INVESTIGATION OF ACID TOLERANT PROTEIN OF SOYBEAN *BRADYRHIZOBIUM*

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การปลูกพืชถั่วเหลืองในพื้นที่ที่เป็นกรดพบว่าไม่สามารถเจริญเติบโตได้ดี ซึ่งสาเหตุเกิดจากเชื้อไรโซเบียมที่ทำหน้าที่สร้างปม และตรึงไนโตรเจนจากอากาศให้กับถั่ว มีความไวต่อสภาวะที่เป็นกรด ดังนั้นการแก้ไขปัญหาการปลูกพืชตระกูลถั่วในพื้นที่ที่มีสภาวะที่เป็นกรด คือการคัดเลือกเชื้อไรโซเบียมที่มีความสามารถในการเจริญเติบโต และตรึงไนโตรเจนได้ดีในสภาวะดังกล่าว โดยในการศึกษาครั้งนี้ได้ทำการคัดเลือกไรโซเบียมสายพันธุ์ DASA01007 โดยใช้ความสามารถในการเจริญเติบโตในสภาวะที่เป็นกรด และประสิทธิภาพในการตรึงไนโตรเจนเป็นเกณฑ์ เพื่อให้เข้าใจกลไกของเชื้อที่สามารถปรับตัวให้เจริญในสภาวะที่เป็นกรดได้ จึงได้ทำการศึกษาโดยใช้วิธีการวิเคราะห์การแสดงออกของโปรตีนโดยรวม (proteomic) ผลการทดลองพบโปรตีนทั้งหมด 29 ชนิด จากการแบ่งกลุ่มตามกลุ่มโปรตีนที่เป็นออร์โทโลกัสกัน สามารถแบ่งออกเป็น 8 กลุ่ม และไม่สามารถแบ่งกลุ่มได้ 1 กลุ่ม โดยโปรตีนที่มีระดับการแสดงออกที่สูงขึ้นในสายพันธุ์ DASA01007 ที่มีการเจริญแบบปรับตัวที่ระดับความเป็นกรด-ด่าง 4.5 (pH 4.5A) ประกอบด้วยโปรตีนที่เกี่ยวกับกระบวนการระดับเซลล์ (cellular processes) (60 kDA chaperonin 6 และ 10 kDa chaperonin), conserved hypothetical protein,โปรตีนที่เกี่ยวกับกระบวนการแปลรหัส (30 ribosomal protein S6), โปรตีนที่เกี่ยวกับกระบวนการเมทาบอลิซึม (ATP synthaes subunit beta), โปรตีนที่มีหน้าที่เกี่ยวกับกระบวนการควบคุม (Two-component response regulator, *tcs*R), กลุ่ม interconversions และ salvage protein ของ นิวคลีโอไซด์, และ นิวคลีโอไทด์ (nucleoside diphosphate kinase, *ndk*) ส่วนโปรตีนที่ไม่พบในสายพันธุ์ DASA01007 ที่มีการเจริญแบบปรับตัวที่ระดับความเป็นกรด-ด่าง 4.5 (pH 4.5A) ประกอบด้วยโปรตีนที่เกี่ยวข้องกับการลำเลียง และขนส่งโปรตีนที่มีระดับการแสดงออกที่สูงขึ้นในสายพันธุ์ DASA01007 ที่มีการเจริญแบบปกติที่ระดับความเป็นกรด-ด่าง 4.5 (pH 4.5N) ประกอบด้วย hypothetical protein,โปรตีนที่เกี่ยวกับการลำเลียงและขนส่ง (ABC transporter sugar-binding protein, ABC transporter amino acid-binding protein) และโปรตีนที่เกี่ยวกับกระบวนการแปลรหัส (elongation factor Tu) ส่วนโปรตีนที่มีระดับการแสดงออกที่สูงขึ้นในสายพันธุ์ DASA01007 ที่มีการเจริญแบบปรับตัวที่ระดับความเป็นกรด-ด่าง 5.5 (pH 5.5A) ประกอบด้วยโปรตีนที่เกี่ยวกับกระบวนการระดับเซลล์ (cellular processes) (10 kDa chaperonin และ10 kDa chaperonin1) ส่วนโปรตีนที่มีระดับการแสดงออกที่สูงขึ้นในสายพันธุ์ DASA01007 ที่มีการเจริญแบบสภาวะปกติที่ระดับความเป็นกรด-ด่าง 6.8 นั้นได้แก่โปรตีนที่

เกี่ยวกับกระบวนการระดับเซลล์ (60 kDa chapoeronin 1 และ 60 kDa chaperonin 6) และโปรตีนที่เกี่ยวกับการแปลรหัส (elongation factor Tu) นอกจากนี้ได้ทำการคัดเลือกยีนที่คาดว่าเกี่ยวข้องกับโปรตีนที่ตอบสนองในสภาวะกรด ได้แก่ *bll*5845, *bll*1317, *blr*5625 และ *bsr*7532 เพื่อโคลนเข้าไปใน *Escherichia coli* สายพันธุ์ DH5α จากผลการทดลองพบว่า *E. coli* ที่ผ่านการโคลนด้วยยีนที่คัดเลือกมาดังกล่าว สามารถเจริญเติบโตได้ดีกว่าสายพันธุ์ปกติเมื่อเลี้ยงที่ระดับความเป็นกรด-ด่างที่ 4.0 ดังนั้นอาจสรุปได้ว่ายีนที่นำมาศึกษาเกี่ยวข้องกับความสามารถในการเจริญในสภาวะที่เป็นกรด

สาขาวิชาเทคโนโลยีชีวภาพ
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MONCHAI MANASSILA : INVESTIGATION OF ACID TOLERANT PROTEIN OF SOYBEAN *BRADYRHIZOBIUM*. THESIS ADVISOR : ASSOC. PROF. NEUNG TEAUMROONG, Dr. rer. nat. 133 PP.

ACID TOLERANCE/ADAPTIVE RESPONSE/*BRADYRHIZOBIUM*/ACID RESPONSE

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. *Bradyrhizobium* sp. DASA01007 was selected on the basis of acid tolerant and symbiosis efficiency. To understand the acid tolerant strain, proteomic analysis was used in this study. The 29 identified proteins were grouped into 8 categories based on Category Orthologous Group (COG) and one group of unknown categories: Proteins produced from pH 4.5A (adaptive) consisting of proteins in cellular processes (60 kDA chaperonin 6 and 10 kDa chaperonin), conserved hypothetical protein, translation (30 ribosomal protein S6), energy metabolism (ATP synthaes subunit beta), regulatory functions (Two-component response regulator, *tcs*R), and interconversions and salvage of nucleosides and nucleotides (nucleoside diphosphate kinase, *ndk*) group were up-regulated. Transport and binding proteins were absent in pH 4.5A (adaptive) condition. These proteins including, hypothetical protein, transport and binding proteins (ABC transporter sugar-binding protein and ABC transporter amino acid-binding protein), and translation protein (elongation factor Tu) were up-regulated at pH 4.5N (normal). At pH 5.5A (adaptive), proteins up-regulated in cellular processes (10 kDa chaperonin

and 10 kDa chaperonin 1) were detected. Finally, in normal condition (pH 6.8), the up-regulated proteins, such as cellular processes (60 kDa chapoeronin 1, 60 kDa chaperonin 6) and translation protein (elongation factor Tu) were found. The 4 putative genes derived from identified proteins (*bll*5845, *bll*1317, *blr*5625 and *bsr*7532) were cloned into *Escherichia coli* DH5α. Four *E. coli* transformants generated higher growth in acid condition at pH 4.0 than the wild type. This implied that these genes might be involved in acid tolerance.

School of Biotechnology

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LIST OF ABBREVIATIONS

bp	=	base pair
°C	=	degree celcius
COG	=	category orthologous group
dNTP	=	deoxynucleotide 5’ triphosphate
et al.	=	Et alia (and other)
g	=	gram
h	=	hour
kDa	=	kilodalton
kV	=	kilovolt
LC-MS/MS	=	liquid chromatography mass spectrometry
l	=	liter
M	=	mole
mA	=	milliamp
mg	=	milligram
min	=	minute
ml	=	millilitre
mM	=	millimole
ng	=	nanogram
nm	=	nanometer
PCR	=	Polymerase Chain Reaction

LIST OF ABBREVIATIONS (Continued)

PHBA	=	polymeric beta-hydroxybutyric acid
pmol	=	picomol
ppm	=	parts per million
RNA	=	ribonucleic acid
rRNA	=	ribosomal RNA
rpm	=	revolution per minute
µg	=	microgram
µl	=	microlitre
UV	=	ultraviolet
V	=	volt
W	=	watt
W/V	=	weigth per volume

CHAPTER I

INTRODUCTION

Soil acidity is a major problem to agricultural productivity in many areas of the world. Legume pastures and grains crops often perform poorly on acid-soil because of the acid sensitivity of their associated root nodule bacteria (Lie, 1981). Soil acidity is a complex problem due in part to hydrogen ion concentration and especially as pH drops below pH 5, to toxicities of aluminium and manganese, and limited availability of calcium, phosphorus and molybdenum (Coventry et al., 1989). Acid tolerance in rhizobia has been considered as a key phenotypic characteristic in that it enables the bacteria to perform well under the otherwise restrictive conditions of excessive acidity (Howieson et al., 1988). The screening for acid-tolerant isolates that can colonize and/or persist in acidic soils thus gave rise to novel strains with enhanced survival and/or symbiosis under moderately acid conditions. The fixed nitrogen produced by this bacterium is subsequently assimilated by the host, and improves plant growth and productivity. Overall, N_2 fixation supplied ~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. This is a continuing need for the identification of acid tolerant inoculum strains of rhizobia for increasing yields of legume crops on acid soil or in environments in which acidification is a problem (Watkin et al., 2003). The survival or growth of bacteria can be adversely affected by low pH and cells need to adapt to a changing environment to

survive. 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). An understanding of the response of bacteria to low pH is particularly relevant to interactions between bacteria and their host plant. Bacteria of medical importance must survive harsh acidic environments such as those encountered in the stomach or macrophage phagolysosome while agriculturally important bacteria like root nodule bacteria must first survive in acidic soil before they can successfully invade legume roots (Munns, 1986). 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 (Booth, 1985), DNA repair (Foster, 1995), ion cycling (Booth, 1985), and pH amelio ration (Stim and Bennett, 1993). Among the stress-inducible genetic system is the acid habituation or acid-tolerance response (ATR). This is an important response that enables cell exposed to mildly acid pH to cope more effectively with subsequent more low-pH conditions than cells previously grown at neutral pH. Several genes involved in acid tolerance in *Sinorhizobium meliloti* WSM419 were identified by Tn 5 mutagenesis. The process by which this strain senses and/or responds to low pH appears to involve a signal transuduction system (Acts, histidine protein kinase ‘sensor’, and ActR, its cognate regulator). By analogy to the enteric organism, the existence of an ATR system in root nodute bacteria (O'Hara and Glenn, 1994) suggested that synthesis of new protein

induced under acidic condition. Fenner et al., 2004 reported that ActS/ActR system is essential for acid tolerance in *S. medicae* and ActS is involved in responses to both microaerobic and acidic conditions. The isolation, sequencing and characterization of the RegBA homologs ActS and ActR from the root nodule microsymbiont *S. medicae*, both of which are essential for growth at low pH. 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* spp. is a multigenic phenotype in which the genetic determinants appear to be associated with diverse cellular functions. *B. japonicum*, the nitrogen-fixing symbiont of soybean, is among the most agriculturally important plant-associated bacteria. *B. japonicum* is soil bacterium that has ability to infect the roots of soybean and enter into a symbiotic, nitrogen-fixing association with the host plant. However, detail of genes controlling diverse cellular functions of *B. japonicum* in acid condition was not clearly investigated.

According to the bradyrhizobial strains isolated from soil in Thailand, it was found few number of isolates enable to survive under low pH (4.5) condition. Thus, in order to enhance inocula of bradyrhizobial cell survival in acidic soil, ATR might be an alternative approach. The present research aims also to observe the genes in which response to low pH condition.

Research objectives

1. To compare whether cultivation of *B. japonicum* based on acid-tolerance response (ATR) promote better symbiotic response with soybean or not.
2. To determine protein production, genes expression and relate genes in low pH condition.
3. To identify the genes which response to low pH condition in acid tolerance *B. japonicum*.

CHAPTER II

REVIEW OF THE LITERATURE

2.1 Characteristics of the rhizobia

Rhizobia or root nodule bacteria are middle size, rod shaped cell, 0.5-0.9 μm in width and 1.2-3.0 μm in length. They do not from endospores, are gram-negative, and are mobiled by a single polar flagellum or two to six peritrichous flagella. Uneven gram staining is frequently encountered with rhizobia, depending on the age of the culture. Cell from a young culture and nodule bacteroids usually show even gram stianing while older and longer cell give a banded appearance with unstained area. These unstained areas have been identified to be large granules of polymeric beta-hydroxybutyric acid (PHBA). The PHBA is refractile under phase contrast microscopy. Rhizobia are predominantly aerobic chemoorganotrophs and are relatively easy to culture. They grow well in the presence of O_2 and utilize relatively simple carbohydrates and amino compounds. With the exception of a few strains, they have not been fix N in the free-living from except under special conditions. Rhizobia can combine nitrogen gas from air to nitrogenous compound that plant can utilize as a direct nitrogen source. The enzyme nitrogenase is a complex of two enzymes, an Fe-containing protein and an Fe-Mo protein. It is responsible for the conversion (reduction) of atmospheric N into NH_4^+, and is synthesized in the cytosol of the bacteroids. The legume utilizes NH_4^+ to convert certain precursor metabolites such as α-ketoglutarate, phosphoenolpyruvate into amino acid, which, in turn, are synthesized

into protein. The complex biochemical reaction whereby the inert atmospheric N is enzymatically reduced into a utilizable form for plant by the nitrogenase enzyme complex of the bacteroids is called biological nitrogen fixation. The symbiosis between leguminous plant and rhizobia is a complex interaction. This is a result from an effect between the legume and specific strain of rhizobium and legume are neither productive nor persistence without effective nodulation by appropriate rhizobia (Somasegaran and Hoben, 1994).

Members of genus *Bradyrhizobium* are a symbiotic nitrogen- fixing soil bacterium that has the ability to form nodules on the root or stem of specific leguminous plants. *Bradyrhizobium* species are Gram-negative bacilli (0.5-0.9 x 1.2-3.0 μm) with a single subpolar or polar flagellum. These bacteria are nonspore-forming and aerobic, and possess a respiratory type of metabolism with oxygen as the terminal electron acceptor. They usually contain granules of poly-β-hydroxybutyrate, which are refractile by phase-contrast microscopy. Colonies are circular, opaque, rarely translucent, white, and convex, and tend to be granular in texture. They are slow growing in contrast to *Rhizobium* species, which are considered fast growing rhizobia. In a liquid media broth, it takes *Bradyrhizobium* species 3-5 days to create a moderate turbidity and 6-8 hours to double in population size. They tend to grow best with pentoses as a carbon source. Colonies often do not exceed 1 mm in diameter within 5-7 days incubation on yeast-mannitol-mineral salts agar. They produce an alkaline reaction in mineral salts medium containing mannitol and several other carbohydrates. Growth on carbohydrate media is usually accompanied by the production of extracellular polysaccharide slime. *B. japonicum* is the most agriculturally important species because it has the ability to form root nodules on

soybeans (*Glycine max*). *B. japonicum* USDA110, which was originally isolated from soybean nodule in Florida, USA in 1957, has been widely used for the purpose of molecular genetics, physiology, and ecology, because of its superior characteristics regarding symbiotic nitrogen fixation. The genome of *B. japonicum* USDA110 is a single chromosome 9,105,828 bp in length. The average G+C content of the genome is 64.1 mol %. Fifty-two percent of the 8,317 potential protein-coding genes are like genes of known function, 30% of the genes are hypothetical, and 18% have no similarity to any reported genes. In addition, 34% of the genes were like genes in *M. loti* and *S. meliloti*, and 23% of the genes were unique to *B. japonicum*. The genome structure of *B. japonicum* USDA110 is similar to that of *M. loti* in that many of the genes for symbiotic nitrogen fixation are clustered on the chromosome.

To date, the genus *Bradyrhizobium* consists of 9 species including *B. japonicum* (type species) nodulating *Glycine* (Jordan, 1982), *B. betae* from the roots of *Beta vulgaris* afflicted with tumor-like deformations (Rivas et al., 2004), *B. canariense* from genistoid legumes from the Canary Islands (Vinuesa et al., 2005), *B. elkanii* nodulating *Glycine* (Kuykendall et al., 1992), *B. iriomotense* from a Tumor-Like Root of the *Entada koshunensis* from Iriomote Island (Islam et al., 2008), *B. jicamae* and *B. pachyrhizi* from nodules of *Pachyrhizus erosus* (Ramirez-Bahena et al., 2009), *B. liaoningense* nodulating *Glycine* (Xu et al., 1995), and *B. yuanmingense* nodulating *Lespedeza* (Yao et al., 2002), In addition to the species subdivision, a number of serogroups have been described among slow-growing soybean symbionts. Many other slow-growing rhizobia have been isolated from other legume hosts and are commonly referred to as *Bradyrhizobium sp.*, followed by the name of the legume host. A special feature of the *Bradyrhizobium*–legume symbiosis is that

some strains of *Bradyrhizobium sp.*, such as BTai1 and ORS278 can form stem nodules on some plant species, produce bacteriochlorophyll and perform photosynthesis (Molouba et al., 1999). In addition, photosynthetic *Bradyrhizobium* strains have also been reported as endophytes of African wild rice (Chaintreuil et al., 2000).

2.2 Soybean

Soybeans (*Glycine max*) are an important global crop, providing oil and protein. Soybean was considered for its use in crop rotation as a method of fixing nitrogen. Soybean varies in growth and habit. The height of the plant varies from below 20 cm up to 2 m. The pods, stems, and leaves are covered with fine brown or gray hairs. The leaves are trifoliolate, having 3 to 4 leaflets per leaf, and the leaflets are 6-15 cm long and 2-7 cm broad. The leaves fall before the seeds are mature. The fruit is a hairy pod that grows in clusters of 3-5, each pod is 3-8 cm long, usually contains 2-4 (rarely more) seeds and 5-11 mm in diameter. Soybeans occur in various sizes, and in many hull or seed coat colors, including black, brown, blue, yellow, green and mottled.

The genus *Glycine* are divided into two subgenera, *Glycine* and *Soja*. The subgenus *Soja* (Moench) F.J. Herm, includes the cultivated soybean, *Glycine max* (L.) Merr., and the wild soybean, *G. soja* Sieb. & Zucc. Both species are annual. *G. soja* is the wild ancestor of *G. max* and grows wild in China, Japan, Korea, Taiwan and Russia. The subgenus *Glycine* consists of at least 16 wild perennial species: for example, *G. canescens* F.J. Herm. and *G. tomentella* Hayata, both found in Australia and Papua New Guinea (Newell and Hymowitz, 1983).

Cultivation is successful in climates with hot summer, with optimum growing conditions in mean temperatures of 20 to 30 C; temperatures of below 20 °C and over 40 °C retard growth significantly. They can grow in a wide range of soils, with optimum growth in moist alluvial soils with a good organic content. Soybeans, like most legumes, perform nitrogen fixation by establishing a symbiotic relationship with the bacterium *Bradyrhizobium japonicum* (syn. *Rhizobium japonicum*; (Jordan, 1982). However, for best results an inoculum of the correct strain of bacteria should be mixed with the soybean (or any legume) seed before planting. Modern crop cultivars generally reach a height of around 1 m, and take 80–120 days from sowing to harvesting. The soybean planted area has expanded to the lower north, northeast and central plain of Thailand. The planted area reached of 815,940 rai in 2007 and decreased to about 752,668 and 758, 041 rai in 2008 and 2009, respectively (http://www.oae.go.th/download/article/2553). Due to the increasing of planted area of the second rice (irrigated crop) in dry season and sugarcane in rainy season, the total product of soybean is get along with the planted area about 201,291 ton in 2007 and decreased to 186,598 and 190,480 ton in year 2008 and 2009, respectively. Although the planted area and the total product was decreased, the national productivity of soybean was increased from 247 kg/rai in 2007 to 248 and 249 kg/rai in 2008 and 2009, respectively (http://www.oae.go.th/download/article/2553). This was probably associated with the release of high yielding varieties combined with appropriate management techniques. At present, the popular varieties are SJ5, Chiang Mai 60, SJ4, Sukhothai 2 and Sukhothai 1, respectively.

2.3 Biological Nitrogen Fixation (BNF)

Nitrogen is commonly the most limiting element in agricultural production and or the most of expensive to purchase as fertilizer. There is an abundant supply form air (78% air is nitrogen gas, amounting to about 8,000 pounds nitrogen in the air over every area at land). However, the nitrogen in air is a stable gas normally unaviable to plant. Biological Nitrogen Fixation (BNF) involves the enzymatic reduction of nitrogen gas to ammonia. The ammonia produced then can be incorporated by enzymatic for the growth and maintenance of the cell. BNF is unique to bacteria, animal and plants that fix N_2 must be do in associate with bacteria (Stacey et al., 1992). Many leguminous plants are able to utilize this atmospheric nitrogen through an association with rhizobia, bacteria that are hosted by the root system of certain nitrogen fixing plants. This self sufficiency, with many free the plant from the need amendment with manufacture N fertilizers, is achieved by symbiotic fixation of inert gas from atmospheric into NH_4^+ in the soil that is used in amino acid and protein synthesis. Nitrogen reduction is a very complex mechanism not as yet fully elucidated. The result of net reduction of molecular nitrogen to ammonia is generally accounted for by the following equation.

$$\mathbf{N_2 + 8H^+ + 8e^- + 16\ MgATP \xrightarrow{Nitrogenase} 2NH_3 + H_2 + 16\ MgADP + 16Pi}$$

The components of N_2-fixation process were nitrogenase enzyme and leghemoglobin, in nodules of legumes, nitrogenase synthesis normally follow very shortly after bacteria are released from infection threads. The enzyme nitrogenase is comprised of two easily separable proteins designated the iron (Fe) protein component II and the molybdenum-iron (MoFe) protein or component I. The MoFe

protein is a tetramer ($\alpha 2\beta 2$) of 220 kD Molecular mass. The MoFe protein contain 2 atoms of Mo and 24 to 32 atoms of Fe and S per molecule. The role of MoFe protein is to transfer electrons to N_2 and H^+. The Fe-protein is a homodimer. The Fe-protein has two Mg-ATP binding site and as ATP binds to these sites, the potential of electron at the (4Fe-4S) clster is reduced, allowing to Fe-protein to donate electrons to the MoFe protein (Vance et al., 1998). It is responsible for conversion (reduction) the atmospheric N to NH_4^+ and synthesized in the bacteroid cytosol. The assimilation of N_2 fixed in the bacteroids is exported through the inner and outer membrane of the microsymbiont and through the peribacteroid membrane into host plant cytosol. The nitrogenase enzyme complex is rapidly and irreversibly denatured by O_2. Thus the enzyme is functional only in low O_2 environments. The important contributor to solving the O_2 dilemma is leghemoglobin an O_2 binding protein found within nodule infected cells. This plant protein which is very similar to animal hemoglobin gives nodules their pink color. Leghemoglobinfaccilitates the diffusion of available O_2 though the plant cell cytoplasm to bacterial cells at concentrations which allow oxidative phosphorylation to occur without inactivation of nitrogenase activity (Vance et al., 1998).

2.4 Acid soil

Soil acidity is a major growth-limiting factor for plant in many parts of the world. Acid soil toxicity is not a single factor but a complex of factors that may affect the growth plants through different physiological and biochemical pathways. The specific causes of poor plant growth on acid soils may vary with soil pH, clay mineral types and amounts, organic matter contents and kinds, levels of salts, and particularly, with plant species or

genotypes (Clark, 1982). Growth-limiting factors that have been associated with the acid soil infertility complex include toxicities of Al^{3+}, Mn^{2+} and other metal ions, low pH (H^+ toxicity), and deficiencies or unavailabilities of certain essential element, particularly Ca, Mg, P and Mo. Acid soil factors may act somewhat independently, or more often together, to affect the growth of higher plants (Foy and Fleming, 1978).

The direct effects of the H^+ ion on plant growth are difficult to determine in acid soils, because at soil pH levels where it is considered harmful, Al, Mn and other mineral elements may also be soluble in toxic concentrations, and the availabilities of essential elements, particularly Ca, Mg, P and Mo may be suboptimal. In most acid soils (pH > 4), Al^{3+} and Mn^{2+} toxicities more important than H^+ ion toxicity in limiting the growth to higher plants. However, H^+ ion toxicity may restrict the survival and activity of rhizobia or other soil microorganisms (Kamprath and Foy, 1985). The effects of the H^+ ion are confouned with other factors in acid soils. In general, the root of plants damage by low pH (<4.0) are short, thickened, fewer in number and discolored brown or dull gray (Islam et al., 1980). Excess H^+ ions have marked effects on root membrane permeability. Roots may lose previously absorbed cations as well as organic substances, and prolonged exposure to low pH may reduce their capacities for subsequent absorption of nutrients. Its effect on nutrient uptake and retention by plant roots, the H^+ ion can increase plant requirement for Ca and perhaps other nutrient in growth medium. For example, Lund (1970) found that soybean taproots growing in the nutrient solution portion of a split medium required higher Ca levels at pH 4.5 than pH 5.6.

2.5 Effect of acid soil on rhizobia-host plant

Soil acidity effects on legume nodulation include reduced survival and growth of rhizobia in soil and seed, reduced attachment and root-hair infection and poor plant growth. Actually, acid pH has a much effect on the fast growing rhizobia than bradyrhizobia. (Bryan, 1923) reported that alfalfa bacteria were killed at a soil pH of 5.0 those of red clover at pH 4.5 to 4.7 and those of soybean at pH 3.5 to 3.9. Nodules formed at any pH where plants grew, but their numbers were greatest at a pH near 7.0. Doolas (1930) found that soybean rhizobia remained viable in soil at pH 4.8 to 8.3. Soybean roots showed depressed nodulation within the pH range of 3.8 to 4.6 but were healthy at pH 5.6. The H^+ ion is particularly important in legumes grown without fertilizer N. It affects rhizobial survival and multiplication in soils, root infection and nodule initiation, legume rhizobial efficiency, and nutrition of host plant. The root infection process is particularly sensitive to acidity, requiring a higher pH than that for rhizobial survival (Munns, 1978). Glycine was also pH sensitive and showed a steady increase in nodulation between pH 4 and 6. Introduction of rhizobial strains with acid tolerance property has been conducted in the central region of Argentina. This area was found become progessively more acidic over the last two decades. The inoculant is applied for alfalfa, however, the response to inoculation with specific rhizobia is strongly by native soil rhizobia that compete with the introduced strains (Broughton et al., 2003). Munns (1978) noted that wide variability in H^+ ion tolerance within both host plant and rhizobial specie provide an opportunity to develop more effective rhizobial host plant combinations but that better screening guidelines are needed for evaluating the effectiveness of such under field conditions.

2.6 Bacterial physiology in acid condition

Enterobacteriaceae have been widely investigated regarding the existence of inducible acid survival mechanisms. Since, they are constantly under assault by a wide array of environmental stresses. One of the most frequently encountered hostile conditions is acid stress. Neutralophiles like *E. coli*, *Samonella typhimuium* or *Shigella flexneri* while travelling through the gastrointestinal tract must endure extreme low pH in the stomach as well as volatile fatty acids present in the intestine and faeces. Acid survival responses in their group of bacterial are contained components of inducible acid tolerance and acid resistance. The lowing of internal pH (pH_i) by proton leak at low outside pH (pH_o) will induce several amino acid decarboxylases, particularly arginine and glutamate decarboxylases. These enzymes are membrane embedded, function best at $pH_i \sim 5.0$, and are important in the buffering of intracellular H^+. This system appear to act as inducible pH homeostasis system, elevating pH_i by consuming a proton during decarboxylation and then exchanging the decarboxylation endproduct for new substrate via membrane bound anti-proton (Bearson et al., 2006). Another acid tolerance mechanism requires induction and is referred to as the ATR (acid tolerance response). The ATR will protect cell below pH4 and requires protein synthesis to be effective. Furthermore, the ATR requires the Mg^{2+}-dependent protein-translocating ATPase (atp) as well as the *fur* gene product, while nonadaptive acid tolerance require neither (Foster and Hall, 1991). In addition, Jonge et. al. (2003) also demonstrated that the amount of proton pumping H^+/ATPase, both in *E. coli* 0157 and *S. typhimurium* strains, was low when grown at pH value less than 6 after growth at pH 7.5. Cyclo fatty acid construct of membranes of bacteria grown at pH value less than 6 was higher than that of membranes of bacteria grown at pH 7.5. In case of *Helicobacter* which colonies the human stomach must facing acidic condition all the time.

Most of *Helicobacter* harbor urease enzyme which able to convert urea to ammonia and CO_2 for buffering both pH in cytoplasm and periplasm. However, the mechanism to survive in acid condition is more complicated than previously discussed. Wen et. al. (2003) demonstrated that about 200 groups were up-regulated at pH 4.5. These genes include pH homeostatic genes were overlap with some genes induced by temperature stress. Thus, *Helicobacter* has evolved multifaceted acid-adaptive mechanisms enabling it to colonize the stomach that may be targets for eliminatory infection.

For rhizobia, several researchers have shown that the cytoplasmic pH of acid-tolerant strains is strongly affected by external acidity. The hight cytoplasmic potassium and glutamate level in acid-stress cells of *R. leguminosarum* bv. fasiloi, a response which is similar to that found somatically stressed cells. Differences in lipopolysaccharide (LPS) composition, proton exclusion and extrusions, accumulation of cellular polyamines and synthesis of acid shock protein have been associated with the growth of cell at acidic pH. Moreover, the composition and structure of the outer membrane as well as nod factor could also be a factor in pH tolerance (Morón et al., 2005; Zahran, 1999).

2.7 Proteomics

Proteomics, an abbreviation of the words protein and genomics (Wilkins et al., 1995), is an advancing branch of molecular biology that deals with the systematic, large-scale analysis of proteins. Proteins play major roles in almost every biological function, so an extensive study of the proteins in the cell contributes a unique global perspective on how these molecules interact and cooperate to create and protect a working biological system. The cell corresponds to internal and external effects by adjusting the level and activity of its proteins, so changes in the proteome, either

qualitative or quantitative, provide a snapshot of the cell in action. The proteome is a complex and dynamic entity that can be defined in terms of the sequence, structure, abundance, localization, modification, interaction and biological function of its components, providing a rich and varied source of data. An equally diverse range of technologies are required for the analysis of these various properties of the proteome (Twyman, 2004). The terms “proteomics’’ and “proteome’’ were first used by Marc Wilkins and colleagues in the early 1990s and reflect the terms “genomics’’ and “genome’’, which describe the entire collection of genes in an organism. These “-omics’’ terms represent a redefinition of how to approach to biology and the workings of living systems (Liebler, 2002) (Figure 2.1).

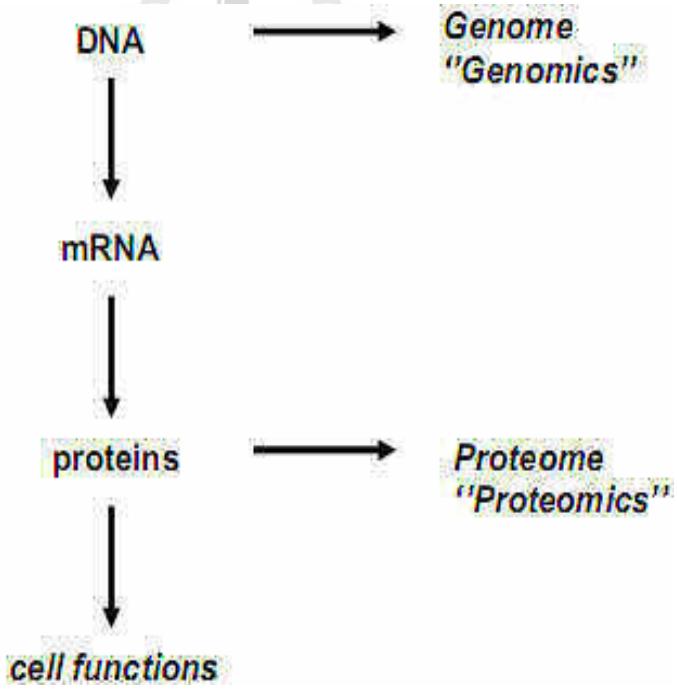

Figure 2.1 Biochemical context of genomics and proteomics (Liebler, 2002).

Proteome analyses are accompanied by two-dimensional gel electrophoresis (2DE) for separation of proteins followed by protein identification by mass spectrometry (MS) and database searches. Due to the introduction of soft ionization methods to mass spectrometry like matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) together with developments in sample preparation techniques and rapid increase of sequential information in databases, mass spectrometry has admitted of protein identification and characterization during the last decade (Ashcroft, 2003).

Proteomics, especially 2DE, has been used from the outset to investigate the bacterial proteome under different growth conditions and various external stress factors (Krueger and Walker, 1984; Young and Neidhardt, 1978). Nevertheless, Fleischmann and colleges (1995) opened a new field for bacterial proteomics by completing first genome sequence of a bacterium, Haemophilus influenzae strain RD KW20. On the basis of a good-elucidated genomic sequence, introduction of a large-scale MS techniques emerged to identify protein spots on a 2 dimensional gel. The complete genomic sequences of around 350 bacteria have been identified, so that one can have the opportunity to choose between a diversity of bacteria for proteomic studies based on scientific interest.

2.8 Two-dimensional gel electrophoresis

2DE, first introduced for protein separation in the early 1970s by O'Farrell (1975), is a powerful and widely used electrophoretic technique that analyzes thousands of individual protein species extracted from cells, tissues, or other biological samples. Spot patterns are formed in 2DE analysis in which every single

spot indicates an individual protein species to its specific coordinates. The intensity of an individual spot implies how much the cell has produced of that actual protein (Bendixen, 2005). This method is actually a combination of two different and independent types of separations. The first dimension is isoelectric focusing (IEF), which separates proteins according to their isoelectric points (pI); the second dimension is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their molecular weights (MW).

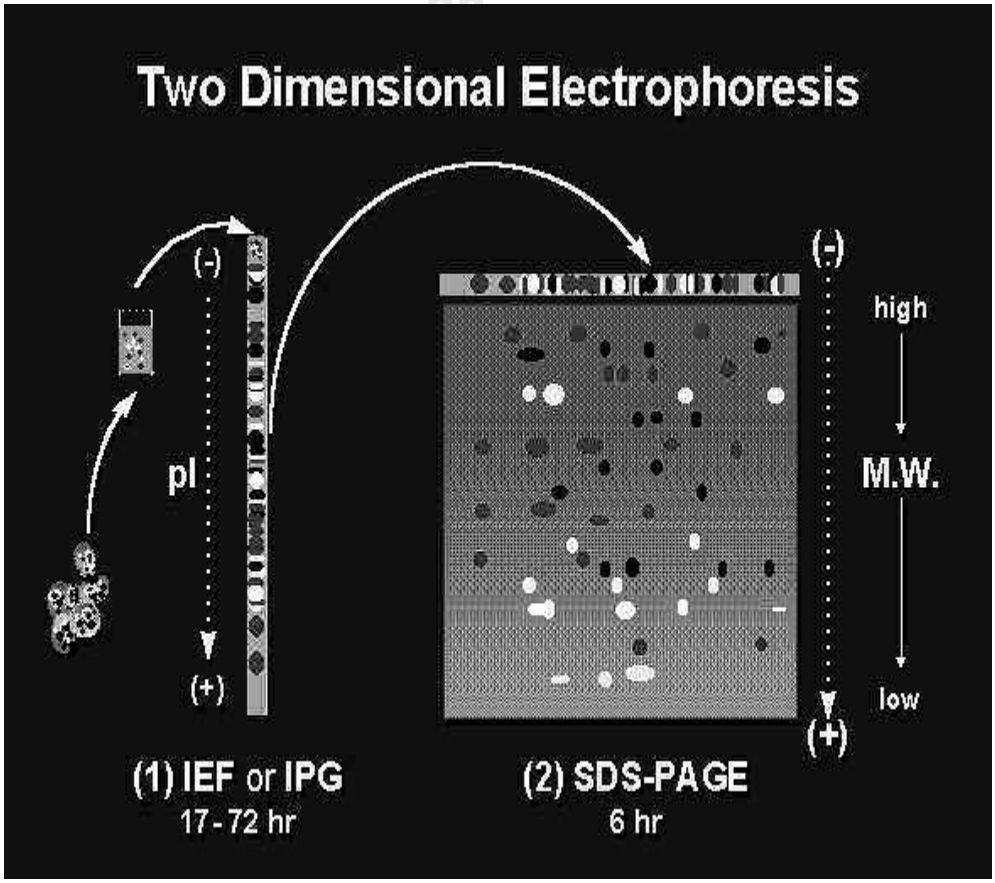

Figure 2.2 A model of two-dimensional gel electrophoresis (resource: http://en.wikibooks.org/wiki/File:2D_electrophoresis.gif).

2.8.1 First dimension: IEF

IEF is an electrophoretic method that distinguishes proteins based on their isoelectric points pI. Proteins are amphoteric molecules (zwitterions); they carry either positive, negative, or zero net charge, depending on the pH of their surroundings. The net charge of a protein is the sum of all the negative and positive charges of its amino acid side chains and amino- and carboxyl-termini. The pH at which a protein has no net charge is called the “isoelectric point’’ or “pI’’ of that IEF-focuses proteins. Equilibrate in SDS and reducing agent to give uniform protein shape, single subunits, uniform negative charge/mass ratio SDS-charged proteins in IPG strip. Apply to SDS-PAGE gel SDS-charged proteins resolved according to size in SDS-PAGE. Proteins are positively charged at pH values below their pI and negatively charged at pH values above their pI.

2.8.2 pH Gradients

The existence of a pH gradient is crucial to the IEF technique. When an electric field is applied, a protein will move to the position in the pH gradient where its net charge is zero. A protein carrying a positive net charge will move toward the cathode, becoming incrementally less positively charged as it moves through the pH gradient until it reaches its pI. A protein with a negative net charge will move toward the anode, becoming less negatively charged until it also reaches zero net charge. If a protein should diffuse away from its pI, it immediately gains charge and migrates back. This behaviour is called focusing effect of IEF, which concentrates proteins at their pIs and lets proteins be separated dependent upon very small charge differences (Berkelman and Stenstedt, 2001).

2.8.3 Second dimension: SDS-PAGE

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, is an electrophoretic technique that separates proteins according to their molecular weights irrespective charge. The main principle underlying this technique is the exposure of denatured proteins to the anionic detergent (SDS) within the polyacrylamide gel, which binds stoichiometrically to the polypeptide backbone and carries a large negative charge. The separation is not influenced by the intrinsic electrical charge of the proteins due to the presence of SDS in the sample and the gel. When in solution, SDS forms spherical micelles composed of 70-80 molecules with the dodecyl hydrocarbon in the core and hydrophilic sulfate groups in the head. When SDS binds to proteins, it creates complexes with a necklace-like structure composed of protein-decorated micelles connected by short flexible polypeptide segments (Ibel et al., 1994). In the consequence of this necklace structure, great amounts of SDS are involved in the SDS-protein complex in a ratio roughly 1.4 g SDS/g protein. The existence of tens or hundreds of SDS molecules on each polypeptide eclipses any intrinsic charge of the proteins themselves so that anionic complexes formed have a constant net negative charge per unit mass. SDS molecules binds stoichiometrically to the proteins, that means the larger the protein, the higher binding capacity of SDS it has. This event brings two significant outcomes assuring molecular weight based-separation only, which are the facts that all SDS-protein 28 complexes have primarily the same charge density, and they sustain relative differences in molecular weights between proteins. As mentioned in one dimensional gel electrophoresis, the gel enables the size-based separation by sieving the proteins as they migrate. This sieving effect is based on the pore size of the gel, which is then dependent on the gel

concentration. Total acrylamide content of the gel is referred to as T%. For the polyacrylamide gels, the monomer is made up of the gelling agent acrylamide and also the cross-linking agent bis-acrylamide. Thus, the pore size becomes also dependent on bis-acrylamide content (C%) (Twyman, 2004). Generally, pore size is controlled by varying the T% between 4 % and 20% while keeping C% constant. So gel conditions can be chosen according to molecular weights of the proteins to be separated and the desired degree of restriction. Gels having high T% (i.e.12%) have small pore sizes making them restrictive and favouring the movement of smaller proteins while little or no movement of larger proteins, and vice versa (Kinter and Sherman, 2000). The molecular weights of the proteins in the sample can be predicted by including, in one of the lanes of the gel, a series of protein markers whose masses are already known. The most frequently used buffer system to run second dimension SDS-PAGE is the tris-glycine electrophoresis buffer system which is described by Laemmli (1970). This buffer system separates proteins at high pH that brings the benefit of minimal protein aggregation and clean separation even at relatively high protein loads. On the other hand, the Laemmli buffer system has disadvantages in terms of a limited gel shelf life.

2.8.4 Protein detection and image acquisition

The last step of 2D-PAGE is the detection of proteins that are separated and planned for in-gel digestion. Visualization of proteins can be commonly achieved by three well known techniques, namely Coomassie brilliant blue-staining (CCB), silver-staining and fluorescent-staining. In general, amount of protein in the sample determines which types of staining method were applied. CBB is an organic

dye used for colouring proteins in polyacrylamide gels. It is commercially available with different labels as G-250, R-250, and R-350 according to detection sensitivity. In spite of various staining procedures, standard staining is usually performed mixing the dye with concentrated acid in ethanol or methanol. So, a colloidal suspension is generated, that develops intensely coloured complexes with proteins. Overnight immersion of the gel in this solution, saturates the gel and stains proteins. CCB is a commonly used protein detection technique due to its ease of use, high compatibility with mass spectrometry, and linear response for most proteins, it lacks the sensitivity for proteomic analysis with a detection limit below 10 ng. Moreover, interpretation of mass spectrometry data can be obstructed in situations where possible modifications of glutamic acid side chains occur by the dye. CBB also does not necessarily offer a successful staining for highly acidic, highly basic, or glycosylated proteins. Another commonly used method is silver staining which is based on reactions known from photography. The basic principle is diffusion of silver ions into the gel at acidic pH, their binding to sulfhydryl and carboxyl groups of amino acid side chains (Rabilloud, 1990), and reduction to metallic form at basic pH. Protein spots are black or brown coloured due to the mediation of silver crystallization by peptide backbone and functional groups of amino acids (Moritz and Meyer, 2003). Silver staining is about 100 times more sensitive than CBB staining (Switzer et al., 1979) giving detection limits for 2D gel protein bands in the range of 0.5-1.0 ng. Despite this, silver staining protocols suffer from the disadvantage of limited compatibility with downstream mass spectrometry since cysteine residues and alkylate-exposed amino groups can be modified by silver stains. It can also be troublesome due to the fact that precise timing is required between staining steps to get reproducible and coherent results.

Recently, a number of fluorescent dyes have been introduced into detection methods. These dyes consist of complexes of an organic compound and a heavy metal component (e.g. ruthenium; Sypro Ruby). Fluorescent staining combines the advantages of high sensitivity and compatibility with mass spectrometry (Berggren et al., 2000; Mackintosh et al., 2003). However, these techniques require a high expense of hardware since fluorescent signal is not visible to human eye directly. Additionally, gels stained with fluorescent dyes can not be stored for long terms because they are temporarily light sensitive (Granvogl et al., 2007). Following to the staining procedure, the abundance of different proteins on a 2D-gel is specified in terms of intensity, size and shape of the related spots. Hence, protein quantitation necessitates the conversion of an analogue gel image into digital data. In this way, objective comparisons of equivalent protein spots on different gels can be performed in order to observe up-regulated and/or down-regulated proteins as well as newly produced proteins. Since human eye is very subjective when judging protein spots, 2D-gels stained with CBB or silver are scanned by a charge-coupled device (CCD) camera or a densitometer. The image from a CCD camera is then analyzed by computer based software tools, such as DECODON Delta2D, Bio-Rad's PDQuest and Image Master Platinum for spot detection, matching and quantitation.

2.8.5 In-gel digestion

The in-gel digestion, a method introduced by Rosenfeld et al (1992), is a critical step of sample preparation for the mass spectrometric analysis of proteins. After visualization of the gel, protein of interest requires to be divided into its peptides for protein identification by mass spectrometry. Because generated peptides

have molecular weights within the mass range of mass spectrometers. Mass spectrometry can still produce measurement errors whose magnitude increases as the length of the peptide chain gets longer. In addition, the fact that protein databases are constructed according to the peptide masses makes peptides preferable against intact proteins. The term “in-gel’’ represents that the protein is processed and digested while contained in the polyacrylamide gel piece. Although several modifications have contributed to increasing peptide yield and improving the quality of MS data, essential steps of the technique have remained unchanged. Basically, in-gel digestion includes cut of protein spots of interest from the gel, its destaining, reduction and alkylation of cysteines, and treatment of proteins with a specific protease. In this manner, the gel matrix is penetrated by the enzyme, which leads to digestion of the protein to peptides. For most proteome studies, trypsin is a universal choice due to its exclusive properties as a protease. Specifically, it cleaves an amino bond on the C-terminal side of lysine and arginine residues. The spacing of these two residues in many proteins provides the generation of the peptide masses that fits the range required for MS analysis (Olsen et al., 2004). Peptide fragments having 6-20 amino acids are optimum for MS analysis and database searches.

2.9 Mass Spectrometry (MS)

MS has been accepted as the most comprehensive and all-purpose analytical technique that measures mass-to-charge ratio (m/z) of molecules and atoms since the early 1900s. It has come into prominence in the fields of chemistry, physics, geology, archaeology, nuclear science, material science, environmental science, forensic science, and petroleum industry. MS major serves the purposes of molecular mass

determination, structure elucidation, quantification at trace levels, and mixture analysis. High resolution mass spectrometry made exact mass measurements possible in 1950s while development of gas chromatography (GC) MS facilitated the analysis of complex mixtures in the following decade (Watson and Biemann, 1964). Previously, the application of mass spectrometry to biology was ambiguous due to the absence of ionization methods applicable to biological compounds. Fortunately, introduction of soft ionization techniques have paved the way for mass analysis of large molecular mass biological compounds so that mass spectrometry has become an essential tool in biological research. More specifically, MS satisfies all basic requirements of proteomic studies, which are a broad dynamic detection range, high throughput and accurate protein identification, absolute protein quantification, the ability to cope with multiple proteins in a single spot, and characterization of post-translational modifications.

As mentioned previously, MS is an analytical technique that can measure the m/z of individual molecules and atoms. The first fundamental step in MS analysis is to convert the neutral analytic molecules into gas-phase ionic species to be able to control their motion. Fragmentation takes place if energies higher than ionization energies are used during ionization process. Then, ions are accelerated through a voltage difference towards a mass analyzer, which separates these molecular ions and their charged fragments based on m/z ratio. Finally, ion currents generated by these mass separated ions are recorded by a convenient detector and monitored in the form of a mass spectrum, a plot of m/z values versus abundance. A high vacuum (10^{-4}-10^{-8} torr) is maintained throughout all components of the system to let ions move freely in space without colliding or interacting with other species.

2.10 Ionization methods

Sample ionization is a keystone of mass spectrometry since a compound needs to be charged and ionized to be analyzed by a mass spectrometer. Ionization sources enable the conversion of neutral molecules into gas phase ionic species so that electric and magnetic fields can be used to exert forces on charged particles in a vacuum for mass analysis. The selection of ionization method is dependent on the nature of the sample investigated. In the case of biomolecules, soft ionization methods which provide mass spectra with less or no fragment ion content are being used. The most common of these methods include ESI and MALDI.

2.10.1 Electrospray Ionization (ESI)

Electrospray ionization (ESI) has become a popular mode of ionization technique for qualitative analysis of a broad variety of compounds in solution. The invention of ESI has given rise to a respectable progress in mass spectrometry. Dole laid the foundations of ESI in 1968 by producing gas phase ions from electrically charged liquid droplets (Dole et al., 1968). The work of Dole then gave inspiration to Yamashita and Fenn for combining ESI with mass spectrometry (Yamashita and Fenn, 1984). The gentile nature of this ionization explains the versatility of ESI-MS combination for the measurements of molecular masses of nonvolatile and thermally unstable compounds. Furthermore, it serves as an excellent interface to couple high performance liquid chromatography (HPLC) with MS for the analysis of complex samples. In ESI process, solution of the sample is first mixed with a continuously flowing suitable solvent in a capillary tube that is a 1:1 mixture of water and an organic solvent (methanol, acetonitrile, or isopropanol). A potential difference of

3-4 kV applied between the capillary and the inlet to the mass spectrometer generates a fine spray of charged droplets forming a cone shape, known as a Taylor cone (Wilm and Mann, 1996), while emerging from the tip of the capillary. A drying gas at the interface, usually nitrogen, helps the evaporation and removal of the solvent from the charged droplets. While the diameter of the charged droplet decreases, the charge density on its surface increases. When surface tension of the droplet and electrostatic repulsion on it are equalized, charged droplet explodes to produce daughter droplets which are exposed to further evaporation. This cycle repeats itself until evaporation is completely finished. In the end, resulting desolvated, gas-phase ions are accelerated through mass analyzer and separated based on m/z ratio and all separated ions are detected with the detector.

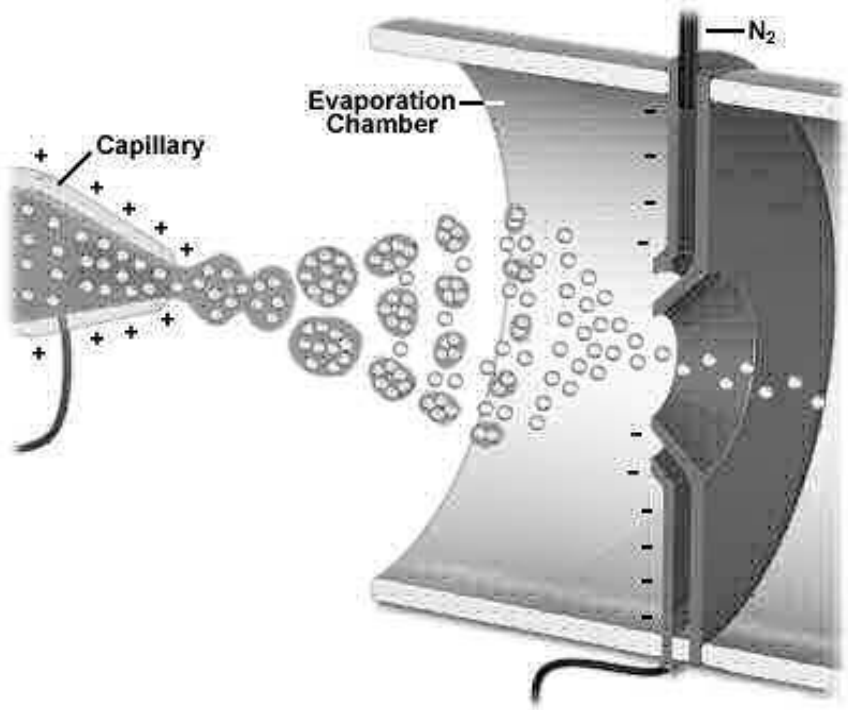

Figure 2.3 ESI process. (resource: http://www .magnet.fsu.edu/education/tutorials/tools/ionization_esi.html).

2.10.2 Matrix-Assisted Laser Desorption/Ionization (MALDI)

MALDI mass spectrometry is an established ionization technique, especially for macromolecules of biological importance. The invention of MALDI was first reported nearly at the same time by two groups, Karas and Hillenkamp (1988) and Tanaka and co-workers (1988). This approach has gained a wide acceptance for analysis of large biopolymers with masses up to 500 kDa. MALDI can also provide characteristics of speed, high sensitivity, ease of use while tolerating small amounts of contaminants like salts and surfactants. An astonishing aspect elevating MALDI to a level where it has become an indispensible technique is mixing of the sample with a "matrix'', a low-molecular weight energy-absorbing organic acid. Matrix is the heart of MALDI process since it serves as laser energy absorbent and an energy transfer agent in order to prevent direct interaction of laser energy with the sample. Principally, the analyze is dispersed in a large excess of matrix material (in the ratio of one-to-several thousands) which can strongly absorb energy at the wavelength of the laser radiation (typically a nitrogen laser at 337 nm). The analyze and matrix is then dissolved in an organic solvent, placed on a metallic probe or sample target, and allowed to dry. Evaporation of the solvent leaves matrix crystals surrounding analyze molecules. After the target is placed in the vacuum chamber of the mass spectrometer, a high energy laser beam is directed at the sample plate with about 106 W/cm ^{2} irradiance power. Most of the laser energy is efficiently absorbed by the matrix crystals causing evaporation of the matrix, and this energy is transferred to the analyze as heat in a controlled manner such that no fragmentation occurs. So analyze molecules are converted into gas-phase ions by gas-phase proton-transfer reactions. Once in the gas phase, analyze ions are then directed electro statically into the mass analyzer.

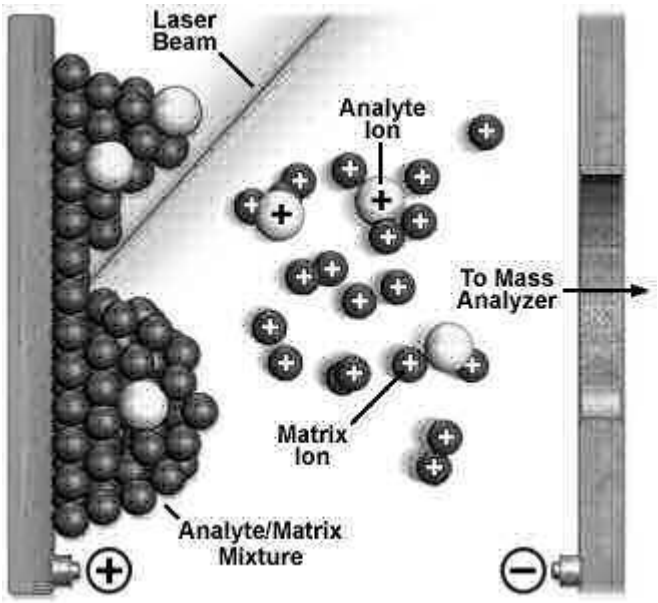

Figure 2.4 MALDI process. (http://www.magnet.fsu.edu/education/tutorials/tools/Ionization_maldi.html).

2.11 Mass analyzers

At the end of ionization process, a mass analyzer is required to separate ions produced at the ion source according to their m/z ratios. Applying suitable electric and magnetic fields, ion motion can be controlled and manipulated in the mass analyzer in order to detect the number of ions at each individual m/z value. The principle of distinguishing the motion of a charged particle relies on kinetic energy, momentum and velocity of the ion so that an analyzer can mass-resolve the ions. Mass analyzers maximize the transmission of all ions entering from ion source and help to focus all mass-resolved ions at a single focal point for the ease of detection. Fundamental instrumental parameters assigning the performance of mass analyzers include mass range, mass resolution, mass accuracy, detection sensitivity, and scan speed as much as design and associated ion optics. For proteomics research, different types of mass

analyzers can be used, such as time-of-flight (TOF), quadrupole ion trap, quadrupole linear, and Fourier transform ion cyclotron resonance (FT-ICR) analyzers. They are quite different in terms of experimental design and performance parameters like resolution, sensitivity, and mass accuracy. Intended for improved overall capability and making the use of different strengths, mass analyzers can be combined in tandem to build hybrid instruments, called tandem mass analyzers (MS/MS) such as quadrupole/quadrupole, magnetic sector/quadrupole, quadrupole/TOF, and ion trap/TOF geometries, etc. Tandem mass analyzers contain three main parts: a first mass analyzer can be used to isolate the ion of interest (precursor ion or parent ion) and second part can be used as a collision cell to fragment the parent (precursor) ions, a collision-induced dissociation device (CID) fragmenting the selected parent ions to form daughter (fragment) ions, and a third part can be used to separate daughter ions according to mass or energy. So an MS/MS spectrum from which structural assignments can be drawn is generated.

2.11.1 Time-of-Flight (TOF)

TOF mass spectrometer, theoretically proposed in 1946 (Stephens, 1946), is a simple type of mass analyzer. By the development of MALDI, TOF has been undergoing a renaissance in the field of biomedical sciences, and being used in coupling with MALDI to handle the pulse of ions. TOF takes the advantage of singly charged peptide ions produced by MALDI to relate the flight time with molecular mass. TOF serves as a kind of velocity analyzer which separates ions based on their different velocities. A short pulse of ions exiting the source is given the same kinetic energy to accelerate them towards the detector; hence, it is obvious that ions carrying

the same charge with different mass will travel through a flight tube at different velocities; thus, it takes different amount of time to travel the same distance. The lighter ions travel down a field-free region faster and reach the detector earlier due to their greater velocity while it takes longer time for heavier ions because of their lower velocity. In this way, flight times of ions required to move through a field-free region between the source and the detector are measured, and packets of isomass ions are quantified. Conversion of time spectrum into a mass spectrum is managed by calibrating the analyzer through measuring the flight times of ions of known mass. So, mass spectrum is acquired. In the past, TOF suffered from the limitation of poor resolution because of the ions' kinetic energy inhomogeneity, which results in recording different arrival times for ions of same mass. This kinetic energy (energy of ion formation) distribution is reduced by use of an energy-correcting device, named as "reflectron''. A retarding field created by the reflectron enables larger pathways for more energetic ions to reach the detector at the same time with less energetic ions of the same mass (Figure 2.5). There exist also spatial (location of ion formation) and temporal (time of ion formation) distributions affecting mass resolution inversely. By a technique known as "delayed extraction'', a time delay is set between ion formation and ion extraction from the source so that spatial and temporal distributions can be prevented. To obtain more detailed structural information for the characterization of proteins, further analysis can be accomplished in reflectron mode MALDI-TOF MS by the production of fragment ions following the ionization, a method called "post-source decay'' (PSD). Applying different voltages, reflectrons can differentiate the fragment and precursor ions of same velocity based on their different kinetic energies. In the end, a very useful mass spectrum of fragment ions is obtained.

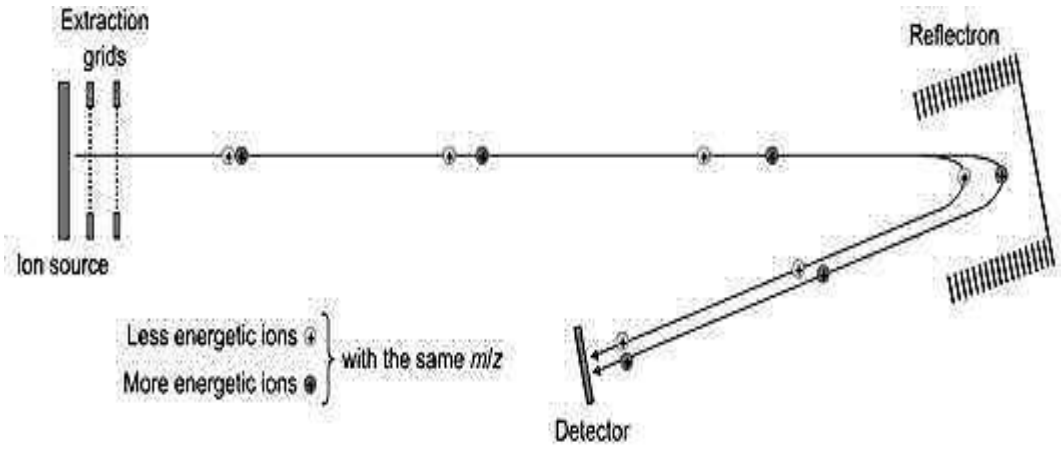

Figure 2.5 A sketch of a reflectron time-of-flight mass analyzer (Lane, 2005).

2.11.2 Quadrupole ion-trap

Quadrupole ion-traps are very compact, versatile and robust mass analyzers, first introduced in the early 1950s (Paul and Steinwedel, 1953). The working principle is first trapping the ions and then detecting them according to their m/z ratios. The trap typically consists of three hyperbolic electrodes, a ring electrode and two end-cap electrodes. By applying DC and RF voltages to the electrodes, ions with a wide m/z range can be trapped within the space between the electrodes. So ions are confined by the RF field and they follow an eight-shaped oscillating trajectory related to their m/z ratio. Helium gas is introduced inside the trap to remove excess energy from the ions as the RF potential increases so that ions can remain closer to the centre of the electrodes. Increasing the DC and RF potentials makes ions of higher m/z unstabilized so that they are ejected from the trap along the axis of the end-caps. Oscillating frequencies are a function of ion masses; thus, ions with different m/z leave the trap at different voltages and times. A matchless virtue of an ion-trap is its ability to store fragment ions for further fragmentation analysis.

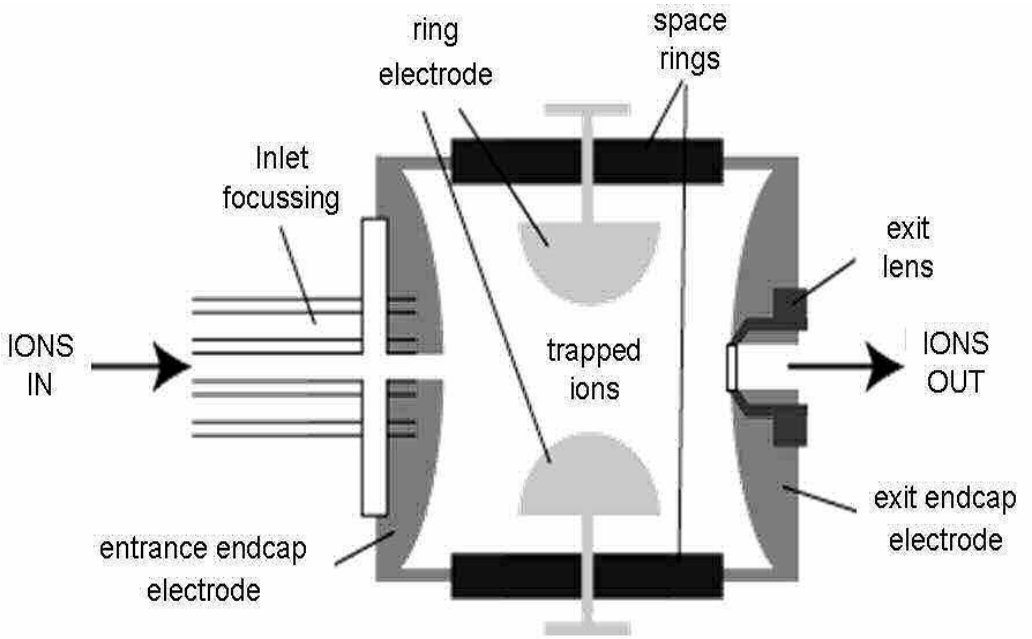

Figure 2.6 A Schematic (cutaway view) of a quadrupole ion trap mass analyser. (http://www.chm.bris.ac.uk/ms/theory/qit-massspec.html).

2.12 Ion detectors and protein identification

Following to the mass analysis, ions reach the ion detector for the detection of their mass and abundance. Ion current generated by the number of ions striking the detector is measured and amplified when necessary by electrometer tubes and vibrating reed electrometers. Electron multiplier tube is a common type of detectors used in MS. Faraday cup, photomultiplier conversion dynode, multichannel plate, charge detector are other detector types. There exist two basic routes by which proteins are identified using MS. These are peptide mass fingerprinting (PMF) by MALDI-MS and peptide sequence tagging by ESI-MS. PMF is related to the identification of proteins using data from intact peptide masses. In this approach, proteins are identified by comparing the list of peptide masses obtained from

proteolytic digestion of an unknown protein with a calculated list of all expected peptide masses for each entry in a protein database. If the theoretical peptide masses are well-correlated with the experimental ones, protein is said to be identified. The major drawbacks of PMF include its incompatibility with protein mixtures and relatively pure sample requirement. On the other hand, peptide sequence tagging is well-suited with analysis of protein mixtures. This technique is based on fragmentation of peptides which produces a short stretch of amino acids. A partial amino acid sequence of a peptide is then obtained by the interpretation of the MS/MS spectrum. In both approaches, database searching benefits from algorithms that have been constructed simultaneously from the beginnings of 1990s.

CHAPTER III

MATERIALS AND METHODS

3.1 Bacterial strains

Acid tolerance (DASA 01001, 01002, 01005, 01006, 01007, 01011, 01013, 01024, 01050) strains obtained from Department of Agricultural culture collection and *B. japonicum* USDA 110 were used in this study.

3.2 Strains selection

Prior to investigate the gene response in bradyrhizobial strains in low pH condition, an appropriate rhizobial strain was selected on the basis of growth rate and plant growth promotion under acidic condition. Acid tolerance, sensitive strains and *B. japonicum* USDA 110 were grown in HM media (Cole and Elkan, 1973). For normal growth analysis, HM medium was adjusted to pH 4.5, 5.0, 5.5, 6.0 and 6.8 by 0.1 M NaOH or 0.1 M HCl. To investigate an adaptive ATR in the bradyrhizobial strains, exponential phase culture was used as inoculum. Bacterial culture was centrifuged at 5,000 rpm for 5 min at room temperature and resuspended in equal volume of HM medium. The bacterial cultures were inoculated to final cells concentration at 10^3 cell/ml in HM medium. The medium was adjusted to pH 6, 5.5, 5.0 and 4.5 respectively. The flasks were incubated at 28°C on a rotary shaker at 200 rpm. The growth pattern was determined on the basis of optical density measurement at 600 nm.

3.3 Plant test

Soybean seeds were surface sterilized in 95% ethanol for 10 sec 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 plate containing wet tissue and kept in the dark place for 1-2 days. Germinated seeds were grown in leonard jar containing sterilized vermiculite then inoculated with 1 ml of rhizobial culture (10^6 cell/ml/seed). Nodulation and nitrogen fixation were tested in Leonard jar containing nitrogen free nutrient and supplement with 20 mM PIPES (pH 6.8) and 20 mM MES (pH 4.5). Soybean were planted in normal condition (pH 6.8) and acidic condition (pH 4.5) (Somasegaran and Hoben, 1994). The leonard jars were put on the shelf light that set up 12 h in light and 12 h in dark. Plants were harvested after 28 days and data such as ARA activity, nodules number, nodule dry weight and plant dry weight were recorded.

3.4 Analysis of protein-profile

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was used to performed protein profile. 1.5 ml of bacterial samples were centrifuged at 12,000 rpm for 10 min and resuspended in 100 µl lysis solution (40 mM Tris-HCl (pH 7.5), 50 mM Dithiothreitol, 2% (w/v) Triton X-100). Cell suspension was heated at 95°C for 10 min prior to electrophoresis. Proteins were separated on 12.5% polyacrylamide gel containing 0.2% SDS and stained with Coomassie brilliant blue R-250 as described by Unni and Rao (2001). SDS-PAGE employed a Tris-Glycine running buffer. Samples were loaded with 100 µg of protein /lane and electrophoresis

was performed with 15 mA for 30 min and 30 mA until tracking dye reached the bottom of gel.

3.5 Two-dimensional gel electorphoresis

Approximately 100 ml packed volume of 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 separated the two 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 in 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, 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 5-8 and 0.25% of 3-10 ampholines). The proteins were dissolved at room temperature with gentle vortexing for 1 h, followed by ultracentrifugation at 100,000×g for15 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) and incubated for overnight at 4°C. The sample was focused on 18 cm. pH 3-10 IPG strip (Amersham

Biosciences) for 90,000 V/h Multiphor™ II Electrophoresis System (GE Healthcare). The strip was removed and incubated for 20 min in SDS equilibration buffer (50 mM Tris-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 x 20 x 18 cm). The gels were visualized by Coomassie brilliant blue staining (Sarma and Emerich, 2006). The gel was scanned and the image analysis was done using ImageMaster 2D Platinum 7.0 (GE Healthcare).

3.6 Sample preparation for LC-MS/MS

Selected proteins spots were excised from stained gels and then, washed twice in MillQ 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) 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. 30 µl MilliQ water was added to the gel pieces at room temperature for 1 hour. Following this, the two supernatants were pooled together (Sarma and Emerich, 2006).

3.7 Protein identification using LC-ESI MS and database search

Mass spectrometric analyses were conducted by nanoflow-LC-ESI-MS/MS (Bruker Esquire 3000 plus Ion Trap; Bruker Daltonics). Peptides were separated by chromatography on a 75 µm × 15 cm Pep-Map nanocolumn (LC Packings) at a flow

rate of 7 l/min using a linear gradient of acetonitrile (5-95% in 60 min) in 0.1% formic acid. The column effluent was sprayed directly into the ion trap which was set to scan the m/z range from 400 to 1,500 in positive ion mode, capturing MS and MS2 data automatically. Instrument operation, data acquisition, and analyses were performed using HyStarTM V2.3 and DataAnalysis 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 as 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. Routine protein identification required sequence-confirmed data for a minimum of one peptides with recognition as the top ranking match in the Mascot Standard scoring system (Li et al., 2010).

3.8 Genomic DNA extraction

Prior to isolate the genomic DNA, bradyrhizobial strains were cultured in HM medium. After cultivation for 5-7 days on rotary shaker for 200 rpm, at 28°C, bacterial cells were pelleted before resuspened in 0.5 ml TEN buffer (0.1 M NaCl, 10 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8)) and centrifugation at 13,000 rpm for 2 min. Bacterial pellets were resuspended in 200 µl of 20% sucrose in TEN buffer, then 100 µl of 10% SDS was added prior to incubated with 20 µl of 2.0 mg/ml lysozyme for 30 min at 37°C. Then, 75 µl of 5 M NaCl, was added and the mixtures were extracted twice with saturated phenol and followed with phenol: chloroform:

isoamylalcohol (25:24:1). The upper phase was taken. DNA was precipitated with two volumes of cold 99% ethanol and 50 µl of 3M sodium acetate. DNA pellets were dried under vacuum before dissolved in TE buffer and treated with 10 mg/ml of RNaseA at 55°C for 15 min.

3.9 Primer design and PCR amplifications

Primer pairs were designed from 4 protein-coding open reading frames (*bll*5845: hypothetical protein, *bll*1317: peroxiredoxin, *blr*5625:10 kDa chaperonin and *bsr*7532:10 kDa chaperonin). Parameters for primer pairs were set to a primer length of 18-20 nucleotides, a melting temperature of 55 ± 5 ºC. Calculation of primer sequences was based on the Primer3 software (http://frodo.wi.mit.edu/primer3).

Table 3.1 Primer pairs use for acid response gene

gene	Fw	Rw
*bll*5845	5’-ATGTCCGGTATCGTTCTC-3’	5’-CTCCAGCTGCTCCGCTAA-3’
*bll*1317	5’-ATGGCGATCCAGACTGGC-3’	5’-CTGCTCGGGCAGCTCTGA-3’
*blr*5625	5’-ATGAAATTCCGTCCGCTT-3’	5’-AAGAAGAAGGCGGCCTAA-3’
*bsr*7532	5’-ATGGCTAAATCCAAATTT-3’	5’-ATGGGCGTGATGGCCTAA-3’

To amplify gene specific PCR, each PCR reaction contained 50 ng of DNA template, 10 pmol of each primer, 2.5 mM of dNTP, 1X PCR buffer and 2.5 U GOTaq® DNA polymeraes (Promega, USA) in total volume of 25 µl. The PCR reaction condition was used as follows; 95°C for 3 min 1 cycle, 95°C for 30 sec, 55°C for 1 min, 72°C for 3 min 30 cycles and final 72°C for 7 min 1 cycle.

3.10 Nucleotide sequences

Generally only one strand of acid response gene fragments were sequenced with the automated sequencer 3730XL (Macrogen, Korea). Each PCR product was sequenced in both directions and the sequences were assembled and checked with the autoassembler 1.4 program (Perkin Elmer) and transferred directly to a sequence analysis program, BlastN 2.0.13 were employed. In this study, to search for homologous sequence in the data bank, Gene Bank (www.ncbi.nlm.nih.gov /genbank) was used.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Growth of bradyrhizobial at various pH conditions

In order to select the appropriate acid tolerant strains of bradyrhizobia that can grow and promote plant growth under acid conditions, acid tolerant *Bradyrhizobium* sp. strains DASA01001, 01002, 01005, 01006, 01007, 01011, 01013, 01024, and 01050 obtained from DOA culture collection were cultured in medium at optimum condition (pH 6.8) and at acidic condition (pH 4.5) compared with reference strain of *Bradyrhizobium japonicum* USDA110. To compare the growth ability in acid condition, the growth of each bradyrhizobia strain was calculated in term of specific growth rate (μ), which is defined as the increase of cell mass per unit of time. The specific growth rate of bradyrhizobia was in range of 0.03-0.079 d^{-1} and 0.005-0.076 d^{-1} at optimum condition (pH 6.8) and acid condition (pH 4.5), respectively (Fig. 4.1). These results indicated that the acid condition had adversely affected on cell growth. This effect was obviously found in *Bradyrhizobium* sp. DASA01002, 01005 and 01006, since their specific growth rate were clearly reduced under acid condition. The specific growth rate of *B. japonicum* USDA110 was also reduced when grow at acid condition. Interestingly, the specific growth rate of *Bradyrhizobium* sp. DASA01001, 01007, 01024 and 01050 were increased when grew at acid condition. These results indicated that these four strains of bradyrhizobial may have potential to survive and promote the growth of soybean under acid soil condition. Thus,

Bradyrhizobium sp. DASA01001, 01007, 01024 and 01050 were selected for plant experiment.

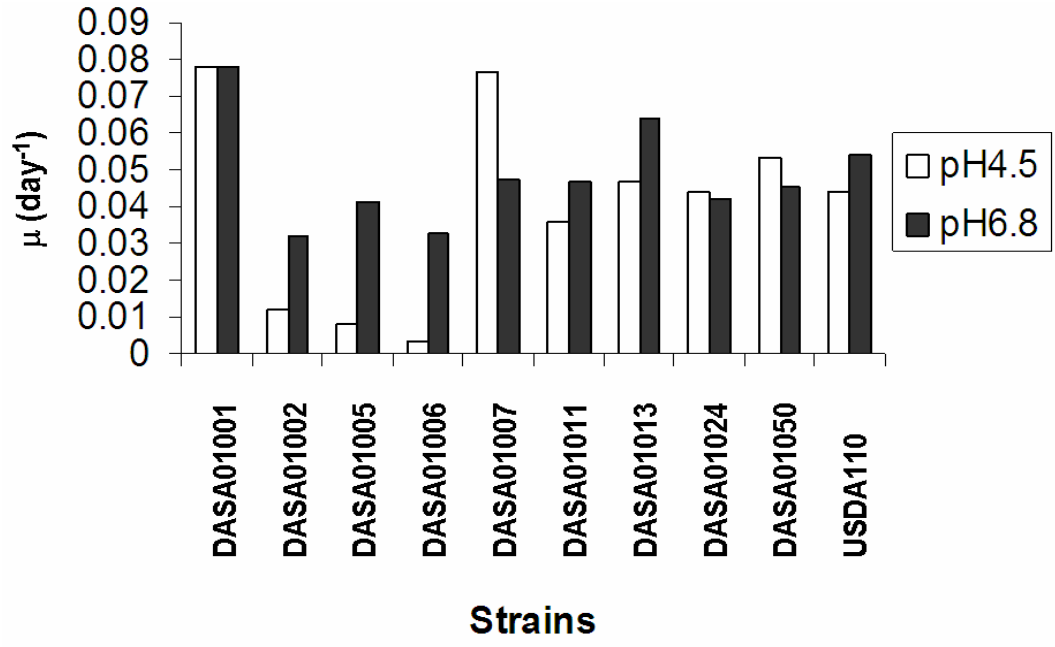

Figure 4.1 Specific growth rates of different bradyrhizobial strains at optimum condition (pH 6.8) and acid condition (pH 4.5).

4.2 Symbiotic efficiency of selected bradyrhizobial strains

Soybean (*Glycine max*, Chieng Mai 60) was used in this study. The selected bradyrhizobial strains DASA01001, 01007, 01024 and 01050 were inoculated into soybean seed (10^6 cells/seed) and planted in both acid (pH 4.5) and normal (pH 6.8) conditions. After 4 weeks of growth under nitrogen-free condition, all the uninoculated plants (control treatment) were distinctly chlorotic, yellow and stunted, while inoculated plant showed different level of greenish leaves as well as different number of nodule forming (Fig 4.2). Gwata et. al. (2003) demonstrated that plant was classified into one of two categories; vigorous plant with dark green leaves indicating

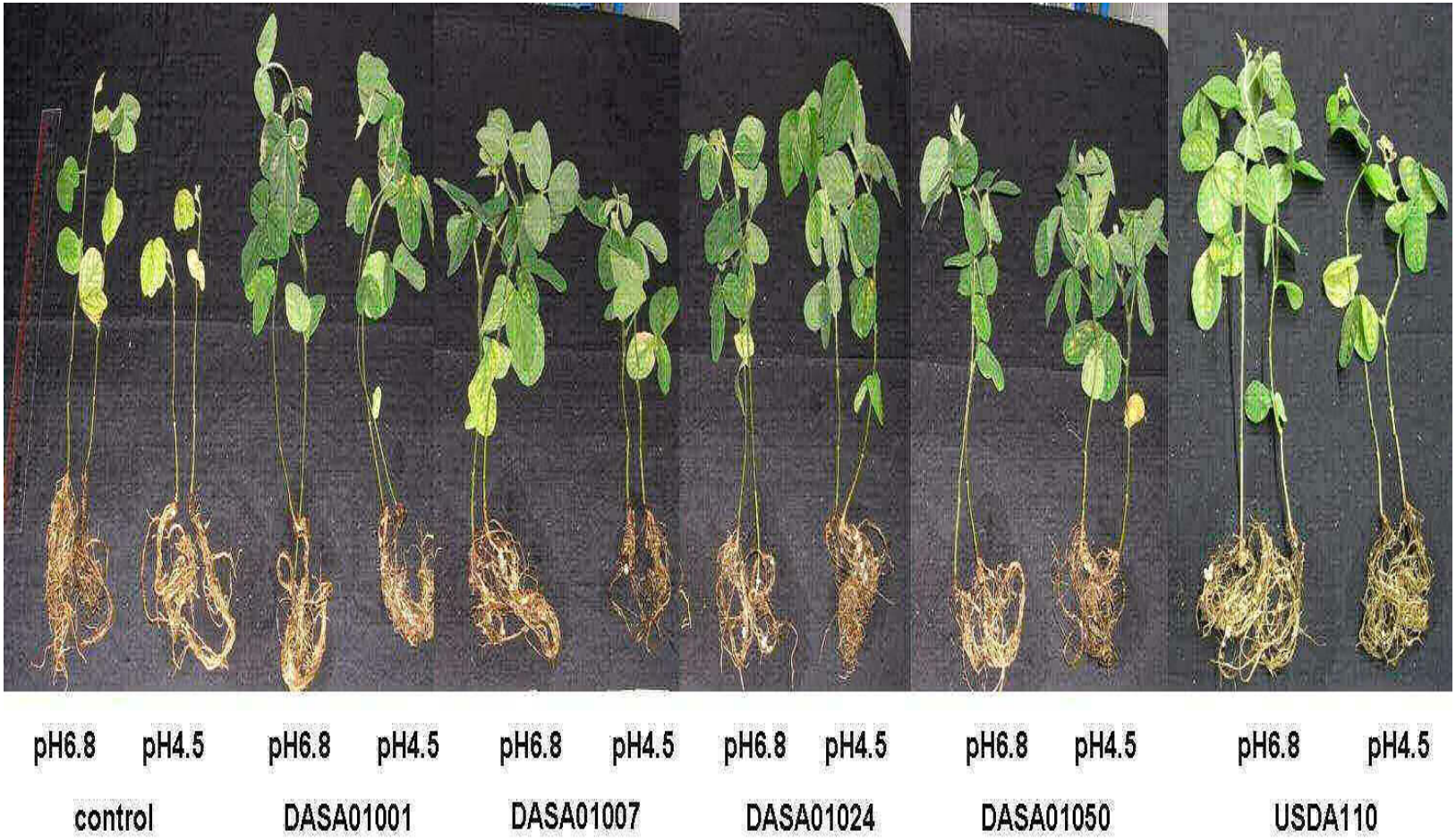

Figure 4.2 Soybean inoculated with different bradyrhizobial strains grown in acid (pH 4.5) and normal (pH 6.8) condition.

effective nodulation and N_2-fixation (+), and stunted plant with chlorotic yellow leaves indicating ineffective nodulation and no N_2-fixation (-).

The effects of inoculation of acid tolerant bradyrhizobia on soybean were presented in Figure 4.3 and 4.4. The data of nodules number, nodules dry weight, dry matter production, and nitrogenase activity were collected and statistically analyzed.

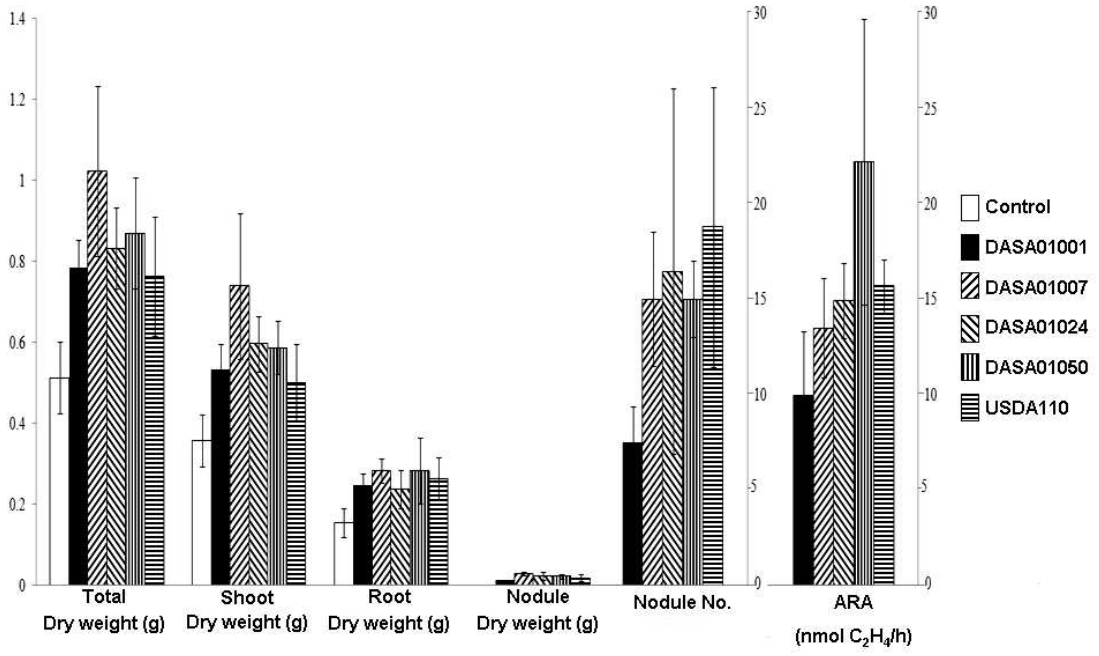

Figure 4.3 Effect of different bradyrhizobial inoculation on symbiotic efficiency with soybean at pH 6.8.

At pH 6.8 (Fig. 4.3), soybean inoculated with *Bradyrhizobium* sp. DASA01007 provided the highest value of total plant dry weight and nodule dry weight, but these data were not significantly different from soybean inoculated with other strains including *B. japonicum* USDA110. Soybean inoculated with *Bradyrhizobium* sp. DASA01007 also provided the highest shoot dry weight, which was significantly different ($p \geq 0.5$) from soybean inoculated with *Bradyrhizobium* sp.

DASA01001 and *B. japonicum* USDA110. However, soybean inoculated with *Bradyrhizobium* sp. DASA01050 provided the highest value of root dry weight and also performed highest nitrogenase activity, however the data were not significantly different from soybean inoculated with other strains. On the other hands, soybean inoculated with *B. japonicum* USDA110 performed highest number of nodule with plant, but the data were not significantly different from soybean inoculated with other strains. From these information revealed that *Bradyrhizobium* sp. DASA01007 and 01050 had symbiotic efficiency similar to *B. japonicum* USDA110 when grow at neutral condition.

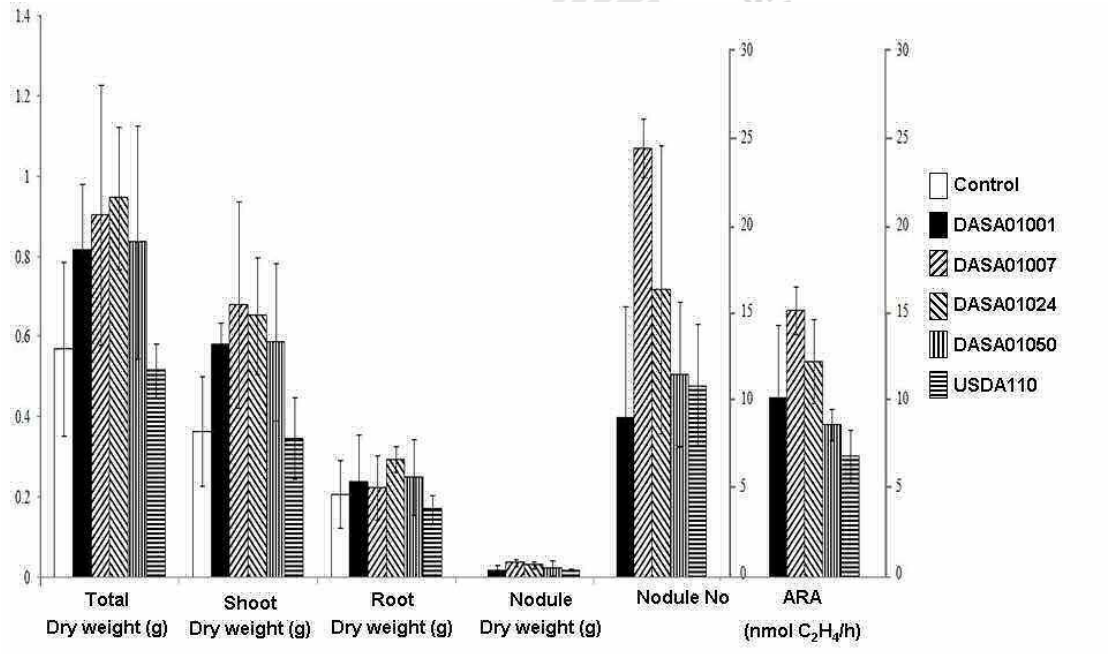

Figure 4.4 Effect of different bradyrhizobial inoculation on symbiotic efficiency with soybean at pH 4.5.

However, once soybean was grown in acid condition (pH 4.5) (Fig. 4.4), soybean inoculated with *Bradyrhizobium* sp. DASA01007 provided highest value of

nitrogenase activity, nodule dry weight, and nodules number. Even the data were not significantly different from soybean inoculated with other strains, *Bradyrhizobium* sp. DASA01007 tended to perform better symbiosis than other strains including *B. japonicum* USDA110. It could be possible that *Bradyrhizobium* sp. DASA01007 could tolerate to acid condition or have acid adaptive tolerant response that allow cells able to survived in acid condition and finally lead to successfully symbiosis with plant. Thus, *Bradyrhizobium* sp. DASA01007 was used to verify the growth in acidic pH under normal and acid adaptive conditions in the next experiments.

4.3 Growth of bradyrhizobia under normal and adaptive conditions

To investigate the growth efficiency of bradyrhizobia, *Bradyrhizobium* sp. strain DASA01007 and *B. japonicum* USDA110 were grown in HM medium at pH 6.8, 6.0, 5.5, 5.0 and 4.5 in both normal (N) and adaptive (A) conditions. The results showed that *Bradyrhizobium* sp. DASA01007 and *B. japonicum* USDA110 performed similar growth in HM medium at pH 5.0, 5.5, 6.0 and 6.8 in both normal (N) and adaptive (A) conditions (Fig. 4.5 and Fig. 4.6). These two strains grow in HM medium at pH 5.0-5.5 better than pH 6.8. Many researches have been proposed that bacteria could develop acid tolerance to 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 more acid condition. This response is known as acid adaptive tolerant response (ATR) (Foster and Hall, 1990; Foster and Hall, 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., 2006a; Tittabutr et al., 2006b), as well as in *Bradyrhizobium* sp. (O'Hara and Glenn, 1994). This phenomenon was also found by Puranamaneewiwat et al., (2006), the growth of ATR *B. japonicum* USDA110 at pH 5.5 in HM medium was higher than those grown at the other pH, including at pH 6.8. This phenomenon did not find when grew in YEM medium. Calcium, one of HM medium components, might be the key role for promoting cell growth in acid condition. Maccio et al., (2002) found that the growth of peanut *Bradyrhizobium* sp. had better growing in calcium-added medium at acid condition. Increasing concentration of calcium significantly improved the rhizobial growth under acid condition stress. This result demonstrated the important role of calcium for *Bradyrhizobium* sp. growth and viability under acid stress. It would indicate that there is a strong dependence between calcium and the capacity of the rhizobia grow at low pH. 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).

However, *Bradyrhizobium* sp. DASA01007 and *B. japonicum* USDA110 could not grow in HM medium at pH 4.5 when grew in normal condition (Fig. 4.5). Interestingly, *Bradyrhizobium* sp. DASA01007 could adapt itself to grow at pH 4.5 when ATR was applied, while *B. japonicum* USDA110 could not grow in pH 4.5 even grown in adaptive condition (Fig. 4.6).

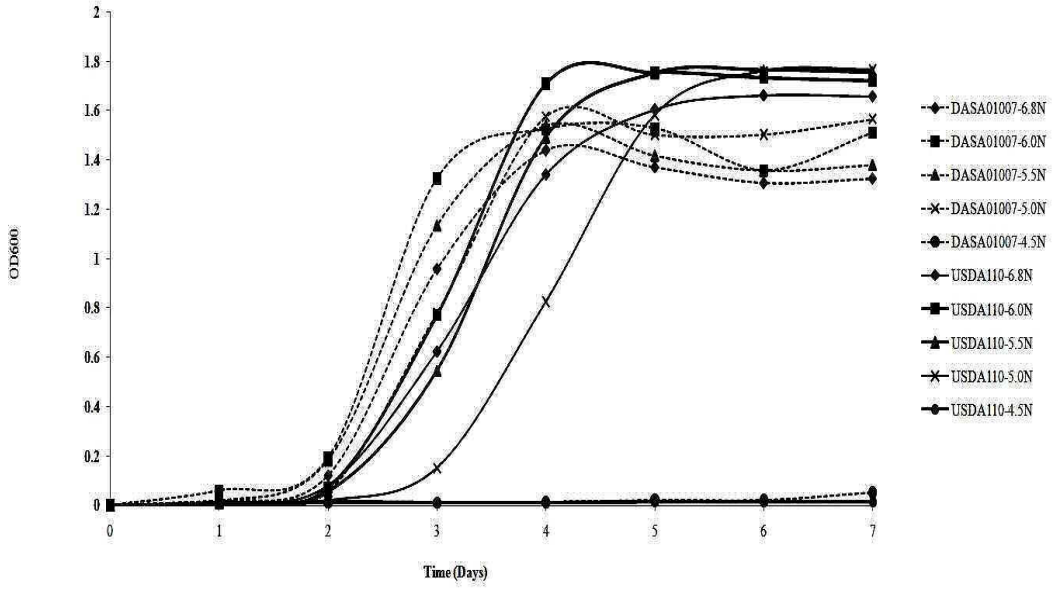

Figure 4.5 Growth of *Bradyrhizobium* sp. DASA01007 and *B. japonicum* USDA110 growing in HM medium at normal condition.

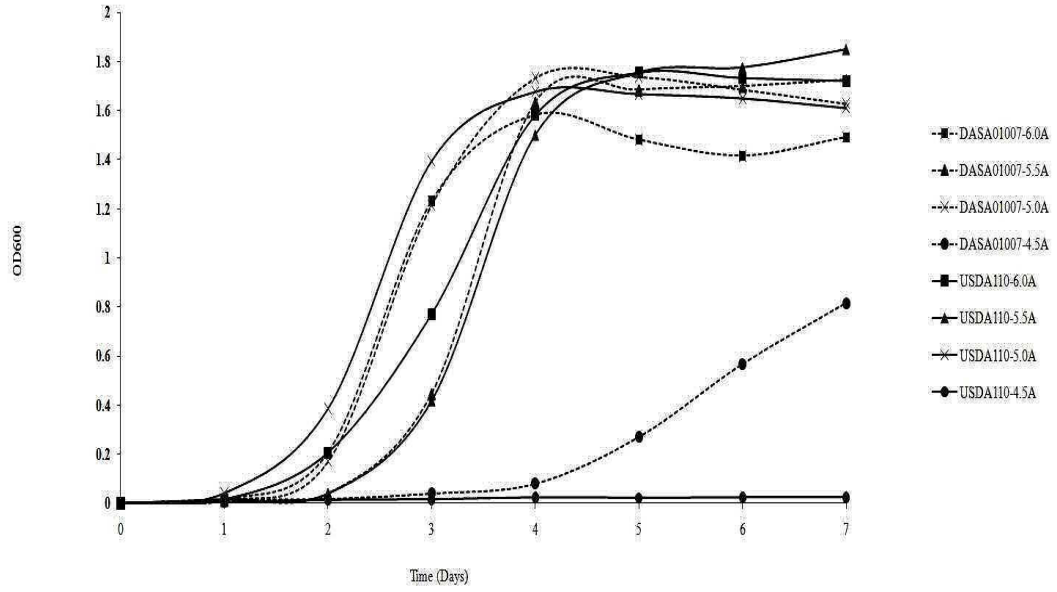

Figure 4.6 Growth of *Bradyrhizobium* sp. DASA01007 and *B. japonicum* USDA110 growing in HM medium at adaptive condition.

As well as, the result of specific growth rate determined from these two strains at pH 4.5 were clearly shown that *Bradyrhizobium* sp. DASA01007 had ability to grow in extreme acid condition when cells were grown under adaptive condition (Fig. 4.7). However, the specific growth rate of *Bradyrhizobium* sp. DASA01007 and *B. japonicum* USDA110 were similar when grown at mild acid condition (pH 5.5 and 6.0) under both normal and adaptive conditions. Although *B. japonicum* USDA110 also showed acid adaptive response at pH 5.0 due to its specific growth rate of cell grew under adaptive condition was higher than those in normal condition. This adaptive response of *B. japonicum* USDA110 was not found at pH 4.5, while found in *Bradyrhizobium* sp. DASA01007 (Fig. 4.7). These results indicated that *Bradyrhizobium* sp. DASA01007 have better induction of acid adaptive tolerant responses which may be one of mechanisms that allow bradyrhizobial cell grow under extreme acid condition.

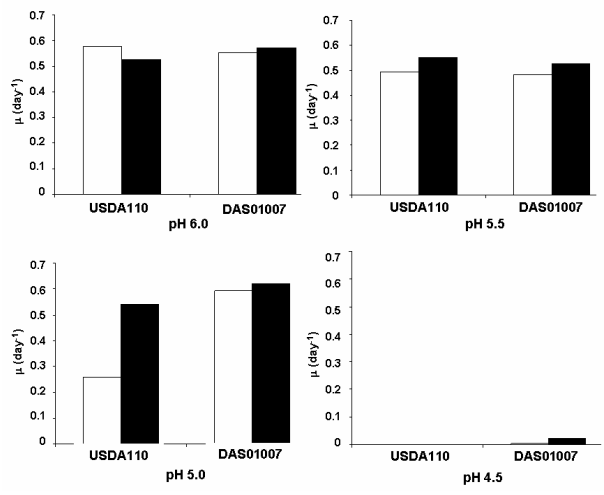

Figure 4.7 Plot of the specific growth rates of *Bradyrhizobium* sp. DASA01007 and *B. japonicum* USDA110 in various conditions (■ adaptive condition and □ normal condition).

4.4 Protein profile analysis using SDS-PAGE

Since *Bradyrhizobium* sp. DASA01007 showed acid tolerant and acid adaptive tolerant response, it is interesting to investigate whether the same group of proteins was involved in these responses. SDS-PAGE technique was used to preliminary observe the profiles of protein extracted from cells grown at different pH under normal or adaptive conditions. For adaptive response, cell grown at pH 4.5 and 5.5 were used to analyze while the cells in normal conditions were grown at pH 4.5 and 6.8. Total cellular proteins of acid tolerance strain were subjected to SDS-PAGE. The result showed that different protein patterns were expressed differently in each growth conditions (Fig. 4.8).

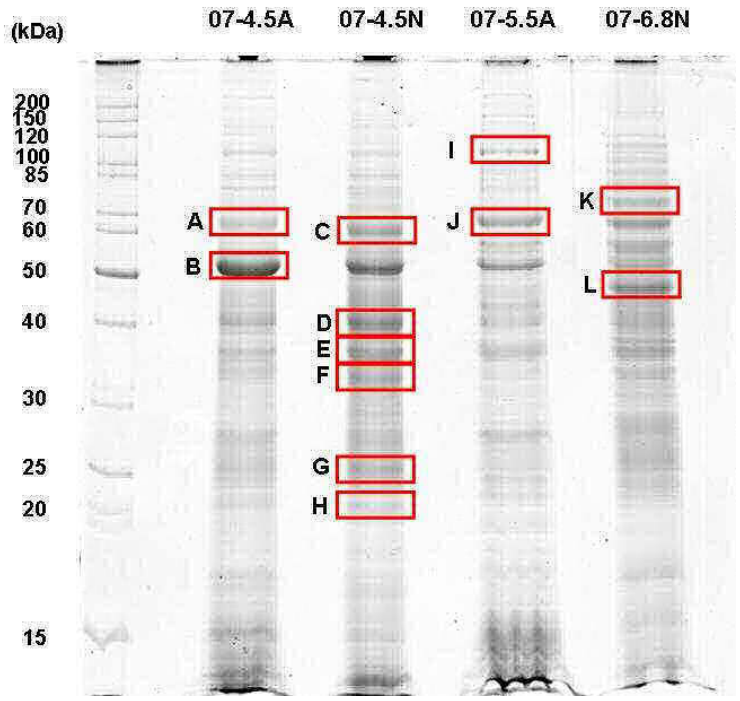

Figure 4.8 Protein profiles of *Bradyrhizobium* sp. DASA 01007 grown in HM medium at pH 4.5, 5.5 and 6.8 in adaptive (A) and normal (N) conditions.

Based on high protein expression level and the presence of extra protein bands, 12 bands (A-L) were preliminary selected for further analysis by LC/MS-MS. The result of top hit protein identification was summarized in Table 4.1. The identified proteins were classified into 4 categories based on Clusters of Orthologous Groups of proteins (COGs) (http://genome.kazusa.or.jp/rhizobase), (i) cellular processes, (ii) conserved hypothetical protein, (iii) transport and binding proteins, and (iv) translation. The best matched protein identified from band A, C and J was GroEL protein, which is a chaperonin 60 heat-shock protein. This protein is expressed when the cell encountering to stress conditions, such as heat or salt stress (Kilstrup et al., 1997). This kind of stress protein was generally involved in the maturation of newly synthesized proteins, and assists in the refolding or degradation of denaturated proteins (Georgopoulos and Welch, 1993; Hartl, 1996). This protein may be important during stress due to rescuing the stress-denatured proteins (Kilstrup et al., 1997). This might be the reason why this protein presented in all growth conditions even grew at pH 6.8. More detail of this protein was discussed in the next section. Interestingly, band B, a hypothetical protein (bll5843) was highly expressed when cells were grown at pH 4.5 under adaptive condition, while the intensity of band tends to be reduced when grown at higher pH under normal condition or at pH 6.8. Moreover, the transport and binding proteins including ABC transporter substrate-binding protein (band D), ABC transporter sugar-binding protein (band E), ABC transporter amino acid-binding protein (band F), and ABC transporter molybdenum-binding protein (band G) could be detected with high intensity of band at pH 4.5 under normal condition. The ABC transporter proteins were involved in transport and binding proteins. Most ABC transporter proteins were up-regulated in cellular extract. This

result was similar to ABC transporter sugar binding protein was up-regulated in *Sinorhizobium medicae* as shown by transcriptional and proteomic analyses (Reeve et al., 2002; Tiwari et al., 2004). 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. Moreover, the proteins involved in translation process including the 50S ribosomal protein L5 (band H), Ribosomal protein S1 (band K) and Elongation factor Tu (band L) were also detected. The 50S ribosomal protein L5 has been detected in acid tolerant *Bacillus cereus*, however this protein was deficient in the acid-sensitive mutant of this strain (Browne and Dowds, 2002). Thus, it is possible that this protein may also be required for acid tolerant response. On the other hands, ribosomal protein S1 has been reported to be induced when exposed to cadmium stress. Interestingly, the ribosomal protein S1 was proportionally induced as the amount of cadmium in the medium, suggesting that S1 may be required for the repair of cadmium-mediated cellular damage (Mohamed Fahmy Gad El-Rab et al., 2006).

This protein may play similar mechanism to acid stress cellular damage. Finally, the elongation factor Tu (EF-Tu) is the protein involved in binding and transporting the appropriate codon-specified aminoacyl-tRNA to aminoacyl site of the ribosome. However, it has been reported that the EF-Tu of *Escherichia coli* has chaperone-like function that interact with denatured proteins for protein renaturation after stress (Caldas et al., 1998), suggesting that EF-Tu might has a role in cell protection in stress condition. Although the ribosomal protein S1 and EF-Tu may play an important role for protecting cell damage during stress condition, these two proteins found to be expressed highly at pH 6.8 and slightly expressed in acid

condition in (Fig. 4.8, band K and L). It could be possible that the growth of cells is slow under stress condition, less protein is required, thus the protein for translation process would be decreased under stress condition. Even the different profiles of protein expression were detected in this experiment, the expression level of these proteins could not be exactly determined from 1D SDS-PAGE, since one protein band usually contain several types of protein that have similar molecular weight. Therefore, the expression levels of interested proteins were examined by using 2D gel to ensure the expression of proteins that would be involved in acid tolerant or acid adaptive tolerant mechanism of *Bradyrhizobium* sp. DASA01007.

Table 4.1 Proteins involved in acid tolerance identified from SDS-PAGE of *Bradyrhizobium* sp. DASA 01007.

Band	Top hit protein	Gene names[a]	Locus Names[b]	Organism	Functional category[c]	Protein Score[d]	Queries Match[e]
A	60 kDa chaperonin 6	*groEL*	blr5626	*Bradyrhizobium japonicum* USDA 110	Cellular processes	746	15
B	hypothetical protein	-	bll5843	*Bradyrhizobium japonicum* USDA 110	Conserved hypothetical protein	344	11
C	60 kDa chaperonin 6	*groEL*	blr5626	*Bradyrhizobium japonicum* USDA 110	Cellular processes	977	18
D	ABC transporter substrate-binding protein	-	blr5675	*Bradyrhizobium japonicum* USDA 110	Transport and binding proteins	212	3
E	ABC transporter sugar-binding protein	-	blr3208	*Bradyrhizobium japonicum* USDA 110	Transport and binding proteins	507	16
F	ABC transporter amino acid-binding protein	-	blr4446	*Bradyrhizobium japonicum* USDA 110	Transport and binding proteins	365	6
G	ABC transporter molybdenum-binding protein	*modA*	blr8160	*Bradyrhizobium japonicum* USDA 110	Transport and binding proteins	263	4
H	50S ribosomal protein L5	*rplE*	bll5388	*Bradyrhizobium japonicum* USDA 110	Translation	231	7
I	hypothetical protein	-	blr0521	*Bradyrhizobium japonicum* USDA 110	Conserved hypothetical protein	242	7

Table 4.1 (continued).

Band	Top hit protein	Gene names[a]	Locus Names[b]	Organism	Functional category[c]	Protein Score[d]	Queries Match[e]
J	60 kDa chaperonin 6	*groEL*	blr5626	*Bradyrhizobium japonicum* USDA 110	Cellular processes	623	13
K	Ribosomal protein S1	-	SKA58_10470	*Sphingomonas* sp. KA58	Translation	150	4
L	Elongation factor Tu	*tuf*	bll5402	*Bradyrhizobium japonicum* USDA 110	Translation	366	7

[a]Genes name obtain from rhizobase (http://genome.kazusa.or.jp/rhizobase/Bradyrhizobium).

[b]Locus name obtain from rhizobase (http://genome.kazusa.or.jp/rhizobase/Bradyrhizobium).

[c]Functional category obtain from rhizobase (http://genome.kazusa.or.jp/rhizobase/Bradyrhizobium).

[d]Protein score from Mascot search (http://www.matrixscience.com)

[e]Queries matce from mascot search (http://www.matrixscience.com)

4.5 Two-dimensional gel analysis and identification of proteins in *Bradyrhizobium* sp. DASA01007 growing under normal and adaptive conditions in response to acidic pH

To identify proteins involving in acid tolerant or acid adaptive tolerant response of *Bradyrhizobium* sp. DASA01007, 2-D gel electrophoresis was carried out. Total protein extracted from *Bradyrhizobium* sp. DASA01007 cells grown in different conditions were separated by 2-D PAGE using IPG strips (non-linear gradient between pH 3 to 10). Based on image analysis of protein spot on 2-D PAGE by using Image Master 2D Platinum 7.0 program (GE healthcare), there were 651, 475, 638 and 745 protein spots could be detected from bacterial cell grown at pH 6.8, pH 5.5A, pH 4.5A and pH 4.5N, respectively. The intensity of interested protein spot was analyzed by comparison with intensity of protein presented in cell grown at pH 6.8. The different intensities of protein spot demonstrated up and down regulated of those specified proteins. Based on the protein profile presented in pH 6.8 condition, 14, 6, and 3 spots of protein which obviously up-regulated were selected from protein profile of cell grown at pH 4.5A, pH 4.5N and pH 5.5A, respectively (Fig. 4.9, 4.10, 4.11). While other 6 protein spots which highly expressed in cells grown at pH 6.8 but down-regulated in cell grown at acid condition, were also selected (Fig. 4.12). These 30 spot of proteins were further analyzed by LC-MS/MS in order to identify the protein by comparison with *B. japonicum* USDA110 database. The peptide mass fingerprinting (PMF) searches were performed with the MSDB databases through the Mascot server. Only proteins identified with at least two peptide hits in duplicate analyses were accepted. The results of identified proteins were summarized in Table 4.2.

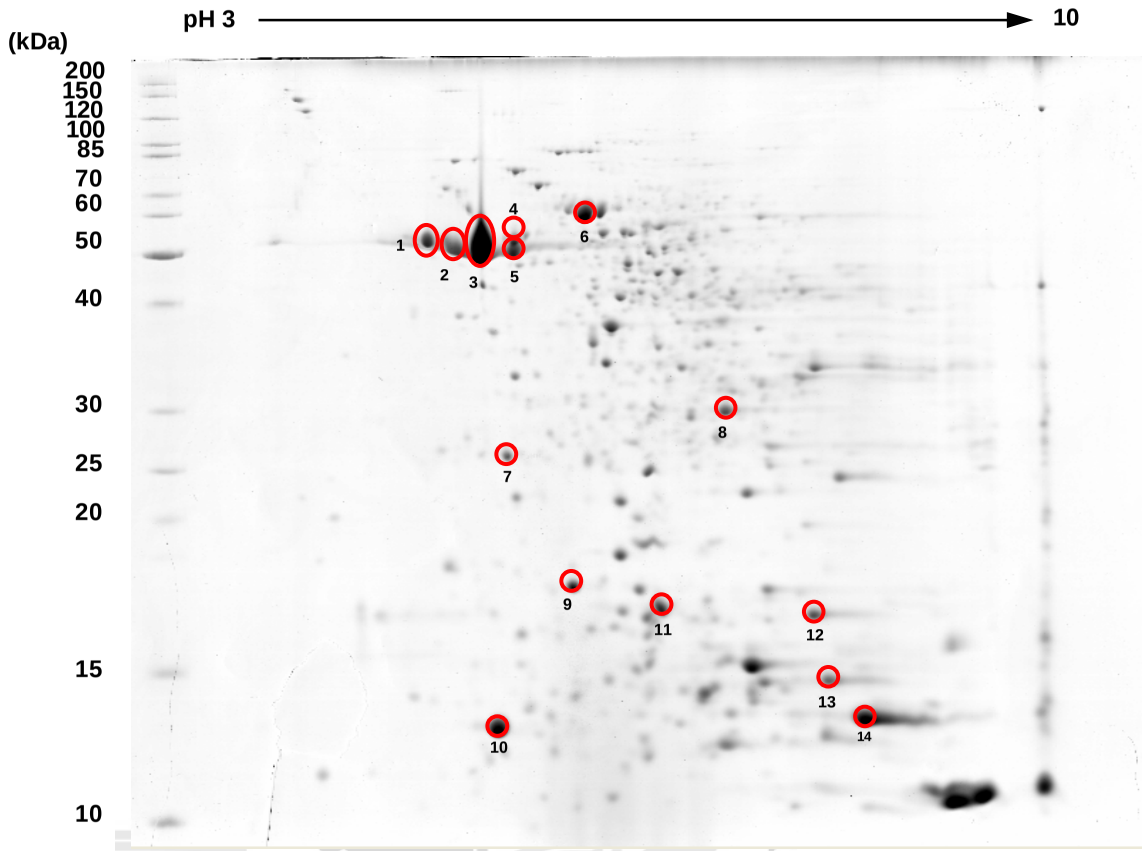

Figure 4.9 Protein spots from *Bradyrhizobium* DASA01007 growing at pH 4.5A.

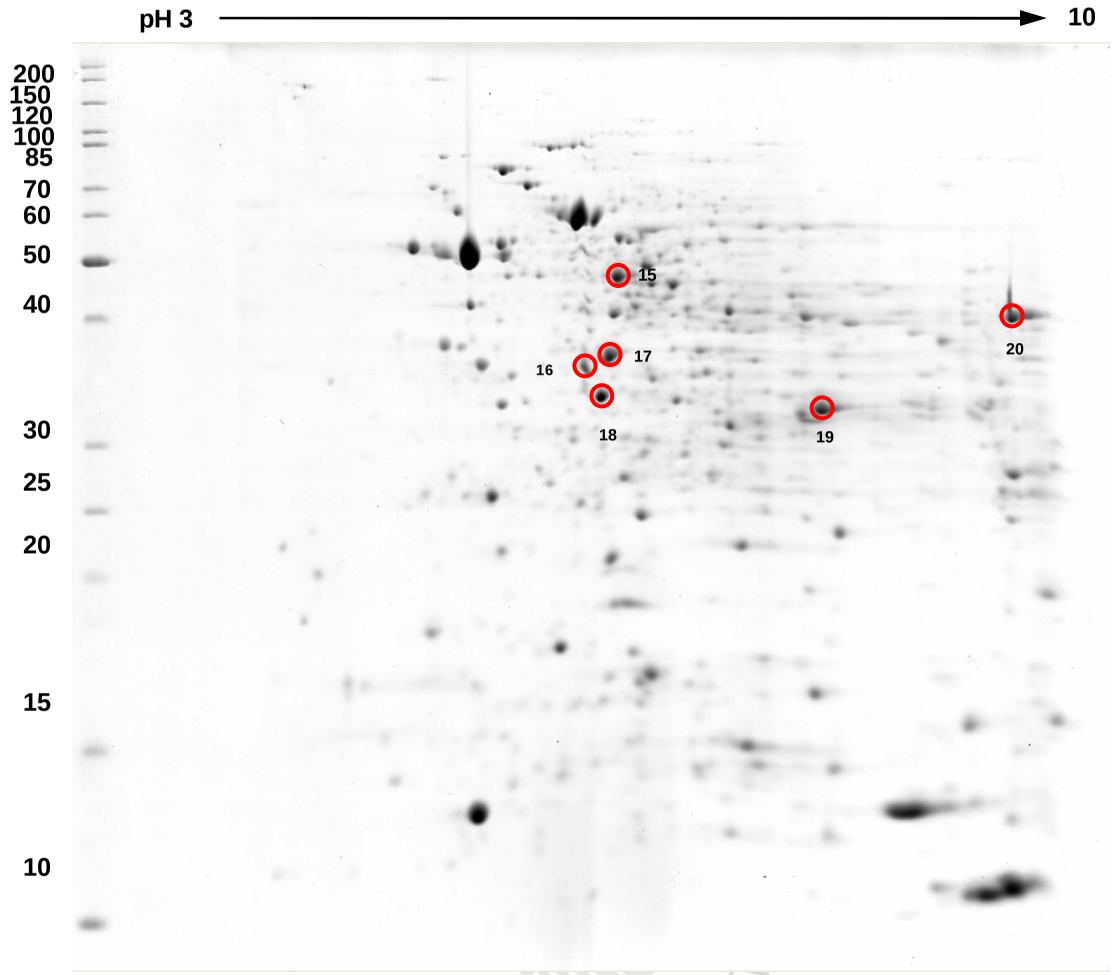

Figure 4.10 Protein spots from *Bradyrhizobium* DASA01007 growing at pH 4.5N.

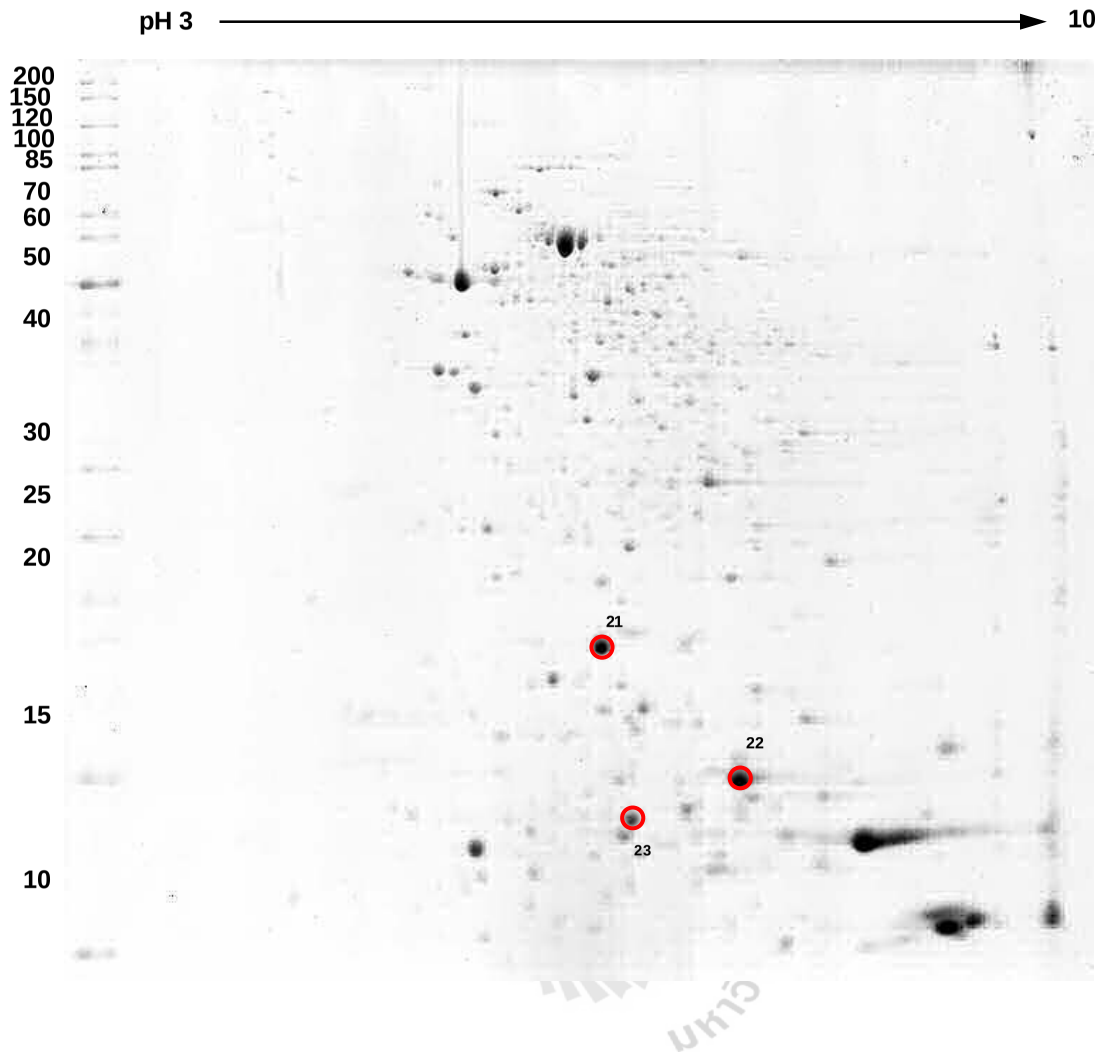

Figure 4.11 Protein spots from *Bradyrhizobium* DASA01007 growing at pH 5.5A.

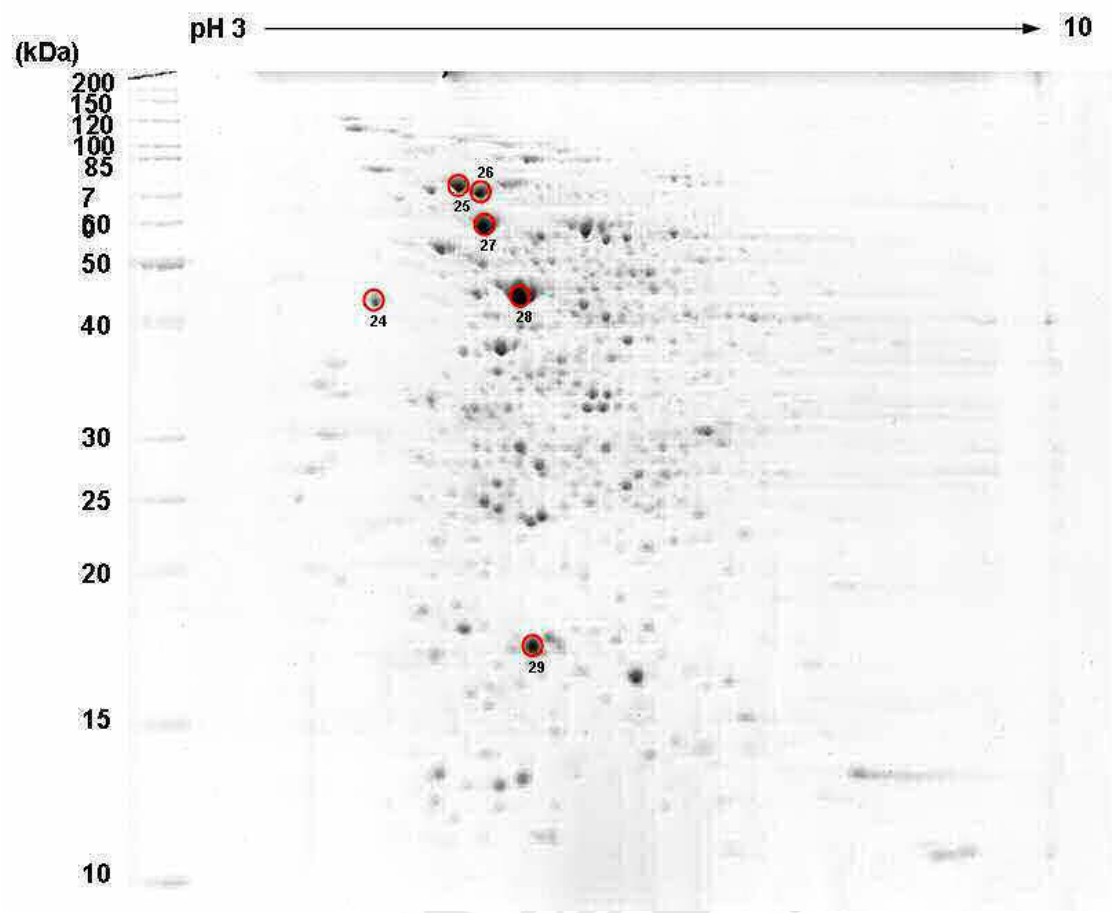

Figure 4.12 Protein spots from *Bradyrhizobium* DASA01007 growing at pH 6.8.

Table 4.2 Proteins involved in acid tolerance identified from 2D-PAGE of *Bradyrhizobium* sp. DASA01007.

Spot no.	Tophit protein	Gene code[a]	Locus name[b]	Organisms	Functional category[c]	Peptide matched[d]	Mascot score[e]	MW[f] (kDa)	PI[g]	Predicted localization[h]	Fold change	Up/down regulation	
												N	A
1	hypothetical protein	-	bll5843	*Bradyrhizobium japonicum* USDA 110	Conserved hypothetical protein	7	314	75,633	4.87	Outer Membrane	1.93 (pH4.5A)	⁄	⁄
2	hypothetical protein	-	bll5843	*Bradyrhizobium japonicum* USDA 110	Conserved hypothetical protein	10	349	51,090	4.97	Extra cellular	2.98 (pH4.5A)	⁄	⁄
3	hypothetical protein	-	bll5845	*Bradyrhizobium japonicum* USDA 110	Conserved hypothetical protein	18	260	51,090	4.97	Extra cellular	45.63 (pH4.5A)	⁄	⁄
4	ATP synthase subunit beta	*atpD*	bll0440	*Bradyrhizobium japonicum* USDA 110	Energy metabolism	20	712	50,987	5.13	Cytoplasmic membrane	2.08 (pH4.5A)	⁄	⁄
5	hypothetical protein	-	bll5843	*Bradyrhizobium japonicum* USDA 110	Conserved hypothetical protein	15	330	51,090	4.97	Extra cellular	1.06 (pH4.5A)	⁄	⁄
6	60 kDa chaperonin 6	*groEL*	blr5626	*Bradyrhizobium japonicum* USDA 110	Cellular processes	66	1764	57716	5.45	Cytoplasmic	1.57 (pH4.5A)	⁄	⁄
7	Two-component response regulator	*tcsR*	blr1194	*Bradyrhizobium japonicum* USDA 110	Regulatory functions	5	310	23,989	5.07	Cytoplasmic	1.19 (pH4.5A)	⁄	⁄
8	Oxido reductase	-	blr2928	*Bradyrhizobium japonicum* USDA 110	Other categories	7	357	31,520	6.92	Unknown	1.09 (pH4.5A)	⁄	⁄

Table 4.2 (Continued).

Spot no.	Tophit protein	Gene code[a]	Locus name[b]	Organisms	Functional category[c]	Peptide matched[d]	Mascot score[e]	MW[f] (kDa)	PI[g]	Predicted localization[h]	Fold change	Up/down regulation	
												N	A
9	30S ribosomal protein S6	*rpsF*	bll4079	*Bradyrhizobium japonicum* USDA 110	Translation	11	257	18,616	5.46	Cytoplasmic	14.69 (pH4.5A)	⁄	⁄
10	Ribosomal protein L7/L12	-		*Rhodopseudomonas palustris* BisA53	Translation	14	310	12,694	5.02	Periplasmic	4.71 (pH4.5A)	⁄	⁄
11	Peroxi redoxin	-	bll1317	*Bradyrhizobium japonicum* USDA110	Other categories	28	626	17,414	6.11	Unknown	4.98 (pH4.5A)	⁄	⁄
12	Nucleoside diphosphate kinase	*ndk*	blr4119	*Bradyrhizobium japonicum* USDA 110	Inter conversions and salvage of nucleosides and nucleotides	11	277	15,050	6.75	Cytoplasmic	3.61 (pH4.5A)	⁄	⁄
13	hypothetical protein	-	bll2431	*Bradyrhizobium japonicum* USDA 110	Conserved hypothetical protein	7	216	16,778	7.85	Unknown	2.84 (pH4.5A)	⁄	⁄
14	10 kDa chaperonin	*groES*	blr5625	*Bradyrhizobium japonicum* USDA 110	Cellular processes	19	229	11,170	7.93	Cytoplasmic	20.03 (pH4.5A)	⁄	⁄
15	Elongation factor Tu	*tuf*	bll5402	*Bradyrhizobium japonicum* USDA 110	Translation	27	907	43,569	5.78	Cytoplasmic	1.92 (pH4.5N)	⁄	⁄
16	hypothetical protein	-	blr0205	*Bradyrhizobium japonicum* USDA 110	Conserved hypothetical protein	31	898	34,868	6.30	Periplasmic	1.91 (pH4.5N)	⁄	⁄

Table 4.2 (Continued).

Spot no.	Tophit protein	Gene code[a]	Locus name[b]	Organisms	Functional category[c]	Peptide matched[d]	Mascot score[e]	MW[f] (kDa)	PI[g]	Predicted localization[h]	Fold change	Up/down regulation	
												N	A
17	ABC transporter sugar-binding protein	-	blr3208	*Bradyrhizobium japonicum* USDA 110	Transport and binding proteins	27	666	38,378	7.63	Periplasmic	1.51 (pH4.5N)	⁄	⁄
18	ABC transporter amino acid-binding protein	-	blr4446	*Bradyrhizobium japonicum* USDA 110	Transport and binding proteins	28	903	36,860	6.21	Periplasmic	2.52 (pH4.5N)	⁄	⁄
19	ABC transporter sugar-binding protein	-	blr3200	*Bradyrhizobium japonicum* USDA 110	Transport and binding proteins	22	492	33,968	7.66	Periplasmic	5.63 (pH4.5N)	⁄	⁄
20	ABC transporter substrate-binding protein	-	blr5675	*Bradyrhizobium japonicum* USDA 110	Transport and binding proteins	11	384	40,020	8.95	Periplasmic	1.55 (pH4.5N)	⁄	-
21	hypothetical protein	-	bll6649	*Bradyrhizobium japonicum* USDA 110	Conserved hypothetical protein	13	223	18,033	6.74	Unknown	6.47 (pH5.5A)	-	⁄
22	10 kDa chaperonin	*groES*	bsr7532	*Bradyrhizobium japonicum* USDA 110	Cellular processes	21	360	10,708	6.59	Cytoplasmic	9.44 (pH5.5A)	⁄	⁄
23	10 kDa chaperonin 1	*groES1*	blr5226	*Bradyrhizobium japonicum* USDA 110	Cellular processes	6	81	11,130	6.10	Cytoplasmic	2.43 (pH5.5A)	-	⁄
24	Peptidoglycan-associated protein	-	ZMO 1354	*Zymomonas mobilis*	-	2	76	27,353	6.92	Outer Membrane	-6.02 (pH4.5A)	⁄	⁄

Table 4.2 (Continued).

Spot no.	Tophit protein	Gene code[a]	Locus name[b]	Organisms	Functional category[c]	Peptide matched[d]	Mascot score[e]	MW[f] (kDa)	PI[g]	Predicted localization[h]	Fold change	Up/down regulation	
												N	A
25	unknown protein	-	-	*Zymomonas mobilis* subsp. mobilis ZM4	-	14	562	68,508	4.81	Cytoplasmic	-308 (pH4.5N)	/	/
26	Ribosomal protein S1	-	-	*Sphingomonas* sp. SKA58	-	7	245	61,616	5.01	Cytoplasmic	-292 (pH4.5N)	/	/
27	Chaperonin GroEL	*groEL*	-	*Sphingopyxis alaskensis* RB2256	Cellular processes	27	617	57,917	5.07	Cytoplasmic	-2.12 (pH5.5A)	/	/
28	Translation elongation factor Tu	-	-	*Sphingopyxis alaskensis* RB2256	Translation	20	594	43,040	5.11	Cytoplasmic	-3.12 (pH4.5A)	/	/
29	Hypothetical protein	-	-	*Prochlorococcus marinus* subsp. pastoris	-	4	54	17,200	9.12	Unknown	-4.42 (pH4.5N)	/	/

[a]Genes Mascot code obtain from rhizobase (http://genome.kazusa.or.jp/rhizobase/Bradyrhizobium).

[b]Locus name obtain from rhizobase (http://genome.kazusa.or.jp/rhizobase/Bradyrhizobium).

[c]Functional category obtain from rhizobase (http://genome.kazusa.or.jp/rhizobase/Bradyrhizobium).

[d]Peptide match from Mascot search (http://www.matrixscience.com).

[e]Mascot score obtain from Mascot search (http://www.matrixscience.com).

[f]MW obtain from search (http://www.matrixscience.com).

[g]PI value obtain from mascot search (http://www.matrixscience.com).

[h]Predicted localization obtained from PSORTb version 3.0.0 (http://www.psort.org/psortb/).

The 29 identified proteins were grouped into 8 categories based on COGs and one group of unknown categories: (i) Cellular processes, consisted of 6 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, consisted of 7 hypothetical proteins (spot no.1, 2, 3, 5, 13, 16 and 29); (iii) Transport and binding proteins, consisted 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, consisted of 4 proteins including 30S ribosomal protein S6 (spot no.9), Ribosomal protein L7/L12 (spot no.10), two protein 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) inter conversions and salvage of nucleosides and nucleotides, nucleoside diphosphate kinase (spot no.12); (viii) other categories, consisted of 2 proteins including oxidoreductase (spot no.8), and peroxiredoxin (spot no.11); and one group of unknown categories, consisted 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). More details of protein characteristic were discussed below according to their response to acid pH under normal and adaptive growth conditions.

The protein profile derived from cells grown at pH 4.5 under adaptive growth condition (pH 4.5A) revealed six major spots were up-regulated. The hypothetical protein (spot no. 3) was 45.63-fold up-regulated in this condition when compared with growth at pH 6.8. This protein was also up-regulated in cells grown at pH 4.5N. The

result indicated that this protein would play an important role in acid tolerant in *Bradyrhizobium* sp. DASA01007. However, the function of this protein would necessary be further identified. The second major spot was the 10 kDa chaperonin (spot no.14), which was 20.03-fold up-regulated in pH 4.5A condition. The chaperonin molecules are required for correct folding and assembly of some protein during normal cell growth. These protein were also induced by several stress conditions for stabilization and protection the disassembled polypeptides under stress conditions (Schmidt et al., 1992). The 10 kDa chaperonin or GroES protein exists as a ring-shaped oligomer with 6-8 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). Chaperonin 60 kDa or GroEL (spot no.6) was also up-regulated in cells grown at acid pH under both normal and adaptive conditions.

This result indicated that GroEL and GroES probably play an important role during growth under acid condition. GroEL has been extensively studied in great detail. It interacts with a wide range of unfolded proteins. BLAST searches of their complete genomes, together with cloning and genetic analysis described many bacteria contain several copies of *cpn*60 gene, with the highest record currently held by *B. japonicum* USDA110 at seven copies (Lund, 2009). Interesting, GroEL protein is involved in *nif* gene regulation in *B. japonicum* (Rodrigues et al., 2006). The simplest model was proposed that one or more of chaperonin proteins assembled with one or more of nitrogenase component and assists the proper folding of nitrogenase protein complex, and finally link to nodulation and nitrogen fixation efficiency of bacteria (Lund, 2009). This model could also be linked to previous plant experiment inoculated with *Bradyrhizobium* sp. DASA01007. The nitrogen fixation efficiency of

this strain tended to be higher than other strains when soybean was grown under acid condition, it may be due to up-regulation of chaperonin in this strain, which resulting in assist the proper protein folding of nitrogenase component. However, this model need to be clarified since the specificity between chaperonin and nitrogenase protein may affect the protein folding and its function (Fischer et al., 1999). The third major protein spot found to be up-regulated at pH 4.5A was 30s ribosomal protein S6 (spot no. 9), which was 14.69-fold up-regulated at pH 4.5A. The 30s ribosomal protein S6 was incorporated with S18 to 16S ribosomal RNA during translation process (Wilson and Nierhaus, 2005). However, 30S ribosomal protein S6 has been identified as a cold shock protein in *E. coli* and *B. subtilis*, suggesting that 30S ribosomal protein S6 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 control some protein synthesize under acid stress condition.

The fifth major protein spot was peroxiredoxin (spot no. 11), which was 4.98-fold up-regulated at pH 4.5A. Peroxiredoxins are antioxidant enzymes that control cytokine-induced peroxide levels which mediate signal transduction in mammalian cells (Wood et al., 2003). However, peroxiredoxin has been reported to be strongly induced during symbiosis with common bean and involved in the defence of *R. etli* bacteroids against oxidative or hydrogen peroxide stress (Dombrecht et al., 2005). Therefore, it is interesting that this protein was up-regulated in free living of *Bradyrhizobium* sp. DASA01007. It could be possible that peroxiredoxin may have other roles in protecting cell against acid stress condition, however the mechanism is unclear. The last major protein that highly expressed in pH 4.5A was ribosomal

protein L7/L12 (spot no.10), which showed 4.71-folds up-regulated in this condition. The ribosomal protein L7/L12 forms a functionally important domain in the ribosome. This domain is involved in interaction with translation factors during protein biosynthesis (Gudkov, 1997). However, there was no clearly evidence showing that this protein is involved in stress tolerance.

On the other hands, the up-regulated proteins in cells grown at pH 4.5N were different from the protein up-regulated in pH 4.5A. Protein involved in transport and binding protein including ABC transporter sugar-binding protein, ABC transporter amino acid-binding protein, and ABC transporter substrate-binding protein were up-regulated. This group of protein may play an important role in exchange and accumulate the nutrient or compatible solute that necessary for cell survival under stress condition. The elongation factor Tu (EF-Tu) was also up-regulated in pH 4.5N. 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. More information of this protein has been described in 1D-PAGE experiment. Nevertheless, some proteins were down-regulated when grown at acid condition. These proteins were peptidoglycan-associated protein, ribosomal protein S1, chaperonin GroEL, elongation factor Tu, hypothetical protein, and unknown protein. However, it should be notified that these down-regulated proteins were matched with protein presented in other bacteria, not in *Bradyrhizobium*. This result may be implied that all up-regulated proteins detected in this research would be involved in acid tolerant or acid adaptive tolerant response. However, the acid tolerant function of these proteins needs to be verified through molecular approached in further

experiment. Protein localization of this protein was predicted by PSORTb version 3.0.0 (http://www.psort.org/psortb/), and the result showed that this protein might be local proteins.

Protein produced from pH 4.5A consist of proteins in cellular processes (60 kDA chaperonin 6 and 10 kDa chaperonin), conserved hypothetical protein, translation (30 ribosomal protein S6), energy metabolism (ATP synthaes subunit beta), regulatory functions (Two-component response regulator (*tcs*R)), and interconversions and salvage of nucleosides and nucleotides (Nucleoside diphosphate kinase (*ndk*)) group were up regulated. Transport and binding proteins were absence in pH 4.5A condition. In pH 4.5N were found up-regulated proteins such as hypothetical protein, transport and biding proteis (ABC transporter sugar-binding protein, ABC transporter amino acid-binding protein), and translation protein (elongation factor Tu). As for pH 5.5A were found protein up-related in cellular processes (10 kDa chaperonin and 10 kDa chaperonin1). Finally, in normal condition (pH6.8) up-regulated proteins were found such as cellular processes (60 kDa chapoeronin 1, 60 kDa chaperonin 6), translation protein (EF-Tu).

However, most study about genes involving the acid stress response in rhizobia have been conducted with *Sinorhizobium medicae* (former *S. meliloti* WSM419). By using a transposon mutagenesis system, a functionally diverse set of pH responsive and acid tolerance related genes could be identified. Gene products required for acid tolerance in *S.mediceae* are for example ActP and an apolipoprotein acyltransferase. A gene coding for a regulatory protein known to be required for the acid tolerance in *S. mediceae* is *actR*. The encoded response regulatory ActR is activated by its corresponding sensor histidine kinase ActS, whose loss also lead to

susitivity to low pH. This somewhat similar to our finding that two component response regulator (*tcsR*) function regulation was found. In addition, the transcriptomic response of *S. meliloti* 1021 following a shift to acidic pH was conducted recently (Hellweg et al., 2009). The result reviewed that strong permanent up-regulation protein such as signal peptide for secretion, protease, lyzozyme and two component system as well as *lpiA* which is necessary for the lipid lysl-phosphatidylglycerol formation in *R. tropici* in low pH medium. This point was a modification of the exterior cell wall by a change of the lipid–structure. Another early induction was observed for *exoV* and *exoH* coding for proteins of the exopolysaccharide I (EPSI) biosynthesis but could not be found in *Bradyrhizobium* sp. DASA01007. For the intermediated permanent up-regulation proteins of *S. meliloti* 1021 in acid condition were again produced from *exo* and *katC* genes. The gene *katC* was annotated as catalase. The induction of a catalase in response in low pH seems reasonable to decompose hydrogen peroxide, since a lowered pH favors the generation of radicals by the Fenton reaction. This is also similar to our result which showed up-regulation of peroxiredoxins in acid condition. Peroxiredoxins also known as thiol-specific antioxidants which detoxifies hydrogen peroxide, akyl hydroperoxides, and peroxynitrite was a major protein in *Bradyrhizobium* (Sarma and Emerich, 2006).

4.6 Acid response genes isolation

To verify the function of up-regulated proteins of *Bradyrhizobium* sp. DASA01007 in response to acid condition, 4 protein spots (Hypothetical protein, bll5845, spot no.3; Peroxiredoxin, bll1317, spot no.11; 10 kDa chaperonin, blr5625,

spot no.14; and 10 kDa chaperonin, bsr7532, spot no.22) that highly be expressed when grown under adaptive to acid condition were selected for gene isoaltion (Fig. 4.13). Genes encoding these four proteins were isolated from genomic DNA of *Bradyrhizobium* sp. DASA01007 by PCR technique. The primer pairs and PCR condition were optimized to obtain the whole open reading frame of interested genes, and the expected size of PCR products were obtained (Fig. 4.14).

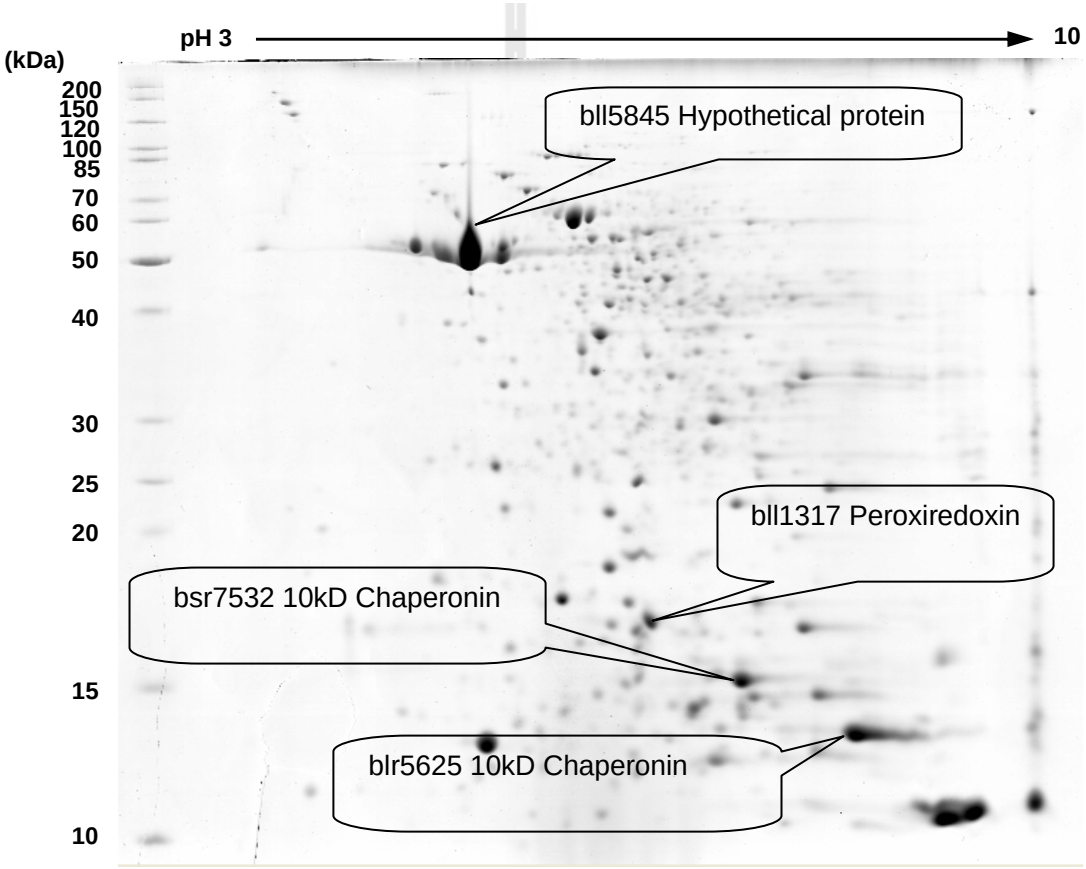

Figure 4.13 Protein spots selected from *Bradyrhizobium* sp. DASA 01007.

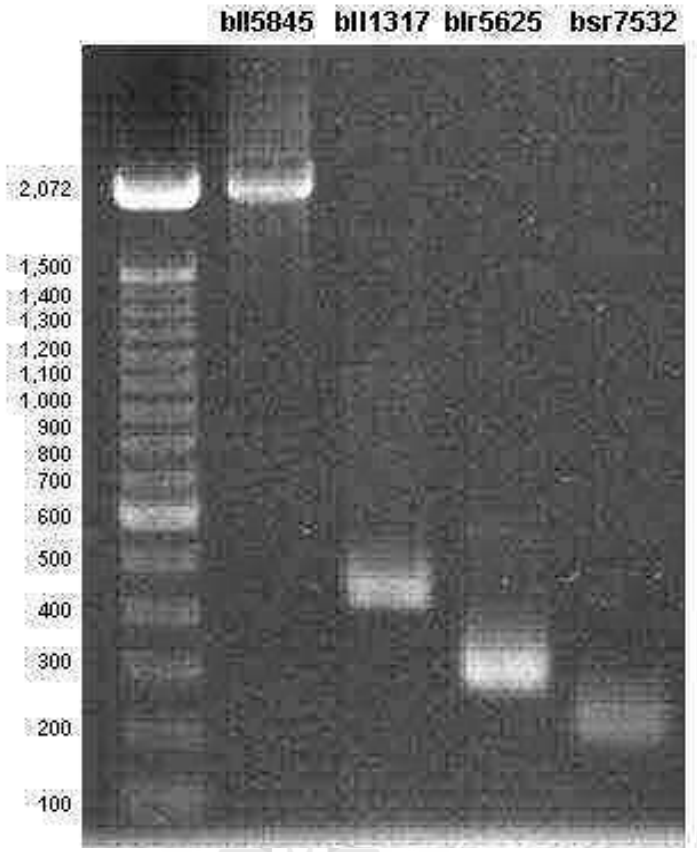

Figure 4.14 PCR products from selected genes of *Bradyrhizobium* sp. DASA 01007.

The derived PCR products were sequenced and aligned with the sequence of genes of interest from *B. japoncum* USDA110 to ensure the accuracy of isolated genes. Figure 4.15-4.18 showed the nucleotide and their deduced amino acid alignments of isolated genes with gene and proteins from *B. japonicum* USDA110. The derived PCR product of hypothetical protein (spot no.3) showed 92% and 60% identity with gene locus bll5845 and its amino acid sequence of *B. japonicum* USDA110. The low identity of DNA and amino acid sequence may due to incomplete sequencing of PCR product which needed to be verified by primer walking. However, the peptides sequence derived from 2D-PAGE were presented in

the deduced amino acid of isolated gene, which ensure that the correct gene was isolated. On the other hands, the derived PCR product of peroxiredoxin (spot no.11), 10 kDa chaperonin (spot no.14) and 10 kDa chaperonin (spot no.22) showed 96, 93, and 98% identity at nucleotide level with gene locus bll1317, blr5625, and bsr7532, respectively of *B. japonicum* USDA110. While the deduced amino acid from these genes showed 95, 94, and 96% identity at amino acid sequence, respectively with proteins in *B. japonicum* USDA110. The high identity of nucleotide and amino acid sequences between isolated gene and gene from *B. japonicum* USDA110 ensure the accuracy of isolated gene to be used in further experiment.

(A) DNA alignment of bll5845 open reading frame

Identities = 405/437 (92%)

```
01007: 45      atgtccggtatcgttctctcctcgtcggttcgtcagaacctgctctccctccagtccacc 104
               ||||||||||||||||||| || |||||||||||||||| || || ||||||||||||
U110 : 6416220 atgtccggtatcgttctctcttcctcggttcgtcagaaccttctttctctccagtccacc 6416161

01007: 105     gctgaccttctcgccaccacacagaaccgtctgtcgaccggcaagagcgtcaactcggcc 164
               |||||||| |||||||||||| |||| ||||||||||||||||||||||||||||||||
U110 : 6416160 gctgacctcctcgccaccacgcagagccgtctgtcgaccggcaagagcgtcaactcggcc 6416101

01007: 165     ctggacaatcccaccaacttcttcaccgcccagtcgctcgacaaccgcgccagcgacatc 224
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U110 : 6416100 ctggacaatcccaccaacttcttcaccgcccagtcgctcgacaaccgcgccagcgacatc 6416041

01007: 225     aacaatctgctcgacggcatcgccaacggcgtgcaggtgctgcaggccgccaacaccggc 284
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U110 : 6416040 aacaatttgctcgacggcatcgccaacggcgtgcaggtgctgcaggccgccaacaccggc 6415981

01007: 285     atcacctcgctgcagaagctgatcgacagcgccaagtcgatcgccaaccaggcgctgcag 344
               |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
U110 : 6415980 atcacctcgctgcagaagctgatcgacagcgcgaagtcgatcgccaaccaggcgctgcag 6415921

01007: 345     accaccgtcggctactccaccaagtccaacgtctccaccacgattgctggtgcgacggct 404
               ||||| |||||||||||||||||||||||||||||||| ||  | |||||||| ||
U110 : 6415920 accacggtcggctactccaccaagtccaacgtctccaccaccatctcgggtgcgaccgcc 6415861

01007: 405     tcggacctgcgtggcacgaccagtttcaccagcgcggatgcgctgagcaacgtgctctat 464
                | || ||||| |||||||| || ||| ||||||||   |||   ||||||||| | |||
U110 : 6415860 gctgatctgcgcggcacgacgagcttcgccagcgcgaccgcgagcagcaacgtggtgtat 6415801

01007: 465     agcggcgcggccggcgg 481
               |||||||||||||||||
U110 : 6415800 agcggcgcggccggcgg 6415784
```

(B) Protein alignment of bll5845 open reading frame

Identities = 193/317 (60%), Positives = 213/317 (67%), Gaps = 15/317 (4%)

```
01007: 45  MSGIVLSSSVRQNLLSLQSTADLLATTQNRLSTG**KSVNSALDNPTNFFTAQSLDNR**ASDI 224
           MSGIVLSSSVRQNLLSLQSTADLLATTQ+RLSTGKSVNSALDNPTNFFTAQSLDNRASDI
U110 : 1   MSGIVLSSSVRQNLLSLQSTADLLATTQSRLSTG**KSVNSALDNPTNFFTAQSLDNR**ASDI 60

01007: 225 NNLLDGIANGVQVLQAANTGITSLQKLIDSAKSIANQALQTTVGYSTKSNVSTTIAGATA 404
           NNLLDGIANGVQVLQAANTGITSLQKLIDSAKSIANQALQTTVGYSTKSNVSTTI+GATA
U110 : 61  NNLLDGIANGVQVLQAANTGITSLQKLIDSAKSIANQALQTTVGYSTKSNVSTTISGATA 120

01007: 405 SDLRGTTSFTSADALSNVLYXXXXXXXXXXXXXXXXXXXVQGTNTGTVINA-----ATTGA 569
           +DLRGTTSF SA A SNV+Y                    G+   T   A     A TGA
U110 : 121 ADLRGTTSFASATASSNVVYSGAAGGTTAASGTTTLGASIGSFASTGATAGDGTTALTGA 180

01007: 570 SLL---NG-TXXXXXXXXXXXXXDTLTVNGKTIXXXXXXXXXXXXXXXXXXXXXGITTGN 737
             L   NG T             DTLTVNGKTI                          +GN
U110 : 181 ITLIATNGTTATGLAGNAQPADGDTLTVNGKTITFRSGAAPASTAVPSGSG-----VSGN 235

01007: 738 VFTDNSTGNVTVYLGSGTKAAVGDVLTAIDLASGVQ----SNVAGTLTVNSGQTISSVS- 902
           + TD + GN TVYL S T   V D+L+AIDLASGV+    S+ A T+ V++ Q  ++VS
U110 : 236 LVTDGN-GNTTVYLASAT---VNDLLSAIDLASGVKTVSISSGAATIAVSASQPGAAVST 291

01007: 903 -GGGALVLKSTYGSDLS 950
              GA+ LKS+ G+DLS
U110 : 292 AAAGAVTLKSSTGADLS 308
```

Figure 4.15 Alignment of DNA (A) and protein (B) of bll5845 open reading frame *B. japonicum* USDA110 and from *Bradyrhizobium* sp. DASA01007. The identified peptides from 2D-PAGE were highlighted in bold and underline.

(A) DNA alignment of bll1317 open reading frame

Identities = 441/459 (96%)

```
01007: 48      gcctgacacctcgaccttgccgggcatcgcttcgaggttcagcttcttgaccacgccgtc 107
               ||||||||||||||||||||||||||||||| |||||||||||||||| ||||||||||
U110 : 1429738 gcctgacacctcgaccttgccgggcatcgcctcgaggttcagcttcttcaccacgccgtc 1429797

01007: 108     ctcgaccagcatcgaatagcgcttggagcggatgccgaggccattgccggaggcgtccag 167
               |||||||||||||||||||||||||||||||||||||||| ||| |||||||||| ||
U110 : 1429798 ctcgaccagcatcgaatagcgcttggagcggatgccgaggccgttggcggaggcgtcgag 1429857

01007: 168     ctccatgccgatcgccttggcgaagtcggcattgccgtcggcgaggaagatggcctcgtc 227
               |||||||||||||||||| |||||||||||||||||||||||| | |||||||||
U110 : 1429858 ctccatgccgatcgccttggtgaagtcggcattgccgtcggcgaggaatacggcctcgtc 1429917

01007: 228     gcgctggtcggtatcgcgcttccaggcgttcatgacgaaagcgtcgttgacggagacgat 287
               |||||||||||| |||||||||||||||||||||||| |||||||||||||||| |||
U110 : 1429918 gcgctggtcggtgtcgcgcttccaggcgttcatgacgaaggcgtcgttgacggagatgat 1429977

01007: 288     ggcgatggtgtcgacgcccttgtccttcatggcataggcgttgaggaagatgctcggcag 347
               |||||||||||||||||||||||||| ||||| ||||||||||||||||||||||||
U110 : 1429978 ggcgatggtgtcgacgcccttgtccttgatggcgtaggcgttgaggaagatgctcggcag 1430037

01007: 348     atgcatcttgtggcaggtgccggtgtaggcgccgggcactgcgaacagcgccacttttctt 407
               |||||||||||||||||||||||||||||||||||||||| ||||||||||||| |||||
U110 : 1430038 atgcatcttgtggcaggtgccggtgtaggcgccgggcaccgcgaacagcgccaccttctt 1430097

01007: 408     gcccttgaagatgtcgtcggtggtcttcacctgcgggccttccgccgtcatcacgcggaa 467
               |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U110 : 1430098 gcccttgaagatgtcgtcggtggtcttcacctgcgggccttccgccgtcatcacgcggaa 1430157

01007: 468     tttcgccccgggtagcttttcgccagtctggatcgccat 506
               ||||||| |||| ||||| ||||||||||||||||||||
U110 : 1430158 tttcgcctcgggcagcttgtcgccagtctggatcgccat 1430196
```

(B) Protein alignment of bll1317 open reading frame

Identities = 146/153 (95%), Positives = 150/153 (98%)

```
01007: 506 MAIQTGEKLPGAKFRVMTAEGPQVKTTDDIFKGKKVALFAVPGAYTGTCHKMHLPSIFLN 327
             MAIQTG+KLP AKFRVMTAEGPQVKTTDDIFKGKKVALFAVPGAYTGTCHKMHLPSIFLN
U110 : 1   MAIQTGDKLPEAKFRVMTAEGPQVKTTDDIFKGKKVALFAVPGAYTGTCHKMHLPSIFLN 60

01007: 326 AYAMKDKGVDTIAIVSVNDAFVMNAWKRDTDQRDEAIFLADGNADFAKAIGMELDASGNG 147
           AYA+KDKGVDTIAI+SVNDAFVMNAWKRDTDQRDEA+FLADGNADF KAIGMELDAS NG
U110 : 61  AYAIKDKGVDTIAIISVNDAFVMNAWKRDTDQRDEAVFLADGNADFTKAIGMELDASANG 120

01007: 146 LGIRSKRYSMLVEDGVVKKLNLEAMPGKVEVSG 48
           LGIRSKRYSMLVEDGVVKKLNLEAMPGKVEVSG
U110 : 121 LGIRSKRYSMLVEDGVVKKLNLEAMPGKVEVSG 153
```

Figure 4.16 Alignment of DNA (A) and protein (B) of bll1317 open reading frame *B. japonicum* USDA110 and from *Bradyrhizobium* sp. DASA01007. The identified peptides from 2D-PAGE were highlighted in bold and underline.

(A) DNA alignment of blr5625 open reading frame

Identities = 296/315 (93%)

```
01007: 47      atgaaattccgtccgcttcacgaccgcgtcgtggtcaagcgcatcgacgcagaagagaag 106
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U110 : 6186325 atgaaattccgtccgcttcacgaccgcgtcgtggtcaagcgcatcgacgcagaagagaag 6186384

01007: 107     accgctggcggcatcatcattcccgacactgccaaggaaaagccctcccagggcgaagtc 166
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U110 : 6186385 accgctggcggcatcatcattcccgacactgccaaggaaaagccctcccagggcgaagtc 6186444

01007: 167     gtcgccgttggccccggtggccgcgacgaagccggcaagctgatcccgatcgacctgaag 226
               |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
U110 : 6186445 gtcgccgtcggccccggtggccgcgacgaagccggcaagctgatcccgatcgacctgaag 6186504

01007: 227     gtcggcgaccgcgtgctgttcggcaagtggtccggcaccgaggtcaagatcgacggtcag 286
               |||||||| |||||||||||||||||||||||||||||||||||||||||||||||| |
U110 : 6186505 gtcggcgatcgcgtgctgttcggcaagtggtccggcaccgaggtcaagatcgacagcgtc 6186564

01007: 287     gacctgctgatcatgaaggagagcgacgtgatgggcgttctcgaagtcagcgagtccaag 346
               || ||  |||||||||||||| |||||| | |||||||| ||||| |||  ||  ||||||
U110 : 6186565 gatctcttgatcatgaaggaaagcgacatcatgggcgtcctcgacgtccccgcttccaag 6186624

01007: 347     aagaaggcggcctaa 361
               |||||||||||||||
U110 : 6186625 aagaaggcggcctaa 6186639
```

(B) Protein alignment of blr5625 open reading frame

Identities = 98/104 (94%), Positives = 100/104 (96%)

```
01007: 47  MKFRPLHDRVVVKRIDAEEK**TAGGIIIPDTAKEKPSQGEVVAVGPGGRDEAGK**LIPIDLK 226
           MKFRPLHDRVVVKRIDAEEKTAGGIIIPDTAKEKPSQGEVVAVGPGGRDEAGKLIPIDLK
U110 : 1   MKFRPLHDRVVVKRIDAEEK**TAGGIIIPDTAKEKPSQGEVVAVGPGGRDEAGK**LIPIDLK 60

01007: 227 VGDRVLFGK**WSGTEVK**IDGQDLLIMK**ESDVMGVLEVSESKK**KAA 358
           VGDRVLFGKWSGTEVKID  DLLIMKESDVMGVL VSESKKKAA
U110 : 61  VGDRVLFGK**WSGTEVK**IDSVDLLIMK**ESDIMGVLDVPASKK**KAA 104
```

Figure 4.17 Alignment of DNA (A) and protein (B) of blr5625 open reading frame *B. japonicum* USDA110 and from *Bradyrhizobium* sp. DASA01007. The identified peptides from 2D-PAGE were highlighted in bold and underline.

(A) DNA alignment of bsr7532 open reading frame
Identities = 242/246 (98%)

```
01007: 51      gttgtcgatcttgacctcggtgcccgaccacttgccgaacagcacgcggtcgccgacctt 110
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
U110 : 8265622 gttgtcgatcttgacctcggtgcccgaccacttgccgaacagcacgcggtcgccgacctt 8265563

01007: 111     gaggtcgatcgggatcagcttgccggcctcgtcgcggccgccggggccgacggcgacgac 170
               |||||||||||||||||||||||| |||||| ||||||||||||||||||||||||||
U110 : 8265562 gaggtcgatcgggatcagcttgccggtctcgtcacggccgccggggccgacggcgacgac 8265503

01007: 171     ctcgccctgggacggcttttccttggcggtgtccggaatgatgatgccgcccttggtctt 230
               |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
U110 : 8265502 ctcgccctgggagggcttttccttggcggtgtccggaatgatgatgccgcccttggtctt 8265443

01007: 231     ttcctcggcgtcgatacgtttgaccacgacacggtcatgcggcggacgaaatttggattt 290
               |||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
U110 : 8265442 ttcctcggcgtcgatacgtttgaccacgacacggtcatgcagcggacgaaatttggattt 8265383

01007: 291     agccat 296
               ||||||
U110 : 8265382 agccat 8265377
```

(B) Protein alignment bsr7532 open reading frame
Identities = 80/83 (96%), Positives = 81/83 (97%)

```
01007: 296 MAKSKFRPPHDRVVVKRIDAEEKTKGGIIIPDTAKEKPSQGEVVAVGPGGRDEAGKLIPI 117
           MAKSKFRP HDRVVVKRIDAEEKTKGGIIIPDTAKEKPSQGEVVAVGPGGRDE GKLIPI
U110 : 1   MAKSKFRPLHDRVVVKRIDAEEKTKGGIIIPDTAKEKPSQGEVVAVGPGGRDETGKLIPI 60

01007: 116 DLKVGDRVLFGKWSGTEVKIDNK 48
           DLKVGDRVLFGKWSGTEVKIDN+
U110 : 61  DLKVGDRVLFGKWSGTEVKIDNE 83
```

Figure 4.18 Alignment of DNA (A) and protein (B) of blr7532 open reading frame *B. japonicum* USDA110 and from *Bradyrhizobium* sp. DASA01007. The identified peptides from 2D-PAGE were highlighted in bold and underline.

4.7 Growth of *E. coli* transformants in acid condition

Each PCR product was cloned into pRK404A, a medium copies number plasmid (produced 3-7 copies per cells), in order to verify acid tolerant function in *E. coli*. The PCR product derived from hypothetical protein, spot no.3; Peroxiredoxin, spot no.11; 10 kDa chaperonin, spot no.14; and 10 kDa chaperonin, spot no.22 were cloned into pRK404A and named as 5845pRK404A, 1317pRK404A, 5625pRK404A, and 7532pRK404A, respectively. Each constructed plasmid was transformed into *E. coli* DH5α by electroporation resulting in 4 *E. coli* transformants. To verify the acid tolerant function of constructed plasmids, all *E. coli* transformants were grown in minimal medium at pH 7.0, 4.5, and 4.0 compared with wild-type *E. coli* DH5α (Fig. 4.19). The growth of transformant *E. coli* harboring 5625pRK404A had highest cell concentration when grown in minimal medium at pH 7.0, while the growth of *E. coli* harboring 5845pRK404A, 1317pRK404A, 7532pRK404A, as well as wild-type *E. coli* were similar when grown at pH 7.0. This result indicated that harboring of constructed plasmid 5625pRK404A could promote the growth, while other constructed plasmids did not affect the growth of cell when grown at neutral pH. Then, the growth of cell was determined in acid conditions at pH 4.5, and 4.0. The results showed that all *E. coli* transformants performed better growth than wild-type in all acid conditions. *E. coli* harboring plasmid 5625pRK404A had highest cell concentration when grown in minimal medium at pH 4.5 and 4.0. These results revealed the potential of isolated genes for promoting acid tolerance in *E. coli* transformants. However, the acid tolerant function of these genes is necessary to be further verified by trasfer into *Bradyrhizobium* sp. DASA01007 as well as gene knock out to construct the mutants.

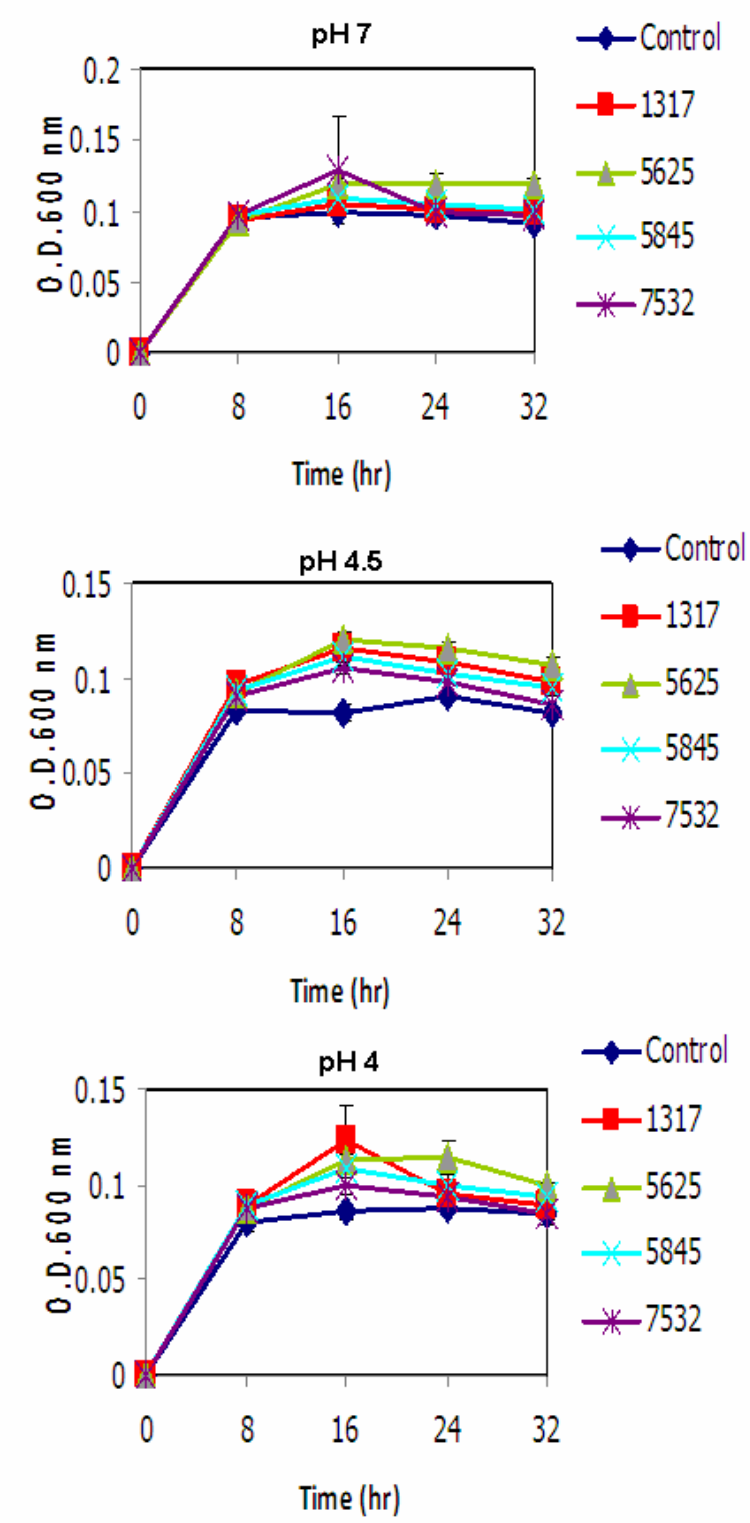

Figure 14.9 Growth of *E. coli* DH5α (control) and *E. coli* transformants in minimal medium at pH 7.0, 4.5, and 4.0.

CHAPTER V

CONCLUSION

This research provided interesting information of acid tolerant *Bradyrhizobium* and its symbiotic efficiency with soybean, as well as the proteins expression in response to acid condition. *Bradyrhizobium* sp. DASA01007 performed the best growth ability among other bradyrhizobia including *B. japonicum* USDA110 in acid condition (pH 4.5). Acid tolerant *Bradyrhizobium* sp. DASA01007 provided the highest symbiotic efficiency with soybean, especially nitrogen fixation. This strain performed better growth at pH 4.5 when grown in adaptive condition indicating an acid adaptive tolerant response mechanism is presented in the cell. To investigate proteins involving in acid tolerant or acid adaptive tolerant response, 1D- and 2D-PAGE techniques were used to determine proteins expression in acid pH under normal or adaptive conditions. Total of 29 identified proteins were identified in response to acid condition, these proteins were grouped into 8 categories based on their function and 1 group of unknown categories. Among these proteins, 23 proteins were up-regulated, while 6 proteins were down-regulated in acid condition when compared to protein expressed at neutral pH Chaperonin (GroEL and GroES) was another group of protein that highly expressed in cells grown at acid condition. Chaperonin assembled with one or more of nitrogenase component and assists the proper folding of nitrogenase protein complex. This model linked to plant experiment inoculated with *Bradyrhizobium* sp. DASA01007 could promote nitrogen fixation of

soybean grown under acid condition. Peroxiredoxins was aslo highly expressed under acid condition. It could be possible that peroxiredoxin may have other roles in protecting cell against acid stress condition. To verify the function of up-regulated proteins of *Bradyrhizobium* sp. DASA01007 in response to acid condition, 4 protein spots (Hypothetical protein, bll5845; Peroxiredoxin, bll1317; 10 kDa chaperonin, blr5625; and 10 kDa chaperonin, bsr7532) that highly expressed when grew under adaptive to acid condition were selected for gene isolation. PCR products derived from selected spots were cloned into pRK404A and transformed to *E. coli* DH5α. All *E. coli* transformants performed better growth than wild-type at all acid conditions. These results revealed the potential of isolated genes for promoting acid tolerance in *E. coli* transformants. However, the acid tolerant function of these genes is necessary to be further verified by transfer into *Bradyrhizobium* sp. DASA01007 as well as gene knock out to construct the mutants.

This research would be useful for agriculture applications. Acid torelant *Bradyrhizobium* can be used directly for soybean in acidic soil. As well as the strategy of growing cell in mild acid condition before exposure to extreme acid soil wil increase the symbiotic efficiency of soybean bradyrhizobium inoculant. The protein expression profiles in acid condition aslo provide useful information for further study of acid tolerant protein function in *Bradyrhizobium.*

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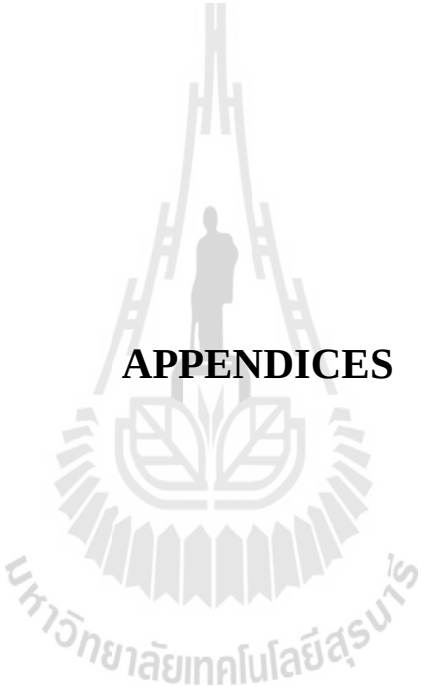
APPENDICES



APPENDIX A

Protein search results by Mascot search and Psortb search

Sequences of matched peptide were underline

Mascot search obtain from mascot search (http://www.matrixscience.com).

Predicted localization obtained from PSORTb version 3.0.0

(http://www.psort.org/psortb/).

Spot 1

Match to: **Q89HZ6_BRAJA** Score: **314**

Bll5843 protein *Bradyrhizobium japonicum.*

Nominal mass (M_r): **75633**; Calculated pI value: **4.87**

Sequence Coverage: **5%**

Matched peptides shown in **Bold and underline**

```
   1 MSNIVLSASV RQNLLSLQST ADLLATTQER LSTGKKVNTA LDNPTNFFTA
  51 QGLDNRASDI SNLLDGINNG VQVLQAANTG ITSLQKLIDS AKSIANQALQ
 101 TTVGYSTKSN VSTTIPGATP ADLRGTTSYA SATANSNVLY TGAAGGVTPV
 151 TGTAALGASL GSNAGSAVGF AATAADGTTV LSGTATLLGT TASTTFGAPP
 201 ADGDTITVNG KTITFRTGIP PTTQPTGWGL DASGHIATDG NGNSIVYEGT
 251 AVAPRATVND VLSAIDLASG VKTATISAGA AAIAVSGSTG PIGTLQVASS
 301 ISGGGQVTLK SSTGADLSVT GKADFLNKLG LTTATGAGNA NVTANRSTTA
 351 GSLGTLVQDG STLNIDGHTI TFKNAQTPQS AASVTSGGVN GNIVTDGNGN
 401 STVYIQSATL TDLLNSIDLA TGVKTASIFN GAASLTTTAG QIPSSVNSSG
 451 QLALSTGINA DLSITGTGNA LHAFGLSGNT GTATAFTAAR TSGVGGVSGK
 501 TLTFTSFNGG TPVNVTFGDG TNGTVKTLDQ LNAQLQANHL TATIDANGLL
 551 TVTTVNEYAS STLGSTTAGG TVGGTITGIL AFTTAQPPVQ DPVAQTARSN
 601 LVNQFNNILA QIDTTSQDSS FNGVNLLNGD TLKLVFNETG SSTLGINGVV
 651 FNAAGLGLSN LVNGVDFIDN GATNKVLTSL NAASSTLRSE GSALGSNLSI
 701 VQVRQDFSKN LINVLQTGSS NLTLADTNEE AANSQALSTR QSIAVSALSL
 751 ANQSQQSVLQ LLR
```

Localization Scores:

Cytoplasmic	0.00
CytoplasmicMembrane	0.00
Periplasmic	0.00
OuterMembrane	5.87
Extracellular	4.13

Final Prediction:

Unknown (This protein may have multiple localization sites.)

Secondary localization(s):

S-layer

Spot 2

Match to: **Q357K5_9BRAD** Score: **349**

Hypothetical protein. *Bradyrhizobium* sp. BTAi1.

Nominal mass (M_r): **51090**; Calculated pI value: **4.97**

Sequence Coverage: **14%**

Matched peptides shown in **Bold and underline**

```
   1 MSGIVLSASV RQNLLSLQST AQLLATTQNN LATGKKVNSA LDNPTNFFTA
  51 QGLDNRASDI SNLLDGIGNG VQVLQAANTG ITSLQKLVDS AKSIANQVLQ
 101 SSVGYSTKSN VTSAALAGAT ASSLIGASTT AVTGSVVLND NTSSAVAITG
 151 TTKLSGTPGT SSNDLASSIT TGDTLVVNGT TFTFIAGTSS SGTNIGVGDT
 201 VTNLLSTIQS ATGVTSSITA GAITLTPPAA GLTLSGTSLA KLGLSAVGNS
 251 LSGQTLTIAA TGGGTATSIT FGLGTGQVNS LNDLNTKLAA NNLQASFDTS
 301 SGKISITTTN DAASATIGAI GGTAAASSQS FNGLTAAAPV ADATAQSQRS
 351 SLVAQYNNVL QQINTTAADA SFNGVNLLNG DTLKLTFNET GKSSLSITGV
 401 TFNIAGLGLS NLTAGTDFLD NNSANKVLNV LNTASSTLRS EASTLGSNLS
 451 VVQIRQDFNK NLINVLQTGS SNLTLADTNE EAANSQALST RQSIAVSALS
 501 LANQSQASVL QLLR
```

Localization Scores:

Cytoplasmic	0.00
CytoplasmicMembrane	0.00
Periplasmic	0.00
OuterMembrane	0.04
Extracellular	9.96

Final Prediction:

Extracellular	9.96

Secondary localization(s):

Flagellar

Spot 3

Match to: **Q357K5_9BRAD** Score: **260**

Hypothetical protein. *Bradyrhizobium* sp. BTAi1.

Nominal mass (M_r): **51090**; Calculated pI value: **4.97**

Sequence Coverage: **8%**

Matched peptides shown in **Bold and underline**

```
   1 MSGIVLSASV RQNLLSLQST AQLLATTQNN LATGKKVNSA LDNPTNFFTA
  51 QGLDNRASDI SNLLDGIGNG VQVLQAANTG ITSLQKLVDS AKSIANQVLQ
 101 SSVGYSTKSN VTSAALAGAT ASSLIGASTT AVTGSVVLND NTSSAVAITG
 151 TTKLSGTPGT SSNDLASSIT TGDTLVVNGT TFTFIAGTSS SGTNIGVGDT
 201 VTNLLSTIQS ATGVTSSITA GAITLTPPAA GLTLSGTSLA KLGLSAVGNS
 251 LSGQTLTIAA TGGGTATSIT FGLGTGQVNS LNDLNTKLAA NNLQASFDTS
 301 SGKISITTTN DAASATIGAI GGTAAASSQS FNGLTAAAPV ADATAQSQRS
 351 SLVAQYNNVL QQINTTAADA SFNGVNLLNG DTLKLTFNET GKSSLSITGV
 401 TFNIAGLGLS NLTAGTDFLD NNSANKVLNV LNTASSTLRS EASTLGSNLS
 451 VVQIRQDFNK NLINVLQTGS SNLTLADTNE EAANSQALST RQSIAVSALS
 501 LANQSQASVL QLLR
```

Localization Scores:

Cytoplasmic	0.00
CytoplasmicMembrane	0.00
Periplasmic	0.00
OuterMembrane	0.04
Extracellular	9.96

Final Prediction:

Extracellular	9.96

Secondary localization(s):

Flagellar

Spot 4

Match to: **Q89X74_BRAJA** Score: **712**

ATP synthase beta chain. *Bradyrhizobium japonicum*.

Nominal mass (M_r): **50987**; Calculated pI value: **5.13**

Sequence Coverage: **38%**

Matched peptides shown in **Bold and underline**

```
   1 MAAQSGRVTQ VIGAVVDVQF EGHLPAILNS LETKNGGNRL VLEVAQHLGE
  51 STVRTIAMDT TEGLVRGQEV TDTGSPIRVP VGEGTLGRII NVIGEPIDEA
 101 GPVKSEGLRA IHQEAPTYTD QSTEAEILVT GIKVVDLLAP YAKGGKIGLF
 151 GGAGVGKTVL IQELINNVAK AHGGYSVFAG VGERTREGND LYHEFIESKV
 201 NADPHNPDPS VKSKCALVFG QMNEPPGARA RVALTGLTIA EDFRDKGQDV
 251 LFFVDNIFRF TQAGSEVSAL LGRIPSAVGY QPTLATDMGA LQERITTTQK
 301 GSITSVQAIY VPADDLTDPA PATSFAHLDA TTTLSRSIAE KGIYPAVDPL
 351 DSTSRMLSPL VVGEEHYAVA RQVQQVLQRY KALQDIIAIL GMDELSEEDK
 401 LTVARARKVE RFMSQPFHVA EIFTGSPGKF VDLADTIKGF KGLVEGKYDH
 451 LPEAAFYMVG TIEEAVEKGK KLAAEAA
```

Localization Scores:

Cytoplasmic	3.70
CytoplasmicMembrane	6.29
Periplasmic	0.01
OuterMembrane	0.00
Extracellular	0.00

Final Prediction:

Unknown (This protein may have multiple localization sites.)

Spot 5

Match to: **Q357K5_9BRAD** Score: **330**

Hypothetical protein. *Bradyrhizobium* sp. BTAi1.

Nominal mass (M_r): **51090**; Calculated pI value: **4.97**

Sequence Coverage: **14%**

Matched peptides shown in **Bold and underline**

```
   1 MSGIVLSASV RQNLLSLQST AQLLATTQNN LATGKKVNSA LDNPTNFFTA
  51 QGLDNRASDI SNLLDGIGNG VQVLQAANTG ITSLQKLVDS AKSIANQVLQ
 101 SSVGYSTKSN VTSAALAGAT ASSLIGASTT AVTGSVVLND NTSSAVAITG
 151 TTKLSGTPGT SSNDLASSIT TGDTLVVNGT TFTFIAGTSS SGTNIGVGDT
 201 VTNLLSTIQS ATGVTSSITA GAITLTPPAA GLTLSGTSLA KLGLSAVGNS
 251 LSGQTLTIAA TGGGTATSIT FGLGTGQVNS LNDLNTKLAA NNLQASFDTS
 301 SGKISITTTN DAASATIGAI GGTAAASSQS FNGLTAAAPV ADATAQSQRS
 351 SLVAQYNNVL QQINTTAADA SFNGVNLLNG DTLKLTFNET GKSSLSITGV
 401 TFNIAGLGLS NLTAGTDFLD NNSANKVLNV LNTASSTLRS EASTLGSNLS
 451 VVQIRQDFNK NLINVLQTGS SNLTLADTNE EAANSQALST RQSIAVSALS
 501 LANQSQASVL QLLR
```

Localization Scores:

Cytoplasmic	0.00
CytoplasmicMembrane	0.00
Periplasmic	0.00
OuterMembrane	0.04
Extracellular	9.96

Final Prediction:

Extracellular	9.96

Secondary localization(s):

Flagellar

Spot 6

Match to: **BAC50891** Score: **1764**

BA000040 NID: *Bradyrhizobium japonicum* USDA 110

Nominal mass (M_r): **57716**; Calculated pI value: **5.45**

Sequence Coverage: **51%**

Matched peptides shown in **Bold and underline**

```
  1 MAAKEVKFSV DARDKMLRGV DILANAVKVT LGPKGRNVVL DKSFGAPRIT
 51 KDGVTVAKEI ELEDKFENMG AQMVREVASK SADAAGDGTT TATVLAQAIV
101 REGAKSVAAG MNPMDLKRGI DLAVEAVVAD LVKNSKKVTS NDEIAQVGTI
151 SANGDAEIGK FLADAMKKVG NEGVITVEEA KSLETELDVV EGMQFDRGYI
201 SPYFVTNADK MRVEMDDAYI LINEKKLSSL NELLPLLEAV VQTGKPLVIV
251 AEDVEGEALA TLVVNRLRGG LKVAAVKAPG FGDRRKAMLQ DIAILTGGQA
301 ISEDLGIKLE NVTLNMLGRA KKVMIDKENT TIVNGAGKKA DIEARVSQIK
351 AQIEETTSDY DREKLQERLA KLAGGVAVIR VGGATEVEVK ERKDRVDDAM
401 HATRAAVEEG IVPGGGVALL RASEQLKGLR TKNDDQKTGV EIVRKALSAP
451 ARQIAINAGE DGSVIVGKIL ENKTYAYGFD SQTGEYVNLV TKGIIDPTKV
501 VRTAIQNAAS VAALLITTEA MVAELPKKGG AGPAMPPGGG MGGMDF
```

Localization Scores:

Cytoplasmic	9.26
CytoplasmicMembrane	0.24
Periplasmic	0.48
OuterMembrane	0.01
Extracellular	0.01

Final Prediction:

Cytoplasmic	9.26

Spot 7

Match to: **Q89V62_BRAJA** Score: **310**

Two-component response regulator. *Bradyrhizobium japonicum*.

Nominal mass (M_r): **23989**; Calculated pI value: **5.07**

Sequence Coverage: **24%**

Matched peptides shown in **Bold and underline**

```
   1 MNAPSTHLVI ADDHPLFRDA LRQAVASVLT SARIDEAGSF EDLTKLLEQT
  51 SDVDLILLDL SMPGISGFSG LIYLRAQYPA IPVVIVSASD DSATIRRSLD
 101 FGASGFIPKR FGVETLRDAI LKVMEGDVWV PADTDLSAAT DPDMTRLRDR
 151 LVTLTPQQVR VLMMLSEGLL NKQIAYELGV SEATIKAHVS AILQKLGVES
 201 RTQAVIAAAR IAGGQWKQGT STG
```

Localization Scores:

Cytoplasmic	9.26
CytoplasmicMembrane	0.24
Periplasmic	0.48
OuterMembrane	0.01
Extracellular	0.01

Final Prediction:

Cytoplasmic	9.26

Spot 8

Match to: **Q89R44_BRAJA** Score: **357**

Oxidoreductase. *Bradyrhizobium japonicum.*

Nominal mass (M_r): **31520**; Calculated pI value: **6.92**

Sequence Coverage: **27%**

Matched peptides shown in **Bold and underline**

```
   1 MSASTSQNQR IAVVGLGSMG FGMATSLKRA GHAVTGCDVS ADAVARFVKD
  51 GGAGAKTPAE AARGADVVVS VVVNAAQTET ILFGKDGVAE TMPKDSVFLS
 101 SATMDPDVAR RLAKQLEATG RHYLDAPISG GAQRAAQGEL TILASGSPAA
 151 FAKARPALDA MAAKLYELGD AAGQGAAFKM INQLLAGVHI AAASEAMAFA
 201 AKQGLDIRKV YEVITASAGN SWMFENRMPH VLDGDYTPRS AVEIFVKDLG
 251 IIQDMARSAR FPVPVSAAAL QMFLMTAAAG MGRDDDASVA RMYAQVTGVK
 301 LPGDK
```

Localization Scores:

Cytoplasmic	2.00
CytoplasmicMembrane	2.00
Periplasmic	2.00
OuterMembrane	2.00
Extracellular	2.00

Final Prediction:

Unknown

Spot 9

Match to: **BAC49344** Score: **257**

BA000040 NID: *Bradyrhizobium japonicum* USDA 110

Nominal mass (M_r): **18616**; Calculated pI value: **5.46**

Sequence Coverage: **34%**

Matched peptides shown in **Bold and underline**

```
  1 MALYEHVFLA RQDASPQQVE ELTAQMTGIV EGLGGKVTKT ENWGVRSLTY
 51 RMNKNRKAHF VLLNIDAPSA AIAEIERQER ISEDVIRYLS VRVEELEEGP
101 SAMMRKADRD RERDDRGGGF RGEREGGFRG DREGGFRGGD RDGGGFRGDR
151 GPRRPREEAE TATDGE
```

Localization Scores:

Cytoplasmic	9.26
Cytoplasmic Membrane	0.24
Periplasmic	0.48
OuterMembrane	0.01
Extracellular	0.01

Final Prediction:

Cytoplasmic	9.26

Spot 10

Match to: **Q373S9_RHOPA** Score: **310**

Ribosomal protein L7/L12.- *Rhodopseudomonas palustris* BisA53.

Nominal mass (M_r): **12694**; Calculated pI value: **5.02**

Sequence Coverage: **43%**

Matched peptides shown in **Bold and underline**

```
   1 MADLQKIVDD LSSLTVLEAA ELAKLLEEKW GVSAAAAVAV AGPAAAAAAP
  51 AEEKTEFTVV LASAGDKKIE VIKEVRAITG LGLKEAKDLV EGAPKPVKEG
 101 VNKDEAEKVK AQLEKAGAKV ELK
```

Localization Scores:

Cytoplasmic	0.00
CytoplasmicMembrane	3.24
Periplasmic	6.49
OuterMembrane	0.14
Extracellular	0.14

Final Prediction:

Spot 11

Match to: **Q89UU2_BRAJA** Score: **626**

Peroxiredoxin. *Bradyrhizobium japonicum*.

Nominal mass (M_r): **17414**; Calculated pI value: **6.11**

Sequence Coverage: **50%**

Matched peptides shown in **Bold and underline**

```
  1 MAIQTGDKLP EAKFRVMTAE GPQVKTTDDI FKGKKVALFA VPGAYTGTCH
 51 KMHLPSIFLN AYAIKDKGVD TIAIISVNDA FVMNAWKRDT DQRDEAVFLA
101 DGNADFTKAI GMELDASANG LGIRSKRYSM LVEDGVVKKL NLEAMPGKVE
151 VSGGDTLLGQ L
```

Localization Scores:

Cytoplasmic	2.00
CytoplasmicMembrane	2.00
Periplasmic	2.00
OuterMembrane	2.00
Extracellular	2.00

Final Prediction:

Unknown

Spot 12

Match to: **BAC49384** Score: **277**

BA000040 NID: *Bradyrhizobium japonicum* USDA 110

Nominal mass (M_r): **15050**; Calculated pI value: **6.75**

Sequence Coverage: **33%**

Matched peptides shown in **Bold and underline**

```
   1 MAIERTFSII KPDATARNLT GAVNAVIEKA GLRIVAQKRI RMTKEQAETF
  51 YAVHKARPFF GELVEFMTSG PVVVQVLEGE NAVAKYRDAM GATDPSKAAE
 101 GTIRKLYAKS IGENSAHGSD APETAAIEIA QFFSGNEIVG
```

Localization Scores:

Cytoplasmic	9.26
CytoplasmicMembrane	0.24
Periplasmic	0.48
OuterMembrane	0.01
Extracellular	0.01

Final Prediction:

Cytoplasmic	9.26

Spot 13

Match to: **Q89SG9_BRAJA** Score: **216**

Bll2431 protein. *Bradyrhizobium japonicum*.

Nominal mass (M_r): **16778**; Calculated pI value: **7.85**

Sequence Coverage: **26%**

Matched peptides shown in **Bold and underline**

```
   1 MTINRRDLAL STLAVPALAI SALALTTPAL AAAADEEAVA KKVEAFRLAQ
  51 IAADPKALGA LCWDDLSYSH SSGKVEDKAT FITNATDGKS KFLSIEYKDP
 101 TIKVVGPAAI VRFHWVAEQE MAADGKKVPT NLHILMNWQK QGDDWKLLSR
 151 AATKL
```

Localization Scores:

Cytoplasmic	0.00
CytoplasmicMembrane	2.50
Periplasmic	2.50
OuterMembrane	2.50
Extracellular	2.50

Final Prediction:

Unknown

Spot 14

Match to: **Q89IK9_BRAJA** Score: **299**

10 kDa chaperonin (Protein Cpn10) (groES protein). ***Bradyrhizobium japonicum*****.**

Nominal mass (M_r): **11170**; Calculated pI value: **7.93**

Sequence Coverage: **52%**

Matched peptides shown in **Bold and underline**

```
   1 MKFRPLHDRV VVKRIDAEEK TAGGIIIPDT AKEKPSQGEV VAVGPGGRDE
  51 AGKLIPIDLK VGDRVLFGKW SGTEVKIDSV DLLIMKESDI MGVLDVPASK
 101 KKAA
```

Localization Scores:

Cytoplasmic	9.26
CytoplasmicMembrane	0.24
Periplasmic	0.48
OuterMembrane	0.01
Extracellular	0.01

Final Prediction:

Cytoplasmic	9.26

Spot 15

Match to: **Q89J82_BRAJA** Score: **907**

Elongation factor TU. *Bradyrhizobium japonicum*.

Nominal mass (M_r): 43569; Calculated pI value: 5.78

Sequence Coverage: 46%

Matched peptides shown in **Bold and underline**

```
  1 MAKAKFERNK PHCNIGTIGH VDHGKTSLTA AITKILAETG GATFTAYDQI
 51 DKAPEEKARG ITISTAHVEY ETKNRHYAHV DCPGHADYVK NMITGAAQMD
101 GAILVVSAAD GPMPQTREHI LLARQVGVPA LVVFLNKCDM VDDPELLELV
151 ELEVRELLSK YEFPGDKIPI IKGSALAALE DSDKKLGHDA ILELMRNVDE
201 YIPQPERPID QPFLMPVEDV FSISGRGTVV TGRVERGIVK VGEEIEIVGL
251 RATQKTTVTG VEMFRKLLDQ GQAGDNIGAL LRGTKREDVE RGQVLAKPGS
301 VKPHTKFKAE AYILTKEEGG RHTPFFTNYR PQFYFRTTDV TGVVHLPEGT
351 EMVMPGDNIA MEVHLIVPIA MEEKLRFAIR EGGRTVGAGV VASIIE
```

Localization Scores:

Cytoplasmic	9.26
CytoplasmicMembrane	0.24
Periplasmic	0.48
OuterMembrane	0.01
Extracellular	0.01

Final Prediction:

Cytoplasmic	9.26

Spot 16

Match to: **Q89XV0_BRAJA** Score: 898

Blr0205 protein. *Bradyrhizobium japonicum*.

Nominal mass (M_r): 34868; Calculated pI value: 6.30

Sequence Coverage: 38%

Matched peptides shown in **Bold and underline**

```
   1 MSAPLEQPPA STKGFSMTTS FPVTKTGLRF GLATALVGCL ALALIAGPGR
  51 AADDPVLAKV NGAEIKKSDV AMAEEELGPS LAQMDPATKD ENVLSFLIDM
 101 KIVSKAAEDK KVADSEEFKK RLAFARNRLL MDSLLANEGK AATTPDAMKK
 151 VYEEASKQIT GEQEVRARHI LVETEDEAKA VKAELDKGAD FAELAKKKSK
 201 DPGSADGGDL GFFTKEQMVP EFSAVAFALE PGKISDPVKS QFGWHIIKVE
 251 EKRNRKAPDF EQVKAQIEQY VTRKAQADYV AKLRTEAKVE RLDQPAADAK
 301 PADAAKPSDA KPSDSKMAPP AKK
```

Localization Scores:

Cytoplasmic	0.00
CytoplasmicMembrane	0.00
Periplasmic	7.40
OuterMembrane	2.49
Extracellular	0.11

Final Prediction:

Unknown

Spot 17

Match to: **Q89QC0_BRAJA** Score: 666

ABC transporter sugar-binding protein. ***Bradyrhizobium japonicum***.

Nominal mass (M_r): 38378; Calculated pI value: 7.63

Sequence Coverage: 41%

Matched peptides shown in **Bold and underline**

Peptide sequence

```
   1  MLKLKTTFLA LALAGAATMA AGVTASAQDK ATVGIAMPTK SSARWIDDGN
  51  NMVKVLKERG YNTDLQYAED DIPNQLSQVE NMVTKGAKAL VIAAIDGTTL
 101  SDVLKQAKAK GITVIAYDRL IRGTPNVDYY ATFDNFQVGV LQAESLVQGL
 151  GLKDGKGPFN IELFGGSPDD NNAYFFYNGA MSVLKPYIDS GKLVVVSGQM
 201  GMDKVATLRW DGATAQARMD NLLSAYYGNK KVNAVLSPYD GLSIGIISSL
 251  KGVGYGSADQ PMPVISGQDA EVPSIKAMLR GDQYSTIFKD TRDLAKVTAD
 301  MVDAALAGKQ VTVNDTNTYE NGVKKVPSYL LKPVVVYKDN WEKVLVDSGY
 351  YKKSQFQ
```

```
  1 MLKLKTTFLA LALAGAATMA AGVTASAQDK ATVGIAMPTK SSARWIDDGN
 51 NMVKVLKERG YNTDLQYAED DIPNQLSQVE NMVTKGAKAL VIAAIDGTTL
101 SDVLKQAKAK GITVIAYDRL IRGTPNVDYY ATFDNFQVGV LQAESLVQGL
151 GLKDGKGPFN IELFGGSPDD NNAYFFYNGA MSVLKPYIDS GKLVVVSGQM
201 GMDKVATLRW DGATAQARMD NLLSAYYGNK KVNAVLSPYD GLSIGIISSL
251 KGVGYGSADQ PMPVISGQDA EVPSIKAMLR GDQYSTIFKD TRDLAKVTAD
301 MVDAALAGKQ VTVNDTNTYE NGVKKVPSYL LKPVVVYKDN WEKVLVDSGY
351 YKKSQFQ
```

Localization Scores:

Cytoplasmic	0.00
CytoplasmicMembrane	0.00
Periplasmic	10.00
OuterMembrane	0.00
Extracellular	0.00

Final Prediction:

Periplasmic	10.00

Spot 18

Match to: **Q89LU7_BRAJA** Score: 903

ABC transporter amino acid-binding protein. *Bradyrhizobium japonicum*.

Nominal mass (M_r): 36860; Calculated pI value: 6.21

Sequence Coverage: 54%

Matched peptides shown in **Bold and underline**

```
  1 MKRVTLALTL ALAAGLTAQA ADAQTLKTVK DRGTLSCGVS QGLPGFSAPD
 51 DKGNWTGLDV DVCRAIAAAI FNDPTKVKFV PTSAKDRFTA LQSGEIDVLS
101 RNTTWTISRD TSLGANFTGV TYYDGQGFMV KKSLKVNSAL ELNSASVCVQ
151 TGTTTEQNLA DYFKANNMKY EVIAFGTNDE TVKAYEAGRC DVFTTDQSGL
201 YANRLKLANP NDHMVLPEII SKEPLGPMVR HGDDQWFDIV KWTLFALVTT
251 EELGVTSKNV DEKAKLESPE LKRVLGSDGN FGEQLGLTKD WVVRIVKAVG
301 NYGEVFDRNV GAGSPLAINR GLNNLWNKGG LQYAPPIR
```

Localization Scores:

Cytoplasmic	0.00
CytoplasmicMembrane	0.00
Periplasmic	10.00
OuterMembrane	0.00
Extracellular	0.00

Final Prediction:

Periplasmic	10.00

Spot 19

Match to: **Q89QC8_BRAJA** Score: 492

ABC transporter sugar-binding protein. ***Bradyrhizobium japonicum***.

Nominal mass (M_r): 33968; Calculated pI value: 7.66

Sequence Coverage: 31%

Matched peptides shown in **Bold and underline**

Peptide sequence

```
  1 MILKALFAAS ATAALLLALP ANAAELTIGF SQIGSESGWR AAETSVSKQE
 51 AAKRKVNLKI ADAQQKQENQ IKAIRSFIAQ NVDAIFLAPV VSTGWDSVLK
101 EAKEAKIPVV LLDRDIDPSG KELYLTAVTS DSVHEGEVAG DWLAKTVGGK
151 ACNIVELQGT VGASVAANRK KGFDTAIAKH ANLKVVRSQT GDFTRAKGKE
201 VMESFIKAEG GGKSICAVYA HNDDMMVGAI QAMKEAGLKP GKEILTVSID
251 AVPDIFKAMA AGEANATVEL TPNMAGPALD AIAAFKDKGT VPPKWIQTES
301 KLYTAADDPQ KIYDSKKGLG Y
```

Localization Scores:

Cytoplasmic	0.00
CytoplasmicMembrane	0.00
Periplasmic	10.00
OuterMembrane	0.00
Extracellular	0.00

Final Prediction:

Periplasmic	10.00

Spot 20

Match to: **Q89IG4_BRAJA** Score: 384

ABC transporter substrate-binding protein. *Bradyrhizobium japonicum*.

Nominal mass (M_r): 40020; Calculated pI value: 8.95

Sequence Coverage: 20%

Matched peptides shown in **Bold and underline**

```
   1 MKSLKLIGLA FGASIALSSA AFAQDVTIAV AGPMTGTESA FGRQMKNGAE
  51 MAVADINTAG GINGKKLALN VEDDACDPKQ ARSIAEKIAG AKIPFVAGHY
 101 CSSSSIPASE AYADGNVLQI TPASTNPLFT ERKLWNVARV CGRDDQQGLI
 151 AAQYIAKNYK GKNIAILNDK TTYGKGLADE TKKALNKAGI TEKMYESYNK
 201 GDKDFNAIVS RLKRDNIDLV YVGGYHQESG LILRQMRDQG LKTVLMAGDA
 251 LADKEYASIT GPAGEGTLFT FGPDPRNKPT AKKIVDAFKA KNIDPEGYTL
 301 YTYAAMQVWS QAAKKAGTTD AKKVMEAMKA GKWDTVIGPI EYDAKGDIKQ
 351 IDYVVYKWDA KGGYAEIKGN GT
```

Localization Scores:

Cytoplasmic	0.00
CytoplasmicMembrane	0.00
Periplasmic	10.00
OuterMembrane	0.00
Extracellular	0.00

Final Prediction:

Periplasmic	10.00

Spot 21

Match to: **Q89FQ0_BRAJA** Score: 223

Bll6649 protein. ***Bradyrhizobium japonicum***.

Nominal mass (M_r): 18033; Calculated pI value: 6.74

Sequence Coverage: 23%

Matched peptides shown in **Bold and underline**

```
     1 MLMKSIAAGL AGTALLATAA FAQSPTATTD KAPTAATTTT TTSASGEWRT
    51 SKMPGLKIYN DANENIGSIN DLLMDKSGAI KIAVIGVGGF LGMGEHLVAV
   101 PYDKLKFVNE AVAYTGAAGT NPNAKPATTT TTGAATGTDK TATTVTASSK
   151 WYPDHAVFNA SKDELKNMPE FKYSE
```

Localization Scores:

Cytoplasmic	0.00
CytoplasmicMembrane	2.50
Periplasmic	2.50
OuterMembrane	2.50
Extracellular	2.50

Final Prediction:

Unknown

Spot 22

Match to: **Q89DA7_BRAJA** Score: 360

10 kDa chaperonin (Protein Cpn10) (groES protein). ***Bradyrhizobium japonicum***.

Nominal mass (M_r): 10708; Calculated pI value: 6.59

Sequence Coverage: 69%

Matched peptides shown in **Bold and underline**

```
   1 MAKSKFRPLH DRVVVKRIDA EEKTKGGIII PDTAKEKPSQ GEVVAVGPGG
  51 RDETGKLIPI DLKVGDRVLF GKWSGTEVKI DNEELLIMKE SDIMGVMA
```

Localization Scores:

Cytoplasmic	9.26
CytoplasmicMembrane	0.24
Periplasmic	0.48
OuterMembrane	0.01
Extracellular	0.01

Final Prediction:

Cytoplasmic	9.26

Spot 23

Match to: **AAC44752** Score: 81

BJU55047 NID: *Bradyrhizobium japonicum*

Nominal mass (M_r): 11300; Calculated pI value: 6.10

Sequence Coverage: 21%

Matched peptides shown in **Bold and underline**

```
   1 MHFRPLHDRV LVRRIDAEEK TAGGIIIPDT AKEKPQEGEI IAAGSGGRNE
  51 QGQLIPIDVK PGDRVLFGKW SGTEVKIDGQ DYLIMKESDL LGVVDKTGSV
 101 KKAA
```

Localization Scores:

Cytoplasmic	9.26
CytoplasmicMembrane	0.24
Periplasmic	0.48
OuterMembrane	0.01
Extracellular	0.01

Final Prediction:

Cytoplasmic	9.26

Spot 24

Match to: **Q5NMT2_ZYMMO** Score: 76

Peptidoglycan-associated protein. *Zymomonas mobilis*.

Nominal mass (M_r): 27353; Calculated pI value: 6.92

Sequence Coverage: 4%

Matched peptides shown in **Bold and underline**

```
  1 MLNGMFDFGG KNDGVSGFIG GGVGVARVAL NHYSLGGSGS FANDNDAHFA
 51 WQVIAGIRKP VTKMIDFELK YRFFNVNGLN FRTTDMGNMS GRYRSHSVLA
101 GLVFNFGEPK AAQPAPPPMP APPPPTPPAP PPPEEPPAPP VPAIPGPFLV
151 FFDFDKYNIT PEAASILDNV ASSYAQTGQA RVVVAGYTDT AGPAKYNMGL
201 SQRRADSVKA YLVGKGVPDD AMATEAYGKT HLLVQTADGV REPQNRRVEI
251 TFGPGSGQ
```

Localization Scores:

Cytoplasmic	0.01
CytoplasmicMembrane	0.01
Periplasmic	0.03
OuterMembrane	9.92
Extracellular	0.03

Final Prediction:

OuterMembrane	9.92

Spot 25

Match to: **AAV89284** Score: 562

AE008692 NID: *Zymomonas mobilis* subsp. mobilis ZM4

Nominal mass (M_r): 68508; Calculated pI value: 4.81

Sequence Coverage: 19%

Matched peptides shown in **Bold and underline**

```
   1 MGKVIGIDLG TTNSCVAVME GGQPKVIENA EGARTTPSIV AFTKDSERLI
  51 GQPAKRQAVT NSENTIFAVK RLIGRRFDDP VTKRDTELVP YHIVRGSNGD
 101 AWVKAGGQDY SPSQISAFIL QKMKETAESY LGETVDQAVI TVPAYFNDAQ
 151 RQATKDAGKI AGLEVLRIIN EPTAAALAYG LDKNDGKTIA VYDLGGGTFD
 201 ISILEIGDGV FEVKATNGDT FLGGEDFDTK IVSYLAEEFK KAEGIDLTKD
 251 RLALQRLKEA AEKAKIELSS AQTTEVNLPF ITADATGPKH LVKTISRAEL
 301 ERLVADLIDR TLEPVKKALA DAGVKASDID DVVMVGGMTR MPKVRQVVKE
 351 FFGKEPHTGV NPDEVVAMGA AIQAGVLQGD VKDVLLLDVT PLSLGIETLG
 401 GVFTRMIDRN TTIPTKKSQV YSTAEDNQNA VTIRVFQGER EMAADNKLLG
 451 QFDLVGIPPA PRGVPQIEVT FDIDANGIVN VSAKDKGTGK EQQIRIQASG
 501 GLSEGDIDKM VKDAEKFAAD DKHRRELAEA KNNGDSLVHT TERQLTELGD
 551 KVDAALKTEV EAAVAAVKTA LEGEDVAQIN EKTQALGQVA MKLGQALYEQ
 601 DQANNERHDT PETEKAEGDN VVDAEFQEID DQDKK
```

Localization Scores:

Cytoplasmic	9.26
CytoplasmicMembrane	0.24
Periplasmic	0.48
OuterMembrane	0.01
Extracellular	0.01

Final Prediction:

Cytoplasmic	9.26

Spot 26

Match to: **Q1N9C2_9SPHN** Score: 245

Ribosomal protein S1. *Sphingomonas* sp. SKA58.

Nominal mass (M_r): 61616; Calculated pI value: 5.01

Sequence Coverage: 7%

Matched peptides shown in **Bold and underline**

```
   1 MASTAFPSRD DFAALLNDSL GGEDGGFEGR VVKGTVTGIE NDLAVIDVGL
  51 KSEGRVPLRE FAMPGQKADL KVGDEVEVYV DRVENAHGEA MLSRDRARRE
 101 AAWDKLEAEF TESARVEGVI FGRVKGGFTV DLDGAVAFLP GSQVDIRPVR
 151 DVTPLMDIPQ PFQILKMDRR RGNIVVSRRA ILEETRAEQR SGLIQTLAEG
 201 QIIEGVVKNI TDYGAFVDLG GIDGLLHVTD LSYKRINHPN EMINIGDTVK
 251 VQIIRINRDT QRISLGMKQL ESDPWEGASA KYPVGAKLTG RVTNITEYGA
 301 FVELEPGIEG LVHVSEMSWT KKNVHPGKIV STSQEVEVLV LEVDPEKRRI
 351 SLGLKQAQSN PWDSFAERHP VGSTVEGEVK NATEFGLFIG LDGDVDGMVH
 401 MSDIAWGISG EDALALHRKG ETVQAVVLDI DVEKERISLG MKQLERGGPA
 451 AGGTAAAAAG LNKNAIVTVT VLEVRDGGLE VQAGEDGAAG FIKRSDLGRD
 501 RDEQRPERFQ VGQKFDAMVT GFDRAKKPTF SVKAMQIAEE KQAVAQYGSS
 551 DSGASLGDIL GEALKAKNEG
```

Localization Scores:

Cytoplasmic	9.26
CytoplasmicMembrane	0.24
Periplasmic	0.48
OuterMembrane	0.01
Extracellular	0.01

Final Prediction:

Cytoplasmic	9.26

Spot 27

Match to**: Q1GVZ9_9SPHN** Score: 617

Chaperonin GroEL.- *Sphingopyxis alaskensis* RB2256.

Nominal mass (M_r): 57917; Calculated pI value: 5.07

Sequence Coverage: 22%

Matched peptides shown in **Bold and underline**

```
   1 MAAKDVKFSR DARERILKGV DILADAVKVT LGPKGRNVVI DKSFGAPRIT
  51 KDGVSVAKEI ELKDKFENMG AQMLREVASK ANDKAGDGTT TATVLAQAIV
 101 REGMKSVAAG MNPMDLKRGI DLAVTKVVED LKARSTPVSG SSEIAQVGII
 151 SANGDVEVGE KIAEAMEKVG KEGVITVEEA KGLEFELDVV EGMQFDRGYL
 201 SPYFITNPEK MIVELTDPYI LIFEKKLSNL QSMLPILEAV VQSGRPLLII
 251 AEDIEGEALA TLVVNRLRGG LKVAAVKAPG FGDRRKAMLQ DIAILTKGEM
 301 ISEDLGIKLE NVTLNMLGQA KRVTIDKDNT TIVDGAGDAE AIKGRVEQIR
 351 AQIETTTSDY DREKLQERLA KLAGGVAVIK VGGATEVEVK ERKDRVDDAL
 401 HATRAAVEEG IVPGGGTALL YATKALEGLK GANDDQTRGI DIIRKAIETP
 451 LRQIAANAGH DGAVVAGNLL RVGDVEQGFN AATDVYENLK AAGVIDPTKV
 501 VRTALQDAAS VAGLLITTEA AVSELPEDKP AMPMGSGGMG GMGGMDF
```

Localization Scores:

Cytoplasmic	9.26
CytoplasmicMembrane	0.24
Periplasmic	0.48
OuterMembrane	0.01
Extracellular	0.01

Final Prediction:

Cytoplasmic	9.26

Spot 28

Match to: **Q1GP97_9SPHN** Score: **594**

Translation elongation factor Tu.- *Sphingopyxis alaskensis* RB2256.

Nominal mass (M_r): **43040**; Calculated pI value: **5.11**

Sequence Coverage: 26%

Matched peptides shown in **Bold and underline**

```
   1 MAKAKFERTK PHCNIGTIGH VDHGKTSLTA AITKVLAENV AGNAAVDFAN
  51 IDKAPEERER GITISTAHVE YETESRHYAH VDCPGHADYV KNMITGAAQM
 101 DGAILVVSAA DGPMPQTKEH ILLAKQVGVP TMVVFLNKVD QLDDPELLEL
 151 VELEIREELS KRDFDGDNIP IIAGSALAAL EGRDDNIGKD AILKLMAAVD
 201 EWIPQPERPL DKPFLMPIED VFSISGRGTV VTGRVETGVV KVGEEVEIVG
 251 IKDTKKTVVT GVEMFRKLLD QGQAGDNIGA LIRGVGREEV ERGQVLAKPG
 301 SITPHTEFTS EVYVLSKDEG GRHTPFFANY RPQFYFRTTD VTGEVILPEG
 351 TEMVMPGDNV QLSVKLIAPI AMDPGLRFAI REGGRTVGAG VVATVTK
```

Localization Scores:

Cytoplasmic	9.26
CytoplasmicMembrane	0.24
Periplasmic	0.48
OuterMembrane	0.01
Extracellular	0.01

Final Prediction:

Cytoplasmic	9.26

Spot 29

Match to: **Q7V285_PROMP** Score: **54**

Hypothetical protein.- ***Prochlorococcus marinus*** **subsp. pastoris (strain CCMP 1378 / MED4).**

Nominal mass (M_r): **17200**; Calculated pI value: **9.12**

Sequence Coverage: 5%

Matched peptides shown in **Bold and underline**

```
  1 MSNSNYDNNY GQENYRSRGN NDRSNFRNRS GGNRDGGGFR IRLSDNEMKA
 51 VRSIQEAFQL KSTVAVLGFS VRTLSEMIED KDLMESITKF ARNNKNTSSP
101 NKATASENRS KKVVPDPFAR PVKNTPSEQT QPNKEEIKEE IKKEQEEVDD
151 K
```

Localization Scores:

Cytoplasmic	9.26
CytoplasmicMembrane	0.24
Periplasmic	0.48
OuterMembrane	0.01
Extracellular	0.01

Final Prediction:

Cytoplasmic	9.26

APPENDIX B

DNA and Protein search from BlastN 2.0.13

DNA alignment of bll1317 open reading frame

Bradyrhizobium japonicum USDA 110 DNA, complete genome

Length = 9105828 Score = 767 bits (387) Expect = 0.0

Identities = 441/459 (96%)

```
Query: 48      gcctgacacctcgaccttgccgggcatcgcttcgaggttcagcttcttgaccacgccgtc 107
               ||||||||||||||||||||||||||||||| |||||||||||||||| ||||||||||
Sbjct: 1429738 gcctgacacctcgaccttgccgggcatcgcctcgaggttcagcttcttcaccacgccgtc 1429797
Query: 108     ctcgaccagcatcgaatagcgcttggagcggatgccgaggccattgccggaggcgtccag 167
               |||||||||||||||||||||||||||||||||||||||||||| ||| |||||||||| ||
Sbjct: 1429798 ctcgaccagcatcgaatagcgcttggagcggatgccgaggccgttggcggaggcgtcgag 1429857

Query: 168     ctccatgccgatcgccttggcgaagtcggcattgccgtcggcgaggaagatggcctcgtc 227
               |||||||||||||||||| |||||||||||||||||||||| | |||||||||
Sbjct: 1429858 ctccatgccgatcgccttggtgaagtcggcattgccgtcggcgaggaatacggcctcgtc 1429917

Query: 228     gcgctggtcggtatcgcgcttccaggcgttcatgacgaaagcgtcgttgacggagacgat 287
               |||||||||||| |||||||||||||||||||||||| |||||||||||||||| |||
Sbjct: 1429918 gcgctggtcggtgtcgcgcttccaggcgttcatgacgaaggcgtcgttgacggagatgat 1429977

Query: 288     ggcgatggtgtcgacgcccttgtccttcatggcataggcgttgaggaagatgctcggcag 347
               |||||||||||||||||||||||||| ||||| |||||||||||||||||||||||||
Sbjct: 1429978 ggcgatggtgtcgacgcccttgtccttgatggcgtaggcgttgaggaagatgctcggcag 1430037

Query: 348     atgcatcttgtggcaggtgccggtgtaggcgccgggcactgcgaacagcgccactttctt 407
               |||||||||||||||||||||||||||||||||||||||| ||||||||||||| |||||
Sbjct: 1430038 atgcatcttgtggcaggtgccggtgtaggcgccgggcaccgcgaacagcgccaccttctt 1430097

Query: 408     gcccttgaagatgtcgtcggtggtcttcacctgcgggccttccgccgtcatcacgcggaa 467
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1430098 gcccttgaagatgtcgtcggtggtcttcacctgcgggccttccgccgtcatcacgcggaa 1430157

Query: 468     tttcgccccgggtagcttttcgccagtctggatcgccat 506
               ||||||| |||| ||||| ||||||||||||||||||
Sbjct: 1430158 tttcgcctcgggcagcttgtcgccagtctggatcgccat 1430196
```

Protein alignment of bll1317 open reading frame

peroxiredoxin *Bradyrhizobium japonicum* USDA 110]

Length = 161 Score = 295 bits (755) Expect = 1e-78

Identities = 146/153 (95%) Positives = 150/153 (98%) Frame = -1

```
Query: 506 MAIQTGEKLPGAKFRVMTAEGPQVKTTDDIFKGKKVALFAVPGAYTGTCHKMHLPSIFLN 327
           MAIQTG+KLP AKFRVMTAEGPQVKTTDDIFKGKKVALFAVPGAYTGTCHKMHLPSIFLN
Sbjct: 1   MAIQTGDKLPEAKFRVMTAEGPQVKTTDDIFKGKKVALFAVPGAYTGTCHKMHLPSIFLN 60
Query: 326 AYAMKDKGVDTIAIVSVNDAFVMNAWKRDTDQRDEAIFLADGNADFAKAIGMELDASGNG 147
           AYA+KDKGVDTIAI+SVNDAFVMNAWKRDTDQRDEA+FLADGNADF KAIGMELDAS NG
Sbjct: 61  AYAIKDKGVDTIAIISVNDAFVMNAWKRDTDQRDEAVFLADGNADFTKAIGMELDASANG 120
Query: 146 LGIRSKRYSMLVEDGVVKKLNLEAMPGKVEVSG 48
           LGIRSKRYSMLVEDGVVKKLNLEAMPGKVEVSG
Sbjct: 121 LGIRSKRYSMLVEDGVVKKLNLEAMPGKVEVSG 153
```

DNA alignment of blr5625 open reading frame

Bradyrhizobium japonicum USDA 110 DNA, complete genome

Length = 9105828 Score = 474 bits (239) Expect = e-130

Identities = 296/315 (93%)

```
Query: 47      atgaaattccgtccgcttcacgaccgcgtcgtggtcaagcgcatcgacgcagaagagaag 106
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6186325 atgaaattccgtccgcttcacgaccgcgtcgtggtcaagcgcatcgacgcagaagagaag 6186384

Query: 107     accgctggcggcatcatcattcccgacactgccaaggaaaagccctcccagggcgaagtc 166
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6186385 accgctggcggcatcatcattcccgacactgccaaggaaaagccctcccagggcgaagtc 6186444

Query: 167     gtcgccgttggccccggtggccgcgacgaagccggcaagctgatcccgatcgacctgaag 226
               |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6186445 gtcgccgtcggccccggtggccgcgacgaagccggcaagctgatcccgatcgacctgaag 6186504

Query: 227     gtcggcgaccgcgtgctgttcggcaagtggtccggcaccgaggtcaagatcgacggtcag 286
               |||||||| |||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct: 6186505 gtcggcgatcgcgtgctgttcggcaagtggtccggcaccgaggtcaagatcgacagcgtc 6186564

Query: 287     gacctgctgatcatgaaggagagcgacgtgatgggcgttctcgaagtcagcgagtccaag 346
               || ||  |||||||||||||| |||||| | |||||||| ||||| |||  ||  ||||||
Sbjct: 6186565 gatctcttgatcatgaaggaaagcgacatcatgggcgtcctcgacgtccccgcttccaag 6186624

Query: 347     aagaaggcggcctaa 361
               |||||||||||||||
Sbjct: 6186625 aagaaggcggcctaa 6186639
```

Protein alignment of blr5625 open reading frame

10 KD chaperonin *Bradyrhizobium japonicum* USDA 110]

Length = 104 Score = 195 bits (495) Expect = 2e-48

Identities = 98/104 (94%) Positives = 100/104 (96%) Frame = +2

```
Query: 47  MKFRPLHDRVVVKRIDAEEKTAGGIIIPDTAKEKPSQGEVVAVGPGGRDEAGKLIPIDLK 226

Sbjct: 1   MKFRPLHDRVVVKRIDAEEKTAGGIIIPDTAKEKPSQGEVVAVGPGGRDEAGKLIPIDLK 60

Query: 227 VGDRVLFGKWSGTEVKIDGQDLLIMKESDVMGVLEVSESKKKAA 358

Sbjct: 61  VGDRVLFGKWSGTEVKIDSVDLLIMKESDIMGVLDVPASKKKAA 104
```

DNA alignment of bll5845 open reading frame

Bradyrhizobium japonicum USDA 110 DNA, complete genome

Length = 9105828 Score = 613 bits (309) Expect = e-172

Identities = 405/437 (92%)

```
Query: 45      atgtccggtatcgttctctcctcgtcggttcgtcagaacctgctctccctccagtccacc 104
               ||||||||||||||||||||| || |||||||||||||||||| || || ||||||||||||
Sbjct: 6416220 atgtccggtatcgttctctcttcctcggttcgtcagaaccttctttctctccagtccacc 6416161

Query: 105     gctgaccttctcgccaccacacagaaccgtctgtcgaccggcaagagcgtcaactcggcc 164
               |||||||| |||||||||||| |||| ||||||||||||||||||||||||||||||||
Sbjct: 6416160 gctgacctcctcgccaccacgcagagccgtctgtcgaccggcaagagcgtcaactcggcc 6416101

Query: 165     ctggacaatcccaccaacttcttcaccgcccagtcgctcgacaaccgcgccagcgacatc 224
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6416100 ctggacaatcccaccaacttcttcaccgcccagtcgctcgacaaccgcgccagcgacatc 6416041

Query: 225     aacaatctgctcgacggcatcgccaacggcgtgcaggtgctgcaggccgccaacaccggc 284
               |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 6416040 aacaatttgctcgacggcatcgccaacggcgtgcaggtgctgcaggccgccaacaccggc 6415981

Query: 285     atcacctcgctgcagaagctgatcgacagcgccaagtcgatcgccaaccaggcgctgcag 344
               |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct: 6415980 atcacctcgctgcagaagctgatcgacagcgcgaagtcgatcgccaaccaggcgctgcag 6415921

Query: 345     accaccgtcggctactccaccaagtccaacgtctccaccacgattgctggtgcgacggct 404
               ||||| |||||||||||||||||||||||||||||||| ||  | |||||||| ||
Sbjct: 6415920 accacggtcggctactccaccaagtccaacgtctccaccaccatctcgggtgcgaccgcc 6415861

Query: 405     tcggacctgcgtggcacgaccagtttcaccagcgcggatgcgctgagcaacgtgctctat 464
                | || ||||| |||||||| || ||| ||||||||   |||   ||||||||| | |||
Sbjct: 6415860 gctgatctgcgcggcacgacgagcttcgccagcgcgaccgcgagcagcaacgtggtgtat 6415801

Query: 465     agcggcgcggccggcgg 481
               |||||||||||||||||
Sbjct: 6415800 agcggcgcggccggcgg 6415784
```

Protein alignment of bll5845 open reading frame

Bradyrhizobium japonicum USDA 110]

Length = 757 Score = 296 bits (757) Expect = 3e-78

Identities = 193/317 (60%) Positives = 213/317 (67%)

Gaps = 15/317 (4%) Frame = +3

```
Query: 45  MSGIVLSSSVRQNLLSLQSTADLLATTQNRLSTGKSVNSALDNPTNFFTAQSLDNRASDI 224
           MSGIVLSSSVRQNLLSLQSTADLLATTQ+RLSTGKSVNSALDNPTNFFTAQSLDNRASDI
Sbjct: 1   MSGIVLSSSVRQNLLSLQSTADLLATTQSRLSTGKSVNSALDNPTNFFTAQSLDNRASDI 60

Query: 225 NNLLDGIANGVQVLQAANTGITSLQKLIDSAKSIANQALQTTVGYSTKSNVSTTIAGATA 404
           NNLLDGIANGVQVLQAANTGITSLQKLIDSAKSIANQALQTTVGYSTKSNVSTTI+GATA
Sbjct: 61  NNLLDGIANGVQVLQAANTGITSLQKLIDSAKSIANQALQTTVGYSTKSNVSTTISGATA 120

Query: 405 SDLRGTTSFTSADALSNVLYXXXXXXXXXXXXXXXXXXVQGTNTGTVINA-----ATTGA 569
           +DLRGTTSF SA A SNV+Y                    G+   T   A     A TGA
Sbjct: 121 ADLRGTTSFASATASSNVVYSGAAGGTTAASGTTTLGASIGSFASTGATAGDGTTALTGA 180

Query: 570 SLL---NG-TXXXXXXXXXXXXXDTLTVNGKTIXXXXXXXXXXXXXXXXXXXXXGITTGN 737
             L   NG T             DTLTVNGKTI                        +GN
Sbjct: 181 ITLIATNGTTATGLAGNAQPADGDTLTVNGKTITFRSGAAPASTAVPSGSG-----VSGN 235

Query: 738 VFTDNSTGNVTVYLGSGTKAAVGDVLTAIDLASGVQ----SNVAGTLTVNSGQTISSVS- 902
           + TD + GN TVYL S T   V D+L+AIDLASGV+    S+ A T+ V++ Q  ++VS
Sbjct: 236 LVTDGN-GNTTVYLASAT---VNDLLSAIDLASGVKTVSISSGAATIAVSASQPGAAVST 291
Query: 903 -GGGALVLKSTYGSDLS 950
              GA+ LKS+ G+DLS
Sbjct: 292 AAAGAVTLKSSTGADLS 308
```

DNA alignment of bsr7532 open reading frame

Bradyrhizobium japonicum USDA 110 DNA, complete genome

Length = 9105828 Score = 456 bits (230) Expect = e-125

Identities = 242/246 (98%) Strand = Plus / Minus

```
Query: 51      gttgtcgatcttgacctcggtgcccgaccacttgccgaacagcacgcggtcgccgacctt 110
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 8265622 gttgtcgatcttgacctcggtgcccgaccacttgccgaacagcacgcggtcgccgacctt 8265563

Query: 111     gaggtcgatcgggatcagcttgccggcctcgtcgcggccgccggggccgacggcgacgac 170
               ||||||||||||||||||||||||| |||||| ||||||||||||||||||||||||||
Sbjct: 8265562 gaggtcgatcgggatcagcttgccggtctcgtcacggccgccggggccgacggcgacgac 8265503

Query: 171     ctcgccctgggacggcttttccttggcggtgtccggaatgatgatgccgcccttggtctt 230
               |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 8265502 ctcgccctgggagggcttttccttggcggtgtccggaatgatgatgccgcccttggtctt 8265443

Query: 231     ttcctcggcgtcgatacgtttgaccacgacacggtcatgcggcggacgaaatttggattt 290
               |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct: 8265442 ttcctcggcgtcgatacgtttgaccacgacacggtcatgcagcggacgaaatttggattt 8265383

Query: 291     agccat 296
               ||||||
Sbjct: 8265382 agccat 8265377
```

Protein alignment bsr7532 open reading frame

10 KD chaperonin (protein CPN10) [*Bradyrhizobium japonicum* USDA110]

Length = 98 Score = 162 bits (411) Expect = 9e-39

Identities = 80/83 (96%) Positives = 81/83 (97%) Frame = -1

```
Query: 296 MAKSKFRPPHDRVVVKRIDAEEKTKGGIIIPDTAKEKPSQGEVVAVGPGGRDEAGKLIPI 117
           MAKSKFRP HDRVVVKRIDAEEKTKGGIIIPDTAKEKPSQGEVVAVGPGGRDE GKLIPI
Sbjct: 1   MAKSKFRPLHDRVVVKRIDAEEKTKGGIIIPDTAKEKPSQGEVVAVGPGGRDETGKLIPI 60

Query: 116 DLKVGDRVLFGKWSGTEVKIDNK 48
           DLKVGDRVLFGKWSGTEVKIDN+
Sbjct: 61  DLKVGDRVLFGKWSGTEVKIDNE 83
```

BIOGRAPHY

Mr. Monchai Manassila was born on March 16, 1976 at Nakhon Ratchasima, Thailand. He received his Bachelor degree of Science in Animal Production Technology, Suranaree University of Technology, 1998 and he received his Master degree of Science from School of Biotechnology, Suranaree University of Technology, 2003. Then, he had continued his Doctoral degree in 2004 at School of Biotechnology, Suranaree University of Technology, Nakhon Ratchasima. During his study, he had experience on his thesis work in investigation of acid tolerance protein of soybean *Bradyrhizobium*. He presented research work in 16th International Congress on Nitrogen Fixation in the topic of proteins involved in adaptive acid tolerant response of soybean *Bradyrhizobium* (poster presentation), June 14-19, 2009. Big Sky, Montana, USA.