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Original Article

Multiple Mechanisms of *Enterobacter asburiae* strain RS83 for Plant Growth Enhancement

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Abstract

Enterobacter asburiae strain RS83 having the capacity of promoting plant growth and yield of various vegetable crops was isolated from the rhizosphere of cassava in Phitsanulok province. The objective of this study was to investigate possible bacterial determinants of strain RS83 involved with plant growth enhancement. Different methods such as using specific media, biochemical tests and high performance liquid chromatography technique were used to analyze bacterial determinants of interest relevant to plant growth promotion. Results revealed several bacterial determinants of strain RS83 implicated in plant growth improvement. These determinants released from strain RS83 included indole-3 acetic acid (IAA; plant-hormone like), catechol siderophore enterobactin (iron-chelating agent), and gluconic acid relating to phosphate solubilization. Moreover, the presence of pellicles in a semi-solid nitrogen-free medium inoculated with strain RS83 indicated possible nitrogen-fixing capacity of the bacterium. In conclusion, strain RS83 possesses several bacterial determinants involved with plant growth promotion.

Keywords: *Enterobacter asburiae* strain RS83, bacterial determinants, plant growth enhancement

1. Introduction

The important role of free-living plant growth-promoting rhizobacteria (PGPR) in agriculture and horticulture for improving crop productivity has been demonstrated (Lucy *et al.*, 2004). Effects of PGPR on various crop plants have been studied for more than three decades. Plants receive the benefits of growth and yield promotion from PGPR in several ways ranging from enhancing germination rates (Nezarat and Gholami, 2009), promoting vegetative development (Dobbelaere *et al.*, 2001; Jetiyanon and Plianbangchang, 2010), and stimulating crop productivity (Alam *et al.*, 2001; Jetiyanon and Plianbangchang, 2012; Kloepper *et al.,* 1989).

Several mechanisms for plant growth and yield enhancement by PGPR have been explored to explain how plants respond and earn benefits from the bacteria (Vessey, 2003). In addition, bacterial determinants of PGPR for plant growth promotion have been investigated in different plant-microbe systems. 1-Aminocyclopropane-1-carboxylate (ACC) deaminase enzyme released from certain microorganisms could hydrolyze ACC in the plant into ammonia and α-ketobutyrate (Glick *et al.*, 1998) resulting in lowered plant ethylene level. This facilitates the formation of longer roots which enhance survival of seedlings during the first few days of germination (Glick, 2005). Significant change of root growth and morphology by the action of indole-3-acetic acid (IAA) released from PGPR (German *et al.*, 2000) influences plants'nutrient uptake potentials. Increasing the availability of nutrients in the rhizosphere for plants by PGPR could involve phosphate solubilization of unavailable forms of nutrients (Richardson, 2001) and/or by facilitating the transport of ferric iron by siderophore released from PGPR (Masalha *et al.*, 2000). Some PGPR exert beneficial effects on plants by the process of nitrogen fixation by delivering combined nitrogen to the plant (Ashraf *et al.*, 2011). However, most studies mentioned above presented a single mode of

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action executed by a specific PGPR strain for plant growth promotion. Until now, little evidence has demonstrated multiple modes of actions for plant growth promotion executed by a single PGPR strain.

Enterobacter asburiae strain RS83 was originally isolated from the rhizosphere of cassava in Phitsanulok, Thailand. The strain RS83 has the capacity to induce resistance to disease against several diseases (Jetiyanon and Wittaya-areekul, 2009). Recently, lipopolysaccharide of strain RS83 was reported to trigger early defensive-related enzymes accountable for disease reduction (Jetiyanon and Plianbangchang, 2013). In the absence of pathogen, the bacterium demonstrated growth benefits for various vegetables such as fruit vegetables (cucumber, tomato, pepper) and leafy vegetables (lettuce and green kuang futsoi) (Jetiyanon, 2002; Figure 1). Therefore, the objective of this study was to explore possible bacterial determinants of strain RS83 for plant growth enhancement.

2. Materials and Methods

2.1 Bacterial maintenance and culture

E. asburiae strain RS83 was maintained in tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD, USA) supplemented with 20% glycerol at -80°C for long-term storage. For experimental use, the frozen bacterium was transferred onto tryptic soy agar (TSA; Becton Dickinson) and was incubated at 30°C for 24 h before use.

2.2 IAA measurement and analysis

Bacillus cereus strain RS87, previously shown to produce IAA and to promote growth in cucumber, pepper, and rice was included in this study as the reference strain (Jetiyanon *et al.*, 2008). *E. asburiae* strain RS83 *and B. cereus* strain RS87 were grown in a modified Nutrient Broth-M26 (NB-M26) for 24 h in the Environmental Shaker-Incubator ES-20 (BIOSAN, Riga, Latvia) at 200 r/min at 30°C as seed culture. The procedure of IAA measurement was conducted as described by Gordon and Weber, 1951. The amount of IAA produced per milliliter culture was estimated using the IAA standard curve. A standard absorption curve was obtained from authentic IAA (Sigma-Aldrich Co., USA) dissolved in absolute ethanol at different concentrations. The test was replicated three times. The appearance of a pink color in the solutions after reaction time represents the existence of IAA. The more intense the pink color in the solution the higher the IAA amount present.

To analyze and quantify IAA amount produced by strain RS83, the bacterium was cultured in 20 mL of modified NB-M26 for 24 h as a seed culture and then 1mL of a culture was inoculated to 100 mL of MS medium amended with 5 mM L-tryptophan. The culture was incubated for another 48 h on the shaker. Bacterial broth culture was centrifuged at 12,000*g* for five min. The supernatant was acidified to pH 2.8 with 1N HCl. IAA was then extracted from supernatant three times with ethyl acetate (1:3). The extract was filtered through 0.45-µm membrane. The ethyl acetate was evaporated at 40°C using BÜCHI Rotavapor model R200 (BÜCHI Labortechnik GmbH, Deutschland, Germany). The extracted residue was immediately dissolved in 2 ml absolute methanol, kept in a small vial and maintained at 4°C until used. HPLC chromatograms were produced by injecting 30µl of 20 times dilution culture extract into a VertiSep™ Silica-Based IRS C18 HPLC column (3.9 mm x 300 mm, 10 µm, Vertical Chromatography Co., Ltd, Thailand) using the equipment of Shimadzu CLASS-VP V6.14 SP1 (Tokyo, Japan). The concentration of authentic IAA was diluted 10 times before injecting into HPLC column. Two solvent systems were used to separate indole compounds. Solvent A was water:acetonitrile:acetic acid (85:15:1 [vol/vol]), the flow rate was 1.5 mL/min, and the operating pressure was 1,400 lb/in^2. Solvent B was 30% methanol in water, the flow rate was 1.5 mL/min, and the operating pressure was 1,600 lb/in^2. IAA was quantified with UV detector set at 254 nm (modified procedure from Tien *et al.*, 1979). The concentration of IAA in the culture extract was acquired by comparing its respective peak area with the corresponding area obtained by the authentic IAA (0.13 mg/mL; Sigma- Aldrich Co., USA). At least three independent replicate extracts were analyzed.

2.3 Phosphate solubilization test and analysis

A qualitative phosphate solubilization test was carried out in National Botanical Research Institute's phosphate growth medium containing bromophenol blue (NBRIP-BPB) modified from Mehta and Nautiyal (2001) procedures. The strain RS83 was prepared by culturing the bacterium for 2

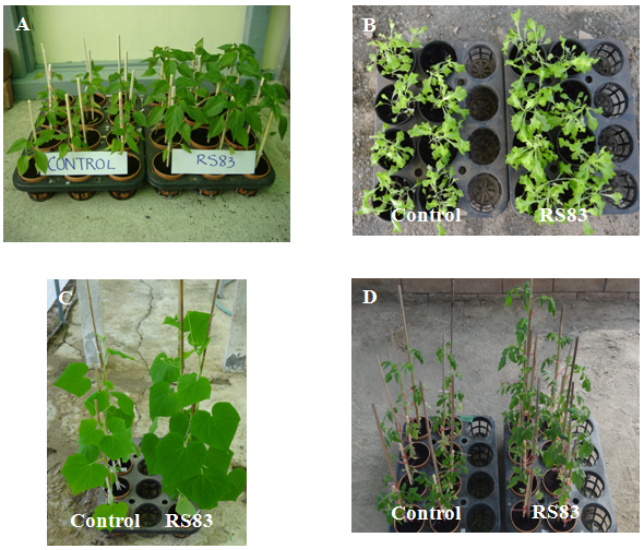

Figure 1. The capacity of *E. asburiae* strain RS83 for growth enhancement in different plant families. (A) Peppers, (B) lettuces, (C) cucumbers, and (D) tomatoes.

days in TSB medium. Then, 50 ml of strain RS83 (log 8.0 CFU/ml) was transferred to 5 mL of NBRIP-BPB medium in a 30-mL test tube. The test tubes were incubated at a constant temperature of 30°C in Shaker-Incubator for 5 days. The cultures were harvested by centrifugation at 10,000*g* in a refrigerated table-top centrifuge for 10 minutes at 25°C. The culture supernatant was obtained. The optical density of the culture supernatant was measured at 600 nm by using a DR/4000U spectrophotometer. The pH of the culture supernatant was also measured by a Sartorius Docu-pH meter (Goettingen, Germany). The reduction of blue color in the solution and a drop in pH of the culture supernatant after incubation indicate the capacity of the bacterium to solubilize phosphate. *B. cereus* strain RS87 previously shown to solubilize phosphate was used as a reference strain (Jetiyanon and Plianbangchang, 2010). Sterile TSB without a bacterium served as the control. The test was replicated three times.

For determination and quantification of organic acid type in the culture medium relating to phosphate solubilization executed by the strain RS83, the bacterium was cultured in 50 mL of Luria Bertani (LB; Becton Dickinson, Sparks, MD, USA) contained in a 250 mL Erlenmeyer flask. The flask was incubated in Shaker-Incubator at 30°C for 24 h. The bacterial cells were washed with 10mM potassium phosphate buffer (pH 7.5) two times. Then 10 mL of bacterial suspension in the buffer was inoculated to 100 mL of GSM medium and incubated at 37°C for 24 h (Safura *et al.*, 1995). The supernatant was obtained by centrifugation at 10,000*g* for 10 min and filtered through 0.2 µm Sartorius filter. The organic acid in filtrates was identified by HPLC with a VertiSep™ Silica-Based IRS C18 HPLC column. Organic acid was monitored using a UV detector at 220 nm. The mobile phase consisted of 50mM sodium phosphate and 5 mM tetra-butyl-ammonium hydrogen sulfate, pH 6.5 (95%), plus acetonitrile (5%) and a flow rate of 0.25 mL/min·(modified from Song *et al.*, 2008). Fifty percent gluconic acid solution in water (1,240 mg/mL; Merck, Switzerland) was run as a positive control to identify its peak. The tests were repeated independently three times in different culture replicates.

2.4 Siderophore production test and analysis

Chrome azurol S (CAS) agar medium was used for testing the capacity of strain RS83 to produce siderophores. CAS agar was prepared from four solutions which were sterilized separately before mixing. The procedure was conducted according to Alexander and Zuberrer (1991). The bacterial strain RS83 grown on TSA medium for 24 h was transferred and streaked onto CAS agar medium in a petri dish. The bacterium was incubated in the dark at 30°C. The orange halo around the bacterial colony was observed daily for 3 days. The test was repeated three times.

To analyze the siderophore type, a starter culture was prepared by transferring one bacterial colony grown on TSA medium to 100 mL of LB medium in 250-mL Erlenmeyer flask. The culture was incubated in the shaker-incubator for 24 h at 30°C. One milliliter of the starter culture was then used to inoculate a 1,000-mL Erlenmeyer flask containing one-fifth volume of the second basal medium. The procedure was modified from Poole *et al.* (1993). The culture was incubated in a shaker incubator at 35°C until the cells were in the late logarithmic phase of growth. The bacterial culture was harvested by centrifugation at 9,000*g* for 10 min, and the supernatant was decanted and saved. The supernatant was acidified by 10N HCl to pH 2.5. Siderophore was extracted from the culture supernatant with 1 volume of ethyl acetate washed with 0.1M citrate buffer (pH 5.5). The method was modified from Costa and Loper (1994). The extracted residues were re-suspended in 2 mL absolute methanol, kept in a small vial, and maintained at 4°C until use. HPLC was used to examine and quantitatively assess the released siderophore from strain RS83. The extracted sample from strain RS83 was diluted with absolute HPLC methanol grade 2,000 times before injecting 30µl of the sample into a VertiSep™ Silica-Based IRS C18 HPLC apparatus. The sample was separated by linear gradient elution using methanol with 0.1% formic acid as solvent A and acetonitrile as solvent B at a flow rate of 1 ml/min (modified from Fiedler *et al.*, 2001). Siderophore was quantified with fluorescence detector set at 280 nm and 440 nm (excitation and emission, respectively) using HPLC equipment. A hundred times dilution of purified enterobactin (10mg/1mµl) from *Escherichia coli* (Sigma-Aldrich; USA) was run as a positive control to identify its peak. The experiment was repeated three times.

2.5 Nitrogen fixation test

The strains RS83 and RS87 were grown on TSA medium at 30°C for 24 h. About ¼ of a sterile loop of the bacterial culture was scraped from the medium and then inoculated into 5 mL semi-solid JNFb medium in a sterile test tube (15 mm x 150 mm) by gently spinning the loop to disperse bacterial cells into ¾ of the culture medium. The procedure of media preparation was executed according to Olivares *et al.*, 1996. The un-inoculated medium served as a control.

Veil-like pellicles appearing in the semisolid JNFb medium 4 days after inoculation were transferred and streaked onto JNFb agar plates (Baldani *et al.*, 1992). If the bacterium could produce pellicles in a semi-solid JNFb medium and if the transferred pellicles could grow on JNFb agar, this would indicate nitrogen fixation by the bacterium since the media are both nitrogen-free. The experiment was repeated 5 times.

3. Results

3.1 Indole-3-acetic acid

Results from colorimetric analysis demonstrated that strain RS83 produced IAA at amounts (68 µmol/mL) 2.3 times greater than produced by strain RS87 (29 µmol/mL) as shown

Table 1. Indoleacetic acid production by *Enterobacter asburiae* strain RS83 and *Bacillus cereus* strain RS87.

Bacterial strains	IAA production (µmol/mL)
Enterobacter asburiae strain RS83	68.29
Bacillus cereus strain RS87	29.43

Note: IAA production from each bacterial strain was performed at least 3 times and the data shown in the table are the means of IAA production in each strain.

in Table 1.

HPLC analysis revealed that the culture extract of strain RS83 showed two prominent peaks appearing at different retention time. One of them was IAA peak presented at the retention time 18.276 min relevant to the retention time of the authentic IAA peak which was at 18.272 min (Figure 2). The area under the authentic IAA peak was 97% of the total peak area, while the IAA peak extracted from the culture of strain RS83 was 30% of the total peak area. Once calculated back to the original concentration of the culture extract based on comparison with the known authentic IAA concentration, it was found that the strain RS83 produced approximately 118 µmol/mL of IAA. The sensitivity of IAA amount detected by HPLC equipment was about 1.7 times greater than the amount detected with colorimetric assay.

3.2 Phosphate solubilization

There was a decrease in blue color of NBRIP-BPB medium overtime in the inoculated test tubes, while consistent color appeared in the un-inoculated control. Five days after bacterial inoculation, partial disintegration of $Ca_3(PO_4)_2$ was observed at the bottom of the inoculated tubes of both strain RS83 and strain RS87 (Figure 3). The optical density (OD) of the solution inoculated with strain RS83 (OD=0.713) and strain RS87 (OD=0.746) was approximately 17% and 14% less than the un-inoculated control (OD=0.863), respectively. In addition, the pH of the culture supernatant inoculated with strain RS83 and strain RS87 was reduced to 3.27 and 4.39, respectively, while the pH of the un-inocualted control was 6.47, almost similar to the pH before incubation (Table 2).

Chromatograms of the standard 50% gluconic acid , demonstrated by HPLC method, showed two peaks at different retention times but only one prominent, a sharp peak at the retention time 13.502 min corresponding to gluconic acid (Figure 4). The area under the standard gluconic acid peak was 55% of the total peak area which was comparable to the proportion of a standard 50% gluconic acid solution. The culture supernatant inoculated with strain RS83 exhibited several chromatograms at different retention times, however only one outstanding peak was presented at the retention time 13.501 min. The peak was at a similar position shown in

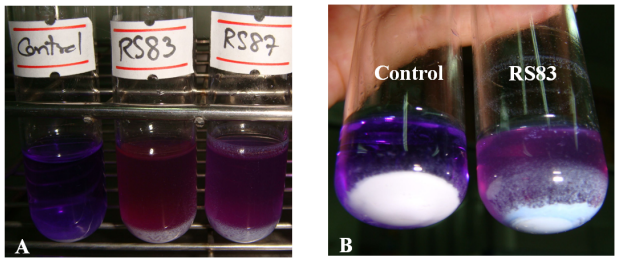

Figure 3. A) decrease in blue color 5 days after incubation in test tubes inoculated with *E. asburiae* strain RS83 and *B. cereus* strain RS87 comparing with uninoculated control (left). B) Partial disintegration of $Ca_3(PO_4)_2$ at the bottom of a test tube inoculated with strain RS83 compared with an uninoculated control.

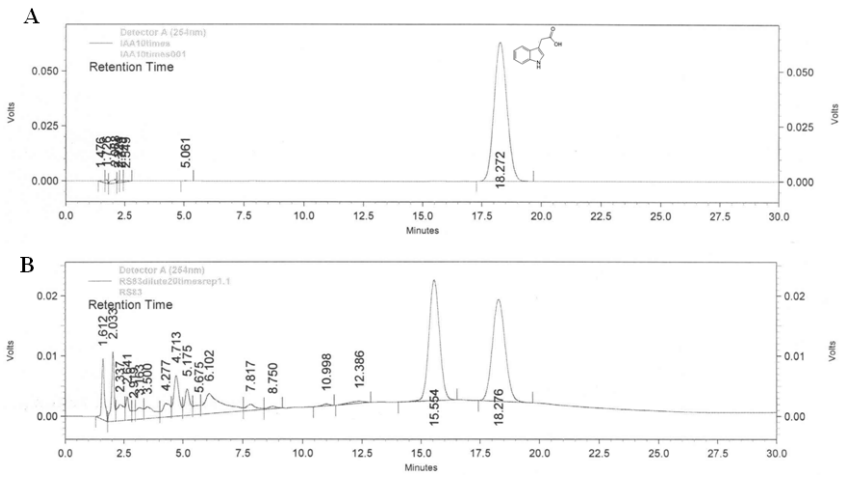

Figure 2. HPLC analysis of indole-3-acetic acid (IAA) produced from *Enterobacter asburiae* strain RS83. (A) Authentic IAA (1.3mg/mL; Sigma-Aldrich, USA) used as a standard, diluted 10 times (B) Culture extract of strain RS83, diluted 20 times.

the standard 50% gluconic acid mentioned above with the area under the peak 46% of the total area. After calculation of the culture supernatant from strain RS83 based on comparison with the standard 50% gluconic acid used in this study, it was found that strain RS83 generated about 279 mg/mL gluconic acid.

3.3 Siderophore

Forty eight hours after incubation of strain RS83 on CAS medium, an orange halo was noticeable around the bacterial colony (Figure 5). The presence of the halo indicates the capacity of strain RS83 for siderophore production.

The peak of a purified enterobactin, analyzed with HPLC method, notably appeared at the retention time 1.276 min (Figure 6A). The area under the peak was approximately 79% of the total peak area. The crude extract from medium cultured with strain RS83 demonstrated a notable peak at the retention time 1.353 min (Figure 6B). The area under the peak was 94% of the total area. Combinations of the purified enterobactin and extracted sample from strain RS83 in 1:2 ratios gave the outstanding peak at the retention time 1.347 min (Figure 6C). This confirmed that the extract cultured with strain RS83 contained an enterobactin type. When compared with the known purified enterobactin concentration, the result indicated that the strain RS83 released about 120 mg/1 mL of extracted culture medium.

Table 2. Optical density (OD) and pH of NBRI-BPB culture supernatant inoculated with *E. asburiae* strain RS83 and *B. cereus* strain RS87 5 days after incubation

Treatment	OD at 600nm	pH
Uninoculated control	0.863	6.47
strain RS83	0.713	3.27
strain RS87	0.746	4.39

Note: Each treatment was performed at least 3 times and the data shown in the table are the means in each strain.

3.4 Nitrogen fixation

Twenty four hours after inoculation with strain RS87, a distinct veil-like pellicle appeared 0.5 cm below the surface of a semi-solid JNFb medium. Concurrently, in the tubes inoculated with strain RS83, a light blue-green color occurred at the medium surface in combination with a faint veil-like pellicle. This blue-green color changed in intensity from the surface downward to the bottom of the medium 3-4 days later accompanied by the presence of the obvious pellicle at the same position as strain RS87. The un-inoculated medium control exhibited a clear-light yellow color (Figure 7).

After transferring pellicles from the inoculated tubes

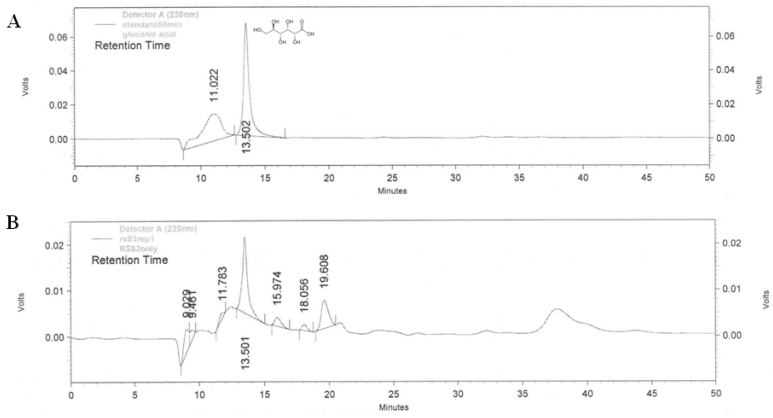

Figure 4. HPLC analysis of gluconic acid produced from *Enterobacter asburiae* strain RS83. (A) Standard gluconic acid (50% solution in water; 1,240mg/1mL; Merck KGaA, Darmstadt, Germany) (B) Culture supernatant inoculated with strain RS83.

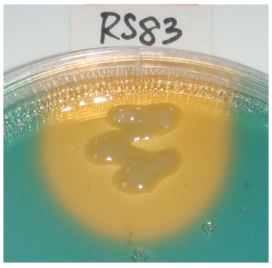

Figure 5. An orange halo around bacterial colony of *E. asburiae* strain RS83 streaked onto CAS medium 48 h after incubation.

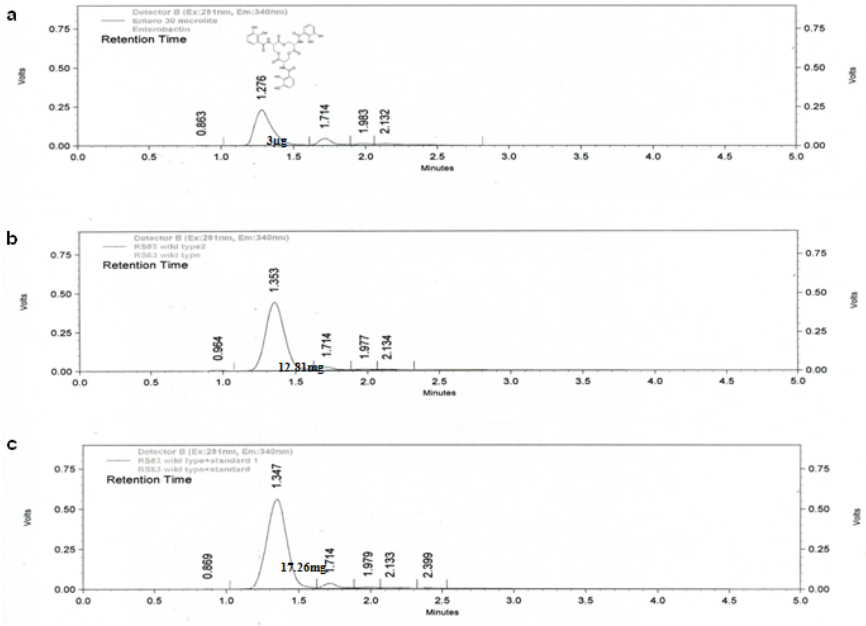

Figure 6. HPLC analysis of enterobactin (Ent) secretion in wild type and mutant strains of RS83. (A) Purified Ent (1mg/100µg) from *E. coli* (Sigma-Aldrich, USA) used as a standard; diluted 100 times (B) Extracted BM medium cultured with wild type strain; diluted 2,000 times. (C) Mixture of standard Ent and extracted BM medium cultured with strain RS83. Note: Numbers with the unit at the top-right of enterobactin peak represented the calculated enterobactin amount.

of a semi-solid JNFb medium to JNFb agar, bacterial colonies of both strains grew on the medium within 48 hours. In addition, the further outgrowth of the both strain RS83 and strain RS87 was observed on the medium (Figure 8).

4. Discussion

In a previous study, overproduction of IAA (8.28 mg/mL or 47 µmol/mL) of the mutant *Pseudomonas putida* GR 12-2/*aux*1 somehow inhibited root elongation under gnotobiotic conditions (Xie *et al.*, 1996). Although the bacterium, strain RS83, studied here produced higher IAA at higher levels (68.29 µmol/mL) than the mutant strain GR12-2/*aux*, plants treated with strain RS83 still had better root growth and morphology during seedling growth resulting in greater root surface area compared with non-bacterized control treatment plants (data not shown). These high amounts of IAA released from strain RS83 would be diminished due to environmental processes such as dilution, leaching, and oxidation so that the concentrations were appropriate for plant uptake.

Rodríguez and Fraga (1999) reported that several rhizobacteria can solubilize phosphate with strains from genera *Bacillus*, *Pseudomonas* and *Rhizobium* being the most powerful phosphate solubilizers. The main mechanism for mineral phosphate solubilization is the production of low molecular weight organic acids in which acid phosphatases play a major role in the mineralization of organic phosphorous in soil. In addition, different kinds of organic acids released from rhizobacteria i.e., citric acid, gluconic acid, lactic acid, succinic acid and propionic acid. It was confirmed that the strain RS83 studied here produced organic acid by the appearance of partial disintegration of insoluble tricalcium phosphate in the NBRIP-BPB medium at the bottom of the

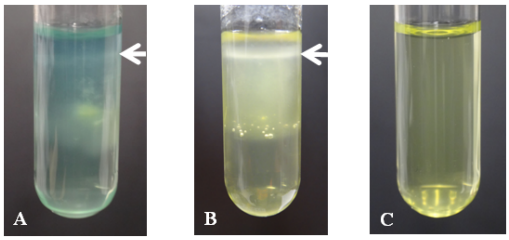

Figure 7. Veil-like pellicle appeared in the semisolid JNFb medium 3 days after culture with the strain RS83 (a) and the strain RS87 (b) comparing with the non-inoculated test tube (c). The arrows indicate the presence of the pellicle.

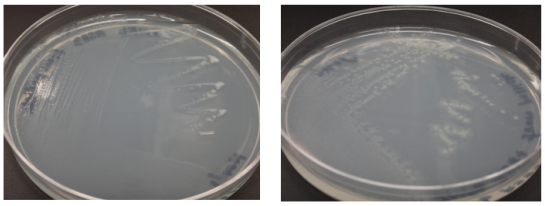

Figure 8. Bacterial colony appearance of *E. asburia* strain RS83 (left) and *B. cereus* strain RS87 (right) on JNFb agar 7 days after transferring pellicles from the semi-solid JNFb agar tube inoculated with strain RS83 and strain RS87.

tube. The HPLC method revealed that the organic acid type released from strain RS83 was gluconic acid.

Different types of siderophores may be liberated from different kinds of rhizobacteria (Crosa and Walsh, 2002). Positive effects on alfalfa plantlet growth had been observed after inoculation of siderophore producing *Azospirllum*, *Pseudomonas*, and *Rhizobium* grown in iron limited cultures. Inoculated alfalfa seeds also increased their germination as well as the root and stem dry weight (Carrillo-Castañeda *et al.*, 2002). The catechol siderophore enterobactin (also known as enterochelin) is commonly released by species classified in the Enterobacteriaceae such as *Escherichia coli* and *Enterobacter cloacae* strain EcCT-501 (Costa and Loper, 1994). This study was the first to discover that *Enterobacter asburiae* strain RS83 produced catechol siderophore enterobactin which would also account for plant growth enhancement.

In general, the bacteria utilize the enzyme nitrogenase to catalyze the conversion of atmospheric nitrogen (N_2) to ammonia (NH_3). Then, plants can readily assimilate NH_3 to produce the aforementioned nitrogenous biomolecules. Diverse genera of diazothrophs including *Azospirillum* spp., *Klebsiella* spp., and *Enterobacter* spp. can associate at the rhizoplane, invade damaged cells, or sometimes be found distributed in plant cells as endophytes without special compartmentation. Previous investigation has shown that rifampicin resistance (rif^r) *Enterobacter asburiae* strain RS83 colonized tomato plants at the rhizosphere, rhizoplane, and root cells. In these studies the highest population of strain RS83 was located at the rhizosphere, with the lowest population found inside the root cells (personnel communication). In this study, an occurrence of pellicles in a semi-solid JNFb medium inoculated with strain RS83 indicated the possible nitrogen-fixing capacity of the bacterium. Estimating nitrogenase enzyme activity will be further investigated to confirm the capacity of strain RS83 for biological nitrogen fixation.

In a review of mechanisms for plant growth promotion with *Azospirillum*, Bashan and de-Bashan (2010) reported the possibility of several mechanisms that function simultaneously or sequentially to benefit plant growth. In the current study with strain RS83, results demonstrated that there are at least four bacterial determinants involved in plant growth promotion. However, the exact contribution to optimum plant growth of each bacterial determinant released by strain RS83 could not be determined. Other factors such as environmental effects and plant growth stages would affect the bacterial determinants of strain RS83. These other factors may synergistically assist bacterial mechanisms in providing great benefits to the plants.

Furthermore, each bacterial determinant of strain RS83 may contribute to plant growth improvement at different plant growth stages. Meanwhile, some of the bacterial determinants may act together under certain circumstance. Therefore, molecular approaches in combination with investigations at different plant growth stages and under differing environmental conditions are needed in the future. These investigations could explore the exact modes of actions of strain RS83 for plant growth promotion.

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