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Burkholderia sp. KCTC 11096BP as a Newly Isolated Gibberellin Producing Bacterium

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We isolated 864 bacteria from 553 soil samples and bioassayed them on cucumber and crown daisy for plant growth promotion. A new bacterial strain, *Burkholderia* sp. KCTC 11096BP gave maximum growth promotion and was selected for further investigations. The culture filtrate of this bacterium was thus analyzed for the presence of gibberellins and we found physiologically active gibberellins were found (GA_1, 0.23 ng/100 ml; GA_3, 5.11 ng/100 ml and GA_4, 2.65 ng/100 ml) along with physiologically inactive GA_9, GA_{12}, GA_{15}, GA_{20}, and GA_{24}. The bacterial isolate also solubilised tricalcium phosphate and lowered the pH of the medium during the process. The isolate was identified as a new strain of *Burkholderia* through phylogenetic analysis of 16S rDNA sequence. Gibberellin production capacity of genus *Burkholderia* is reported for the first time in current study.

***Keywords*: *Burkholderia*, gibberellins, phosphate solubilization, plant growth, phylogenetic analysis**

Bacteria are abundantly present in the soil, interact with plant roots in the rhizosphere and enhance plant growth and development in certain instances. The plant growth-promoting rhizobacteria (PGPR) develop a mutualistic relationship with the host plants and gives a benefit to them through N_2 fixation by nitrogenase, nitrate reductase activity, siderophore production, and phytohormone secretion in the rhizosphere (Fulchieri *et al*., 1993; Cassán *et al*., 2001a, 2001b). Gibberellins production by PGPR promote the growth and yield of many crop plants, deconjugation of gibberellin-glucosyl conjugates exuded by the roots, or in the plant (Piccoli *et al*., 1997), and 3β-hydroxylation by bacterial enzymes of inactive 3-deoxy gibberellins present in roots, to active forms such as GA_1, GA_3, and GA_4 (Piccoli *et al*., 1996; Cassán *et al*., 2001a, 2001b). PGPR have been reported to directly enhance plant growth by a variety of mechanisms, i.e. fixation of atmospheric nitrogen that is transferred to the plant, production of siderophores that chelate iron and make it available to the plant root, solubilization of minerals such as phosphorus, and synthesis of phytohormones (Glick, 1995). Direct enhancement of mineral uptake due to increases in specific ion fluxes at the root surface in the presence of PGPR has also been reported (Bertrand *et al*., 2000). The PGPR indirectly enhance plant growth via suppression of phytopathogens by a variety of mechanisms. These include the ability to produce siderophores that chelate iron, making it unavailable to pathogens; the ability to synthesize antifungal metabolites such as antibiotics, fungal cell wall-lysing enzymes, or hydrogen cyanide, which suppress the growth of fungal pathogens; the ability to successfully compete with pathogens for nutrients or specific niches on the root; and the ability to induce systemic resistance (Bloemberg and Lugtenberg, 2001; Persello-Cartieaux *et al*., 2003). GAs have been identified and isolated from higher plants, fungi and bacteria. It was reported that 136 GAs from higher plants (128 species), 28 GAs from fungi (7 species), and only 4 GAs (GA_1, GA_3, GA_4, and GA_{20}) from bacteria (7 species) have been identified till recently (MacMillan, 2002).

Phosphorus is an essential macronutrient for plant growth and development. However, estimates showed that in some soils, up to 75% of applied phosphate fertilizer may become unavailable to the plant due to mineral phase re-precipitation (Sundara *et al*., 2002). PGPR constitutes phosphate- solubilizing bacteria (PSB), which convert insoluble phosphates to soluble forms (Peix *et al*., 2002; Sudhakara *et al*., 2002) and therefore can also be used to enhance the solubilization of reprecipitated soil P for crop improvement (Shekhar *et al*., 2000). It was observed that the release of low molecular weight organic acids by the PSB was responsible for solubilization of insoluble phosphate (Kim *et al*., 1997; Hilda and Fraga, 1999).

The current study was carried out to find the gibberellin producing capacity of soil bacteria. Furthermore, phosphate solubilization activity of bacterial isolates was also investigated.

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Materials and Methods

Collection, isolation, and preservation of bacteria from soil

Soil samples were collected from Geongbuk Province, Korea and 10 g of soil was transferred to 250 ml flasks containing 100 ml of sterile Amies solution (Amies, 1967). Resulting suspensions were serially diluted (10^{-4}) and 0.1 ml aliquots were plated on tryptic soy agar (TSA; Merck Co., Germany), for isolation of bacteria. Bacterial cultures were incubated for 24 h at 30°C and, bacterial colonies differentiated by their morphology, pigmentation, and growth rate were selected, counted, and re-streaked on fresh TSA medium. For long term preservation, bacteria were stored in 50% glycerol at -20°C.

Plant growth promoting capacity of *Burkholderia* sp. KCTC 11096BP

Seeds of cucumber and crown daisy were purchased from Seminis Korea Co. (Korea) were surface sterilized with NaOCl (5%) for 10 min and thoroughly rinsed with distilled water. Seeds were sown in plastic pots under controlled greenhouse conditions at 30±2°C. Plant seedlings were treated with 5 ml of bacterial suspension after 14 days of sowing, and the growth attributes i.e. plant length, shoot length, plant fresh weight, plant dry weight were recorded after 14 days of treatment. The bacterial culture suspension was incubated for 3 days at 30°C on a shaking incubator at 200 rpm. Distilled water and nutrient broth (NB, 5 ml) were used as control for this experiment.

Phosphate solubilization capacity of *Burkholderia* sp. KCTC 11096BP

Burkholderia sp. KCTC 11096BP was inoculated on 0.5% National Botanical Research Institute's Phosphate (Nautiyal, 1999) media plates, and incubated for 7 days at 30°C. The bacterial isolate formed the clarification halos, which indicated P solubilization. The P solubilization process was confirmed by checking pH of the media on daily basis.

Extraction and quantification of gibberellins

Bacterial gibberellins (GAs) were extracted from culture filtrates after 3 days of incubation, according to an established protocol (Lee *et al*., 1998). Extracted GAs were subjected to reverse-phase C_{18}-HPLC. The GAs were chromatographed on a 3.9×300 m Bondapak, C_{18} column (Waters Corp., USA) and eluted at 1.5 ml/min with the following gradient: 0 to 5 min, isocratic 28% MeOH in 1% aqueous acetic acid; 5 to 35 min, linear gradient from 28 to 86% MeOH; 35 to 36 min, 86 to 100% MeOH; 36 to 40 min, isocratic 100% MeOH. Up to 48 fractions of 1.5 ml each were collected. The fractions were then prepared for injection in gas chromatograph/mass spectrometer (GC/MS) with selected ion monitoring mode (SIM) (6890N network GC system, and 5973 network mass selective detector; Agilent Technologies, USA). For each GA type, 1 μl of sample was injected in a 30 m×0.25 mm (i.d.), 0.25 μm film thickness DB-1 capillary column (J & W Scientific Co., USA). The GC oven temperature was programmed for a 1 min hold at 60°C, then to rise at 15°C/min to 200°C followed by 5°C/min to 285°C. Helium carrier gas was maintained at a head pressure of 30 kPa. The GC was directly interfaced to a Mass Selective Detector with an interface and source temperature of 280°C, an ionizing voltage of 70 eV and a dwell time of 100 ms. Full scan mode (the first trial) and three major ions of the supplemented [$^2H_2$] GAs internal standards (obtained from Prof. Lewis N. Mander, Australian National University, Canberra, Australia) and the bacterial gibberellins were monitored simultaneously. The retention time was determined using hydrocarbon standards to calculate the KRI (Kovats Retention Index) value, while the GAs quantification was based on peak area ratios of non-deuterated (extracted) GAs to deuterated GAs.

Identification of *Burkholderia* sp. KCTC 11096BP

The bacterial isolate SE4 was identified as a new strain of *Burkholderia*, on the basis of partial 16S ribosomal DNA (rDNA) sequence. The chromosomal DNA was isolated through standard procedures (Sambrook and Russel, 2001). The almost complete 16S rDNAs were PCR amplified using the 27F primer; 5'-AGAGTTTGATC(AC)TGGCTCAG-3' and 1492R primer; 5'-CGG(CT)TACCTTGTTACGACTT-3', which were complementary to the 5' end and 3' end of the prokaryotic 16S rDNA, respectively. The amplification reaction was performed as previously described (Adachi *et al*., 1996). The BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/) was used to look for nucleotide sequence homology of this bacterial isolate. The closely related sequences were aligned by CLUSTAL W using MEGA version 4.0 software, and the neighbor-joining tree was generated using same software. Bootstrap replication (1,000 replications) was used for a statistical support for the nodes in the phyloge-

Table 1. Effect of *Burkholderia* sp. KCTC 11096BP on growth attributes of the cucumber and crown daisy

Plant material	Treatment	Plant length (cm/plant)	Shoot length (cm/plant)	Plant fresh weight (g)	Plant dry weight (g)
Cucumber	Control (DW)	$26.34^a \pm 1.03$	$16.08^a \pm 0.81$	$3.27^a \pm 0.04$	$0.24^a \pm 0.03$
	NB media	$31.1^b \pm 1.25$	$16.35^a \pm 0.68$	$3.99^b \pm 0.05$	$0.25^a \pm 0.02$
	Culture suspension	$43.54^c \pm 0.91$	$21.29^b \pm 0.33$	$4.94^c \pm 0.12$	$0.30^b \pm 0.03$
Crown daisy	Control (DW)	$18.62^a \pm 0.63$	$7.67^a \pm 0.23$	$0.66^a \pm 0.11$	$0.18^a \pm 0.01$
	NB media	$18.8^a \pm 0.54$	$7.41^a \pm 0.22$	$0.68^a \pm 0.12$	$0.19^a \pm 0.02$
	Culture suspension	$24.55^b \pm 1.0$	$11.22^b \pm 0.50$	$1.85^b \pm 0.21$	$0.23^b \pm 0.04$

In a column, treatment means having a common letter(s) are not significantly different at the 5% level by DMRT. A 10 ml culture suspension was given to plant seedlings after 14 days of sowing and the resultant growth promotion was measured. DW, distilled H_2O; NB, nutrient broth.

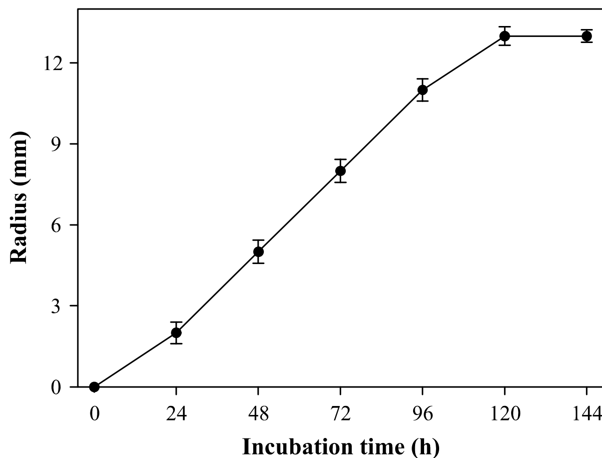

Fig. 1. P solubilization capacity of *Burkholderia* sp. KCTC 11096BP. *Burkholderia* sp. KCTC 11096BP was inoculated on national botanical research institute's phosphate (NBRIP) growth media plates, and incubated for 7 days at 30°C. Values given are means of three replicates. Error bars indicate standard deviations.

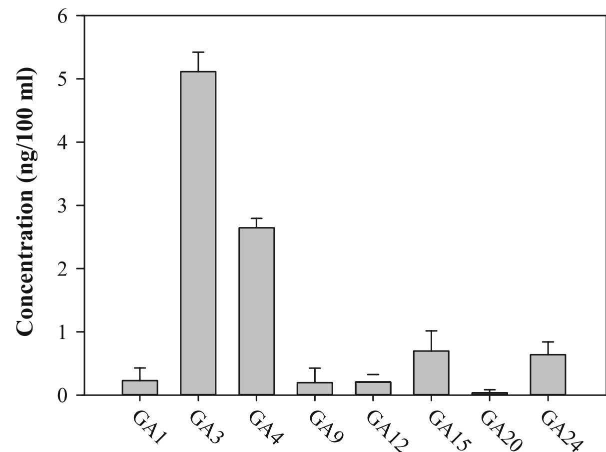

Fig. 3. Gibberellins production by *Burkholderia* sp. KCTC 11096BP. The bacterial culture was centrifuged and 100 ml of culture filtrate was analyzed for the presence of GAs, using GA extraction protocol (Lee *et al*., 1998). Values given are means of three replicates, while error bars show standard deviations.

netic tree.

Statistical analysis

The data were analyzed statistically for standard deviation by using the sigma plot software (2004). The mean values were compared, using Duncan's multiple range test at $P<0.05$ (ANOVA SAS release 9.1; SAS, USA).

Results

Bioassay of *Burkholderia* sp. KCTC 11096BP on Cucumber and Crown daisy

The fungal isolate SE4 was bioassayed on cucumber and crown daisy for its growth promoting capacity. The bacterial suspension of SE4 significantly promoted growth of cucumber and crown daisy seedlings. In both vegetables, the shoot length, plant length, fresh weight, and dry weight parameters were significantly promoted compared to control treatment. The control included both distilled water and nutrient broth treatments (Table 1).

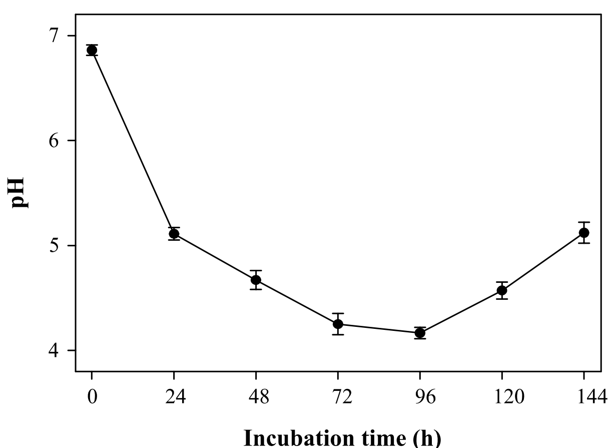

Fig. 2. P solubilization was confirmed by checking pH of the media on daily basis, using liquid NBRIP medium for bacterial growth. The pH of the medium was observed to fall continuously up to 96 h. Values given are means of three replicates. Error bars indicate standard deviations.

Phosphate solubilization capacity of *Burkholderia* sp. KCTC 11096BP

Phosphate solubilization activity of SE4 using insoluble tricalcium phosphate was determined by a plate-screening method. The maximum size of the clearing zone was reached after 120 h (Fig. 1). In a separate experiment using liquid NBRIP medium, the pH of the medium was observed to fall continuously up to 96 h (Fig. 2) and this was attributed to the solubilization process taking place in the medium.

Gibberellins analysis

Gibberelins analysis of the culture filtrate of bacterial isolate SE4 showed the presence of GA_1, GA_3, GA_4, GA_9, GA_{12}, GA_{15}, GA_{20}, and GA_{24}. These gibberellins were identified and quantified by comparing their mass spectra and KRI with those available from spectral library. The bioactive GA_3 and GA_4 were the most abundant GA types and were present in concentrations of 5.11 ng and 2.65 ng per 100 ml of culture filtrate respectively. The physiologically inactive GAs were present in lesser amounts (Fig. 3).

Identification of fungal isolate SE4

The bacterial strain SE4 was sequenced, which showed 98.8% homology with *Burkholderia cepacia.* The phylogenetic analysis of partial 16S rDNA sequence of bacterial isolate SE4 was performed by constructing Neighbor Joining (NJ) tree for 14 taxa (13 references and 1 clone) with 1,000 bootstrap replications using Maximum Composite Likelihood method.

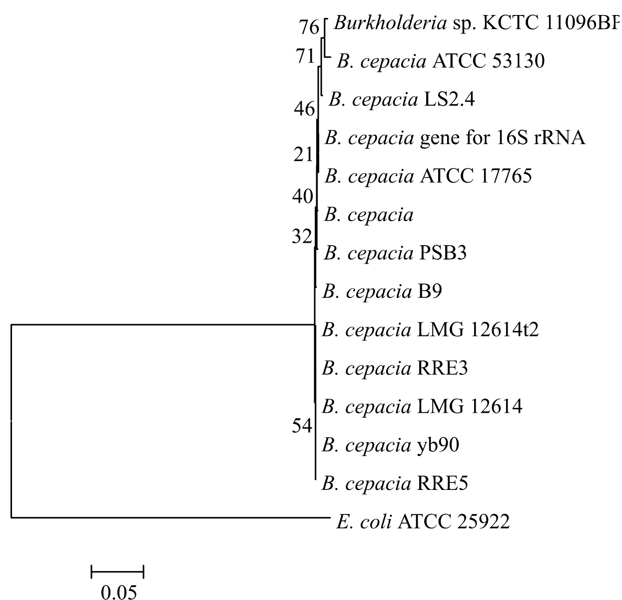

Fig. 4. Identification of bacterial isolate SE4 by phylogenetic analysis. Neighbor joining (NJ) distance tree with 1,000 replications was constructed using 14 taxa (13 reference sequences and 1 clone). *Burkholderia* sp. KCTC 11096BP formed a subclade with *B. cepacia* ATCC 53130 with a bootstrap support of 75%, which confirm *Burkholderia* sp. KCTC 11096BP as a new strain of genus *Burkholderia*. The analysis was done using Mega4 software.

These strains were selected through BLAST search showing maximum sequence homology percentage and query coverage, and lowest E values. *E. coli* was used as out group (Fig. 4). The phylogenetic analysis of 16S rDNA sequence confirmed bacterial isolate SE4 is a new strain of *Burkholderia*. The bacterial strain was thus named as *Burkholderia* sp. KCTC 11096BP. The 16S rDNA sequence of this strain has been submitted to GenBank database (accession no. FJ445745). The bacterial isolate was also deposited to the Korea Collection for Type Cultures (KCTC) and was allotted no. KCTC 11096BP.

Discussion

Plant growth promotion by PGPR species with gibberellins production capacity have been reported in several previous studies (Bastián *et al*., 1998; Gutierrez-Manero *et al*., 2001). In current study, bacterial culture suspensions significantly promoted growth parameters of cucumber and Crown daisy, which may be due to the GA production capacity of the newly isolated *Burkholderia* sp. KCTC 11096BP. In a similar study, *Phaseolus lunatus* inoculated with a specific strain of *Bradyrhizobium* sp. showed a marked internode elongation that was not observed in plants inoculated with other compatible bradyrhizobia (Dobert *et al*., 1992). Yanni *et al*. (2001) observed that inoculation of rice with *Rhizobium* strains promoted seedling vigor, root length, shoot length and grain yield of rice. They reported that pure cultures of these *Rhizobium* strains produced auxin (IAA) and gibberellins (tentatively identified as GA_7).

The process of traditional P fertilizer production is environmentally undesirable, not least because of the release of contaminants into the main product, gas stream and by-products (Song *et al*., 2008). For over a century agricultural microbiologists and microbial ecologists have been interested in the ability of some bacteria to dissolve poorly soluble mineral phosphates such as tricalcium phosphate or hydroxyapatite (Goldstein *et al*., 1993), as most of the phosphorus in soils is present in insoluble form (Abd-Alla, 1994). The phosphate solubilization capacity of *Burkholderia* sp. KCTC 11096BP was observed for 7 days in liquid NBRIP medium. The measurement of clearing zone formed, which showed that maximum phosphate solubilization took place until 120 h of bacterial inoculation. An increase in clearing halos indicated an increased acidic secretion by the bacterial strain, which converted insoluble phosphate to soluble one. The plate screening method used in this experiment can be generally regarded as reliable technique for isolation and preliminary characterization of phosphate-solubilizing microorganisms (Illmer and Schinner, 1992). The pH of the media was also analyzed at 24 h interval and the pH was found to decrease until 96 h of bacterial inoculation. This decrease in pH clearly indicated the production of acids, which are considered to be responsible for phosphate solubilization. The extent of pH decline correlates with the phosphate solubilization potential of the bacterial strain as acidic secretions by the bacterial strain are responsible for the dissolution of tricalcium phosphate in the culture medium. Kpomblekou and Tabatabai (1994) reported that microorganisms which decrease the medium pH during growth are efficient P solubilizers.

Gibberellins producing ability is inherent in all groups of microorganisms (Mitter *et al*., 2002) including epiphytic and rhizospheric bacteria i.e. the representatives of the genera *Azotobacter*, *Arthrobacter*, *Azospirillum*, *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Flavobacterium*, *Micrococcus*, *Agrobacterium*, *Clostridium*, *Rhizobium*, and *Xanthomonas*). However, *Burkholderia* was never reported for gibberellins production earlier. The bacterial isolate *Burkholderia* sp. KCTC 11096BP was found to produce 8 types of GAs, including bioactive GA_1, GA_3, and GA_4. It was also observed that the relative content of the 3β-hydroxylated GAs (GA_1, GA_3, and GA_4) was higher than that of other GAs in the culture broth of the PGPR, which confirm earlier reports for 3β-hydroxylated GAs in *B. cereus* MJ-1, *B. macroides* CJ-29, and *B. pumilus* CJ-69 (Joo *et al*., 2004). The GAs were analyzed through GC/MS in selected ion monitoring (SIM) mode, which provides a more reliable GAs quantification technique as compared to TLC, bioassays or HPLC-UV that give poor resolution and least degree of reliability. The major advantage of GC/MS is its unbiased character. In comparison with non-MS detection based chromatographic techniques (HPLC-DAD, GC-FID), where only compounds targeted by a special analytical protocol are found, GC-MS analysis can result in interesting and unexpected new knowledge about a particular extract (Franck *et al*., 2005).

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