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Extracellular Proteome of *Bacillus amyloliquefaciens* KPS46 and Its Effect on Enhanced Growth Promotion and Induced Resistance Against Bacterial Pustule on Soybean Plant

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ABSTRACT

Bacillus amyloliquefaciens strain KPS46 is gram positive that has been found to be effective in controlling various diseases and improving crop yield under greenhouse and field conditions. This study was investigated for KPS46 ability to activate extracellular protein elicitors in enhanced plant growth and induced systemic resistance of soybean plants. Soybean cv. Spencer seeds treated with KPS46 increased root and shoot length, biomass by more than 20-40% and the reduced bacterial pustule disease severity up to 50% when compared with the non-treated control. Bioassay data showed that seedling growth phenotype increased by KPS46 was significant upregulation of 20- elicited extracellular protein spots, whereas sterile distilled water had no effect on either enhanced plant growth or induced resistance. To investigate the proteins involved in growth promotion and induced resistance mechanisms, two-dimensional gel electrophoresis was used to separate extracellular proteins secreted by KPS46 wild type and by N19G1, a UV-derived mutant of KPS46 with reduced production of extracellular proteins and lacking growth promotion and induced resistance activity. Using the 2D-PAGE, the extracellular protein of *B. amyloliquefaciens* KPS46 grown in nutrient glucose broth was studied. The identity and putative function of twenty proteins secreted by KPS46 but not by N19G1 were determined. The 20 extracellular protein spots were identified that were known to be secreted by various mechanisms. These extracellular proteomes of the strain KPS46 included proteins from different functional classes and some proteins of yet unknown function. To our knowledge, this was the first two-dimensional extracellular proteome map of a PGPRs, KPS46. The analysis revealed a number of proteins which might be involved in plant growth promotion and induced resistance by acting as plant growth regulators, accumulating bio-fertilizer/ nutrients, producing antibiotic compounds, stimulating metabolism or functioning in defense against stress factors.

Key words: secretome system, 2D-PAGE, UV-mutagenesis, possible elicitors, biocontrol

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INTRODUCTION

Bacillus amyloliquefaciens KPS46, a strain of plant growth promoting rhizobacteria (PGPR) isolated from healthy soybean rhizosphere in central Thailand, can protect soybean and other crop plants against multiple plant pathogens by induced resistance and secretion of antimicrobial metabolites (Prathuangwong and Kasem, 2004; Prathuangwong *et al*., 2005). When applied as a seed treatment, it can also promote the growth (Buensanteai *et al*., 2008) and induce resistance against several diseases of soybean plants (Prathuangwong and Buensanteai, 2007). 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 and disease protection (Kloepper *et al*., 2004). Extrapolating from the accumulative literature on these two related species and on other species of PGPRs, numerous mechanisms are possible in the strain 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 and induced resistance.

As a starting point towards understanding how KPS46 causes enhanced plant growth and induced resistance, we focused this study on extracellular protiens secreted by KPS46 that might directly and indirecly affect the physiology and biochemistry of soybean plants. Strains in the *Bacillus* group secrete high levels of extracellular proteins as enzymes and secondary metabolites (Tjalsma *et al*., 2004; Voigt *et al*., 2006). Some proteins secreted by *Bacillus spp*. are involved in key ecological functions such as biofilm formation (Tjalsma *et al*., 2004), but extracellular proteins, as a group, have not yet been investigated in the context of plant growth promotion and induced resistance mechanisms. Also, the basic of understanding molecular and protein related secondary metabolites, and the secretome system of the strain KPS46 and other PGPRs are not clear. An understanding of the protein content of bacilli group PGPRs, including the extracellular secretome, is required to identify determinants playing a role in biological control. In a wider context, effective protein separation and identification in the bacilli group PGPRs can provide researchers with targets for anti-fungal activity, enhanced growth promotion and induced resistance for plant disease management. Proteomics is one of the best strategies used to reveal the expressions of whole proteins in cells and their interactions. The term proteome is used here to describe the complex state of an organism under defined conditions rather than its complete protein repertoire. Due to its high resolution, two dimensional polyacrylamide gel electrophoresis (2D-PAGE), combined with high throughput mass spectrometry and protein bioinformatics, is widely used for protein separation and identification, which is considered discriminating to allow the unique identification of unknown proteins (Voigt *et al*., 2006).

Xanthomonas axonopodis pv. *glycines* (Sinclair, 1982; Vauterin, *et al.,* 1995) causes bacterial pustule is common pathogen of soybean (*Glycine max* L.) in Thailand. Damage and losses can vary greatly (from 15 to 45%) from field to field depending on the level of cultivar resistance, amount of over-season inoculums, and environmental condition (Prathuangwong and Amnuaykit, 1989). The disease occurs annually when weather is hot and humid which may result in limited development of raised lesions from leaf surface. Foliar symptoms include small pale green spots with raised centers on either or both leaf surfaces at early stage of infection. The spots turn reddish brown enter and may enlarge and coalesce. Heavy infection on resistance cultivars (e.g. SJ2 and AGS292 local cultivars) will lead to premature defoliation (Sinclair, 1982). Conventional management of bacterial pustule relies on preventative copper sprays that continuous use leads to resistant bacterial strains. Biological control using antagonist microbes is thought to be precisely alternative method that *B. amyloliquefaciens* KPS46 reveals high efficacy on reduced bacterial pustule infection by *X. axonopodis* pv. *glycines* of soybean plant (Prathuangwong and Kasem, 2004; Prathuangwong and Buensanteai, 2007).

The primary aim of this study was to describe whether or not extracellular proteins secreted by KPS46 had direct and indirect role in regulating plant growth and induced resistance mechanisms. One objective was to determine if extracellular proteins produced by KPS46 in liquid culture could enhance growth and induced resistance against bacterial pustule caused by *X. axonopodis* pv. *glycines* when applied to soybean seeds. The proteomic approach was used in analyzing extracellular proteins secreted by KPS46 to identify those extracellular proteins that might be important in plant growth promotion and induced resistance. To narrow the spectrum of proteins, secreted proteins from wildtype KPS46 were compared with those from a UV-mutant strain affected in growth promotion and induced resistance traits. Protein bioinformatics tools were employed to identify the proteins and understand the proteomics of the strain KPS46.

MATERIALS AND METHODS

Bacterial strains and culture conditions

B. amyloliquefaciens KPS46 was isolated from soybean plant grown at research station, Kasetsart University, Kamphaeng Saen Campus, Thailand (Prathuangwong and Kasem, 2004). *B. amyloliquefaciens* strain KPS46, *X. axonopodis* pv. *glycines* strain 20036a (gift from Prof. Anne K. Vidaver from Department of Plant Pathology, University of Nebraska-Lincoln), the reference PGPR strains as *Psedomonas fluorescens* WCS417r and *B. amyloliquefaciens* IN937a (provided by Prof. Gary Y. Yuen from Department of Plant Pathology, University of Nebraska-Lincoln), stored in nutrient broth with 10% glycerol at -80 °C were retrieved by streaking them from nutrient broth into nutrient glucose agar (NGA) at 28 ± 2 °C for 48 h. The all bacterial cultures were propagated in 500 ml of nutrient broth containing 2% glucose (NGB) culture medium 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 resuspended 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.

Plant cultivation and induction treatment

The first series of experiments was conducted to KPS46 culture fluid fractions and extracted extracellular proteins for enhancement of soybean growth. Seeds of soybean cv. Spencer (seeds provided by G. Hartman, University of Illinois) were surface-disinfested by treatment with 95% ethanol (v/v) for 2 min, followed by soaking in 20% commercial bleach (v/v) 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 treatment included a whole culture of KPS46 in NGB, cell-free fluid from a KPS46 culture in NGB, suspension of live cells collected from a KPS46 culture, and a suspension of heat-killed cells. The cell concentration in the whole culture and cell suspensions were adjusted to $1{\times}10^8$ cfu

ml^{-1} on the basis of absorbance, while culture fluid was diluted by the same dilution factor as the whole culture. Other treatments included suspensions of extracellular proteins (250 ug/ml) extracted from KPS46 culture fluids. Other seed treatments were also conducted with abiotic inducer of commercial salicylic acid and a biotic inducer reference PGPR including *P. fluorescens* WCS417r and *B. amyloliquefaciens* IN937a to compare with the same condition of strain KPS46. In the greenhouse experiment, soybean seeds treated with KPS46 culture fractions and 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 replication 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 60-75% relative humidity during the light period, 15 °C and >93% relative humidity during the dark period). 14 days after seedling emergence, seedlings were harvested for measurements of root and shoot lengths and fresh and dry weights. The experiment was conducted three times.

The other experiment is the inoculation of soybean with KPS46 bacterial strains for study on induction of resistance mechanism. The experiment was conducted at University of Nebraska-Lincoln. Bacterial pustule-susceptible soybean cultivar Spencer was used. Seeds were surface- disinfested, treated by each elicitor before experiments, planted and grown same as the condition above. Soybean plants were grown for approximately 2 weeks to the two-fully-expanded-leaf stage. At this time, plants were challenge-inoculated with *X. axonopodis* pv. *glycines* strain 20036a. The pathogen cell suspensions were sprayed onto plants using hand-held spray bottles. Plants treated with distilled water served as the no-pathogen control. Put the pathogen treated plants in a moisture chamber. Remaining pots of plants were maintained in the greenhouse for 7 days. Disease severity (percent leaf area exhibiting lesions and chlorosis) was estimated for each leaf using the method described by Prathuangwong *et al*. (1993). Measurements from all leaves in each pot were averaged prior to statistical analysis. The experiment was performed 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 $P \leq 0.05$.

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 ($\lambda = 600$ 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 ul) 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 (Buensanteai *et al*, 2008).

More than 2,490 irradiated strains were screened for loss of or decrease in extracellular protein production (Buensanteai *et al*, 2008). Fluid from cultures of these strains in NGB was conducted to determine whether or not extracellular proteins produced by UV mutant strain N19G1 derived from KPS46 could enhance plant growth and induced resistance. As in the 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 planted in pots of planting medium. Biomass, the number of lateral root, and root and shoot lengths were determined 14 days after seed germination. Moreover, soybean plants were grown for approximately 2 weeks to the two-fully-expanded-leaf stage. At this time, plants of cv. Spencer were challenge-inoculated with *X.axonopodis* pv. *glycines* strain 20036a the same as described above. Disease severity (percent leaf area exhibiting lesions and chlorosis) was estimated for each leaf of KPS46 wildtype and UV-mutant treatments using the method described by Prathuangwong *et al.* (1993). Each experiment was conducted three times.

Extracellular sample preparation for the proteome analysis

A final concentration of 5 mM PMSF (phenylmethylsulphonyl fluoride, Sigma P7626) was added to *B. amyloliquefaciens* KPS46 culture medium (250 ml) at the late-exponential phase of growth to prevent proteolytic digestion, then cells were removed by centrifugation at 12,000 rpm at 4 °C for 10 min followed by filtration through a 0.2 nm nitrocellulose filter. After that, 10 ml 50% TCA [trichloroacetic acid, Sigma (T6399)] was added to the culture filtrated, 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). Total protein content of the samples was quantified by the Bradford (1976).

Two-dimensional polyacrylamide gel electrophoresis (2D–PAGE), mass spectrometry and protein database searching

The intent of this experiment was to identify some of the specific components of the extracellular proteome of KPS46 that might have some relationship to plant growth promotion and induced resistance against bacterial pustule disease. 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 and induced resistance ability. The extracellular protein samples were used directly to passively rehydrate isoelectrically focused on an Electro Immobiline Dry Strip pH 4–7 (11 cm; Bio-Rad, USA) by applying 185 Ïl of each sample (equivalent to 250 Ïg 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 (Voigt *et al.*, 2006). 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)]. 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 strain KPS46. 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). The sequence data were compared with the NCBI and SwissProt databases using local MASCOT to identify possible protein names and functions.

RESULTS AND DISCUSSION

Effects of KPS46 extracellular proteins on plant growth and induced resistance

Extracellular proteins extracted from cultures of strain KPS46 was effective in promoting the growth of soybean seedlings under greenhouse conditions. These culture fractions, when applied to soybean seed, significantly 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). All of the treatments involving KPS46 culture fractions, except the heat-killed cells, increased shoot and root lengths and plant biomass (Figure 1B) compared to the distilled water control. Two weeks after the beginning of seed treatment in greenhouse trails, soybean seedlings treated with active elicitors of KPS46 were averaged in each treatment of more than 33.0 and 15.0 gram whereas non-treated seedlings were less than 24.0 and 11.5 gram of fresh and dry weights respectively. According to DMRT ($P \leq 0.05$), the stimulation of seedling growth with KPS46's active elicitor was significant different from KPS46 killed cell and ddH_2O negative control.

In the experiments using soybean cultivars Spencer challenge-inoculated with *X.*

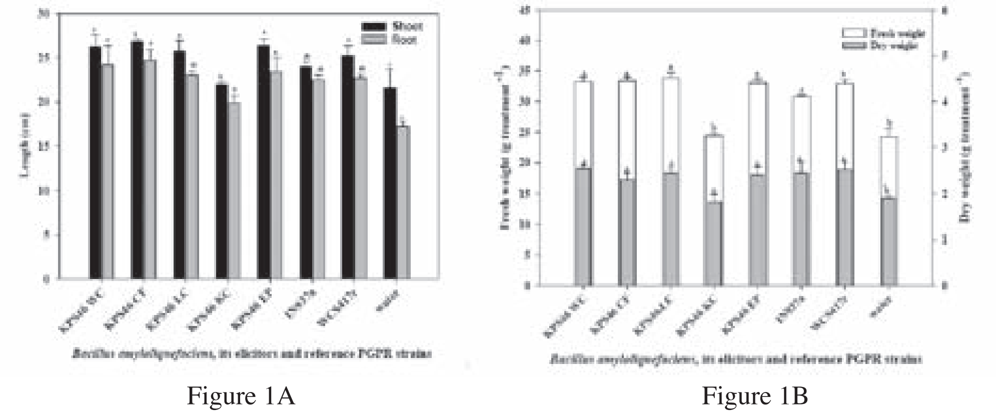

Figure 1A Figure 1B

Figure 1 Effects of cellular fractions and extracellular protein extracted from cultures of *Bacillus amyloliquefaciens* KPS46 on the growth of soybean under greenhouse conditions, as measured at 14 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, EP = extracellular proteins extract, *Pseudomonas fluorescens* wcs417r (WCS417r), *Bacillus amyloliquefaciens* IN937a (IN937a), and sterile distilled water. 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.

axonopodis pv. *glycines* strains 20036a, seed treatment with the strain KPS46 reduced the severity of bacterial pustule in the foliage, confirming that induction of systemic resistance occurred. The ability of KPS46 showed the most effective in reducing bacterial pustule disease severity more than 50 % (Table 1). However *P. fluorescens* wcs417r and salicylic acid could reduce disease severity of soybean bacterial pustule the same as KPS46 but not *B. amyloliquefaciences* IN937a. There was no significant effect of the treatments treated with KPS46 which killed cell and distilled water on all parameters were determined (Table 1). These results showed that the strain KPS46 systemically reduced disease severity to a level that was not significantly different from the protection afforded by salicylic acid, a chemical elicitor of systemic acquired resistance. In this investigation, extracellular metabolites from the strain KPS46 were also found to reduce the incidence of bacterial pustule disease in soybean plants.

UV mutant strain N19G1 phenotype and proteomic analysis of extracellular proteins of KPS46 wildtype

Strain N19G1 exhibited significantly ($P \leq 0.05$) reduced excretion of extracellular protein in NGB medium compared to wildtype KPS46. When extracellular proteins extracted from KPS46 and N19G1 were applied as a seed treatment in greenhouse experiments (Figure 2A, 2B), the strain N19G1 had little or no affect on either soybean growth parameters or induced bacterial pustule disease resistance compared to the distilled water control. In contrast, the KPS46 and its proteins secreted by KPS46 elevated all growth parameters.

The 2D-PAGE of extracellular proteins excreted by the strain KPS46 revealed around 190 detectable spots (Figure 3). The large majority of the identified proteins were homologous to

Table 1 Effects of *Bacillus amyloliquefaciens* strain KPS46 compared with two reference PGPR and salicylic acid on severity of bacterial pustule disease caused by *Xanthomonas axonopodis* pv. *glycines* (Xag) in soybean cv. Spencer.

Treatment[1/]	Disease severity[2/] (%)	Disease reduction (%)
1. KPS46 whole-cell (WC)	35.1^{a}	55.2
2. KPS46 culture filtrate contained extracellular protein (CF)	35.7^{a}	54.4
3. KPS46 live cell (LC)	36.9^{a}	55.5
4. KPS46 killed cell (KC)	77.4^{cd}	4.9
5. KPS46 extracellular proteins (EP)	33.9^{a}	60.8
6. Salicylic acid	38.2^{ab}	53.0
7. WCS417r[3/]	34.5^{a}	57.6
8. IN937a[4/]	46.2^{b}	43.3
9. N19G1	73.6^{c}	9.6
10. Positive Control (water + Xag)	81.4^{d}	0.0
11. Negative Control (water alone)	0	-

[1/] Seed treatment with inducer and challenged inoculation with pathogen (Xag) at 14 days after planting.

[2/] Disease severity was expressed as percent leaf area infection with *Xanthomonas axonopodis* pv. *glycines* evaluated by the method of Prathuangwong *et al*. (1993) at 7 days after inoculation. Means in the column followed by the same letter are not significantly different according to Duncan's Multiple Rang Test.

[3/] WCS417r = *Pseudomonas fluorescens* wcs417r.

[4/] IN937a = *Bacillus amyloliquefaciens* IN937a.

proteins produced by *B. subtilis*. The remainders being homologous to proteins were reported from other *Bacillus* spp., or other strains of *B. amyloliquefaciens* (Table 2). Twenty proteins samples are placed in six categories based on predicted function in Table 2. Four spots were involved in detoxification and adaptation of cellular processing including catalase, two forms of superoxide dismutase, and general stress protein. Six proteins were in the transporter binding protein and lipoprotein 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 subtilisin, a serine endopeptidase, while other were homologous to neutral proteases and the fifth to a protease that was toxic to

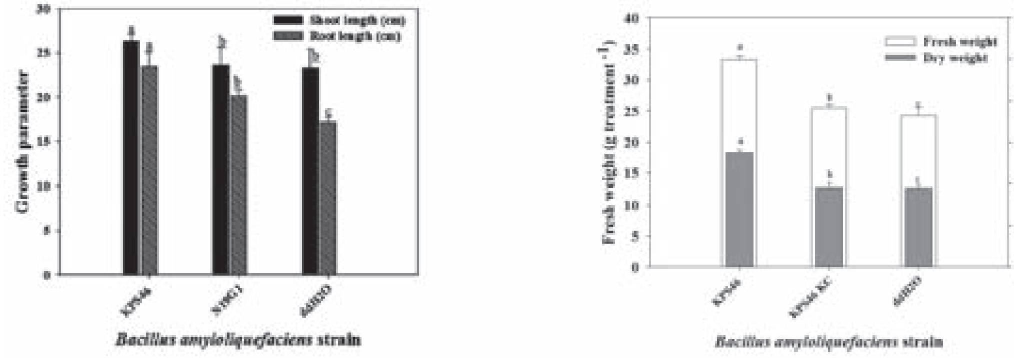

Figure 2A

Figure 2B

Figure 2 Effects of extracellular protein extracts from *Bacillus amyloliquefaciens* wildtype 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 ($P \leq 0.05$).

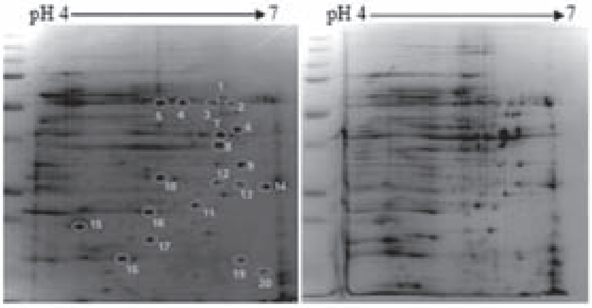

Figure 3 Two-dimensional gels of extracellular proteins extracted from *Bacillus amyloliquefaciens* KPS46: gels of proteins from wildtype strain KPS46 (left) and mutant strain N19G1 (right) run on 11 cm of 4–7 IPG strips in the first dimension and 14% SDS-PAGE in the second dimension. Circled spots correspond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

Table 2 Upregulated proteins from the extracellular secretome of *Bacillus amyloliquefaciens* KPS46.

Spot number coverage (%)	Protein name[1/]		Species	Accession number	Molecular mass (Da)	*pI*	Score	Sequence
Functional category 1 : Detoxification and adaptation of cellular processing								
1	KatA	Catalase	*B. subtilis*	Q3HNK4_BACSU	54503	6.45	2021	75
13	SodA	Manganese 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.licheniformis*	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. amyloliquefaciens*	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.

phytopathogenic two 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.

This study represented the first analysis of protein metabolite production by *B. amyloliquefaciens* KPS46 in relations to plant growth promotion and induction of resistance. It was found that KPS46 culture fluid extracts containing secreted proteins individually could influence the growth and induce resistance of soybean to the same degree as cells of KPS46 washed free of pre-formed exoproducts. Whether or not the same type of compounds were secreted by KPS46 cells while existing in the spermosphere or rhizosphere remained to be determined, nevertheless, the results were consistent with the hypothesis that strain KPS46 promoted the growth and induced the bacterial pustule disease resistance of soybean by secretion of several types of compounds. Because the protein extracts affected soybean growth development and induced resistance under greenhouse conditions, we could not rule out the possibility that under natural soil conditions the same compounds or other compounds within each extract might influence plant growth and induced resistance 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.

We also found that reduced production of the extracellular proteins of compounds in the UV mutant strain N19G1 was associated with reduced capacity to promote plant growth. This set of results also supported the hypothesis that these groups of compounds might 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 could not conclude which of this factor was most important in growth promotion. Nevertheless, the results from experiments with wildtype KPS46 and mutant N19G1 opened several potential avenues for further research.

The deduced identity and function of proteins secreted by KPS46 revealed some that potentially could be involved in soybean growth enhancement and induced resistance. Whether any of proteins were active on the root surface or in plant cells would have to be determined by testing each protein in purified form. Three proteins in the protein and lipopeptide transport groups were homologous to the same oligopeptide permease (Opp) of *B. subtilis*, 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, inducer resistance such as fengycin lipopeptide production (Rudner *et al.*, 1991; Monnet, 2003) 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 is a 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. 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 (Idriss *et al.*, 2002).

One protein was homologous to inosine monophosphate dehydrogenase (IMPDH), a key enzyme involved in the biosynthesis of nucleotides, purines and pyrimidines. It has been reported to be associated with biofertilization and plant growth promotion mechanism. This protein is involved in active dinitrogenase reductase (NifH) and nitrogen metabolisms across in *Rhizobium tropici* strain CIAT8999-10.T (Christiansen-Weneger, 1992; O'gara *et al.*, 1997; Collavino *et al.*, 2005). Thus it might be important in nitrogen and nitrate uptake as a biofertilizer and bioremediation to plant growth enhancement (Wang *et al.*, 1988; Mantelin and Touraine, 2003; Vessey, 2003).

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* was essential to ensure complete protection of cells against oxidative stress (Naclerio *et al.*, 1995; Voigt *et al.,* 2006) associated with colonization and the competition mechanisms of PGPR, which is one of the generally accepted concepts in that beneficial PGPR are effective when they successfully colonize and persist in the plant rhizosphere. Two of the protein spots had homology to the same protein: the extracellular superoxide dismutase (SOD). It 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 photosystemI, photosystemII reaction. Moreover, Beyer and Fridovich (1987) 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 were also some amino acid metabolism proteins present in the KPS46 extracellular medium. This protein spot is a regulator of nitrogen metabolism proteins in bacteria and is closely associated 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).

This study represented the first analysis of the extracellular proteome of a *Bacillus* species in relation to plant growth enhancement and induced resistance. 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. (Tjalsma *et al.*, 2004). Apart from sample preparation issues, the biggest limitation in the using the proteomic approach to identify 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 marked the beginning of the development of a protein map for strain KPS46 and would 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 enhanced 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.

CONCLUSION

The extracellular proteome of the plant growth promoting bacteria, *B. amyloliquefaciens* KPS46 was studied for the first time. The work provided an initial extracellular protein reference map for the plant growth promoting bacteria, *B. amyloliquefaciens* KPS46 and would aid the future proteomic-based studies of this microorganism and other plant growth promoting rhizobacterium/ bacterium. In light of the available evidences of this study we suggest that the beneficial effect of seed treatment like increased disease control with *B. amyloliquefaciens* KPS46 contributing to induced resistance and enhanced growth promotion of crop plants. Thus, the use of *B. amyloliquefaciens* KPS46 may minimize the cost of control strategies and reduce the risk associated with the high use of chemical pesticides and fertilizer in commercial soybean production.

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