugarcane Growth-Promoting *Microbispora* sp. Strain GKU 823

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ABSTRACT The endophytic plant growth-promoting *Microbispora* sp. strain GKU 823 was isolated from the roots of sugarcane cultivated in Thailand. It has an estimated 9.4-Mbp genome and a G+C content of 71.3%. The genome sequence reveals several genes associated with plant growth-promoting traits and extensive specialized metabolite biosynthesis.

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Endophytic actinomycetes are free-living filamentous bacteria that mutually colonize inside plants and facilitate plant growth through direct and indirect mechanisms employing plant growth-promoting (PGP) traits (1). The endophytic *Microbispora* sp. strain GKU 823 was isolated from roots of sugarcane cultivated in Thailand and has been shown to enhance sugarcane growth (2). This strain exhibits PGP traits, including the production of indole-3-acetic acid (IAA) and siderophores, phosphate solubilization, and suppression of fungal growth (2). Based on 16S rRNA gene sequence analysis, *Microbispora* sp. GKU 823 is closely related to *Microbispora hainanensis* 211020^T (99.09% similarity, GenBank accession no. KR560040). The whole-genome sequence of *Microbispora* sp. GKU 823 was obtained, and genes associated with plant growth promotion and specialized metabolite biosynthesis were identified.

Total genomic DNA of *Microbispora* sp. GKU 823 was extracted using the Isolate II genomic DNA extraction kit (Bioline, UK), according to the manufacturer's instruction. The genome was sequenced using the Ion PGM system, generating 1,230,781 reads (with approximately 30× coverage) with an average read length of 225 bp. The genome was assembled using SPAdes version 3.9 (3) and evaluated by QUAST version 3.2 (4). Reads were assembled into 262 contigs (coverage, ≥10×; length, ≥1,000 bp), with an N_{50} of 69,483 bp. The largest contig obtained was 321,219 bp in length. The draft genome is estimated to be 9,430,099 bp, with a G+C content of 71.3%.

Functional gene annotation of the assembled genome was carried out by the Rapid Annotations using Subsystems Technology (RAST) server (5). rRNA and tRNA genes were determined by RNAmmer (6) and tRNAscan-SE (7), respectively. The annotation predicted a total 9,248 coding sequences, 58 tRNA genes, and 3 rRNA genes. The average nucleotide identity values of the genome by BlastN (ANIb) were calculated in JSpeciesWS (8). The genome comparison revealed that *Microbispora* sp. GKU 823 had an ANIb value of 92.47% similar to *Microbispora rosea* NRRLB-2630, 90.70% to *M. rosea* NRRL B-2631, and 81.20% to *Microbispora* sp. strain ATCC PTA-5024.

Genes related to PGP traits, including phosphate solubilization (alkaline phosphatase and isocitrate dehydrogenase [9, 10]), indole-3-acetic acid (IAA) production (tryptophan 2-monooxygenase [11]), and genes involved in fungal cell wall degradation (family 18 chitinase [12]) were detected in the genome of *Microbispora* sp. GKU 823. Moreover, genes involved in stress tolerance (betaine aldehyde dehydrogenase, proline

dehydrogenase, superoxide dismutase, and trehalose synthase [13]) were also present. These genes may provide *Microbispora* sp. GKU 823 with the capability to promote the growth of sugarcane (2). antiSMASH version 3.0 (14) predicted 23 secondary metabolite gene clusters in the genome of *Microbispora* sp. GKU 823, including seven gene clusters of nonribosomal peptide synthetase, four gene clusters of type I polyketide synthase and terpene, three gene clusters of bacteriocin, two gene clusters encoding siderophores (including desferrioxamine E), and a single gene cluster encoding a lanthipeptide. These secondary metabolite gene clusters indicate that endophytic *Microbispora* species are potential sources of novel specialized metabolites.

Accession number(s). The draft genome sequence of *Microbispora* sp. GKU 823 has been deposited in the DDBJ/ENA/GenBank databases under the accession number MWJN00000000.

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PROKARYOTES

Draft Genome Sequence of Plant Growth-Promoting Endophytic *Streptomyces* sp. GKU 895 Isolated from the Roots of Sugarcane

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ABSTRACT *Streptomyces* sp. GKU 895 is an endophytic actinomycete isolated from the roots of sugarcane. GKU 895 has a genome of 8.3 Mbp and the genome exhibits adaptations related to plant growth-promoting activity. It also has extensive specialized metabolite biosynthetic gene clusters apparent in its genome.

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Sugarcane is an economically important crop for the production of sugar and biofuels. Plant growth-promoting endophytes are heterogeneous groups of bacteria that reside mutually within plant tissues and appear to provide many benefits to the plant host (1). *Streptomyces* sp. GKU 895 is a root-associated bacterium isolated from sugarcane plants cultivated in Thailand (2). Based on 16S rRNA sequence analysis, strain GKU 895 is closely related to *Streptomyces canus* NRRLB-1989^T (99.4% similarity, GenBank accession no. KP637153). *Streptomyces* sp. GKU 895 shows the ability to solubilize phosphate and produce indole-3-acetic acid (IAA), siderophores, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase. It is also capable of suppressing the growth of *Bacillus cereus* ATCC 11778 and the sugarcane red rot fungal pathogens *Colletotrichum falcatum* and *Fusarium moniliforme* (3). Moreover, *Streptomyces* sp. GKU 895 enhances sugarcane growth when applied in pot experiments (2). Here, we present the draft genome sequence of *Streptomyces* sp. GKU 895 determined using a combined sequencing approach using the Ion PGM and MinION.

Total DNA of *Streptomyces* sp. GKU 895 was extracted using an ISOLATE II genomic DNA extraction kit (BIOLINE, United Kingdom). The Ion PGM system produced 1,019,643 reads (with approximately 30× coverage) with an average read length of 240 bp. MinION reads were obtained using a ligation sequencing kit 2D (SQK-LSK208, R9.4) and spot-on flow cell Mk1 (R9.4) and were extracted using Poretools (4) which generated 21,275 2D MinION reads with an average length of 2,979 bp. The sequences were assembled using SPAdes version 3.9 with the nanopore option (5) and determined by QUAST version 3.2 (6). The input sequences were assembled to 190 contigs (coverage ≥10 and length ≥1,000) with an N_{50} of 61,010. The largest contig is 213,532 bp in length. The draft genome contains 8,296,413 bp with a G+C content of 70.7%.

The genome was annotated using the Rapid Annotations using Subsystems Technology (RAST) server (7), which identified 8,208 coding sequences. RNAmmer (8) and tRNAscan-SE (9) revealed 6 rRNA genes and 64 tRNA genes. RAST annotation revealed that the genome of *Streptomyces* sp. GKU 895 possesses genes associated with plant growth promotion including *acdS* gene-encoding ACC deaminase; genes involved in IAA synthesis [indoleacetamide hydrolase and nitrilase (10, 11)]; genes which assist in mineral phosphate solubilization including isocitrate dehydrogenase, citrate synthase,

and purple acid phosphatase (12, 13); genes for the degradation of fungal cell walls [family 18 and 19 chitinases (14)]; and genes involved in host plant colonization such as salicylate hydroxylase (15). AntiSMASH (version 3.0) analysis (16) predicts 28 gene clusters involved in specialized metabolite production in the *Streptomyces* sp. GKU 895. These consist of six gene clusters encoding putative butyrolactones, three gene clusters of siderophores (including desferrioxamine E) and terpenes, two gene clusters for bacteriocins, melanins, type II polyketide synthases, and a single gene cluster encoding ectoine, nonribosomal peptide synthetase (NRPS), NRPS-bacteriocin, type III polyketide synthase (T3PKS), T3PKS-NRPS, T3PKS-terpene-butyrolactone, and terpene-butyrolactone-NRPS.

Accession number(s). The draft genome sequence of *Streptomyces* sp. GKU 895 has been deposited in the DDBJ/ENA/GenBank database under the accession number MWJO00000000.

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Diversity of Culturable Plant Growth-Promoting Bacterial Endophytes Associated with Sugarcane Roots and Their Effect of Growth by Co-Inoculation of Diazotrophs and Actinomycetes

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Abstract Application of environmentally friendly agents to reduce the use of chemicals and to enhance growth of plants is an ultimate goal of sustainable agriculture. The use of plant growth-promoting endophytes has become of great interest as a way to enhance plant growth and additionally protect plants from phytopathogens. In this study, 135 isolates of endophytic bacteria including actinomycetes were isolated from roots of commercial sugarcane plants cultivated in Thailand and were characterized for plant growth-promoting (PGP) traits. Based on morphological and 16S rRNA sequence analysis, the endophytes were distributed into 14 genera of which the most dominant species belong to *Bacillus*, *Enterobacter*, *Microbispora*, and *Streptomyces*. Two strains of endophytic diazotrophs, *Bacillus* sp. EN-24 and *Enterobacter* sp. EN-21; and two strains of actinomycetes, *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895, were selected based on their PGP traits including 1-aminocyclopropane-1-decarboxylate deaminase, indole-3-acetic acid, nitrogen fixation, phosphate solubilization, and siderophore production for evaluation of sugarcane growth enhancement by individual and co-inoculation. Sixty days after co-inoculation by endophytic diazotrophs and actinomycetes, the growth parameters of sugarcane plants were significantly greater than that of individual and un-inoculated plants. The results indicated that these endophytes have high potential as PGP agents that could be applied to promote sugarcane growth and could be developed as active added value biofertilizers in the future.

Keywords Actinomycete · Diazotroph · Endophyte · Diversity · Plant growth promotion · Sugarcane

Introduction

Sugarcane (*Saccharum officinarum* L.) is a tropical plant belonging to the grass family, Gramineae, as well as maize, rice, sorghum, and wheat. Sugarcane is the world's largest crop and is economically important in Thailand, which ranks fourth among sugarcane producers on the world market, after Brazil, India, and China (Food and Agriculture Organization of the United Nations 2015). Although sugar is the primary product from sugarcane, ethanol has become a new high-value product because of the worldwide interest in replacing gasoline with biofuel.

For several decades, boosting crop yields by chemical fertilizers and various nutrients and pesticides has dominated agronomy (Tilman 1998). Now, the use of environmentally friendly strategies for reducing the use of chemicals to enhance growth of sugarcane plants is an ultimate goal of sustainable agriculture. One of the alternative ways to promote plant growth and protect plants from diseases is the application of bacterial endophytes. These are free-living bacteria that colonize the inside of the plants and can affect plant growth by direct and indirect mechanisms (Gupta and others 2000; Glick 2012). They

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are termed plant growth-promoting endophytes (PGPE) (Taulé and others 2011) with various PGP traits including solubilization of rock phosphates, production of siderophores to scavenge iron, production of phytohormones, fixation of nitrogen, secretion of 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce ethylene levels in plants, and production of specialized metabolites to protect plants from phytopathogens (Gupta and others 2000; Mendes and others 2007; Glick 2012). A range of PGPE including endophytic actinomycetes significantly increase vegetative growth and grain yield of several crops, including jatropha, maize, rice, sorghum, sugarcane, and wheat (Olivares and others 1997; Yanni and others 2001; Govindarajan and others 2007; Luna and others 2010; Rungin and others 2012; de Jesus Santos and others 2014; Alves and others 2015; Qin and others 2015) by increasing shoot and root lengths as well as biomass through the secretion of plant growth-promoting substances, increasing the availability of nutrients, or promoting growth by combined modes of action (Vessey 2003). Nevertheless, the application of single bacterial strains resulted in inconsistencies in the field (Bashan and Holguin 1997). Therefore, co-inoculation of compatible bacteria has been found to more significantly enhance the growth of plants than individual inoculation. For example, co-inoculation of mixtures of diazotrophic bacteria gave better growth of rice and sugarcane (Govindarajan and others 2007); and co-culture of diazotrophs and fungal endophytes revealed better growth and yield of chickpea (Verma and others 2014).

This study aimed to select powerful endophytic PGP diazotrophs and actinomycetes isolated from sugarcane plants in Thailand. The diversity of culturable root-associated endophytic bacteria and actinomycetes was systematically analyzed and their PGP traits and plant growth-promoting effects on sugarcane plants were thoroughly evaluated after individual and co-culture inoculation.

Materials and Methods

Isolation of Culturable Endophytic Bacteria and Actinomycetes from Sugarcane

Five-month-old healthy sugarcane plant cultivars LK92-11 and KK3, commercial canes mainly planted in Thailand for sugar industry, were obtained from Kud Chock and Nongkonthai sugarcane plantations, Phu Khiao, Chaiyaphum, Thailand through Mitr Phol Sugarcane Research Center. The plant samples were vigorously shaken to loosen attached soil from the roots and air-dried at room temperature (RT) overnight, then kept at 4 °C till use.

To isolate root-associated endophytic bacteria, sugarcane roots were surface sterilized using the method of Rachniyom and others (2015). The roots were first rinsed under running tap water for 2–3 min to remove soil particles, and then immersed with shaking in sterilized 0.1 % (v/v) Tween 20 for 5 min, followed by 70 % ethanol for 5 min, 1 % sodium hypochlorite solution for 10 min, 10 % (w/v) sodium hydrogen carbonate solution for 10 min, and finally washed three times with sterile distilled water.

Endophytic bacteria were isolated according to the method for isolation of nitrogen-fixing bacteria described by Cavalcante and Dobereiner (1988). The roots were crushed in 5 % sucrose solution and ten-fold serial dilutions were made. A 100 µl sample of each dilution was dropped into semisolid LGI medium (Baldani and others 2014) and incubated at 28 °C for 7–10 days. A veil-like pellicle near the surface of the medium appeared and was streaked onto LGI agar and nutrient agar (NA) supplemented with 50 µg ml^{-1} nystatin and 50 µg ml^{-1} cycloheximide and incubated at 28 °C for 3–7 days. The final rinsing water was also spread on both media to confirm surface sterilization. Colonies of bacteria were picked and purified on LGI agar and NA. The pure cultures were classified by Gram staining and morphological observation under the light microscope. Bacteria were stored in 40 % glycerol solution at −80 °C.

For isolation of endophytic actinomycetes, the surface-sterilized roots were macerated in ¼ Ringer's solution (0.9 % NaCl, 0.042 % KCl, 0.048 % $CaCl_2$, and 0.02 % $NaHCO_3$). The root materials and the liquid solution were, respectively, placed and spread onto starch casein agar (SCA) (Küster and Williams 1964) and water agar (WA) supplemented with 50 µg ml^{-1} nalidixic acid, 100 µg ml^{-1} ampicillin, 50 µg ml^{-1} nystatin, and 50 µg ml^{-1} cycloheximide and incubated at 28 °C for 2–4 weeks. The final rinsing water was also spread on both media to confirm surface sterilization. Colonies of endophytic actinomycetes were picked and purified on mannitol soya (MS) agar (Hobbs and others 1989). The pure cultures were stored in 20 % glycerol solution at −80 °C.

Identification of Endophytic Bacteria

All the bacterial endophytes were grouped based on their colony morphology on agar plates. Endophytic bacteria were grown on NA and Tryptic Soy Agar (TSA) media at 28 °C for 2 days. Endophytic actinomycetes were grown on International *Streptomyces* Project (Shirling and Gottlieb 1966) Media No. 2, 3, and 4 at 28 °C for 14 days. The representatives of endophytic bacteria and actinomycetes were selected based on morphological characteristics for 16S rRNA gene sequencing and analysis. Genomic DNA of bacteria was prepared by a standard protocol (Green and

Sambrook 2012). The 16S rRNA gene was amplified using universal primers and the PCR protocol of Lane (1991). For actinomycetes, genomic DNA was prepared according to Kieser and others (2000). 16S rRNA genes were amplified using primers and the PCR protocol of Rachniyom and others (2015). PCR products were purified using Gel/PCR DNA fragment extraction kit (Geneaid, Taiwan) according to the manufacturer's protocol and subjected to DNA sequencing at Macrogen Inc. (Korea). All sequences obtained from the 16S rRNA genes were edited manually using BioEdit (Hall 1999), then compared with type strains using the EzTaxon–e server (Kim and others 2012a). The sequences of the isolates and the most similar species were aligned using CLUSTAL X program version 2.0 (Larkin and others 2007). Phylogenetic tree was constructed using neighbor-joining algorithm (Saitou and Nei 1987) in MEGA software version 6.0 (Tamura and others 2013). The topology of tree was evaluated with bootstrap analysis based on 1000 resamplings (Felsenstein 1985). A distance matrix was generated using Kimura's two-parameter model (Kimura 1980).

Characterization of Plant Growth-Promoting Traits

Indole-3-Acetic Acid (IAA) Production

IAA production was determined by a colorimetric method (Pilet and Chollet 1970). Endophytes were inoculated into glucose-beef extract broth supplemented with 10 mM L-tryptophan and incubated at 28 °C for 7 days in the dark. The culture was then centrifuged and 1 ml of the supernatant was transferred into a vial containing 2 ml of Salkowski's reagent (Pilet and Chollet 1970). The mixture was left at RT for 30 min in the dark. Development of a pink-red color indicated IAA production.

Siderophore Production

An overnight-NB culture of bacteria (10^7 CFU ml^{-1}) or an agar plug of the 14-day growth of actinomycetes on MS agar was, respectively, dropped or placed on chrome azurol S medium (Schwyn and Neilands 1987) and incubated at 28 °C for 7 days for non-actinomycete bacteria and 14 days for actinomycetes. An orange halo indicated siderophore production.

Phosphate Solubilization

Phosphate solubilization was detected by the modified method of Rodríguez and Fraga (1999). An overnight-NB culture of bacteria (10^7 CFU ml^{-1}) or an agar plug of 14-day growth of actinomycetes on MS agar was, respectively, dropped or placed on Pikovskaya's agar (Pikovskaya 1948) containing 0.5 % (w/v) $Ca_3(PO_4)_2$ and incubated at 28 °C for 7 days for non-actinomycete bacteria and 14 days for actinomycetes. Formation of a clear zone indicated solubilizing ability.

1-Aminocyclopropane-1-Carboxylate (ACC) Deaminase Activity

ACC deaminase activity was determined by the method of Glick and others (1995) using N-free medium (Nfb) (Dobereiner and Day 1976) for bacteria and minimal medium (MM) (Kieser and others 2000) for actinomycetes containing 0.3 m mol l^{-1} ACC (Sigma, USA) as a sole nitrogen source. MM with 0.1 % (w/v) $NH_4(SO_4)_2$ was used as a positive control and cultivation without ACC was used as a negative control. After incubation at 28 °C for 7 days for non-actinomycete bacteria and 14 days for actinomycetes, colony growth on Nfb or MM with addition of ACC indicated ACC deaminase activity.

Nitrogen Fixation and nifH Gene Amplification

Nitrogenase activity was determined by the acetylene reduction assay (ARA) according to Hardy and others (1973). An overnight-NB culture of bacteria (10^7 CFU ml^{-1}) was washed three times with distilled water to remove NB before transferring to 5 ml of semisolid Nfb medium and incubated at 28 °C for 2 days. Next, 10 % (v/v) of gas in the headspace of the test tube was replaced by acetylene and left at RT for 1 h. Gas was collected from the headspace and analyzed for ethylene by gas chromatography (Hewlett Packard HP Series II, Japan). The nitrogen-fixing *Azospirillum brasilense* TS13 (Meunchang and others 2004) was used as a positive control and an uninoculated tube served as a negative control. All of the bacterial isolates and *Azospirillum brasilense* TS13 as positive control were subjected to *nifH* gene amplification using primers nifH-F (5′-AAAGGNGGNATCGGNA ANTCCACCAC-3′) and nifH-R (5′-TTGTTNGCNGCN TACATNGCCATCAT-3′) and the PCR protocol described by Rösch and others (2002).

Evaluation for Antimicrobial Activity

To evaluate antimicrobial activity, seven test microbes, *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 259233, *Aspergillus niger* ATCC 6275, *Colletotrichum falcatum* DOAC 1655, and *Fusarium moniliforme* DOAC 1224, were used. Antibacterial activity was evaluated by an overlay method (Anand and others 2006). An overnight-NB culture of bacteria (10^7 CFU ml^{-1}) or a 14-day-old MS agar plug of actinomycetes was,

respectively, dropped or placed on NA plates and incubated for 24 h. The assay plates were overlaid with test bacteria (10^7 CFU ml^{-1}). Appearance of an inhibition zone indicated positive activity.

Antifungal activity was evaluated by dual-culture assay (Fokkema 1976). An overnight-NB culture of bacteria (10^7 CFU ml^{-1}) was spotted on potato dextrose agar and incubated for 24 h. Actinomycetes were streaked on one-third of the area of PDA and allowed to grow for 7 days. An agar plug of the test fungus was prepared using a sterile cock borer (diameter 8 mm), placed opposite the bacterial colony and incubated at RT for 7 days. Inhibition of fungal growth was compared to the growth of the fungal control.

Evaluation of Plant Growth Promotion by Individual and Co-Inoculation

Endophytic bacteria and actinomycetes were selected based upon their PGP traits to evaluate sugarcane growth enhancement by individual and co-culture inoculation. To abnegate the effect of antagonistic activity among them, the selected isolates were tested for antibacterial activity against each other, and only the strains showing no activity were used to inoculate sugarcane. Tissue cultured sugarcane plants of cultivar LK92-11 were obtained from the Plant Tissue Culture Unit at Central Laboratory and Greenhouse Complex, Kasetsart University (Kamphaengsaen Campus). Sugarcane plantlets were rinsed with sterile distilled water to remove remaining agar from the roots. The height of the plants was normalized to 6.5 cm by truncating shoots and roots. Plants were then inoculated with bacteria either individual or co-culture (10^7 CFU ml^{-1} of each strain) by the root dip method (Musson and others 1995) in a tissue culture bottle containing Murashige and Skoog (MS0) medium (Murashige and Skoog 1962) and immersed at RT for 24 h. Inoculated sugarcane plants were transferred to cleaned mini-pot (6 × 5.5 cm) containing sterilized hydroponic materials (perlite:vermiculite, 3:1). The experiment was set up in completely randomized design (CRD) using five replicate mini-pots per treatment and each treatment was replicated twice. Plants were maintained in the greenhouse and supplied with sterilized sucrose-free MS0 broth twice a day for 60 days. The plants were then harvested and examined for root and shoot lengths, and root and shoot fresh/dry weights. Plants were surface sterilized and the inoculated endophytes were re-isolated according to the procedure described above. Plant growth parameters were statistically analyzed by one-way ANOVA and Tukey's multiple range tests (TMRT) using SPSS (version 16.0) at $p = 0.05$ to determine the efficacy of un-inoculated, individual, and co-culture inoculation to promote growth of sugarcane plants.

Results

Distribution of Sugarcane Root-Associated Bacterial Endophytes

One hundred and thirty-five culturable root-associated bacterial isolates were obtained from sugarcane. They comprised 52 isolates of endophytic bacteria and 83 isolates of actinomycetes. According to the preliminary dereplication by morphological criteria and cultural characteristics, 19 and 11 isolates of endophytic bacteria and actinomycetes were assigned to the genus level using 16S rRNA sequence analysis, respectively. Sequence similarity of 16S rRNA genes of these endophytes (ranging from 1282 to 1513 bp) to the closest type strains from the EzTaxon–e server was 98.4–100 % (Table 1). All of the representative strains displayed considerable diversity and were distributed under 11 families of *Bacillaceae*, *Enterobacteriaceae*, *Micrococcaceae*, *Micromonosporaceae*, *Moraxellaceae*, *Paenibacillaceae*, *Pseudomonadaceae*, *Staphylococcaceae*, *Streptomycetaceae*, *Streptosporangiaceae*, and *Thermomonosporaceae* comprised 10 genera of bacteria and 4 genera of actinomycetes (Fig. 1). *Streptomyces* was the most frequently isolated genus ($n = 47$, 34.81 %) followed by *Microbispora* ($n = 29$, 21.48 %), *Enterobacter* ($n = 23$, 17.04 %), *Bacillus* ($n = 14$, 10.37 %), *Actinomadura* ($n = 5$, 3.70 %), *Pantoea* ($n = 4$, 2.96 %), *Acinetobacter* ($n = 2$, 1·48 %), *Kosakonia* ($n = 2$, 1.48 %), *Micromonospora* ($n = 2$, 1.48 %), *Paenibacillus* ($n = 2$, 1.48 %), *Staphylococcus* ($n = 2$, 1.48 %), *Lysinibacillus* ($n = 1$, 0.74 %), *Micrococcus* ($n = 1$, 0.74 %), and *Pseudomonas* ($n = 1$, 0.74 %). 16S rRNA gene sequence similarity values of four strains, *Actinomadura* sp. GKU 822, *Microbispora* sp. GKU 823, GKU 898, and *Pantoea* sp. EN-29 with closest type strains were lower than 99 % (Table 1) and therefore probably represent new species.

Plant Growth-Promoting Traits of Sugarcane Endophytes

The plant growth-promoting (PGP) traits, including antagonistic activity, were examined from 135 strains of sugarcane endophytes (Table 2). Among them, three strains of actinomycetes, *Streptomyces* sp. GKU 833, GKU 879, and GKU 895, and two strains of bacteria, *Bacillus* sp. EN-27 and *Enterobacter* sp. EN-21, showed the highest activity of PGP traits. The highest frequency of PGP trait possessed by both endophytic bacteria and actinomycetes in this study was siderophore production, followed by IAA production and phosphate solubilization (Table 2). Most of the siderophore-producing species belong to the genera

Table 1 Colony morphology and 16S rRNA sequence similarity of plant growth-promoting bacterial endophytes from sugarcane to the closest type strains of validly described species

Isolates (*n*)	Colony morphology[a]	Closest type strains		Accession no.
		Species	Similarity (%)	
Actinomycetes				
GKU 822 (5)	Dv, Dv	*Actinomadura rayongensis* RY35-68^T	98.7	KF638418
GKU 824 (9)	Syb, Syb	*Microbispora amethystogenes* JCM3021^T	99.1	KP637152
GKU 898 (8)	So, Mo	*Microbispora amethystogenes* JCM3021^T	98.6	KP702933
GKU 823 (12)	Myp, Myp	*Microbispora hainanensis* 211020^T	98.8	KR560040
GKU 885 (2)	Gy, Gy	*Micromonospora carbonacea* DSM 43815^T	99.6	KP637151
GKU 869 (3)	Lgo, Lgo	*Streptomyces angustmycinicus* NBRC 3934^T	99.9	KP702932
GKU 895 (23)	Lgrb, Mr	*Streptomyces canus* NRRLB-1989^T	99.4	KP637153
GKU 867 (2)	Gg, Ly	*Streptomyces lannensis* TA4-8^T	99.9	KP702931
GKU 866 (5)	Lgg, Dgy	*Streptomyces tendae* ATCC 19812^T	99.1	KP637154
GKU 810 (2)	Pg, Lgyb	*Streptomyces somaliensis* NBRC 12916^T	99.9	KP640619
GKU 883 (12)	Pg, Bg	*Streptomyces coerulescens* ISP 5146^T	99.5	KP765677
Bacteria				
EN-5 (1)	C, E, Co, Cr, −ve	*Acinetobacter nosocomialis* NIPH 2119^T	100.0	KP702924
EN-41 (1)	C, E, P, W, −ve	*Acinetobacter oleivorans* DR1^T	100.0	KP702928
EN-24 (2)	I, L, Um, Cr, +ve	*Bacillus amyloliquefaciens* subsp. *plantarum* FZB42^T	100.0	KP702917
EN-25 (7)	C, E, Co, Cr, +ve	*Bacillus aryabhattai* B8W22^T	99.9	KP702925
EN-27 (2)	I, Cu, Co, Cr, +ve	*Bacillus safensis* FO-36b^T	100.0	KP702915
EN-14 (1)	C, U, Co, Cr, +ve	*Bacillus aerophilus* 28K^T	100.0	KP702929
EN-7 (2)	C, U, R, Cr, +ve	*Bacillus subtilis* subsp. *inaquosorum* KCTC 13429^T	99.7	KP702936
EN-21 (20)	C, E, Co, O, −ve	*Enterobacter asburiae* JCM6051^T	99.0	KP765678
EN-23 (3)	C, E, Co, W, −ve	*Enterobacter cloacae* subsp. *dissolvens* LMG2683^T	100.0	KP765679
EN-17 (1)	C, U, Co, Cr, −ve	*Kosakonia cowanii* CIP 107300^T	99.8	KP702937
EN-20 (1)	C, E, Co, W, −ve	*Kosakonia oryzae* Ola 51^T	99.7	KP702913
EN-9 (1)	I, E, Co, Cr, +ve	*Lysinibacillus macroides* LMG 18474^T	100.0	KP702920
EN-31 (1)	C, E, Co, Y, +ve	*Micrococcus yunnanensis* YIM65004^T	99.9	KP702921
EN-26 (2)	C, E, Co, O, +ve	*Paenibacillus illinoisensis* NRRL NRS-1356^T	99.8	KP702927
EN-29 (2)	I, E, R, Cr, −ve	*Pantoea cypripedii* ATCC29267^T	98.4	KP702918
EN-4 (1)	I, E, R, Cr, −ve	*Pantoea dispersa* LMG 2603^T	99.8	KP702916
EN-37 (1)	I, E, R, W, −ve	*Pantoea dispersa* LMG 2603^T	99.3	KP702922
EN-34 (1)	C, E, Co, Cr, −ve	*Pseudomonas guariconensis* PCAVU11^T	99.6	KP702919
EN-28 (2)	C, E, Co, Y, +ve	*Staphylococcus cohnii* subsp. *cohnii* ATCC 29974^T	100.0	KP702923

[a] Color of aerial and substrate mycelia of endophytic actinomycetes on ISP2 medium. *Bg* blackish green, *Dgy* dark grayish yellow, *Dv* dark violet, *Gg* greenish gray, *Gy* grayish yellow, *Lgg* light greenish gray, *Lgo* light grayish olive, *Lgrb* light grayish reddish brown, *Lgyb* light grayish yellowish brown, *Ly* light yellow, *Mo* moderate orange, *Mr* moderate reddish, *Myp* moderate yellowish pink, *Pg* paul green, *So* strong orange, *Syb* strong yellowish brown. Colony morphology of endophytic bacteria on NA medium. Form of colony (C/I; circular/irregular), margin of colony (E/Cu/U/L; entire/curled/undulate/lobate), elevation (Co/Um/R/P; convex/umbonate/raised/pulvinate), color (Cr/Y/W/O; cream/yellow/white/off-white), Gram's straining (−ve/+ve; Gram negative/Gram positive). *n* number of isolates

Streptomyces, *Microbispora*, and *Bacillus*; while IAA producing dominant species were *Streptomyces*, *Microbispora*, and *Enterobacter*. Members of the genera *Streptomyces* and *Microbispora* were also the majority species capable of phosphate solubilization. However, the highest activity of phosphate solubilization was found in *Pantoea* sp. EN-29 and EN-39 (data not shown). Besides, ACC deaminase-production was mainly detected in species belonging to *Streptomyces* and *Bacillus* (Table 2).

To evaluate nitrogenase activity, endophytic bacteria were screened using the ARA method. Only six out of the 52 strains belonging to *Bacillus*, *Enterobacter*, *Lysinibacillus*, and *Pantoea* could reduce acetylene gas, ranging between 14.59 and 25.39 nmol C_2H_4 h^{-1} $(10^7$ cells$)^{-1}$.

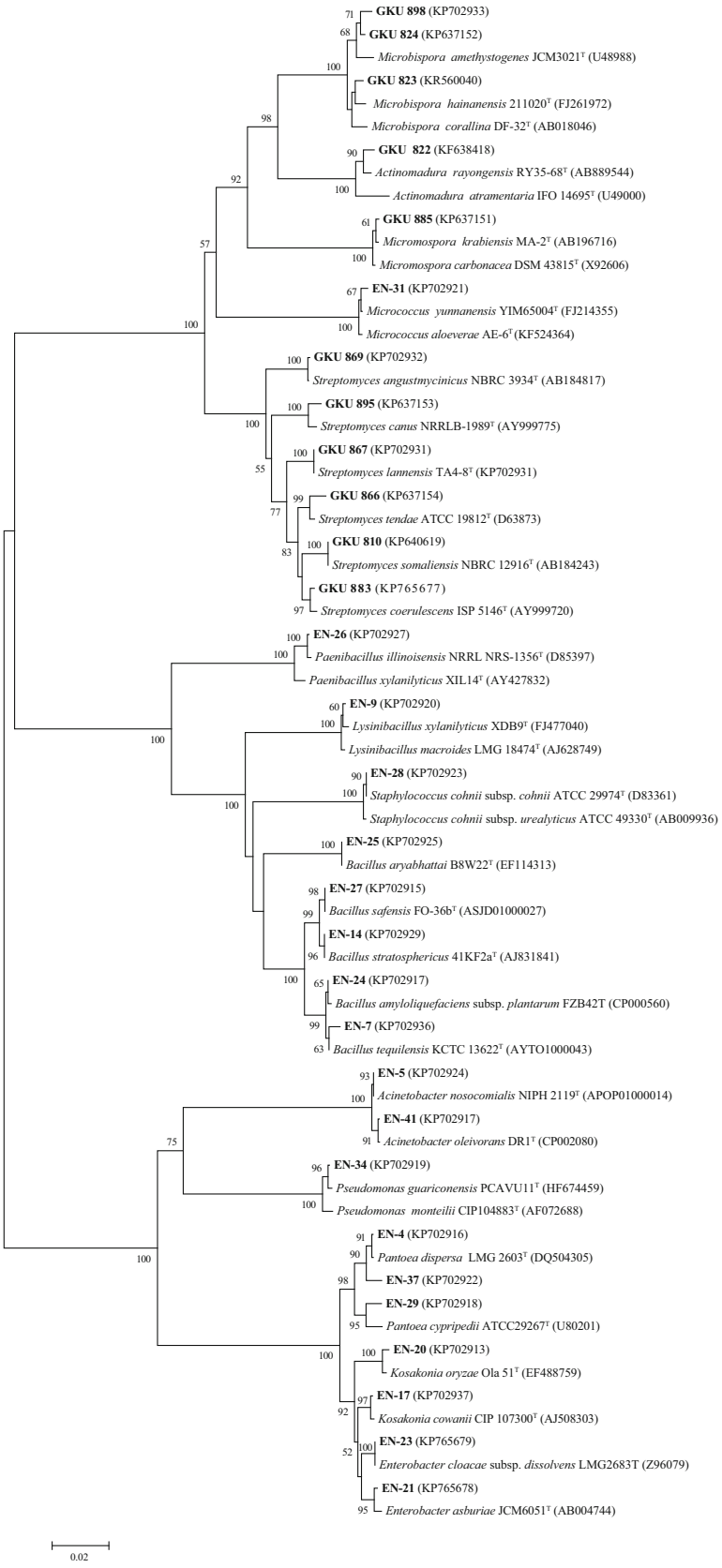

Fig. 1 Neighbor-joining tree based on 16S rRNA gene sequences showing relationships between the representative endophytic bacteria and actinomycetes isolated from sugarcane roots and the nearest type strains. Number of branch points indicates bootstrap value based on 1000 replications; only values above 50 % are shown. *Bar*, 0.01 substitutions per nucleotide position

Table 2 Number of positive isolates of sugarcane endophytic bacteria and actinomycetes with plant growth-promoting traits

Genus (*n*)	Plant growth-promoting traits[a] (%)					Antagonistic activity (%)						
	IAA	SD	P	ACC	ARA	BC	EC	PA	SA	AN	CF	FM
Actinomycetes												
Actinomadura (5)	2 (40)	4 (80)	–	–	ND	–	–	–	–	2 (40)	2 (40)	3 (60)
Microbispora (29)	14 (48.3)	20 (68.9)	19 (65.5)	–	ND	1 (3.4)	–	–	–	4 (13.8)	2 (6.9)	17 (58.6)
Micromonospora (2)	–	2 (100)	–	–	ND	–	–	–	–	2 (100)	1 (50)	2 (100)
Streptomyces (47)	26 (55.3)	33 (70.2)	18 (38.3)	11 (23.4)	ND	12 (25.5)	1 (2.1)	–	4 (8.5)	21 (44.6)	22 (46.8)	23 (48.9)
Total (83)	42 (50.6)	59 (71.0)	37 (44.6)	11 (13.2)	–	13 (15.7)	1 (1.2)	–	4 (4.8)	29 (34.9)	27 (32.5)	45 (54.2)
Bacteria												
Acinetobacter (2)	–	–	–	1 (50)	–	–	–	–	–	–	–	–
Bacillus (14)	3 (21.4)	10 (71.4)	3 (21.4)	7 (50)	2 (14.3)	5 (35.7)	6 (42.8)	–	1 (7.1)	4 (28.6)	8 (57.1)	8 (57.1)
Enterobacter (23)	11 (47.8)	9 (39.1)	2 (8.7)	3 (13)	2 (8.7)	–	–	–	–	–	4 (17.4)	5 (21.7)
Kosakonia (2)	1 (50)	–	–	–	–	–	–	–	–	–	–	–
Lysinibacillus (1)	–	–	–	1 (100)	1 (100)	–	–	–	–	–	–	–
Micrococcus (1)	1 (100)	–	–	–	–	–	–	–	–	–	–	–
Paenibacillus (2)	1 (50)	2 (100)	1 (50)	–	–	1 (50)	–	–	–	–	2 (100)	2 (100)
Pantoea (4)	2 (50)	–	3 (75)	–	1 (100)	–	–	–	–	–	–	3 (75)
Pseudomonas (1)	1 (100)	–	–	–	–	–	–	–	–	–	–	–
Staphylococcus (2)	–	–	–	1 (50)	–	–	–	–	–	–	1 (50)	1 (50)
Total (52)	20 (38.5)	21 (40.4)	9 (17.3)	13 (25)	6 (11.53)	6 (11.5)	6 (11.5)	–	1 (1.9)	4 (7.7)	15 (28.8)	19 (36.5)

[a] *IAA* indole-3-acetic acid, *SD* siderophore, *P* phosphate solubilization, *ACC* 1-aminocyclopropane-1-carboxylate deaminase, *ARA* acetylene reduction assay, *BC B. cereus* ATCC 11778, *EC E. coli* ATCC 8739, *PA Ps. aeruginosa* ATCC 15442, *SA Staph. aureus* ATCC 259233, *AN A. niger* ATCC 6275, *CF C. falcatum* DOAC 1655, *FM F. moniliforme* DOAC 1224, *n* number of strains, *ND* not determined, – none

Table 3 Identification and PGP traits of selected sugarcane diazotrophic bacteria and endophytic actinomycetes

Strains	Accession no.	Closest type strains	Similarity (%)	Plant growth-promoting traits[a]						Antagonistic activities			
				IAA	SD	P	ACC	ARA[b]	*nifH*	BC	AN	CF	FM
EN-21	KP765678	*Enterobacter asburiae* JCM6051^T	99.00	+	+	−	+	25.39	+[c]	−	−	−	−
EN-24	KP702917	*Bacillus amyloliquefaciens* subsp. *plantarum* FZB42^T	99.52	−	+	−	−	20.11	−	+	+	+	+
GKU 823	KR560040	*Microbispora hainanensis* 211020^T	98.77	+	+	+	−	ND	ND	−	+	−	−
GKU 895	KP637153	*Streptomyces canus* NRRL B-1989^T	99.36	+	+	+	+	ND	ND	+	−	+	+

[a] *IAA* indole-3-acetic acid, *SD* siderophore, *P* phosphate solubilization, *ACC* 1-aminocyclopropane-1-carboxylate deaminase, *ARA* acetylene reduction assay, *nifH* dinitrogenase reductase encoding gene, *BC B. cereus* ATCC 11778, *AN A. niger* ATCC 6275, *CF C. falcatum* DOAC 1655, *FM F. moniliforme* DOAC 1224, + positive activity, – negative activity, *ND* not determined

[b] ARA values represent activity expressed as nmol C_2H_4 h^{-1} $(10^{-7}$ cells$)^{-1}$

[c] Accession No. KT275189

The highest level of nitrogenase activity was found in *Enterobacter* sp. EN-21 (25.39) and *Bacillus* sp. EN-24 (20.11). To evaluate nitrogen fixation ability, *nifH* gene amplification was performed. Ten *nifH*-positive strains (19.23 % of the total) were obtained. There were five strains, namely *Bacillus* sp. EN-27, *Enterobacter* sp. EN-21, *Enterobacter* sp. EN-30, *Lysinibacillus* sp. EN-9, and *Pantoea* sp. EN-39 that exhibited both nitrogenase activity and a *nifH* gene (Fig. S1). However, the *nifH* gene was absent in an ARA-positive endophyte, *Bacillus* sp. EN-24.

Antagonistic potential tests conducted against seven test strains of bacteria and fungi revealed that the most susceptible fungi that interacted with the endophytes were *Fusarium moniliforme*, followed by *Aspergillus niger* and *Colletotrichum falcatum*, while the most susceptible bacterium was *Bacillus cereus* (Table 2). Surprisingly, none of the endophytes inhibited *Pseudomonas aeruginosa*. Six strains of endophytes, *Bacillus* sp. EN-8, *Bacillus* sp. EN-15, *Bacillus* sp. EN-24, *Actinomadura* sp. GKU 870, *Streptomyces* sp. GKU 833, and *Streptomyces* sp. GKU 878, effectively inhibited growth of *A. niger* and the pathogenic fungi, *F. moniliforme* and *C. falcatum*, causing red rot disease in sugarcane (Alexander and Viswanathan 2002). *B. cereus* and *Escherichia coli* were the test bacteria most frequently inhibited by the endophytic bacteria as well as actinomycetes (Table 2).

Sugarcane Plant Growth Enhancement

Based on PGP traits, two strains of endophytic diazotrophs, *Bacillus* sp. EN-24 and *Enterobacter* sp. EN-21, and two strains of actinomycetes, *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895, were selected to evaluate growth enhancement of sugarcane in pot experiments (Table 3). Antagonistic test between each strain was performed and reviewed no effect to each other; therefore, they were suitable for co-culture inoculation. Nine treatments included (T1) *Enterobacter* sp. EN-21, (T2) *Bacillus* sp. EN-24, (T3) *Microbispora* sp. GKU 823, (T4) *Streptomyces* sp. GKU 895, (T5) EN-21 + EN-24, (T6) GKU 823 + GKU 895, (T7) GKU 823 + EN-21 + EN-24, (T8) GKU 895 + EN-21 + EN-24, (T9) GKU 823 + GKU 895 + EN-21 + EN-24 and un-inoculated control were conducted to investigate individual and co-culture inoculation. Two-month-old sugarcane plants treated by either individual or co-inoculation gave statistically significantly ($p \leq 0.05$) greater root and shoot lengths, and root and shoot biomass than un-inoculated plants (Table 4; Fig. 2). Either individual endophytic diazotroph or actinomycete inoculated plants (T1, T2, T3, and T4) gave measurable increments in root length (51–89 %), shoot fresh weight (67–106 %), and shoot dry weight (76–146 %) but remarkably increased in root fresh weight (147–278 %; Table 4). The increase of root fresh weight was due to increased lateral root production (data not shown). These parameters were not significantly different when two strains of diazotrophs (T5) and actinomycetes (T6) were inoculated except shoot fresh (126 %) and dry weight (180 %) that T6 gave notably increased. The percentage increase in shoot length of individual and co-inoculated sugarcane were higher than that of the un-inoculated controls, especially T4, T5, T7, and T9, which were significantly (44–59 %) greater; while significant increase in root length was noticed in T7, T8, and T9 (125–128 %; Table 4). The most significant enhancement of growth appeared in T7 and T8 in which each endophytic

Table 4 Effect of individual and co-inoculation of endophytic diazotrophs and actinomycetes on growth of sugarcane plants at 60 days after inoculation

Treatments	Root			Shoot			Root re-isolation (CFU g FW^{-1})	
	Length (cm)	FW (g)	DW (g)	Length (cm)	FW (g)	DW (g)	Bacteria	Actinomycetes
Un-inoculated control	6.25 ± 0.96^{b}	1.04 ± 0.02^{f}	0.13 ± 0.02^{e}	30.58 ± 4.66^{b}	2.65 ± 0.08^{d}	0.30 ± 0.01^{e}	–	–
T1: *Enterobacter* sp. EN-21	9.45 ± 1.54^{ab}	3.24 ± 0.10^{de}	0.25 ± 0.03^{cd}	42.62 ± 6.64^{ab}	4.88 ± 0.16^{abc}	0.70 ± 0.03^{bc}	$5.27 \pm 0.35 \times 10^{4}$	–
T2: *Bacillus* sp. EN-24	11.43 ± 1.31^{ab}	3.93 ± 0.13^{abcd}	0.37 ± 0.01^{ab}	42.66 ± 3.48^{ab}	5.07 ± 0.22^{abc}	0.74 ± 0.04^{abc}	$8.22 \pm 0.17 \times 10^{4}$	–
T3: *Microbispora* sp. GKU 823	9.82 ± 1.53^{ab}	2.57 ± 0.05^{e}	0.16 ± 0.02^{e}	39.82 ± 2.22^{ab}	4.43 ± 0.10^{c}	0.53 ± 0.03^{d}	–	$9.47 \pm 0.07 \times 10^{3}$
T4: *Streptomyces* sp. GKU 895	11.85 ± 3.83^{ab}	3.34 ± 0.05^{cde}	0.26 ± 0.03^{cd}	43.98 ± 7.82^{a}	5.48 ± 0.12^{abc}	0.72 ± 0.05^{bc}	–	$2.05 \pm 0.50 \times 10^{4}$
T5: EN-21 + EN-24	12.05 ± 1.87^{ab}	3.79 ± 0.14^{abcd}	0.22 ± 0.05^{de}	45.74 ± 6.30^{a}	4.62 ± 0.11^{bc}	0.63 ± 0.03^{cd}	$8.27 \pm 0.70 \times 10^{4}$	–
T6: GKU 823 + GKU 895	11.49 ± 3.48^{ab}	4.24 ± 0.08^{abc}	0.33 ± 0.05^{abc}	40.64 ± 5.84^{ab}	5.99 ± 0.08^{a}	0.84 ± 0.09^{a}	–	$9.22 \pm 0.10 \times 10^{3}$
T7: GKU 823 + EN-21 + EN-24	14.25 ± 3.71^{a}	4.70 ± 0.10^{a}	0.36 ± 0.09^{ab}	48.22 ± 6.56^{a}	5.73 ± 0.07^{ab}	0.74 ± 0.07^{ab}	$6.67 \pm 0.81 \times 10^{3}$	$5.31 \pm 0.15 \times 10^{3}$
T8: GKU 895 + EN-21 + EN-24	14.30 ± 5.24^{a}	4.33 ± 0.05^{ab}	0.40 ± 0.04^{a}	41.92 ± 4.60^{ab}	5.98 ± 0.05^{a}	0.78 ± 0.06^{ab}	$4.72 \pm 0.65 \times 10^{3}$	$4.35 \pm 0.73 \times 10^{3}$
T9: GKU 823 + GKU 895 + EN-21 + EN-24	14.12 ± 2.95^{a}	3.46 ± 0.10^{bcde}	0.28 ± 0.03^{bcd}	48.64 ± 2.21^{a}	5.29 ± 0.07^{abc}	0.73 ± 0.02^{bc}	$1.49 \pm 0.11 \times 10^{3}$	$3.01 \pm 0.85 \times 10^{3}$

Data are means ± standard deviations (SD) of five replicates. Means designated with same letters do not differ significantly ($p \leq 0.05$) according to the Tukey's multiple range test

FW fresh weight, *DW* dried weight

actinomycete was co-inoculated with both *Enterobacter* sp. EN-21 and *Bacillus* sp. EN-24. T7 and T8 caused drastic increases of 128–129 % in root length, 316–352 % in root fresh weight, 177–208 % in root dry weight, 37–58 % in shoot length, 116–126 % in shoot fresh weight, and 146–160 % in shoot dry weight over those of un-inoculated treatments. The results suggested that the enhancement of growth in sugarcane was due to multiple growth-promoting traits from the mixture of the bacterial endophytes (Table 3). Our results indicated that co-inoculation of either endophytic *Microbispora* or *Streptomyces* combined with the mixture of *Enterobacter* and *Bacillus* gave higher growth of sugarcane than individual inoculation.

Remarkably, when four strains of endophytes were co-inoculated onto sugarcane plants (T9), some growth parameters such as root and shoot fresh/dried weights were significantly lower than those of T7 and T8 and some (root fresh/dried weight) were even lower than single-strain inoculation (Table 4). Re-isolation experiments of the endophytes from two-month-old sugarcane plants were then performed and the identity of isolates was proved by examination of colony morphology and 16S rRNA sequencing. Inoculated endophytic diazotrophs and actinomycetes abundantly appeared in roots but less in stems in every treatment (data not shown). About 10^4 CFU g fresh weight^{-1} were found in sugarcane roots inoculated with individual (T1, T2, T3, T4) and combinations of two bacterial strains (T5 and T6; Table 4). However, the viable cell numbers of the endophytes decreased 10 times when more than two strains of bacteria were co-inoculated (T7, T8, and T9); particularly T9 gave the lowest. This might be explained by a reduction of plant biomass in T9 (Table 4) due to the low population of the endophytes. In addition, this re-isolation experiment confirmed that *Enterobacter* sp. EN-21, *Bacillus* sp. EN-24, *Microbispora* sp. GKU 823, and *Streptomyces* sp. GKU 895 are true endophytes of sugarcane.

Discussion

Plant growth-promoting endophytes (PGPE) are heterogeneous groups of bacteria that beneficial to host plants (Gaiero and others 2013). Several PGPE can encourage plant growth through the release of phytohormones, increment of nutrients, and protection from phytopathogens. In this work, culture-dependent approach was used to isolate culturable root-associated bacteria including actinomycetes from sugarcane cultivated in Thailand. The sugarcane roots used for isolation in this study were successfully surface disinfected in which no colonies were detected from the final rinses on any medium agar used;

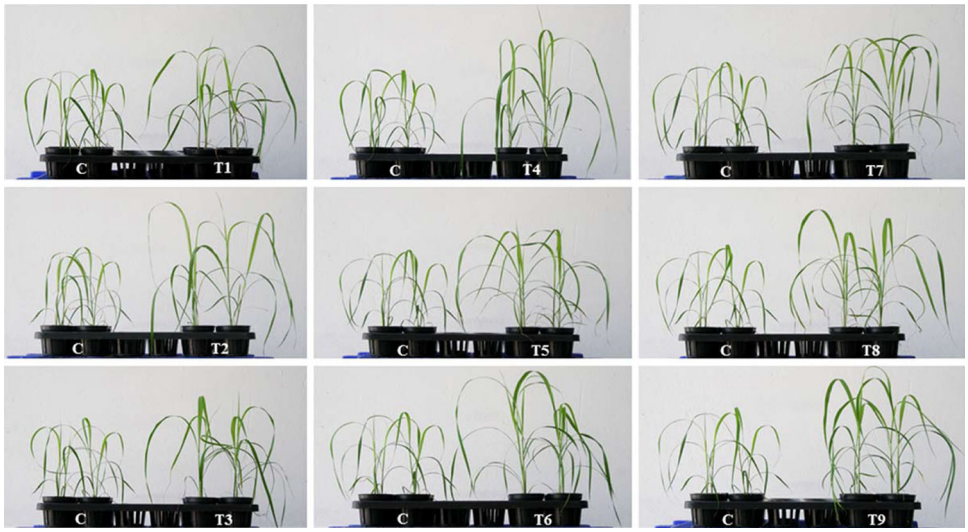

Fig. 2 Effects of individual and co-inoculation of endophytic bacteria and actinomycetes on growth promotion of sugarcane plants at 60 days after inoculation. T1, *Enterobacter* sp. EN-21; T2, *Bacillus* sp. EN-24; T3, *Microbispora* sp. GKU 823; T4, *Streptomyces* sp. GKU 895; T5, EN-21 + EN-24; T6, GKU 823 + GKU 895; T7, GKU 823 + EN-21 + EN-24; T8, GKU 895 + EN-21 + EN-24; T9, GKU 823 + GKU 895 + EN-21 + EN-24 and C, un-inoculated control

thus, the isolated bacteria were true endophytes. From the isolation procedures used in this work, 38.52 % of endophytic bacteria and 61.58 % of actinomycetes were obtained. Fourteen genera were systematically identified and the most dominant species were *Bacillus*, *Enterobacter*, *Microbispora*, and *Streptomyces*.

Diazotrophs appeared to be the most numerous bacteria so far isolated from sugarcane including *Azospirillum* spp., *Burkholderia* spp., *Enterobacter* spp., *Gluconacetobacter diazotrophicus*, *Herbaspirillum* spp., *Klebsiella* spp., *Pantoea* spp., and *Pseudomonas* spp. (Asis and others 2000; Suman and others 2001; Perin and others 2006; Govindarajan and others 2007; Mendes and others 2007; Castro-González and others 2011; Taulé and others 2011; Lin and others 2012). Surprisingly, the well-known sugarcane diazotrophic species, including *Azospirillum* spp., *Burkholderia* spp., *G. diazotrophicus*, and *Herbaspirillum* spp. (Govindarajan and others 2007; Taulé and others 2011), were not found in this work. A plausible explanation for their notable absence could be that the composition of the endophytic bacterial community depends to some degree on a specificity of particular bacterial genotypes for particular sugarcane cultivars, different geographic origins, and level of fertilizers used (Reis-Junior and others 2000; Taulé and others 2011). Moreover, the sugarcane root-associated nitrogen-fixing bacteria in this work were isolated without using the enrichment method of reseeding in the N-free medium (Castro-González and others 2011), the amount of diazotrophic bacteria could, therefore, be affected and resulted in less population. The majority of endophytic bacteria isolated in this study were mainly found as members of the genera *Bacillus* and *Enterobacter*. The results were in agreement with several reports of which *Bacillus* and *Enterobacter* were dominant sugarcane endophytes (Velazquez and others 2008; Magnani and others 2010; Taulé and others 2011).

Although endophytic actinomycetes have been isolated from several different kinds of plants including a variety of medicinal and crop plants (Coombs and Franco 2003; Tian and others 2007; Zhao and others 2011), this is the first report to describe the diversity of endophytic actinomycetes from sugarcane. The dominance of *Streptomyces* spp. in the culturable diversity of root-associated endophytes in this report has been noticed in several previous studies from various plant species, that is, wheat, rice, and herbaceous and medicinal plants (Coombs and Franco 2003; Tian and others 2007; Zhao and others 2011; Kim and others 2012; Li and others 2012). However, we found different genera of non-streptomycetes (*Actinomadura*, *Microbispora*, and *Micromonospora*) associated with roots of sugarcane (Fig. 1). The presence of *Actinomadura*, *Microbispora*, and *Micromonospora* was in agreement with previous reports that they are common genera found in root tissues of plants (Qin and others 2011; Mingma and others 2014). Although *Streptomyces* spp. was predominantly associated with sugarcane roots, we observed a large number of colonies of *Microbispora* spp. (21.48 %). The results suggested that *Microbispora* spp. also largely occupied in root tissues of sugarcanes. This work indicated that sugarcanes are rich bio-resources for diversity of endophytic bacteria including diazotrophs and actinomycetes.

PGP traits were examined in sugarcane endophytes and revealed that strains belong to genera *Streptomyces*, *Bacillus*, and *Enterobacter* showed the highest activity of PGP traits. *Bacillus* and *Enterobacter* have been previously reported to promote the growth of maize, sugarcane, and tobacco (Kloepper and others 2004; Lin and others 2012; Naveed and others 2014). The highest frequency of PGP

trait possessed by sugarcane endophytes in this study was siderophore production, followed by IAA production and phosphate solubilization. It has been suggested that siderophores are involved in both plant growth and health protection by chelating iron (Jaber and others 2002; Rungin and others 2012; Radzki and others 2013). Recent work of Rungin and others (2012) also confirmed that a siderophore-producing endophytic *Streptomyces* increased root/shoot lengths and biomass of rice and mung bean compared to the deficient mutant. It was demonstrated that bacterial endophytes carrying IAA trait could increase root elongation and could affect the development of lateral roots, which improves the plant's nutrient uptake from the rhizosphere (Idris and others 2007; Goudjal and others 2013). A recent report showed that *Microbispora* spp. and *Streptomyces* spp. exhibited a high variability in IAA production and resulted in significant promotion of shoot length of mandarin seedlings (Shutsrirung and others 2013). Members of PGPE in the genera *Bacillus*, *Enterobacter*, *Pseudomonas*, *Micromonospora*, and *Streptomyces* are known as phosphate solubilizers (Hamdali and others 2008; Bashan and others 2013) with the ability to convert insoluble compounds of phosphorus into available phosphates that enhance nutrient availability to plants (Son and others 2006). However, the highest activity of phosphate solubilzation in this work was found in *Pantoea* spp. Recently, phosphate-solubilizing *Pantoea* has been reported as a plant growth promoter which increased height and dry weight of *Lotus tenuis* cv. Pampa INTA (Castagno and others 2011). PGPE harboring ACC deaminase in sugarcane could facilitate plant growth by conversion of ACC to ammonia and α-ketobutyrate, which bacteria can consume and consequently lower the ethylene level in plants (Glick 2005).

Most of the endophytic bacteria isolated from sugarcane have been reported as diazotrophs, with effects on plant growth promotion (Govindarajan and others 2007; Lin and others 2012). In this work, only 11.53 % out of sugarcane endophytic bacteria showed nitrogenase activity belonging to *Bacillus*, *Enterobacter*, *Lysinibacillus*, and *Pantoea* (Table 2). Although the standard protocol for isolation of diazotrophic bacteria was employed (Cavalcante and Dobereiner 1988), fewer ARA positives were often obtained from plants such as rye grass (Habibi and others 2014) and rice (Rangjaroen and others 2015). Our ranges of ARA activity were in accordance with previous reports of *Enterobacter* spp. and *Bacillus* spp. (Habibi and others 2014). When *nifH* gene amplification was investigated to evaluate nitrogen fixation ability of ARA-positive endophytes, 19.23 % of the total was detected (Table 2). There were only five strains which exhibited both nitrogenase activity and a *nifH* gene (Fig. S1). Our results were in agreement with Yim and others (2009) in which the *nifH* gene could not be amplified from all ARA positives. Although, ARA is a common method to detect nitrogenase activity of microbial cultures and has been widely used to consequently identify nitrogen-fixing bacteria (Hardy and others 1973), it was suggested that such activity varies with growth stage, culture condition, and media composition. Hence, amplification of the *nifH* gene, encoding dinitrogenase reductase, was additionally used to perform the possible nitrogen-fixing ability. Although the *nifH* gene is the most widely used marker gene for identification of nitrogen-fixing bacteria, nucleotide sequences of *nifH* genes were diverse in many microorganisms (Waugh and others 1995; Zehr and others 2003). In this experiment, degenerate primers were used for amplification of *nifH* gene (Rösch and others 2002), nevertheless, it was yet absent in an ARA-positive *Bacillus* sp. EN-24 (Fig. S1). The absence of the *nifH* gene could therefore be explained by the variability of this gene (Waugh and others 1995; Zehr and others 2003). It is suggested that the other nitrogenase genes, *nifD* and *nifK*, could be used to confirm the nitrogen-fixing ability since *nifHDK* genes are known to encode the components of the nitrogenase enzyme complex (Howard and Rees 1996).

Some recent reports have indicated that the root nodule-associated *Micromonospora* has the possible ability to fix atmospheric nitrogen (Trujillo and others 2010; Lorena and others 2012), but endophytic *Micromonospora* from medicago plants apparently had no functional nitrogenase and consequently no nitrogen fixation occurred (Martunez and others 2014). Because nitrogen fixation in endophytic actinomycetes has not yet been definitively explained and there is very little information about it, ARA assays were not performed on actinomycetes in this work.

Antagonistic tests revealed susceptible bacteria and fungi including *F. moniliforme* and *C. falcatum* causing red rot disease in sugarcane (Alexander and Viswanathan 2002) that interacted with the endophytes. *B. cereus* and *E. coli* were most frequently inhibited (Table 2), which was consistent with a previous report (Hassan and others 2010). The results of this study indicated that endophytic bacteria and actinomycetes from sugarcane carried multiple traits of PGP and are therefore considered to be safe agents to apply for plant growth enhancement and to control phytopathogens in sugarcane plantations.

Based on PGP traits, two endophytic diazotrophs, *Bacillus* sp. EN-24 and *Enterobacter* sp. EN-21, and two strains of actinomycetes, *Microbispora* sp. GKU 823 and *Streptomyces* sp. GKU 895, were selected to evaluate growth enhancement of sugarcane plants by individual and co-inoculation. Two-month-old sugarcane plants treated by either individual or co-inoculation gave significantly greater biomass and lengths of root and shoot than uninoculated control (Table 4; Fig. 2). The increase of root

fresh weight was due to increased lateral root production, which might be the effect of plant hormone production by the endophytes (Idris and others 2007; Goudjal and others 2013). The most significant enhancement of growth appeared in the treatment that each endophytic actinomycete was co-inoculated with both *Enterobacter* sp. EN-21 and *Bacillus* sp. EN-24. Because some *Enterobacter* species are known to be human pathogens, *Enterobacter* sp. EN-21 should be further investigated concerning human health if it is to be applied at the field level. It has been demonstrated that several diazotrophic bacteria, including *Bacillus* and *Enterobacter*, could promote growth of sugarcane (Lin and others 2012) as well as jatropha, maize, rice, sorghum, sugarcane, and wheat (Olivares and others 1997; Yanni and others 2001; Govindarajan and others 2007; Luna and others 2010; Rungin and others 2012; de Jesus Santos and others 2014; Alves and others 2015; Qin and others 2015). Furthermore, co-inoculation of five diazotrophs (*Azospirillum*, *Burkholderia*, *G. diazotrophicus*, *Klebsiella*, and *Pseudomonas*) was reported to give better growth of rice (Govindarajan and others 2007); while, co-culture of diazotrophs and a fungal endophyte, *Trichoderma*, revealed better growth and yield of chickpea (Verma and others 2014). It was suggested that the combination of phosphate-solubilizing, nitrogen-fixing, and phytohormone producing bacteria could provide a more balanced nutrition for plants and stimulate more growth of plants. For example, the combination of a phosphate-solubilizing *B. megaterium*, nitrogen-fixing *B. subtilis*, and *Rhizobium leguminosarum bv. phaseoli* caused a significant enhancement of seed yield and an uptake of macronutrients and micronutrients elements in common bean (Elkoca and others 2010). Mixed inoculation of *Rhizobium*, a phosphate-solubilizing *B. megaterium* subsp. *phospaticum* strain-PB, and a biocontrol fungus *Trichoderma* sp. was reported to increase seed germination, nutrient uptake, plant height, number of branches, nodulation, pea yield, and total biomass of chickpea compared to either individual inoculation or an un-inoculated control (Rudresh and others 2005). Therefore, the plant growth-promoting properties of endophytic bacteria and actinomycetes suggest that these bacteria merit further investigation for potentially safe and environmentally friendly biofertilizers which can help us limit the use of chemical fertilizers in agriculture.

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Compliance with Ethical Standards

Conflict of interest None.

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