Proteomic Analysis of *Bradyrhizobium japonicum* USDA110 in Acidic Condition

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ABSTRACT

Bradyrhizobium japonicum is an agriculturally important bacterium due to its capacity of nitrogen-fixing symbiosis with leguminous plants, especially soybean (*Glycine max*). In this report, we studied acid-tolerance metabolism of *B. japonicum* USDA110 using proteome analysis. Two-dimensional electrophoresis gel image analysis revealed 568 and 628 protein spots of cells grown at pH 4.7 and pH 6.8, respectively. Only 84 protein spots with at least 3-fold differential expression were further identified by MALDI-TOF MS. The annotated proteins were assigned to four different classes: (i) proteins produced only at pH 4.7 condition (15 proteins such as D-alanine aminotransferase, 2-haloalkanoic acid dehalogenase and periplasmic mannitol-binding protein); (ii) proteins produced under both conditions but strongly induced at pH 4.7 (27 protein spots such as triosephosphate isomerase, UTP-glucose-1-phosphate uridylyltransferase and glyceraldehyde 3-phosphate dehydrogenase); (iii) proteins down-regulated during growth at pH 4.7 (25 proteins such as GroEL, acyl-CoA dehydrogenase and ATP synthase beta chain), and (iv) proteins specific to growth at pH 6.8 (17 proteins such as ATP-dependent protease ATP-binding subunit, N-utilization substance protein A and 2-isopropylmalate synthase). The data of the differential protein expression can be a basis for mechanism elucidation of the acid response in *B. japonicum* USDA110.

Keywords: acidic condition, *Bradyrhizobium japonicum*, proteomic, rhizobium, two-dimensional gel electrophoresis.

1. INTRODUCTION

The acid soil appears widely in the world. In tropical regions, soil acidity is one of important problems influencing legumes and biological nitrogen fixation of rhizobia [1]. Low pH in soil is often associated with not only toxicity of high levels of aluminium (Al) and manganese (Mn), but also nutrient deficiency by low levels of phosphorus (P), potassium (K), calcium (Ca), molybdenum (Mo), and magnesium (Mg). These stresses can affect all aspects of nodulation and nitrogen fixation of rhizobia and also their survival and multiplication in soil [2]. The scientists have tried to isolate the effective plants and rhizobia that can resist soil acidity. Concerning rhizobia, the best strains from acid soil have been isolated from various places in the world. *Bradyrhizobium japonicum* is one of the most effective species among various rhizobia [3].

B. japonicum (formerly *Rhizobium japonicum*)

belongs to genus *Bradyrhizobium*. It is a slow-growing rhizobium that has an ability to form the nodules of soybean (*Glycine max*) [1]. Inside the nodules, rhizobia reduce dinitrogen to ammonium, which is secreted to the leguminous plants in exchange for a carbon and energy source. Therefore, *B. japonicum* is a very important factor in soybean production. Many properties of *B. japonicum* were studied such as salt tolerance [4], desiccation tolerance [5], antibiotic tolerance [6] and acid tolerance [3]. The scientists have tried not only to isolate the best strains, but also to study mechanisms of these properties via both genetic and proteomic approaches [7-9].

The proteome analysis is a powerful method to study expression of proteins in a genome-wide scale. The technique generally used in proteomic analysis is two-dimensional gel electrophoresis (2-DE). This technique is to separate proteins from samples in two steps. The first step is to separate proteins according to the difference in isoelectric points. The second step is to re-separate in perpendicular direction according to the difference in molecular weights. The separated proteins are further identified by mass spectrometry [10].

The aims of this research are to determine the growth of *B. japonicum* USDA110 at various pHs of medium and to identify the proteins related to acid tolerance property in *B. japonicum* USDA110 using proteome analysis methods. To our best knowledge, this is the first report on proteome analysis of *B. japonicum* USDA110 in acid condition.

2. MATERIALS AND METHODS
2.1 Bacterial Culture

The *B. japonicum* USDA110 was inoculated in 200 ml of HM medium [11] (per liter of deionized water: 125 mg Na_2HPO_4, 250 mg Na_2SO_4, 320 mg NH_4Cl, 180 mg $MgSO_4 . 7H_2O$, 6.6 mg $FeCl_3 . 6H_2O$, 13 mg $CaCl_2 . 2H_2O$, 1300 mg HEPES, 1100 mg MES, 250 mg yeast extract, 1000 mg L-arabinose, pH 6.8) under shaking condition at 28°C for 3 days.

For growth curve analysis, the precultures were inoculated to give final cell densities *ca* 10^3 cells ml^{-1} into flasks of HM medium adjusted to pH 4.5, 4.7, 5, 5.5, 6 and 6.8 by 0.1 M NaOH or 0.1 M HCl. The flasks were held at 28°C on a rotary shaker maintained at 200 rpm. The growth pattern was determined every 12 hours for 120 hours on the basis of the optical density measurement at 660 nm.

2.2 Two-Dimensional Gel Electrophoresis

For proteomics, bacterial cell pellets were harvested in an early logarithmic phase. Proteins from the bacteria were extracted by freeze-thawing in the lysis solution (40 mM Tris-HCl (pH 7.5), 50 mM dithiothreitol, 2% (w/v) Triton X-100). One hundred eighty micrograms of total protein were loaded for analytical gels stained with silver and 1.2 mg of protein were used for preparative gels stained with Coomassie brilliant blue (CBB). The first-dimension of electrophoresis was performed using a 18-cm immobilized pH gradient (IPG) gel strip (non-linear gradient between 3 and 10) running on IPGphor (Amersham Bioscience) for 51 kVh and the second dimension was analyzed on 12.5% SDS-PAGE (0.15x20x18 cm) at constant voltage according to the method of Kruft et al [12]. The gels were visualized by silver staining method for image analysis [13] and CBB staining for mass spectrometry (MS) analysis. At least 2 gels were performed for each treatment.

2.3 Peptide Mass Fingerprinting

The silver-stained gels were scanned and analyzed using ImageMasterí 2D software version 3.01 (Amersham Bioscience). Selected protein spots with at least 3-fold differential expression were excised from CBB-stained gels and identified by MALDI-TOF mass spectrometer.

For peptide mass fingerprint analysis, sample preparation was carried out based on the method reported by Millar et al [14]. MS analysis was performed on an Autoflex

MALDI-TOF MS (Bruker Daltonik GmbH, Germany). The peptide mass fingerprint spectra were used to search NCBInr database with Mascot search engine. Background and trypsin autodigestion peaks were omitted from the data. The confidence of matches relied on the number of peptide matches (usually more than three) and the overlap of matched peptide masses with the major peaks of the mass spectra as described by Mathesius et al [15].

3. RESULTS AND DISCUSSION

3.1 Growth Curves of *Bradyrhizobium japonicum* USDA110 at Various pHs

The *B. japonicum* USDA 110 could grow at 6.8, 6, 5.5, 5 and 4.7. But, at pH 4.5, they did not grow (Fig. 1). The growth rate of bacteria at pH 5.5 in HM medium was higher than those grown at the other pHs, including the widely recommended culturing pH, 6.8. The final pH in every treatment has been decreased (data not shown). Macció et al. [16] studied the growth of *Bradyrhizobium sp.* of peanut growing in calcium-added MSM (liquid minimal saline medium) at acid condition. The bacteria demonstrated a growth and visibility diminution at low pH (5.0) and at a calcium concentration of 0.05 mM. Moreover, increasing concentration of calcium significantly improved the rhizobial growth under acid stress conditions. Molecules regulated to the host plant-rhizobium recognition, such as exopolysaccharides, showed changes at different pH values and calcium concentrations [16]. Calcium is likely to improve the ability of rhizobium to attach to plant roots [17]. The lowest tolerant pH values are closed to that observed in our result of which 0.08 mM (13 mg/l) calcium was added. In addition, Raza et al. [6] concluded that the *Bradyrhizobium* sp. (Lupini) stains survived in acidic condition which was pH 4-5. These pH values are close to the lowest pH which *B. japonicum* USDA110 can tolerate. This result showed that *B. japonicum* USDA110 was able to grow in mild acid condition (pH 4.7).

3.2 Two-Dimensional Electrophoresis of Proteins from *Bradyrhizobium japonicum* USDA110 Growing in Normal and Acid Conditions

Even though silver staining is sensitive but conventional silver staining methods is not compatible with MS. The combination of silver in the first analytical 2-DE and
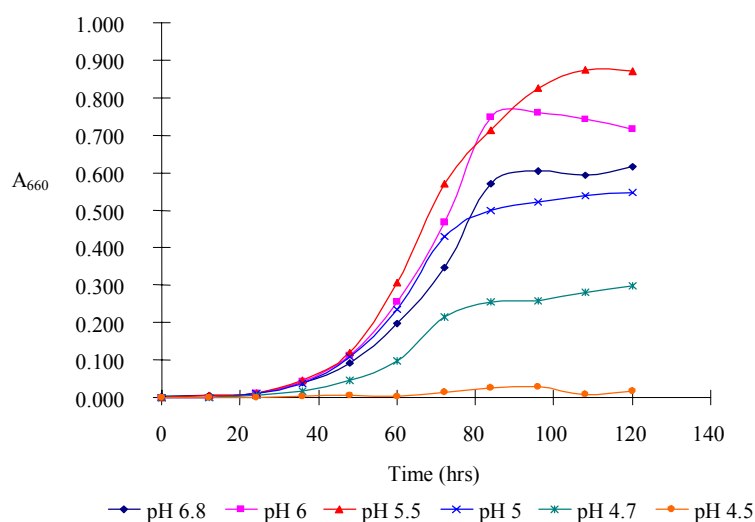

Figure 1. Growth curve of *B. japonicum* USDA110 growing at 28ºC in HM medium of various pHs.

Coomassie staining in the second preparative 2-DE, was then used in our experiment. Another solution is the use of fluorescent, non-covalent staining methods, e.g. SYPRO® Ruby stain, more ideal especially for high-throughput gel staining. Alternatively, the relatively new variant of 2-DE, differential in-gel electrophoresis (2D-DIGE), can be used. In 2D-DIGE technique, two or three protein samples are differentially labeled with resolvable fluorescent dyes prior to 2-DE on a single gel [18].

The complete genomic sequence of *B. japonicum* [19] has supplied chances for examination of the gene expression and the function of each gene to understand the molecular mechanism, especially acid tolerance. The database of gene and protein sequences have been employed for the proteome analysis of the differentiation in protein display between cultured cells of *B.*

japonicum growing at normal (pH 6.8) and acid (pH 4.7) conditions. The size of its genome is about 9.1 Mbp that comprises about 8300 potential protein-coding genes [19]. However, the 2-DE gel patterns of bacteria covered approximately 600 protein spots (Figs. 2 and 3). If we simply assume that one gene encodes one protein, we could calculate that about 7% of whole genome-coding capacity of *B. japonicum* USDA110 was detected on one 2-D gel within pH range of 3-10 and molecular weight range of 34-110 kDa. It showed that the 2-DE technique has some limitations in the analysis of total cellular protein. The encoding genes may be under-expressed or the proteins are present in relatively low amounts in the experimental condition. Moreover, some protein spots may migrate together with more dense protein spots or have very low molecular weights and are not resolved in the second dimension. Alternatively,
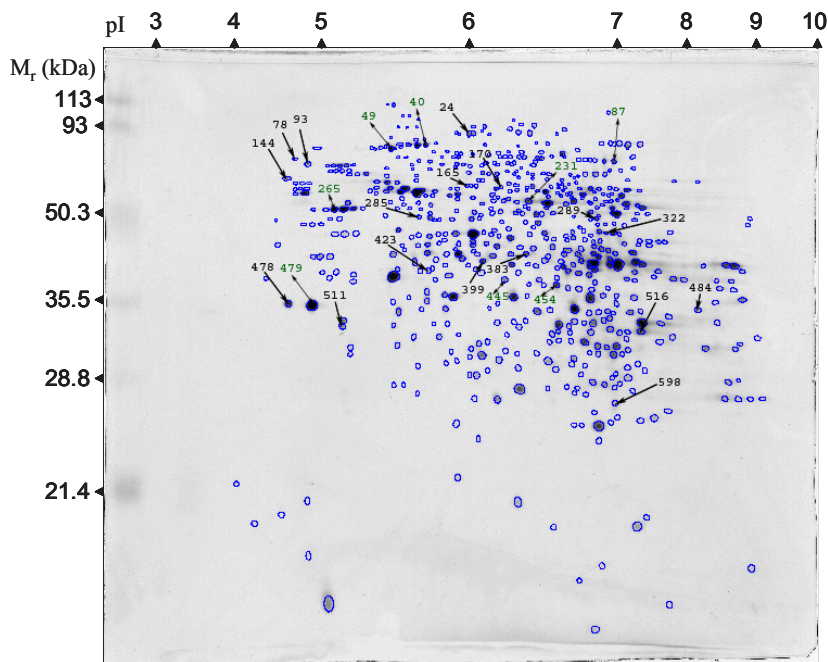

Figure 2. Identified protein spots from *B. japonicum* USDA110 growing at the normal condition (pH 6.8). Arrows with double heads indicate protein spots down-regulated by acidic pH, arrows with single head pointed inwards show specific proteins in normal condition.

the proteins may not be solubilized using the current sample preparation protocol. The running first dimension with narrow pI range, increasing concentration of sample, more effective sample preparation protocol, and more sensitive detection techniques could solve the limitation problem in the number of detected proteins as well as the problem encountered with the characterization of low-abundance and poorly resolved proteins.

3.3 Protein Identification by MALDI-TOF Mass Spectrometry

From the 2-DE gel patterns, 628 and 568 protein spots were detected from bacteria grown in normal condition and acid condition, respectively. After identification by MALDI-TOF MS and data searching against NCBInr database using the Mascot search engine, the selected 84 protein spots were assigned to 70 *B. japonicum* USDA110 proteins.
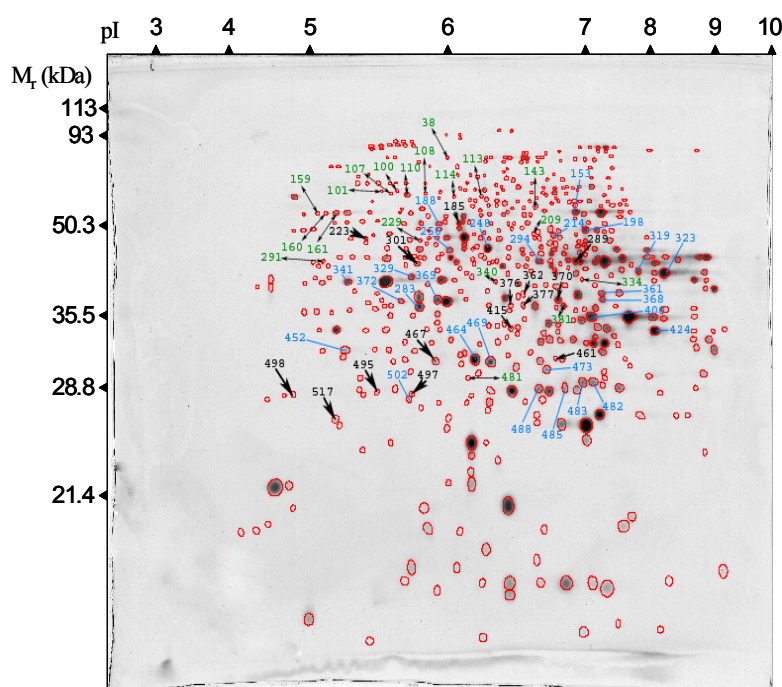

Figure 3. Identified protein spots from *B. japonicum* USDA110 growing at the acid condition (pH 4.7). Lines indicate protein up-regulated by acidic pH, arrows with double heads indicate protein spots down-regulated by acidic pH, arrows with single head pointed inwards show specific proteins in acid condition.

Some protein spots were found in different positions in the gels. It is possible that there are posttranslational modification and the presence of isoforms [20]. Multiple spots had similar observed molecular weight but different pI, signifying covalent attachment of small functions (phosphorylation, glycosylation, acetylation etc.) such as ATP synthase beta chain (spot 261/161, 264/159, and 265/160), bll2743 (spot -/376, and -/377), and 60 kDa Chaperonin (196/108, and 200/107). Some other spots were significantly different in M_r and pI, indicating modification by proteolytic processing such as 3-hydroxybutyrate dehydrogenase (-/467, and 554/464), succinyl-diaminopimalate desuccinylase (293/188, and -/185), and acyl-CoA dehydrogenase (212/113, and 170/-).

From clusters of orthologous groups of proteins (COG) database, most proteins of *B. japonicum* USDA110 were classified into 4 categories while the rest are unclassified (as shown in Table 1). The classified proteins were further grouped according to their function such as transport and metabolism of carbohydrate, amino acid and lipid.

Among transport and binding proteins, several ABC transporter proteins were detected such as ABC transporter sugar-binding protein, ABC transporter amino acid-binding protein, ABC transporter substrate-binding protein, and ABC transporter phosphate-binding protein. Most ABC transporter proteins were represented among the up-regulated proteins in the cellular extracts of *B. japonicum*, except ABC transporter amino acid-binding protein (gene code: bll7600). This result was similar to ABC transporter sugar binding protein up-regulated and another ABC transproter down-regulated in *Sinorhizobium medicae* as showed by transcriptional [8] and proteomic [9] analyses and three homologues of distinct ABC transporters up-regulated in *Streptococcus oralis* [20]. The ABC transporters constitute a superfamily of diverse membrane proteins which utilize the energy derived from ATP hydrolysis to fuel the transport of substrate; such as monosaccharide, amino acid, and ion, across the cell membrane [20, 21].

Glyceraldehyde 3-phosphate dehydrogenase, a glycolytic enzyme, was up-regulated at low pH. Wilkins et al. [20] found that it was also up-regulated when *S. oralis* grew at pH 5.2. If increasing amount of glyceraldehyde 3-phosphate dehydrogenase is reflected by improved glycolytic flux, the resulting increase in ATP production may support increased H^+ extrusion under acidic conditions. On the other hand, ATP synthase (also called H^+-ATPase) beta chains were identified to be a down-regulated protein. However, in other studies of some species (*Streptococcus oralis* [20], *Listeria monocytogenes* [22], and *Streptococcus mutans* [23]), it was up-regulated. The H^+-ATPase facilitates the physical extrusion and active efflux of H^+

ions, and plays a role in response of bacteria to low external pH, especially anaerobic bacteria [20, 22]. For *B. japonicum* USDA110, an aerobic bacterium, this mechanism may not be used. In aerobic bacteria, a proton-motive force is created during the process of electron transport from NAD-linked substrates to oxygen. Dehydrogenases, quinines and oxydoreductases are three major constituents of the respiratory chain [22]. In our result, some dehydrogenases were up-regulated proteins suggesting that *B. japonicum* USDA110 use this process, at least partly, to maintain its pH homeostasis.

Concerning 60 kDa chaperonin (GroEL), some researchers suggested that it be up-regulated protein [20, 22, 23] but it was identified to be down-regulated in our experiment. The chaperonins are a class of the proteins expressed in response to a range of environmental stresses, playing a role in refolding or degradation of denatured proteins, and it may be that GroEL homolog, not detected in our experiment, is produced in increased amounts to counteract the effects of acidification of environment. It should be noted that GroEL levels remained unchanged in *Sinorhizobium medicae* exposed to an acid treatment (pH 5.7) while GroES levels were elevated [9]. At this point, the mechanisms are unclear. Thus, more experiments are needed.

Other acid-responsive genes whose roles were elucidated in *Sinorhizobium medicae* and *Rhizobium leguminosarum* bv. *viciae* e.g. two-component sensor regulator system *actS-actR,* low pH-induced regulator gene *phrR,* pH-regulated structural gene *lpiA* [24], were not detected in our experiment. It is possible that our 2-DE protocol was not appropriated to recover them. For example, PhrR and ActR proteins are too basic to be resolved in our IEF condition. Basic IPG strips should be used. LpiA protein, on the other hand, is so highly hydrophobic that may not be solubilized well during sample preparation [9].

Proteome analysis has provided a powerful and high resolution tool for

Table 1. COG classification of proteins from *B. japonicum* USDA110 growing in normal condition and acid condition.

Spot[1] N/A	Accession no.[2]	Gene code[3]	Protein identity	Type of protein spot

Category I. Cellular mechanism

Group A. Carbohydrate transport and metabolism

469/372[b]	NP_768909	blr2269	ABC Transporter Sugar-Binding Protein	Up-regulated protein spot
463/369[b]	NP_772422	bll5782	ABC Transporter Sugar-Binding Protein	Up-regulated protein spot
434/341[b]	NP_769848	blr3208	ABC Transporter Sugar-Binding Protein	Up-regulated protein spot
484[a]/-	NP_773074	bll6434	C_4-Dicarboxylate Binding Protein	Specific spot in normal condition
438/319[b], 446/323[b]	NP_768163	bll1523	Glyceraldehyde 3-Phosphate Dehydrogenase	Up-regulated protein spot
589/485[b]	NP_771447	bll4807	Triosephosphate Isomerase	Up-regulated protein spot
-/289[b]	NP_769846	blr3206	blr3206 (Probable Aldose 1-Epimerase Precursor)	Specific spot in acid condition

Group B. Amino acid transport and metabolism

-/301[b]	NP_769562	blr2922	ABC Transporter Amino Acid-Binding Protein	Specific spot in acid condition
516[a]/-	NP_774240	bll7600	ABC Transporter Amino Acid Binding protein	Specific spot in normal condition
364248[b]	NP_773086	blr6446	ABC Transporter Substrate-Binding Protein	Up-regulated protein spot
-/415[b]	NP_774236	bll7596	D-Alanine Aminotransferase	Specific spot in acid condition
285[a]/-	NP_767162	bll0522	Argininosuccinate Synthase	Specific spot in normal condition
165[a]/-	NP_773075	bll6435	2-Isopropylmalate Synthase	Specific spot in normal condition
454[a]/347	NP_773137	bll6497	Ketol-Acid Reductoisomerase	Down-regulated protein spot
293/188[b], -/185[b]	NP_769704	blr3064	Succinyl-Diaminopimalate Desuccinylase	Up-regulated protein spot Specific spot in acid condition
465/361[b]	NP_768053	bll1413	bll1413 (Probable Threonine Ammonia-Lyase)	Up-regulated protein spot

Group C. Lipid transport and metabolism

87[a]/44	NP_767213	blr0573	Acetyl-CoA Synthetase	Down-regulated protein spot
212/113[b], 170[a]/-	NP_767798	blr1158	Acyl-CoA Dehydrogenase	Down-regulated protein spot Specific spot in normal condition
580/482[b]	NP_772013	bll5373	bll5373 (Probable Short-Chain Dehydrogenase)	Up-regulated protein spot

Group D. Nucleotide transport and metabolism

322[a]/-	NP_772330	blr5690	Adenylosuccinate Lyase	Specific spot in normal condition
554/464[b], -/467[b]	NP_768128	blr1488	3-Hydroxybutyrate Dehydrogenase	Up-regulated protein spot Specific spot in acid condition
209/114[b]	NP_767649	bll1009	Sulfur Oxidation Protein	Down-regulated protein spot
577/483[b]	NP_767323	blr0683	Orotidine 5´-Monophosphate	Up-regulated protein spot

Group E. Coenzyme transport and metabolism

316/198[b]	NP_770774	blr4134	Aminotransferase	Up-regulated protein spot
423[a]/-	NP_767586	bll0946	Geranyltransferase	Specific spot in normal condition
495/400[b]	NP_773327	blr6687	blr6687(Putative Phosphoglycerate Dehydrogenase)	Up-regulated protein spot
573/481[b]	NP_767206	bll0566	bll0566 (Putative Uroporphyrinogen III Synthase)	Down-regulated protein spot

Table 1 (cont.)

Spot[1] N/A	Accession no.[2]	Gene code[3]	Protein identity	Type of protein spot
Group F. Secondary metabolites biosynthesis, transport and catabolism				
-/362[b]	NP_770383	blr3743	Periplasmic Mannitol-Binding Protein	Specific spot in acid condition
590/502[b],	NP_767477	bll0837	bll0837 (Putative Carboxymethylenebutenolidase)	Up-regulated protein spot
-/495[b],				Specific spot in acid condition
-/497[b]				
Group G. Energy production and conversion				
269/153[b]	NP_769456	blr2816	Aldehyde Dehydrogenase	Up-regulated protein spot
231[a]/128	NP_771424	bll4784	Aldehyde Dehydrogenase	Down-regulated protein spot
261/161[b],	NP_767080	bll0440	ATP Synthase Beta Chain	Down-regulated protein spot
264/159[b],				
265[a]/160[b]				
321/209[b]	NP_767091	bll0451	Dihydrolipoamide S-Succinyltransferase	Down-regulated protein spot
511[a]/-	NP_768018	blr1378	Electron Transfer Flavoprotein Large Subunit	Specific spot in normal condition
289[a]/-	NP_771756	blr5116	Nrd Protein	Specific spot in normal condition
383[a]/-	NP_769556	blr2916	Tartrate Dehydrogenase	Specific spot in normal condition
240/143[b]	NP_774247	bll7607	bll7607 (Putative Aldehyde Dehydrogenase)	Down-regulated protein spot
Group H. Inorganic ion transport and metabolism				
531/424[b]	NP_767731	blr1091	ABC Transporter Phosphate-Binding Protein	Up-regulated protein spot

Category II. Cellular processes and signaling

Group A. Signal transduction mechanisms				
598[a]/-	NP_767026	bll0386	Two-Component Response Regulator	Specific spot in normal condition
Group B. Cell wall/membrane biogenesis				
354/256[b]	NP_767636	blr0996	HlyD Family Secretion Protein	Up-regulated protein spot
477/368[b]	NP_768139	blr1499	UTP-Glucose-1-Phosphate Uridylyltransferase	Up-regulated protein spot
-/223[b]	NP_768912	blr2272	blr2272 (Putative Porin Precursor)	Specific spot in acid condition
Group C. Cell mobility				
479[a]/394	NP_773505	bll6865	Flagellin	Down-regulated protein spot
478a/-	NP_773506	bll6866	Flagellin	Specific spot in normal condition
Group D. Posttranslational modification, protein turnover, chaperones				
557/469[b]	NP_771948	blr5308	Anti-Oxidant Protein	Up-regulated protein spot
24a/-	NP_768044	blr1404	ATP-Dependent Protease ATP-Binding Subunit	Specific spot in normal condition
195/101[b],	NP_772266	blr5626	60 kDa Chaperonin	Down-regulated protein spot
196/108[b],				
200/107[b],				
201/110[b]				
177/100[b]	NP_774173	blr7533	60 kDa Chaperonin	Down-regulated protein spot

Table 1 (cont.)

Spot[1] N/A	Accession no.[2]	Gene code[3]	Protein identity	Type of protein spot

Category III. Information processing

Group A. Transcription

390/291[b]	NP_772016	bll5376	DNA-Directed RNA Polymerase Alpha Subunit	Down-regulated protein spot
144[a]/-	NP_767425	bll0785	N-Utilization Substance Protein A	Specific spot in normal condition

Group B. Translation

72/38[b]	NP_769176	blr2536	Glycyl-tRNA Synthetase Beta Chain	Down-regulated protein spot
40[a]/12	NP_767419	bll0779	Polyribonucleotide Nucleotidyltransferase	Down-regulated protein spot
49[a]/20	NP_772043	bll5403	Translation Elongation Factor G	Down-regulated protein spot
452/334[b]	NP_767442	bll0802	Tryptophan-tRNA Ligase	Down-regulated protein spot

Category IV. Poorly characterized (General function prediction only)

445[a]/340[b]	NP_769845	blr3205	Dehydrogenase	Down-regulated protein spot
567/473[b]	NP_766862	blr0222	Glyoxalase II	Up-regulated protein spot
-/498[b]	NP_774200	blr7560	2-Haloalkanoic Acid Dehalogenase	Specific spot in acid condition
-/370[b]	NP_771986	blr5346	blr5346 (Putative Hydrolase)	Specific spot in acid condition
344/214[b]	NP_774125	blr7485	Hypothetical Zinc Protease	Up-regulated protein spot

Unclassified proteins

424/329[b]	NP_767453	bll0813	bll0813 (Hypothetical Protein)	Up-regulated protein spot
540/452[b]	NP_768131	bll1491	bll1491 (Hypothetical Protein)	Up-regulated protein spot
482/381[b]	NP_769383	bll2743	bll2743 (Hypothetical Protein)	Down-regulated protein spot
-/376[b]	NP_769383	bll2743	bll2743 (Hypothetical Protein)	Specific spot in acid condition
-/377[b]	NP_769383	bll2743	bll2743 (Hypothetical Protein)	Specific spot in acid condition
78[a]/-	NP_772483	bll5843	bll5843 (Hypothetical Protein)	Specific spot in normal condition
93[a]/-	NP_772486	bll5846	bll5846 (Hypothetical Protein)	Specific spot in normal condition
402/283[b]	NP_766845	blr0205	blr0205 (Hypothetical Protein)	Up-regulated protein spot
-/517[b]	NP_766867	blr0227	blr0227 (Hypothetical Protein)	Specific spot in acid condition
397/294[b]	NP_770666	blr4026	blr4026 (Hypothetical Protein)	Up-regulated protein spot
337/229[b]	NP_769839	blr3199	blr3199 (Hypothetical Protein)	Down-regulated protein spot
586/488[b]	NP_769127	blr2487	blr2487 (Hypothetical Protein)	Up-regulated protein spot
399[a]/-	NP_770027	bll3387	bll3387 (Unknown Protein)	Specific spot in normal condition
-/461[b]	NP_769505	blr2865	blr2865 (Unknown Protein)	Specific spot in acid condition

[1]Spot N/A, the spot number in normal condition (N) and acid condition (A) as given in Figure 2 and 3, respectively. [a]Protein spots identified from *B. japonicum* USDA110 growing at the normal condition (Figure 2); [b]Protein spots identified from *B. japonicum* USDA110 growing at the acid condition (Figure 3). [2]Accession no., the accession number of identified protein from NCBI database. [3]Gene code, Locus-tag of the encoding gene in the genome of *B. japonicum* USDA110 [19].

allocating functions to many new genes arising from the genome sequencing experiments. To our knowledge, this is the first report on 2-DE of proteins from acid-induced *B. japonicum* USDA110. Some proteins, found in this experiment, may relate to the important functions of acid tolerant metabolism. However, there are still some protein spots of which their functions are still unclear and needed to be characterized further.

4. CONCLUSION

From studying growth curves and pH curves of *B. japonicum* USDA110 at various pHs, *B. japonicum* USDA110 was able to grow in mild acid condition (pH 4.7). The 2-DE, a proteomic technique, has provided many details about protein compositions of bacterial cells in different pH conditions. Protein identification using MALDI-TOF MS showed that the acid tolerant property has relationship with wide range of bacterial metabolic pathway including transport system, β-oxidation of fatty acid, posttranslational modification, glycolytic pathway and ATP synthesis.

The data from this study can be a basis for mechanism elucidation of acid tolerance in *B. japonicum* USDA110 and, hence, leading to improved production of legume cultivated in acid soil.

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