The Biocontrol Bacterium *Bacillus amyloliquefaciens* KPS46 Produces Auxin, Surfactin and Extracellular Proteins for Enhanced Growth of Soybean Plant

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Abstract

Bacillus amyloliquefaciens strain KPS46, a plant growth promoting rhizobacterium isolated from soybean, was investigated for the secretion of compounds that might be involved in plant growth promotion. Extracts containing indoles, lipopeptides, and proteins were made from the cell-free fluid of KPS46 broth cultures. These extracts, along with a whole culture of KPS46 and the raw fluid and cellular fractions from a KPS46 culture, were applied separately to soybean seeds. The seeds were planted into plates of a plant growth medium and kept in a growth chamber or into pots of pasteurized planting media maintained in a greenhouse. Under both conditions, all of the extracts and fractions, with the exception of a treatment with heat-killed KPS46 cells, increased root and shoot lengths and plant biomass as compared to distilled water control. Thus, indoles, lipopeptides, and proteins secreted by KPS46 can directly influence plant growth and development. Analysis of the indole and lipopeptide extracts by HPLC revealed the auxin indole-3-acetic acid and the antibiotic surfactin to be the primary compounds, respectively. To investigate the proteins involved in growth promotion, two-dimensional gel electrophoresis was used to separate proteins secreted by KPS46 and by N19G1, a UV-derived mutant of KPS46 with reduced production of auxins, lipopeptides, extracellular proteins, and lacking growth promotion activity. The identity and putative function of twenty proteins secreted by KPS46 but not by N19G1 were determined. The analysis revealed a number of proteins which may be involved in plant growth promotion by acting as plant growth regulators, stimulating metabolism or functioning in defense against stress factors.

Keywords: signal molecules, elicitors, PGPR, proteomic analysis

Introduction

Bacillus amyloliquefaciens KPS46, a strain of plant growth promoting rhizobacteria (PGPR) isolated from a soybean plant in central Thailand (Prathuangwong and Kasem, 2004; Prathuangwong et al., 2005), can protect soybean and other crop plants against multiple plant pathogens by induced resistance and secretion of antimicrobial metabolites (Buensanteai et al., 2007a; Prathuangwong and Buensanteai, 2007; Prathuangwong and Kasem, 2004; Prathuangwong et al., 2004, 2005). When applied as a seed treatment, it can also promote the growth of soybean (Buensanteai et al., 2007b, 2008). The mechanisms by which KPS46 promotes plant growth are not well understood.

In addition to KPS46, other strains classified in *B. amyloliquefaciens* and in the closely related species *B. subtilis* have been reported to be effective for the biocontrol of phytopathogens and for plant

growth promotion (Araujo et al., 2005; Kloepper et al., 2004; Idriss et al., 2007). Extrapolating from the accumulative literature on these two related species and on other species of PGPR, numerous mechanisms are possible in KPS46 as to antagonism against pathogens, alteration of nutrient availability, and direct interactions with plants. All of these interactions could potentially lead to plant growth promotion.

As a starting point towards understanding how KPS46 causes enhanced plant growth, we focused this study on factors secreted by KPS46 that might directly affect the physiology of soybean plants. In this context, the production of auxins, indolic compounds with phytohormone activity, by *Bacillus* spp. is well known (Araujo et al., 2005; Kloepper et al., 2004; Idriss et al., 2007). Indole-3-acetic acid (IAA), the main auxin in plants, controls important processes including cell enlargement and division and tissue differentiation. Regulation of these processes requires a balance between auxins and other phytohormones. Thus, IAA production by strains of PGPR can contribute sufficient auxin to the plant's auxin pool to have profound effects on these processes and potentially allow the microorganisms to redirect a plant's physiology and biochemistry for their own benefit (Idriss et al., 2007; Leveau and Lindow, 2005; Patten and Glick, 2002).

Other groups of compounds commonly reported to be secreted by *Bacillus* spp. include the lipopeptide families, iturin and surfactin that are well known for their strong antibiotic activity (Araujo et al., 2005; Bonmatin et al., 2003; Jacques et al., 1999; Ongena et al., 2007). Surfactin, because of its biosurfactant quality, is directly toxic to diverse microorganisms, including fungi, bacteria, and virus (Bonmatin et al., 2003). It was recently to induce resistance in plants (Ongena et al., 2007), and thus, there is evidence that surfactin also has the potential to directly affect the physiology of plants.

Another large group of bacteria-secreted compounds is extracellular proteins. Strains in the *Bacillus* group secrete high levels of extracellular proteins as enzymes and secondary metabolites (Tjalsma et al., 2004). Some proteins secreted by *Bacillus* spp. are involved in key ecological functions such as biofilm formation (Oosthuizen et al., 2002), but extracellular proteins, as a group, have not yet been investigated in the context of plant growth promotion.

The primary goal of this study was to determine whether or not compounds secreted by KPS46 have a direct role in regulating plant growth, i.e., promoted plant growth under optimal nutrient conditions and in the absence of plant-deleterious organisms. One objective was to determine whether lipopeptides, indoles, and extracellular proteins produced by KPS46 in liquid culture could enhance growth of soybean when applied to soybean seeds. Another objective was to analyze the lipopeptide and indole extracts from KPS46 to identify the primary components. Lastly, the proteomic approach was used in analyzing extracellular proteins secreted by KPS46 to identify those that might be important in plant growth promotion. To narrow the spectrum of proteins, secreted proteins from wildtype KPS46 were compared with those from a mutant strain affected in growth promotion traits. Preliminary results have been published (Buensanteai et al., 2007b, 2008).

Materials and Methods

Culture Conditions and Preparation of Extracellular Extracts

Cells of *B. amyloliquefaciens* strain KPS46 stored in nutrient glucose broth with 10% glycerol at -80°C were revived by streaking onto nutrient glucose agar (NGA) and cultured at 28°C ± 2°C for 48 h. To prepare cultures for extraction of extracellular factors, the strain was transferred to 500 mL volumes of nutrient broth containing 2% glucose (NGB) and incubated for 48 h at 28 ±2°C with constant shaking at 180 rpm. The cultures were centrifuged at 13,000 rpm at 4°C for 20 min and the supernatants were passed through 0.2 nm nitrocellulose filters and retained for further extraction.

The bacterial cell pellet was washed three times in sterile saline (0.85% NaCl). The cells were re-suspended in sterile distilled water and the density of the suspension was adjusted to 10^8 cfu mL^{-1} based on optical density (OD of 0.2 at 600 nm). A portion of the cell suspension was used in experiments in live form, while another portion was heated in a water bath at 100°C for 30 min to kill the cells prior to use.

Extracts containing indoles were made from the cell-free culture supernatant using the method described by Araujo et al. (2005). Cell-free culture

fluid was extracted three times with ethyl acetate after adjusting the pH to 2.8 and then evaporated at 40°C for 20 min. The material was solubilized in sterile distilled water for use in experiments.

Extracellular lipopeptides were extracted from KPS46 culture supernatant by acidifying the fluid to pH 2.0 with concentrated HCl and allowing the formation of a precipitate at 4°C overnight. The precipitate was collected by centrifugation (12,000 rpm, 4°C, 15 min), washed three times with distilled water, and dried by vacuum lyophilization. The dried lipopeptide was extracted three times with 100% methanol for 3 h. The methanol was removed with a rotary evaporator under reduced pressure, yielding a brown-colored crude lipopeptide extract. The extract was solubilized in sterile distilled water prior to use in experiments.

To prepare the extracellular protein extract, 20 mL 50% trichloroacetic acid (Sigma T6399) was added to 500 mL of culture supernatant, mixed well and placed on ice for 30 min. The aggregated proteins were precipitated by centrifugation at 12,000 rpm at 4°C for 15 min, washed three times in cold 70% ethanol (-20°C), dried and dissolved in IEF (isoelectric focusing) sample buffer consisting of 8 M urea (Sigma U6504), 2 M thiourea (Sigma T7875), 2% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, Sigma C9426), 2% Triton X-100 (Sigma T8532), 50 mM DTT (dithiothreitol, Sigma D9163), and 0.5% ampholytes (Bio-Rad 163-1152) (Buensanteai et al., 2008), the protein extract was solubilized in sterile distilled water. Total protein content of the sample was quantified by the Bradford assay (Bradford, 1976) prior to use in experiments.

Plant Bioassays for Growth Promotion

Two sets of experiments were conducted to evaluate KPS46 culture fluid extracts for effects on soybean growth. Seeds of soybean cv. Spencer were surface disinfested by treatment with 95% ethanol for 2 min, followed by soaking in 20% (v/v) solution of commercial bleach for 20 min. The seeds were then washed with sterile distilled water 5 times in order to remove the bleach. Before planting, 30 g of soybean seeds were mixed thoroughly with 5 mL of a liquid treatment. The treatments included a whole culture of KPS46 in NGB, cell-free fluid from a KPS46 culture in NGB, a suspension of live cells collected from a KPS46 culture, and a suspension of heat-killed cells. Sterile distilled water was used as the control. Cell concentrations in the whole culture and cell suspensions were adjusted with sterile distilled water to 1×10^8 cfu mL^{-1}, based on absorbance, while culture fluid was diluted by the same dilution factor as the whole culture. Other treatments included extracts of extracellular proteins (250 μg mL^{-1}), lipopeptides (50 μg mL^{-1}) and indoles (50 μg mL^{-1}) extracted from KPS46. Distilled water was used to dilute the raw extracts to these concentrations, which roughly correspond to those found in NGB cultures with 1×10^8 cfu KPS46 mL^{-1}.

In one set of experiments conducted under gnotobiotic conditions, treated seeds were placed onto 0.5X MS medium (Murashige and Skoog basal salt mixture, Sigma M5524) in square plates. There were four replicate plates per treatment with three seeds per plate. The plates were incubated on edge at a 65◦ angle to allow root growth along the agar surface and unimpeded growth of the tops of the seedlings after the seed had germinated. The plates were maintained in a growth chamber with a photoperiod of 16 h of light, 8 h of darkness, light intensity of 200 μmol m^2s^{-1}, and constant temperature of 24°C. At 7 days after germination, seedlings were harvested for measurement of growth parameters (root and shoot lengths; fresh and dry weights; and numbers of lateral roots). The experiment was performed three times.

In another set of experiments, soybean seeds treated with KPS46 culture extracts were planted in pots (30 cm diameter) containing a steam-pasteurized potting medium of Sharpsburg silt clay loam, vermiculite, and sand mixed in equal volumes. There were 10 replicate pots per treatment with two seeds per pot. The pots were watered daily with a nutrient solution (20-10-20 Peat-Lite Special, Scotts-Sierra Horticultural Co., Marysville, OH) and kept in a greenhouse with a 12-h photoperiod (25°C and 60 to 75% relative humidity during the light period, 15°C and >93% relative humidity during the dark period). At 14 days after seedling emergence, seedlings were harvested for measurements of root and shoot lengths, along with fresh and dry weights. The experiment was conducted three times.

Data from each experiment was subjected to analysis of variance using SAS version 9.1. Separation

of treatment means was accomplished by Duncan's Multiple Range Test, and all tests for significance were conducted at $P \leq 0.05$.

Analysis of Indoles

Supernatants from two types of cultures of KPS46 were extracted and assayed for indoles. In one, KPS46 was grown in NGB for 24, 48, 72, 96 and 120 h. In the other, KPS46 was cultured at 28±2°C for 48 h on a shaker incubator in DF salt minimal medium amended with 0, 125, 250, 500, 2,500 and 5,000 mM tryptophan obtained from a filter-sterilized 2 mg mL^{-1} stock of L-tryptophan prepared in warm water. The indole extract was extracted from cell-free culture supernatants as described above. Indole concentrations were determined based on the method described by Patten and Glick (2002) with slight modifications. For the colorimetric Salkowski assay for indoles, 1 mL of culture fluid was mixed with 4 mL of Salkowski's reagent and incubated at room temperature for 20 min. The absorbance was measured at 535 nm using a spectrophotometer (Spectronic 20D, Rochester, NY, USA) (Crozier et al., 1988). The quantity of indoles was determined by comparison with a standard curve using purified IAA in the concentration range of 0-50 μg mL^{-1}. Concentration of IAA in the extracts also was determined by reverse phase-HPLC by injecting 60 μL aliquots of extracts into an Alltech, type Econosphere C185U column (250 by 4.6 mm) equipped with a differential UV detector absorbing at 280 nm. The isocratic solvent used for reverse-phase chromatography was acetonitrile-glacial acetic acid (1%) in water (1:9). The flow rate was adjusted to 1 mL min^{-1}. Peak retention times were compared with those of chemically-synthesized IAA standards and quantified by comparison of peak areas.

Analysis of Lipopeptides

A lipopeptide-enriched extract was obtained from 20 mL of the crude lipopeptide extract from cultures of KPS46 in NGB by purification on an ISOLUTE C-18 CE type cartridge (International Sorbent Technology Ltd., Hengoed, UK) following a modification of the method described by Jacques et al. (1999) and Araujo et al. (2005). The enriched extract was dissolved in butanol and loaded onto a column of a reverse-phase HPLC system. The system was operated at a flow rate of 2.0 mL min^{-1} with 90% methanol as the mobile phase. HPLC spectra were detected by a UV monitor at 210 nm. The presence of surfactin-type lipopeptides was determined on the basis of retention times compared with those of purified surfactin standards (Sigma S3523).

Mutagenesis of KPS46 and Selection of Mutant Strains

Cells were obtained from a culture of strain KPS46 in NGB at stationary phase, 4 h after the optical density (λ = 600 nm) of cultures stopped increasing. The cells were washed and suspended in 0.05 M potassium phosphate buffer, pH 6.8, to 1×10^8 cfu mL^{-1}. Volumes (200 μL) of this suspension were exposed to UV irradiation for various durations in glass petri dishes at a constant distance from the UV source (λ = 254 nm, 10 erg mm^{-2}; BII Illuminator). The samples were frequently agitated during exposure. Aliquots (100 μL) were removed from the irradiated samples at different time intervals and spreaded onto nutrient glucose agar plates. Colonies arising from surviving cells were selected following 48 h of incubation in the dark condition (Whistler et al., 2000; Saxena et al., 2002).

More than 2,490 irradiated strains were screened for loss of or decrease in indole production. Fluid from cultures of these strains in NGB was tested for indole concentration via the Salkowski assay. Those strains in which indole production was decreased by more than 10% compared with wildtype KPS46 were considered to be indole-reduced mutants and were retained for further study. Selected indole-reduced mutants were compared further to wildtype KPS46 with respect to excretion of IAA, using reverse-phase HLPC as described above, and production of lipopeptides. Relative lipopeptide production was accessed on the basis of swarming motility across a solid surface, this assay predicated by lipopeptides being required for swarming motility (Kearns and Losick, 2003). This trait was evaluated by seeding cell suspensions of stationary-phase cultures (50 μL; 1×10^8 cfu mL^{-1}) onto the centers of motility test plates (NGB with 1.5% agar; and LB with 0.3, 0.7, or 1.5% agar). Plates were incubated at 37°C, and the diameters of halos due to bacterial migration were measured 24 to 48 h postinoculation, and

swarm cell differentiation was analyzed (Senesi et al., 2002; Kearns and Losick, 2003).

In addition, an experiment was conducted to determine whether or not extracellular proteins produced by UV mutant strain N19G1 derived from KPS46 can enhance plant growth. As in the gnotobiotic and greenhouse experiments described above, 30 g of soybean seeds was mixed thoroughly for 5 min with 15 mL of protein extract from cultures of wild type strain KPS46 and mutant strain N19G1, and the seed germinated and seedlings grown in plates with 0.5X MS medium or planted in pots of planting medium. Biomass, number of lateral root, and root and shoot lengths were determined at 7 days after seed germination. Each experiment was conducted three times.

Proteomic Analysis of Extracellular Proteins of KPS46

The intent of this experiment was to identify some of the specific components of the extracellular proteome of KPS46 that may have some relationship to IAA biosynthesis, lipopeptide production and plant growth promotion. To reduce the range of proteins to those more closely involved in these processes, extracellular proteins of KPS46 were compared with those secreted by mutant strain N19G1 which was reduced in growth promotion ability. Phenylmethylsulphonyl fluoride (Sigma) was added to late-exponential phase cultures of KPS46 and N19G1 to a final concentration of 5 mM to prevent proteolytic digestion. Proteins were then extracted from the culture fluids as described above. The extracellular protein samples were used directly to passively rehydrate isoelectrically focused on an Electro Immobiline Dry Strip pH 3–10 and pH 4-7 (11 cm; Bio-Rad, USA) by applying 185 μL of each sample (equivalent to 250 μg of protein). Extracellular preparations dissolved in the 10X of IEF sample solution was applied to the first dimension. The IPG strips were focused for 15 h at 400V followed by 1 h at 600V using a Multiphor II (Amersham Pharmacia). After placing IPG strips in equilibration buffer A [50 mM Tris/HCl, pH 6.8, containing 8 M urea, 30% glycerol, 2.5% SDS (sodium dodecyl sulfate, Sigma-Aldrich 436143) and 0.25% DTT] for 15 min, they were transferred into buffer B [50 mM Tris/HCl, pH 6.8, 8 M urea, 30% glycerol, 2.5% SDS, 0.25% DTT and 4.5% iodoacetamide (Sigma, 1149)] for 15 min. The isoelectric focusing gels were embedded in gels (0.25 M Tris/HCl, pH 6.8, 0.25% SDS, 1% agarose onto 14% SDS polyacrylamide). The proteins were resolved in the second dimension by a constant current of 100 volts until the bromphenol blue marker entered the stacking gel, followed by 140 volts until the blue dye reached the bottom of the gel (Antelmann et al., 2003; Duy et al., 2007; Lai et al., 2003). The 2D gels were visualized by staining with colloidal Coomassie blue G-250 [17% ammonium sulphate, 34% methanol, 3.6% orthophosphoric acid, 0.1% Coomassie blue G-250 (Sigma B0770)] (Voigt et al., 2006). The gels were fixed in destaining solution (80% ethanol and 20% acetic acid mixture) and washed with 70% ethanol. The gels were analyzed using ImageMaster software for protein spots visible exclusively or at higher intensity in the gel containing extracellular proteins from wildtype strain KPS46 as compared to the UV mutant strain N19G1. Proteins showing more than 2-fold increase in expression in the wildtype compared with the mutant were selected for identification. Amino acid sequences of the protein spots were identified by the Protein and the Mass Spectrometry Core Facility of the Center for Biotechnology, University of Nebraska-Lincoln, using mass spectrometry analysis methods modified from those described by Voigt et al. (2006) and Domon and Aebersold (2006). The sequence data were compared with the NCBI and SwissProt databases using local MASCOT to identify possible protein names and functions.

Results

Effects of KPS46 Extracellular Factors on Plant Growth

Crude extracellular lipopeptide, proteins, and indole extracts from cultures of strain KPS46 were effective in promoting the growth of soybean seedlings under gnotobiotic conditions. These culture extracts, when applied to soybean seeds, increased root and shoot lengths, by more than 40 and 20%, respectively (Figure 1A), and increased fresh and dry weights by more than 30% (Figure 1B) compared to the distilled water control. Seed treatment with the extracted factors of KPS46 also increased the number of lateral root more than 20% compared to the control (Figure 2). Treatment with the lipopeptide,

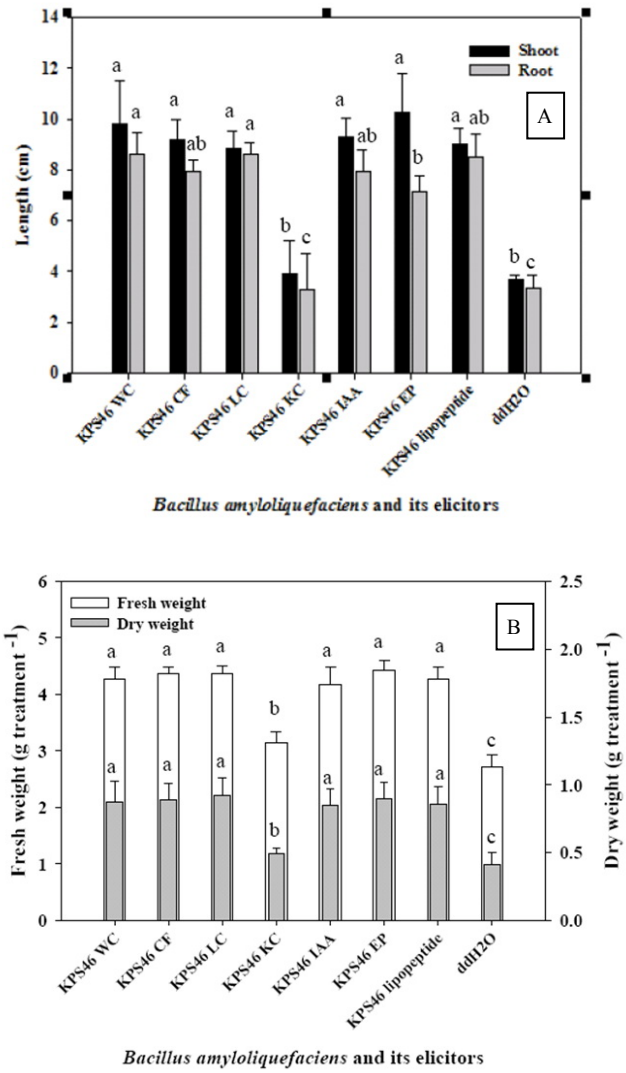

Figure 1 Effects of cellular fractions and extracellular extracts from cultures of *Bacillus amyloliquefaciens* KPS46 on the growth of soybean under gnotobiotic conditions, as measured at 7 days after inoculation in: A) shoot and root length; B) fresh and dry weight. WC = whole culture, CF = culture fluid, LC = live cell suspension in distilled water, KC = heat killed cell suspension, IAA = indole extract, EP = extracellular proteins extract. The data are the average of four replications (three plants per replication) for each treatment. Error bars represent the standard deviation. For each growth parameter, different letters indicate significant differenced ($P \leq 0.05$) among treatments.

Protein, and indole extracts had similar effects on soybean growth as seed treatments with a whole culture of KPS46, the cell-free fluid fraction from a KPS46 culture, and a suspension of live cells from the culture (Figures 1 and 2). There was no significant effect of seed treatment with a suspension of heat-killed cells of KPS46 compared with distilled water for any soybean plant growth parameter (Figures 1 and 2). Similar results were obtained when the experiment was repeated.

Seed treatments with extracted lipopeptides, proteins, IAA, and other fractions from a culture of KPS46 yielded similar results in greenhouse pot experiments (Figure 3). All of the treatments involving KPS46 culture fractions, except the heat-killed cells, increased shoot and root lengths (Figure 3A) and plant biomass (Figure 3B) compared to the distilled water control. Repetition of the experiment yielded similar results.

Indole-3-Acetic Acid Analysis

Strain KPS46 secreted IAA as the major auxin, as determined by HPLC, when cultured in NGB medium, with the highest IAA concentration (31.0 μg mL^{-1}) detected at stationary phase (Figure 4). In mineral medium amended with various concentrations of tryptophan, strain KPS46 secreted higher amounts of IAA with increasing tryptophan concentration, while growth of the bacterium was unchanged across tryptophan concentrations (Figure 5), revealing that IAA production in KPS46 is dependent upon tryptophan as a precursor to IAA rather than as a nutrient source for growth.

Lipopeptide Production

When the lipopeptide extract from the culture fluid of KPS46 was analyzed by HPLC, high concentrations of bioactive non-polar antibiotics were detected (Figure 6A). Surfactin-type lipopeptides were identified in the extract on the basis of their retention times being similar to those of purified surfactin standards, such as surfactin produced by *B. subtilis* ATCC21332 (Figure 6B). C18 homologues represented together more than 50% of the total amount of lipopeptides present in the extract. Based on HPLC peak areas of the lipopeptide extract compared with values obtained for standards, the total amount of surfactins produced by strain KPS46 was 550 mg L^{-1} ± 20.267 mg L^{-1} (mean and standard deviation calculated from three independent cultures).

UV Mutant Strain N19G1 Phenotype

Strain N19G1 exhibited significantly ($P \leq 0.05$) reduced excretion of IAA in NGB medium compared to wildtype KPS46 (Figure 7). The mutant strain also

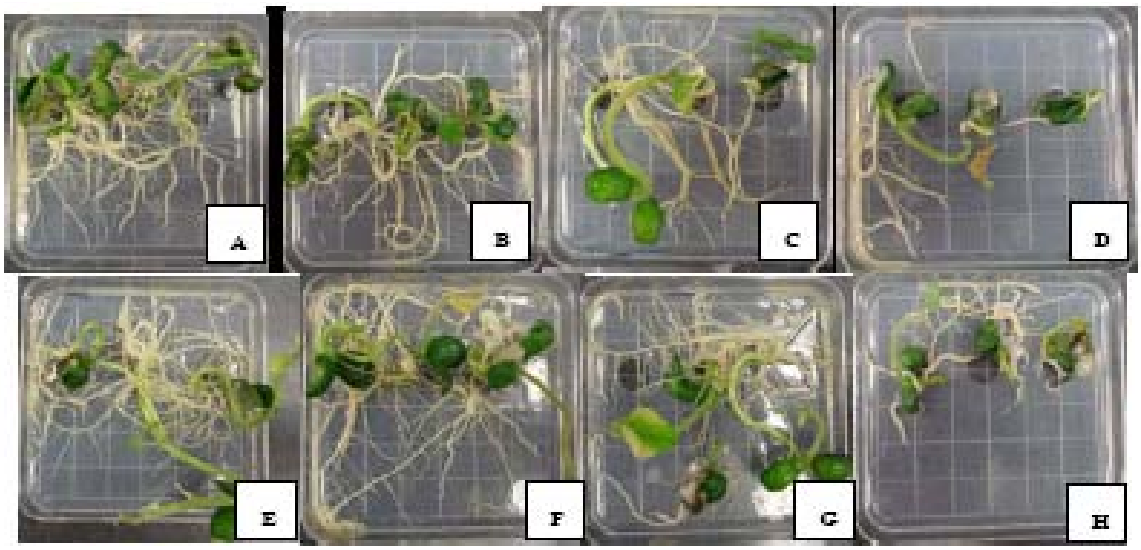

Figure 2 Effects of cellular fractions and extracellular extracts from cultures of *Bacillus amyloliquefaciens* KPS46 on root development in soybean under gnotobiotic conditions, as observed at 7 days after treatment of soybean 'Spencer' seeds with a whole culture (A), culture fluid (B), live cell suspension in distilled water (C), heat killed cell suspension (D), indole extract (E), extracellular protein extract (F), lipopeptide extract (G) and distilled water (H).

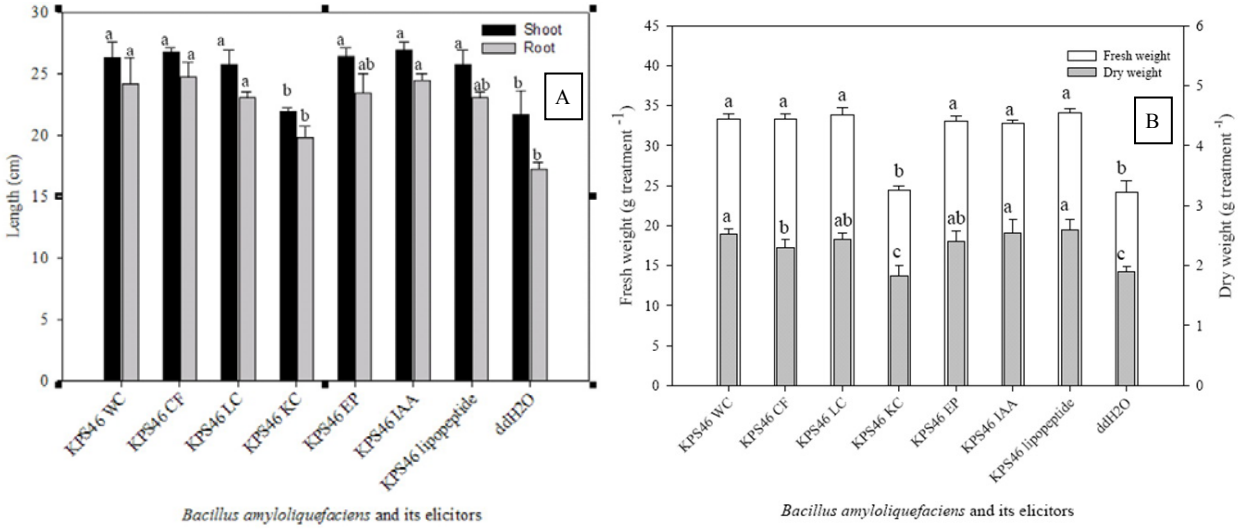

Figure 3 Effects of cellular fractions and extracellular extracts from cultures of *Bacillus amyloliquefaciens* KPS46 on the growth of soybean under greenhouse conditions, as measured at 14 days after treatment in: A) shoot and root length; B) fresh and dry weight. WC = whole culture, CF = culture fluid, LC = live cell suspension in distilled water, KC = heat killed cell suspension, IAA = indole extract, EP = extracellular protein extract. The data are the average of four replications (three plants per replication) for each treatment. Error bars represent the standard deviation. For a growth parameter, bars with the same letter are not significantly different ($P \leq 0.05$).

exhibited decreased swarming motility, resulting in altered colony morphology on all test media compared with wildtype KPS46 (Figure 8). When extracellular proteins extracted from KPS46 and N19G1 were applied as a seed treatment in gnotobiotic (Figure 9) and greenhouse experiments (Figure 10), proteins from strain N19G1 had little or no affect on soybean growth parameters compared to the distilled water control. In contrast, proteins secreted by KPS46 elevated all growth parameters under both experimental conditions.

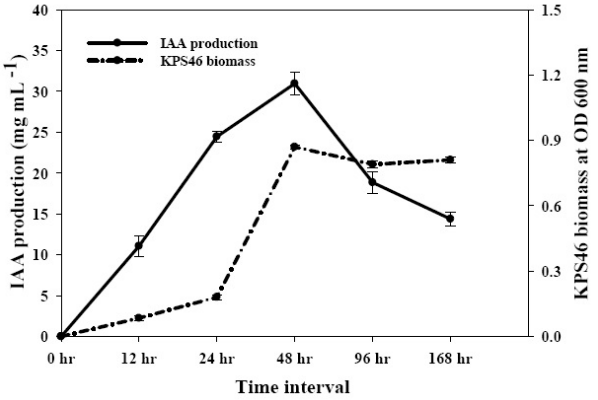

Figure 4 Concentrations of indole-3-acetic acid (IAA) secreted by *Bacillus. amyloliquefaciens* KPS46 into NGB medium, as measured by reverse phase-HPLC, and concurrent relative population levels of the bacterium measured by absorbance. Data are the means and standard deviations of three replicates. Error bars represent the standard deviation.

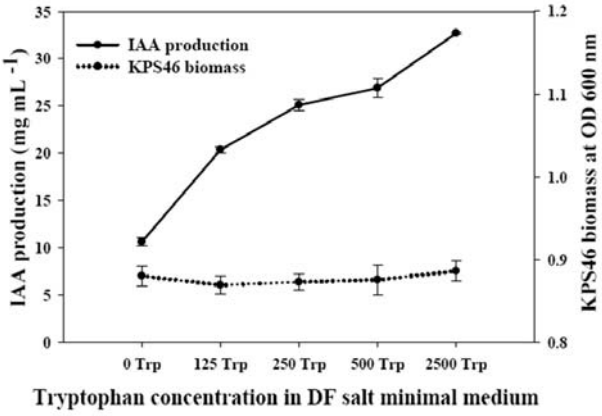

Figure 5 Production of indole-3-acetic acid (IAA) by *Bacillus amyloliquefaciences* KPS46 and populations of KPS46 in DF salt minimal medium amended with increasing concentrations of L-tryptophan. IAA and bacterial populations were determined 24 h after inoculation of each medium with the same starting population of KPS46. Data are the means and standard deviations of three replicates. Error bars represent the standard deviation.

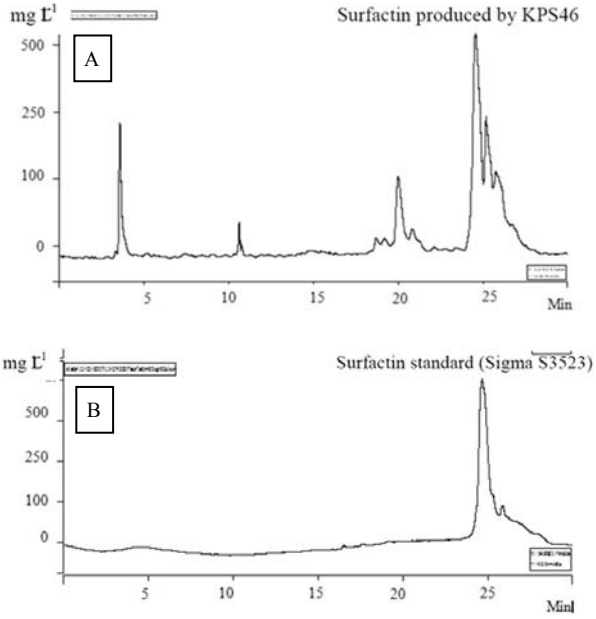

Figure 6 Reverse phase-HPLC profile of lipopeptides produced by *Bacillus amyloliquefaciences* KPS46 in NGB medium (A) and surfactin standard produced by *B. subtilis* (Sigma S3523) (B.).

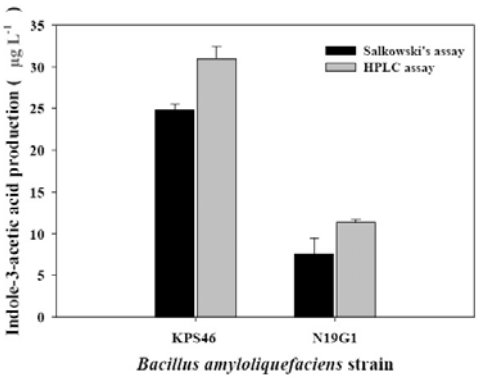

Figure 7 Concentrations of indole-3-acetic acid secreted by wildtype strain *Bacillus amyloliquefaciens* KPS46 and UV mutant strain N19G1 cultures grown in NGB medium. The concentration values were standardized to 10^8 cfu mL^{-1} of cells in the medium. The data are the means and standard deviations of three replicates. Error bars represent the standard deviation. Bars with the same letter are not significantly different ($P \leq 0.05$).

Proteomic Analysis

Two dimensional polyacrylamide gel electrophoresis of extracellular proteins secreted by wildtype KPS46 revealed around 190 detectable spots (Figure 11). Among these, twenty corresponding to upregulated proteins, as compared to proteins from mutant strain N19G1, were assigned numbers as shown in Figure 11 and identified on the basis of amino acid sequence. The large majority of the identified proteins were homologous to proteins produced by *B. subtilis*, the remainder being homologous to proteins reported from other *Bacillus* spp. or other strains of *B. amyloliquefaciens* (Table 1). The twenty proteins were placed in six categories based on predicted function. Four were involved in detoxification and adaptation of cellular processing including catalase, two forms of superoxide dismutase, and general

Table 1 Upregulated proteins from the extracellular secretome of *Bacillus amyloliquefaciens* KPS46.

Spot number	Protein name[1/]		Species	Accession number	Molecular mass (Da)	*pI*	Score	Sequence coverage (%)
Functional category 1 : Detoxification and adaptation of cellular processing								
1	KatA	Catalase	*B. subtilis*	Q3HNK4_BACSU	54503	6.45	2021	75
13	SodA	Manesium superoxide dismutase (MnSOD)	*B. vallismortis*	Q6XZA2_BACVA	16176	5.27	540	44
14	SodF/ SodC	Copper/ Zinc superoxide dismutase (FeSOD, Cu/ZnSOD)	*B. cereus*	BCE33L4639	16176	8.24	235	29
18	YhdN	General stress protein 69	*B.subtilis*	G16U_BASU	20607	4.49	121	6
Functional category 2 : Transport/binding protein and lipopeptide								
2	OppA-F	Oligopeptide permease	*B. subtilis*	CAA39787	61543	5.61	933	35
3	OppA-F	Oligopeptide permease	*B. subtilis*	CAA39787	61543	5.61	1095	33
4	OppA-F	Oligopeptide permease	*B. subtilis*	CAA39787	61543	5.61	809	33
5	AppA	Oligopeptide ABC transporter oligopeptide binding protein	*B. subtilis*	I40545	61965	5.84	1379	34
8	YufN	ABC transporter (lipoprotein) homolog yufN	*B. subtilis*	C70009	37383	5.03	355	20
12	PstB	Phosphate ABC transporter (binding protein) homolog yqgG	*B. subtilis*	A69956	31721	5.04	326	19
Functional category 3 : Protein and amino acid biosynthesis								
6	NprE	Neutral protease or extracellular neutral protease (Fragment)	*B. subtilis*	BSU1110	59317	7.89	779	45
7	NprB	Neutral protease B, Bacillolysin	*B. subtilis*	Q1L026_BACSU	56785	8.65	755	31
11	AprE	Serine alkaline protease (Pro-subtilisin)	*Bacillus sp. DJ-4*	Q6IT79_9BACI	39071	9.23	538	33
16	AprE	Subtilisin	*B. subtilis*	Q4ZIL5_BACSU	27367	6.64	671	50
19	YraA	Probable intracellular protease	*B.lichenifor mis*	Q65FN6_BACLD	19604	5.30	160	30
Functional category 4 : Amino acid metabolism								
9	GlnA/ GlnR	Glutamine synthetase	*B. subtilis*	BAA00730	50590	5.02	726	28
Functional category 5 : Energy metabolism								
10	LplD	Lytic enzyme or hydrolytic enzyme	*B. subtilis*	Q9R7J4_BACSU	27479	6.65	927	60
15	FbaA	Fructose-bisphosphate aldolase	*B. subtilis*	D32354	30552	5.19	595	38
17	Csn	Chitosanase precursor	*B. amyloliquefa ciens.*	Q9ET84_BACAM	31442	8.83	706	42
Functional category 6 : Nucleotide and nucleic acid metabolism								
20	GuaB	Inosine-5-monophosphate dehydrogenase	*B. subtilis*	DEBSMP	53129	6.18	865	36

[1/] Source of reference: NCBI and SwissProt database.

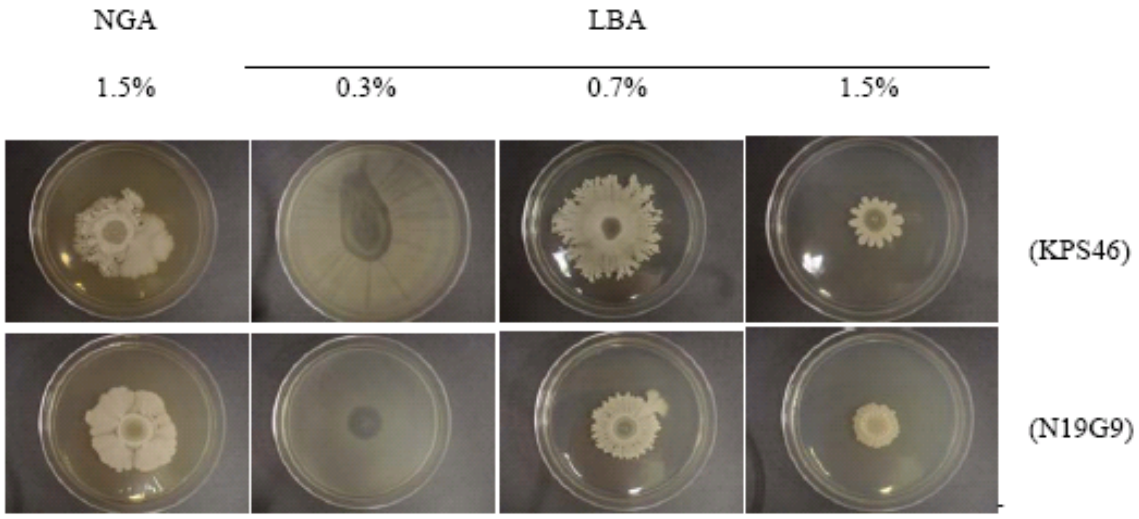

Figure 8 Swarming motility of *Bacillus amyloliquefaciens* wild type KPS46 and UV mutant strain N19G1 on NGB with 1.5% agar and LBA with 0.3, 0.7, and 1.5% agar.

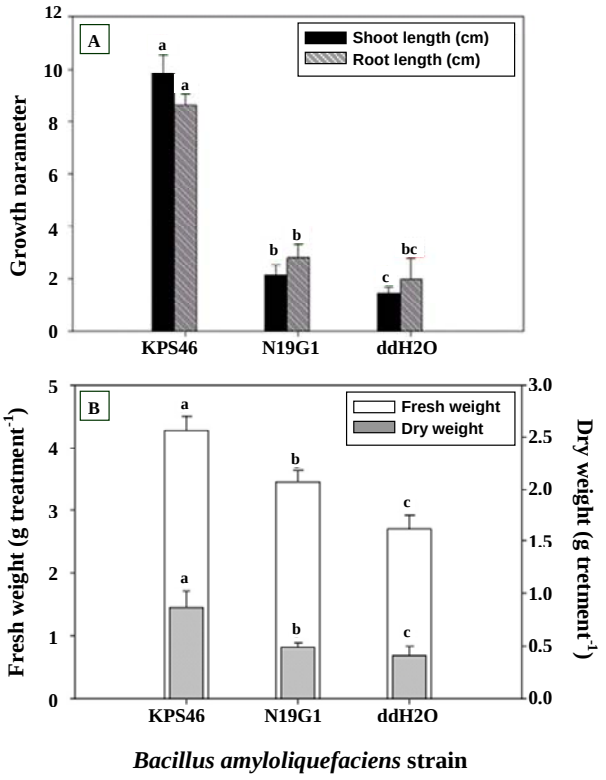

Figure 9 Effects of extracellular protein extracts from *Bacillus amyloliquefaciens* wild type strain KPS46 and UV mutant strain N19G1 in gnotobiotic experiment on soybean shoot and root length (A) and fresh and dry weight (B), as measured at 7 days after inoculation. The data are the average of four replications (three plants per replication) for each treatment. Error bars represent the standard deviation. For a growth parameter, bars with the same letter are not significantly different ($P \leq 0.05$).

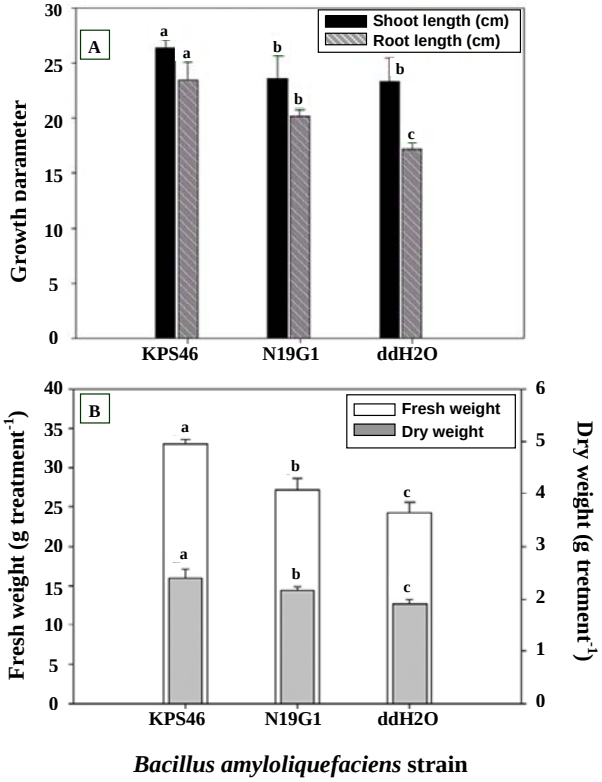

Figure 10 Effects of extracellular protein extracts from *Bacillus amyloliquefaciens* wild type strain KPS46 and UV mutant strain N19G1 in greenhouse experiment on soybean shoot and root length (A) and fresh and dry weight (B). The data are the averages of four replications (three plants per replication) for each treatment, as measured at 14 days after treatment. Error bars represent the standard deviation. For a growth parameter, bars with the same letter are no significantly different ($P \leq 0.05$).

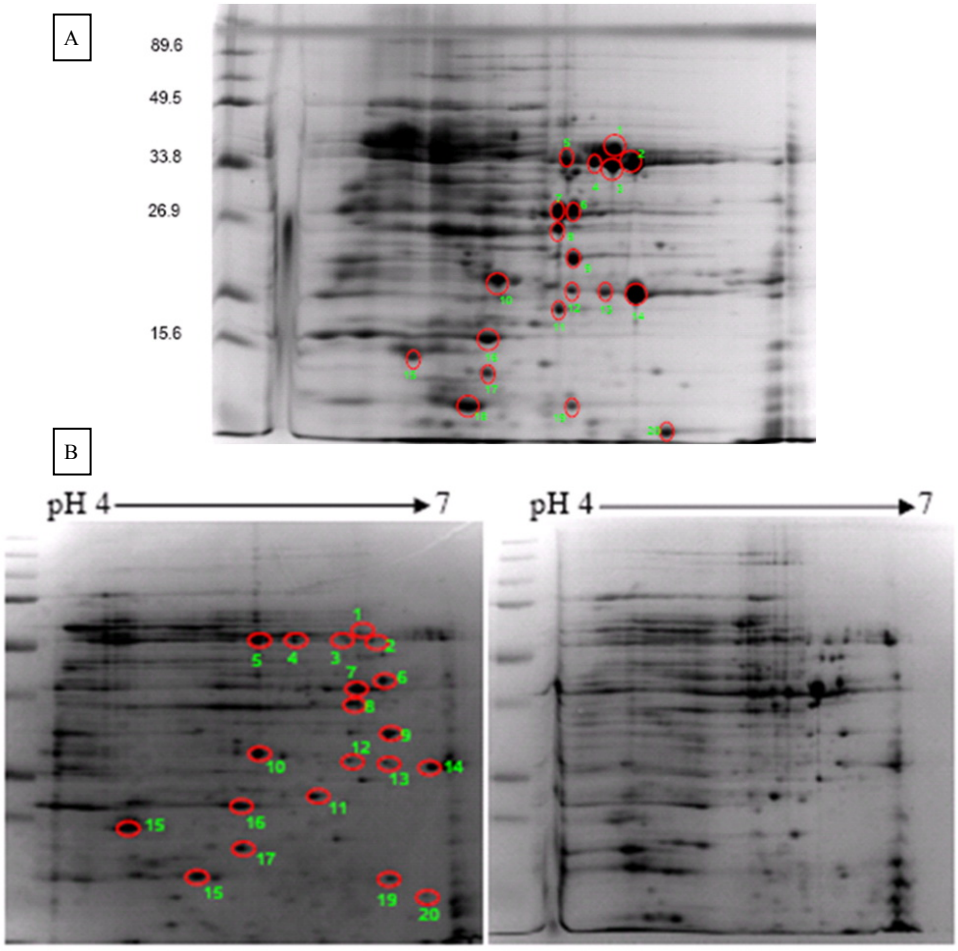

Figure 11 Two-dimensional gels of extracellular proteins extracted from *Bacillus amyloliquefaciens* KPS46: A) gel run on 11 cm of 3-10 IPG strips in the first dimension and 14% SDS-PAGE in the second dimension; B) gels of proteins from wildtype strain KPS46 (left) and mutant strain N19G1 (right) run on 11 cm of 4-7 IPG strips in the first dimension and 14% SDS-PAGE in the second dimension. Circled spots correspond to up-regulated proteins, identified by numbers, that were selected for analysis of amino acid sequence. *kD* = KiloDal.

stress protein. Six proteins were in the protein- and lipoprotein-binding transporter category, three of them (spot numbers 2, 3 and 4) were found to be homologous to the same oligopeptide permease of *B. subtilis*. The other proteins in this category were homologs of three different ABC transporter proteins. Among the five proteins in the protein and amino acid biosynthesis group, two were homologous to form subtilisin, a serine endopeptidase, while two other were homologous to neutral proteases and the fifth to a protease that is toxic to phytopathogenic microorganisms. The remaining proteins fell into three categories: amino acid metabolism, energy metabolism and nucleotide and nucleic acid metabolism. Notable in the energy metabolism category was a chitosanase homologue.

Discussion

This study represents the first analysis of metabolite production by *B. amyloliquefacians* KPS46 in relations to plant growth promotion. We found that KPS46 culture fluid extracts containing secreted indoles, lipopeptides and proteins individually can influence the growth of soybean to the same degree as cells of KPS46 washed free of pre-formed exoproducts. Whether or not the same type of compounds are secreted by KPS46 cells while existing in the spermosphere or rhizosphere remains to be determined, but nevertheless, the results are consistent with the hypothesis that strain KPS46 promotes the growth of soybean by secretion of several types of compounds.

Because each of the three crude extracts affected soybean growth and development under gnotobiotic condition, we surmise that components within each extract had direct effects on soybean, perhaps acting as signaling compounds. At the same time, we cannot rule out the possibility that under natural soil conditions the same compounds or other compounds within each extract may influence plant growth indirectly by altering soil nutrient availability or by inhibiting deleterious microorganisms. Analysis of the constituents of the extracts revealed some compounds that potentially could play dual roles.

Because IAA was the predominant component in the indole extract and its role in plant growth stimulation by PGPR has been well established (Kutschera and Briggs, 1987; Vande Broek et al., 1999; Lambrecht et al., 2000; Patten and Glick, 2002; Martinez-Morales et al., 2003; Araujo et al., 2005; Vande Broek et al., 2005; Idriss et al., 2007; Spaepen et al., 2007), it was most likely the compound responsible for the activity of that extract. The effects of the indole extract on root development apparent in the gnotobiotic experiments are consistent with the effects of exogenous IAA. The peak concentrations of IAA detected in the indole extract of KPS46 were higher than that reported for any other strains of PGPR (Araujo et al., 2005; Leveau and Lindow, 2005; Kang et al., 2006). As in other IAA-producing microorganisms, IAA production by KPS46 is tryptophan inducible (Folke et al., 2000). For example, *B. amyloliquefaciens* FZB42 also exhibited increased IAA production with greater tryptophan availability (Idriss et al., 2007). Soybean seeds contained over 0.5 g tryptophan per 100 g seed (Padgette et al., 1996); assuming that a portion of the tryptophan pool is exuded out of the seed and available to bacteria in the spermosphere, it is certainly possible that the all or some of the growth promotion activity of live KPS46 cells applied to soybean seed can be attributed to IAA production.

Although surfactin was a major component of the lipopeptide extract, its role in causing the growth promotional effects of that extract needs to be confirmed. If purified surfactin proves to have this effect, then this would be a new function for a secondary metabolite that is better known for its effects on surface hydrophobicity, bacterial adhesion and movement on surfaces, and antimicrobial activity (Ahimou et al., 2000; Bonmatin et al., 2003; Kinsinger et al., 2003). There is evidence that certain synthetic surfactants can stimulate plant growth by synergizing auxin action, activating certain plant enzyme systems, or affecting plant cell membrane permeability, thereby increasing water or nutrient uptake or excretion of plant factors such as riboflavin (Ernst et al., 1971; Parr and Norman, 1965). But other than a report that surfactin can induce resistance in bean (Ongena et al., 2007), there is no precedence for surfactin having a direct effect on plants leading to elevated growth.

We also found reduced production of these groups of compounds in the UV mutant strain N19G1 which was associated with reduced capacity to promote plant growth. This set of results also supports the hypothesis that these groups of compounds may be involved in growth promotion. But because N19G1 was altered in multiple traits as compared to the wildtype, presumably through simultaneous mutations in multiple genes or through a mutation in a regulatory gene, we cannot conclude which of the three factors is most important in growth promotion. Nevertheless, results from experiments with wildtype KPS46 and mutant N19G1 open several potential avenues for further research.

The deduced identity and function of proteins secreted differentially between KPS46 and N19G1 revealed some that potential could be involved in soybean growth enhancement. Whether any of proteins are active on the root surface or in plant cells will have to be determined by testing each protein in purified form. Three proteins in the protein and lipopeptide transport group were homologous to the same oligopeptide permease (Opp) of *B. subtilis*, which has been reported to be a plant growth regulator protein likely related to peptide plant hormones (Ryan and Pearce, 2004). It is also reported to be an essential component of enzyme systems for the biosynthesis of membrane components and related to a large family of membrane transport signaling systems, antifungal lipopeptide production (Rudner et al., 1991; Kuwana et al., 2002; Lai et al., 2003; Monnet, 2003; Rodionov et al., 2006) and thermotolerance as membrane-components are in contact with high temperature condition to increase its PGPR competition (Ryan and Pearce, 2004). Another protein in the same

group, ABC transporter (lipoprotein) homology yufN protein, is not only one of the key enzymes of cell membrane metabolism, but is also important for binding tryptophan, a precursor of plant growth regulator IAA. Yet another protein in the group, ABC transporter phosphate binding protein pstB. The *pst* operon is a member of the PhoP-PhoR two-component signal transduction system, which controls the phosphate response in *B. subtilis*. The phosphate ABC transporter binding protein could bind with phytases (myo-inositol hexakisphosphate phosphohydrolase) (Idriss et al., 2002; Konietzny and Greiner, 2002). For these reasons, phosphate ABC transporter binding protein could be related to the phosphate solubilizing abilities of plant growth promoting organisms such as *Bacillus* spp. and *Trichoderma* spp., and thus is associated with enhanced phosphorus uptake by plants (De Freitas et al., 1997).

One protein was homologous to inosine monophosphate dehydrogenase, a key enzyme involved in the biosynthesis of nucleotides, nucleosides, purines and pyrimidines. The IMPDH protein required by *Rhizobium tropici* strain CIAT8999-10.T could be activated dinitrogenase reductase protein (NifH) (Christiansen-Weneger, 1992; Collavino et al., 2005; O'gara et al., 1997). This protein also associated with the iron of the nitrogenase complex and nitrogen metabolisms. Thus it might be important in nitrogen and nitrate uptake as a biofertilizer and bioremediation to enhance plant growth and development (Fisher, 1999; Mantelin and Touraine, 2003; Vessey, 2003; Wang et al., 1988).

Regarding, the remaining proteins have been known as a fundamental principle underlying all groups of these molecules to adapt the PGPR survival with highest competition. They could contribute to lipopeptide production, nitrogen fixation, production of siderophores, phosphorus solubilization, release of volatile compounds, increase in nitrate uptake, survival, colonization, competition, biofertilization, biodegradation and bioremediation (Vessey, 2003). This study found that, four proteins extracted from KPS46 involved in detoxification and adaptation of cellular processing that differentially regulated the three proteins including: catalase, superoxide dismutase, and general stress protein. The catalase, as was described for *B. licheniformis* and *B. subtilis* is essential to ensure complete protection of cells against oxidative stress (Naclerio et al., 1995; Yoshida et al., 2001; Voigt et al., 2006) that is associated with colonization and the competition mechanisms of PGPR, because one of the generally accepted concepts is that beneficial PGPR are effective when they successfully colonize and persist in the plant rhizosphere (Yan et al., 2003). The extracellular superoxide dismutase (SOD), is a free radical metabolizing enzyme that protects the cell membrane from damage by the highly reactive superoxide free radicals (Bowler et al., 1990). Superoxide dismutases are metalloenzymes, which catalyse the dismutation of the superoxide anion radical into molecular oxygen and hydrogen peroxide (H_2O_2). There are four types of enzymes with Mn, Fe, Cu/Zn or Ni, as the prosthetic group that trigger photosystem I, photosystem II reaction. Moreover, Beyer and Fridovich (1987) have suggested that the reaction of H_2O_2 with FeSOD also attacked tryptophan amino acid residues, a precursor of phytohormone, IAA, in the *E. coli* enzyme.

There was also some amino acid metabolism protein present in the KPS46 extracellular medium. This protein spot is a regulator of nitrogen metabolism proteins in bacteria and is closely connected with the intracellular levels of glutamine and glutamate, the main nitrogen donors in the cell. Glutamine is formed from glutamate and ammonium by glutamine synthetase (GlnA), which is a major way for the cell to assimilate ammonium (Fisher, 1999; Larsen et al., 2006). In the gram-positive model organism *B. subtilis*, the two transcriptional regulators as the nitrogen transcription factor (TnrA) and glutamine synthetase (GlnA/ GlnR) are important for the regulation of the nitrogen metabolism and biofertilization (Schreier and Sonenshein, 1986; Vessey, 2003).

This study represents the first analysis of the extracellular proteome of a *Bacillus* species in relations to plant growth enhancement. While the genome of a *B. amyloliquefaciens* strain, FZB42, has been sequenced (Chen et al., 2007), there is no other extensive proteomic study with *B. amyloliquefaciens* as reported for other *Bacillus* spp. (Gohar et al., 2005; Oosthuizen et al., 2002; Tjalsma, et al., 2004). Thus, apart from sample preparation issues, the biggest limitation in the

using the proteomic approach to identifying proteins from *B. amyloliquefaciens* is the limited information available in protein databases from *B. amyloliquefaciens*. Most of the characterized proteins in the Swiss-Prot and EMBL protein databases are from *B. subtilis*. While it is likely that proteins from the two *Bacillus* species with similar amino acid sequences would have homologous function, this has to be confirmed empirically. Nevertheless, this research presented here marks the beginning of the development of a protein map for strain KPS46 and will encourage future proteomic studies with other PGPR strains. Ultimately, the approaches used in this study could increase understanding of the modes of action by which *B. amyloliquefaciens* KPS46 by enhances plant growth at molecular and biochemical levels. With such information, we could potentially enhance the efficacy of KPS46 and other PGPR strains or better exploit such strains as sources of new bioproducts.

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