Extracellular Proteome of Plant Growth Promoting-bacteria, *Bacillus amyloliquefaciens* KPS46 and Its Effect on Enhanced Growth Promotion and Induced Systemic Resistance on Soybean

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ABSTRACT

*Bacillus amyloliquefaciens* KPS46 is gram positive and as a plant growth promoting bacteria, it has well document on enhance plant growth promotion and systemic protection against bacterial pustule and several diseases in soybean. The strain KPS46 promotes plant growth by synthesizing growth-stimulating phytohormones, secondary metabolite, extracellular protein to enhanced growth promotion and protect plant from pathogen infection. This study determined a protein secretion which allows prediction of the amino sequence involved in extracellular protein of KPS46. Using the 2-DE the extracellular protein of *B. amyloliquefaciens* KPS46 grown in NGB media was studied. The 20 were identified that are known to be secreted by various mechanisms. The extracellular proteome of KPS46 includes proteins from different functional classes and some proteins of yet unknown function. To our knowledge, this is the first two-dimensional extracellular proteome map of a PGPB, KPS46. Furthermore, evaluating the efficacy of KPS46 in promoting the growth of soybean, the strain increased in root elongation by up to 17%, shoot elongation by up to 35%, fresh weight by up to 32% and dry weight by up to 32%. And the strain reduce disease severity up to 55%. These results show the potential of using KPS46 to enhance growth of

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soybean, as well as use as a biocontrol agent for plant diseases management. Thus, the use of 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 in the future.

**Key words:** *Bacillus amyloliquefaciens*, extracellular protein secretion, induced systemic resistance, enhanced growth promotion

**Introduction**

Plant growth promoting rhizobacteria/bacteria (PGPR/PGPB) could enhance plant growth and induced systemic resistance (ISR) via direct and indirect modes of action, but the specific mechanisms involved have not all been well-characterized and understand (Chinnasany, 2006). In the previous report, the *Bacillus* group is well documented for enhanced growth promotion and induced systemic resistance (Ryu et al., 2004). *B. amyloliquefaciens* KPS46 is a plant growth promoting-bacterium, originally isolated from the soybean plant at the agricultural research station of central of Thailand (Prathuangwong and Kasem, 2003). The strain has been identified as a potential plant growth promoter and biocontrol agent by induced systemic resistance (Prathuangwong and Buensanteai, 2006) as well as the secretion of elicitors to inhibit the phytopathogens in various plants caused by soilborne and airborne pathogens (Prathuangwong and Kasem, 2004). It could promoted plant growth and protect soybean plants from multiple diseases including bacterial pustule caused by *X. axonopodis pv. glycines* (Prathuangwong and Kasem, 2003). However, the basic of understanding molecular and protein related phytohormones, secondary metabolites, and the secretome system of the strain KPS46 is not clear. An understanding of the protein content of bacilli group PGPR/PGPB, 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 PGPR/PGPB can provide researchers with targets for anti-fungal activity, enhanced growth promotion and induced systemic resistance for plant disease management.

Proteomics is one of the best strategies used to reveal the dynamic 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 PAGE, combined with high throughput mass spectrometry and bioinformatics, is widely used for protein separation and identification, which is considered sufficiently discriminating to allow the unique identification of unknown proteins (Luo et al. 2007). The aim of this study was to describe the extracellular proteins that are secreted by *B. amyloliquefaciens* KPS46 via two-dimensional PAGE technology from a species of *B. amyloliquefaciens* KPS46 with well established biocontrol, enhanced growth promotion and induced systemic resistance properties to produce an initial protein reference map. Protein bioinformatics tools were employed to identify the proteins to understand
the proteomics of plant growth promoting-bacterium. To our knowledge, this is a new report on extracellular protein secretion analysis of plant growth promoting-bacteria, B. amyloliquefaciens 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, 2001). B. amyloliquefaciens strain KPS46 and X. axonopodis pv. glycines strain 20036a (gift from Prof. Anne K. Vidaver 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 agar at 28 + 2 °C for 48 h. The B. amyloliquefaciens strain KPS46 was propagated in 500 ml of NGB culture medium for 48 h at 28 + 2 °C. Bacterial cultures were centrifuged at 13,000 rpm for 20 min and the bacteria cell pellet was washed three in sterile saline water (0.85% NaCl). They were resuspended in 100 ml sterile distilled water and the density of the suspension was adjusted to 10^8 CFU/ml by measuring optical density (OD) of 0.2 at 600 nm.

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. Then 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)

The above samples were used directly to passively rehydrate isoelectrically focussed on an Electro Immobiline Dry Strip pH 3–10 (11 cm; Bio-Rad, USA) by applying 185 ul of each sample (equivalent to 250 ug of protein). Extracellular preparations containing 250 µg protein mixture dissolved in the 10X of IEF sample solution was applied to the first dimension. IPGs were focused for 15 h at 400V followed by 1 h at 600V using a Multiphor II (Amersham Pharmacia). After placing in equilibration buffer A [50 mM Tris/HCl, pH 6.8, containing 8 M urea, 30 % (v/v) glycerol, 2.5% SDS (Sodium dodecyl sulfate,
Sigma-Aldrich 436143) and 0.25% DTT] for 15 min and buffer B [50 mM Tris/HCl, pH 6.8, 8 M urea, 30% (v/v) glycerol, 2.5% SDS, 0.25% DTT and 4.5% iodoacetamide (Sigma, 1149)] for 15 min, the isoelectric focusing gels were embedded in 0.25 M Tris/HCl, pH 6.8, 0.25% SDS, 1% agarose onto 14% SDS polyacrylamide gels (20x20x0.1 cm), and the proteins were resolved in the second dimension by a constant current of 20 mA until the bromphenol blue marker entered the stacking gel, followed by 45 mA until the blue dye reached the bottom of the gel (Luo et al., 2007). The 2D–PAGE gels were visualized by staining with colloidal Coomassie blue G-250 [17% ammonium sulphate, 34% methanol, 3.6% orthophosphoric acid, 0.1% Coomassie G-250 (Sigma B0770)] (Voigt et al., 2006). The gels were fixed in destaining solution (80% ethanol and 20% acetic acid mixture) and wash by 70% ethanol.

**In-gel digestion and MS**

Place a clean glass plate on the light box, rinse gel briefly in sterile distilled water and place on the glass plate and excise spot with the spot picker and place into a microfuge tube. Add 200 ul of Modified De-stain and fill out form for MS/MS. Gel pieces were washed three times with 100 ul of 50 mM ammonium bicarbonate, pH 8.2, 50% ACN and dried using a SpeedVac centrifuge for 20 min. Trypsin in 50 mM ammonium bicarbonate (20 ug/ul) was added to each gel piece and incubated at 30 °C for 16 h. The peptides were extracted by sonication. The peptide solution was automatically desalted and concentrated using ZipTips from Millipore (Bedford, Mass. USA) in a Map II/8 (Bruker–Daltonik, Karlsruhe, Germany) liquid handling unit and spotted onto the Axima matrix-assisted laser desorption/ionisation (MALDI) target plate with 1.5 ul alpha-cyano-4-hydroxy-cinnamic acid. Peptide mass fingerprints of tryptic peptides were generated by MALDI time-of-flight mass spectrometry (MALDI-TOF-MS) using an Axima CFR (Kratos, Manchester, UK). Peptide mass fingerprints were searched proteins from all Bacteria species using the Mascot peptide mass fingerprint, where a modified MOWSE scoring algorithm was used to rank results (http://www.matrixscience.com/help/scoring_help.html).

**Database searching**

PMF data were generated from raw MALDI-MS data using the SNAP algorithm (Bruker) with a signal/noise threshold of 6 and a minimum quality factor of 30. The PMF data were used to screen the NCBInr database dated 2005/1/25 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein) and MUMDB dated 2006/02/22 (http://mips.gsf.de/genre/proj/Ustilago/) using local MASCOT (MASCOT 2.1.0, Matrix Science). The MIPS database, together with the TargetP server (http://www.cbs.dtu.dk/services/TargetP/) and the WoLF PSORT server (http://wolfpsort.seq.cbrc.jp) was also used to annotate identified proteins and to assign their predicted subcellular localization. Functional categories were assigned according to the Functional Catalogue Database of MIPS
When multiple functional categories were allotted to an individual protein, the category with the lowest expectation value was chosen.

**Plant cultivation and induction treatment**

Soybean seedlings were produced from seeds of cv. Spencer (gift from Prof. G. Hartman, University of Illinois) and surface sterilized by treatment with 95% ethanol (v/v) for 20 s, followed by soaking in 20% bleach (v/v) for 20 min. Seeds were washed with sterile distilled water 5 times in order to remove excess bleach. The seeds were then air dried by placing them in a laminar flow hood for 24 h. Before planting, 30 g of soybean seeds were mixed thoroughly with 5 ml of whole cell (WC), culture filtrate (CF), live cell (LC) and killed cell (KC) of KPS46 bacterial suspension as described above and 5 min air dried. Other seed treatments were also conducted with abiotic inducer of commercial salicylic acid and a biotic inducer reference PGPR including *Pseudomonas fluorescens* WCS417r, *Lysobacter enzymogenes* C3 and *B. amyloliquefaciens* IN937a to compare with the same condition of strain KPS46. Four replicates were performed (2 seeds per pot and 20 pots per treatment). Soybean seeds were grown in 12 inch pots containing sterile pasteurize soil mixture in a greenhouse with day and night temperatures of 27 °C and 24 °C, respectively, and with a 16/8-h light/dark cycle. Each treatment was challenge inoculated with *X. axonopodis* pv. *glycines* after 14 days of planting. The bacterial suspension of *X. axonopodis* pv. *glycines* strain 20036a with $10^8$ CFU/ml were spray onto plants from hand-hold spray bottles. The plant treated with distilled water was served as control. Disease severity was assessed with the method described by Prathuangwong et al. (1993). Sampling of enhanced growth promotion of soybean was done at 14 d after seed germination. Root length, root fresh weight, root dry weight, shoot fresh weight and shoot dry weight were determined. The experiment was repeated three times and the data obtained was analyzed using SAS version 9.1 analysis.

**Results and Discussion**

The proteomics of extracellular protein extract from PGPR/PGPB, *B. amyloliquefaciens* KPS46, across a gradient of pH 3–10. The results showed that the strain KPS46 produces highest levels of extracellular proteins secretion at the early stationary phase. We prepared extracellular proteins at the late-exponential phase of growth in nutrient broth medium containing 2% glucose (NGB) to avoid contamination with cytosolic proteins due to cell lysis and toxin production processing. The protein composition of the samples was then examined by two dimentiion gel electrophoresis (Fig. 1). *B. amyloliquefaciens* KPS46 secrete much higher levels of protein when grown in a complex medium (NGB) compared to cells grown in minimal medium, this is not apparent from our gels because the same protein amount was loaded onto each gel (data not shown). From about 189 protein spots visible on the gels, altogether 20 proteins could be identified that are known to be secreted by other
mechanisms such as transient export signals are twin-arginine signal peptides (RR/KR), lipoprotein signal peptides (Lipo), and transmembrane domains (TM) more than the Secretion-type signal peptide (Sec) pathway. In addition, some of the proteins, occur as multiple protein spots on the 2D-PAGE. The almost of extracellular proteins of *B. amyloliquefaciens* KPS46 identified were homologous to proteins secreted by *B. subtilis*. The extracellular proteome of *B. amyloliquefaciens* KPS46 includes proteins from different functional classes, such as enzymes for the degradation of various macromolecules, proteins involved in oligopeptide accumulation, lipopeptide production, lytic enzymes, antimicrobial-related proteins, carbohydrate degrading enzymes, several proteases and peptidases, enzymes involved in nucleic acid degradation, phosphodiesterases and phosphatases, enzymes involved in cell wall function, transport related proteins, proteins involved in sporulation and membrane bioenergetics and some proteins of yet unknown function. In addition, 20 proteins were identified in the extracellular proteome of *B. amyloliquefaciens* KPS46, which were not expected to be secreted because they have no predicted signal for secretion through one of the known systems, and which, taking into account their function, in most cases would be expected to have a cytoplasmic localisation (data not shown). The secretion of proteins without known export signals have been also described in other studies of the extracellular proteome (Voigt *et al.*, 2006). It is not yet known whether these proteins are secreted via an unknown secretion mechanism, but more likely they have been released into the medium by cell lysis.

To our knowledge, this is the first two-dimensional extracellular protein map of a PGPB *B. amyloliquefaciens* KPS46.

In addition, when applied as a seed treatment in greenhouse experiments, the strain KPS46 increased root elongation by up to 17%, shoot elongation by up to 35%, fresh weight by up to 32% and dry weight by up to 32% (Fig. 2, 3 and 4). A significant effect of KPS46 WC, LC and CF treatment was observed in greenhouse studies of soybean cv. Spencer when all trials were analyzed together for all growth and disease severity parameters measured. *B. amyloliquefaciens* KPS46 was most effective in reducing bacterial pustule disease severity of 54.8 % compared with negative control treatment (Table 1). However *P. fluorescence* wcs417r, *B. amyloliquefaciences* IN937a and salicylic acid could reduced disease severity of soybean bacterial pustule as same as KPS46. There was no significant effect of the treatments that treated with KPS46 KC and distilled water on all parameter were determined (Table 1).

From this results shown that *B. amyloliquefaciens* 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. This indicates the potential of using strain KPS46 for protection of soybean bacterial pustule disease and production at a commercial scale for a biological control product. In this investigation, extracellular metabolites from *B. amyloliquefaciens* KPS46 were also found to reduce the incidence of bacterial pustule disease in soybean plants. The mode of action of *B.
*B. amyloliquefaciens* KPS46 via induced systemic resistance of soybean against bacterial pustule disease that it could enhance peroxidaes, 1,3-β-glucanases, phenylalanine ammonia lyase and phenolic compounds in soybean treated with *B. amyloliquefaciens* KPS46 (Buensanteai and Pathuangwong, 2006; Buensanteai et al., 2007). These produces appear to be one mechanism of biological control by strain KPS46 and may play a role in plant defense against pathogen infection (Prathuangwong and Buensanteai, 2006). Further studies have to be performed on the duration of ISR by biocontrol agent and to find out the signaling molecules and gene or protein functions involved in *B. amyloliquefaciens* KPS46 mediated ISR in soybean. In addition to the study of proteome and transcriptome induced resistance, increase the efficacy of strain KPS46 with understanding of various factors that will influence the beneficial expression including selected mixtures of different mode of action of PGPRs/ PGPBs strains, application of booster inoculants, and formulation technologies , is an required need of knowledgeable information.

![Fig. 1](image)

**Fig. 1** Two-dimensional map of extracellular protein extract of *B. amyloliquefaciens* KPS46. This gel was run on 11 cm 3–10 IPG strips in the first dimension and 14% SDS-PAGE in the second dimension. kD KiloDaltons

![Fig. 2](image)

**Fig. 2** Effect of PGPR difference strains on growth promotion in greenhouse experiment. Soybean cv Spencer seed treated by *B. amyloliquefaciences* KPS46: whole cell (WC) (A), protein culture filtrate (CF) (B), live cell (LC) (C), killed cell (KC) (D), *P. fluorescence* wcs417r: (WCS417r) (E), *B. amyloliquefaciences*
IN937a: (IN937a) (F), and ddH2O (G).

Fig. 3 Effect of PGPR/PGPB difference strains on soybean shoot and root length in greenhouse experiment. Soybean cv Spencer seed treated by *B. amyloliquefaciens* KPS46: WC, CF, LC, KC, *P. fluorescence* wcs417r (WSC417r), *B. amyloliquefaciens* IN937a (IN937a), *L. enzymogenese* C3 (C3) and ddH2O.

Table 1 Effect of plant growth promoting rhizobacteria/bacteria, *B. amyloliquefaciens* KPS46 compare with two reference PGPR and salicylic acid on reduction disease severity of soybean bacterial pustule caused by *X. axonopodis pv. glycines* (Xag)

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<thead>
<tr>
<th>Treatment</th>
<th>Disease severity (%)</th>
<th>Disease reduction (%)</th>
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<tbody>
<tr>
<td>1. KPS46 whole-cell (WC)</td>
<td>36.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55.2</td>
</tr>
<tr>
<td>2. KPS46 culture filtrate contain extracellular protein (CF)</td>
<td>37.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54.4</td>
</tr>
<tr>
<td>3. KPS46 Live cell (LC)</td>
<td>36.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55.5</td>
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Conclusion

The extracellular proteome of the plant growth promoting bacteria, *B. amyloliquefaciens* KPS46 has been studied for the first time. Twenty protein spots were identified from 22 different genes. The work presented here provides an initial extracellular protein reference map for the plant growth promoting bacteria, *B. amyloliquefaciens* and will aid 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. 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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