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Full Length Research Paper

Growth, symbiotic, and proteomics studies of soybean *Bradyrhizobium* in response to adaptive acid tolerance

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Bradyrhizobial inoculated soybean often performs poorly on acid-soil because of the acid sensitivity of their associated root nodule bacteria. Acid tolerance in rhizobia has been considered as a key phenotypic characteristic in that it enables the bacteria to perform well under the restrictive conditions of excessive acidity. Since bacteria could develop acid tolerance to a more acid condition by using adaptive acid tolerance response (ATR), it is interesting to investigate whether bradyrhizobia could have this response and what proteins are involved in ATR. *Bradyrhizobium* sp. DASA01007 was selected for this study based on its ATR ability and symbiosis efficiency with soybean under acid condition. To establish an ATR in bradyrhizobia, late log phase culture of cell grown in mild acid condition was subsequently used as inoculum to more acid conditions. The 2D-gel and proteomic analyses were used to investigate the proteins response during ATR compared with non-adaptive conditions. The 29 identified proteins were grouped into 8 categories based on category orthologous group (COG) and one group of unknown categories. Hypothetical protein, transport and binding proteins, and translation protein were up-regulated at pH 4.5N (non-adaptive condition). While up-regulated proteins found during growth at pH 4.5A (adaptive condition) consisted of proteins in cellular processes, translation, energy metabolism, regulatory functions, interconversions and salvage of nucleosides and nucleotides, and conserved hypothetical proteins group. However, transport and binding proteins were absent in adaptive condition. At pH 5.5A, proteins involved in cellular processes were also detected. Several proteins overproduced in adaptive condition may be involved in ATR of bradyrhizobia. An importance of ATR in root nodule bacteria would support a better chance of survival in low pH soils than those conventionally grown in neutral pH. These results suggest that the use of ATR condition could provide an improvement in the production of inoculants.

Key words: Adaptive acid tolerance, *Bradyrhizobium*, Soybean, 2D-gel electrophoresis.

INTRODUCTION

Rhizobia are soil bacteria that can elicit the formation of nitrogen-fixing nodules on the legumes. Environmental conditions usually affect symbiosis between rhizobia and its host. The survival or growth of bacteria can be adversely affected by low pH, and cells need to adapt to a changing environment to survive. Thus, acid tolerance in rhizobia has been considered to use as inoculum for acid soil condition (Howieson et al., 1988). The screening for acid-tolerant isolates that can colonize and/or persist in acidic soils thus give rise to novel strains with enhanced survival and/or symbiosis under moderately acid conditions. Since N_2 fixation supply ~50% of nitrogen used in agriculture (Vance et al., 1998), the selection of acid tolerant strain of rhizobia has markedly improved legume productivity on acid-soil. Therefore, this is a continuing need for the identification of acid tolerant inoculums strains of rhizobia for increasing yields of

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legume crops on acid soil or in environments in which acidification is a problem (Watkin et al., 2003).

However, studies on rhizobial tolerance to acidity in soils revealed that an acid-tolerant rhizobium in laboratory cultures does not necessarily insure an outstanding survival and competition of the same rhizobia under comparable acid conditions in soil (Brockwell et al., 1991). Even more uncertain is the correlation between the rhizobial ability to persist in acid soils and the capacity of these bacteria to express their symbiotic phenotype in the same acidity (Howieson et al., 1988). The mechanisms allowing cells to survive and grow at low pH are not yet widely defined, although a number of processes have been proposed to be involved for the enteric, including cytoplasmic buffering, ion cycling (Booth, 1985), DNA repair (Foster, 1995), and pH amelioration (Stim and Bennett, 1993).

Many research studies have proposed that bacteria could develop acid tolerance to a more acid condition when log-phase cells grown at neutral pH were exposed to mild acid conditions for a period of time before challenging to a more acid condition. This response is known as adaptive acid tolerant response (ATR) (Foster and Hall, 1990, 1991; Goodson and Rowbury, 2008). ATR was also found in several rhizobia, such as *Rhizobium leguminosarum* (O'Hara and Glenn, 1994), *Mesorhizobium huakuii* LL56, *Mesorhizobium* sp. LL22 (Rickert et al., 2000), *Sinorhizobium* sp. BL3 (Tittabutr et al., 2006), as well as in *Bradyrhizobium* sp. (O'Hara and Glenn, 1994). Complementary to this approach, the identification of the genetic determinants of acid tolerance in rhizobia has also been considered as a key strategy in the attempt to manipulate and improve bacterial survival and symbiosis at low pH. The available evidence indicated that tolerance to acidity in *Bradyrhizobium* sp. is a multigenic phenotype in which the genetic determinants appear to be associated with diverse cellular functions. However, detail of genes or proteins controlling diverse cellular functions of *Bradyrhizobium japonicum* growing in acid condition especially under ATR condition has not been clearly investigated.

The present research aimed to investigate the ATR ability and the proteins which response to adaptive acid tolerance of soybean *Bradyrhizobium* sp. DASA01007, an acid tolerant strain isolated from acidic soil in Thailand by using 2D-Gel and proteomic analyses. An ATR in root nodule bacteria is that inoculants grown at acid conditions would have a better chance of survival in low pH soils than those conventionally grown at pH 6.8.

MATERIALS AND METHODS

Bacterial strains and growth medium

Bradyrhizobium sp. DASA01007 (acid tolerant strain) was provided by Soil Microbiology Research Group, Department of Agriculture, Thailand, while *B. japonicum* USDA110, was provided by Prof. K. Minamisawa (Graduate School of Life Science, Tohoku University, Japan), were used as standard strain for comparison in this study. HM medium (per liter of deionized water: 1.0 g Sodium glutamate, 0.125 g Na_2HPO_4, 0.25 g Na_2SO_4, 0.32 g NH_4Cl, 0.18 g $MgSO_4 \cdot 7H_2O$, 0.004 g $FeCl_3$, 0.013 g $CaCl_2 \cdot 2H_2O$, 1.3 g HEPES (N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)), 1.1 g MES (2-(N-Morpholino)ethanesulfonic acid), 1.0 g yeast extract, 1.0 g L-arabinose, pH 6.8) was used for all experimental growth conditions (Cole and Elkan, 1973).

Adaptive acid tolerance and non-adaptive acid tolerance assay

For non-adaptive growth conditions, acid tolerant bradyrhizobial strains DASA01007 and *B. japonicum* USDA110 were grown in HM medium (pH 6.8) and inoculated directly to HM medium which already adjusted the pH at pH 4.5, 5.5, and 6.8. For adaptive growth condition, late log phase of bacterial starter culture (4 days after inoculation) in HM medium at pH 6.8 was inoculated to a more acid condition in HM medium (pH 5.5) to get the final cells concentration at 10^3 cells/ml. Bacterial cultures were incubated at 28°C on a rotary shaker at 200 rpm. When the culture reached late log phase, bacterial culture was used as inoculum and subsequently inoculated to HM medium at pH 4.5. The growth pattern was determined on the basis of optical density measurement at 600 nm for 7 to 14 days.

Plant test

Soybean seeds (*Glycine max* Chiang Mai 60) were surface sterilized in 95% ethanol for 10 s before adding 3% sodium hypochlorite to immerse the seed completely. After 5 min drain of the sterilant, seeds were rinsed six times with sterilized water. The sterilized seeds were put on sterilized plate containing wet tissue and kept in a dark place for 1 to 2 days. Germinated seeds were grown in Leonard's jar containing sterilized vermiculite then inoculated with bradyrhizobial culture (10^6 cells/seed). Since the initial pH of vermiculite is 7.15, thus acidity of vermiculite was adjusted by soaking vermiculite in nutrient solution at different pH 24 h before planting. Then, acid conditions of vermiculite were maintained by different pH of nutrient solution supplemented with Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) and MES as buffer.

Nodulation and nitrogen fixation were tested in Leonard's jar containing nitrogen free nutrient solution which was adjusted to normal condition (pH 6.8) and acidic condition (pH 4.5), and supplemented with 20 mM PIPES (pH 6.8) and 20 mM MES (pH 4.5). The Leonard's jars were put on the shelf light and were set up 12 h in light and 12 h in dark at 25°C. Plants were harvested after 28 days and data such as acetylene reduction activity (ARA), nodules number, nodule dry weight, and plant dry weight were recorded (Somasegaran and Hoben, 1994). Data were analyzed by SPSS 16.0.2 (SPSS Inc., Chicago, USA).

Protein extraction and separation by 2D-gel electrophoresis

To investigate proteins involved in acid tolerant or adaptive acid tolerant response of *Bradyrhizobium* sp. DASA01007, total proteins were extracted from cells grown in different conditions. Approximately, 100 ml packed volume of selected bacterial suspension was resuspended in Buffer A (0.1 M Tris-HCl, pH 8.8). An equal volume of phenol (saturated with Buffer A) was added into the bacterial suspension and the mixture was vortexed for 5 min at room temperature.

Centrifugation at 5000×*g* for 5 min at room temperature resulted in two separating phases. The phenol phase was re-extracted two more times to remove most of the nucleic acids and polysaccharide

contaminants. Five volumes of methanol containing 0.1 M ammonium acetate was added into the final phenol phase, mixed and incubated at -20°C for overnight. The precipitated protein was collected by centrifugation at 5000×*g* for 15 min and washed twice with methanol containing 0.1 M ammonium acetate.

To remove the ammonium acetate, the precipitated protein was further washed with ice-cold absolute ethanol and centrifuged, and the pelleted protein was air dried to remove the traces of ethanol. The dried pellet was solubilized in IEF buffer (8.0 M urea, 2.0 M thiourea, 4.0% CHAPS, 2.0% Triton X-100, 50 mM DTT, 0.75% of 5 to 8, and 0.25% of 3 to 10 ampholines, GE Healthcare, USA). The proteins were dissolved at room temperature with gentle vortexing for 1 h, followed by ultracentrifugation at 100,000×*g* for 15 min to remove the insoluble material. The protein concentration was determined using the method described by Bradford (1976).

The sample was then immediately diluted to 1 mg/ml for a total volume of 500 µl using DeStreak rehydration solution (Amersham Biosciences, USA) and incubated for overnight at 4°C. The sample was focused on 18 cm, pH 3 to 10 IPG strip (Amersham Biosciences, USA) for 90,000 V/h Multiphor™ II Electrophoresis System (GE Healthcare, USA).

The strip was removed and incubated for 20 min in SDS equilibration buffer (50 mMTris-HCl, pH 8.8; 6 M urea; 4% w/v SDS; 2% v/v glycerol) containing 2% w/v DTT, then for 20 min in SDS equilibration buffer containing 2.5% w/v iodoacetamide. The second dimension was analyzed on 12.5% SDS-PAGE (0.15×20×18 cm). The gels were visualized by Coomassie brilliant blue staining (Sarma and Emerich, 2006). The gel was scanned and image analysis was done using Image Master 2D Platinum 7.0 (GE Healthcare, USA).

The intensity of interesting protein spot was analyzed by comparison with intensity of protein presented in cell grown at pH 6.8. The different intensities (2-fold) of protein spot demonstrated up and down regulation of those specified proteins, which were picked up for further analysis. Protein extraction and 2D-Gel analysis from bacteria grown in each condition were repeated 2 times.

Sample preparation for Liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS)

The twenty nine selected protein spots were excised from stained gels, then washed twice in MilliQ water for 15 min. The washed gel pieces were subjected to two cycles of dehydration with 50% acetonitrile followed by rehydration with 50 mM ammonium bicarbonate solution for 15 min per cycle and digested for overnight at 37°C in 20 µl of sequencing grade trypsin (Sigma-Aldrich, USA), according to the manufacturer's instructions (1 µg in 100 µl of 50 mM ammonium bicarbonate). The supernatants were transferred into a fresh tube and stored at room temperature until required. Thirty µl MilliQ water was added to the gel pieces at room temperature for 1 h. Following this, the two supernatants were pooled together (Sarma and Emerich, 2005).

Protein identification using LC-MS/MS and peptide mass fingerprint database search

Mass spectrometric analyses were conducted by nanoflow-LC-ESI-MS/MS (Bruker Esquire 3000 plus Ion Trap; Bruker Daltonics, Germany). Peptides were separated by chromatography on a 75 µm × 15 cm Pep-Map nanocolumn (LC Packings). Instrument operation, data acquisition, and analyses were performed using HyStarTM V2.3 and Data Analysis V3.1 software. Data captured by either LC-ESI-MS/MS were matched using the MASCOT version 2.2.03 (Matrix Science, UK (http://www.matrixscience.com) against MSDB database). Carbamidomethyl (Cys) and oxidation (Met) were considered as variable modifications and a single missed cleavage was permitted. For LC-MS/MS data, peptide mass tolerance was set at 3.0 Da and MS/MS ion mass tolerance was set at 1.5 Da. Peptide charge states (+1, +2, +3) were taken into account (Li et al., 2010).

RESULTS

Growth of bradyrhizobia and symbiotic efficiency of bradyrhizobial strains under acid condition

Bradyrhizobium sp. DASA01007 and *B. japonicum* USDA110 were grown in HM medium at pH 6.8, 5.5, and 4.5 in non-adaptive condition. The specific growth rate of each culture was determined, and the growth curves were plotted as shown in Figure 1. These results show that acid condition had an effect on cell growth. Although, there were no significant difference between the specific growth rate of both strains at pH 6.8 and 5.5, the growth rates of *Bradyrhizobium* sp. DASA01007 and *B. japonicum* USDA110 were reduced when bradyrhizobial strains grown at a strong acid condition. There was almost no growth of both cultures under pH 4.5, which was significantly different from the growth of bacteria cultured at pH 6.8 and 5.5 (Figure 1). These results emphasized the importance of strain selection for legume inoculum.

To investigate the symbiotic efficiency of bradyrhizobia, *Bradyrhizobium* sp. DASA01007 and *B. japonicum* USDA110 were inoculated into soybean seed (10^6 cells/seed) and planted in both acid (pH 4.5) and normal (pH 6.8) conditions. At pH 6.8, soybean inoculated with *Bradyrhizobium* sp. DASA01007 provided the highest value of total plant dry weight, shoot dry weight, root dry weight, and nodule dry weight, but these data were not significantly different from those of soybean inoculated with *B. japonicum* USDA110 ($P \leq 0.05$). On the other hand, although soybean inoculated with *B. japonicum* USDA110 performed highest nitrogenase activity in terms of ARA, the data was not significantly different from soybean inoculated with *Bradyrhizobium* sp. DASA01007 ($P \leq 0.05$). These pieces of information revealed that *Bradyrhizobium* sp. DASA01007 had a similar symbiotic efficiency to *B. japonicum* USDA110 when *Bradyrhizobium* sp. DASA01007 grown at neutral condition.

Once soybean was grown in acid condition (pH 4.5), soybean inoculated with *Bradyrhizobium* sp. DASA01007 provided the highest value of total dry weight and nitrogenase activity, and the data were significantly different from soybean inoculated with *B. japonicum* USDA110. *Bradyrhizobium* sp. DASA01007 tends to perform better symbiosis than *B. japonicum* USDA110 (Table 1). It would be possible that *Bradyrhizobium* sp. DASA01007 could tolerate in acid condition or have adaptive acid tolerance response that allows cells to survive in acid condition and finally lead to successful symbiosis with plant. Thus, *Bradyrhizobium* sp.

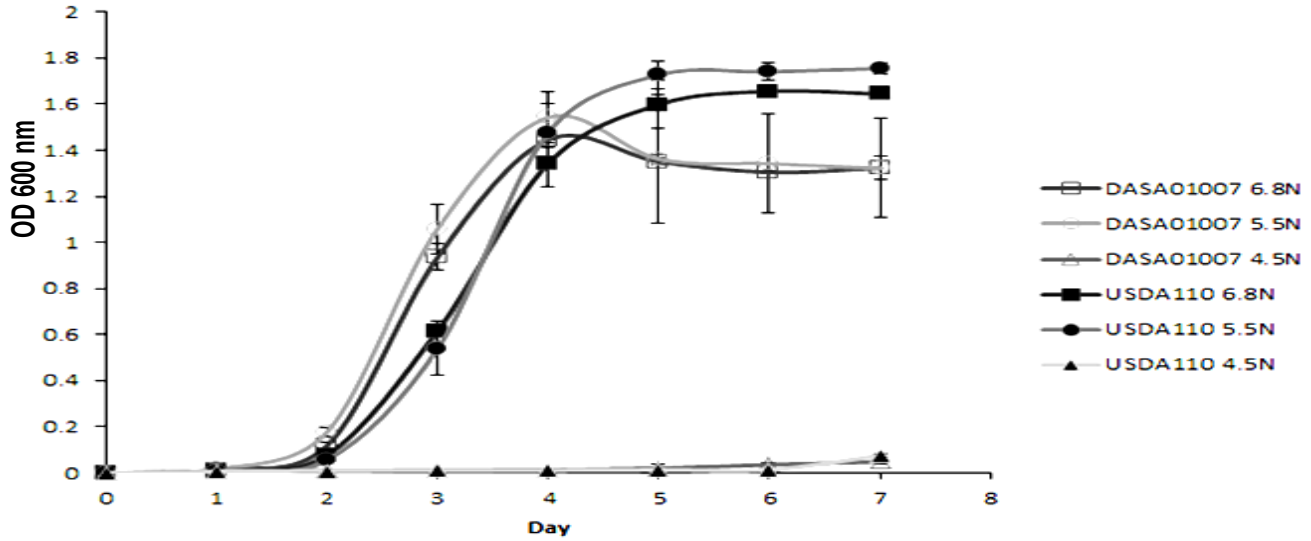

Figure 1. Growth of *Bradyrhizobium* sp. DASA01007 and *B. japonicum* USDA110 growing in HM medium at pH 4.5, 5.5, and 6.8 under non-adaptive condition (N).

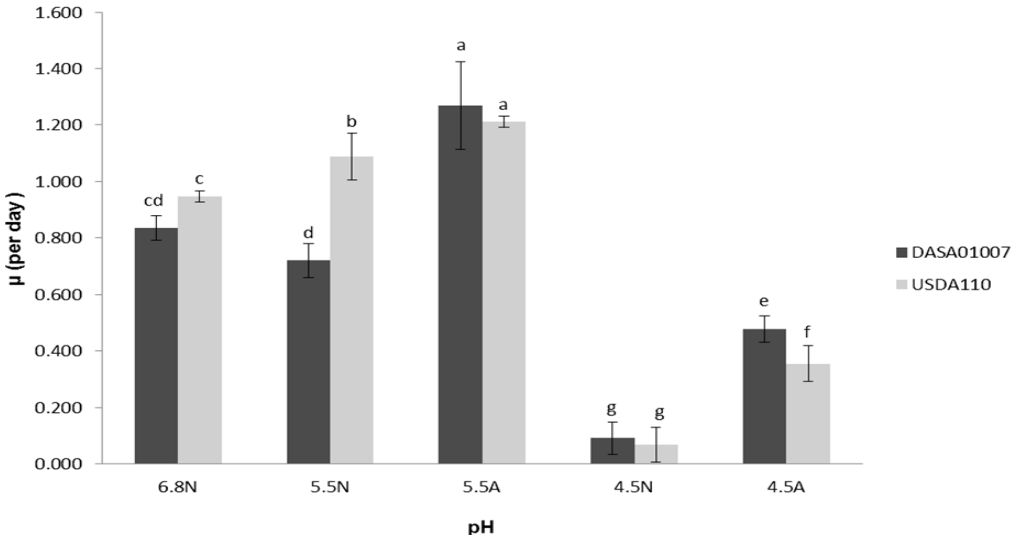

Figure 2. Specific growth rates of *Bradyrhizobium* sp. DASA01007 and *B. japonicum* USDA110 growing in HM medium at pH 4.5, 5.5, and 6.8 under non-adaptive (N) and adaptive (A) conditions. Results of the experiments represent triplicates.

DASA01007 was used to verify the growth in acidic pH under non-adaptive (N) and acid adaptive (A) conditions in the next experiments.

Growth of bradyrhizobia under non adaptive (N) and acid adaptive (A) conditions

Bradyrhizobium sp. strain DASA01007 and *B. japonicum* USDA110 were grown in HM medium at pH 5.5 and 4.5 in both non-adaptive (N) and adaptive (A) conditions. The specific growth rates of bradyrhizobia were in range of 0.069 to 1.089 per day and 0.355 to 1.270 per day at non adaptive and adaptive conditions, respectively (Figure 2). It was clearly indicated that the specific growth rates of bradyrhizobia cultured under adaptive condition were significantly better than those cultured under non adaptive condition at pH 4.5 and 5.5.

The results showed that *Bradyrhizobium* sp. DASA01007 significantly grew better than *B. japonicum* USDA110 in HM medium at pH 4.5 under adaptive acid conditions. However, the specific growth rates of *Bradyrhizobium* sp. DASA01007 and *B. japonicum* USDA110 were not significantly different when grown at

Table 1. Effect of different bradyrhizobial inoculations on symbiotic efficiency with soybean at pH 6.8 and 4.5.

Strains	pH	Total plant dry weight (mg/pot)	Shoot dry weight (mg/pot)	Root dry weight (mg/pot)	Nodule dry weight (mg/pot)	Nodule number	ARA (µM C_2H_4/h/pot)
Un-inoculated	4.5	570.00^{b*}±216.33	363.33^{b}±136.50	206.67^{ab}±85.05	0.00	0.00	0.00
DASA01007	4.5	903.33^{a}±177.86	646.67^{a}±187.70	256.66^{ab}±23.09	26.67^{a}±8.33	14.61^{a}±1.67	15.29^{a}±1.39
USDA110	4.5	516.67^{b}±66.58	346.67^{b}±101.16	170.00^{b}±34.64	18.33^{a}±2.08	11.00^{a}±3.50	6.94^{b}±1.51
Un-inoculated	6.8	512.67^{b}±89.81	356.67^{b}±63.51	154.33^{b}±35.80	0.00	0.00	0.00
DASA01007	6.8	1023.33^{a}±210.08	740.00^{a}±180.00	283.33^{a}±30.55	28.33^{a}±3.79	15.00^{a}±3.50	13.48^{a}±2.61
USDA110	6.8	763.33^{ab}±146.40	500.00^{ab}±95.39	263.33^{ab}±51.32	17.67^{a}±8.08	18.78^{a}±7.33	15.70^{a}±1.40
			ns	ns	ns	ns	

ns = Not significantly different at $P \leq 0.05$ level. Data are means of three replicates ± SD. *Same letters are not significantly different at $P \leq 0.05$ level.

acid condition (pH 5.5) under adaptive conditions (Figure 2). These results indicated that *Bradyrhizobium* sp. DASA01007 have better induction of adaptive acid tolerance response (ATR) which may be one of the mechanisms that allow bradyrhizobial cell to grow under extreme acid condition.

2D-gel analysis of *Bradyrhizobium* sp. DASA01007 in response to acidic pH

To identify proteins involved in acid tolerance or adaptive acid tolerance response of *Bradyrhizobium* sp. DASA01007, 2D-gel electrophoresis was carried out. There were 651, 475, 638, and 745 protein spots that could be detected from bacterial cells grown at pH 6.8, 5.5A, 4.5A, and 4.5N, respectively. Based on the protein profile present in pH 6.8 condition, 15, 6, and 3 spots of protein which were obviously up-regulated, were selected from protein profile of cells grown at pH 4.5A, 4.5N, and 5.5A, respectively. While other 5 protein spots, which were highly expressed in cells grown at pH 6.8 but down-regulated in cells grown at acid condition were also selected (Figure 3). These 29 spots of protein were analyzed by Liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) and the peptide mass fingerprinting (PMF) searches were performed with the MSDB databases through the Mascot server (http://www.matrixscience.com). Only proteins identified with at least two peptide hits in duplicate analyses were accepted.

In order to identify the protein, amino acid sequence fragments were compared with *B. japonicum* USDA110 database (http://genome.kazusa.or.jp/rhizobase/Bradyrhizobium). The results of identified proteins are summarized in Table 2.

Based on the protein profile, the 29 selected proteins were grouped into 8 categories based on COGs and one group of unknown categories: (i) Cellular processes consisting of 5 proteins including 60 kDa chaperonin 6 (spot no.6), two proteins of 10 kDa chaperonin (spot no.14 and 22), 10 kDa chaperonin 1 (spot no.23), and chaperonin GroEL (spot no.27); (ii) Conserved hypothetical protein, consisting of 7 hypothetical proteins (spot no.1, 2, 3, 5, 13, 16, and 21); (iii) Transport and binding proteins, consisting of 4 proteins including two proteins of ABC transporter sugar-binding proteins (spot no.17 and 19), ABC transporter amino acid-binding protein (spot no.18), and ABC transporter substrate-binding protein (spot no.20); (iv) Translation, consisting of 4 proteins including 30S ribosomal protein S6 (spot no.9), Ribosomal protein L7/L12 (spot no.10), two proteins of elongation factor Tu (spot no.15 and 28); (v) Energy metabolism, ATP synthase subunit beta (spot no.4); (vi) Regulatory functions, two-component response regulator (spot no.7); (vii) Interconversions and salvage of nucleosides and nucleotides, nucleoside diphosphate kinase (spot no.12); (viii) Other categories, consisting of 2 proteins including oxidoreductase (spot no.8), and peroxiredoxin (spot no.11); and one group of unknown categories, consisting of 4 proteins including peptidoglycan-associated protein (spot no.24), ribosomal protein S1 (spot no.26), unknown protein (spot no.25), and hypothetical protein (spot no.29).

Proteins produced from pH 4.5A consist of proteins in cellular processes (60 kDa chaperonin 6 and 10 kDa chaperonin), conserved hypothetical protein, translation (30S ribosomal protein S6), energy metabolism (ATP synthase subunit beta), regulatory functions (two-component response regulator), and interconversions and salvage of nucleosides and nucleotides (nucleoside diphosphate kinase) group were up-regulated.

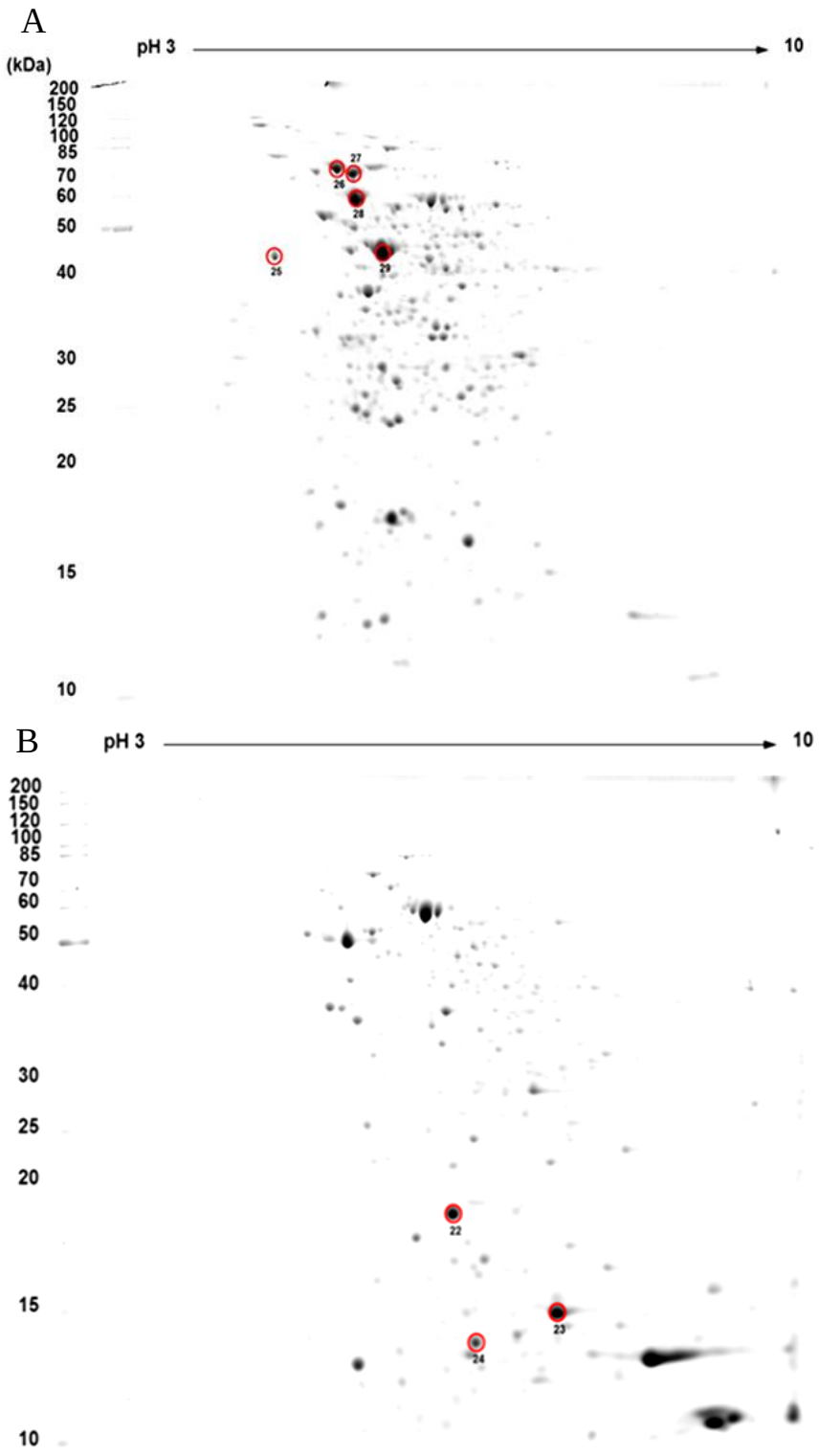

Figure 3. 2D-Gel analyses of proteins from *Bradyrhizobium* sp. DASA01007. Selected protein spots showing up- or down-regulation were circle in each condition: A) Protein spots (spot no. 25 to 29) from *Bradyrhizobium* sp. DASA01007 growing at pH 6.8, B) Protein spots (spot no. 22 to 24) from *Bradyrhizobium* sp. DASA01007 growing at pH 5.5A (adaptive condition), C) Protein spots (spot no. 1 to 15) from *Bradyrhizobium* sp. DASA01007 growing at pH 4.5A (adaptive condition), D) Protein spots (spot no. 16 to 21) from *Bradyrhizobium* sp. DASA01007 growing at pH 4.5N (non-adaptive condition).

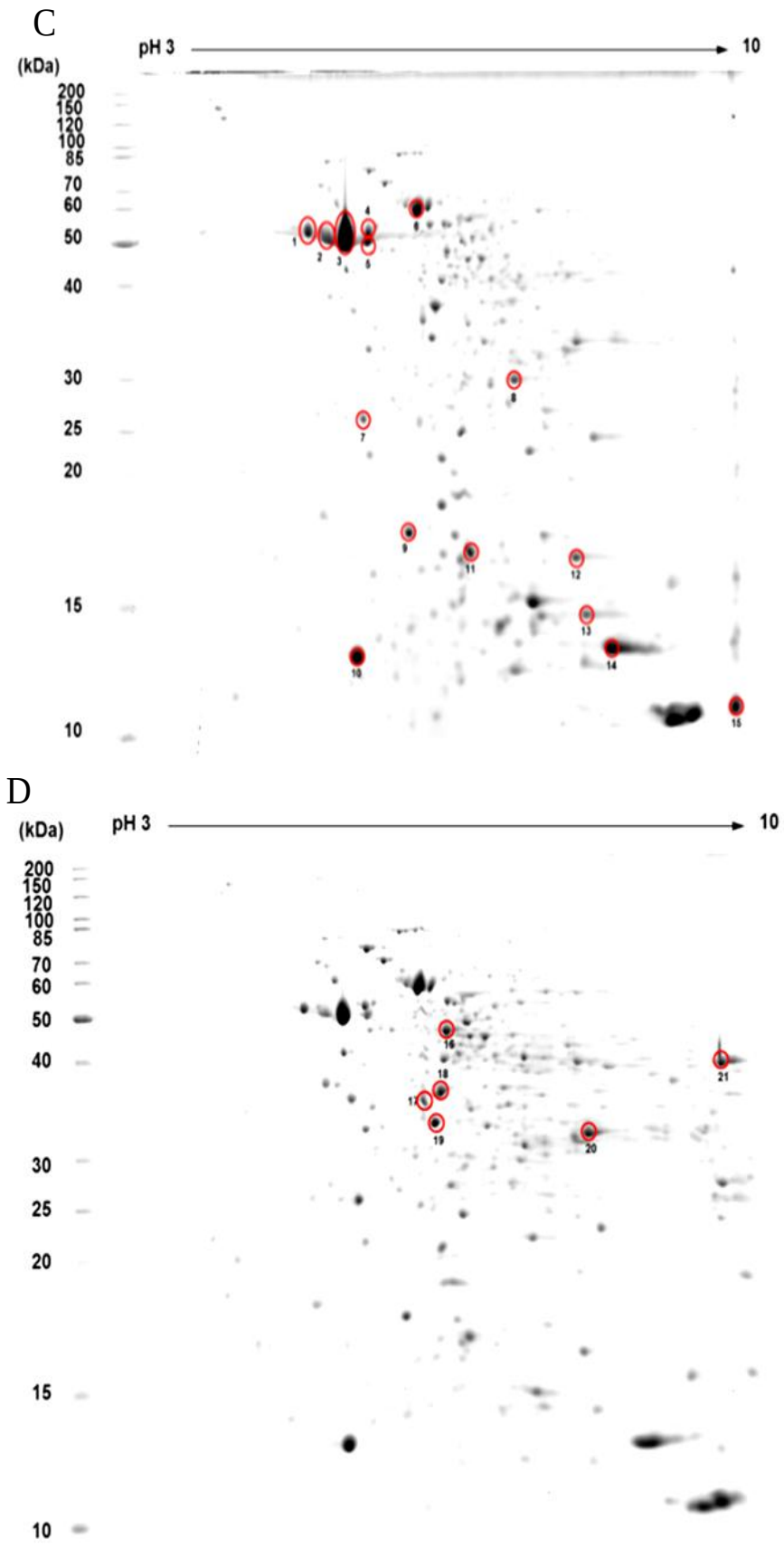

Figure 3. Contd.

Table 2. Proteins involved in acid tolerance of *Bradyrhizobium* sp. DASA01007 identified from 2D-PAGE.

Spot No.	Functional category[c]	Tophit protein	Gene code[a]	Locus name[b]	Organisms	Peptide matched[d]	Mascot score[e]	MW[f] (kDa)	PI[g]	Predicted localization[h]	Fold change[i]		
											pH 5.5A	pH 4.5A	pH 4.5N
6	Cellular processes	60 kDa chaperonin 6	*groEL*	blr5626	*B. japonicum* USDA110	66	1764	57,716	5.45	Cytoplasmic		1.57	
14	Cellular processes	10 kDa chaperonin	*groES*	blr5625	*B. japonicum* USDA110	19	229	11,170	7.93	Cytoplasmic		20.03	
22	Cellular processes	10 kDa chaperonin	*groES*	bsr7532	*B. japonicum* USDA110	21	360	10,708	6.59	Cytoplasmic	9.44		
23	Cellular processes	10 kDa chaperonin 1	*groES1*	blr5226	*B. japonicum* USDA110	6	81	11,130	6.10	Cytoplasmic	2.43		
27	Cellular processes	Chaperonin GroEL	*groEL*	-	*S. alaskensis* RB2256	27	617	57,917	5.07	Cytoplasmic	-2.12		
1	Conserved hypothetical protein	Hypothetical protein	-	bll5843	*B. japonicum* USDA110	7	314	75,633	4.87	Outer membrane		1.93	
2	Conserved hypothetical protein	Hypothetical protein	-	bll5843	*B. japonicum* USDA110	10	349	51,090	4.97	Extracellular		2.98	
3	Conserved hypothetical protein	Hypothetical protein	-	bll5845	*B. japonicum* USDA110	18	260	51,090	4.97	Extracellular		45.63	
5	Conserved hypothetical protein	Hypothetical protein	-	bll5843	*B. japonicum* USDA110	15	330	51,090	4.97	Extracellular		1.06	
13	Conserved hypothetical protein	Hypothetical protein	-	bll2431	*B. japonicum* USDA110	7	216	16,778	7.85	Unknown		2.84	
16	Conserved hypothetical protein	Hypothetical protein	-	blr0205	*B. japonicum* USDA110	31	898	34,868	6.30	Periplasmic			1.91
21	Conserved hypothetical protein	Hypothetical protein	-	bll6649	*B. japonicum* USDA110	13	223	18,033	6.74	Unknown			6.47
17	Transport and binding proteins	ABC transporter sugar-binding protein	-	blr3208	*B. japonicum* USDA110	27	666	38,378	7.63	Periplasmic			1.51
18	Transport and binding proteins	ABC transporter amino acid-binding protein	-	blr4446	*B. japonicum* USDA110	28	903	36,860	6.21	Periplasmic			2.52

Table 2. Contd.

19	Transport and binding proteins	ABC transporter sugar-binding protein	-	blr3200	*B. japonicum* USDA110	22	492	33,968	7.66	Periplasmic		5.63
20	Transport and binding proteins	ABC transporter substrate-binding protein	-	blr5675	*B. japonicum* USDA110	11	384	40,020	8.95	Periplasmic		1.55
9	Translation	30S ribosomal protein S6	*rpsF*	bll4079	*B. japonicum* USDA110	11	257	18,616	5.46	Cytoplasmic	14.69	
10	Translation	Ribosomal protein L7/L12	-		*R. palustris* BisA53	14	310	12,694	5.02	Periplasmic	4.71	
15	Translation	Elongation factor Tu	*tuf*	bll5402	*B. japonicum* USDA110	27	907	43,569	5.78	Cytoplasmic	2.14	
28	Translation	Translation elongation factor Tu	-		*S. alaskensis* RB2256	20	594	43,040	5.11	Cytoplasmic	-3.12	
4	Energy metabolism	ATP synthase subunit beta	*atpD*	bll0440	*B. japonicum* USDA110	20	712	50,987	5.13	Cytoplasmic membrane	2.08	
7	Regulatory functions	Two-component response regulator	*tcsR*	blr1194	*B. japonicum* USDA110	5	310	23,989	5.07	Cytoplasmic	1.19	
12	Interconversions and salvage of nucleosides and nucleotides	Nucleoside diphosphate kinase	*ndk*	blr4119	*B. japonicum* USDA110	11	277	15,050	6.75	Cytoplasmic	3.61	
8	Other categories	Oxidoreductase	-	blr2928	*B. japonicum* USDA110	7	357	31,520	6.92	Unknown	1.09	
11	Other categories	Peroxiredoxin	-	bll1317	*B. japonicum* USDA110	28	626	17,414	6.11	Unknown	4.98	
24	-	Peptidoglycan-associated protein	-	ZMO 1354	*Z. mobilis*	2	76	27,353	6.92	Outer membrane	-6.02	
25	-	Unknown protein	-	-	*Z. mobilis* subsp. *Mobilis* ZM4	14	562	68,508	4.81	Cytoplasmic		-3.08
26	-	Ribosomal protein S1	-	-	*Sphingomonas* sp. SKA58	7	245	61,616	5.01	Cytoplasmic		-2.92

Table 2. Continued.

29	-	Hypothetical protein	-	-	*P. marinus* subsp. *pastoris*	4	54	17,200	9.12	Unknown	-4.42

[a]Genes Mascot code obtained from rhizobase (http://genome.kazusa.or.jp/rhizobase/Bradyrhizobium). [b]Locus name obtained from rhizobase (http://genome.kazusa.or.jp/rhizobase/Bradyrhizobium). [c]Functional category obtained from rhizobase (http://genome.kazusa.or.jp/rhizobase/Bradyrhizobium). [d]Peptide matched from Mascot search (http://www.matrixscience.com). [e]Mascot score obtained from Mascot search (http://www.matrixscience.com). [f]MW obtained from search (http://www.matrixscience.com). [g]PI value obtained from mascot search (http://www.matrixscience.com). [h]Predicted localization obtained from PSORTb version 3.0.0 (http://www.psort.org/psortb/). [i]Fold change compared with pH 6.8

Note that the proteins in cellular processes were also up-regulated in cell at pH 5.5A. Transport and binding proteins were absent in pH 4.5A condition.

DISCUSSION

Bradyrhizobium sp. generally tolerates to mild acid condition. *B. japonicum* USDA110 was able to grow in HM medium at pH 4.7 (Puranamaneewiwat et al., 2006). In this study, the growth rate of *Bradyrhizobium* sp. DASA01007 at pH 4.5 was better than *B. japonicum* USDA110 in HM medium might be the effect of calcium, one of HM medium components might play the key role in promoting cell growth in acid condition. Maccio et al. (2002) found that peanut *Bradyrhizobium* sp. has better growing in calcium-added medium and increasing concentration of calcium significantly improved the growth under acid condition. The role of calcium has been suggested in maintaining cell envelop stability, specifically in the LPS structure and the expression of outer membrane protein (Ballen et al., 1998).

The growth of *Bradyrhizobium* under low pH condition would benefits the symbiotic efficiency and growth of soybean grown in acid soil. Although the optimum pH for soybean growth is 5.6 to 7.0, soybean could grow in wide range of acid soil condition (pH 4.9 to 6.0); however, with decline of growth and yield (Fageria and Baligar, 1999). Soybean variety Chiang Mai 60 used in this study could not grow well under pH 4.5. Thus, we selected pH in range of 4.5 to 7.0 for this study. Inoculation of *Bradyrhizobium* sp. DASA01007 could significantly promote plant dry weight and nitrogen fixation ability of soybean grown under acid condition when compared with plant inoculated with *B. japonicum* USDA110 or uninoculation. This result revealed the potential of using *Bradyrhizobium* sp. DASA01007 as inoculation for soybean grown under mild acid condition. *Bradyrhizobium* sp. DASA01007 tends to perform better symbiosis than *B. japonicum* USDA110 (Table 1). It would be possible that *Bradyrhizobium* sp. DASA01007 could tolerate in acid condition or have adaptive acid tolerance response that allows cells to survive in acid condition and finally lead to successful symbiosis with plant.

From this study, some proteins produced from *Bradyrhizobium* sp. DASA01007 under acid adaptive condition, pH 4.5A may play an important role in acid tolerance. The 10 kDa chaperonin was 20.03-fold up-regulated in pH 4.5A condition. The chaperonin molecules are required for correct folding and assembly of some proteins during normal cell growth. These proteins were induced by several stress conditions for stabilization of the disassembled polypeptides (Schmidt et al., 1992). The 10 kDa chaperonin or GroES protein exists as a ring-shaped oligomer with 6 to 8 identical subunits, which interact with chaperonin 60 kDa or GroEL as a co-chaperonin to assist the function of chaperonin in active state (Lund, 2009). GroEL was also up-regulated in cells grown at acid pH under both non-adaptive and adaptive conditions. It interacts with a wide range of unfolded proteins. Interestingly, GroEL protein is involved in *nif* gene regulation in *B. japonicum*.

It was proposed that one or more of chaperonin proteins assembled with nitrogenase and assisting the proper folding of nitrogenase complex, and finally link to nodulation and nitrogen fixation efficiency of bacteria (Lund, 2009). This model could also be linked to plant experiment inoculated with *Bradyrhizobium* sp. DASA01007 in this study in which up-regulation of chaperonin in the cell may results in assisting the proper protein folding of nitrogenase component. However, this model needs to be clarified since the specificity between chaperonin and nitrogenase protein may affect the protein folding and its function (Fischer et al., 1999).

The 30S ribosomal protein S6 was 14.69-fold up-regulated at pH 4.5A. This was incorporated with S18 to 16S ribosomal RNA during translation process (Wilson and Nierhuas, 2005). However, the 30S ribosomal protein S6 has been identified as a cold shock protein in *Escherichia coli* and *Bacillus subtilis*, suggesting this protein may play a unique role in sensing temperature differences to control ribosome function (Otani et al., 2001). It is possible that the up-regulation of this protein in *Bradyrhizobium* sp. DASA01007 may also play a role in sensing pH differences and controlling

some protein synthesize under acid stress condition. Another highly expressed ribosomal protein under pH 4.5A was ribosomal protein L7/L12 (spot no.10), which showed 4.71-folds up-regulated in this condition. This protein forms a functionally important domain in the ribosome and involved in interaction with translation factors during protein biosynthesis (Gudkov, 1997).

Peroxiredoxin is antioxidant enzyme that control cytokine-induced peroxide levels which mediate signal transduction in mammalian cells (Wood et al., 2003). It was a thiol-specific antioxidants which detoxify hydrogen peroxide, alkyl hydroperoxides, and peroxynitrite. However, peroxiredoxin has been reported to be strongly induced during symbiosis with common bean and involved in the defense of *Rhizobium etli* bacteroids against oxidative or hydrogen peroxide stress (Dombrecht et al., 2005). Therefore, it is interesting due to this protein was 4.98-fold up-regulated at pH 4.5A in free living of *Bradyrhizobium* sp. DASA01007. It could be possible that peroxiredoxin may have other roles in protecting cell against acid stress condition, since a lowered pH favors the generation of radicals by the Fenton reaction (Sauviac et al., 2007). However, the mechanism is unclear.

On the other hand, the up-regulated proteins in cells grown at pH 4.5N were different from those in pH 4.5A. It revealed the different cell mechanisms used for regular acid tolerance and ATR. In pH 4.5N, the proteins involved in transporting and binding protein were up-regulated. This group of proteins may play an important role in exchanging and accumulating the nutrient or compatible solutes that are necessary for cell survival under stress condition (Sarma and Emerich, 2005). However, the elongation factor Tu (EF-Tu) was also up-regulated in both pH 4.5N and 4.5A.

The EF-Tu is the protein involved in binding and transporting codon-specified aminoacyl-tRNA to aminoacyl site of the ribosome. This protein also has chaperone-like function that interacts with denatured proteins for protein renaturation after stress (Nomura et al., 2010). Nevertheless, some proteins were down-regulated when grown at strong acid condition. However, it should be noted that these down-regulated proteins were matched with proteins present in other bacteria, not in *Bradyrhizobium*. These results imply that all up-regulated proteins detected in this research may be involved in acid tolerance or ATR.

This research revealed the presence of ATR in soybean *Bradyrhizobium*. Development of ATR in *Bradyrhizobium* sp. DASA01007 could promote cell growth under acid condition, which is useful for agricultural applications. The strategy of developing ATR in bradyrhizobia by growing cell in mild acid condition before exposure to extreme acid soil would increase the acid tolerance and symbiotic efficiency of inoculant. Moreover, this research also revealed several proteins of soybean *Bradyrhizobium* that responded during growth under acid conditions.

Different groups of protein were up-regulated in cell grown under adaptive and non-adaptive to acid conditions. Thus, it was indicated that the mechanism of ATR is different from the mechanism of regular cell acid tolerance. The protein expression profiles in different acid conditions provided useful information for further study of proteins that have roles in acid tolerance or ATR of *Bradyrhizobium.*

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