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Nature of a Root-Associated *Paenibacillus polymyxa* from Field-Grown Winter Barley in Korea

RYU, CHOONG-MIN[1,2], JINWOO KIM[1], OKHEE CHOI[1], SOO-YOUNG PARK[2], SEUNG-HWAN PARK[2], AND CHANG-SEUK PARK[1]*

[1]*Department of Applied Biology and Environmental Science, College of Agriculture and Life Sciences, Gyeongsang National University, Jinju 660-700, Korea*
[2]*Laboratory of Microbial Genomics, Korea Research Institute of Bioscience & Biotechnology, Daejeon 305-333, Korea*

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Abstract Soil or seed applications of plant growth-promoting rhizobacteria (PGPR) have been used to enhance growth of several crops as well as to suppress the growth of plant pathogens. In this study, we selected a PGPR strain, *Paenibacillus polymyxa* strain E681, out of 3,197 heat-stable bacterial isolates from winter wheat and barley roots. Strain E681 inhibited growth of a broad spectrum plant pathogenic fungi *in vitro*, and treatment of cucumber seed with E681 reduced incidence of damping-off disease caused by *Pythium ultimum*, *Rhizoctonia solani*, or *Fusarium oxysporum*. When inoculated onto seeds as vegetative cells or as endospores, E681 colonized whole cucumber root systems and root tips. Different temperatures such as 20°C and 30°C did not affect root colonization by strain E681. This colonization was associated with a consistent increase in foliar growth of cucumber in the greenhouse. These results indicate that strain E681 is a promising PGPR strain for application to agricultural systems, particularly during the winter season.

Key words: Plant growth-promoting rhizobacteria, *Paenibacillus polymyxa*, root colonization

Plant growth-promoting rhizobacteria (PGPR) colonize the rhizosphere of many plant species and confer beneficial effects on plants [21, 22]. Many mechanisms such as production of plant hormones and hormone-like compounds or antibiotics by these bacteria have been suggested. to understand the beneficial effects by PGPR strains [9, 10, 34, 36]. For the past decades, many researchers have studied the capacity of these PGPR to increase plant productivity and control plant pathogens under greenhouse or field conditions [3, 23, 24, 37]. Although previous data from different experiments were often inconsistent, up to 20–70% yield increases were reported [21, 22]. Inconsistency of results in the field are probably due to the lack of understanding of the detailed mechanisms of plant growth promotion by PGPR and of optimal conditions for bacterial root colonization and the root competency of target crops [45].

To date, fluorescent pseudomonads have received considerable attention from many research groups because this bacterial group possesses a versatile metabolism and utilizes various substrates released by the root [42, 45]. Moreover, *Pseudomonas fluorescens* and *P. putida* have short generation times and a strong mobility, allowing them to rapidly colonize roots where they directly protect against soilborne pathogens and to adapt their fitness to the rhizosphere [5, 29, 45]. In contrast, *Bacillus* spp. and *Paenibacillus* spp. have been considered less as potential PGPR strains than Gram-negative bacteria, because bacilli typically have longer generation times and are isolated at lower population densities from plant roots than *Pseudomonas* spp. [7, 45]. However, recent reports provided evidence that *Bacillus* and *Paenibacillus* spp. also elicited plant growth promotion or suppression of plant diseases [6, 7, 12, 14, 17, 19, 23, 26, 27, 35]. The major advantage of using bacilli in this respect is their formation of endospores that are more stable and durable under starvation or unfavorable conditions [7, 27]. Endospore formation can make bacilli easy to formulate and commercialize, with a long-term shelf-life. Several commercial products such as Quantum, Kodiak, BioYield, Epic, Rhizo-Plus, Serenade, Subtilex, and System 3 originated from *Bacillus* spp. are commercially available in the U.S.A. and other countries [5, 27, 28].

Paenibacillus polymyxa as the type species of *Paenibacillus* spp. has been considered as a biochemically and biologically important bacterium that produces a broad spectrum of antibiotics such as the polymyxin group [29, 44], plant growth regulators like cytokinin and auxin [25, 40], an elicitor of

*Corresponding author
Phone: 82-55-751-5442; Fax: 82-55-758-5110;
E-mail: changpark@nongae.gsnu.ac.kr

growth promotion and induced systemic resistance like 2,3-butanediol [30, 34, 36]. *P. polymyxa* is also known as a free-living nitrogen fixer, and many strains contain the *nifH* gene, an indicator of nitrogen fixation in many plant species [14]. Production of the plant hormone cytokinin by a *P. polymyxa* strain was reported to be a mechanism underlying growth promotion in *Arabidopsis thaliana* [40]. The same strain elicits induced resistance against the soft-rot disease caused by *Erwinia carotovora* subsp. *carotovora* and tolerance to drought stress *in vitro* [41]. In the field, *P. polymyxa* increased plant performance and yield. In many cases, *P. polymyxa* strains were rhizosphere-competent, when isolated from maize and wheat [13, 32, 33, 43].

The objectives of this study were 1) to isolate endospore-forming rhizobacteria that produce a broad spectrum of antibiosis against fungal pathogens and have capacity to colonize plant root, 2) to assess selected strain(s) that suppress disease incidence caused by major soilborne pathogens in a greenhouse, and 3) to characterize root colonization of selected strains on different parts of the root and at different temperatures.

MATERIALS AND METHODS

Isolation of Endospore-Forming Rhizobacteria

Endospore-forming rhizobacteria were isolated from barley or wheat roots, which grew at 66 different locations in southern Korea, Gyeongsangnamdo and Chunranamdo provinces, from December 1995 through May 1997. Isolation of endospore-forming rhizobacteria from these plants was done by a modified protocol of Kang *et al.* [18]. Root systems of two or three plants were cut with flame-sterilized scissors and then washed with tap water to remove soil and plant particles. The washed roots were transferred into 250-ml flasks containing 100 ml of 0.1 M $MgSO_4$ and then shaken at 250 rpm at room-temperature for 30 min. The washed roots were macerated in a sterile mortar with pestle, transferred to 30 ml of 0.1 M $MgSO_4$ solution in a test tube, and heat-treated at 80°C for 30 min in a water bath. A 100 μl aliquot of the suspension was plated on 1/10 strength trypticase soy agar medium (1/10 TSA; 3 g of BBL Trypticase soy broth, 22 g of agar per L). After two days, colonies on 1/10 TSA were randomly selected with sterile toothpicks and transferred to new 1/10 TSA for further assay. Selected bacterial colonies were stored at -80°C in trypticase soy broth (TSB) amended with 20% glycerol.

Preparation of Bacterial Strains and Cucumber Seeds

Pseudomonas fluorescens strain B16 was grown on King's B media (KB; 1.5 g K_2HPO_4, 1.5 g $MgSO_4$, 10 ml glycerol, 10 g proteose pepton No 3, 22 g agar, in 1 l sterile water) and *Bacillus subtilis* strain E606 and *P. polymyxa* strains on TSA at 28°C [4]. For collecting endospores, *Paenibacillus polymyxa* strain E681 was grown on the TSA containing 50 mg of $MnSO_4{\cdot}H_2O$/l for 5 days, and resuspended in 0.1 M $MgSO_4$. To remove vegetative cells from this suspension, the suspension was heat-treated at 100°C for 10 min and cooled at room temperature for 30 min. To collect vegetative cells, bacterial cells grown in PD broth (Potato dextrose agar medium; Difco, Detroit, MI, U.S.A.) containing 5 g/l peptone for 1–2 days were harvested by centrifugation and resuspended in 0.1 M $MgSO_4$.

All cucumber (*Cucumis sativus* cv. Shinpung; Hungnong Seed Co., Korea) seeds were surface-sterilized with 1% NaOCl for 2 min and washed three times with distilled water prior to assay. Fifteen cucumber seeds were soaked in 10 ml of 10^8–10^9/ml bacterial solution. The control treatment was done with 0.1 M $MgSO_4$ buffer without bacteria. Bacteria-treated seeds were sterile-air dried for 30 min on the sterile filter paper in a laminar air hood prior to use in experiments.

In vitro Screening of Antibiotic Capacity Against Plant Pathogenic Fungi

To select bacterial isolates that showed any antagonism against plant pathogenic fungi, we determined the antibiotic capacity of the selected bacterial colonies for inhibition against the soilborne pathogenic fungi, *Rhizoctonia solani*, *Pythium ultimum*, and *Fusarium oxysporum* f. sp. *cucumerium*. Bacteria were spot-inoculated on the edge with equal spacing around the perimeter of PDA (Potato dextrose agar medium). After one day, a mycelial plug of each fungi grown on PDA was placed on the center of the plate. Five days after incubation, suppression of fungal growth was measured as the length of clear zone between bacterial colony and each fungus.

Assessment of Root Colonization *In Vitro*

Pseudomonas fluorescens strain B16 and *B. subtilis* strain E606 were used as positive and negative control, respectively, in the root colonization experiment [4, 18]. To assess root colonizing capacity, we used the Double Layer Filter Paper (DLF) method as described previously [4, 18, 31]. Five seeds soaked in the bacterial suspension or buffer were placed on the bottom layer of filter paper (Whatman No .2) in a Petri plate (d=11 cm) and covered with the same sized filter paper. Sterile water (4.5 ml) was added onto two layers of filter papers and the Petri plates were sealed with commercial polypropylene wrap to maintain constant moisture. The sealed Petri plates were incubated horizontally at 20°C or 30°C in the dark to allow straight growth of the emerging roots. After 72 h at 30°C and 90 h at 20°C, four 1 cm root segments from the basal part to the root tip were cut with a sterile scalpel. Each 1 cm root section was vigorously stirred with a vortex mixer for 20 sec in a test tube containing 10 ml of 0.1 M

$MgSO_4$, and plated on bacterial culture media (1/10 TSA for *P. polymyxa* E681 and KB for *P. fluorescens* B16). After 3 days at 30°C, the colony forming units (cfu)/root in the plates were determined as root colonization capacity. This experiment was conducted three times.

Assessment of Root Colonization *In Vivo*

To test root colonization of strain E681 in the soil, we modified the protocol previously described [2, 31]. Each polypropylene tube (10 cm long×3 cm diameter) was filled with sandy loam soil with about 20% (w/w) moisture contents, on which cucumber seeds treated with strains, E681, E606, or B16 were planted (1 cm below the surface). Four tubes per treated soil were placed horizontally inside the plastic boxes (12×12×10 cm) containing the same sandy loam soil with the same water content. Each plastic box was covered with the same size of plastic box and sealed with parafilm to maintain a constant soil matrix potential. The boxes were placed in a growth chamber with a 14-h photoperiod at 25°C for 7 days. After 7 days, 1 cm root tip segments were collected from each tube, resuspended in 10 ml of 0.1 M $MgSO_4$, and spread on KB containing rifampicin (100 μg/ml). Soil particles on the roots were removed gently, and the root tips were then collected to reisolate the introduced bacteria. The root segments were transferred into a test tube containing 10 ml of 0.1 M $MgSO_4$ solution. *In vivo* root colonization of these bacteria was determined by counting the numbers of bacterial colonies on the plates. This experiment was conducted three times.

Biological Control Assay Against Cucumber Damping-Off

Preparation of the bacterial inocula was done as previously described [10]. Mycelial plugs of the damping-off fungi, *F. oxysporum*, *P. ultimum*, and *R. solani*, which were grown on PDA at 27°C for 5 days, were added into the mixture of air-dried sand and 3% ground oatmeal (w/v). Each inoculum (30 g) was incubated in 20% humidity at 25°C–27°C for 3 weeks, and then was mixed thoroughly with 1,000 ×*g* of natural field soil. Ten cucumber seeds treated with strains E681, E606, and B16 were sown in each rectangular pot (16×10×6.5 cm) carrying the infested soil. The pots were kept in a greenhouse and managed using standard nursery practices for the Jinju area in Korea. Ten days after seeding, plants were observed carefully for typical symptoms of damping-off, and the number of symptomatic plants was counted. This experiment was conducted three times.

Assay for Cucumber Growth Promotion by Seed Treatment

Cucumber seeds coated with strain E681 at 10^5–10^6 cfu/seed were sown in a pot (16×10×6.5 cm) with four replicates in a greenhouse condition. No nutrients were added in this experiment. After three weeks, the fresh weight of cucumber was measured. This experiment was conducted six times.

Identification of *Paenibacillus polymyxa* Strain E681

P. polymyxa strain E681 was identified, based on the 8th edition of *Bergey's Manual of Systematic Bacteriology* [39] and the 2nd edition of *Plant Pathogenic Bacteria* [38]. Spore formation and morphology were observed under a light microscope and a scanning electron microscope after 10 days of incubation on TSA, as previously described by Choi *et al.* [4].

Identification of strain E681 was done by the fatty acid methyl ester analysis procedure in the Sherlock system (Microbial ID, Inc., Newark, DE, U.S.A.). The other procedures were followed by the standard protocols described in several previous reports (Microbial ID, Inc., Newark, DE, U.S.A.; http://www.midi-inc.com/pages/GC.html).

To confirm the identification of strain E681, we compared nucleotide sequences of 16S rRNA from E681 with those of other known bacteria. For PCR amplification, reaction mixtures (20 μl in total) containing 50 pmol of each primer, BSF 8F (5'-agagtttgatcctggctcag-3') and BSR 1541R (5'-aaggaggtgatccagccgca-3'), were prepared using the AccuPower PCR system (Bioneer, Daejon, Korea), and 100 ng of template DNA. The PCR conditions were 2 min at 95°C, 33 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 45 s. Cycling was completed by a final elongation step at 72°C for 7 min. After amplification, PCR products were separated on 1.0% agarose gel, and approximately 1,500 bp of the amplified fragment was excised from the gel and purified using the QIAquick gel extraction kit (LRS Laboratories, Korea). The nucleotide sequences of the products were directly determined with primers BSF 784F, BSF 1099F, BSR 534R, and BSR 1114R. The PCR product was resequenced with primers T7 and SP6 after being cloned into pGEM T vector.

Data Analysis

Data were subjected to analysis of variance using JMP software (SAS Institute Inc., Cary, NC, U.S.A.). Significance of PGPR effects was determined by the magnitude of the F value at P=0.05. When a significant F value was obtained for treatments, separation of means was accomplished using Fisher's protected least significant difference (LSD) at P=0.05.

RESULTS

Isolation of Endospore-Forming Rhizobacteria from Field-Grown Wheat and Barley

Endospore-forming rhizosphere bacteria (rhizobacteria) were isolated from roots of the barley or wheat seedlings

sampled from 66 different locations in Gyeongsangnamdo and Chunranamdo provinces in Korea from December 1995 to May 1997. According to the morphological difference of colonies on 1/10 TSA medium after heat treatment at 80°C for 30 min, 3,179 isolates were selected, which formed endospores and showed bacilli-like colony growth. The colonies varied in color (mostly light-brown, milky-white, or yellow color) and in morphology (round, round with a scalloped edge, round with a ridge along the edge, and irregular shapes).

In Vitro Screening of Antagonist and Root-Colonizing Rhizobacteria

Out of the 3,179 isolates, 365 isolates had antagonistic capacity against *Rhizoctonia solani*, 101 against *Fusarium oxysporum* f. sp. *cucumerinum*, and 31 against *Pythium ultimum*. The bacterial isolates showing clear zones greater than 3 mm were considered as positive strains in these tests. Thirty-one isolates showed inhibitory activity to all of the three pathogens (data not shown). Any isolates constitutively antagonistic to *P. ultimum* showed inhibitory activity against both *R. solani* and *F. oxysporium* f. sp. *cucumerium*. We determined root colonization capacity on the region 1 cm above the root tip (root tip 1 cm) after seed treatment. Out of thirty-one isolates that colonized the root segment, isolates E681, G157, and H210 were selected for further experiments. After repeated experiments, seed treatment of strain E681 showed greater colonization on the root part than that of isolates G157 or H210 (data not shown).

Identification of Strain E681

According to biochemical tests based on *Bergey's Manual* [39], isolate E681 was Gram-positive; had anaerobic growth; hydrolyzed gelatin, casein, and starch; and produced acid from D-glucose and D-xylose (data not shown). On observation under a scanning electron microscope, the endospore was located in the central or paracentral position of each cell. The shape of endospores was oval and longitudinal and displayed three or four laterally deep grooves or ridges (Fig. 1). Colonies of strain E681 on nutrient agar medium were thin, and often exhibited amoeboid spread, whereas on glucose agar (or potato dextrose agar medium) colonies were usually heaped and mucoid with a matted surface. Throughout these physiological assays, strain E681 was matched with *P. polymyxa* described in *Bergey's Manual*. Fatty acid methyl ester analysis revealed that strain E681 could be identified as *P. polymyxa* with a 0.856 similarity value in the Sherlock system (Microbial ID, Inc., Newark, DE, U.S.A.). The nucelotide sequence of the 16S rDNA from strain E681 had 99% identity to *Paenibacillus polymyxa* strain GBR-1 (AF515611). Taken together, our data concluded that strain E681 is a *P. polymyxa* strain.

Fig. 1. Scanning electron microphotograph of endospores of *P. polymyxa* strain E681.
The white bar=1 μm.

Suppression of Plant Pathogenic Fungi by *P. polymyxa* Strain E681

Cucumber seed treatment with *P. polymyxa* strain E681 reduced the number of seedlings showing damping-off symptom in the soil amended with *P. ultimum, F. oxysporum*, and *R. solani* inocula. The protection rate indicated by healthy stands three weeks after seeding by strain E681 was 80% against *P. ultimum*, 70% against *F. oxysporum*, and 67% against *R. solani*, whereas *P. fluorescens* strain B16 was 70%, 78%, and 87%, respectively, and the control treatment was 55%, 50%, and 33% (Table 1). Thus, strain E681 demonstrated greater biological control capacity against *P. ultimum* than strain B16 and the control. Strains

Table 1. Effect of *P. polymyxa* strain E681 on suppression of plant pathogenic fungi *in vitro* and *in vivo*.

Traits	Treatments		
	E681	B16	Control
Suppression of fungal growth *in vitro*[a]			
P. ultimum	+++	+++	-
R. solani	++++	+++	-
F. oxysporum	++++	+++	-
Suppression of damping-off *in vivo*[b] (Healthy stand (%))			
P. ultimum	80a	70b	55c
R. solani	70a	78a	50b
F. oxysporum	67b	87a	33c

[a]Fungal growth suppression by strain E681 and B16 was measured by the diameter of the clear zone caused by inhibition of fungal growth on PDA. +++, 4–5 mm; ++++, <6 mm.
[b]Numbers represent mean of four replications per treatment, five plants per replication. Healthy stands that did not show any damping-off symptom were counted four weeks after sowing of the bacteria-treated seeds in the soil amended with each pathogen. Different letters indicate significant differences on Fisher's protected LSD test at P=0.05.

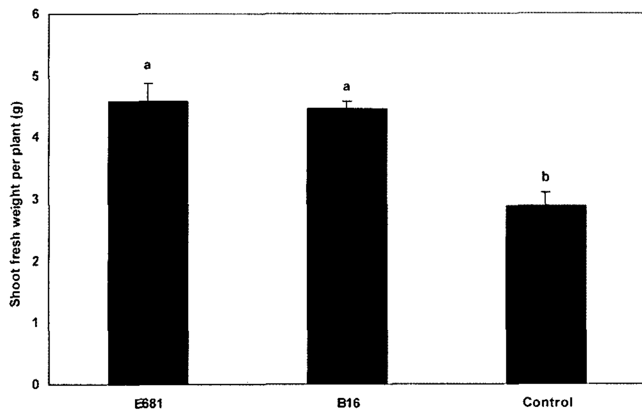

Fig. 2. Enhancement of cucumber seedling growth by *P. polymyxa* strain E681 and *P. fluorescens* strain B16. Numbers represent the mean of four replications per treatment, one shoot fresh weight per replication.
Different letters indicate significant differences on Fisher's protected LSD test at P=0.05.

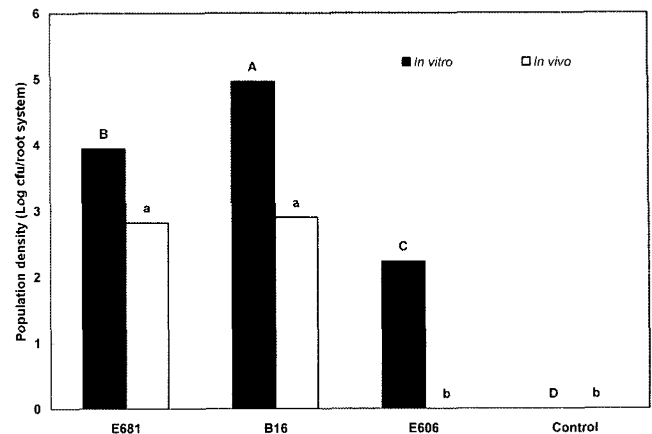

Fig. 3. Root colonization of *P. polymyxa* strain E681 on cucumber *in vitro* and *in vivo*. Numbers represent mean log colony forming units (cfu) of four replications per treatment.
Different letters indicate significant differences on Fisher's protected LSD test at P=0.05.

E681 and B16 treatments did not have different protection rates against *R. solani*, but both treatments resulted in significantly greater protection than the control treatment against the other two pathogens (Table 1).

Plant Growth Promotion of Cucumber Seedling by Strain E681

On growth promotion assay, seeds that had been soaked in strains E681 and B16 suspensions resulted in increased shoot fresh weight of cucumber seedlings at 160% and 155%, respectively, compared with water treatment three or four weeks after sowing (Fig. 2).

Root Colonization of *P. polymyxa* Strain E681 *In Vitro* and *In Vivo*

We assessed the root colonization capacity of strain E681 after seed treatment of cucumber *in vitro* (DLF method) and *in vivo* (soil). In *in vitro* assay, the population densities on the upper 1 cm root (root base 1 cm) were not significantly different between strains E681 (1.27×10^5 cfu/cm root) and B16 (2.0×10^5), whereas strain B16 colonized ten times higher than strain E681 on the root tip (Fig. 3). Under *in vivo* conditions in soil, the population densities of strain E681 and strain B16 were 6.7×10^2 and 8.0×10^2 cfu/cm root, respectively, whereas *B. subtilis* E606 was not detected on the root tip (Fig. 3). On the basal root, root colonization of strains E681, E606, and B16 in soil was not significantly (P=0.05) different.

To assess their practical application, the spermosphere survival rate was tested at room temperature one year after seed treatment. No bacteria were recovered from seed treated with strain B16, whereas strain E681 sustained a population density of 5.6×10^5 cfu/seed one year after treatment. The population density after one year was not statistically (P= 0.05) different from the initial density of 6.0×10^6 cfu/seed.

Root Colonization of Different Cell Types (Vegetative or Endospore Cells) at Different Temperatures (20°C or 30°C)

To test bacterial viability at different temperatures, the population densities of *P. polymyxa* strain E681 on the different positions of cucumber root at 30°C and 20°C were measured using the DLF method. Strain E681 colonized whole cucumber root systems at a range of 10^3-10^5 cfu/cm segment at 30°C, whereas population densities of strain E681 ranged from 2.4×10^2 to 5.0×10^3 at 20°C (Fig. 4). At the lateral, basal, and middle cucumber root, strain E681 at

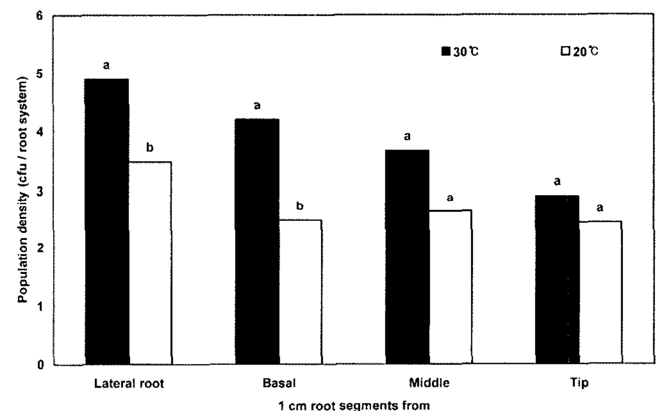

Fig. 4. Population densities of *Paenibacillus polymyxa* strain E681 on cucumber root at 20°C and 30°C, determined by the DLF method. Numbers represent the mean of four replications per treatment, one root segment per replication. Strain E681 was inoculated on the cucumber root.
Different letters indicate significant differences on Fisher's protected LSD test at P=0.05.

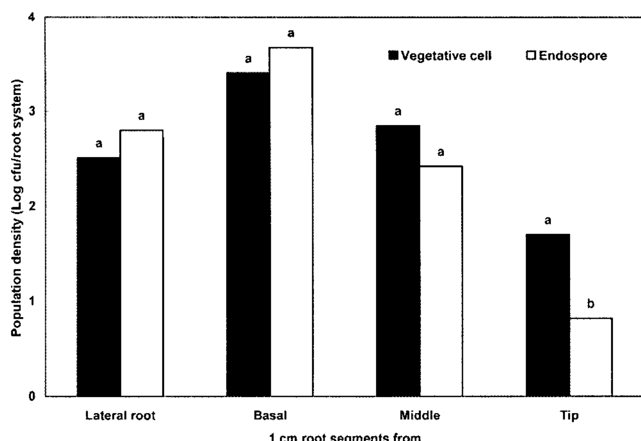

Fig. 5. Root colonization ability of the endospore and vegetative cell of *P. polymyxa* strain E681 on cucumber root, determined by the DLF method. Numbers represent the mean of four replications per treatment, one root segment per replication.
Different letters indicate significant differences on Fisher's protected LSD test at P=0.05.

20°C colonized at a significantly lower level (10 to 100 times) than it did at 30°C (Fig. 4). The colonization on the root tip by strain E681 at both 20°C and 30°C was not statistically (P=0.05) different. The root colonization capacity of vegetative cells and endospores of strain E681 were not significantly different from each other. On root tips, the population densities of vegetative cells were 10 times higher than endospore treatment (Fig. 5). The population density gradually declined from 1,000 in the basal part to 10 cfu/root segment in the root tip part.

DISCUSSION

In recent years, several research groups studying plant growth promoting rhizobacteria (PGPR) have reported advantages to use endospore-forming *Bacillus* and *Paenibacillus* spp. These bacilli have been shown to have promising biological control and to induce systemic resistance in different crop systems, presumably because of their stability and durability under diverse environmental conditions [3, 7, 20, 25, 35, 37]. However, *Bacillus* and *Paenibacillus* spp. have also been considered less rhizosphere competent than *Pseudomonas* spp. [11, 45]. Our study suggested that *Paenibacillus polymyxa* strain E681, screened out of 3,197 heat-stable rhizosphere bacteria from wheat and barley roots, could overcome the problem of rhizosphere competence. Our study also demonstrated that *P. polymyxa* strain E681 has a broad spectrum of antagonism against major soilborne pathogens and promotes growth of cucumber in the greenhouse. In addition, strain E681 colonized cucumber roots even at 20°C, which is a relatively low temperature for *Bacillus* and *Paenibacillus* spp. (Fig. 4). These results suggest that *P. polymyxa* strain E681 is good to be used in cultivation of vegetables in a greenhouse during the cool season in Korea.

Treatment of cucumber seeds with strain E681 increased foliar weight from the spring to next fall seasons in the greenhouse (Fig. 2). We do not have direct evidence on the mechanisms of plant growth promotion by strain E681. *P. polymyxa* has been referred to as a free-living nitrogen fixer using acetylene reduction or the *nifH* gene [1, 15]. However, the *nifH* gene, an indicator of N_2 fixation, could not be found from the strain E681 by an *nifH*-specific primer, and any enzymatic activities could not be detected from strain E681 by the acetylene reduction assay, indicating no possibility of involvement of N_2 fixation in plant growth promotion (S. Y. Ghim, H. Cheong, S. H. Park, unpublished data). Rather, production of plant hormones by rhizobacteria can be more acceptable as a mechanism for plant growth promotion than free-living N_2 fixation.

Recent analysis of compounds secreted from strain E681 detected IAA-like substrates (S. H. Park, unpublished data), resulting in increasing root development, compared with unbacterized roots. In addition to IAA, cytokinin is an important plant growth regulator that acts mainly in a variety of responses in many species, including breaking seed dormancy and delaying leaf senescence. More interestingly, cytokinins were originally defined as hormones that stimulate cell division in culture in the presence of auxin [8]. An earlier report showed that a *P. polymyxa* strain produced detectable amounts of cytokinins resulting in enhancement of plant growth [40]. Therefore, if strain E681 could produce both IAA and cytokinin, a mechanism on consistent plant growth promotion by strain E681 could be explained. To confirm this hypothesis, we need to generate either an auxin or cytokinin defective mutant and compare growth promotion by seed treatment of these mutants and wild-type. We may also try to test if strain E681 enhances growth of auxin or cytokinin insensitive plants. Recently, bacterial volatile organic compounds (VOCs) have been discovered as a bacterial determinant of plant growth promotion [34, 36]. The major substance included 2,3-butanediol, which *P. polymyxa* also produced [30]. Since this compound was identified in the VOC collection of strain E681 (unpublished data), it is possible that 2,3-butanediol can play a role in plant growth promotion. Therefore, further study on the effect of 2,3-butanediol on plant growth should be done, and the role of plant growth promotion by 2,3-butanediol produced by strain E681 needs also to be investigated.

Root colonization is an important capacity of PGPR for consistent effects on plant growth promotion and inhibition of soilborne fungi on the plant root. Strain E681 colonized the whole root system, including lateral roots and root tips, both *in vitro* and in soil. Under *in situ* conditions, plant roots can grow more than 10 cm underground, where

growth of many aerobic bacteria is restricted. Root colonization study with *Bacillus* sp. revealed that the species aggressively colonized at the basal roots. However, *Bacillus* sp. L324-92R_{12} colonized poorly on the root tip (below 5 cm) at below the detection level [20]. The main reason is that most bacilli, including *B. subtilis,* are strictly aerobic bacteria. In contrast, *P. polymyxa* is a facultative anaerobic bacterium that can be active even under anaerobic condition. Thus, *P. polymyxa* can thrive under semi-anaerobic conditions. Moreover, recent observations by scanning electronic microscopy and confocal laser scanning microscopy revealed that strain E681was detected inside the cucumber root tissues, especially the intercellular space between the epidermal and cortical cells [4].

Strain E681 maintained its biological capacity even at the relatively low temperature of 20°C (Fig. 3). Additional studies revealed that the growth rates of strain E681 at 20°C and 30°C were similar (data not shown). It is worth to note that strain E681 can keep the capacity at such a low temperature, allowing more competency against other soil bacteria that are not active at the same temperature. This trait of strain E681 should be useful for the suppression of soilborne pathogens that cause severe economic problems, such as Phytophthora blight of pepper, in the cool season under greenhouse conditions.

Collectively, our results suggest that *P. polymyxa* strain E681 has excellent promise for practical use in field conditions, because it possesses the desired traits of a biological control agent against soilborne diseases, and for plant growth promotion in cultivation at low temperatures.

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