COMPETITION MECHANISMS OF *Bradyrhizobium*

Waraporn Payakapong

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กลไกการสร้างความสามารถในการแข่งขันของแบรดดีไรโซเบียม

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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วัตถุประสงค์ของงานวิจัยนี้เพื่อศึกษาอิทธิพลของปัจจัยทางชีวภาพ และกายภาพต่อความสามารถในการแข่งขันการสร้างปมในรากต้นถั่วเหลืองของแบรดดีไรโซเบียม และปรับปรุงสายพันธุ์แบรดดีไรโซเบียมให้มีความสามารถในการทนเกลือได้สูงขึ้นเพื่อเพิ่มคุณสมบัติในการแข่งขัน ณ สภาวะแวดล้อมที่มีปริมาณเกลือสูง ปัจจัยทางชีวภาพที่ทำการศึกษาได้แก่ สายพันธุ์ของถั่วเหลือง สายพันธุ์และจำนวนเซลล์ของแบรดดีไรโซเบียมที่ใช้ ผลการทดลองพบว่าทั้งสามปัจจัยมีผลต่อความสามารถในการแข่งขันการสร้างปมของแบรดดีไรโซเบียม โดยความสามารถในการแข่งขันโดยรวมของแบรดดีไรโซเบียมทั้งสี่สายพันธุ์พบว่า สายพันธุ์ USDA110 มีความสามารถสูงที่สุด รองลงมาคือสายพันธุ์ THA6, SEMIA5019 และ THA5 ตามลำดับ

อิทธิพลของปัจจัยทางกายภาพต่อความสามารถในการแข่งขันการสร้างปมในการศึกษาครั้งนี้คือปัจจัยของเกลือ ขั้นแรกของการศึกษาได้คัดเลือก *Sinorhizobium* สายพันธุ์ BL3 ซึ่งมีความสามารถในการทนเกลือจากบริเวณพื้นที่ที่มีผลกระทบจากเกลือในเขตจังหวัดนครราชสีมา และได้ทำการคัดเลือกยีนส์ที่ควบคุมคุณสมบัติดังกล่าว โดยใช้เทคนิคการคัดเลือกคอสมิดที่มีชิ้นส่วนของยีนส์ที่ควบคุมการทนเกลือ การทำให้ยีนส์นั้นไม่แสดงออกโดยการกลายพันธุ์ และการหาลำดับเบสของยีนส์ คอสมิดที่คัดเลือกออกมาได้ประกอบด้วยกลุ่มของยีนส์จากสองบริเวณของโครโมโซม จากการวิเคราะห์ลำดับเบส และวิเคราะห์หน้าที่ของยีนส์จากความคล้ายคลึงของโปรตีนอื่น ๆ ในฐานข้อมูลพบว่าคอสมิดชุดที่หนึ่ง (pUHR307) ประกอบไปด้วย antirestriction protein, ATPase, xanthine dehydrogenase, transcriptional regulator syrB (AraC family), DNA methylase, partitioning protein และ conserved hypothetical protein คอสมิดชุดที่สอง (pUHR310) มีความคล้ายกับ choline dehydrogenase และ betaine aldehyde dehydrogenase ของ *S. meliloti* จากนั้นนำคอสมิดทั้งสองชุดนี้ใช้ในการปรับปรุงสายพันธุ์แบรดดีไรโซเบียมสายพันธุ์ THA6 ซึ่งแบรดดีไรโซเบียมที่ได้ (RUH161 และ RUH162) มีความสามารถในการทนเกลือได้สูงขึ้นกว่า THA6 สายพันธุ์เดิม อย่างไรก็ตามความสามารถที่เพิ่มขึ้นนี้ไม่มีผลช่วยปรับปรุงความสามารถในการแข่งขันการสร้างปม ณ สภาวะที่มีเกลือ

นอกจากนี้ได้ศึกษาถึงการตอบสนองของ *Sinorhizobium* สายพันธุ์ BL3 ต่อสภาวะที่มีเกลือ ในระดับการเปลี่ยนแปลงของปริมาณโปรตีน โดยเน้นถึงการเปลี่ยนแปลงของโปรตีนที่แสดงออกที่ผิวเซลล์ จากการวิเคราะห์พบว่าโปรตีนหลายชนิดมีการกระตุ้นหรือลดการแสดงออกในระดับ 1.5 เท่า ซึ่งขึ้นอยู่กับความเข้มข้นของเกลือ และระยะเวลาที่สัมผัสกับสิ่งแวดล้อมนั้น กลุ่ม

โปรตีนที่เกี่ยวข้องกับกระบวนการสร้างพลังงาน การสร้างและซ่อมแซมดีเอ็นเอ และกลุ่มโปรตีนที่เกี่ยวข้องกับการส่งผ่านสารที่ทำหน้าที่ควบคุมระดับความเข้มข้นของเซลล์และส่งผ่านไอออนต่าง ๆ เข้าและออกนอกเซลล์ มีการเพิ่มระดับความเข้มข้นขึ้นทั้งการตอบสนองต่อสิ่งแวดล้อมอย่างฉับพลันหรือภายหลังระยะปรับตัว ดังนั้นเมมเบรนโปรตีนจึงเป็นส่วนสำคัญส่วนหนึ่งในกลไกการป้องกันตัวเองต่อสภาวะที่มีเกลือสูง

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2548 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

WARAPORN PAYAKAPONG : COMPETITION MECHANISMS OF *Bradyrhizobium.* THESIS ADVISOR : PROF. NANTAKORN BOONKERD, Ph.D. 232 PP. ISBN 974-533-445-6

COMPETITION/BRADYRHIZOBIUM/SALT TOLERANCE GENE/MEMBRANE PROTEOMIC

The objectives of this experiment were to examine the influences of biotic and abiotic factors on nodulation competitiveness of bradyrhizobia and the construction of salt tolerant bradyrhizobia in order to achieve the highly nodulation competitor under salt stress condition. The emphasized biotic factors are soybean cultivars, bradyrhizobial strains and proportion of inoculation. These biotic factors exhibited the influence on nodulation competitiveness of *Bradyrhizibium*. Bradyrhizobial strain USDA110 had the highest general competitive ability (GCA), followed by THA6, SEMIA5019 and THA5, respectively.

The abiotic factors affecting nodulation competitiveness was emphasized on salt stress. The salt tolerant *Sinorhizobium* strain BL3 was isolated from salt affected area of Nakhon Ratchasima province of Thailand. In order to investigate salt tolerant mechanism, salt tolerant genes were isolated by the cosmid library isolation technique, random mutagenesis, and DNA sequencing. Cosmid clones containing two different regions of LT11 chromosome were identified. The sequence analysis of first region (pUHR307) exhibited the homology with antirestriction protein, ATPase, xanthine dehydrogenase, transcriptional regulator syrB (AraC family), DNA methylase, partitioning protein and conserved hypothetical protein. Second region of clones (pUHR310) showed a relatively high homolog with choline dehydrogenase and

betaine aldehyde dehydrogenase of *S. meliloti*. Recombinant *Bradyrhizobium japonicum* THA6 (RUH161 and RUH 162) was constructed by introducing such salt tolerant cosmids. Salt tolerant ability of RUH161 and RUH162 was elevated over THA6 wild type. However, nodulation competitiveness under salt stress environment was not improved.

The salt stress response of *Sinorhizobium sp.* BL3 was also analyzed at the protein expression level. Determination of membrane protein expression changes under salt stress using quantitative proteomic analysis revealed that several membrane proteins exhibited up- or down regulation by more than 1.5 folds with the level depend upon salt concentration and exposure time. A group of protein relating to energy metabolism, DNA repair and synthesis, and transportation proteins involved in compatible solute and ion transport across membranes were up-regulated in either immediate or late response. Therefore, membrane proteins are most likely play an important role in salt stress response mechanism.

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Student's Signature _____

Advisor's Signature _____

Co-advisor's Signature _____

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LIST OF ABBREVIATIONS

ABC	=	ATP binding cassette
ACN	=	acetonitrile
Am	=	amplicilin
bp	=	base pair
α-CHCA	=	α-Cyano-4-hydroxycinnamic acid
CID	=	collision induced dissociation
2-D	=	2 dimention
Da	=	dalton
DTT	=	1,4,-dithio-L-Threitol
ESI	=	electrospray ionization
FA	=	Fluorescent antibodies
FITC	=	Fluorescein isothiocyanate
GCA	=	General competitive ability
Gm	=	gentamycins
ha	=	hectare
HPLC	=	high performance liquid chromatography
i.d.	=	inner diameter
IPG	=	immobilized pH gradient
kbp	=	kilobase pair
kDa	=	kilodalton
Km	=	kanamycins
LC-MS/MS	=	liquid chromatography-tandem mass spectrometry

LIST OF ABBREVIATIONS (Continued)

MALDI-MS	=	matrix-assisted laser desorption ionization mass spectrometry
MS	=	mass spectrometry
m/z	=	mass-to-charge
MS/MS	=	tandem mass spectrometry
NAD^+	=	nicotinamide adenine dinucleotide
ηLC	=	nano-liquid chromatography
OD	=	optical density
o.d.	=	outer diameter
omp	=	Outer membrane protein
PAGE	=	polyacrylamide gel electrophoresis
PCR	=	Polymerase Chain Reaction
PMF	=	peptide mass fingerprinting
PMSF	=	phenylmethylsulphonyl fluoride
Rf	=	rifampicin
rRNA	=	ribosomal ribonucleic acid
SCA	=	Specific competitive ability
SCX	=	strong cation exchange chromatography
St	=	streptomycin
Tc	=	tetracyclin
TFA	=	trifluoroacetic acid
TMD	=	transmembrane domain
TOF	=	time of flight (mass spectrometry)
TOF/TOF	=	double time of flight (tandem mass spectrometry)

LIST OF ABBREVIATIONS (Continued)

tolC	=	outer membrane secretion protein
U	=	unit
UV	=	ultraviolet
V	=	volt
X-glc	=	5-bromo-4-chloro-3-indolyl-D-glucuronide

CHAPTER I

INTRODUCTION

Soybean production in Thailand has been applied with symbiotic nitrogen fixing bacteria as a biofertilizer inoculum over the past decade. *Bradyrhizobia* are used as soybean seed inoculants. This microorganism is able to reduce atmospheric dinitrogen gas (N_2) into nitrogenous compound that plant can utilize as direct nitrogen source. Therefore the biofertilizer is an essential factor for increasing crop yield and it provides a cheap environmental friendly alternative to chemical fertilizer.

The symbiosis between leguminous plants and rhizobia is a complex interplay between the two organisms that lead to the formation of a nitrogen-fixing organ, the root nodule. The inoculation of soybean with *Bradyrhizobium* generally increases nodulation, and nitrogen fixation, leading toincrease in soybean yield. However, even when superior nitrogen-fixing strains of bradyrhizobia are used as inoculants, the plants are often nodulated by inferior strains from the indigenous soil populations. This phenomenon has been termed the “competition problem”. This problem causes the decrease in crop yield from ineffective N_2 fixation. Significant efforts have been made to understand and alter the competitiveness of indigenous rhizobia. The biotic and abiotic factors have been reported in relation to nodulation competitiveness. Biotic factors include i) leguminous plant host; ii) rhizobial strain; iii) others soil microorganism (Sadowsky, 2000). Numerous studies have shown that the legume host is the major factors influence competition for nodulation, due to host-controlled selective or restrictive nodulation mechanisms (Bottomey, 1992; Crcgan and Keyser,

1986; Jones and Russel, 1976). Furthermore, intrinsic performance of rhizobia influencing competition have been reported (Zdor and Pueppke, 1991; Liu et al., 1989; Triplette and Barta, 1987; Roberto et al., 1997; Chun and Stacey, 1994; Boundy-Mills et al., 1994). However, most biotic factors on competition for nodulation is not well understood (Sadowsky, 2000).

The abiotic factors involve in the environmental stress. Several environment conditions (e.g., salinity, unfavorable soil pH, nutrient deficiency, mineral toxicity, temperature extremes, insufficient or excessive soil moisture) are severe factors affecting growth and competitive of N_2-fixating bacteria (Dowling and Broughton, 1986; Triplett and Sadowsky, 1992). The correlation evidence has been reported by Hunt and co-worker (1981) that at low water content in soil affect the successful of soybean inoculation in soil with a high indigenous population of *R. japonicum*. Therefore, under such condition, superior inoculums rhizobial strain could not expected to express its full capacity for nitrogen fixation and nodulation competition (Zahran, 1999). The important problem that critically reduce the number of nodulation by superior strains is the rapidly death of inoculant after apply to the field. The death of inoculant depends on the adverse environmental condition in each field. Rice (1997) demonstrated that the population in root rhizosphere of plants inoculated with peat decline more than 90% within 2 days. Correlation reported from Date (1968) demonstrated that as few as 100 rhizobia per seed at sowing can give satisfactory nodulation under favorable conditions while 100,000 cells per seed may not be sufficient where condition are less favorable.

Salinity of soil is one of the most severe environmental effects for the survival of rhizobia. Moreover, salinity has been identified as a potential threat to the

sustainability of agricultural development in Thailand, especially in the Northeast region exhibiting large expanses degradation of land producing salt scalds. Salinity causes the detrimental effect to cell due to the combination of ionic and osmotic stress causes the denaturing of protein and lack of water activity. The loss of even a small fraction of intracellular water is lethal death for most cells (Billi et al., 2000). Salt stress not only affects on bacterial survival but also on all stage of nodule formation; including restriction of root colonization, inhibition of processes of infection and nodule development, or impairment of active nodule functioning. These effects may be mediated through an effect of salt on the hosts or through a specific effect on microsymbiont itself (Abdelmoumen et al., 1999). Rhizobia show marked variation in salt tolerance. A number are growth inhibited by 100 mM salt, especially, *Bradyrhizobia* (Singleton et al., 1982; Yelton et al., 1983; Zhang, et al., 1991), while *Sinorhizobium meliloti* (Graham and Parker, 1964; Sauvage et al., 1983), *S. fredii* (Yelton et al., 1983) and *Rhizobium tropici* (Graham, 1992) have been reported the growth at salt concentrations more than 300 mM.

The mechanisms of cellular adaptation preventing water loss under salt stress condition have been extensively studied in bacteria, fungi, algae, plants, and animals (Martin et al., 1999; Brown, 1976; Somero et al., 1992; Yancey et al., 1982). The well characterized adaptation-strategies to be used to deal with these problems are (i) the intracellular accumulation of anionic ions such as potassium, (ii) the accumulation, either by transport or synthesis, of selected organic molecules termed compatible solute (e.g. proline, glycine betaine, proline betaine, octoine) which may be negative charged (and act as counterions for intracellular K^+) or neutral (e.g., glutamate, trehalose) (Wood, 1999). The osmoprotection mechanism of rhizobial species displays

a large variation. In the presence of high levels of salt (up to 300 to 400 mM NaCl), the intracellular free glutamate and/or K^+ were greatly increased (sometimes up to six fold in a few minutes) in cell of *R. meliloti* (Botsford and Lewis, 1990; Jakobson, 1985; Le Rudulier and Bernard, 1986) *R. fredii* (Fujihara and Yoneyama, 1993; 1994; Yelton et al, 1983), *Sinorhizobium fredii* (Susheng et al, 1993), and rhizobia from the woody legume *Leucaena leucocephala* (Yap and Lim, 1983). K^+ strictly controls Mg^{2+} flux during osmotic shock. Accumulation of several osmolites to maintain cell integrity have been reported in several rhizobia; *N*-acetylglutaminyl-glutamine in *R. meliloti* (Smith et al., 1994; 1989), trehalose in *R. leguminosarum* (Breedveld et al., 1991) and peanut rhizobia (Ghittoni and Bueno, 1996); glycine betaine in almost rhizobium species, except *B. japonicum* (Boncompagni et al., 1999). However, these salt adaptation mechanisms are only one part of the complex mechanisms. Attempt to understand complete mechanism have been intensively studying.

This research was aimed at the study of biotic and abiotic factors controlling nodulation competitiveness of *Bradyrhizobium*. Biotic factors affecting competitiveness was focused on the general competitive ability (GCA) of bradyrhizobia strains, soybean cultivar and proportion of inoculation. Abiotic factor was emphasized only on salinity stress. For the salt tolerant rhizobia to be used to investigate salt adaptation mechanism were prior isolated from nodule of wild weed legume which naturally grown under salt affected area. Due to the complexity of salt stress responses, the mechanisms involving in salt tolerant under genomic and proteomic level were explored. Under molecular level study was performed through the use of techniques analysis of gene expression, random mutagenesis and DNA sequencing. Besides, the investigation in proteomic level was performed by analysis

of membrane proteomic and quantitative analysis to examine the shift of protein abundant at the membrane region.

The discovery of novel genes or proteins could improved the understanding of their roles in salt stress adaptation, consequently, provided the basis of effective engineering strategies leading to greater stress tolerance. Therefore, construction of superior salt tolerant bradyrhizobia in commercial inoculant might be improved a long term survival under stress soil as well as solve nodulation competitiveness problem due to increase the full expression in nodulation, consequently, improve soybean yield as described by Chien and colleagues (1992) that the high-tolerant strains are symbiotically more efficient than salt-sensitive ones under saline conditions.

Research objectives

a) To investigate the effect of biotic factors including intrinsic bradyrhizobia strains, soybean cultivar and proportion of inoculation on nodulation competitiveness of bradyrhizobia.
b) To isolate and characterize of salt tolerance genes from salt tolerant rhizobia.
c) To investigate the expression of membrane proteome of salt tolerant rhizobia under salt stress condition.
d) To genetically engineer salt tolerant bradyrhizobia by using genes involved in salt tolerance and determine the nodulation competitive pattern of such strain under salt stress condition.

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CHAPTER II

REVIEW OF THE LITERATURE

2.1 Rhizobia

Rhizobia are gram-negative chemoheterotrophic organotroph bacilli that live freely in the soil. They have symbiotic relationships with legume plants. The bacteria fix nitrogen from the atmosphere into a plant usable form. In return the plant feeds the bacteria with sugars, proteins, and oxygen. They are capable of forming relationships with a wide variety of legumes such as alfalfa, clover, soybeans and peas. In nitrogen-poor soils, rhizobia give the advantage to their hosts, allowing them to grow in nitrogen poor soil.

Rhizobia have been classified into six different genera according to Bergey' s Manual of Determinative Bacteriology (Jordan, 2001) and 16S rRNA analysis; *Rhizobium* (Frank, 1889); *Bradyrhizobium* (Jordan and allen, 1974); *Sinorhizobium* (Chen et al., 1988); and *Azorhizobium* (Dreyfus et al., 1988); *Mesorhizobium* (Jarvis et al., 1997); and *Allorhizobium* (de Lajudie et al., 1998). They are normally rod-shaped and do not form endospores. They are motile by one polar or subpolar flagellum or by 2–6 peritrichous flagella, and grow by aerobic respiration of various carbon compounds. They are easily cultivated on medium containing carbohydrates and a considerable portion of this carbohydrate is converted to extracellular slime, which presumably serves a role in the environment (Jordan, 1989).

The interaction between a particular strain of rhizobia and the "appropriate" legume is mediated by a "Nod factor" secreted by the rhizobia and transmembrane

receptors on the cells of the root hairs of the legume. Different strains of rhizobia produce different Nod factors, and different legumes produce receptors of different specificity (Roa, 1999). If the combination is correct, the bacteria enter an epithelial cell of the root; then migrate into the cortex. Their path runs within an intracellular channel that grows through one cortex cell after another, then the infection threads developing (Rhijn and Vanderleyden, 1995). Meanwhile, the infection threads make their way into the nodule cells and release rhizobia into the cytoplasm of infected cells. The rhizobia, which act as symbiosomes, enlarge and differentiate into nitrogen-fixing bacteroids, then the nitrogen fixation started (Appelbaum, 1990).

2.2 Competition for nodulation of *Rhizobium* symbiosis

In the context of *Rhizobium*-legume interactions, competition success has been operationally defined as the ability of one or more rhizobial strains to occupy nodules of a legume host when challenged with a mixture strains. The strain occupying a significantly greater number of nodules than the others is said to be more competitive. This definition, however, is relatively narrow in that it only measures the end result of the complex interaction between host and microbe. In natural systems, nodulation competitiveness is most likely due to the summation of many competitive interactions including competition for: nutrients and nod gene-inducing flavonoids in the rhizosphere, attachment to plant root surface interaction sites, and space within nodules. Consequently, a *Rhizobium* strain must be competitive in several ecological areas for its occupy a majority of nodules in a field grown soybean (Sadowsky, 2000).

Nowadays, the use of rhizobial culture in establishment of legumes has been widely recognized. The benefits by the use of *Rhizobium* inoculants shows cost effective by the marginal farmers provided that they use quality tested inoculants on the farm. However, nodule formation by effective inoculant often fail when inoculum is applied into the area where contained indigenous rhizobia. This is called "**competitive problem**". There are a lot of factors controlling this phenomenon. The known factors influencing the competitiveness can be categorized into 2 major groups.

a) Biotic factors including:

1. Leguminous plant host
2. Rhizobial strains
3. Other soil microorganisms

b) Abiotic Factors including:

1. Temperature
2. Plant nutrient limitation and requirement
3. Soil moisture
4. Soil pH
5. Soil salinity

2.2.1 Biotic factors

Rhizobium-Leguminous symbiosis is a complex biological interaction. Therefore, the success in symbiosis depends upon both sides of organisms. The competitiveness for nodulation of rhizobia mainly depends upon biotic factors which are connected to living things. It consists of i) leguminous plant host; ii) rhizobia or bradyrhizobia strains; iii) other soil microorganisms.

2.2.1.1 Leguminous plant host

In many symbiotic partnerships, the host plant exerts a major influence on initiation of symbiosis (Acuna et al., 1987; Bhuvaneswari et al., 1980). Because the competitiveness of indigenous and introduced microorganisms is tied to an organism's ability to nodulate a given legume, host genes influencing nodulation affect competition in a primary manner. Numerous studies have shown that the legume host can dramatically influence the prevalence, types and competitiveness of rhizobia in soils (Bottomey, 1992; Crcgan and Keyser, 1986; Vest et al., 1973). This is thought to be, in part, due to host-controlled selective or restrictive nodulation mechanisms (Cregan, 1989), physiological differences between soybean genotypes and to differential responses of rhizobial strains to *nod* gene-inducing signal molecules (Bottomey, 1992).

In 1954, a single recessive gene, termed *rj,* was reported that produces a nonnodulating condition with all *B. japonicum* strains (Williams and Lynch, 1954). The single dominant gene *Rj,* (Caldwell, 1966) conditions restricted nodulation with all tested strains of the 122 and cl serogroups (Caldwell et al., 1966). The single dominant gene *Rj,* conditions restricted nodulation with only USDA 33 and not with other strains that are serologically related (Vest, 1970). A fourth single dominant gene,

Rj, conditions restricted nodulation with strain USDA 61 (Vest and Calwell, 1972). The reaction of Rj_4 with strains serologically similar to USDA 61 has not been reported, although other strains are reportedly restricted for nodulation (Deving et al., 1990).

Host-controlled nodulation restriction was also found in the *Pisum sativum-R. leguminosarum* bv. *viceae* strain TOM symbiosis. While strain TOM nodulates the pea genotype *P. sativum* cv. Afghanistan (Brevin et al., Gottfert et al., 1990; Davis et al., 1988), most European and North American strains of *R. leguminosarum* bv. *viceae* fail to nodulate this host (Brevin et al., 1980). On the other hand, several Middle Eastern strains of *R. leguminosarum* bv. *viceae* nodulate commercial pea cultivars in addition to cv. Afghanistan (Acuna et al., 1987; Ma and lyer, 1990). A single recessive host gene, *sym-2,* found in cv. Afghanistan, was subsequently shown to condition nodulation restriction (Lie, 1984). A CSN gene, *nodX,* which specifically interacts with the *sym-2* locus, has been isolated from strain TOM (Davis et al., 1988).

Host controlled restriction of soybean nodulation has also been demonstrated in other *B. japonicum* (Ferry et al., 1994) and *R. fredii* symbioses (Meinhardt et al., 1993). Lewis-Henderson & Djordjevic (1991) indicated that a single recessive gene in cv. Woogenellup, *rwt1,* is responsible for conditioning nodulation restriction with strain TA1. Two negatively acting *R. leguminosarum* bv. *trifolii* strain TAl genes, *nodM* and *csn-I,* have been shown to specifically interact (in a gene-for-gene manner) with cv. Woogenellup (Lewis-Henderson & Djordjevic, 1991).

Host preference for nodulation among *B. japonicum* strains has been reported by Bottomley et al (Bottomley et al., (1994). They identified 17 ETs among 95 strains isolated from *Lupinus* and *Ornithopus* species. Of these, 73% fell into two closely related Ets, which dominated in the nodulation of white lupin, seradella and sirato. In addition to their ability to select specific strains of bradyrhizobia, there are several examples where the host plant restricts nodulation by specific strains or serogroups of *B. japonicum* strains. Preempting nodulation by ineffective or inefficient indigenous strains of bradyrhizobia has been proposed as a means to control competition for nodulation (Cregan, 1989).

Strain by cultivar (or genotype) interactions have been demonstrated in the *B. japonicum*-soybean symbiosis and this host-controlled restriction of nodulation occurs at the strain or serogroup level (Sadowsky and Graham, 1998), Lohrke and colleague (1977) showed that single recessive soybean allele and a single *B. japonicum* gene, *noe*D (Lohrke et al., 1998), interact to control selective nodulation specificity.

2.2.1.2 Rhizobial stains

The intrinsic biotic factors of rhizobial strains; genetic and physiological characteristics possessed by rhizobial strain; are involved in competition. However, the competition is challenged between one or more rhizobial strains to occupy nodules of a legume host, thus, inoculants and indigenous rhizobia/bradyrhizobia are involved.

"**Inoculants rhizobial stains**" are the effective nitrogen fixation strain which have been well screened, characterized and selected for the highest performance in nodulation. The criteria of selection are high N_2 fixation levels,

adapted to the set of environment conditions of a specific site and the wide host range preference.

"**Indigenous rhizobial stains**" are the native rhizobia that naturally colonize in soil and are generally highly competitive ability, but ineffective nitrogen fixation. How an organism becomes indigenous is not presently known. It is thought that the primary or early preemptive colonization, followed by prolonged periods of stable maintenance in a soil population leads to the establishment of the "indigenous" state. Several studies suggest that a nonindigenous microbe can become a member of the indigenous, autochthonous, population by prolonged and repeated applications of the microbe in the soil inoculants (Turco and Sadowsky, 1995).

Intrinsic factors influencing competition of both inoculant rhizobia and indigenous rhizobia include: *i*) cell surface molecules *ii*) motility and chemotaxis; *iii*) production of antibiotics; *iv*) nodulation efficiency genes; *v*) speed of nodulation; *vi*) number of indigenous rhizobia.

i) **Cell surface molecules**: Alterations or deletion in genes controlling cell surface characteristics influence competition for nodulation. In *S. fredii,* nonmucoid mutants of strain USDA208 are more competitive for nodulation of "Peking" soybean roots than the wild-type strain (Zdor and Pueppke, 1991). Moreover, transposon *Tn*5 insertion mutants of *B. japonicum* deficient in exopolysaccharide synthesis were less competitive than the wild-type strain (Bhagwat and Keister, 1991).

ii) **Motility and chemotaxis**: The motility and chemotaxis mutants have also been shown to be impaired in competition for nodulation (Caetano-Anolls et al., 1988). Ames & Bergman (1981), who examined nonmotile mutants of *R. meliloti*

that were either flagellated or nonflagellated. Both types of nonmotile mutants were less competitive for nodulation than the wild-type strain but were identical to the wild-type in growth rate and nodule formation. Liu et al. (1989) reported that a nonmotile *Tn*7 mutant of *B. japonicum* was decreased relative to the wild-type strains.

iii) **Production of antibiotic:** Genes encoding the production of antibiotic factors have also been shown to confer increased nodulation competitiveness (Triplett and Sadowsky, 1992; Triplett, 1990). For example, trifolitoxin genes in *R. leguminosarum* bv. *trifolii* have been shown to increase nodulation competitiveness of *R. etli,* and presumably other rhizobia, in soil (Chun and Stacey, 1994). The small bacteriocin; *N*-acyl-L-homoserine lactone quorum sensing molecule; is found in strain *R. leguminosarum*. This antibiotic inhibits a wide range of bacteria (Schripsema et al., 1996).

iv) **Nodulation efficiency genes:** In some cases, the ability of a microsymbiont to efficiently and effectively nodulate it's legume host has been shown to affect competition for nodulation (Sanjuan and Olivares, 1991). Genes influencing the efficiency of nodulation, *nfe* (for nodule formation efficiency), have been identified in *R. meliloti* (Sanjuan and Olivares, 1991) and *B. japonicum* (Chun and Stacey, 1994). In *B. japonicum*, the *nfe*C gene has also been shown to influence competitiveness (Chun and Stacey, 1994). In addition, mutations in the *B. japonicum nodVW* and *R. fredii nolJ* genes have been reported to cause a delay in nodulation (Gottfert, 1993; Boundy-Mills et al., 1994). In *R. meliloti,* the gene cluster involved in host-specific nodulation *(hsn)* has been designated *hsnABCD* [also termed *nodFEGH,* respectively] (Debelle et al., 1986). Genotype-specific nodulation (GSN) genes are those bacterial sequences that allow nodulation of specific plant genotypes within a

given legume species (Lewis-Henderson and Djordjevic, 1991; Sadowky et al., 1991). If the plant genotype is a cultivated variety, the genes are referred to as cultivar-specific nodulation (CSN) determinants (Lewis-Henderson and Djordjevic, 1991). Several other GSN (or CSN) genes have also been reported. In *Sinorhizobium fredii* (formally *R. fredii)* strain USDA257, a single, chromosomally located CSN gene, *nolC,* has been shown to control nodulation of a commercial soybean cultivar (Lewis-Henderson and Djordjevic, 1991). This GSN gene of *B. japonicum* strain USDA110; the *noIA* gene; was found the allowing serocluster 123 isolates to form nodules on serogroup 123-restricting plant genotypes (Sadowky et al., 1991). The 710-bp open reading frame is located approximately 3.6 kb transcriptionally downstream of *nodD* and is presumably transcribed from its own promoter. Translational/transcriptional *lacZ* fusion experiments indicated that *nolA* was moderately induced by *nod* gene transcriptional activators, such as soybean seed extract and the isoflavone genistein (Sadowky et al., 1991). The GSN and CSN genes can affect nodulation competitiveness of an organism by eliminating or inhibiting nodulation ability (which obviously reduces its competitiveness) or by blocking nodulation of other nodulation-competent strains (Dowling et al., 1989; Chatterjee et al., 1990).

v) **Speed of nodulation:** Early rhizobia1 infection of legume roots induces an autoregulatory response in the plant that prevents infection by subsequent inoculations (Pierce and Bauer, 1983; Stephens and Cooper, 1988). This has also been demonstrated in split-root systems in which two sides of a root are spatially separated and inoculated at different time intervals (Kosslak and Bohlool, 1984). Nodulation is prevented on that side of the split root that is inoculated 24 hours after the other. Suppression of nodulation increases as the time interval between inoculation of the

two sides increases (Kosslak and Bohlool, 1983). The suppression of late nodulation occurs at the nodule-meristem stage of development prior to nodule growth (Calvert et al., 1984). The split-root system appears to be a useful screening method for determining the competitiveness of a group of strains (Sargent et al., 1987). However, in each of these studies, unrelated strains were used rather than genetically defined isogenic ones. As a result, no definitive conclusion about the role of speed of nodulation in nodulation competitiveness can be ascertained. Moreover, there are some conflicting reports about the correlation between speed of nodulation and nodulation competitiveness (Trinick and Hadobas, 1989). However, these studies also used genetically unrelated strains.

vi) **Number of rhizobia:** Several studies have shown that number of indigenous rhizobia affect the competition of nodulation. Thies et al. (1991) and Saginga et al. (1996) suggested reduction in the percentage of nodules formed by the inoculant strain when indigenous strains occur at levels of only 10 rhizobia g^{-1}. Frequently, introduced strains are outnumbered by indigenous soil populations by as much as 250:1 are not evenly distributed throughout the soil (Brockwell et al., 1995), and are often not well adapted to general soil conditions. On the other hand, the numbers of inoculant strain are also important in order to overcoming indigenous rhizobia. Weaver & Frederick (1974) have estimated that to obtain 50% occupancy of soybean nodules by the inoculum strain, an inoculant rate 1000 times the soil population would be required. Singleton and Stockinger (1983) inoculated soybeans with various mixtures of effective and ineffective strains of *R. japonicum*. As anticipated, they found that the proportion of effective nodules formed increased as the ratio of effective to ineffective bacteria became greater in the inoculant. However,

the total volume of effective nodules tissue remained approximately constant throughout. This was regarded as a 'compensatory mechanism' for keeping the amount of effective nodules tissue constant even as the proportion of effective nodules declined.

2.2.1.3 Other soil mocroorganisms

The interaction with other root organisms could achieved in either positive advantage or disadvantages. There are some publications reported the enhancing of nodulation. Coinoculation of rhizobia with vescicular mycorrhizae, phosphate solubilizing microbes, plant growth promoting rhizobia, and *Bacillus* increased either nodulation, nitrogen fixation, or root populations (Peterson et al., 1996; Singh and Singh, 1989). Antibiotic-producing *Pseudomonas fluorescens* CHA0 impaired the ability of one of three strains of *S. meliloti* to compete for nodulation (Postma et al., 1990). Inhibition of growth and nodulation of *R. leguminosarum* bv. *trifolii* on subterranean clover was showed to be caused by antibiotic-producing fungi (Postma et al., 1990). Predatory interactions between rhizobium and protozoans have been review by (Danso and Kenya, 1975).

2.2.2 Abiotic factors

Abiotic factors are involved in several substances or environment conditions which affect the nodulation of competition. Several environmental conditions are limiting factors to the growth and activity of the N_2-fixing plants. In the *Rhizobium*-legume symbiosis, which is a N_2-fixing system, the process of N_2 fixation is strongly related to the physiological state of the host plant. Therefore, a competitive and persistent rhizobial strain is not expected to express its full capacity for nitrogen

fixation if limiting factors impose limitations on the vigor of the host legume (Brockwell et al., 1995; Peoples et al., 1995). Some of these factors might directly affect competitiveness, many most likely act by altering the persistence and survival of inoculated strains and only indirectly influence competitive interactions. Abiotic factors have reported in influence the competition including: *i)* temperature; *ii)* plant and microbial nutrient limitations and requirements; *iii)* soil moisture; *iv)* soil pH; *v)* soil salinity.

2.2.2.1 Temperature

Soil temperature has also been shown to greatly influence the growth and survival of rhizobia in soil and competition for nodulation (Sadowsky, 2000). Kennedy and Wollum (1998) reported that population levels of *B. japonicum* decreased in soils that were exposed to elevated temperature and Kluson and coworkers (1986) report the differential competitiveness of *B. japonicum* in response to soil temperature.

2.2.2.2 Plant and microbial nutrient limitations and requirements

A variety of nutritional factors are influence the growth of rhizobia in the rhizosphere, in some instances directly affecting competitive interactions. Brockwell and coworkers (1995) have reviewed the nutritional factors influencing the ecology of rhizobia in soil. Bradyrhizobia is fairly metabolically diverse and has been shown to use a variety of plant-derived compounds for growth. Some compounds have been shown to be chemotactic and induce nod genes in *B. japonicum* (Sadowsky and Graham, 1998). Metabolic engineering of rhizobia for use of specific host-derived nutritional factors in the rhizosphere, e.g. rhizopines produced in a “biased

rhizosphere, has been proposed as one means to alter competitiveness (O'Connell et al., 1996).

2.2.2.3 Soil moisture

Symbiotic N_2 fixation of legumes is also highly sensitive to soil water deficiency. A number of temperate and tropical legumes, e.g., *Medicago sativa* (Abdel-Wahab and Zahran, 1983), *Pisum sativum* (Abdel-Wahab and Zahran, 1979), *Arachis hypogaea* (Simpson and Daft, 1991), *Vicia faba* (Devries et al., 1989) exhibit a reduction in nitrogen fixation when subject to soil moisture deficit. In a recent work, Athar and Johnson (1996) reported that two mutant strains of *R. meliloti* were competitive with naturalized alfalfa rhizobia and were symbiotically effective under drought stress. These results suggest that nodulation, growth, and N_2 fixation in alfalfa can be improved by inoculating plants with competitive and drought-tolerant rhizobia.

2.2.2.4 Soil pH

For bradyrhizobia and rhizobia, competitive interactions have been shown to be influenced by soil pH (Sadowsky and Graham, 1997), due to *Rhizobium* strains are unable to grew in culture media at pH 5.0, while *Bradyrhizobium sp*. are able to tolerate pH 4.5 (Brockwell et al., 1991). Munns et al. (1979) noted that nodulation and nitrogen fixation by some strains of *Bradyrhizobium* at acidic pH differ with the cultivar of mung bean used. Vargas and Graham (1989) examined the cultivar and pH effects on competition for nodule sites between isolates of *Rhizobium* in beans (*P. vulgaris*) under acidic conditions. They found a significant effect of host cultivar, ratio of inoculation, and pH on the percentage of nodule occupancy by each strain.

2.2.2.5 Soil salinity

Salinity affects the competition due to the reduction of survival rate of inoculum. Unsuccessful symbiosis under salt-stress may be due to failure in the infection process because of the effect of salinity on the establishment of rhizobia (Singleton and Bohlool, 1984). However, Chien and colleagues (1992) have shown that highly salt tolerant rhizobial strains are symbiotically more efficient than salt sensitive ones under salt stress. Details of salt affecting N_2 fixation are described in the later part of this review.

2.3 Salt affected area in Thailand

Salinity is not a new problem identified as a potential threat to the sustainability of agricultural development in Thailand. The classification of 6 main problems of land resources in Thailand demonstrated that the main problem is salt affected area. Especially, in the northeastern part of Thailand, this type of soil is scattered all over. The total area ranging from severe, moderate, to low salinity is amounted to 17.8 million rai or 16.73% of the northeastern land area (Table 2.1) (Land Development, www, n.d.). This region composed of claystones and shales interbedded with two to three layers of evaporites (halite, gypsum, anhydrite, carnallite and sylvite) varying in thickness from 10 to 170 m (Japakasetr and Suwanich, 1984). Nakhon Ratchasima province has the highest irrigated area of about 81,250 rai of which 30 percent is already salt-affected with different severity and it keeps increasing year by year (Sustainable use of problem soils in rainfed agriculture, www, 2003).

Increasing in salinity is due to the migration of salts from underlying deposits. Evaporite structures are relatively plastic, and in some areas where the overburden is thinner or of less density the evaporite layer may rise in a dome shape thus increasing the source of localised salt and creating salinity hotspots (Supajanya 1992). Heavy floods particularly in salt-patch areas damaged large areas of the region's vegetative cover, causing severe soil desalinization in rainfed agriculture as well as irrigated salt-affected soils which contributes to the formation of sodic soils; a soil pH above 8.4 and high in salt content; and increases the severity of the problem. Moreover, saline ground water reaches the surface through the openings created by salt mining activities, consequently, major effect saline contamination of landuse.

Table 2.1 Specific problem soils in Thailand.

Problem Soils	**Area (rai)**
1. Salt Affected Soils	21,718,790
1.1 Coastal Saline Soils	3,611,580
1.1.1 Coastal Saline Soils, Potentially Acid	2,885,090
1.1.2 Coastal Saline Soils, Non-potentially Acid	726,490
1.2 Inland Saline/Sodic Soils	18,107,210
1.2.1 extream saline soil	1,771,220
1.2.2 moderate saline soil	3,690,250
1.2.3 low saline soil	12,645,740
2. Sandy Soil	7,127,500
2.1 extream sandy soil, no organic stratum	6,613,530
2.2 extream sandy soil with organic stratum	513,970
3. Acid Sulphate Soil	5,326,790
4. Organic Soil	505,180
5. Shallow Soil	51,291,150
5.1 Laterrite soil and conglomerate soil	31,796,210
5.2 Soil with stone	17,327,600
5.3 Soil with calcium bi carbonate	2,167,340
6. Slope Complex	96,158,200
Total	**182,127,610**

Source: Land Development (www, n.d.)

2.4 Effects of salinity soil on Nitrogen fixation

As with most cultivated crops, the salinity response of legumes varies greatly and depends on such factors as climatic conditions, soil properties, and the stage of growth (Cordovilla and Lluch, 1995; Cordovilla et al., 1995 (A, B)). Therefore, it is important to understand the effect of salinity on soybean cultivation.

2.4.1 Salt tolerant ability of legume

Leguminous plant exhibit differ in their response to salt stress. *Phaseolus vulgaris* tolerates low (48 mM NaCl), but not higher levels (72 and 96 mM NaCl) of salinity stress (Wignarajah, 1990). Some legumes, e.g., *Vicia faba*, *P. vulgaris*, and *Glycine max*, are more salt tolerant than others, e.g., *Pisum sativum* (Delgado et al., 1994). It has been reported that some *V. faba* tolerant lines sustained nitrogen fixation under saline conditions (Abdel-Wahab and Zahran, 1981**;** Cordovilla et al., 1995). Other legumes, such as *Prosopis* **(**Fagg and Stewart**,** 1994**),** *Acacia* **(**Zhang et al., 1991), and *Medicago sativa* **(**Abdel-Wahab and Zahran, 1983), are also reported in tolerate of salt.

Many soybean genotypes are resistant to the salt stress. Soybean cultivar ICAL-132 showed better growth than others tested three cultivars by exhibited a reduction of shoots fresh weight only 33% at 40 mM NaCl (Shereen and Ansari, 2001). In four independent studies, 33 of 66, 6 of 16, 19 of 60, and 10 of 257 U.S. cultivars and breeding lines were identified as resistant to chloride, based on visual leaf scorching ratings and/or reduced chloride levels in the leaf (Parker et al., 1983, 1986; Shao et al., 1995; Yang and Blanchar, 1993). Xu and colleagues (1999) reported that eight Chinese landraces of soybean had high levels of salt tolerance. Some accessions of the wild progenitor of soybean (*G. soja Sieb*. and *Zucc*.), and the

more distantly related perennial accessions have also been classified as chloride excluders or as salt resistant (Li et al., 2000; Pantalone et al., 1997; Wang et al., 1997). More recent work from Kao and co-worker (2005) reported the different ranking of salt tolerant among wild soybean species showing that *G. tomentella* tolerate to salt up to 85 mM while concentration of NaCl up higher than that 17 mM revealed the reduction in biomass. In 1997, some commercial soybean varieties appeared tolerant to salinity (Hilal et al., 1998).

2.4.2 Genetically modified plant against salinity stress

As agricultural land is increasingly salinized through inefficient fertilizer practices, salt-water intrusion, development of salt tolerant cultivars becomes increasingly important as a means of combating salt-related yield losses. Nowadays, efforts to improve plant tolerant to high salinity through breeding and genetic engineering have been well illustrated. Salt tolerance of transgenic tobacco engineered to over-accumulate mannitol was first demonstrated by Tarczynski et al., (1993). It is interesting to note that glycine betaine- (Kishitani et al., 2000) and trehalose- (Garg et al., 2002) overproducing transgenic rice plants accumulated fewer Na^+ ions, and maintained K^+ uptake. Apse and colleagues (1999) demonstrated that the modulate overexpression of homologous cDNA encoding a sodium/proton antiporter can confer improved salinity tolerance on *Arabidopsis.* Eduardo Blumwald and coworkers (1999) have successfully engineered transgenic *Arabidopsis* plants that overexpress *AtNHX1,* a vacuolar Na^+/H^+ antiport, which allowed the plants to grow in 200 mM NaCl. In the 2001, Zhang and Blumwald reported the genetic modification of tomato plants to overexpress the *Arabidopsis thaliana AtNHX1* antiport, which

likewise allowed those plants to grow in the presence of 200 mM NaCl. Similar results have been reported for transgenic canola (*Brassica napus* L.) over-expressing *AtNHX1* (Zhang et al., 2001). The engineering of biosynthetic enzymes for osmoprotectants has been showed to improve salinity tolerant plant (Weretilnyk et al., 1990). Even though, engineering of leguminous plant have not yet been reported, but with this knowledge, the successful construction of highly salt tolerant cultivar will be achieved in near by future.

2.4.3 Salinity affects growth and survival of Rhizobia

In agriculture, leguminous biological nitrogen fixation has been used to improve infertile soils, especially those affected by salinity (Surange et al., 1997; Zhang et al., 1991) However, nodulation and nitrogen fixation in legume-*Rhizobium* associations are adversely affected by salinity (Mohammad et al., 1991). The effects of salt stress on nitrogen fixation have been examined in several studies (Lauter et al., 1981; Rai and Prasad, 1983; Velagaleti et al., 1990). Rhizobial strains are very sensitive to high salt stresses, which affects their growth and survival in soil, restriction on root colonization, inhibition of processes of infection, nodule development, or impairment of active nodule functioning, dinitrogen fixation capacity and hence the productivity of legumes (Abdelmoumen et al., 1999; Athar and Johnson, 1997; Cordvilla, 1996).

Growth of rhizobia under salt stress environment showed high variation. Rhizobia, e.g., *R. meliloti* were tolerant to 300 to 700 mM NaCl (Embalomatis et al., 1994; Helemish et al., 1991; Mohammad et al., 1991; Sauvage et al., 1983). Strains of *R. leguminosarum* have also been reported to be tolerant to NaCl concentrations up to

350 mM NaCl in broth culture (Abdel-Wahab and Zahran, 1979, Botsford and Lewis, 1990). *Rhizobium* strains from *V. unguiculata* tolerant up to 5.5% NaCl, which is equivalent to about 450 mM NaCl (Mpepereki et al., 1997), while some Rhizobia from woody legumes also showed substantial salt tolerance: strains from *Acacia*, *Prosopis*, and *Leucaena* are tolerant to 500 to 850 mM NaCl (Lal and Khanna, 1995; Zahran et al., 1994; Zhang et al., 1991). Soybean and chickpea rhizobia were tolerate upto 340 mM NaCl, with fast-growing strains being more tolerant than slow growing strains (El-Sheikh and Wood, 1990). Similar report from Mohammad and coworker (1991) showed that the slow growing peanut rhizobia are less tolerant when compared to fast-growing rhizobia. Thus, tolerance to salt stress is an important part of saprophytic competence and competitiveness in *Rhizobium* (Yap and Lim, 1983). Many reports have shown that high salt-tolerant strains are symbiotically more efficient than salt-sensitive ones under saline conditions (Chien et al., 1992) and often out competed by indigenous strains that already exist in the field.

2.4.4 Salt tolerant mechanism of rhizobia

Basic mechanism of all bacteria respond in environmental osmolarity upshift are changes in cell structure, organization, and composition that result from transmembrane water flux (Figure 2.1, left column) trigger and are modulated by physiological responses (Figure 2.1, right column). Bacteria respond to osmotic upshifts in three overlapping phases: dehydration (loss of some cell water) (phase I), adjustment of cytoplasmic solvent composition; particular zwitterionic organic cosolvents such as ectoine and glycine betaine are selected for this role over inorganic solutes such as K^+ and rehydration, (phase II), and cellular remodeling by

osmoregulation of uptake, efflux, biosynthesis, and/or catabolism is required to modulate the cytoplasmic levels of these osmoregulatory (compatible) solutes (phase III) (Wood, 1999; Miller and Wood, 1996). These compatible solutes maintain an equilibrium between macromolecule surface areas and the water phase by resisting drastic changes in intracellular water density. These interactions stabilize proteins by raising the chemical potential of the denatured protein, which leads to contraction of the random coil to a folded structure (Qu et al., 1998). A recent study quantifying the stability afforded by compatible solutes showed that the osmolyte trimethylamine oxide can increase the population of folded structures compared to denatured protein by nearly 5 orders of magnitude (Baskakov and Bolen, 1998). Therefore, the potential of compatible solute on prevent denaturing of proteins is base on the ability of compatible solutes to accumulate at these interface regions (Martin et al., 1999; Wiggins, 1990).

The salt adaptation mechanism of rhizobial species displays a large variation. In the presence of high levels of salt (up to 300 to 400 mM NaCl), the intracellular free glutamate and/or K^+ were greatly increased (sometimes up to six fold in a few minutes) in cell of *R. meliloti* (Botsford and Lewis, 1990; Jakobson, 1985; LeRudulier and Bernard, 1986) and *S. fredii* (Fujihara and Yoneyama, 1993; 1994; Yelton et al., 1983; Susheng et al., 1993). Moreover, maintaining cytoplasmic osmolality by accumulation of osmolytes is also well identified in Rhizobia. The accumulation of these osmolytes is dependent on the level of osmotic stress, the growth phase of the culture, the carbon source, and the presence of osmolytes in the growth medium. An osmolyte, *N*-acetylglutaminyl-glutamine amide, accumulates in cells of *R. meliloti* (Smith et al., 1994; 1989). Trehalose accumulates to higher levels in cells of *R.*

leguminosarum (Breedveld et al., 1991) and peanut rhizobia (Ghittoni and Bueno, 1996) under the increasing osmotic pressure of hypersalinity. However, these compounds, unlike other bacterial osmoprotectants, do not accumulate as cytosolic osmolytes in salt-stressed *S. meliloti* cells. Talibart and co-worker (1997) showed that ectoine acts as osmoprotectant for a various rhizobia (*S. meliloti, B. japonicum,* and *R. reguminosarum*), however it does not accumulate within cells. Glycine betaine; the most effective osmotic stress response; was well identified in *R. meliloti* (LeRudulier and Bernard, 1986; Sauvage et al., 1983; Smith et al., 1988). Besides, when externally provided glycine betaine and choline enhance the growth of *R. tropici*, *S. meliloti*, *S. fredii*, *R. galegae*, *Mesorhizobium loti*, *M. huakuii*, and *Agrobacterium tumefaciens* (Boncompagni et al., 1999). Almost all *Rhizobium* species, except *B. japonicum*, are able to adapt well in osmotic stress by accumalating glycine betaine (Boncompagni et al., 1999). The addition of sodium salts to bacteroids of *Medicago sativa* nodules increased the uptake activity of the exogenously added glycine betaine (Fougere and Le Rudulier, 1990). These osmoprotective substances may play a significant role in the maintenance of nitrogenase activity in bacteroids under salt stress.

Base on above discussion, nodulation competitiveness of inoculant rhizobia is one of the major factors that determine nitrogen fixation in legumes. Therefore, the improvement of nodulation competitiveness in intended for legume inoculation under salt stress condition is of considerable practical importance.

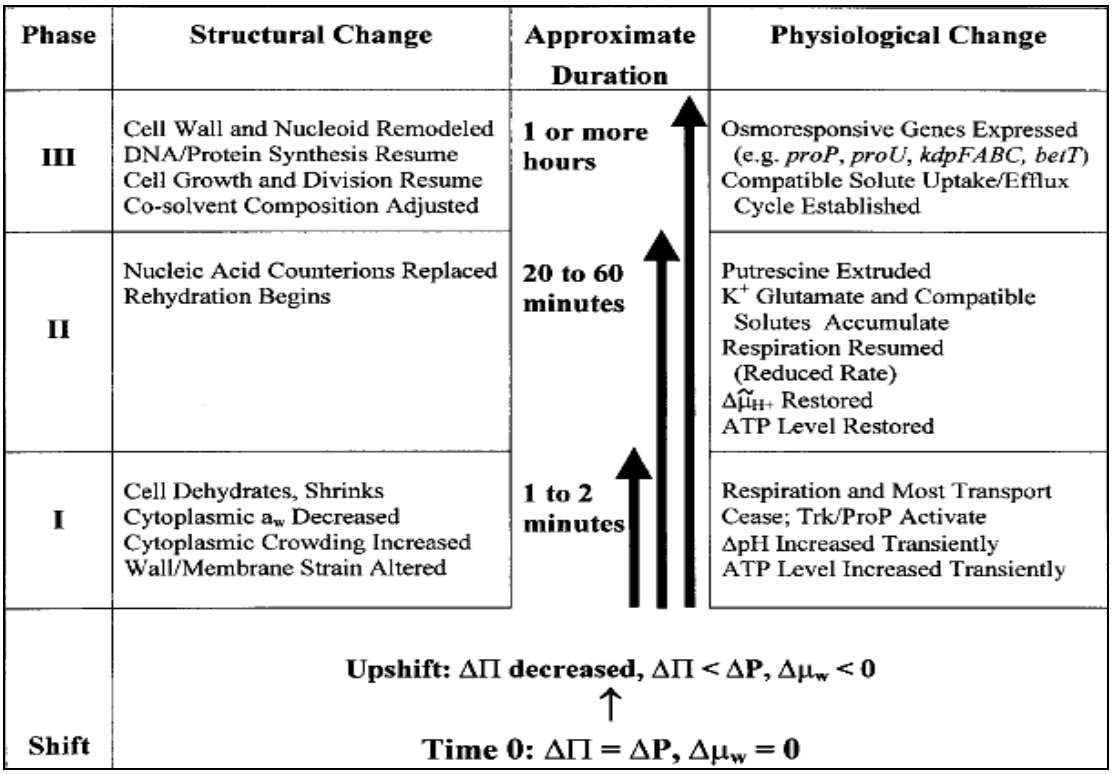

Figure 2.1 Phases of the osmotic stress response for *E. coli* K-12. Structural and physiological responses triggered by osmotic upshifts imposed at time zero proceed in parallel along the indicated, approximate timescales (Wood, 1999).

2.5 Proteins expression change under salt stress condition

A sudden increase in environmental salinity cause severely effect to *Rhizobium* survival. To avoid the cell devastation from such effect, cell response by instantly induce a global network of proteins; salt stress response proteins. Nowadays, the proteomic analysis approach is introduced to examine global protein expression changes. The proteome has been defined as the protein complement expressed by a genome under specific condition (Wilkins et al., 1996; Wasinger et al., 1995;

Hochstrasser et al., 1998; Loo et al., 1996). Proteome reflects the cellular state or the external conditions encountered by a cell, and proteome analysis can be viewed as a genome-wide assay to differentiate and study cellular states and to determine the molecular mechanisms that control them (Haynes et al., 1998). Therefore, the investigation of salt tolerant mechanism can be accomplished under genomic or proteomic level. Besides, working of both strategies in parallel could be proved and clarified the actual mechanisms. Interestingly, the availability of (genomic) DNA databases listing the sequence of every potentially expressed protein and rapid advances in technologies capable of identifying the proteins that are actually expressed now make proteomics a realistic proposition (Aebersold and Goodlett 2001). The investigation of protein expression under salt stress condition has been reported in several publications. Petersohn et al. (2001) have used proteomics, transcriptional analysis, transposon mutagenesis approach to identify the expression profile of general stress genes and proteins controlling by the stress sigma factor σ^B. Result revealed that at least 75 genes were induced under stress condition. Moreover, the most interesting of the σ^B-independent stress phenomena was the induction of the extracytoplasmic function sigma factor σ^W and its entire regulon by salt shock. The proteomic analysis of *L. monocytogenase* exhibited 12 proteins showing high induction after salt stress were similar to general stress proteins (Ctc and DnaK), transporters (GbuA and mannose-specific phosphotransferase system enzyme IIAB), and general metabolism proteins (alanine dehydrogenase, CcpA, CysK, EF-Tu, Gap, GuaB, PdhA, and PdhD) (Duché et al., 2002).

In *Rhizobium* species, the protein wide expression changes under salt stress condition have not yet been determined. Nevertheless, proteomic approach has been

reported in others purpose. For example, *S. medicae* protein response to pH stress by upregulated DegP, fructose bisphosphate aldolase, GroES, malate dehydrogenase and two hypothetical proteins. These findings implicate proteolytic, chaperone and transport processes as key components of pH response in *S. medicae* (Reeve et al., 2004). Proteomic analysis of heat shock protein expression in *B. japonicum* revealed 19 proteins was induced (Munchbach, 1999). Proteome analysis of the model microsymbiont *S. meliloti* could isolate and characterization of novel proteins (Guerreiro et al., 1999). Bolanos et al. (2004) has analyzed the cell surface interactions of *Rhizobium* bacteroids and other bacterial strains with symbiosomal and peribacteroid membrane components of the pea nodules. Moreover, Djordjevic et al. (2003) reported the global analysis protein expression profiles of *S. meliloti.*

In case of membrane proteomic sensing the salt stress is interesting to be explored due to it is the first organelle that interfaces with the surrounding environment. Moreover, membrane proteins play an important role in maintaining normal cell volume and intracellular ion balance involving transport of inorganic and organic molecules (Martin et al., 1999; Wiggins, 1990, Botsford and Lewis, 1990; Boncompagni et al., 1999; Smith et al., 1994; 1989; Breedveld et al., 1991). For example, the control of membrane permeability, Na^+/H^+ antiporters; membrane proteins; are essential for maintenance of the balance between Na^+ and K^+ ions in plant, fungal, and bacterial cells. It is the most important aspect of the acclimation of these organisms to high-salt conditions (Blumwald et al., 1984; Padan and S. Schuldiner, 1994). Additionally, it is wide distribution of proteins at the membrane which is important in biological functions involving the transportation of nutrients to

and from the cell (Klebba, 1998), conjugation (Koebnik, 1999), controlling cell morphology, intercellular communication and cell metabolism.

Rhizobial membrane proteins that are involved in salt stress include: the *bet*S gene, encoding a glycine betaine/proline betaine transporter (Boscari et al., 2002), the *kup* gene, encoding a potassium uptake system protein (Nogales et al., 2002), and the *omp*10, encoding outer membrane lipoprotein (Wei et al., 2004). However, salt tolerant is a complex mechanism which a group of protein network is expressed. Hence, large scale profiling expression change of membrane proteome is of great interest. Therefore, the study of membrane proteins expression changes under salt stress condition might provide better understanding of salt tolerant mechanism of rhizobia.

2.6 Proteomic analysis by mass spectrometry

2.6.1 Nano-liquid chromatography-tandem mass spectrometry (ηLC-MS/MS)

The separation principle of ηLC is almost the same as reversed-phase high-performance liquid chromatography (HPLC); the only difference is that the dimensions and flow rates are much smaller. In a typical ηLC-MS/MS experiment, the analyte is eluted from a reversed-phase column to separate the peptides by hydrophobicity, and is ionized and transferred with high efficiency into the mass spectrometer for analysis (Mann et al., 2001).

2.6.2 Protein identify by Mass spectrometry

Mass spectrometry is essentially a technique for "weighing" molecules. Obviously, this is not done with a conventional balance or scale. Instead, mass

spectrometry is based upon the motion of a charged particle, called an ion, in an electric or magnetic field. The mass to charge ratio (*m/z*) of the ion effects this motion. Since the charge of an electron is known, the mass to charge ratio a measurement of an ion's mass.

2.6.3 Protein identification by database searching

First method, a "protein mass fingerprint (PMF)" is obtained of a protein enzymatically degraded with a sequence-specific protease such as trypsin. This set of masses, typically obtained by mass spectrometry (MS), is then compared to the theoretically expected tryptic peptide masses for each entry in the database. The proteins can be ranked according to the number of peptide matches. More sophisticated scoring algorithms take the mass accuracy and the percentage of the protein sequence covered into account and attempt to calculate a level of confidence for the match (Berndt et al., 1999).

Second method, databases can also be searched by peptide sequence obtained from tandem mass spectrometric data of peptides. In this technique, one peptide species out of a mixture is selected in the first mass spectrometer and is then dissociated by collision with an inert gas, such as argon or nitrogen. The resulting fragments are separated in the second part of the tandem mass spectrometer, producing the tandem mass spectrum, or MS/MS spectrum (Mann et al., 2001). Because the tandem mass spectra contain structural information related to the sequence of the peptide, rather than only its mass, these searches are generally more specific and discriminating (Yates et al., 1997). In this way, large numbers of proteins, up to hundreds, can be identified all at once (Figure 2.2).

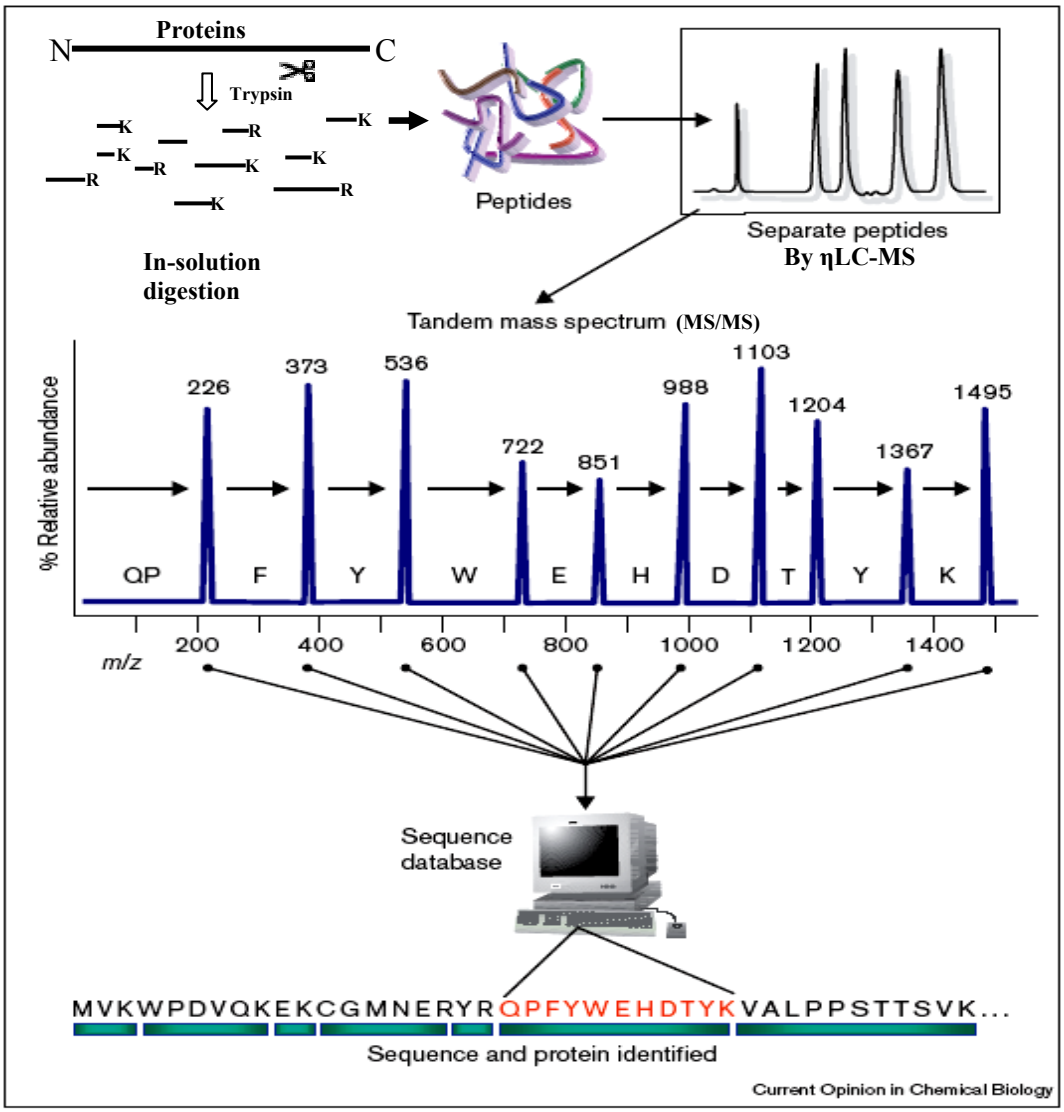

Figure 2.2 Schematic illustration of standard proteome analysis by ηLC-MS/MS. Proteins are in-solution digested with trypsin, and the resulting peptides are separated by on-line ηLC. An eluting peptide is ionized by ESI, enters the mass spectrometer, and is fragmented to collect sequence information (tandem mass spectrum). The spectrum from the selected, ionized peptide is compared with predicted tandem mass spectra that are computer generated from a sequence database to identify the protein. Unambiguous protein identification is accomplished when multiple peptides from the same protein are matched. *m/z*, mass : charge ratio. (Gygi and Aebersold, 2000).

2.6.4 Quantitative analysis

Quantitative protein profiling is therefore accomplished when a protein mixture (reference sample) is compared with a second sample containing the same proteins at different abundances and labeled with heavy stable isotopes. In theory, all the peptides in the sample then exist in analyte pairs of identical sequence but different mass. Because the peptide pairs have the same physico-chemical properties, they are expected to behave identically during isolation, separation and ionization. Thus, the ratio of intensities of the lower and upper mass components provides an accurate measure of the relative abundance of the peptides (and hence the protein) in the original protein mixtures. This approach is the derivatization of lysine with a methoxy-imidazole moiety (2-methoxy-4, 5-dihydro-1H-imidazole) in light (d0) or heavy (d4) form have been used to increase the ionization efficiency of lysine-containing peptides (Figure 2.3) (Peters et al., 2001). The labeling strategy provides a mass difference of 4 Da between the heavy and light versions of the reagent. Therefore, the different peptide abundant can be quantified (Figure 2.4). When proteome extracts are first digested with trypsin or lys-C and then derivatized, all lysine in the digested peptides will be labeled except those arising from the C-termini of proteins.

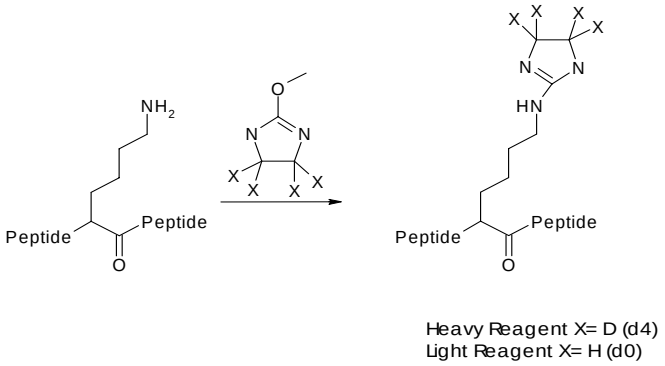

Figure 2.3 Mass Tag derivatisation converts lysine residues into a more basic derivative (Peters et al., 2001).

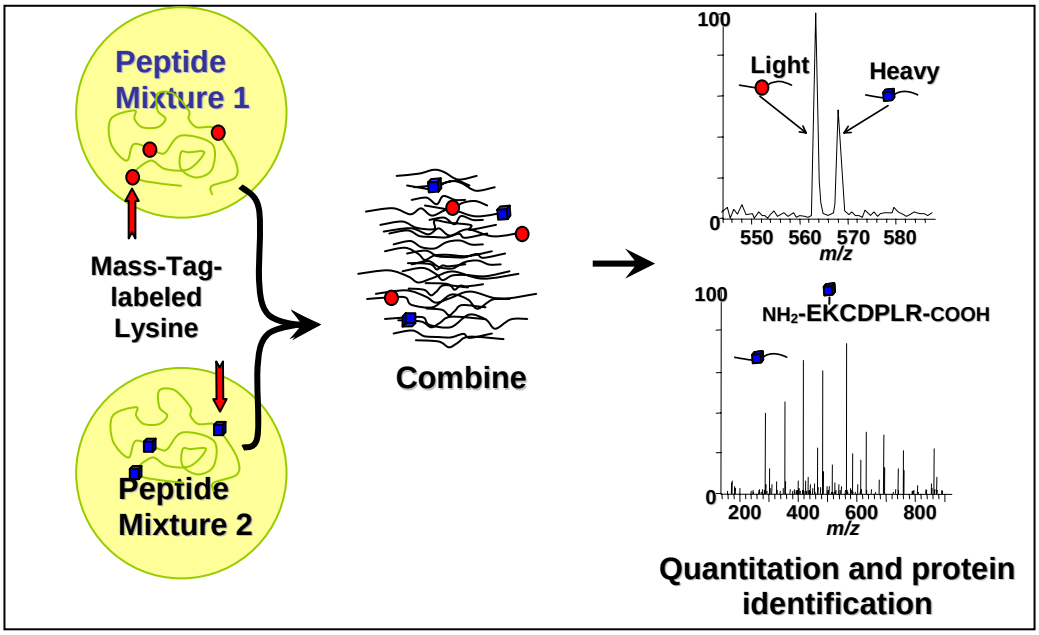

Figure 2.4 Schematic illustration of quantitative analysis by using mass tag derivatisation.

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CHAPTER III

BIOTIC FACTORS AFFECT NODULATION COMPETITION OF *Bradyrhizobium japonicum* STRAINS

ABSTRACT

Direct and indirect fluorescent antibody assay were applied to identify bradyrhizobia in soybean nodules preserved in four different storage conditions. Results showed that soybean nodules dried in the oven, stored under room temperature, or at -20^0C were as suitable as fresh nodules for strain identification using fluorescent antisera. Dried storage nodule was chosen for nodulation competitiveness experiment. The influence of five Thai soybean cultivars on nodulation competitiveness of four *Bradyrhizobium japonicum* strains was investigated. Cultures of *B. japonicum* strains; THA5, THA6, USDA110 and SEMIA5019 were mixed with each other prior to inoculating germinated soybean seeds growing in Leonard jars with nitrogen-free nutrient solution. At harvest, nodule occupancy by each strain was determined by a fluorescent antibody technique. The term 'general competitive ability' was introduced to describe the average competitive nodule occupancy of a strain in paired co-inoculation with a number of strains on soybean. The nodule occupancies by an individual strain were directly correlated with the proportions of that strain in the inoculum mixtures. USDA110 showed higher nodulation competitiveness than the other strains on three of the five cultivars. The Thai strain THA6 appeared to be more competitive than USDA110 on cultivar SJ5.

Thus, nodulation competitiveness of the *B. japonicum* strains was affected by soybean cultivars.

INTRODUCTION

Bradyrhizobium japonicum, is a slow glowing root nodule symbiont, which is widely used as an inoculant in soybean fields thorough the world. Generally, soybean inoculated with *B. japonicum* forms highly effective nodules and frequently increases soybean yields, especially in fields where soybeans are cultivated for the first time (Caldwell and Vest, 1970). The major problem of soybean inoculation is that the existing indigenous strains in the field may often suppress the introduced inoculant strains applied to soybeans subsequently. Therefore, it is necessary that the highly effective introduced strain has also the capacity to compete with the resident ineffective rhizobia in the soil (Dowling and Broughton, 1986). The competitive mechanism is a complex interplay between each strain and the host plant. Numerous abiotic and biotic factors are known to influence the competitiveness of specific rhizobial inoculants (Turco and Sadowsky 1995; Bottomley, 1992; Dowling and Broughton, 1986.)

Soybean cultivars are also known to influence nodulation competition among *B. japonicum* strains (Triplett and Sadowsky, 1992). In Thailand, many soybean cultivars have been developed with characteristics appropriate for different geographical areas. These Thai soybean cultivars may have different selective influences on the soil bradyrhizobia and therefore the nodulation competition of introduced *B. japonicum* strains may be affected by these cultivars. To develop a

successful strategy for inoculation of soybean fields in Thailand, it is necessary to determine how Thai soybean cultivars interact with different *B. japonicum* strains. The usual method for the assessment of competitive ability for nodulation involves the inoculation of a cultivar with equal mixtures of two inoculant strains. The results of such studies may provide the relative nodule occupancies of the two competing strains. However, nodule occupancies from a paired competition assay involving only two strains cannot be used to predict the competitive abilities of these strains in relation to other strains. To predict the competitive ability of a strain with other strains in general, it would be necessary to conduct competition experiment with a number of strains. The 'general competitive ability (GCA)' is introduced to describe of a strain for nodulation as the average nodule occupancy of the strain in paired co-inoculation assays with a number of strains. This is in contrast to the 'specific competitive ability (SCA)', which is a strain for nodulation as the average nodule occupancy of a strain in paired co-inoculation assay with another strain. The objectives of this investigation are to: (i) assess the general competitive abilities of four *B. japonicum* strains for nodulation of soybean using all possible paired combinations of these strains in co-inoculation experiments, (ii) determine the effect of five Thai soybean cultivars on the general nodulation competitive abilities for these strains, and (iii) investigate the influence of relative proportions of co-inoculating inoculum on nodule occupancy.

MATERIALS AND METHODS

B. japonicum strains

Cultures of *B. japonicum* strains USDA110 and SEMIA5019 were obtained from NifTAL Center, University of Hawaii, Honolulu, USA. *B. japonicum* strains THA5, THA6 and THA7 were obtained from the Thailand Department of Agriculture, Bangkok, Thailand.

Specific antisera

Specific antisera for *B. japonicum* strain USDA110, SEMIA5019, THA5, THA6 and THA7 were produced by rabbits (Somasegaran & Hoben 1994). Anti-Rabbit IgG FITC conjugate was purchased from Sigma, Missouri, USA.

Inoculum preparation

B. japonicum strains were cultured with YEM (Vicent, 1970) containing ($g.l^{-1}$) : $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.5; Manitol, 10; Yeast extract, 0.5; NaCl, 0.1. The pH was maintained between 6.5-6.8. Cultures were grown aerobically (agitated at 250 rpm on a rotary shaker) at 28^oC until the cell concentration reached 10^8-10^9 cells/ml. Cultures were collected by centrifugation at 5000 rpm for 10 min. The precipitate was further washed twice with sterilized water. Cells were diluted with sterilized water to provide 10^8cells/ml by direct determination in Petroff-Hauser counting chamber. For paired co-inoculation experiments, the two competing strains were mixed at the ratios of 1:1, 1:9 and 9:1 (v/v).

Soybean cultivars

The soybean cultivars used in this experiment, CM2, CM60, SJ2, SJ4 and SJ5, were obtained from the Department of Agriculture, Bangkok, Thailand. Seeds were rinsed in 95% ethanol for 10 s to remove waxy material and trapped air, further washed twice with sterilized water. Seeds were then surface sterilized by immersion in 6% sodium hypochlorate for 3 min, followed by rinsing six changes with sterile water. Seeds were placed in petri dishes containing water agar until germinated.

Nodulation competition assay by fluorescent antibody technique

Nodules were washed twice with sterilized water to remove dirt, then surface sterilized by immersion in 6% sodium hypochlorate for 3 min, followed by rinsing three changes with sterilized water. Stored soybean nodules were placed one nodule in each well of a microtiter plate. In case of dried nodule, the nodules imbibition was performed prior place to microtiter plate. Thirty microliter of sterilized water was pipetted into each well. Nodules were then pieced and squeezed against the side of the wall using toothpick. Sufficient amounts of bacteriods were loaded onto the end of toothpick to make smears on microscope slides, followed by air dry and heat fix the smear. The tested fluorescent antibody (FA) was diluted 1:100 then drop to completely cover the smear. Slide was then incubated in a moisture chamber at 37^{o}C for 30 minutes. The slide was then washed off the excess FA with water, and then submerged in saline for 10 min, followed by rinsing with water. The assay was performed under a UV microscope equipped with a mercury vapor light source and a filter for FITC excitation (Olympus BX-50-32E01).

Investigation of storage condition for nodule typing

Soybean cultivar used in this experiment was SJ5. The geminated seeds were planted in Leonard's jars and inoculated with ml of 110^8 cells/ml of *Bradyrhizobium* strain SEMIA5019 and THA7. Plants were supplemented with N-free medium and held at 25^{o}C in light room equipped with light source, which provided flux density of light about 450/ μEs^{-1} m^{-2} with a 12 h-12 h light-dark regime. Plants were harvested at four weeks after inoculation. Nodule typing was done by fluorescent antibody assay using antisera conjugated with either a fluorescent dye FITC for detection (direct FA) or using a fluorescent secondary antibody (indirect FA). Fluorescent antisera specific to SEMIA5019 and THA7 were performed according to Somasegaran and Hoben (1994). Bradyrhizobia were assayed in nodules, prepared or preserved in four different ways: (*i*) fresh nodule (examined immediately), (*ii*) frozen nodules (kept at -20^{o}C for 2 weeks), (*iii*) air-dried nodules (left at room temperature until completely dried) and (*iv*) oven-dried nodules (dried at 80^{o}C for 12 h).

Influence of soybean cultivars and *B. japonicum* strains on competition

The germinated seeds of cultivars CM60, CM2, SJ2, SJ4 and SJ5 were grown in Leonard jars with three seeds per jar. Strain USDA110, THA6, THA5 and SEMIA5019 were mixed in different paired combinations at a ratio 1:1, and inoculated with 10^8 cells per seed. Nodulation tests were performed in triplicate. Plants were cultivated as described above. Plants were harvested at four weeks after inoculation. Nodule occupancy was determined through a fluorescent antibody technique applied to nodule contents.

Influence of relative proportions of co-inoculating inoculum on nodule occupancy

Germinated seeds of cultivars CM2 and SJ5 were inoculated with a mixture of two strains at ratios of 1:1, 1:9 and 9:1. All paired combinations of the four strains were applied and the experiment was three replicated. Nodule occupancies were determined after 4 weeks through a fluorescent antibody technique as described above.

Statistical methods

Mean and standard deviations for nodule occupancies were calculated from data obtained from three replications. General competitive ability (GCA) for nodulation was calculated as follows:

$$\mathbf{GCA} = \frac{\mathbf{P1+P2+...P}\boldsymbol{n}}{\boldsymbol{n}}$$

Where P1, P2, P*n* are the proportions of nodule occupied by a strain in paired co-inoculation with strains P1, P2, ...P*n*, respectively; and *n* is the total number of test isolates.

RESULTS AND DISCUSSION

Investigation of storage condition for nodule typing

Soybean nodules preserved under different storage conditions were determined of bradyrhizobia occupancy using specific fluorescent antisera. Results showed that the four preserved conditions; fresh nodule, frozen nodule, air-dried nodule and oven dried nodule did not affect the detection procedure using fluorescent antibody. Fluorescent antiserum specific to SEMIA5019 detected bright green fluorescence in more than 85% nodules in both direct and indirect FA, while antiserum specific to THA7 expressed bright green fluorescence in more than 95% nodules in direct examination and in more than 80% nodules in indirect examination (Table 3.1). When both the bright green (4+) and the light green (2+) flurescences of the bacteroid were taken together, 100% of nodules were detected with these antibodies using either the fluorescent dye or the secondary antibody for detection. Therefore, root nodules dried in the oven, stored under room temperature and frozen were as suitable and reliable as fresh for strain identification by immunofluorescence. In spite of the progressive loss of moisture during the drying, the antigen still retained its specificity and reactivity upon rehydration. When the fluorescent antisera specific for SEMIA5019 was used against bacteroids in the nodules formed by THA7, no fluorescence was detected. Similarly, the antisera specific for THA7 did not cross-react with SEMIA5019. Therefore, these fluorescent antisera can be used as a detection tool in nodule-typing in competitive experiments.

Table 3.1 Percentages of nodules showing bacteroid with bright green (4+) and light green (2+) fluorescence after staining with specific fluorescent antisera.

Strain	Condition	Total-nodules	Direct FA		Indirect FA	
			4+fluorescence	2+fluorescence	4+fluorescenc	2+fluorescenc
SEMIA 5019	Fresh nodule	50	98	2	90	10
	Frozen nodule	50	92	8	98	2
	Air dried nodule	50	98	2	98	2
	Oven dried nodule	50	86	14	98	2
THA7	Fresh nodule	50	96	4	80	20
	Frozen nodule	50	96	4	88	12
	Air dried nodule	50	100	0	100	0
	Oven dried nodule	50	100	0	96	4

Nodulation competitiveness of four *B. japonicum* strains

The nodulation competitiveness of four *B. japonicum* strains was assessed on five soybean cultivars (Table 3.2). The SCAs of all tested strains exhibited differently. THA5 occupied the least number of nodules in paired competition with most other strains. USDA110 showed out-complete over THA6 on soybean cultivars ST2, SJ4 and CM2 while more competitive than SEMIA5019 only with SJ5. THA6 performed better competitiveness than SEMIA5019 on soybean cultivars SJ5, CM2, CM60, besides comparable to USDA110 on soybean SJ5. The GCAs of these strains for nodulation were calculated from 15 paired competition assays involving three co-inoculating strains and five cultivars (Table 3.2). USDA110 had the highest GCA for nodule invasion, followed by THA6, SEMIA5019 and THA5, respectively (Figure 3.1). For double strains occupancies, USDA110 showed the highest percentage,

followed by SEMIA5019, THA5 and THA6, respectively (Figure 3.1). Interestingly, nodule occupancy of all strains showed higher percentages of double occupancies than single occupancies (Figure 3.1). Especially, the co-inoculation of SEMIA5019 and USDA110 showed high double occupancy on all tested soybean cultivars (Table 3.2). The incidence of double occupancy varied depending on the strain used was also exhibited by May and Bohlool (1983).

The concepts of general and specific competitive abilities for nodule occupancy have been introduced to describe nodulation competitiveness of *B. japonicum* strains. A strain with a high general competitive ability is expected to out-compete a large number of competing strains in the soil for occupying nodules. Identification of such a strain would require testing many strains in paired competition against a set of 'test isolates' of *B. japonicum*. The test isolates are strains with known physiological and symbiotic characteristics. In this study, all paired combinations of four strains were tested for nodulation competition, thus, for measuring the general competitive ability of any one strain, the remaining three strains served as the test isolates. Among these four strains, USDA110 and SEMIA5019 have been well characterized for symbiosis, competitiveness ability and other characteristics in USA and Brazil, respectively. (Jordan, 1982; Boddey and Hungria, 1997). However, investigation of competitiveness ability against Thai strains, USDA110 exhibited a highly competitiveness, while SEMIA5019 revealed lower ability than THA6.

Table 3.2 Nodule occupancy by *B. japonicum* strains THA5, THA6, USDA110, SEMIA5019 on five soybean cultivars.

Co-inoculated strains		Soybean cultivar	* Nodules (%) formed by:		
A	B		A	B	Double occupancy
THA5	THA6	ST2	3.3±3.5 ^{f}	66.1±6.5 ^{b}	30.6±3.4 ^{cde}
		SJ4	25.0±3.5 ^{e}	40.0±4.2 ^{c}	35.1±7.7 ^{cd}
		SJ5	1.7±2.9 ^{f}	94.4+5.3 ^{a}	4.0±2.5 ^{f}
		CM2	4.4±0.8 ^{f}	59.9±7.3 ^{b}	34.0±7.4 ^{cde}
		CM60	28.0±5.0 ^{de}	35.5±8.3 ^{cd}	36.5±4.4 ^{cd}
THA5	USDA110	ST2	0.0±0.0 ^{e}	75.4±4.0 ^{a}	24.6±4.0 ^{d}
		SJ4	2.2±3.8 ^{e}	36.9±2.3 ^{c}	60.9±4.3 ^{b}
		SJ5	0.0±0.0 ^{e}	64.2±0.7 ^{b}	35.8±0.7 ^{c}
		CM2	1.4±2.5 ^{e}	59.0±10.3 ^{b}	39.6±9.1 ^{c}
		CM60	1.7±2.9 ^{e}	37.4±7.0 ^{c}	60.9±4.6 ^{b}
THA5	SEMIA5019	ST2	1.7±2.9 ^{j}	73.4±5.2 ^{a}	24.9±4.3 ^{gh}
		SJ4	9.6±9.7 ^{ij}	35.8±6.2 ^{efg}	54.6±6.5 ^{bc}
		SJ5	18.9±5.9 ^{hi}	43.4±7.3 ^{cde}	37.7±11.6 ^{def}
		CM2	4.4±0.7 ^{j}	43.1±11.0 ^{cde}	49.8±9.6 ^{cd}
		CM60	4.3±3.7 ^{j}	65.5±4.0 ^{ab}	30.2±6.4 ^{fgh}
THA6	USDA110	ST2	3.3±3.3 ^{fg}	72.8±4.8 ^{a}	23.9±2.5 ^{cd}
		SJ4	0.0±0.0 ^{g}	80.3±6.3 ^{a}	19.7±6.3 ^{cde}
		SJ5	15.0±5.3 ^{e}	13.4±3.5 ^{e}	71.6±5.3 ^{a}
		CM2	10.2±5.7 ^{ef}	61.4±9.5 ^{b}	28.4±7.6 ^{c}
		CM60	1.9+3.2 ^{fg}	17.8±1.9 ^{de}	80.4±2.8 ^{a}
THA6	SEMIA5019	ST2	16.5±2.1 ^{f}	43.9±8.5 ^{bc}	39.6±6.9 ^{bcd}
		SJ4	31.4±8.0 ^{ed}	47.6±7.1 ^{ab}	21.1±1.1 ^{ef}
		SJ5	58.3±5.9 ^{a}	20.4±5.7 ^{ef}	21.3±5.0 ^{ef}
		CM2	46.0±11.3 ^{b}	4.2±7.2 ^{g}	49.8±4.2 ^{ab}
		CM60	45.3±6.8 ^{b}	22.2±2.7 ^{ef}	32.5±7.9 ^{cde}
SEMIA5019	USDA110	ST2	15.8±3.2 ^{ef}	14.9±4.5 ^{ef}	69.4±4.5 ^{a}
		SJ4	20.0±3.4 ^{e}	30.2±3.7 ^{cd}	49.8±6.8 ^{b}
		SJ5	34.0±4.1 ^{cd}	7.9±6.9 ^{fg}	58.1±10.2 ^{b}
		CM2	25.0±2.9 ^{de}	20.3±2.7 ^{e}	54.7±5.4 ^{b}
		CM60	5.0±4.6 ^{g}	36.9±8.3 ^{c}	58.2±5.4 ^{b}

Means with different letters are significantly different at $p<0.05$.

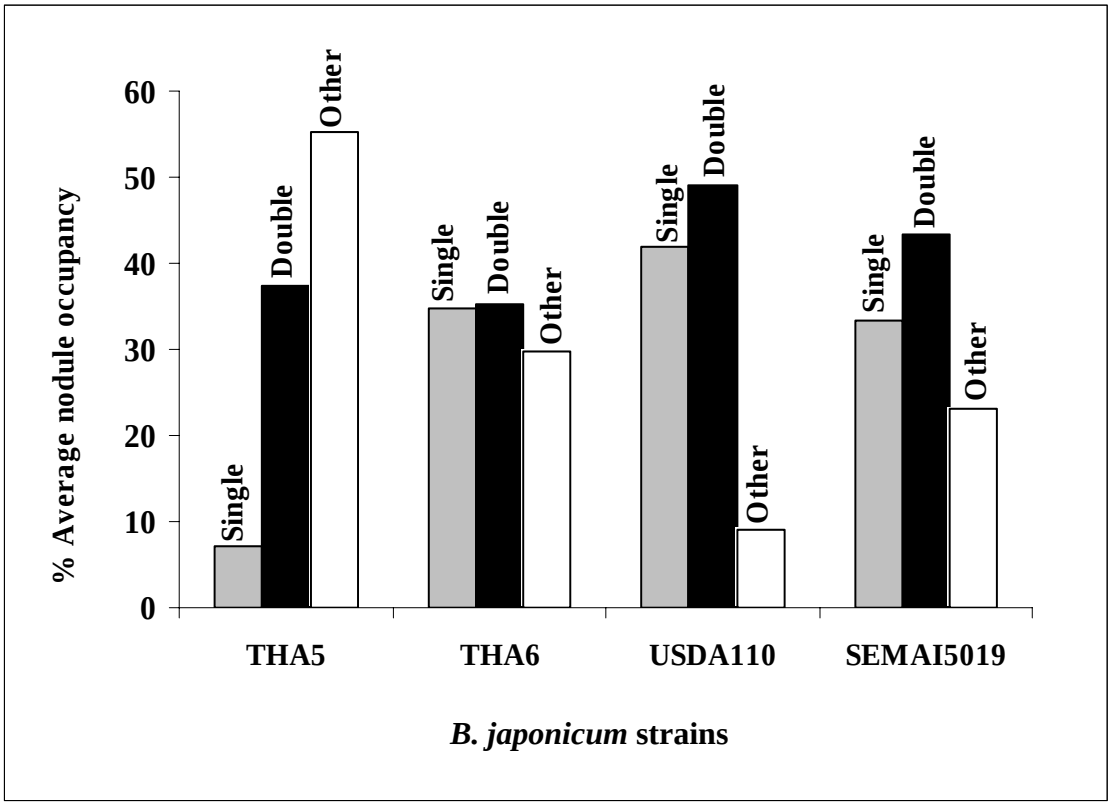

Figure 3.1 Average percentage of nodule occupancies by individual *B. japonicum* strains in experiments using all possible paired co-inoculations involving four strains. For each strain, the single (▩) and double (■) nodule occupancies are indicated. The average nodule occupancy percentages of the other strains (□) in the paired co-inoculations are also indicated.

Influence of soybean cultivars on nodulation competition

From Table 3.2, it appears that the competitive nodule occupancies by the strains were not consistent on all five cultivars. Both general and specific competitive abilities for nodulation were influenced by soybean cultivars. The average percentage of nodule occupancies of four bradyrhizobia strains were calculated from Table 3.2 involving three paired competition assay of one *Bradyrhizobium* strain on each soybean cultivars (Figure 3.2). USDA110 showed higher average nodule occupancies than SEMIA5019 and THA6 on cultivars ST2, SJ4 and CM2, but not on SJ5 and CM60 (Figure 3.2). THA6 appeared to be more competitive than USDA110 and SEMIA5019 on cultivar SJ5 (Figure 3.2). On CM60, THA6 showed lower average nodule occupancies than both USDA110 and SEMIA5019. Soybean cultivars ST2 exhibited nodule occupancy pattern similarly to those SJ4 cultivar, while on SJ5 revealed on the contrary.

The results of this study show that the general competitive ability for nodule occupancy of a strain can be influenced by host cultivars. The conclusion was supported by results of several other studies of *Rhizobium*-host interaction. This was thought to be in part due to host-controlled selective or restrictive nodulation mechanisms (May and Bohlool, 1983; Cregan and Keyser, 1986; Montealegre et al., 1995), physiological differences between soybean phenotypes and, perhaps, to differential responses of *B. japonicum* strains to *nod* gene-inducing signal molecules (Bottomley, 1992). Weiser and colleagues (1990) identified the single dominant genes Rj_2, Rj_3, *and* Rj_4, which restricted nodulation by *B. japonicum* strains 122 and c1 serogroups, USDA33, and USDA61, respectively. Recessive plant genes which restrict nodulation by all bradyrhizobia, Rj_1, Rj_5, and Rj_6 were also been reported

(Pracht et al., 1993). Therefore, Thai-soybean cultivars might have some phenotype specifically to each *B. japonicum* strains.

Therefore, it would be appropriate to test *B. japonicum* strains on several cultivars for selecting the most competitive strains. However, competition experiments are very intensive and it may not be practical to test *B. japonicum* strains on a number of cultivars. Therefore, a well-adapted and widely-grown soybean variety of a region may be selected as the host cultivar of choice for conducting competition experiments with *B. japonicum* strains. In the present study, the five soybean cultivars used are well adapted to different regions of Thailand. From this study, we have identified THA6 as a highly competitive *B. japonicum* strain for the soybean cultivar SJ5. SJ5 has been developed at the Chieng Mai Crops Research Center, Thailand. This cultivar was developed by crossing between SJ2 and Tainuang 4, followed by extensive selection. The major characteristics of this cultivar are high yield, short harvested duration, tolerance to water logging in soil and resistance to several diseases; leaf spot, rust and antractnose. (Department of Agriculture, Thailand, www). This cultivar was selected for appropriated growing in all region of Thailand. THA6 also appeared to be competitive on cultivar CM2, which is mainly grown in the North and Northeastern region of Thailand.

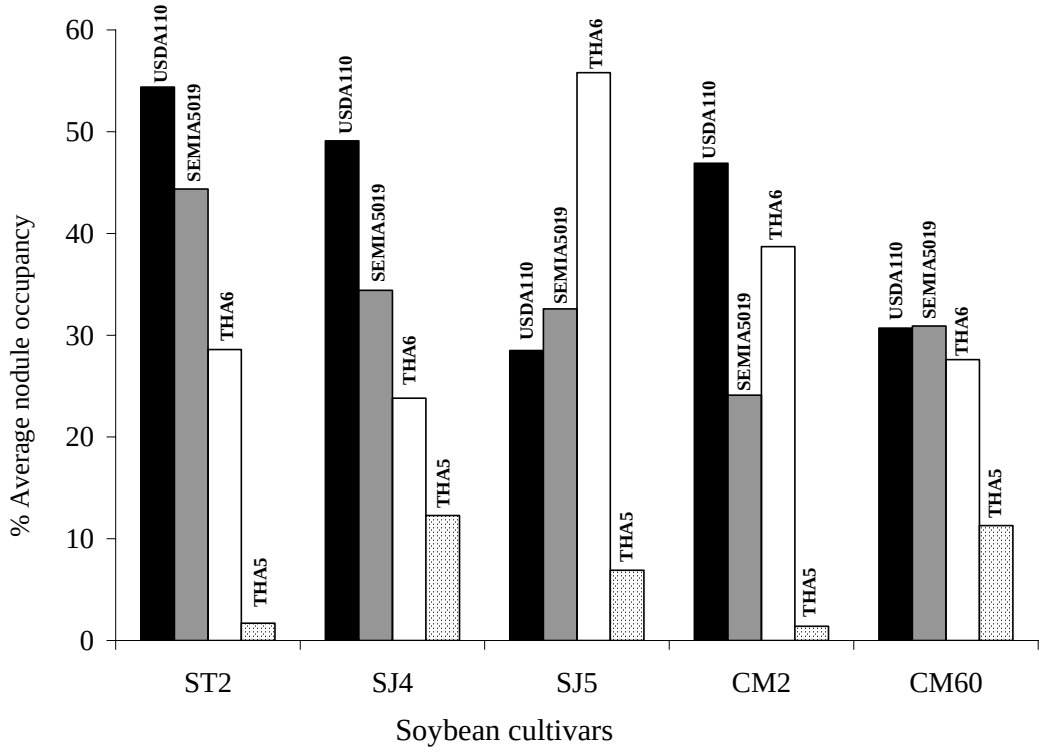

Figure 3.2 Average percentages of nodule occupancies by *B. japonicum* strains USDA110 (■), SEMIA 5019 (▒), THA6 (□) and THA5 (▨) in paired co-inoculation experiments on different soybean cultivars.

Influence of relative proportions of co-inoculating inoculum on nodule occupancy

To determine how the proportion of the two strains in the inoculum mixtures might affect soybean nodule occupancies by these strains, the three possible paired combinations involving four *B. japonicum* strains were used to co-inoculate soybean in three different ratios, 1:9, 1:1 and 9:1. The average nodule occupancies of each strain in paired combination with the other three strains were determined for the three inoculum proportions, 10%, 50% and 90% (Figure 3.3a and 3.3b). Result revealed that the more increase in inoculum proportion; the more nodules achieved. The R^2 values for this relationship ranged from 0.8854 to 0.9987. Therefore, the nodule

occupancies by an individual strain were directly correlated with the proportions of that strain in the inoculum mixtures. The influence of cell number on competition has been reported in several studies. Brockwell and coworker (1995) reported that to success in nodulation competition, the number of introduced strains must be 250:1 of indigenous soil populations. Weaver and Frederick (1974) demonstrated that to obtain 50% occupancy of soybean nodules by the inoculant strain, an inoculation rate at 1,000 times the indigenous population must be applied. Therefore, the number of nodules formed by each strain present depends not only on the nodulation competitiveness of the various strains but also on their number of cells in the inoculant. However, refer to the R^2 values implying that conducting competition experiments with several ratios of the paired inoculants may not be necessary. Result demonstrated that inoculation ratio of only 1:1 can provide reasonable estimates of nodule occupancy by the competing strains of *B. japonicum*.

Additionally, the average nodule occupancies were not only influence by inoculum proportion, but specific competitive ability of individual bradyrhizobium was also involved. It is obviously detected this phenomenon by the paired inoculation of THA5 with other strains, which exhibited the least nodule occupancies even when contained 90% in inoculum. Moreover, the relative nodule occupancy values obtained from competition experiments on two soybean cultivars, CM2 and SJ5, were not similar for the strains at the three inoculum proportions, indicating effects of cultivars on nodule occupancy. These results confirmed the evidence of previous experiment that SCAs, GCAs and soybean cultivars affect nodulation competitiveness of bradyrhizobia.

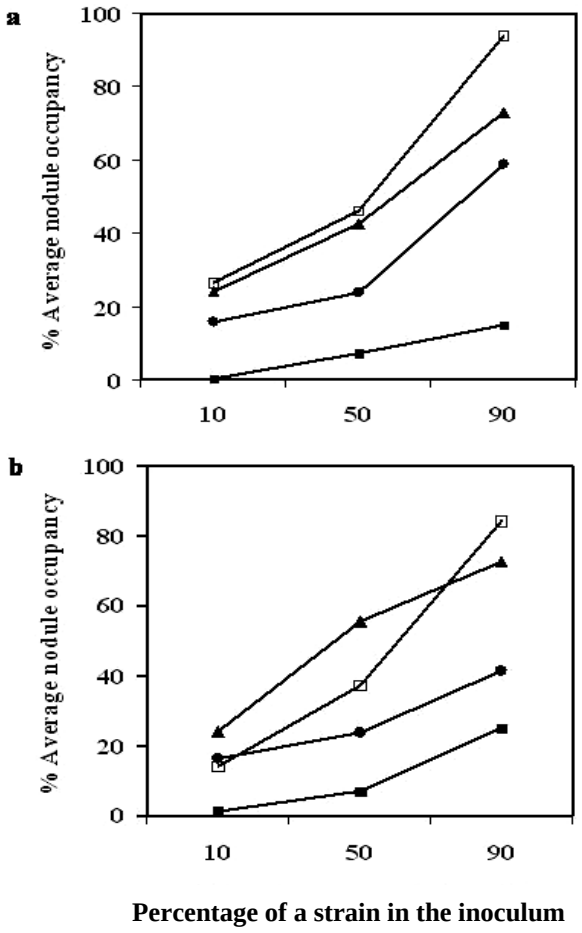

Figure 3.3 Average percentage of soybean nodule occupancies by *B. japonicum* strains USDA110 (□), THA6 (▲), SEMIA5019 (●) and THA5 (■) in paired co-inoculation experiments, where different proportions of a particular strain were used in combination with a competing strain on cultivars CM2 (a) and SJ5 (b).

CONCLUSION

Soybean nodules preserved under different storage conditions; fresh nodule, frozen nodule, air-dried nodule and oven dried nodule did not affect the detection procedure using fluorescent antibody. Thus, the oven dried nodules were applied for nodule typing in all experiments in this chapter. The competitive ability among *B. japonicum* strains depend on the soybean cultivar as well as strain. USDA110 had the highest GCA, followed by THA6, and SEMIA5019. THA5 exhibited the least of SCA and GCA. For double strains occupancies, USDA110 showed the highest percentage, followed by SEMIA5019, THA5 and THA6. The co-inoculation of USDA110 and SEMIA5019 showed high double occupancy on all tested soybean cultivars. Both general and specific competitive abilities for nodulation were also influenced by soybean cultivars. USDA110 showed higher average nodule occupancies than SEMIA5019 and THA6 on cultivars ST2, SJ4 and CM2, but not on SJ5 and CM60. THA6 appeared to be more competitive than USDA110 and SEMIA5019 on cultivar SJ5. On CM60, THA6 showed lower average nodule occupancies than both USDA110 and SEMIA5019. Soybean cultivars ST2 revealed the preference bradyrhizobia occupied pattern similarly to that SJ4 cultivar, which exhibited differently on SJ5. The nodule occupancies by an individual strain were directly correlated with the proportions of such strain in the inoculum mixtures. Additionally, the amount inoculum for success-competition also depends on SCA and GCA of such strain.

To benefit from inoculation, the influence of the biotic factors, including Bradyrhizobia strain, host plant and proportion of inoculum on nodulation competition

are something that needs to be understood. If more information on the competitive ability of individual *B. japonicum* strains and their interaction with soybean varieties are achieved, it would be useful to manage inoculation strategies to enhance nodulation by selected *B. japonicum* strain.

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CHAPTER IV

ISOLATION OF GENES FOR SALT TOLERANCE AND CONSTRUCTION OF SALT TOLERANT DERIVATIVES OF *B. japonicum* THA6

ABSTRACT

Sinorhizobium strain BL3 was isolated from the nodules of the wild bean *Phaseolus lathyroides* that grew in the saline uncultivated areas of Nakhon Ratchasima province in the north-eastern region of Thailand. It can tolerate up to 600 mM NaCl and is effective in N_2 fixation. A genomic DNA clone library of BL3 was constructed and transferred to salt-sensitive rhizobial strain TAL1145 by conjugation and the transconjugants were selected on YEM agar containing 100 mM NaCl. Two hundred colonies that grew under this salt condition were isolated. The recombinant cosmid clones were isolated from twenty four of those colonies. These cosmid clones contained two different regions of the BL3 chromosome. The deduced proteins encoded by the genes of the first region (pUHR307) exhibited homologies with ATPase, xanthine dehydrogenase, transcriptional regulator SyrB (AraC family), DNA methylase, and partitioning protein. The sequence of the cloned DNA representing the second region (pUHR310) showed high similarities with the *bet* gene cluster, involved in glycine betaine biosynthesis in *S. meliloti*. These two clones were introduced into *B. japonicum* THA6 to enhance growth under salt condition. The transconjugants of THA6 containing either of these clones showed better growth than THA6 in the

presence of 50-100 mM NaCl. These transconjugants did not show increased nodulation competitiveness on soybean under salt conditions.

INTRODUCTION

Besides the biotic factors discussed in chapter III, there are many abiotic or environmental factors that influence viability of *Rhizobium* inoculants (Rice, 1977). Environmental factors that limit growth and competitiveness of rhizobia include low or high pH, high temperature, low moisture in soil, nutrient deficiency, mineral toxicity, and soil salinity (Dowling and Broughton, 1986; Triplett and Sadowsky, 1992; Zahran, 1999). Drought and salinity also adversely affect the nitrogen fixation capacity of rhizobia, resulting in lower productivity of legumes (Miller, 1996). Nearly 40% of the world land surface can be categorized as having potential salinity problem (Cordovilla et al., 1994). Moreover, salinity of soil in cultivated area is expected to rise as a result from local salt accumulation due to irrigations and applications of chemical fertilizers (Miller and Word, 1996). In Thailand, there is a widespread salinity problem, especially in the Northeast region of Thailand. The affected areas were estimated to be 17% of the region or 2.85 million ha (DLD, 1989). The process can be related to the occurrence of a salt rock layer, at 80 m depth and its effect on groundwater (Soliman et al., 2004). Saline ground water reaches the surface through natural channels (faults, fractures) or through the openings created by salt mining activities. Salt also moves upwards through bio-climatic factors, which influences the evapotranspiration rate (IRD, 2004). Salinity raises osmolarity of soil and reduces water uptake by plants, resulting in decreased productivity of most crop plants

(Cordovilla et al., 1994). These effects are also obviously found in leguminous plants because *rhizobia* that form nitrogen-fixing symbiosis are also affected by salinity.

Rhizobial strains show marked variation in survival under salt stress. Growth of a number of *rhizobia* was inhibited by 100 mM NaCl (Yelton et al., 1983), while some rhizobia, e.g. *Rhizobium melitoti* were tolerant to 300-700 mM NaCl (Embalomatis et al., 1994; Helemish et al., 1991; Mohammad, 1991 and Muller et al., 1995). Strains of *R. leguminosarum* have been reported to tolerate up to 350 mM NaCl in broth cultures (Abdel-Wahab and Zahran, 1979; Breedveld, 1991). Numerous osmotic adaptation mechanisms for survival under salt stress condition have been reported among microorganisms. Bacterial cells maintain turgor by first increasing their potassium (K^+) content and then replacing part of the accumulated K^+ with compatible solutes in the second phase of osmoadaptation (Wood, 1999). *E. coli* possesses two Na^+/H^+ antiporters (NhaA and NhaB), both of which are implicated in the maintenance of pH homeostasis and salinity tolerance (Padan and Schuldiner, 1994). Many compatible solutes have been reported in providing the protection from salt stress. L-proline has been reported to accumulate in *Salmonella typhimurium* (Dunlap and Csonka, 1985), *E. coli* (Gowrishankar, 1985) and *B. subtilis* (von Blohn et al., 1997). The accumulation of glycine betaine have been demonstrated in *B. subtilis* (Kappes et al., 1996), *L. monocytogenes* (Gerhardt et al., 1996), *Lactobacillus plantarum* (Glaasker et al., 1996), *Corynebacterium glutamicum (*Peter et al., 1996) and *Klebsiella pneumoniae* and other members of the *Enterobacteriaceae* (Le Rudulier and Bouillard, 1983). Potassium glutamate has been shown to accumulate in enteric bacteria (Booth and Higgins, 1990), *C. glutamicum* (Lambert et al., 1995) and *E. coli* (McLaggan et al., 1994). A recent study by Steil et al (2003) demonstrated that

the stress response of *B. subtilis* was controlled by a two-component DegS/DegU system in sensing high salinity. This mechanism displayed immediate expression of 75 genes through a transient induction of the *sigB* gene. Continuous propagation of a *B. subtilis* strain in the presence of 1.2 M NaCl triggered the induction of 123 genes and led to the repression of 101 genes. This provides an insight of a complex mechanism of salt stress response in microorganisms.

Among rhizobia, many strains of *R. fredii* show increased levels of intracellular free glutamate and/or K^+ at high salt concentrations (up to 300 to 400 mM NaCl) (Fujihara and Yoneyama, 1994). Trehalose accumulates to higher levels in cells of *R. leguminosarum* and peanut rhizobia under conditions of hyper-salinity (Streeter and Bhagwat, 1999). *S. meliloti* responded to salt stress by accumulation of several compatible solutes, including glycine betaine, ectoine and disaccharide (Pocard et al., 1997, Talibart et al., 1994, Gouffi et al., 1999). The genes involved in salt tolerance have not been characterized. Cloning and characterization of the genes involved in salt tolerance will provide new understanding on the mechanism of salt tolerance in rhizobia, and help to construct improved recombinant strains.

The objectives of the experiments described in this chapter are to: (i) identify one or more salt tolerant strains of *Sinorhizobium* from Thailand, (ii) construct a gene library of the best salt-tolerant strain, (iii) isolate genes involved in salt tolerance, (iv) transfer the genes for salt tolerance to *B. japonicum* THA6 to improve its salt tolerance ability, and (v) determine the nodulation competitive pattern of such recombinant THA6 under salt stress condition.

MATERIALS AND METHODS

Bacteria and plasmids

Bacterial strains and plasmids used in this study are listed in Table 4.1.

Primer

Primers for PCR amplification and sequencing in this study are listed in Table 4.2

Chemicals and reagents

All chemicals used were analytical grade. The major chemical including Betaine aldehyde chloride, DTT, NAD^+, IPTG, Rifampicin sulfate, streptomycin sulfate, Tetracyclin sulfate, Ampicilin were purchased from Sigma-aldrich, Missouri, USA. The other chemicals and solvents were purchased from Merck, Germany; Fluka, Switzerland; Sigma, USA; QIAGEN, Germany and Claro, USA.

Table 4.1 Bacterial strains and plasmids.

	Relevant characteristics	Marker	Source
Rhizobium strains			
BL3	*Sinorhizobium* sp., wild-type, highly salt tolerant.	Rf^r, St^r	This study
TAL1145	*Rhizobium spp.* Nod^+ on *Leucaena* and bean	Rf^r St^r	Moawad and Bohlool, 1984
THA6	*B. japonicum*	Tc^r, St^r	Department of Agriculture, Thailand
RUH140	TAL1145 transconjugant carrying plasmid pUHR307	Tc^r, St^r	This study
RUH161	THA6 transconjugant carrying plasmid pUHR307	Tc^r, St^r Km^r	This study
RUH162	THA6 transconjugant carrying plasmid pUHR310	Tc^r, St^r Km^r	This study
Escherichia coli			
VCS257	*Eeshericia coli*, host strain for library construction.	-	Gigapack II, STRATAGENE
DH5αMCR	Used for transformation	-	Bethesda Research Laboratories
Plasmid			
pLAFR3	Wide-host-range P1 group cloning vector, used for BL3 genomic DNA library construction.	Tc^r	Staskawicz et al., 1987
pRK2013	Helper plasmid used for mobilizing plasmids of triparental mating	Km^r	Figurski and Helinski, 1979
pPH1J1	P1 group plasmid used to eliminate pLAFR3-based cosmid from *Rhizobium* spp.	Gm^r	Beringer et al., 1978
pUC18	cloning vector.	Ap^r	Maniatis et al., 1982
pUHR305	pLAFR3-based cosmid clone isolated from the genomic library of BL3 containing 20.6 kb of salt tolerant genes.	Tc^r	This study
pUHR306	pLAFR3-based cosmid clone isolated from the genomic library of BL3 containing 31.1 kb of salt tolerant genes.	Tc^r	This study
pUHR307	pLAFR3-based cosmid clone isolated from the genomic library of BL3 containing 18.13 kb of salt tolerant genes.	Tc^r	This study
pUHR308	pLAFR3-based cosmid clone isolated from the genomic library of BL3 containing 23.8 kb of salt tolerant genes.	Tc^r	This study
pUHR309	pLAFR3-based cosmid clone isolated from the genomic library of BL3 containing 29.6 kb of salt tolerant genes.	Tc^r	This study
pUHR310	pLAFR3-based cosmid clone isolated from the genomic library of BL3 containing betaine production operon (bet) with 23.5 kb.	Tc^r	This study
pUHR307-kan	Km^r derivative of pUHR307 containing genes for salt tolerance and a Tn3Ho*gus* insertion	Tc^r Km^r	This study
pUHR310-kan	Km^r derivative of pUHR307 containing genes for salt tolerance and a Tn3Ho*gus* insertion	Tc^r Km^r	This study

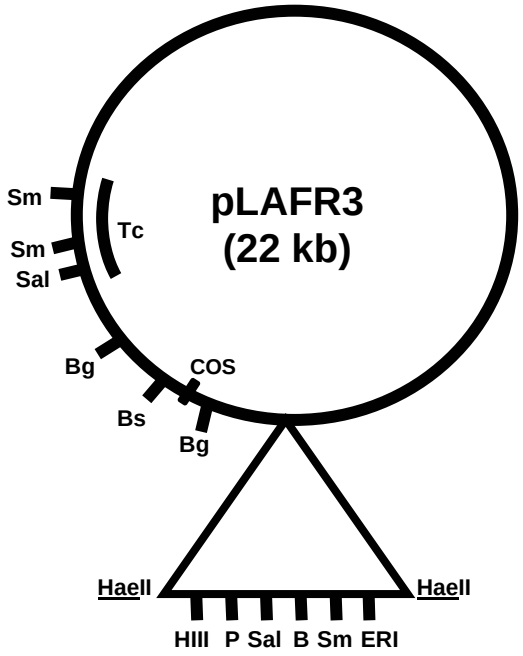

Figure 4.1 Physical map of cosmid pLAFR3 containing tetracycline resistant marker gene, cos site and multiple cloning sites (Staskawicz, et al., 1987). Abbreviations for restriction enzyme sites are as follows: ERI, *Eco*RI; B, *Bam*HI; HIII, *Hin*dIII; P, *Pst*I; Sal, *Sal*I, HaeII; *Hae*II, Sm; *Sma*I, Bg; *Bgl*II, Bs; *Bst*EII.

Table 4.2 Primers used for PCR amplification and DNA sequencing.

Name of primer	Size (bases)	Sequence (5'-3')
3.2 *Hin*dIII Forward_1	19	CAGTCCCTCGATCGCAAAG
3.2 *Hin*dIII Forward_2	19	GATCTGGAGAAGGGGGTGC
3.2 *Hin*dIII Forward_3	21	ACTGAACCGGGATTTGAGTCG
3.2 *Hin*dIII Reverse_1	21	AAGGGCCTACACGTGACGATC
3.2 *Hin*dIII Reverse_2	19	CTTCCGGTTCGTCGGAAAG
3.2 *Hin*dIII Reverse_3	19	TCCAGTTCCGGGACGATTC
4.9 *Hin*dIII Forward_1	21	TCGTCCTGCAAGACATGAAGG
4.9 *Hin*dIII Forward_2	19	AGTGCGGCTTCACAATCCC
4.9 *Hin*dIII Forward_3	20	TTTCACCCTCTACGCGGATG
4.9 *Hin*dIII Forward_4	20	CGGTCAGATTGAGGGTCTCG
4.9 *Hin*dIII Reverse_1	21	CTCTTCGATATCGAGCGAGGC
4.9 *Hin*dIII Reverse_2	21	GATACGACGGTTCATCTTCGC
4.9 *Hin*dIII Reverse_3	22	ATATCCGGCTTCAGGAACTAGC
4.9 *Hin*dIII Reverse_4	20	CGAGACCCTCAATCTGACCG
8.8 *Hin*dIII Forward_1	21	TATTGTGACCTCGCCGACAAG
8.8 *Hin*dIII Forward_2	22	TCCGAAGACCAATTCGGATTAC
8.8 *Hin*dIII Forward_3	22	AACTTCTATTTCGATGTCGGCG
8.8 *Hin*dIII Forward_4	20	CAATCCACCGTTTTCCGATC
8.8 *Hin*dIII Forward_5	20	TCTTCCGGAAGACCGTTACG
8.8 *Hin*dIII Forward_6	21	AGGATTACGATCTCGAAACCG
8.8 *Hin*dIII Forward_7	20	TTCGTCAAGGAGACGATGGG
8.8 *Hin*dIII Forward_8	20	GTTTCTGGGCGCTCTATCCC
8.8 *Hin*dIII Reverse_1	21	GAGTTTCAGGATCTGCATGGG
8.8 *Hin*dIII Reverse_2	20	CACGGTGACTCTCGCATAGC
8.8 *Hin*dIII Reverse_3	20	CCGCTGATACTCAGCCAACC
8.8 *Hin*dIII Reverse_4	20	GAGATCGTCGTCTTGAGCGG
8.8 *Hin*dIII Reverse_5	22	TTGTAATCCTGCATGTAAGGCG
8.8 *Hin*dIII Reverse_6	19	GTTCCTCGATCGCCCTGAT
8.8 *Hin*dIII Reverse_7	20	GACCAAGCGAAGATCGATCG
BetB_1_BamForward	27	GCGGATCCATGAGAGCACAACCCAAAG
BetB_1464_HinReverse	23	GCAAGCTTTCAATACGGCGCCTC
16SrDNA_Forward_37	18	AGAGTTTGATCCTGGCTC
16SrDNA RP2	21	ACGGCTACCTTGTTACGACTT
16SrDNA_Reverse_1430	21	CACCCCAGTCGCTGACCCTAC

Isolation of rhizobia from nodules

Nodules of *P. lathyroides* Linn.f were collected from salt-affected areas in Burawai, Ban Leuam, Nongsuang and Pimai of the Nakhon Ratchasima province of Thailand. Nodules were washed twice with sterilized water to remove dirt, then surface-sterilized by immersion in 6% sodium hypochlorate for 3 min, followed by rinsing six changes of sterilized water. Nodules were squeezed by sterile needles to extract rhizobial cells. *Rhizobia* were isolated following the standard method on YEM medium (g/liter): $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.5; Mannitol, 10; Yeast extract, 0.5; NaCl, 0.1. pH was maintained between 6.5-6.8 (Vincent, 1970).

Screening for salt tolerant rhizobia

All isolates were further screened for salt tolerance by streaking on YEM containing 100, 200, and 300 mM NaCl. Cells were grown at 28°C for 72 h. Isolates, which could grow well in salt media, were selected for further screening. Isolates were precultured in YEM broth to early stationary phase. Fifty microliters of precultures were inoculated to 3 ml of YEM containing 0, 300, and 400 mM NaCl. Cells were grown with agitation at 250 rpm at 28°C. Cell densities were monitored spectophotomatically at 600 nm using Spectronic 21 every 24 h for 5 d.

The isolates growing at the highest level of NaCl were selected for further characterization of growth pattern. Growth patterns were determined by preculturing strains to reach early stationary phase. 0.1 ml volume of cells suspension was inoculated to 250 ml of YEM broth containing 0 to 600 mM NaCl. Treatments were replicated thrice. Cells were grown aerobically at 28°C in a rotary shaker at 250 rpm. Sampling was performed every 4 h by plating onto YEM agar at appropriate dilutions.

Nodulation assay

Nodulation assay of selected isolates was carried out by the standard method (Somasegaran, 1994). Cells were cultured to early stationary phase using YEM medium. Seeds of leguminous plants; *P lathyroides* Linn.f., *Cenjulian cavagia*, *Centrocema pascuorum*, *Macroptilium atropurpureus*, *Vigna radiata*, *V. umbellate*, *V. sinensis*, and *Glycine Max* were surface sterilized with 3.5% sodium hypochloride for 10 min, and then rinsed six times with sterilized water. Seeds were germinated on 1% water agar in petri plates for 2 d at room temperature. Germinated seeds were individually planted into sterilized pouch, and each seed was inoculated with 10^8 cells. Plants were watered with N-free nutrient solution (Somasegaran and Hoben, 1994) and maintained at 25^0C in a growth room equipped with lights, providing a flux density of 450 μ Es^{-1} m^{-2} with a 12 h – 12 h light dark regime. Plants were harvested after four weeks of inoculation.

For the determination of BL3 nodulation under salt stress condition, host plant *P. lathyroides* was used. Seedlings were prepared as for the previous experiment, and grown in modified Leonard jar assemblies containing vermiculite and nitrogen-free nutrient solution, supplemented with 0, 50, 100, and 150 mM NaCl. Inoculation of rhizobia was performed with 10^8 cells per seed. Plants were placed in a control environment as previously described. Nodulation efficiently was assessed by determining the number of nodules, nodule dry weight and plant dry weight after 4 weeks.

For the competition assay between recombinant THA6 and USDA110 was performed using soybean SJ5 variety as the host plant. Strains RUH161, RUH162 and USDA110 were mixed in paired combinations at a ratio of 1:1, and inoculated with

10^8 cells per seed. Leonard jar assemblies contained 0, 25, or 50 mM NaCl in nitrogen-free nutrient solution. Plants were harvested at four weeks after inoculation. Nodulation efficiency was assessed by determining the number of nodules, nodule dry weight and plant dry weight. The competition assay was performed by fluorescent antibody technique as described in Chapter III.

Morphological and biochemical characterization

Rhizobial isolates were scored for number of days to form single colonies, type of colony, gram-straining, carbon source assimilation and others biochemical characteristics according to Bergey's manual of Systematic Bacteriology (Jordan, 2001). Biochemical assays were done by Iapi 20NE from bioMerieux Identification system for non-enteric Gram-Negative bacteria, bioMerieux, Missouri, USA.

Authentication of rhizobial isolates

The rhizobial isolates were authenticated inoculating their respective host legumes. All purified isolates were able to nodulate, confirming that they were true rhizobia and not contaminants.

Amplification of 16s rRNA

Chromosomal DNA of selected isolates was extracted according to standard method (Sambrook and Russell, 2000). Amplification was conducted by PCR reaction performed in a 100 µl mixture containing 200 µM dNTP; 100 pM of each primer; 300 ng of chromosomal DNA, 2.0 mM $MgCl_2$ and 1U of Tag DNA polymerase (Promega, USA). The PCR reactions were performed with the following temperature profile:

initial denaturation at 95 ^{0}C for 10 minutes, 30 cycles at 94 ^{0}C for 1.30 minutes, 55 ^{0}C for 1.30 minutes and 72 ^{0}C for 2 minutes, final extension at 72 ^{0}C for 3 minutes.

DNA sequencing

Plasmid or PCR products were purified for sequencing by a Qiagen plasmid mini prep kit. These plasmids or PCR product were subjected to fluorescently labeled dideoxy termination reactions with Thermal cycler. The sequencing reactions were then separated on an automated DNA sequence (Model 373A; Applied Biosystems, Foster City, CA, USA; at the Biotechnology and molecular biology Instrumentation Facilities of University of Hawaii at Manoa or Model 310A; Applied Biosystems, Foster City, CA, USA at the school of Biotechnology laboratory, Suranaree University of Technology).

Sequence analysis and homology searching

Comparisons of nucleotide sequences and deduced nucleotide sequences were performed by the BLAST algorithms to search the data bases maintained by the National Center for Biotechnology Information (www, 2005)

Cosmid clone library construction

Genomic DNA was prepared according to standard protocol (Sambrook and Russell, 2000). For cosmid library construction, genomic DNA was partially digested with *Sau3*AI. DNA fragments were size-fractionated using a sucrose gradient centrifugation and fragments of 20-30 kb were selected. The fragments were ligated to the cosmid vector pLAFR3 (Figure 4.1), which has previously been digested with *Bam*HI and dephosphorylated. The ligation mixture was packaged *in vitro* with

Gigapack® II XL packaging extracts of lambda phages, and transfected *E. coli* VCS 257 following the protocol specified by manufacturer (STRATAGENE, USA). The transfected library was plated onto LB containing 5 μg/ml tetracycline, and incubated at 37°C for 24 h. Library was composed of 7,231 colonies.

Triparental mating for transferring cloned DNA of cosmid library into TAL1145

To isolate cosmid clones containing genes for salt tolerance, the cosmid clone library was transferred to TAL1145, a salt-sensitive *Rhizobium* strain, by triparental mating (Johnston et al., 1978) using pRK2013 as a helper plasmid (Figurski and Helinski, 1979). The salt tolerant transconjugants were selected by plating on YEM containing 100, 300, and 500 mM NaCl and 10 μg/mL tetracycline, 40 μg/ml rifampicin and 100 μg/mL streptomycin.

Isolation of cosmid clones containing genes for salt tolerance

The cosmid clones from the salt tolerant transconjugants were isolated by alkaline lysis and transformed to *E. coli* DH5αMCR (Sambrook and Russell, 2000). The selected cosmid DNA were isolated from *E. coli* by alkaline lysis and digested with the restriction enzyme *Hin*dIII and *Eco*RI (Sambrook and Russell, 2000). The resulting fragments were ligated to pUC18 plasmid for sequence analysis.

Identification and localization of gene(s) for salt tolerance in cosmid pUHR307

Cosmid pUHR307 (Figure 4.10) , containing genes for salt tolerance, was mutagenized with random insertion of Tn*3*Ho*gus*, a transposon constructed in the laboratory of Prof. Brian Staskawicz, University of California, Berkeley, USA for

making *gus*-fusion mutants (Brian Staskawicz, personal communication). The method for random insertion of Tn*3*Ho*gus* in cloned DNA in a plasmid has been described previously (Borthakur et al., 2003). The derivatives of cosmid pUHR307 were transferred to TAL1145 by triparental mating as described above, and the salt tolerance ability of transconjugants was determined by spotting colonies on duplicate plates of YEM agar and YEM containing 100 mM NaCl. Colonies were incubated at 28°C for 3-7 d. Colonies that showed reduction of growth on YEM containing 100 mM NaCl were selected. From these colonies the pUHR307::Tn*3*Ho*gus* derivatives were isolated and transformed into *E. coli*. The genes involved in salt tolerance in cosmid pUHR307 were localized by determining the position of the transposon in the pUHR307::Tn*3*Ho*gus* derivatives by restriction mapping and sequencing. To knock out the gene function for salt tolerance of the wild type strain BL3, the derivatives pUHR307::Tn*3*Ho*gus* were transferred into the BL3 chromosome by marker exchange using the incompatible plasmid, pPH1J1 (Beringer et al., 1978), and selecting for Km and Gm resistance, but Tc sensitivity, as described by Ruvkun and Ausubel (1981). The resulting knock-out mutants were tested for salt tolerance by growing them in YEM containing 100-300 mM NaCl.

Restriction mapping and sequencing

A restriction map of cloned DNA in cosmid pUHR307 was developed by subcloning the DNA fragments in cosmid pUHR307 at the *Hin*dIII and *Eco*RI sites in pUC18, and analyzing the cloned fragments by restriction digests with both restriction enzymes. The common fragments achieved from comparison of digestion pattern of

similar cosmid clones were sequenced using M13 forward and reverse primers. For complete sequencing of each fragment, primer walking strategy was applied.

Improving salt tolerance of *B. japonicum* THA6

B japonicum THA6 has a relatively high level of a tetracycline resistance (20µg/ml). Therefore, cosmids pUHR307 and pUHR310, containing tet^R as the only antibiotic resistance marker could not be transferred to THA6 using tetracycline as selectable marker. Therefore, two Tn*3*Ho*gus* insertion derivatives of pUHR307 and pUHR310, which contain an additional kanamycin resistance marker (kan^r) were used. The genes involved in salt tolerance in these two derivatives (pUHR307-kan and pUHR310-kan) were not interrupted by the Tn*3*Ho*gus* insertions. pUHR307-kan and pUHR310-kan were transferred to THA6 by triparental mating. Salt tolerance was determined on minimum medium (Howieson, 1985). Media were supplemented with antibiotics at final concentration (µg ml^{-1}); kanarmycin (Km) 25 and tetracycline, (Tc) 10 and Streptomycin (Sm) 100 after autoclaving. The THA6 derivatives containing pUHR307-kan and pUHR310-kan were tested for growth under 0, 50 and 100 mM NaCl.

Betaine aldehyde dehydrogenase activity

For preparation of cell extracts, THA6 and RUH162 were grown in 200 ml of modified minimal medium as previously described. The media were supplemented with 100 mM choline, or 100 mM choline and 50 mM NaCl. Cell cultures were grown under 28^{o}C on rotary shaker at 200 rpm for 96 h. Cell pellets were collected by centrifuging at 7000 g, washed twice with 50 mM phosphate buffer, pH 7, and

resuspended in 1 ml of the same buffer containing 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM EDTA, 1 mM DTT, 4 mg/l DNase and 1 mg/ml lysozyme. Cells were disrupted by sonication on ice using 6-mm diameter probe, 50% duty cycle, amplitude setting 20%, total 10 times. The lysate was centrifuged twice at 12,000 g for 10 min to remove unbroken cells. The supernatant was used for enzyme assays. Protein concentration was measured by Lowry's method (Lowry et al., 1951). Bovine serum albumin was used for standard protein calibration.

Betaine aldehyde dehydrogenase activity was determined spectrophotometrically at room temperature by monitoring the reduction of NAD^+ at 340 nm. The reaction mixture consisted of 50 mM HEPES-KOH pH 8.0, 5 mM DTT, 1 mM EDTA, 10 mM betaine aldehyde, 1 mM NAD^+ and 100 µL of sample solution. A blank was set using the reaction mixture without NAD^+. The reaction was started by adding NAD^+ to the mixture. The enzyme activity was determined by incubation of cell extract with 10 mM betaine aldehyde at 30^{o}C according to the method of von Tigerstrom and Razzell (1968). One unit is defined as the activity that catalyzes the formation of 1 µmol of product (NADH) per minute.

RESULTS AND DISCUSSION

Isolation and screening of salt tolerant rhizobia

Three hundred and seventy three isolates of rhizobia were collected from the nodules of the wild bean *P. lathyroides* that grew in the saline uncultivated areas of the Nakhon Ratchasima province in the north-eastern region of Thailand. The number of isolates collected from Burawai, Ban Leuam, Nongsuang and Pimai were 57, 251,

39 and 26, respectively. The abbreviations, BW, BL, NG, and PM are refer to isolates obtains from Burawai, Banleuam, Nongsuang and Pimai area, respectively. These isolates contained both fast-growing (*Rhizobium and Sinorhizobium*) and slow-growing rhizobia (*Bradyrhizobium)*. None of the slow-growing isolates could grow at 100 mM NaCl of YEM, while 50 of the fast-growing isolates showed some detectable growth within three days. These isolates were then tested on YEM broth containing 300 mM NaCl and eight isolates that grew well were selected. When these isolates were grown in YEM broth containing 400 mM NaCl, only six could grow; two isolates, BL2 and PM1 did not grow (Figure 4.2). Among these six isolates, only one isolate, BL3, grew to the highest cell density (OD_{600} = 0.95) at this salt concentration within three days. BL3 could tolerate up to 600 mM NaCl in YEM broth, although at salt concentrations above 300 mM, its growth was much inhibited (Figure 4.3). Therefore, this strain was selected for further studies on salt tolerance.

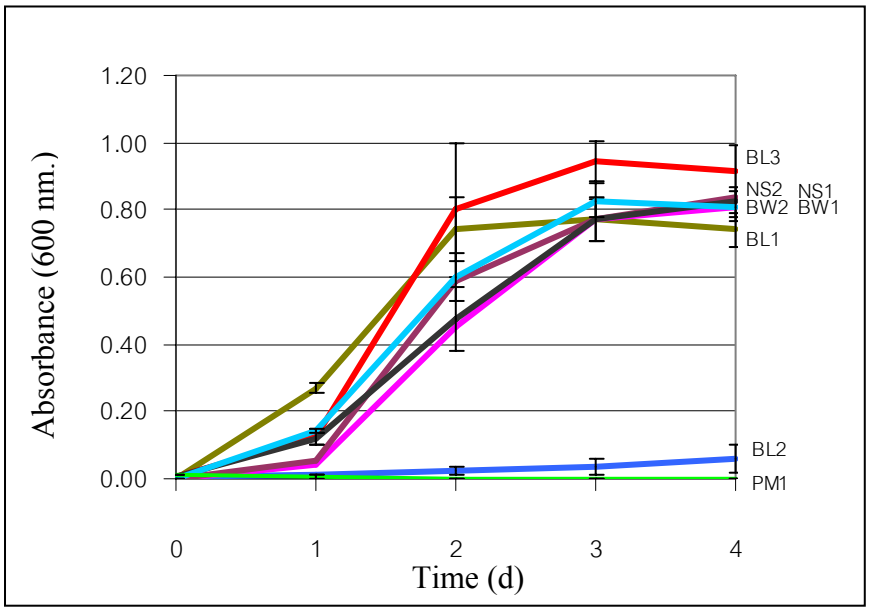

Figure 4.2 Growth pattern examined under YEM containing 400 mM NaCl of BL1 (—), BL2 (—), BL3 (—), NS1 (—), NS2 (—), BW1 (—), BW2 (—), PM1 (—).

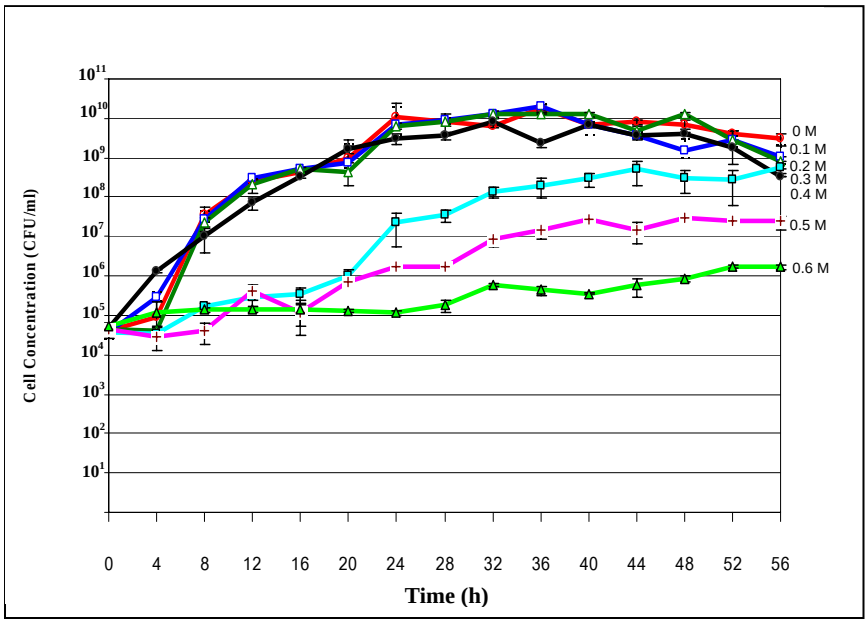

Figure 4.3 Growth of *Sinorhizobium sp.* BL3 in YEM medium containing various salt concentration; 0 mM NaCl (—), 100 mM NaCl (—), 200 mM NaCl (—), 300 mM NaCl (—), 400 mM NaCl (—), 500 mM NaCl (—), 600 mM NaCl (—).

Morphological and biochemical characteristics of the salt-tolerant isolates

The selected 8 isolates were characterized by morphological and biochemical test. All of them are fast-growing and they could form colonies within 3 days. They showed acidic reactions on YEM agar containing bromothymol blue. BL2 and PI1 formed translucent-watery colonies while the remaining six salt-tolerant isolates formed opaque creamy wet colonies. All isolates could utilize esculin, glucose and mannitol. None of these isolates could utilize arginine, gelatin, and citrate. Moreover, indole production could not detect by these isolates. BL1 and BL2 could utilize arabinose, while the other six isolates including BL3 could not utilize arabinose as a sole carbon source (Table 4.3).

Symbiotic characteristics of the salt-tolerant isolates

The eight salt-tolerant isolates formed nitrogen-fixing nodules with *P. lathyroides*, *C. cavagia*, *M. atropurpureus*, while none of them nodulated *Glycine max*. One isolate, BL3 also formed effective nodules on *C. pascuorum* and ineffective nodule on *V. radiata*, *V. umbellate* and *V. sinensis,* while three of these isolates, NS1, NS2 and PI1 did not form nodules on these hosts (Figure 4.4). The host range of the remaining four salt tolerant isolates, BL1, BL2, BW1 and BW2 differed widely in these four host legumes (Table 4.4).

When BL3 was used to inoculate *P. lathyroides* grown in the presence of 100 mM NaCl, the plants formed effective nodules. In the presence of 150 mM salt concentration, plants produced ineffective nodules. This was indicated by the reduction in plant dry mass (Table 4.5).

Table 4.3 Morphological and biochemical characteristics of the salt-tolerant rhizobial isolates.

Characteristic	Strains								
	BL1	BL2	BL3	BW1	BW2	NS1	NS2	PM1	*S. meliloti*
Single colony formed (days)	3	3	3	3	3	3	3	3	3
Type of colony	Medium, creamy opaque	Medium, wet, watery, translucent	Medium, creamy opaque	Medium, creamy opaque	Medium, creamy opaque	Medium, creamy opaque	Medium, creamy opaque	Medium, wet, watery, translucent	Medium, wet, opaque
Gram strain	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
NO_3	-	-	-	-	-	-	-	+	-
Indole production	-	-	-	-	-	-	-	-	-
B-galactosidase	+	+	+	-	-	+	+	+	+
Glucose acidification	-	-	-	-	-	-	-	-	-
Arginine dehydrogenase	-	-	-	-	-	-	-	-	-
Urease	+	+	-	-	-	-	-	-	-
B-glucosidase	+	+	+	+	+	+	+	+	+
Protease (gelatine)	-	-	-	-	-	-	-	-	-
Glucose assimilation	+	+	+	+	+	+	+	+	+
Arabirose assimilation	+	+	-	-	-	-	-	-	+
Manose assimilation	+	+	+	-	-	+	+	-	+
Manitol assimilation	+	+	+	+	+	+	+	+	+
N-acetlyl-glucosamine	+	-	+	+	+	+	+	-	+
Maltose assimilation	+	+	-	+	+	+	+	+	+
Gluconate assimilation	+	+	-	-	-	-	-	-	+
Caprate assimilation	-	+	-	-	-	-	-	-	-
Adiprate assimilation	-	-	-	+	+	-	-	-	-
Malate assimilation	+	+	-	-	-	-	-	-	+
Citrate assimilation	-	-	-	-	-	-	-	-	-
Phenyl-acetate assimilation	-	+	-	-	-	-	-	-	-

Remark; Characterized by Iapi 20 NE from bioMerieux Identification system for non-enteric Gram-Negative rods

(A) (B)

(C)

Figure 4.4 Nodulation test of BL3 on *P. lathyroides*, *C. cavagia*, *M. atropurpureus* and *C. pascuorum* (A), *V. radiata*, *V. umbellate* and *V. sinensis* (B), *G. max* (C).

Table 4.4 Symbiotic characteristics of the salt-tolerant rhizobial isolates.

Isolated strains	Host nodulation test				
	C. pascuorum	*M. atropurpureus*	*V. radiata*	*V. umbellate*	*V. sinensis*
BL1	+	+	+	-	+
BL2	-	+	+	-	+
BL3	+	+	+	+	+
BW1	-	+	-	+	-
BW2	-	+	-	-	+
NS1	-	+	-	-	-
NS2	-	+	-	-	-
PM1	-	+	-	-	-

Table 4.5 Number of nodules, dry weight of nodules and shoots of *P. lathyroides* plants inoculated with *Sinorhizobium sp*. strain BL3 grown in the presence of 0, 50, 100 and 150 mM NaCl.

Condition	Nodules (no. per 5 plants)	Nodule dry mass (mg per 5 plants)	Plant dry mass (g per 5 plants)
Uninoculate	0	0	0.155 ± 0.015^{b}
0 mM NaCl	49.6 ± 7.53^{a}	19.2 ± 4.76^{a}	0.2152 ± 0.014^{a}
50 mM NaCl.	45.2 ± 2.49^{a}	15.8 ± 1.92^{ab}	0.1561 ± 0.017^{b}
100 mM NaCl	44.4 ± 3.28^{a}	15.0 ± 2.10^{b}	0.0934 ± 0.012^{c}
150 mM NaCl	30.6 ± 5.50^{b}	8.8 ± 1.92^{c}	0.0712 ± 0.006^{d}

*Means with different letters are significantly different at $p<0.05$.

16 S rDNA sequence analysis

Since BL3 was found to be both effective for nodulation and salt-tolerant, this strain was selected for further characterization. Chromosomal DNA of BL3 was amplified for the 16S rRNA gene. The 1,315 bp PCR amplified fragment was purified and sequenced. The GenBank accession number for this sequence is AY943949. The sequence showed 99% similarity with the 16S rRNA gene of *Rhizobium sp*. I6, *Sinorhizobium meliloti* strain LMTR32 and *Sinorhizobium sp.* SEMIA 6161. Therefore, it was named *Sinorhizobium sp.* BL3.

Construction of a cosmid clone library of *Sinorhizobium sp.* BL3 DNA

A genomic library of BL3 was constructed by partial digestion of genomic DNA with *Sau*3AI to obtain 20-30 kb fragments, which were then ligated to the *Bam*HI site of cosmid vector pLAFR3. The cosmid library contained 7,231 clones. To verify the presence of insert DNA in the clones, 10 clones were randomly selected from the library for restriction analysis. These cosmids were extracted and digested with *Hin*dIII. Restriction fragments showed that all of them were different, indicating that the library represented random insertion of cloned BL3 DNA (Figure 4.5). These insert fragments ranged between 12.5 kb to 27.8 kb with the average size of 16.81 kb. From *Sinorhizobium* genome projected (www, nd.) illustrated the *S. meliloti* genomes consisting of three replicons; one chromosome of 3.65 Mb, two large plasmid of 1.4 and 1.7 Mb. Thus, this cosmid library exhibits of eighteen-fold coverage of genome of *S. meliloti* as calculated from the insertion length and number of total clones.

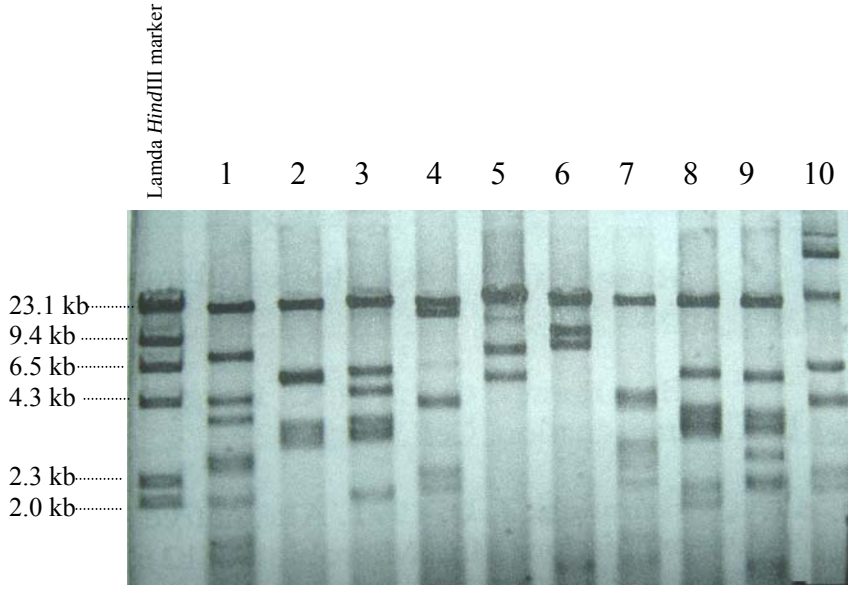

Figure 4.5 Restriction pattern of ten cosmid library digested with *Hin*dIII.

Isolation of cosmid clones harboring genes for salt tolerance

To isolate the genes for salt tolerance, the cosmid clone library of genomic DNA of the salt-tolerant *Sinorhizobium sp*. BL3 was transferred to the salt-sensitive *Rhizobium* strain TAL1145. The transconjugants were plated on YEM agar containing 100 mM NaCl to select salt tolerant colonies. Two hundred such salt tolerant transconjugant colonies were selected. From twenty four of these transconjugants, cosmid DNA was isolated. Restriction analysis of these clones by *Hin*dIII showed two different digestion patterns, indicating that the cloned DNA represented two regions of BL3 genome (Figure 4.6). The first region showed five similar but overlapping restriction patterns (Figure 4.7). Restriction analysis of these overlapping cosmid clones using *Hin*dIII revealed common fragments of sizes 8.8, 4.9, 3.2 and 1.4 kb. Similarly, *Eco*RI digests of these clones showed common fragments of sizes 6.9, 4.8,

4.5 and 2.2 kb (Figure 4.7). Besides this common overlapping fragments, the cosmids contained additional of sizes 3.7, 6.9, 12.7, and 14.1 kb (Figure 4.8). The smallest cosmid, pUHR307, from this group was selected for further study. The second group of cosmid clones, represented by pUHR310, showed only one restriction pattern with five of *Hin*dIII fragments of sizes 6.47, 6.2, 4.79, 3.82 and 2.22 kb (Figure 4.9). The restriction map of pUHR310 is shown in Figure 4.23.

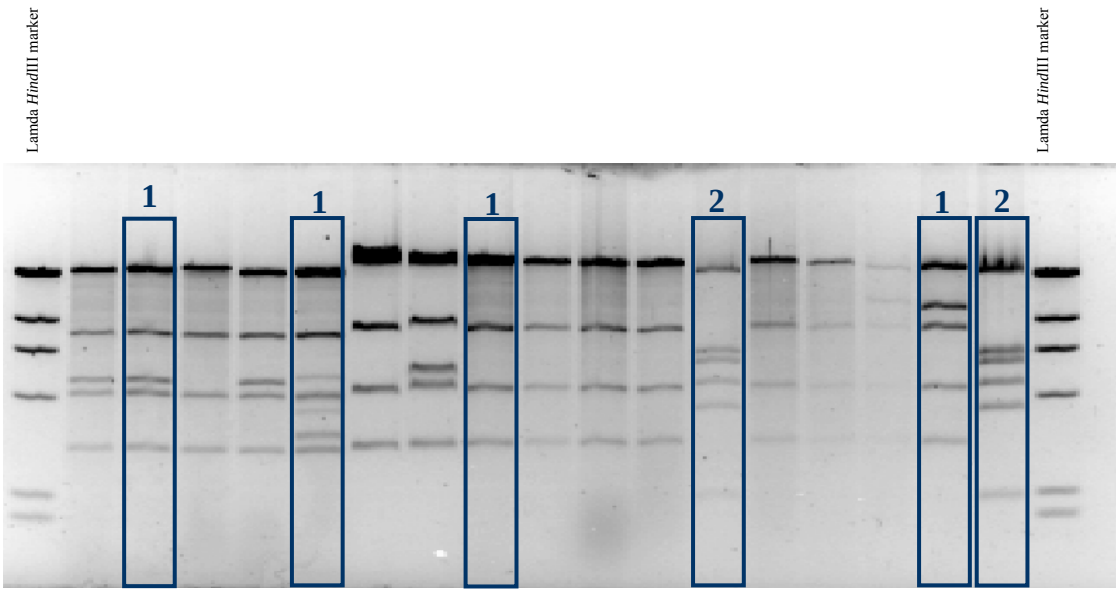

Figure 4.6 Restriction pattern of salt tolerant clones digested with *Hin*dIII revealed two different regions of chromosome. The first salt tolerant genes region exhibited 4 similar restriction patterns, the second salt tolerant region exhibited only one pattern.

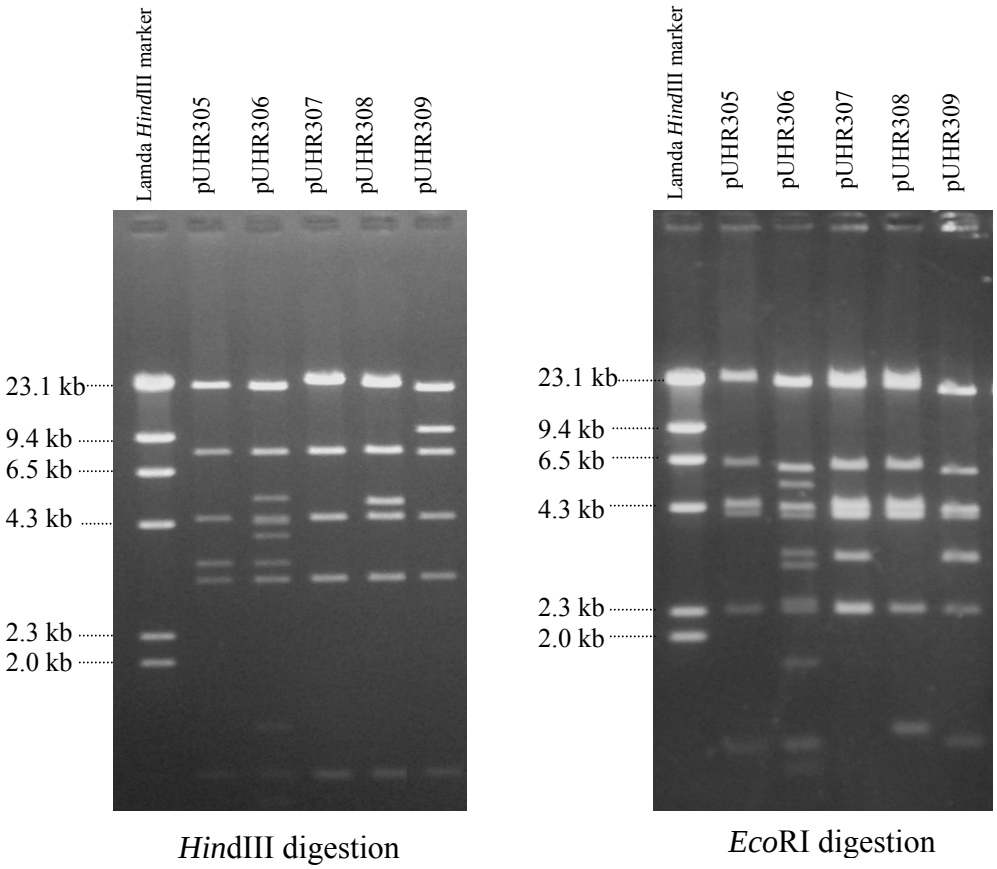

Figure 4.7 Restriction patterns of salt tolerant clones pUHR305, pUHR306, pUHR307, pUHR308, and pUHR309 digested with *Hin*dIII and *Eco*RI.

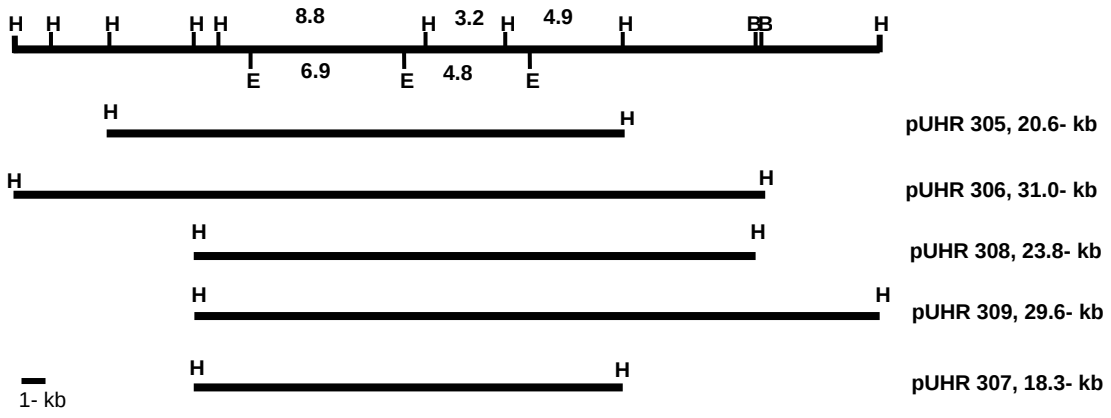

Figure 4.8 Restriction map of salt tolerant clones pUHR305, pUHR306, pUHR307, pUHR308, and pUHR309 showing five overlapping insertion fragment. Restriction sites are: H; *Hin*dIII, E; *Eco*RI, B; *Bam*HI.

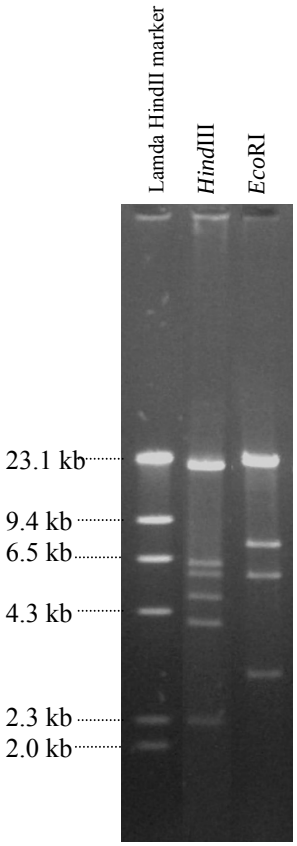

Figure 4.9 Restriction pattern of pUHR310 digested with *Hin*dIII and *Eco*RI.

Identification and characterization of gene(s) involved in salt tolerance in pUHR307

To identify gene(s) involved in salt tolerance, plasmid pUHR307 was mutagenized with random insertion of the transposon Tn*3*Ho*gus*. The position of Tn3Ho*gus* insertions in 58.3% of these pUHR307::Tn*3*Ho*gus* derivatives were determined and found to be randomly inserted throughout the entire insert DNA in pUHR307 (Figure 4.11). Various pUHR307::Tn*3*Ho*gus* derivatives were transferred to TAL1145 expecting that any transconjugant containing Tn*3*Ho*gus*-insertion on a gene for salt tolerance in pUHR307 would be sensitive to salt. Over 3000 colonies were screened and no colony showing reduced growth rate at 100 mM NaCl could be identified. Therefore, an alternative strategy was used to identify genes for salt tolerance in pUHR307. The pool of 3000 pUHR310::Tn3Ho*gus* derivatives were transferred to BL3 by conjugation and then the Tn3Ho*gus* insertions were transferred by marker exchange from pUHR307::Tn3Ho*gus* to the corresponding homologous position in the BL3 chromosome. In this way, 326 knock-out mutants carrying Tn3Ho*gus* insertions in the region of chromosome that was cloned in pUHR307 were created. These mutants were screened for loss of ability to grow at 300-400 mM NaCl. However, none of the mutants had detectable reduction in salt tolerance compared to BL3. These results suggest that the function of the genes in pUHR307 may be substituted by some other genes in BL3. Another possibility is that none of the insertions interrupted any gene required for salt tolerance in pUHR307. Alternatively, the genes in pUHR307 may have regulatory roles for salt tolerance and may not be directly related to salt tolerance in BL3. During selection of TAL1145 transconjugants containing the BL3 clone library for salt tolerance, pUHR307 and other clones that

contain overlapping insert DNA, were isolated from many independent salt tolerant transconjugant colonies. Thus, pUHR307 cannot be a random clone, selected by chance during screening. To determine if pUHR307 contain regulatory genes that may be involved in salt tolerance, it was necessary to determine the nucleotide sequence of the cloned fragment.

Sequence analyses of pUHR307

The 8.8, 4.9, and 3.2-kb *Hin*dIII fragments of pRUH307 that overlapped with the insert DNA in pUHR308 and pUHR309, were subcloned to pUC18 and sequenced. Eleven open reading frames (ORF) were identified by sequence analyses (Figure 4.10). The 8.8-kb and 3.2-kb *Hin*dIII fragments contain three ORFs each, while the 4.9-kb *Hin*dIII fragments have five ORFs. These eleven ORFs are described below (Table 4.6).

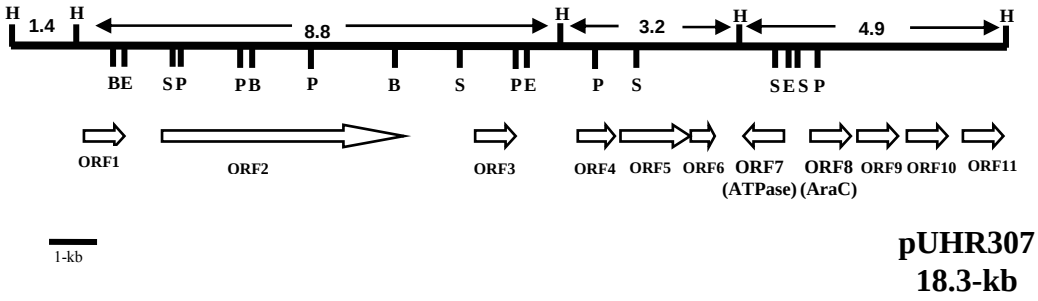

Figure 4.10 Restriction map of pUHR307 containing 11 ORFs. The positions and directions of ORFs are indicated with open arrows. The sizes of the *Hin*dIII fragments are indicated.

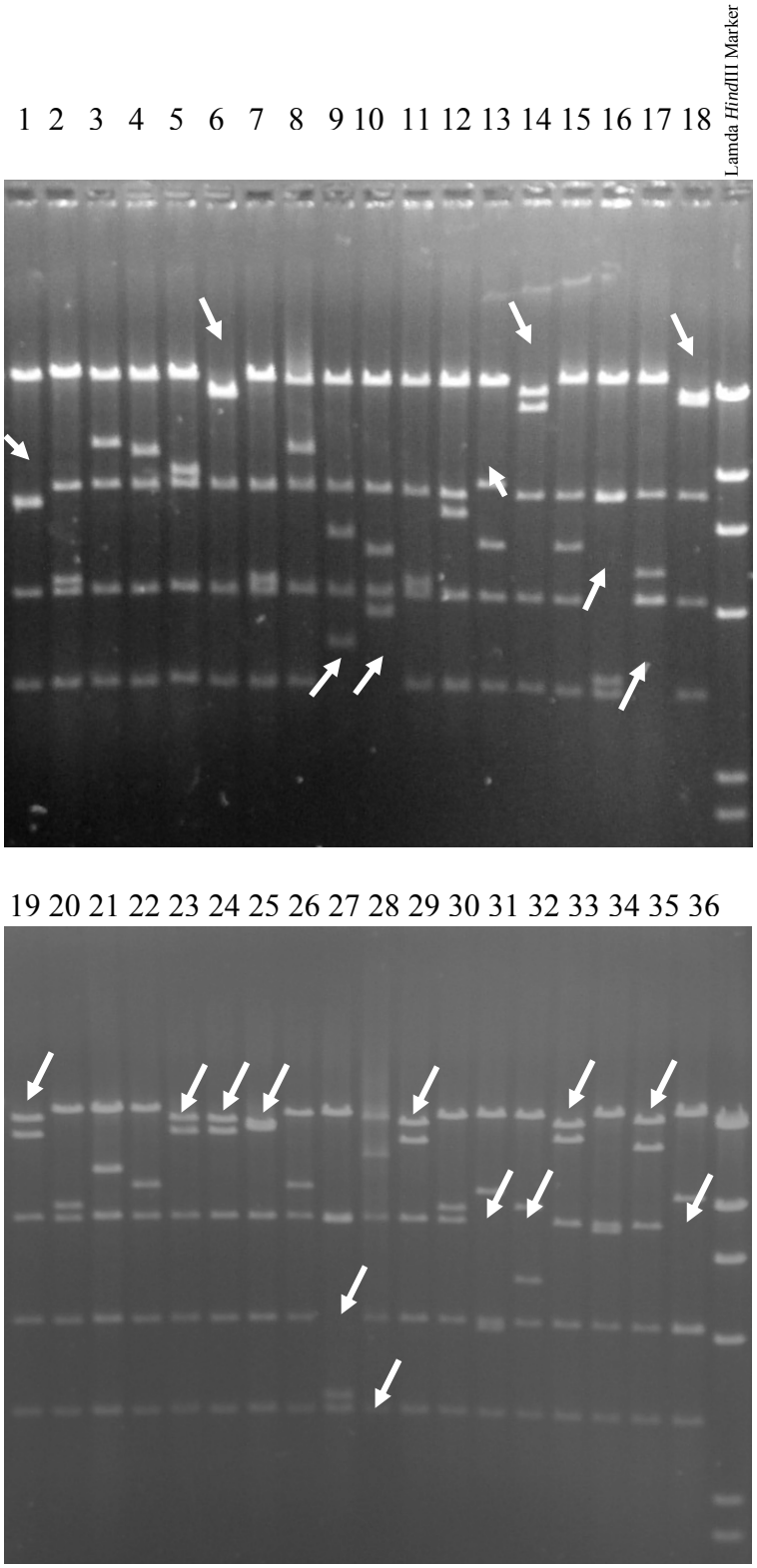

Figure 4.11 Restriction pattern of pUHR307:Tn3Ho*gus* derivatives showing the insertion of *Tn*3 at the random position. Arrows point at the mutagenized fragment.

Table 4.6 Characteristics of the proteins encoded by ORFs identified on 3 *Hin*dIII fragments in cosmid pUHR307.

ORF	Size (bp)	DNA fragment location	Homology with known genes	Possible function
ORF1	605	8.8-kb	60% identities with a hypothetical protein of *Agrobacterium tumefaciens*	Not known
ORF2	4,442	8.8-kb	68% identities to DNA methylase of *A. tumefaciens*	DNA methylase
ORF3	422	8.8-kb	90% identities with partitioning protein (parB)	Chromosome partitioning
ORF4	437	3.2-kb	84% identities with conserved a hypothetical protein Atu6109 of *A. tumefaciens*	Not known
ORF5	929	3.2-kb	64% identities with antirestriction protein gene of *A. tumefaciens*	DNA protection
ORF6	245	3.2-kb	62% identities with hypothetical protein SMb20629 *S. meliloti* 1021	Not known
ORF7	562	4.9-kb	76% of identities with a predicted ATPase of *Rhodospirillum rubrum*	Ion transportation
ORF8	887	4.9-kb	89% identities with probable transcriptional regulator SyrB (AraC family) of *Rhizobium sp.* NGR234	Transcriptional regulation
ORF9	570	4.9-kb	98% identities with Xanthine dehydrogenase, iron-sulfur subunit protein of *S. meliloti* 1021	Purine metabolism
ORF10	976	4.9-kb	74% identities with putative aldehyde or xanthine dehydrogenase, molybdopterin binding subunit protein of *S. meliloti* 1021	Purine metabolism
ORF11	776	4.9-kb	93% identities with xanthine dehydrogenase of *Rhizobium sp.* NGR234	Purine metabolism

Among the eleven ORFs shown in Table 4.6, ORF7 and ORF8 are likely to be involved in salt tolerance, based on their possible functions. The ORF7-encoded ATPase may be involved in Na^+ export channel for enhancing salt tolerance of BL3. The activity of membrane ATPase in active Na^+-export is a well-investigated phenomenon in bacteria (Skulachev, 1994; Ivey et al., 1998, Horikoshi, 1998; Krulwich et al., 1998). Whenever in a saline environment the passive Na^+ flux into the cell increases the cytoplasmic Na^+ concentration above a critical level, Na^+ re-export into the environment is initiated (Na^+ homeostasis) (Raven, 1976; Ritchie, 1992). One obvious possibility of Na^+ retranslocation is the coupling to inverse H^+-gradients created by H^+-ATPases. ATPase was shown to be involved in salt stress response of several other organisms. *S. cerevisiae* plasma membrane H^+-ATPase activity was observed by the addition of NaCl into the culture medium (Watanabe, 1993). Co-expression of the cell membrane associated H^+-ATPase and Na^+/H^+-antiporter can induce the salt tolerant ability in *S. cerevisiae* cells (Watanabe et al., 2005).

The ORF8-encoded protein may be a transcriptional regulator of AraC family. The AraC family of transcriptional activators regulate diverse bacterial functions including sugar catabolism, virulence, and responses to stress. A large number of these proteins were classified as stress response activators. For example, Ada from *E. coli*, *S. typhimurium*, and *Mycobacterium tuberculosis* and *Bacillus subtilis* produced in response to alkylating agents (Demple, 1985; Hakura 1991; Moroshi et al., 1990; Murphy et al., 1996); SoxS from *E. coli* and *S. typhimurium* are synthesized in response to oxidative stress (Ama´bile-Cuevas et al., 1991, Wu and Weiss, 1991); AarP from *Providencia stuartii*, MarA and Rob from *E. coli*, PqrA from *Proteus*

vulgaris, and RamA from *Klebsiella pneumoniae* are involved in tolerance to antibiotics, organic solvents, and heavy metals (George et al., 1995, Ishida et al., 1994, Macinga et al., 1995, Skarstad et al., 1993, Sulavik et al., 1996).

ORF2-encoded DNA methylase may also play an important role in stress response. Methylase or Methyltransferases are employed in restriction-modification and mismatch repair systems in prokaryotes. Methyltransferases recognize specific DNA sequences and transfer a methyl group from the cofactor S-adenosyl-L-methionine (AdoMet) to nitrogenous bases (Guha and Guschlbauer, 1992; Riva et al., 2004, Hale et al., 1994). In *E. coli*, the *ada* gene product has the methyltransferase activity which stimulated under SOS, heat shock, and adaptive response. This protein is a positive regulatory protein that stimulates transcription of the other adaptive response genes including *alkA, alkB, aid,* as well as *ada* itself (Moat and Foster, 1995). It is possible that the ORF-encoded methylase is involved in mismatch repair control the adaptation response under salt stress conditions.

The ORF3-encoded partitioning protein-driven partitioning mechanism is involved in specifically segregating bacterial DNA strands or chromosomes. After DNA replication, bacterial cells must undergo chromosomal segregation; the process whereby new sister chromosomes physically separate and then partition to each pole of the predivisional cell. Some bacteria possess homologs of two genes, *parA* and *parB*, whose products are involved in partitioning (Leonard et al 2005). However, the relationship between this gene and salt stress has still unclear. The *par* gene identified in pUHR307 may not be involved in salt tolerance in BL3.

The ORF5-encoded antirestriction protein may also be related to stress response. Antirestriction mechanism has been reported to avoid degradation of DNA

of many phage and plasmids (Wilkins, 2002). Restriction endonucleases degrade invading double-stranded phage or plasmid DNA that is not appropriately modified. However, under certain stress conditions (for example, induction of the SOS response or heat shock) restriction endonucleases can be temporarily inactivated by antirestriction systems (Kelleher and Raleigh 1994; Barcus and Murray 1995). Therefore, antiresticton protein might prevent the damage DNA to be digested from restriction enzyme before the DNA repair mechanism.

The ORF9-11 encoded protein xanthine dehydrogenase may be an enzyme in purine metabolism. Xanthine dehydrogenase converts hypoxanthine to xanthine. There is a link between nitrogen limitation and purine metabolism (Xi et al., 2000). Therefore, under stress condition if the nitrogen is limited, this mechanism can be enhanced the survival and growth of bacteria.

```
Query: 172 MNLSPIRKVFQGVADRRQMFRMFDRHAQRPNRREGDDSALYRGEWFEIGEASHDYMFEIL 351
           M +S IRKVF+G+ADRRQMFRMFDRHAQRPNR EGDDSALYRGEWFEI +A HDYMFEIL
Sbjct: 1   MTVSSIRKVFEGIADRRQMFRMFDRHAQRPNRWEGDDSALYRGEWFEIAQAQHDYMFEIL 60

Query: 352 PPLWMKAEMFAMREFLTGSITSIFFTLSIDGRIRHFHGYCDLADKGSPERMXXXXXXXXX 531
           PPL+M+ +MFAMREFLTGSITSIFFTL ID R+R+FH YCDL+DKGSPERM
Sbjct: 61  PPLFMRGDMFAMREFLTGSITSIFFTLKIDDRMRYFHAYCDLSDKGSPERMRGAIAERET 120

Query: 532 XXVRAMTREERLEHIWSSADDLSRLRGRALAQRLILASGMVMFFARLRPVPHGKLLEDL 711
             VRAMTREER HIWSS  DD     G    +        V+F+   R     KLL+DL
Sbjct: 121 RPVRAMTREERLDHIWSSSHDDYRGYAGERWPEH-DHGKRTVLFYGG-RQGTGLKLLDDL 178

Query: 712 TDVEIAAKLPVHLRYLPDALAA 777
           TD EIA+KLPVHLRYLPDA+AA
Sbjct: 179 TDAEIASKLPVHLRYLPDAIAA 200
```

Figure 4.12 Blast search analysis of ORF1 shows homologies with hypothetical protein of *A. tumefaciens* with 60% identities and 70% positives. Abbreviations are: + conservative substitution amino acid, -; insertion or deletion of amino acid, X; low complexity of amino acid.

```
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Sbjct: 1    MSND----DPF-TLDLFGNTALSSGLGLGVTAFGD-------EPSPAPVEPEPKTSPQTA 48

Query: 1477 XXXXXXQRSAARRQGDRANFYFDVGEDRGLAASWKERARLNVASILTANEIERHNIPVTR 1656
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Sbjct: 49   PGFQRVDTYAARGE----NFF--LVSDRGLARGWKQRARDNLAAIRLAADIEAADRPATV 102

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            E Q RLIRF+GFGAS+LAN +FRR GE  FR+GWD++G  L+ AV+A +YASL+RCTQY+
Sbjct: 103  EEQARLIRFSGFGASDLANAVFRRPGENGFRKGWDEIGLELQDAVTAQEYASLARCTQYA 162

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Sbjct: 163  HFTPEFIIRAIWAGLQRLGWRGGRVLEPGIGTGLFPALMPEDRRGVSHVTGVEIDPVTAR 222

Query: 2017 IVKLLQPKARIIEGDFAHTDLAPIYDLAIGNPPFRSHRAVRTG-NISSLGLRLHDYFIAR 2193
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Sbjct: 223  IARLVQPRARIVNGDFSCTDLPAYFDLAIGNPPF-SNRTVRSDRTYRSMGLRLHDYFIAR 281

Query: 2194 SIDLLKPGAFAAFVTSSGTLDKADATAREHIAKSADLIAAIRLPEGSFRRDAGTDVVVDL 2373
            S+DLLKPGA AAFVTSSGT+DK D+ AR+HIAKSADLIAA+RLPEGSFR DAGTDVVVD+
Sbjct: 282  SVDLLKPGALAAFVTSSGTMDKTDSIARKHIAKSADLIAAMRLPEGSFRTDAGTDVVVDI 341

Query: 2374 LFFRKRKAGEPEGDLTWLDLEEVRPATDGEGAIRVNRWFAEHPGFVLGDHALTSGPFGET 2553
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Query: 2554 YTCRARAGVELETALKAVISLLPEDRYDGEPTEIDIDLETSSATSSTFAPETEKVREGSF 2733
            Y+C    GV+LE AL A ISLLPE  YDG P EID D   S+       PE   VREGSF
Sbjct: 402  YSCLPHPGVDLERALTAAISLLPEAIYDGAPDEIDHDAAPSADVVDAL-PEGSGVREGSF 460

Query: 2734 FIDNRQGLMQMIDGAPVQIKVRKGRSADGIPEKHVRIIQKLIPVRDAVREVLRCQEQDRP 2913
            F+     L+QMIDGA V I VRKG +++G+PEKH RII+KLIP+RDAVREVL+ QE DRP
Sbjct: 461  FVAKNTALVQMIDGAAVTITVRKGGASEGVPEKHARIIRKLIPIRDAVREVLKAQEFDRP 520

Query: 2914 WKDSQVRLRIAWSSFVRDFGPINHTTVSISEDDETGDVRESHRRPNLQPFLDDPDCWLVA 3093
            WK +QVRLRIAWS+FVRDFGPIN T VS SED ETG+VRE HRRPNLQPFLDDPDCWLVA
Sbjct: 521  WKPAQVRLRIAWSNFVRDFGPINTTVVSTSEDAETGEVREVHRRPNLQPFLDDPDCWLVA 580

Query: 3094 SIEDYDLETDTARPGPIFSEGVISPPAAPVITSAADALAVVLNERGRVDVDHIAELLHRD 3273
            SIEDYDLET+TARPGPIF+E VI+PPAAP+ITSAADALAVVLNERG VDVDHIAELLH D
Sbjct: 581  SIEDYDLETNTARPGPIFTERVIAPPAAPIITSAADALAVVLNERGHVDVDHIAELLHGD 640

Query: 3274 PDDVIAELGDAIFRDPAGGSWQTSDAYLSGPVRTKLTVAQAAAELDPEFRRNVLALQEVQ 3453
             DDVI ELGDAIFRDP  GSW T+DAYLSG VR KL  A+AAA LDP F RNV AL EVQ
Sbjct: 641  VDDVIDELGDAIFRDPETGSWHTADAYLSGQVRDKLKAAKAAAALDPVFERNVRALVEVQ 700

Query: 3454 PADLRPSDITARLGAPWIPASDVTAFVKETMGADIRIHHMPDWGHGRSRQGPLYYTAAGT 3633
            PADLRPSDITARLGAPWIPA+DV AFV+ETM A+IRIHHMP+          L + AAGT
Sbjct: 701  PADLRPSDITARLGAPWIPAADVVAFVQETMSAEIRIHHMPELASWTVEARQLGWMAAGT 760

Query: 3634 SEWGXXXXXXXXXXXXXEQSRAADFRHLQGCDGERRVLNVVDTEAARDKLQRMKEAFQN 3813
            SEWG                    F  ++    E+RVLNVVDTEAA++KLQ++K AFQN
Sbjct: 761  SEWGTDRRHAGELIADALNSRVPQIFDTVKEDHAEKRVLNVVDTEAAKEKLQKIKTAFQN 820
```

Figure 4.13 Blast search analysis of ORF2 shows homologies with DNA methylase of *Mesorhizobium loti*, MAFF303099 with 68% identities and 77% positives. Abbreviations are: + conservative substitution amino acid, -; insertion or deletion of amino acid, X; low complexity of amino acid.

```
Query: 3814 WVWSDPDRTDRLARVYNDRFNNIAPRKFDGSHLKLLGASGAFVLYGHQKRGIWRIISSGS 3993
            W+WSDPDRTDRLARVYNDRFNNIAPR+F+G HL+L GASGAF LYGHQKRGIWRI+S+GS
Sbjct: 821  WIWSDPDRTDRLARVYNDRFNNIAPRRFNGDHLRLPGASGAFSLYGHQKRGIWRIVSAGS 880

Query: 3994 TYLAHAVGAGKTMTMAAAIMEQRRLGLIAKAMLVVPGHCLA 4116
            TYLAHAVGAGKTMT+AAA+MEQRRLGLIAKAMLVVPGHCLA
Sbjct: 881  TYLAHAVGAGKTMTIAAAVMEQRRLGLIAKAMLVVPGHCLA 921

Query: 435  DAWASNFGDEKTELELQPSGKYKPVSRFASFVNVPELIAMFRAFADVVMPEDLRQYVKVP 614
            DAWAS FGD  TELELQPSGKYKPVSRFASFVNVPELIAMFR+FADVVMPEDLRQ+VKVP
Sbjct: 950  DAWASTFGDTTTELELQPSGKYKPVSRFASFVNVPELIAMFRSFADVVMPEDLRQFVKVP 1009

Query: 615  DIATGKRRILTA 650
             I+TGKR+I+T+
Sbjct: 1010 AISTGKRQIITS 1021

Query: 655  PTPAFKAYQQILETRIRAIEEXXXXXXXXXXHLISVITDGRHAAIDLRLVMPAMDDEPEN 834
            PT AFK YQ +L  RI  IE+           L+SVITDGRHAAIDLRLV    D+E +N
Sbjct: 1023 PTQAFKHYQMVLAARIAEIEKRDRPPEPGDDILLSVITDGRHAAIDLRLVDADNDNEADN 1082

Query: 835  KLNLLVRNAHRIWKQTSENTYLRPDGKPYELPGAAQMIFSDLGTINVEKTRGF*AYRWIR 1014
            KLN L+ NA  IW+ T+ + Y+R DGKP+ELPGAAQMIFSDLGTI+VEK+RGF AYRWIR
Sbjct: 1083 KLNALISNAFAIWRATAGHPYVRHDGKPFELPGAAQMIFSDLGTISVEKSRGFSAYRWIR 1142

Query: 1076 RRPRPSSSLFGDVRAGKVRFLIGSSETMGTGVNAQLRLKALHHLDVPWLPSQIEQREGRI 1255
            ++      LFGDVRAGKVRFLIGSSETMGTGVNAQLRLKALHHLDVPWLPSQIEQREGRI
Sbjct: 1162 KKSEAKQRLFGDVRAGKVRFLIGSSETMGTGVNAQLRLKALHHLDVPWLPSQIEQREGRI 1221

Query: 1256 VRQGNQHDEVDIFAYATQGSLDATMWQNNERKARFIAAALSG----RYLQSAGWKI*TKV 1423
            VRQGNQHDEVDIFAYAT+GSLDATMWQNNERKARFIAAALSG    R L+  G     +
Sbjct: 1222 VRQGNQHDEVDIFAYATEGSLDATMWQNNERKARFIAAALSGDTSVRRLEDLG-----EG 1276

Query: 1424 RPISSPWHKAIASGDERLMQKAGL 1495
            +       KAIASGD+RLMQKAGL
Sbjct: 1277 QANQFAIAKAIASGDQRLMQKAGL 1300

Query: 1827 AQDDDLFAVRRQLRDAEREIETANRRIGEIGQDIERLVPTSGDAFAITVMGKPYTERKDA 2006
            A  DD  AVR QLRDAER+IE   RRIGEIGQDI  LVPT+G+AF +TV GK YTERK+A
Sbjct: 1313 AHIDDQHAVRWQLRDAERDIEFCTRRIGEIGQDIGMLVPTTGEAFTMTVAGKVYTERKEA 1372

Query: 2007 GRALMKEILTLVQL 2048
            GRALMKEILTLVQL
Sbjct: 1373 GRALMKEILTLVQL 1386

Query: 2051 QQGEVHIASIGGFDLIYEGERFGRGDNYHYXXXXXXXXXXXXXSDLAVTVTPLGAISRLE 2230
            Q+GE  I S+GGFDL Y+G+RFG+ + Y Y              +LA+TVTPLGAISRLE
Sbjct: 1388 QEGESIIGSVGGFDLEYDGQRFGK-EGYRYTTMLTRTGADSEI-ELAMTVTPLGAISRLE 1445

Query: 2231 HALDGSDXXXXXXXXXXXXXXXXXSYQSRQGGA-FAFADELAEKRRQLREVEEALA 2398
            H+LD  +                  SYQSR  GA FAFA ELA+KRRQL ++E AL+
Sbjct: 1446 HSLDDFEGERERYRQRLADARRRLASYQSRDNGAEFAFAGELADKRRQLGKIEAALS 1502
```

Figure 4.13 Continued.

```
Query: 3404 MQILKLDPRALKDNPDDARRSKSSPQADALLLATVKAVGIIQPPVVSPQTDGGNGYIIQA 3583
            MQILKLDPRALKDNPDD RRSKSSPQ+DALLLATVKAVGIIQPPV+SP+ DGGNGYIIQA
Sbjct: 1    MQILKLDPRALKDNPDDTRRSKSSPQSDALLLATVKAVGIIQPPVISPEVDGGNGYIIQA 60

Query: 3584 GHRRVRQAIAAGLEEIEVIVREAANDNGAMRSMVENIAREPLNGVDQWRGIERLVALGWT 3763
            GHRRV+QAIAAGLEEI V+V  AANDNGAMRSMVENIAREPLN VDQWRGIERLVALGWT
Sbjct: 61   GHRRVKQAIAAGLEEITVLVVAAANDNGAMRSMVENIAREPLNPVDQWRGIERLVALGWT 120

Query: 3764 EEGIGVALALPVRQIRKLACL 3826
            EE IGVALALPVRQIRKL  L
Sbjct: 121  EEAIGVALALPVRQIRKLRLL 141
```

Figure 4.14 Blast search analysis of ORF3 shows homologies with chromosome partitioning protein of *A. tumefaciens* str. C58 with 90% identities and 93% positives. Abbreviations are: + conservative substitution amino acid, -; insertion or deletion of amino acid, X; low complexity of amino acid.

```
Query: 9   AILDSDGIRDMHERLIVETATGLSEGLGERAMQIHLQRIVGAYVGSAHGAGQFYSRAVXX 188
            AILDSDGIRDMHERLIVETATGLS+GLGERAMQIHLQRIVGAYVGSAHGAGQFYS+AV
Sbjct: 50  AILDSDGIRDMHERLIVETATGLSDGLGERAMQIHLQRIVGAYVGSAHGAGQFYSKAVTE 109

Query: 189 XXXXXXXXXXXXXXXDLDGPVGYDSAAQRKREFAADMGVQAHALRMAAEGAVAAYEQIVG 368
                          DLDGPVGYDSAAQRKREFAADMG+QAHALR+AAEGAVAAYEQIVG
Sbjct: 110 ARDATAKGASDARDEDLDGPVGYDSAAQRKREFAADMGIQAHALRLAAEGAVAAYEQIVG 169

Query: 369 EAWKPFDRPVDNPGQSLDRKAAAAQM 446
            EAWKPFDRPVDNPGQ+LDRKAAAAQM
Sbjct: 170 EAWKPFDRPVDNPGQALDRKAAAAQM 195
```

Figure 4.15 Blast search analysis of ORF4 shows homology with conserved hypothetical protein Atu6109 *Agrobacterium tumefaciens* (strain C58, Dupont) plasmid Ti with 84% identities and 88% positives. Abbreviations are: + conservative substitution amino acid, -; insertion or deletion of amino acid, X; low complexity of amino acid.

```
Query: 782  MSRKSESARIDIYARITERIVADLEKGVRPWVQPWSAGHMSGRITRPLRHNGQPYTGLNV 961
            MSRK+ + R DIYARIT+RIVADLEKGVRPWV+PWSA ++SGR++RPLRHNGQ YTGLNV
Sbjct: 1    MSRKTANTRTDIYARITDRIVADLEKGVRPWVRPWSAANLSGRVSRPLRHNGQAYTGLNV 60

Query: 962  LLLWSESIARGFISATWMTLRQANELGAHVRTGESGATVVYASRFTKTEKDAGGGEVERD 1141
            LLLWSES+A GF+S+TWMTLRQANELGAHVR GESGATVVYASRFTKTE DAGGGEVERD
Sbjct: 61   LLLWSESVASGFMSSTWMTLRQANELGAHVRKGESGATVVYASRFTKTEPDAGGGEVERD 120

Query: 1142 IPFLKAYTVFNCDQIEGLPDHYYRRPEPVAEPLERIEHADRFFANTGAVIRHGGSQAFYQ 1321
            IPFLKAYTVFNCDQI+GL DHYY RPEP+A+PLERIEHADRFF NTGAV+R+GG +A+Y
Sbjct: 121  IPFLKAYTVFNCDQIDGLADHYYSRPEPIAKPLERIEHADRFFDNTGAVVRYGGDKAYYS 180

Query: 1322 PSSDSIQMPGFETFRDAESYYAVLGHEVTHWVGASHRLNRDMSRYHKDRTDRAREELCAD 1501
            P+SD IQ+P  E FRD  S+ A   HE  HW G   RLNRD+SRYHKDR +RA EE+  +
Sbjct: 181  PASDHIQLPRPEQFRDMASFVATRAHETLHWAGGPARLNRDLSRYHKDRRERAFEEMLVE 240

Query: 1502 IGACLPLRRSRNRPWNSELAARSCELRGL-SWLKLLDGDXXXXXXXXXXXXXXXYLHDL 1678
            +GA +        P   EL  R      + SW ++L  D                YLHDL
Sbjct: 241  LGAAMICADLGIVP---ELEPRPDHAAYIQSWAEILGSDKRAIFNAAAHAQRAVAYLHDL 297

Query: 1679 QPKAETEREAA 1711
            QP+  + +EAA
Sbjct: 298  QPQPASGQEAA 308
```

Figure 4.16 Blast search analysis ORF5 shows homology with antirestriction protein of *A. tumefaciens* str. C58 with 64% identities and 75% positives. Abbreviations are: + conservative substitution amino acid, -; insertion or deletion of amino acid, X; low complexity of amino acid.

```
Query: 149 MPINVNNPEADALTRKFAHMAGVSITDAIVIAMKEAIERRRHQ*TPLQTAARLRNEHGVK 328
           M IN+N+P+ADALTR FA MAG+SI +AIV AMKEAI+RRR++  PL+TA RLR +HG+
Sbjct: 9   MAININDPQADALTRTFARMAGLSIREAIVTAMKEAIDRRRNREKPLETARRLREKHGIV 68

Query: 329 LDAAARQPLPREAYDELWEKLM 394
           +  AA +PL REAYDE+W+ L+
Sbjct: 69  IGGAASKPLQREAYDEMWDDLV 90
```

Figure 4.17 Blast search analysis ORF6 shows homologies with hypothetical protein SMb20629 of *S. meliloti* 1021 with 62% identities and 81% positives. Abbreviations are: + conservative substitution amino acid, -; insertion or deletion of amino acid, X; low complexity of amino acid.

```
Query: 612 MKVGIDMGTTSEGTSASLDIEELLATRLLVQGNSG 508
           M V IDMGTT  G  A +++EELLATRLLVQGNSG
Sbjct: 1   MTVSIDMGTTRTGEKALMNLEELLATRLLVQGNSG 35

Query: 496 HLLRRLLEQSAPWVQQCIIDPEGDFVTLADRFGHVVVEGE-RTDAELVGIATRIRQHRVS 320
           HLLRRLLEQSAPWVQQ +IDPEGDFVTLAD FGHVVV+    T+A L  IA ++RQHRVS
Sbjct: 40  HLLRRLLEQSAPWVQQAVIDPEGDFVTLADVFGHVVVDASAHTEAALQQIAGKVRQHRVS 99

Query: 319 CVLSLEGLDIEQQMRSAGVFLNAMFDADRDYWYPVLVVVDEAQMFAPSVGGDVSEEARKI 140
            VL+LE L+ E QMR A  FL  +FD DRD+WYP+LVVVDEAQMFAP+  GDV++EARK+
Sbjct: 100 VVLNLENLETELQMRRAAAFLGGLFDMDRDHWYPLLVVVDEAQMFAPAAAGDVADEARKV 159

Query: 139 SLGAMTNLMCRGRKRGLAGVIATQRLAKLA 50
           SLGAMTNLMCRGRKRGLAGVIATQRLAKLA
Sbjct: 160 SLGAMTNLMCRGRKRGLAGVIATQRLAKLA 189
```

Figure 4.18 Blast search analysis of ORF7 shows homology with predicted ATPase of *Rhodospirillum rubrum* with 76% identities and 85% positives. Abbreviations are: + conservative substitution amino acid, -; insertion or deletion of amino acid, X; low complexity of amino acid.

```
Query: 956  MGGNALADTTVHLRRLQGGVSPPVVPEAFSGDTRLVGRWHNKPFEYDLPALEDHILSATY 1135
           MGG+ALADTTVH+RRLQGGVSPPVVPEAFSGDTRLVGRWHNKPFEYDLPALEDHILSATY
Sbjct: 1    MGGHALADTTVHVRRLQGGVSPPVVPEAFSGDTRLVGRWHNKPFEYDLPALEDHILSATY 60

Query: 1136 AGTGTASVKIGRQTISAPARAGMITLWPRGHKGFWRVDGAVEVSNAFLGRSRLVACSDQV 1315
           AGTGTASVKIGRQTISAPAR+GMI+ WPRGH+GFWRVDGAVEVSNAFLGRSR +ACSDQV
Sbjct: 61   AGTGTASVKIGRQTISAPARSGMISFWPRGHRGFWRVDGAVEVSNAFLGRSRFLACSDQV 120

Query: 1316 GNGREPDLLGRVHFSDPKLFTIMRLINDEVSSGDAISHLFIEQXXXXXXXXXRAHSSTS 1495
           GNGREPDLLGRVHFSDPKLFTIM LINDE+SSGDAISHLFIEQ          RAHS+TS
Sbjct: 121  GNGREPDLLGRVHFSDPKLFTIMTLINDEISSGDAISHLFIEQLLDLACLQLLRAHSATS 180

Query: 1496 VPISPGPRRGLSSWQVKRVTTYIREHLAENIRLQELADLVNLSRFHFCTAFRAATGHTPY 1675
           VPISPGPRRGLS+WQV+RVTTY+RE+LA NIR+QELADLV+LSRFHFCTAFR ATGHTPY
Sbjct: 181  VPISPGPRRGLSNWQVRRVTTYMRENLAANIRIQELADLVSLSRFHFCTAFRMATGHTPY 240

Query: 1676 VWLTRQRIAYAKTLLKDRTLRIIDIALIVGYETQSSFSASFRKVVGLTPSEFRRRL 1843
            WLT QRIA+AKTLLKDR LRIIDIALIVGYETQSSFSASFRKVVGLTPSEFRRRL
Sbjct: 241  GWLTHQRIAHAKTLLKDRALRIIDIALIVGYETQSSFSASFRKVVGLTPSEFRRRL 296
```

Figure 4.19 Blast search analysis of ORF8 shows homology with probable transcriptional regulator syrB (AraC family) of *Rhizobium sp*. NGR234 with 89% identities and 94% positives. Abbreviations are: + conservative substitution amino acid, -; insertion or deletion of amino acid, X; low complexity of amino acid.

```
Query: 1997 MAENPSELHPPGPVISRRKXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXMTVNGRAH 2176
            MAENPS LH  GP ISRR+                                  +TVNGR H
Sbjct: 1    MAENPSALHLSGPRISRREALQATAAAAALAALPPGSAAATNAPAAAAVTLTLTVNGRGH 60

Query: 2177 TLTIDPRQSVLDVLRETLNLTGTKKGCNQGACGACTVLVNGKRVVSCLTLASMHD 2341
            TLT+DPRQSVLDVLRETL+LTGTKKGCNQGACGACTVLVNGKR+VSCLTLASM+D
Sbjct: 61   TLTVDPRQSVLDVLRETLDLTGTKKGCNQGACGACTVLVNGKRIVSCLTLASMYD 115

Query: 2346 ARIETIEGLEKDGALHPLQEAFVEHDGLQCGFCTPGQIMSGLGCIAEGHAGSPEEIQFWM 2525
            ARIETIEG+EKDGALHPLQEAFVEHDGLQCGFCTPGQIMSGLGCIAEGHAGSPEEIQFWM
Sbjct: 117  ARIETIEGVEKDGALHPLQEAFVEHDGLQCGFCTPGQIMSGLGCIAEGHAGSPEEIQFWM 176

Query: 2526 SGNICRCGAYPGIV 2567
            SGNICRCGAYPGIV
Sbjct: 177  SGNICRCGAYPGIV 190
```

Figure 4.20 Blast search analysis of ORF9 shows homology with putative aldehyde or xanthine dehydrogenase, iron-sulfur subunit protein of *S. meliloti* 1021with 75% identities and 85% positives. Abbreviations are: + conservative substitution amino acid, -; insertion or deletion of amino acid, X; low complexity of amino acid.

```
Query: 979 MFPFTLERPRSTEDXXXXXXXXXXXXXXGTTLVDLMREEVERPDSLIDINSLPLGGIRVE 800
           M PF  +R  S  +              GTTLVDLMREEVE P+ ++DIN LPL  IR
Sbjct: 1   MRPFEFQRAASESEAIAAGAAGARYLAGGTTLVDLMREEVETPEKIVDINRLPLNYIRAT 60

Query: 799 GEKSGSSAHWRAWPEVARNPNVQRL 725
            E     A  R   EVA N +V+RL
Sbjct: 61  DEAIVIGALAR-MSEVAANQDVRRL 84

Query: 717 LIAESLIEGASPQLRNMASMGGNLLQRVRCPYFRMLDAGCNKRTPGSGCAAIDGLNAGHA 538
           LI E+L EGASPQLRN+AS+GGNLLQRVRCPYFRMLDA CNKR PGSGC+AIDGLNAGHA
Sbjct: 87  LIPETLTEGASPQLRNVASIGGNLLQRVRCPYFRMLDAPCNKRVPGSGCSAIDGLNAGHA 146

Query: 537 ILGTSDHCVATHPSXXXXXXXXXXATMRVKGPQGERSFPVEELFRLPGDMPHLEHTLLPG 358
           ILGTS+HCVATHPS          A + +KGP+GER+ PVEELFRLP   PHLEHTL PG
Sbjct: 147 ILGTSEHCVATHPSDLAVSLVALGAMLSLKGPRGERTIPVEELFRLPESTPHLEHTLEPG 206

Query: 357 ELIVEIRVPGGPHSRGARYLKVRDRASYEFALVSAAAALSIED 229
           ELIVE+ +P GP++R ARYLKVRDR+SYEFALVSAAAAL +E+
Sbjct: 207 ELIVEVHIPNGPYARKARYLKVRDRSSYEFALVSAAAALHVEN 249

Query: 203 AVGGVGTRPWRLRDCEAALVGXXXXXXXXXXXAQLSRQGARPLRHNQFKVELLPRTVVRA 24
           A GGVGTRPWR+   E ALVG           A +S +  RPL HN FKV+LLP T+VRA
Sbjct: 258 AAGGVGTRPWRMNAVEQALVGKPAARASYEIAAAVSTEETRPLSHNGFKVKLLPATIVRA 317

Query: 23  LALAGEV 3
           L +AG+V
Sbjct: 318 LEMAGDV 324
```

Figure 4.21 Blast search analysis of ORF10 shows homology with putative aldehyde or xanthine dehydrogenase, molybdopterin binding subunit protein of *S. meliloti* 1021 with 74% identities and 84% positives. Abbreviations are: + conservative substitution amino acid, -; insertion or deletion of amino acid, X; low complexity of amino acid.

```
Query: 3583 MNVSSIGKPLTRVDGRAKVTGTARYAADFNQPGQLYAVIVSATVGLGRVTEIASTEVERM 3762
            M+VS IGKP+TRVDGRAKVTGTARYAADFNQPGQLYAVIVSATVGLGRVTE+ASTEVERM
Sbjct: 1    MSVSFIGKPVTRVDGRAKVTGTARYAADFNQPGQLYAVIVSATVGLGRVTEVASTEVERM 60

Query: 3763 PGVVALITHRNAQKLPYLPHKGIIDPAVGERLHVLQDDQVHFYGQPVAIVVADNLDHAER 3942
            PGVVA+ITHRNAQKLPYLPHKG+IDPAVGERLHVLQDD+V FYGQPVAIVVADNLDHAER
Sbjct: 61   PGVVAVITHRNAQKLPYLPHKGVIDPAVGERLHVLQDDRVQFYGQPVAIVVADNLDHAER 120

Query: 3943 AAAALRITYVARRPLVDHADQTIERIAPKSADGSRGDADVAVTQAPVMIDETYEIARENH 4122
            AAAALRITYVA+RP+VDHADQT+ERIAPKSADGSRGDAD AVTQAPVMIDETYEIARENH
Sbjct: 121  AAAALRITYVAKRPIVDHADQTMERIAPKSADGSRGDADAAVTQAPVMIDETYEIARENH 180

Query: 4123 NPMEPHATIAAWSGDRLTLWSKSQYLVNEQTEIAAVFGLPVENVEVFCPFIGGAFGTSLR 4302
            NPMEPHATIAAWSGDRLTLWSKSQYLVNEQ EIAAVFGLPV+NVEV CPFIGGAFGTSLR
Sbjct: 181  NPMEPHATIAAWSGDRLTLWSKSQYLVNEQAEIAAVFGLPVDNVEVICPFIGGAFGTSLR 240

Query: 4303 TWPHVTLAALAARQTGRTV 4359
            TWPHVTLAALAARQTGR V
Sbjct: 241  TWPHVTLAALAARQTGRAV 259
```

Figure 4.22 Blast search analysis of ORF11 of 4.9 kb *Hin*dIII fragment shows homologies with Xanthine dehydrogenase of *Rhizobium sp*. NGR234 with 93% identities and 97% positives. Abbreviations are: + conservative substitution amino acid, -; insertion or deletion of amino acid, X; low complexity of amino acid.

Identification and characterization of gene(s) involved in salt tolerance in pUHR310

Sequencing strategy was used for identification of genes involved salt tolerance from the second region of chromosome. Various *Hin*dIII and *Eco*RI fragments of pUHR310 were subcloned in pUC18 and sequenced from both ends. Sequence analysis of one end of 7.68-kb *Eco*RI fragment revealed high homology to the betaine aldehyde dehydrogenase gene of *S. meliloti* (Figure 4.23, 4.24). Furthermore, the sequence from one end of the 3.82-kb *Hin*dIII fragment exhibited 92% identities with the choline dehydrogenase (*betA*) gene of *S. meliloti* (Figure 4.25). From restriction mapping analysis, the arrangement of these genes appears to be the same as the *bet* operon of *S. meliloti*. The pUHR310 may contain the entire *bet*

operon, however, sequencing of the entire insert in pUHR310 will be necessary to identify all these genes of the *bet* operon, required for glycine betaine biosynthesis. Glycine betaine was characterized as osmoprotectant in *Klebsiella pneumoniae* and other members of the *Enterobacteriaceae* (Le Rudulier and Bouillard, 1983). The glycine betaine (*N*,*N*,*N*-trimethyl glycine; GB) *in vivo* is both an effective osmoprotectant (efficient at increasing cytoplasmic osmolality and growth rate) and a compatible solute (without deleterious effects on biopolymer function, including stability and activity) (Felitsky et al., 2004). It appears that glycine betaine strongly stimulates the growth rate of bacteria in high-salt medium (Le Rudulier and Bernard., 1986). Strains of both Gram-negative and Gram-positive bacteria that are well-adapted to high-salt environments are reported to accumulate glycine betaine (Canovas et al., 2000, Mendum and Smith, 2002, Meury, 1988 Peter et al., 1988, Koo et al., 1991, Vijaranakul et al., 1997, and Kappes., et al 1996). The *bet* operon is well characterized in *S. meliloti* (Osteras et al., 1998). Since the *bet* genes have been shown to be required for salt tolerance in many bacteria, it is very likely that the *bet* genes identified in pUHR310 are involved in salt tolerance in BL3.

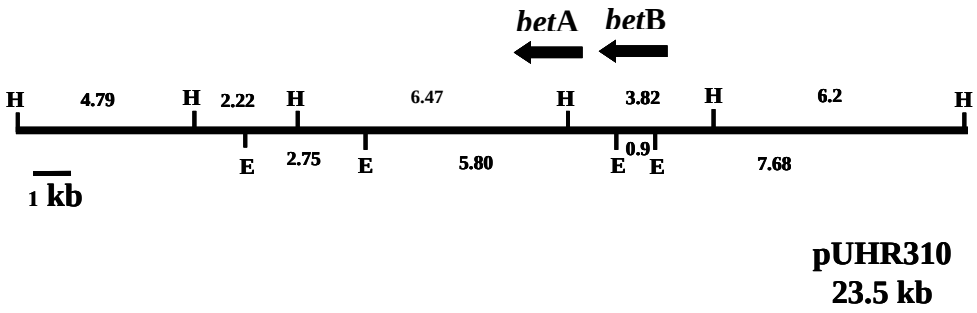

Figure 4.23 Restriction map of pUHR310. The positions and directions of *bet*A and *bet*B genes are indicated with close arrows. The sizes of the *Hin*dIII and *Eco*RI fragments are indicated.

```
1      AGATCCAAACCTTCCCTGGCAAATTTTTGAGGGGCGGTTTTGCCCCGGCAGATTATCATG
61     GAGCCACATCGCCAAAAGGGGCGGCTGCCAGGATTGACGGGCTTTAACATTCCGGACAAA
121    GGCCTGAAATCGGGAGCCGATCCAGCATTGAGACTTCGATCGCGCTGATCTGAGGATTAC
181    TGTGAACGCGCATTTGCGGCCGAATGGAGGATAAGGGGCCGTTTGCCTCGGTCCTGAACA
241    ACAGAGAAGCTGAACGCAAAGCCGAATCCAAATACCGTCCGCGGGGAGAATAAAATGAGA
                                                          M  R
301    GCACAACCCAAAGCGTCGCACTTCATCGATGGCGAATATGTCGAGGACGCGGCCGGCACG
       A  Q  P  K  A  S  H  F  I  D  G  E  Y  V  E  D  A  A  G  T
361    GTGATCGAGAGCATCTATCCGGCGACCGGCGAGGTAATTGCGCGGCTGCATGCCGCGACG
       V  I  E  S  I  Y  P  A  T  G  E  V  I  A  R  L  H  A  A  T
421    CCGGGGATCGTCGAGAAGGCAATCGCAGCCGCGAAGCGGGCACAGCCCGGATGGGCGGCG
       P  G  I  V  E  K  A  I  A  A  A  K  R  A  Q  P  G  W  A  A
481    ATGAGCCCCACGGCGCGTGGCCGCGTCCTGAAGCGAGCCGCCGAGATCATGCGCGAGCGC
       M  S  P  T  A  R  G  R  V  L  K  R  A  A  E  I  M  R  E  R
541    AACCGCGAGCTTTCCGAGCTCGAAACGCTCGACACCGGCAAGCCGATCCAGGAGACGATC
       N  R  E  L  S  E  L  E  T  L  D  T  G  K  P  I  Q  E  T  I
601    GTCGCCGATCCCACGTCCGGCGCGGACAGCTTCGAATTCTTCGGCGGTGTCGCGCCCGCC
       V  A  D  P  T  S  G  A  D  S  F  E  F  F  G  G  V  A  P  A
661    GCCCTCAACGGCGACTATATCCCGCTTGGGCAGGACTTCGCCTATACCAAGCGGGTGCCG
       A  L  N  G  D  Y  I  P  L  G  Q  D  F  A  Y  T  K  R  V  P
721    CTCGGCGTCTGCGTCGGCATCGGCGCCTGGAACTATCCGCAGCAGATCGCCTGCTGGAAG
       L  G  V  C  V  G  I  G  A  W  N  Y  P  Q  Q  I  A  C  W  K
781    GGTGCGCCGGCGCTTGTCGCCGGCAATGCCATGGTTTTCAAGCCGTCGGAGAACACACCG
       G  A  P  A  L  V  A  G  N  A  M  V  F  K  P  S  E  N  T  P
841    CTCGGCGCGCTGAAGATAGCGGAGATCCTGATCGAGGCGGGGCTGCCCAAGGGGCTCTTC
       L  G  A  L  K  I  A  E  I  L  I  E  A  G  L  P  K  G  L  F
901    AATGTCATCCAGGGGGACCGATCGACGGGTCCGCTGCTCGTCAATCACCCGGATGTCGCC
       N  V  I  Q  G  D  R  S  T  G  P  L  L  V  N  H  P  D  V  A
961    AAGGTGTCGCTCACCGGTTCGGTCCCGACCGGCAGACGAGTGGCGGGCGCCGCTGCGGCC
       K  V  S  L  T  G  S  V  P  T  G  R  R  V  A  G  A  A  A  A
1021   GAACTGAAGCACGTGACCATGGAACTCGGCGGCAAATCGCCGCTGATCGTCTTCGACGAT
       E  L  K  H  V  T  M  E  L  G  G  K  S  P  L  I  V  F  D  D
1081   GCCGACCTCGAAAGCGCGATCGGCGGCGCCATGCTCGGCAACTTCTACTCGACCGGGCAG
       A  D  L  E  S  A  I  G  G  A  M  L  G  N  F  Y  S  T  G  Q
1141   GTCTGCTCGAACGGAACCCGGGTGTTCGTGCAGCGCGAGATCAAGGACGCCTTCCTCTCG
       V  C  S  N  G  T  R  V  F  V  Q  R  E  I  K  D  A  F  L  S
1201   CGGCTGAAGGAACGCACCGAGGCGATCGTCATCGGCGACCCGATGGACGAGGCGACGCAG
       R  L  K  E  R  T  E  A  I  V  I  G  D  P  M  D  E  A  T  Q
```

Figure 4.24 Nuclotide sequence of the betaine aldehyde dehydrogenase BADH oxidoreductase NAD protein from *Sinorhizobium* BL3. Amino acid deduced from the nucleotide sequences are specified by standard one-letter abbreviations. Putative Shine-Dalgano sequences are underlined.

```
1261  CTCGGGCCGATGGTCTCCAGGGCCCAGCGCGACAAGGTCTTCTCCTATATCGAAAAGGGC
      L  G  P  M  V  S  R  A  Q  R  D  K  V  F  S  Y  I  E  K  G
1321  AAGGCGGAAGGCGCGCGGCTGCTGACCGGCGGCGGCATTCCGAACCATGTGAGCGGCGAA
      K  A  E  G  A  R  L  L  T  G  G  G  I  P  N  H  V  S  G  E
1381  GGCACCTATATCCAGCCGACGGTCTTTGCCGACGTCACCGACGGGATGACGCACGCGCGC
      G  T  Y  I  Q  P  T  V  F  A  D  V  T  D  G  M  T  H  A  R
1441  GAGGAAATCTTCGGCCCGGTCATGTGCGTGGTCGATTTCGACGACGAGGTGGAAGTCATC
      E  E  I  F  G  P  V  M  C  V  V  D  F  D  D  E  V  E  V  I
1501  GCACGCGCCAACGCCACCGAATTCGGCCTTTCGGCCGGCGTCTTCACCGCCGACCTCACG
      A  R  A  N  A  T  E  F  G  L  S  A  G  V  F  T  A  D  L  T
1561  CGTGCCCACCGCGTCGTCGACCGGCTCGAGGCCGGCACGCTCTGGATCAACACGTATAAT
      R  A  H  R  V  V  D  R  L  E  A  G  T  L  W  I  N  T  Y  N
1621  CTCTGCCCGGTCGAGATCCCCTTCGGCGGATCGAAGCAATCCGGCTTCGGCCGGGAGAAC
      L  C  P  V  E  I  P  F  G  G  S  K  Q  S  G  F  G  R  E  N
1681  TCGGTCGCGGCGCTCGACCACTATACCGAGCTCAAGACCGTCTATGTCGGCATGGGCCCG
      S  V  A  A  L  D  H  Y  T  E  L  K  T  V  Y  V  G  M  G  P
1741  GTCGAGGCGCCGTATTGATGAGTTGATTTGCCCCTCACCTAACCTCTTTCCGCTCGCGGG
      V  E  A  P  Y  *
1801  GCGAGGGGACTGAACGGCGCCGCGAGTCCTTCTCCCCGCCCGCAAATGCAGCGCAGCTGC
1861  TGCGAGACGGTCAGCGGATGAGGCACTCGAAGACAGAAGACACACTATGCAGCAGATTCG
1921  TCATCGTCGTCGGTCGCAGCTCGCGCTCGCTATCGCTGTCGAGACGCAGCATTCGTCATC
1981  GTGCTGATCGCGCACGATATCGCCTCATCAATGCGCAGCGCTTGCTGCGATGAGCATGAC
2041  GCTACATGGCTATC
```

Figure 4.24 Continued.

```
Query: 4   GHAEDLNRK*ELGAQGWAYADVLPYFKRMETSHGGEEDWRGTDGPLHVQRGPVKNPLFHA 183
           GH+ED NR  ELGAQGWAYADVLPY+KRME SHGGEE WRGTDGPLHVQRGPVKNPLFHA
Sbjct: 97  GHSEDFNRWEELGAQGWAYADVLPYYKRMEHSHGGEEGWRGTDGPLHVQRGPVKNPLFHA 156

Query: 184 FIEAGKQAGFELTKDYNGSKQEGFGLMEQTTWKGRRWSAASAYLKPALKRPNVELVRCFA 363
           FIEAGK+AGFE+T+DYNGSKQEGFGLMEQTTW+GRRWSAASAYL+PALKRPNVEL+RCFA
Sbjct: 157 FIEAGKEAGFEVTEDYNGSKQEGFGLMEQTTWRGRRWSAASAYLRPALKRPNVELIRCFA 216

Query: 364 RKVVIENGRATGVEIERGGRIEVVKANHEVIVSASSFNSPKLLMLSGIGPADHLKEMGIE 543
           RK+VIENGRATGVEIERGGRIEVVKAN EVIVSASSFNSPKLLMLSGIGPA HLKEMGI+
Sbjct: 217 RKIVIENGRATGVEIERGGRIEVVKANREVIVSASSFNSPKLLMLSGIGPAAHLKEMGID 276
```

Figure 4.25 Blast search analysis of translated nucleic acid of 3.6 kb *Eco*RI fragment of pUHR310 reveal homology to Choline dehydrogenase (CHD) oxidoreductase flavoprotein fad membrane of *S. meliloti* with 90% identities and 96% positives. Abbreviations are: + conservative substitution amino acid.

The transconjugants of THA6 containing pUHR307 and pUHR310 have higher salt tolerance than THA6

Two regions of *Sinorhizobium sp.* BL3 chromosome, cloned in pUHR307 and pUHR310, were introduced to *B. japonicum* THA6 by conjugation. The resulting transconjugants, RUH161 (THA6 containing pUHR307) and RUH162 (THA6 containing pUHR310), were tested for ability to grow under salt stress conditions in minimum medium. The transconjugants showed higher salt-tolerance ability compared to THA6 under both 50 mM NaCl and 100 mM NaCl conditions (Figure 4.26). This suggests that genes present in pUHR307 or pUHR310 provide some degree of salt tolerance in *Bradyrhizobium*, although the level of salt tolerance in these transconjugants is not as high as in BL3. For high level of salt tolerance both clusters of genes cloned in pUHR307 and pUHR310 may have to be simultaneously expressed in THA6. However, it is not possible to transfer both pUHR307 and pUHR310 into THA6, because they contain the same origin of replication.

The evidence of recombinant organism exhibiting salt tolerance has been reported in yeast and several plants. The co-expression of Na^+/H^+ antiporter and H^+-ATPase genes of the salt tolerant yeast *Zygosaccharomyces rouxii* in *Saccharomyces cerevisiae* resulted in increased salt tolerance (Watanabe et al., 2005). In *Arabidopsis*, the introduction of Na^+/H^+-antiporters derived from the plasma or vacuolar membranes showed enhanced tolerance to salt stress (Ohta et al., 2002 and Gao et al., 2003). Moreover, glycine betaine overproducing transgenic rice plants accumulated fewer Na^+ ions, and maintained K^+ uptake (Kishitani et al., 2000). The betaine aldehyde dehydrogenase genes have been used to transgenetically modify tobacco (Trossat et al., 1997), and spinach (Weretilnyk and Hanson, 1990).

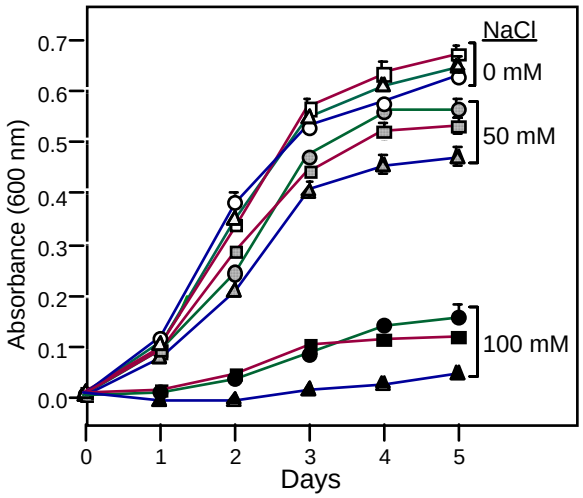

Figure 4.26 Growth of *B. japonicum* THA6 (triangle) and its transconjugant derivatives THA6:pUHR307 (circle) and THA6:pUHR310 (square) in minimal medium containing 0 mM (open symbols), 50 mM (shaded symbols) and 100 mM (close symbols) NaCl. The error bars represent the standard deviation.

Betaine aldehyde dehydrogenase activity of THA6 transconjugants containing pUHR310

The transcojugants of THA6 containing the *bet* gene cluster of BL3 in cosmid pUHR310 were expected to show higher levels of betaine aldehyde dehydrogenase activity. The expression of the *bet* genes was verified by measuring the betaine aldehyde dehydrogenase activity under normal growth condition in media containing 100 mM choline, and 100 mM choline plus 50 mM NaCl. Results revealed that under normal condition in the absence of choline or NaCl, the transconjugants exhibited enzyme activity comparable to THA6. However, when grown in the presence of choline, the transconjugants exhibited 1.35-fold higher enzyme activity than THA6.

In the presence of 100 mM choline plus 50 mM NaCl, the enzyme activity was 1.43-fold higher than THA6 (Table 4.7). These results show that the *bet* genes in pUHR310 are expressed in THA6 transconjugants, resulting in an increase in salt tolerance.

Table 4.7 Betaine aldehyde dehydrogenase activity of THA6 and RUH162:pUHR310.

	Specific activity (U/mg protein) *	
Medium	**THA6**	**RUH162:pUHR310**
Minimal medium (MM)	3.68 ± 0.18 (1x)	3.80 ± 0.27 (1x)
MM+ 100 mM choline	3.75 ± 0.32 (1x)	4.99 ± 0.18 (1.35x)
MM+ 100 mM choline + 50 mM NaCl	3.55 ± 0.57 (0.96x)	5.30 ± 0.10 (1.43x)

* Means ± SD of 3 replications
Fold of enzyme activity was given in the parenthesis

Nodulation competitiveness of transconjugants of THA6 containing pUHR307 or pUHR310

RUH161 (THA6 transconjugant containing pUHR307) and RUH162 (THA6 transconjugant containing pUHR310) showed improved salt tolerance abilities than THA6. Therefore, these transconjugants may also have enhanced nodulation competition ability under salt condition. USDA110 was selected as the competing strain for nodulation competition assay under salt stress condition, because it was shown in the first chapter that THA6 and USDA110 have similar competition abilities on soybean under normal condition. Soybean seedlings grown in Leonard jars were each inoculated with 10^8 cells of 1:1 mixtures of RUH161 and USDA110, and

RUH162 and USDA110. Number and dry weight of nodules, and plant dry weight were measured four weeks after inoculation. Plants inoculated with any single strain exhibited similar nodule and plant dry weights (Table 4.8). Mean nitrogen-fixing efficiency of the recombinant strains RUH161 and RUH162 were not enhanced over THA6 and USDA110 at either normal or salt stress condition. These results suggest that a minor enhancement in salt tolerance does not lead to increase capacity for nodulation under salt stress. These results correlated to a report of Zahan (1999) that the differences in symbiotic efficiency could be due to the genotypic variations of *Rhizobium* but not related to salt tolerance performance. In contrary, Chien and colleagues (1992) reported that highly salt tolerant strains were symbiotically more efficient than salt sensitive ones under salt stress.

One interesting observation was that plants supplied with 0.05% KNO_3 showed a significantly enhanced growth under all salt conditions. Besides, soybean inoculated with *Bradyrhizobium* gave plant yield lower than those supplemented with nitrogen under all salt conditions (Table 4.8). These results implied that when plants are supplied with enough nitrogen, the high salinity stress conditions may not produce detectable adverse effects on growth.

This study hypothesized that increasing survival of rhizobial strains under stress condition would help them to compete with salt-sensitive strains. Results revealed that in paired inoculation, both RUH161 and RUH162 exhibited nodule formation similar to those of USDA110. Interestingly, double occupancy of nodules increased with increasing salt concentrations in all paired inoculations (Table 4.9). The nodule number, nodule dry weight, and plant dry weight decreased with increase in salt concentrations. The dry mass was reduced over 30% with increase in salt

concentration to 25 mM and over 60% at 50 mM NaCl. In this study, the enhanced salt tolerance of RUH161 and RUH162 does not correlate with improvements in nodulation competition under stress conditions. In several reports, the alteration of intrinsic phenotypic characteristics of *Rhizobium* enhanced competitive ability. An example of genetically modified *S. meliloti* with the introduction of multicopy plasmids containing *putA* increased nodulation competitiveness on alfalfa grown in nonsterile soils and subjected to drought stress (Dillewijn et al., 2001). Robleto et al. (1998) demonstrated that a trifolitoxin-producing strains showed increased nodulation competitiveness in the field. The competitiveness of these strains increased because trifolitoxin production conferred a competitive advantage over the rhizosphere microorganisms. In the present study, although the salt tolerance of THA6 transconjugants was enhanced, the competitiveness did not improve. It might be due to each of these two clusters of genes confers only a low level of tolerance to the THA6 transconjugants. Therefore, the ability to compete the nodulation might not be fully expressed. For high level of salt tolerance, both clusters of genes may have to be transferred to salt-sensitive strains, which might lead to enhance nodulation competitiveness.

Table 4.8 Nodule and shoot dry mass of soybean SJ5 growth at 0, 25, and 50 mM.

Sample	NaCl (mM)	AVG.nodule/plant	Average of nodule Dry weight (mg)	Average of plant Dry weight (mg.)
Control	0	0 d	0 e	257.80 ± 27.54 g
	25	0 d	0 e	158.26 ± 21.26 h
	50	0 d	0 e	138.76 ± 12.67 h
Control:	0	0 d	0 e	706.02 ± 53.31 a
KNO_3 0.05%	25	0 d	0 e	377.60 ± 11.87 de
added	50	0 d	0 e	295.94 ± 31.23 fg
USDA110:THA6(wt)	0	18.1 ± 0.8 a	48.87 ± 1.52 ab	489.64 ± 10.69 b
	25	12.8 ± 1.1 b	18.84 ± 2.54 d	289.24 ± 27.22 fg
	50	2.2 ± 0.7 d	0.64 ± 0.22 e	162.86 ± 6.60 h
USDA110	0	17.0 ± 1.0 a	39.34 ± 3.97 c	401.16 ± 23.44 cd
	25	7.2 ± 0.7 c	13.57 ± 4.14 d	270.26 ± 16.55 fg
	50	2.2 ± 1.5 d	0.86 ± 1.11 e	161.97 ± 3.67 h
RUH161	0	18.0 ± 1.0 a	41.11 ± 10.41 c	405.42 ± 75.98 cd
	25	9.0 ± 0.7 c	15.61 ± 5.18 d	275.26 ± 9.88 fg
	50	1.4 ± 0.5 d	0.58 ± 0.37 e	169.22 ± 28.82 h
RUH162	0	17.0 ± 3.7 a	42.46 ± 13.34 bc	416.12 ± 70.75 cd
	25	9.3 ± 1.7 c	16.03 ± 2.73 d	298.10 ± 48.87 fg
	50	1.0 ± 0.0 d	1.17 ± 0.36 e	172.97 ± 3.84 h
USDA110:RUH162	0	17.6 ± 1.6 a	49.00 ± 3.71 ab	426.97 ± 15.77 cd
	25	11.6 ± 2.3 b	20.52 ± 5.10 d	330.00 ± 49.06 ef
	50	2.2 ± 1.1 d	0.93 ± 1.13 e	174.84 ± 8.65 h
USDA110:RUH161	0	19.2 ± 1.6 a	50.58 ± 2.41 a	453.51 ± 29.02 cb
	25	11.6 ± 1.4 b	18.81 ± 6.22 d	290.18 ± 34.13 fg
	50	1.3 ± 0.3 d	0.64 ± 0.27 e	181.26 ± 6.52 h

Means with different letters are significantly different at $p<0.05$.

Table 4.9 Average percentage of nodule occupancy by *B. japonicum* strains on soybean SJ 5 cultivar at 0, 25, and 50 mM NaCl.

Co-inoculated strains		**NaCl (mM)**	**Nodules (%) formed by :**					
A	B		A		B		Double occupancy	
USDA110	RUH161	0	14.86±3.25	a	13.27±6.50	a	71.88±8.46	bc
		25	13.83±5.86	a	15.09±9.14	a	71.08±12.91	bc
		50	0.00±0.00	b	0.00±0.00	b	100.00±0.00	a
USDA110	RUH162	0	20.0±4.31	a	18.98±2.54	a	60.95±6.44	c
		25	15.20±4.6	a	17.48±2.16	a	67.31±2.69	c
		50	3.70±6.42	b	7.84±6.85	ab	88.43±11.14	ab
USDA110	THA6	0	14.66±2.74	a	16.57±4.9	a	68.76±7.05	c
		25	15.59±1.49	a	16.86±6.44	a	67.54±5.87	c
		50	0.00±0.00	b	0.00±0.00	b	100±0.00	a

Means with different letters are significantly different at $p<0.05$.

CONCLUSION

Salt stress condition is one of the abiotic factors affecting rhizobial inoculant survival, consequently affecting nodulation competitiveness. In an attempt to understand salt tolerance mechanism, salt tolerant rhizobial strains were isolated from salt-affected areas at Nakhon Ratchasima, north-eastern region of Thailand. Three hundred and seventy three isolates were screened to identify the most salt tolerant strain. At 100 mM NaCl concentration, all slow-growing strains could not survive, while 50 fast-growing strains grew. Eight isolates were identified on the basis of growth at 300 mM NaCl. The outstanding isolate, BL3, exhibited the best growth over other strains at 400 mM NaCl and tolerated up to 600 mM NaCl. BL3 was selected for isolation of genes for salt tolerance. The characterization of this isolate by

full-length 16S rRNA gene sequencing demonstrated that it belonged to the genus *Sinorhizobium*. BL3 forms effective nodules on *P. lathyroides* under salt stress conditions. The genes for salt tolerance from BL3 were isolated by constructing a cosmid clone library of this strain, then transferring these clones to the salt sensitive *Rhizobium* strain TAL1145, and selecting transconjugants that grew under salt stress conditions. Two hundred such salt tolerant transconjugants were selected. From twenty-four of these transconjugants, clones containing insert DNA from two different regions of BL3 genome were isolated. The first region was represented by five overlapping cosmids, having similar restriction patterns. Restriction analysis of these cosmid clones using *Hin*dIII showed three common fragments of sizes 8.8, 4.9 and 3.2 kb. Sequence analyses of these fragments revealed eleven ORFs, which showed high homologies with bacterial genes with known functions, including ATPase, transcriptional activator proteins of AraC family and xanthine dehydrogenase. The second region of BL3 chromosome containing genes for salt tolerance was represented by insert DNA in cosmid pUHR310. Sequence analysis revealed that pUHR310 contained genes for biosynthesis of glycine betaine, which is known to be an osmoprotectant in *S. meliloti* and other bacteria. Cosmids pUHR307 and pUHR310 were transferred to *B. japonicum* THA6 by conjugation to develop salt-tolerant derivatives. The resulting transconjugants containing either pUHR307 or pUHR310 showed enhanced salt tolerance and grew in medium containing 100 mM NaCl. However, nodulation competitiveness of these transconjugants was not improved. Therefore, improvement in salt tolerance ability of THA6 did not enhance its nodulation competitiveness under stress condition.

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CHAPTER V

PROTEOMIC ANALYSIS OF *Sinorhizobium sp.* STRAIN BL3 MEMBRANE PROTEINS AND QUANTITATIVE PROTEOMIC ANALYSIS OF SALT STRESS RESPONSE

ABSTRACT

The symbiotic relationship between the plant root and the microbe is critically dependent on the environmental conditions, including salt concentration. The protein content of a membrane enriched fraction obtained from *Sinorhizobium sp.* BL3 was investigated for identifing membrane proteins involved in salt stress response. Using an optimized membrane protein extraction protocol and nanoflow liquid chromatography interfaced to electrospray ionization tandem mass spectrometry (LC-MS/MS) 751 proteins were initially identified of which 412 proteins corresponded to membrane proteins which contained at least one transmembrane domain or localized in the membrane region. The membrane proteome could be categorized into 5 different functional groups where three major groups are cell process (30%), small molecule metabolism (27%) and hypothetical/global homology (26%). Over a quarter of the identified proteins have been previously reported as being salt tolerant proteins. Quantitative proteomic analysis was performed using an amine reactive stable isotope

labeled reagent allowing the study of changes in protein abundance as a function of salt stress. Several membrane proteins exhibited up- or down regulation by more than 1.5 fold with the level depend upon salt concentration and exposure time. Proteins involved in energy metabolism, DNA repair and synthesis and transportation proteins involved in compatible solute and ion transport across membranes were up-regulated in either immediate or late response. This study demonstrates that a mass spectrometry based proteomic approach is useful in the systematic study of membrane proteins and their abundance in microbes under various stress conditions.

INTRODUCTION

Salinity is one of the environmental factors that adversely affect Rhizobium-legume symbiosis at all stages of Rhizobium-legume interaction. Salt stress causes a reduction in the numbers of rhizobia attached to root hairs, a decrease in nodule number, and a decrease in the proportion of the nodules that are initiated in saline conditions that are able to differentiate fully into active N_2-fixing nodules (Tu, 1981). The mechanisms involving in-cell adaptation or survival under salt stress condition have been intensively studied. Under salt stress environments, outer membrane proteins (OMPs) are key molecules that interface the cell with the surrounding environment. Moreover, membrane proteins play an important role in maintaining normal cell volume and intracellular ion balance involving transport of inorganic and organic molecules (Martin et al., 1999; Wiggins, 1990; Botsford and Lewis, 1990; Boncompagni et al., 1999; Smith et al., 1994; 1989; Breedveld et al., 1991). Additionally, it is wide distribution of proteins at the membrane which is important in

biological functions involving the transportation of nutrients to and from the cell (Klebba, 1998), conjugation (Koebnik, 1999), controlling cell morphology, intercellular communication and cell metabolism. Rhizobial membrane proteins that are involved in salt stress include: the *bet*S gene, encoding a glycine betaine/proline betaine transporter (Boscari et al., 2002), the *kup* gene, encoding a potassium uptake system protein (Nogales et al., 2002), and the *omp*10, encoding outer membrane lipoprotein (Wei et al., 2004). Hence, large scale profiling expression change of membrane proteome is of great interest. The first insights on a global scale study of gene expression under salt stress of *Sinorhizobium meliloti* has been reported by Ruberg et al (2003), using whole genome transcriptomic approach which provided a comprehensive data of a global change in mRNA level. The interesting result exhibited 15 genes involved in ion uptake were reduced in expression whereas 14 genes involved in transport of small molecules were induced. Therefore, it is interesting to examine the correlation between mRNA and protein expression level. Especially, membrane proteomic analysis could provide the actual evidence of membrane global networks response to such stress.

Membrane proteome analysis is a great challenge to achieved complete proteome, due to it has extreme physicochemical properties that obstacle the successful analyses. Initial membrane proteomic studies were employed by applying two-dimensional (2-D) gel electrophoresis followed by spot identification with MALDI/TOF-TOF. The analysis using such methodology has been reported the identification of 37 membrane proteins from *Escherichia coli* (Molloy et al., 2000), 21 OMPs from *E. coli*, 23 OMPs of *Salmonella typhimurium* and 14 OMPs of *Krebsella pneumoniae* (Molloy et al., 2001) and 15 membrane proteins from green-sulfur

bacterium *Chlolobium tepidum* (Aivaliotis et al., 2004). Attempt to improve membrane protein detection was further performed by 1-D SDS-PAGE combination with LC-MS/MS. One hundred and fourteen integral membrane proteins could be observed from *Halobacterium salinarum* (Klein, 2005) and 79 membrane proteins detected from *Mycobacterium tuberculosis* (Gu et al., 2003). Another approach which has removed the requirement for separation using PAGE is the so called 'shot gun' methodology which couples ion-exchange and reverse phase chromatography (Washburn et al., 2000). The emerging gel-free proteomic approaches have provided powerful tools for the analysis of complex mixtures and allowed previously difficult analytes of membrane proteins to become accessible for mass spectrometric proteomic analysis (Link et al., 1999; Washburn and Yetes, 2000; Washburn et al., 2001; Blonder et al., 2004; Prinz et al., 2004). Three hundred and thirty three membrane proteins achieved from *Pseudomonas aeruginosa* using online-LC-tandem massspectrometry analysis (Blonder et al., 2004). Moreover, the introducing of multidimentional chromatography allows an effective separation of complex mixtures (Fujii et al., 2004, Washburn et al., 2001). 1484 proteins were identified from *Saccharomyces cerevisiae* proteome whereas 131 proteins contained three or more transmembrane proteins (Washburn et al., 2001).

In this investigation, the first proteomic study for identifying and quantitating membrane proteins involved in salt stress of *Sinorhizobium sp.* BL3 have provided insights and further understanding of the functional protein distribution and the roles of membrane proteins in the cell. The effort to achieved large scale membrane proteome, a combination of high resolution separation using off-line strong cationic column (SCX) with nanoflow liquid chromatography/tandem mass spectrometric

have been applied. A total 751 proteins could be identified whereas over half of proteins were identified as membrane proteins. The successful membrane proteins identification provided the further examination of expression shift under the salt stress which mass tag was used to derivatise for monitoring. The quantitative validation was performed by VEMS v.3 program.

MATERIALS AND METHODS

Chemicals and reagents

HPLC-grade acetonitrile (ACN), formic acid, and trifluoroacetic acid (TFA) were purchased from Fisher Scientific. Water was obtained from a milli-Q Plus purification system (Millipore, Bedford, MA). α-Cyano-4-hydroxycinnamic acid (α-CHCA) were purchased from Sigma-Aldrich.

Sinorhizobium sp. BL3 culturing

Inoculum of *Sinorhizobium sp*. BL3 were grown aerobically at 28°C in yeast extract mannitol (YEM) medium (Vincent, 1970) containing ($g.l^{-1}$): $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.5; Manitol, 10; Yeast extract, 0.5; NaCl, 0.1. The pH was maintained between 6.5-6.8.

For quantitative experiments, *Sinorhizobium sp.* BL3 strain was grown in modified minimal salts medium (Howieson, 1985), which contained and ($mg.l^{-1}$) Na_2SO_4, 100; $MgSO_4.7H_2O$, 200; $CaCl_2.2H_2O$, 5; $MnSO_4.4H_2O$, 1.11; K_2HPO_4, 4.35; KH_2PO_4, 3.4; $ZnSO_4.7H_2O$, 1; $CuSO_45H_2O$, 0.5; $FeSO_47H_2O$, 5; NaEDTA, 1; thiamine HCl, 1; pantothenic acid, 1; biotin, $2x10^{-3}$. For carbon and nitrogen source

were added ($g.l^{-1}$) sodium glutamate, 2.5; sodium succinate, 2.0; and KNO_3, 0.05. Media were supplemented with antibiotics at final concentration ($\mu g.ml^{-1}$); streptomycin 100.

Preparation of membrane fractions

Sinorhizobium sp. BL3 cells from 1 liter of cell culture in minimal growth medium were used for each independent experiment (with or without salt). For salt stress condition, cell cultures were grown to reach OD_{600} of 0.6, then sodium chloride solution was added to final concentration of 0.4 and 0.5 M in the media. Cells were then further cultured for 1 h and 6 h. For control condition, cell cultures were grown at the same condition except sodium chloride solutions was not added. Cells were collected by centrifugation at 7000 g and washed twice with 10 mM Tris-Cl, pH 7.5. The cell pellet was resuspended in 10 mL of 10 mM Tris-Cl, pH 7.5 containing 1 mM phenylmethylsulphonyl fluoride (PMSF). A cell volume of 10 mL were disrupted by passing through an Aminco French press at 1,200 psi twice. The lysate was centrifuged twice at 7000 g for 10 min. to remove unbroken cells. Membrane protein extraction was performed according to Molloy et al. (2000). Briefly, the supernatant was diluted with ice cold 0.1 M sodium carbonate (pH 11) to a final volume of 60 mL and stirred slowly on ice for 1 h. The carbonate treated membranes were collected by ultracentrifugation in a Sorval 55.2 Ti rotor at an average of 100,000 g for 1.5 h at 4°C. The membrane pellet was washed twice by sonication in 2 ml of 10 mM Tris-Cl, pH 7.5, containing 1 mM PMSF and ultracentrifugation (14 000 g for 30 min at 4^oC in a Eppendrof table centrifuge). The membrane pellet was then solubilized in a 9 M

urea solution. Protein concentration was measured by the Bradford method (Bradford, 1976) where bovine serum albumin was used for standard calibration.

In-solution digestion of membrane proteins

Ten μg of soluble membrane protein was resuspended in 21 μl 400 mM NH_4HCO_3 (pH 7.8) in 8 M urea. DTT 45 mM was added to the final concentration of mixture, then incubated for 15 min at 56^{o}C. Subsequently the solution was chilled and 5 μL of iodacetamide (100 mM) was added, followed by incubation in the dark at room temperature for 15 min. To the resulting mixture, Lys C protease (Calbiochem, San Diego, CA) was added (15 ng) and further incubated at 37^{o}C for 6 hours. Followed by the addition of 140 μl of H_20 and 20 pmole of sequence grade trypsin (Promega, Madison, WI) in 5 μl and incubation for overnight at 37^{o}C.

Derivatisation

Forty μl of a solution containing (d_0) 2-methyoxy-4, 5-dihydro-1H-imidazole (1M) either (d_4) 2-methyoxy-4,5-dideutero-1H-imidazole was added to 2 μg of digested protein that had been dried in vacuum. The reaction was allowed to proceed for 3 hours at 55^{o}C. An equal volume of 5% formic acid was added to quench the reaction followed by the reaction mixture being de-salted using micro-columns as described by Rappsillber et al. (2003).

Peptides separation by strong cation exchange chromatography (SCX)

SCX was performed using a micro column packed with Self Pack POROS 20 (Applied Biosystems, Framingham, MA) as described in Gobom et al (1999). SCX

columns were conditioned by washing with 4 x 20 µl of 30% acetonitrile, ammonium formate (1M, pH3.0). The tryptic peptides were dissolved in 30% acetonitrile, ammonium formate (1M, pH3.0) and loaded onto the SCX-packed column. The flow-through was collected. A step-wise elution was performed using 20 µl of 30% acetonitrile in ammonium formate (20, 30, 40, 50, 60 and 120 mM, pH3.0). All fractions were dried in vacuum and resuspended in 5% formic acid to allow analysis by nanoLC-MS/MS.

Peptide mass fingerprinting (PMF)

Samples were desalted and concentrated with a self packed C18-columns as described by Gobom et al. (1999). Peptides were eluted in a volume of 0.5 µl using a concentrated solution of α-CHCA in 70% acetonitrile and 0.1% trifluoroacetic acid in water and deposited directly onto the MALDI target plate. Trypsin digested BSA peptides was used for closing external calibration. PMF analysis was performed on a Bruker ultraflex MALDI-TOF-TOF mass spectrometer (Bruker Daltonik GmbH). The MALDI-generated ions were extracted and then accelerated to 25 kV. The TOF was operated in the reflectron mode.

Peptides separation by nanoflow liquid chromatography

Automated nanoflow liquid chromatography/tandem mass spectrometric analysis was performed using a QTOF Ultima mass spectrometer (Micromass UK Ltd., Manchester, UK) employing automated data dependent acquisition (DDA). A nanoflow-HPLC system (Ultimate; Switchos2; Famos; LC Packings, Amstersdam, The Nederlands) was used to deliver a flow rate of 2 $\mu l.min^{-1}$ (loading) and 100

nl.min^{-1} (elution). Loading was accomplished by using a low rate of 2 µl.min^{-1} onto a homemade 2 cm fused silica precolumn (75 µm i.d.; 375 µm o.d.; Resprosil C18-AQ, 3 µm (Ammerbuch-Entringen, DE) using autosampler essentially as described by Licklider et al. (2002). Sequential elution of peptides was accomplished using a linear gradient from Solution A (0.6% acetic acid) to 40% of solution B (80% acetonitrile 0.5% acetic acid) in 90 minutes over the precolumn in-line with a homemade 10 cm resolving column (50 µm i.d.; 375 µm o.d.; Resprosil C18-AQ, 3 µm (Ammerbuch-Entringen, DE). The resolving column was connected using a fused silica transfer line (20 µm i.d.) to a distally coated fused silica emitter (New Objective, Cambridge, MA, USA) (360 µm o.d. / 20 µm i.d. / 10 µm tip i.d.) biased to 1.8 kV.

The mass spectrometer was operated in the positive ion mode with a resolution of 9,000-11,000 full-width half-maximum using a source temperature of 80 °C and a counter current nitrogen flow rate of 150 l/h. Data dependent analysis was employed (five most abundant ions in each cycle were subjected to MS/MS): 1 second MS (m/z 350-1500) and 5 x 1 second MS/MS (m/z 50-2000, continuum mode), 30 seconds dynamic exclusion. A charge state recognition algorithm was employed to determine optimal collision energy for low energy CID MS/MS of peptide ions. External mass calibration using NaI resulted in mass errors of less than 50 ppm, typically 5-15 ppm in the m/z range 50-2000. Raw data was processed using ProteinLynx Global Server ProteinLynx (smooth 3/2 Savitzky Golay and center 4 channels/80% centroid) and the resulting MS/MS data set exported in the Micromass pkl format.

Computational analysis

The raw data was processed using the fast de-convolution algorithm in Protein Lynx Global Server v 2.0.5 (Waters/Micromass UK Ltd., Manchester, UK). The resulting pkl files were imported into Mascot and VEMS v 3.0 for database dependent searching of the *S. meliloti* proteome. A small program which is part of the VEMS v3.0 package was used to automatically extract the retention times of each MS/MS spectrum from the raw data. The pkl files, the extracted retention times, and the raw data were imported into VEMS for the quantitative analysis. All low confident identifications and quantifications were manually validated using the visual tools in the VEMS program. The quantitative score represented a degree of expression changes which calculated by dividing the intensity of the heavy with the total intensity of heavy and light and multiplying by 100. This means that a quantitation value of 50% corresponds to a ratio of 1:1. A ratio of light:heavy of 1:2 and 2:1 corresponds to 66% and 33%, respectively. Functional categorization, prediected transmembrane domains (TMDs) and localization of membrane proteins were performed according to Riley rules (Karp et al., 1999) at *Sinorhizobium* genome project (www, n.d) and using PSORT program to predict TMDs and localization.

RESULTS AND DISCUSSION

Membrane protein identification of *Sinorhizobium sp.* BL3

The membrane sub-proteome, which mainly contains hydrophobic proteins, is one of the most difficult analyte species to characterise. Initially, identification of membrane proteins was performed by applying a 2D-PAGE/MALDI/TOF-TOF

strategy (figure 5.1). However the total identified proteins observed was less than 100. Possible explanations for the poor return include (i) loss of membrane proteins during IEFgel separation, (ii) aggregation of high molecular weight membrane proteins at the top of gel which therefore, could not be separated and characterized, (iii) low abundance protein could not be detected/identified by MALDI-TOF/TOF. These obstacle evidences was also documented that protein losses with the use of immobilized pH gradients (IPGs) due to hydrophobic interactions between certain protein domains and the polymers used to form the pH gradient (Adessi et al., 1997), the reproducibility and load capacity offered by IPGs (Satoni et al., 2000 and Molloy, 2000), there are difficulties in separating proteins with extreme physicochemical properties and of course the dynamic range issue (Jenkins and Pennington, 2001). Therefore, the analytical strategy has been changed to the alternative gel free analysis using multidimensional ηLC-MS/MS. Alongside the well documented adavantages of using 2D-LC, there was an additional advantage that derivatisation of peptides is more efficient than that of protein level and thus introducing quantification by derivatising with isotopic labels is made easier. The peptide separation efficiency of the off-line SCX self packed column was tested using MALDI MS before combining with ηLC/ESI MS/MS (Figure 5.2). The figure clearly shows that the mass fingerprints for

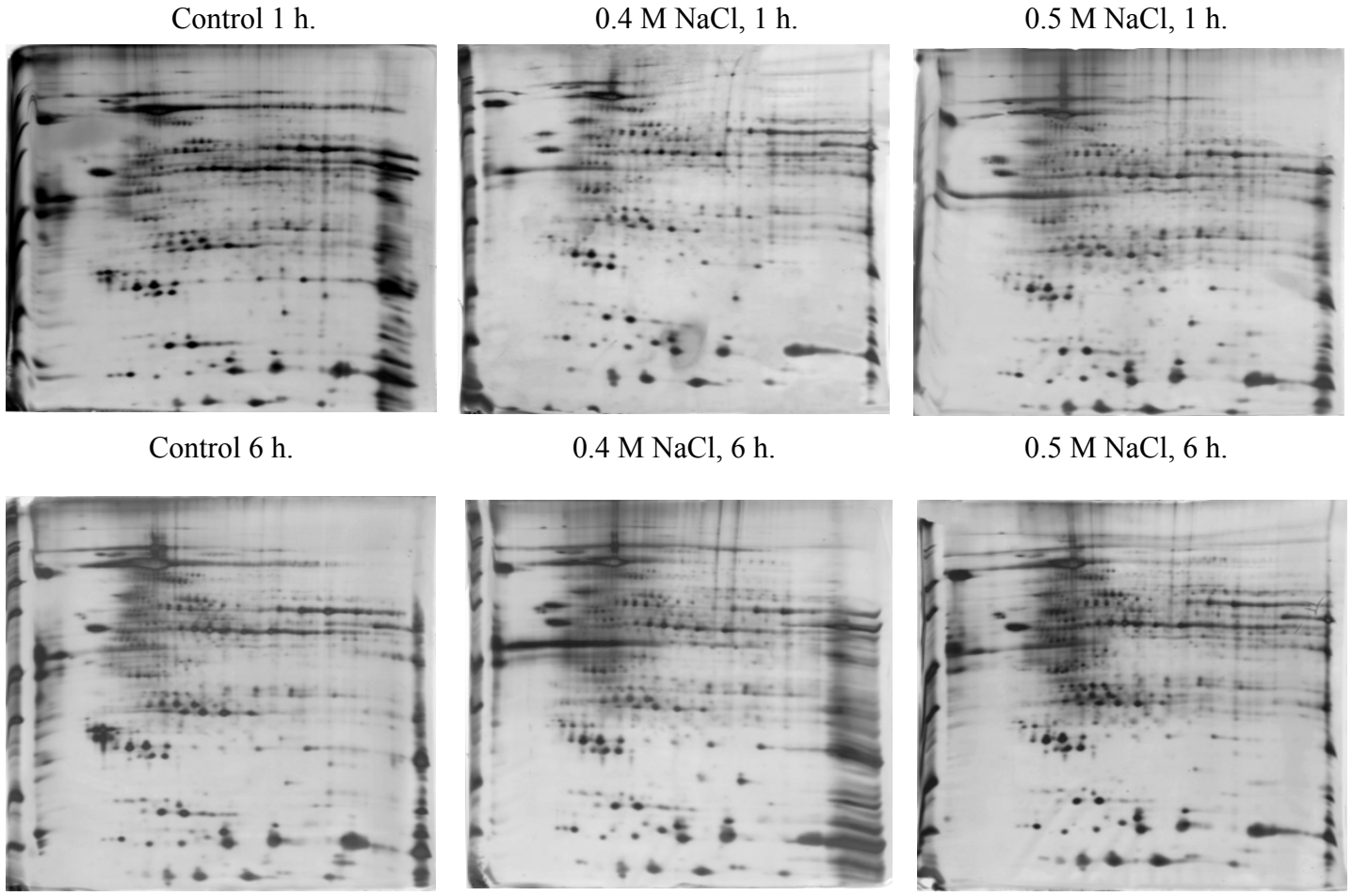


Figure 5.1 Silver stained 2-D gels of membrane proteins from *Sinorhizobium sp.* BL3 under 0.4 M NaCl and 0.5 M NaCl salt condition after shift to salt condition 1 and 6 hours.

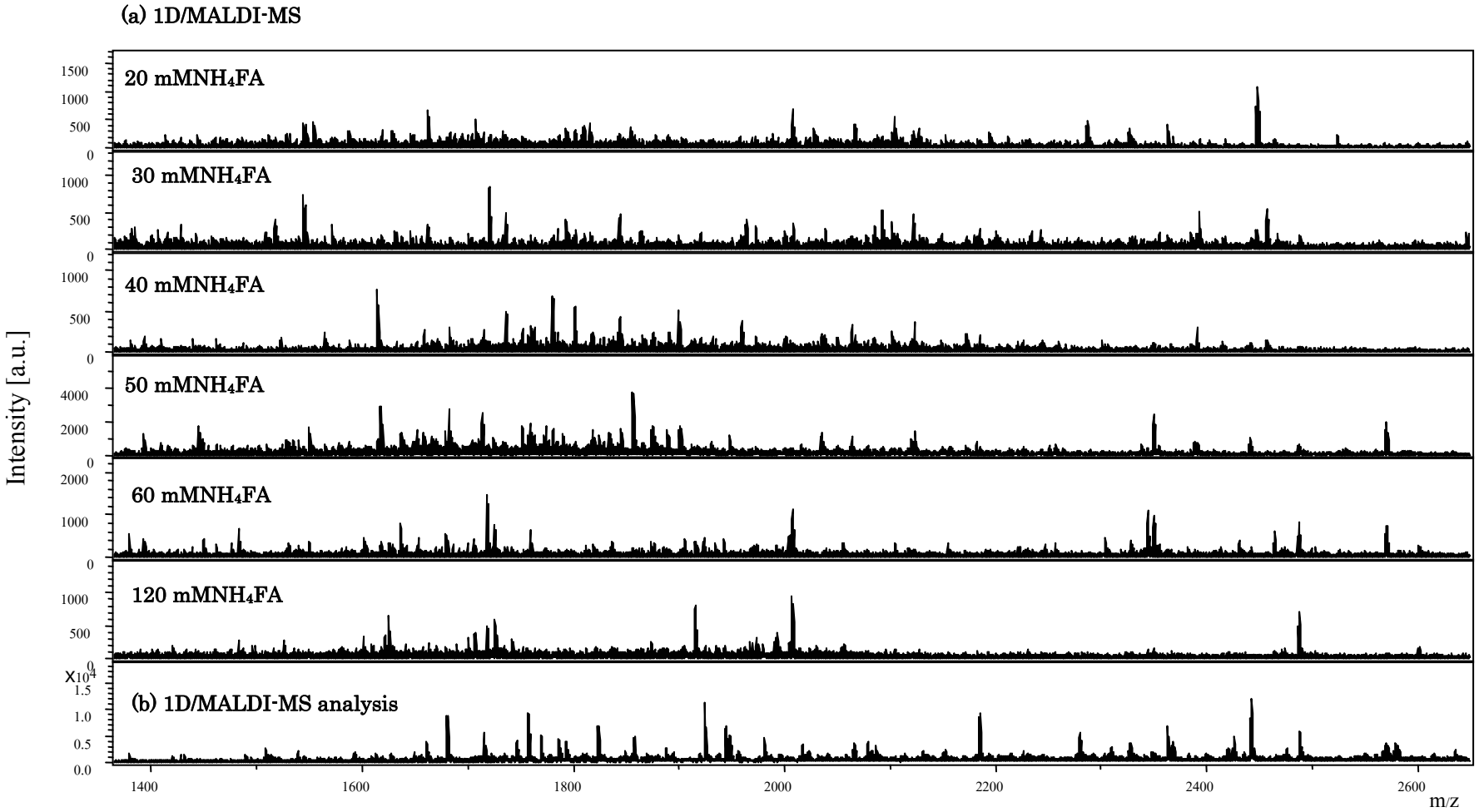


Figure 5.2 Base-peak chromatograms of the digested membrane proteins analyzed by the (a) SCX/MALDI-MS systems and (b) 1D/MALDI-MS systems.

each fraction are quite different indicating offline micro-cloumn SCX fractionation has been successed.

Identification of proteins in *Sinorhizobium* BL3 membrane enriched fraction by LC-MS/MS

A total of 12,685 MS/MS spectra were obtained for identification of peptides and proteins. The data searched with VEMS v3.0 (Matthiesen et al., 2004) against protein sequences based on the complete *S. meliloti* provided 751 confident protein identification with 412 membrane proteins based on either contain TMDs or membrane localization as shown in appendix. Proteins containing from one and up to 16 transmembrane domains were identified. A total of 248 proteins (72.1%) contained one TM domain and 44 (12.8%) contain 2 TMDs (Figure 5.3). Proteins with high numbers of transmembrane domains (TMDs) include transmembrane hypothetical protein SMb20291 with 16 TMDs, probable NADH dehydrogenase I chain L with 15 TMDs, transmembrane proteintransmembrane transport protein with 12 TMDs, putative drug resistance protein with 11 TMDs, and protein-export membrane protein with 10 TMDs. The use of SCX in combination with ηLC allowed a higher number of membrane proteins to be identified compared to the 2-D gel method.

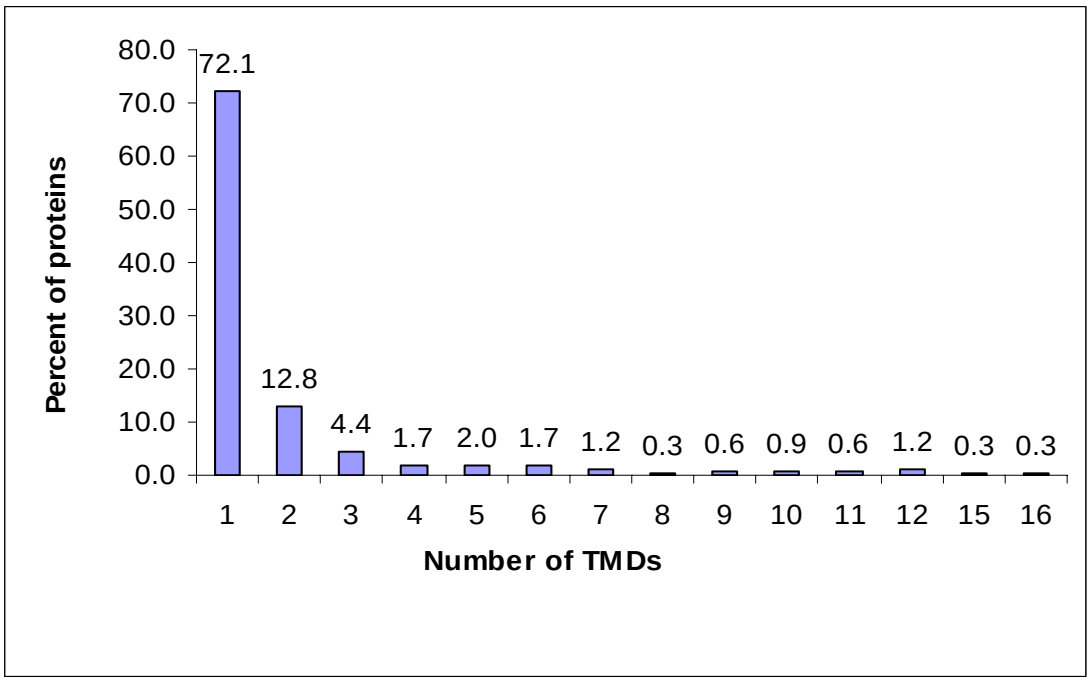


Figure 5.3 Predicted transmembrane domains of identified membrane proteins

The 412 identified membrane proteins from 2D-LC-MS/MS experiment could be categorized into five major functional groups and 39 subgroups based on the classification according to the Riley rules (Karp et al., 1999). The classification of the membrane proteins in these main and sub-functional groups are showed in Figure 5.4. The three largest groups are cell process (Group IV) contained 124 proteins (30%), small molecule metabolism of 110 proteins (27%) and, hypothetical/global homology (Group VI) of 109 proteins (26%), respectively.

Group IV, cell process, identified as a majority group of membrane proteins which involved in transportation of several kinds of molecules through the cell membrane which has also been reported in a major group of the *P. aeruginosa* membrane proteome (Blonder, 2004). In contrast, the membrane proteome of *M.*

tuberculosis detected only 6.6% of cell process proteins among protein identified (Gu et al., 2003). The most numerous transporters belong to the transport of small molecules with 50 identified proteins, the second largest group is that for the transport of carbohydrates, organic acids, alcohols with 26 identified proteins. Nevertheless, many of ABC transporter ATP-binding proteins have no transmembrane domains, however it was classified as peripheral membrane proteins, which are subunits of membrane-associated complexes. The architecture of proteins complexes is species-specific, assignment of proteins to this group of membrane proteins are more or less imprecise (Klein et al., 2005). Furthermore, the periplasmic substrate- binding proteins which are also peripheral membrane proteins are frequently have a predicted membrane anchor as identified by the presence of a LAGC motif (Hayashi and Wu, 1990; Wu and Yates, 2003). This group of proteins are well documented in related to osmo-adaptation in several organism; *S. cerevisiae* (Yale & Bohnert, 2001; Rep et al., 2000); *Lactobacillus plantarum* (Angelis and Gobbetti, 2004), *S. meliloti* (Djordjevic et al., 2003; Guerreiro et al., 1999; Talbart et al., 1997), *B. subtilis* (Steil et al., 2003; Petersohn et al., 2001).

Small molecule metabolism constituted the second largest group of 110 (27%) proteins (Figure 5.4 (B)). The list could be sectioned into; central intermediary metabolism, energy synthesis, and amino acid biosynthesis of 21%, 20%, and 18%, respectively. Almost all the proteins in tricarboxilic acid cycle were identified including pyruvate dehydrogenase alpha2 subunit, citrate synthase, probable succinate dehydrogenase membrane anchor subunit protein, succinyl-coa synthetase alpha chain, probable dihydrolipoamide dehydrogenase (E3 component of 2-oxoglutarate dehydrogenase complex) transmembrane protein, malate dehydrogenase and NADP-

dependent malic enzyme. It is well understood that TCA related proteins basically localized at mitochondria; a subcellular organelles which highly membranous structures of Eukaryotic cells responsible for energy metabolism. Therefore, the identification of these proteins could be implied that at membrane region of prokaryote can compromise mitochondrial function. Moreover, a cytochrome systems and oxidative phosphorelation which are important protein localized at membrane of mitochrondia were also detected including putative cytochrome C oxidase chain II, cytochrome C oxidase subunit II, putative cytochrome c oxidase polypeptide I transmembrane protein, probable cytochrome B transmembrane protein. Oxidoreductase from 4 different gene loci was identified from this fraction including nifE oxidoreductase (SMa0830), putative oxidoreductase (SMb20648), Oxidoreductase small molecule metabolism (SMc00985), and Oxidoreductase (SMc00410). This protein in *B. subtilis* was induced 2.4x under salt stress which believed that it performs functions in maintaining the redox balance of the cell (Petersohn et al., 2001).

The most abundant proteins detected in this experiment are the probable outer membrane protein (OMP) and the transmembrane outer membrane protein, which giving the outstanding Mascot score of 1,832 and 1,343, respectively (Appendix A) and also obviously detected in initial works with 2D-gel based method. It was classified in Group III of cell structure (Figure 5.4D). In *E. coli* exhibited 20 abundant OMPs (Molloy et al., 2000; Wimley, 2003). The outer membrane protects the cell against toxic agents: the combination of a highly charged sugar region and tightly ordered, gel-like hydrocarbon chains results in low permeability. Nevertheless, to aid in the exchange of nutrients and waste, the membrane is rendered selectively

permeable to solutes smaller than ca. 600 Da by pore-forming OMPs called porins (Koebnik et al., 2000; Koronakis et al., 2000; Vandeputte-Rutten et al., 2001; Prince et al., 2002; Chimento et al., 2003).

Twenty six percent of hypothetical/global homology proteins (Group VI) have been detected. This group composes of conserved hypothetical proteins and a group of unclassified regulator proteins. Three proteins among them were reported the increase expression under salt stress condition in *S. meliloti* (Ruberg et al., 2003) including SMa1043; 37% identities with cation efflux system transmembrane protein, SMc01827; contained ABC-type nitrate/sulfonate/bicarbonate transport domain, and SMc02634; 77% identities with predicted phosphatase of *Mesorhizobium sp.* BNC1 (NCBI search domain homology, www, 2005). Additionally, the regulators of several pathways were also identified; e.g. the GTP binding protein; hflX; involved in regulator of metalloprotease; FtsH. Intersetingly, FtsH documented to be related to stress response (Deuerling., 1995; Ge and Taylor, 1996; Narberhaus et al., 1999; Bourdineaud, 2003). Therefore, hflX might be one important regulator for stress response. Furthermore, the transcription regulator syrM involving in positive controls for nod D3 is important for nodulation process. The sensory transduction histidine kinase ;cheA, which is involved in chemotaxis and chemoreceptor protein might be important for cell survival due to chemotaxis need for localization of bacteria to sites on the legume roots (Barbour et al., 1991; Caetano et al., 1991 and Dowling et al., 1986). Plant root exudates can stimulate growth of rhizobia, which including several substances, for example, amino acids, dicarboxylic acids, flavonoids and homoserine (Aguilar et al., 1988; Armitage et al., 1988; Caetano et al., 1988; Kape et al., 1991).

This dataset represents the most comprehensive characterization of the *S. melliloti* membrane proteome. Among the 412 membrane proteins, approximately 26 membranes proteins have been earlier detected in *S. meliloti* by either transcriptomic analysis or molecular and proteomic analysis (Table 5.1) (Djordjevic et al., 2003; Guerreiro et al., 1999; Ruberg, 2003; Wei et al., 2004; Talbart et al., 1997). Beside, more or less 117 membrane proteins were identified as related to salt stress in other organisms in which 12, 4, 62, 52 and 7 membrane proteins were reported the upregulate in *S. meliloti*, *Lactobacillus*, *Bacillus*, yeast and plant, respectively (Table 5.1) (Yale and Bohnert, 2001; Rep et al., 2000; Angelis and Gobbetti, 2004; Djordjevic et al., 2003; Guerreiro et al., 1999; Ruberg, 2003; Wei et al., 2004; Talbart et al., 1997; Steil et al., 2003; Petersohn et al., 2001; Yan et al., 2004).

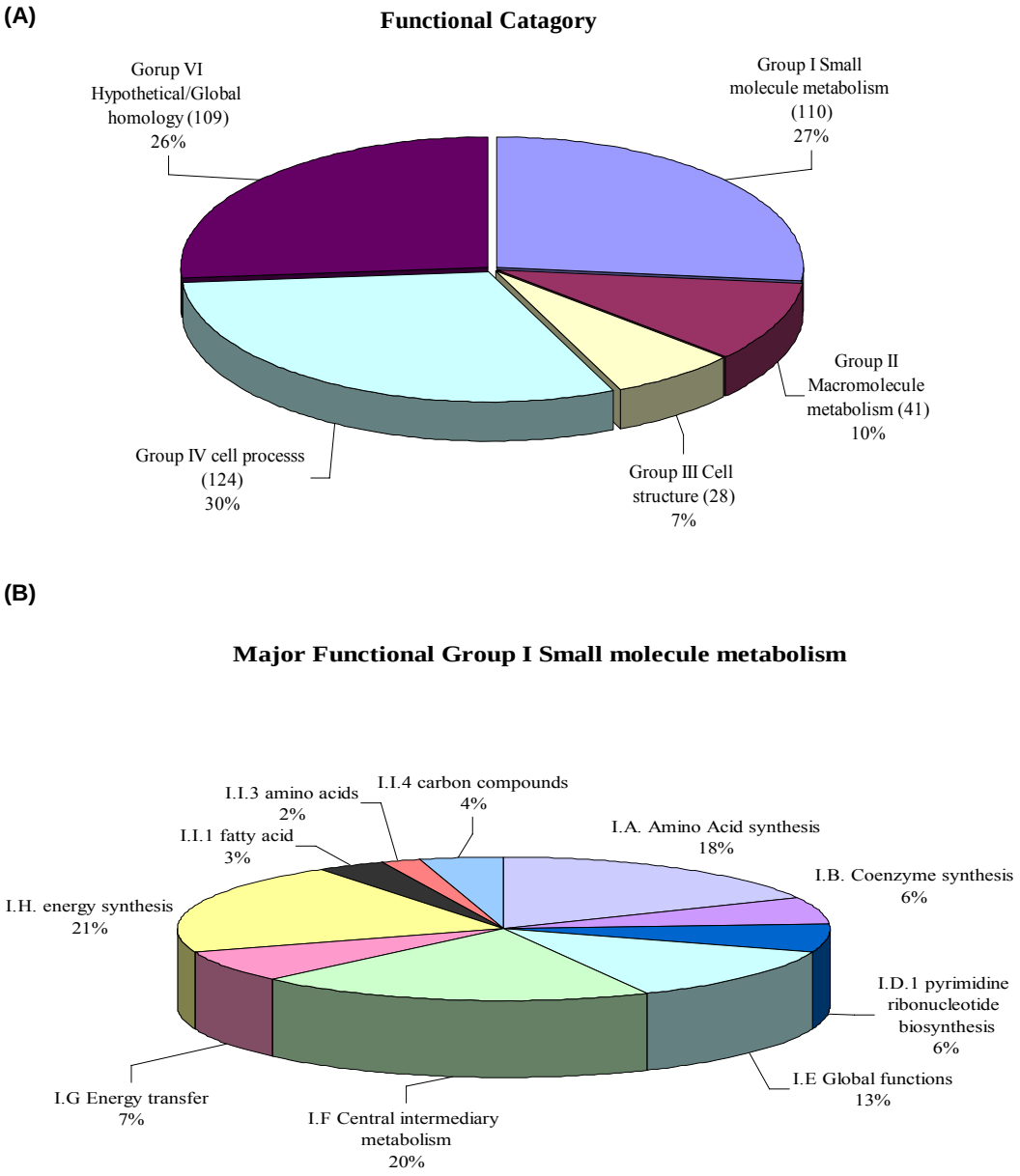
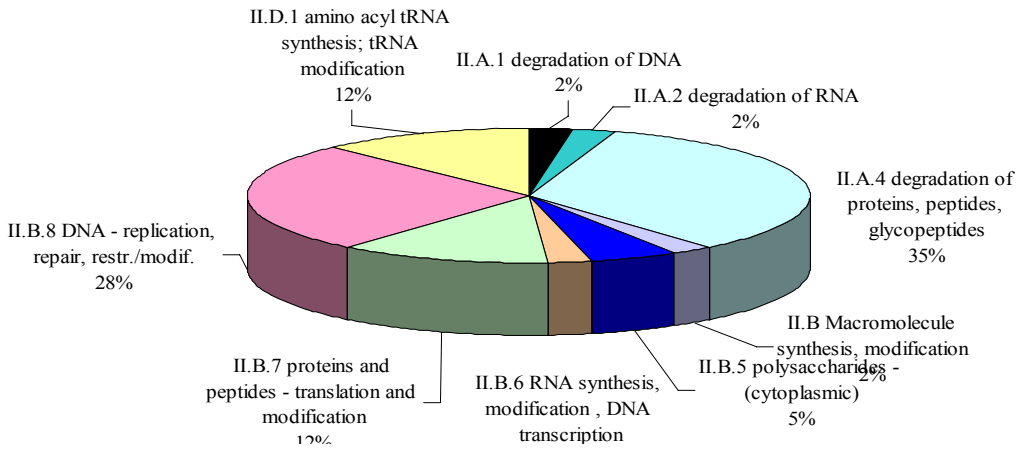


Figure 5.4 The functional category distribution of the 412 identified membrane proteins. The major functional category (A); The subgroup I Small molecule metabolism (B); The subgroup II macro molecule metabolism (C); The subgroup III Cell structure (D); The subgroup IV Cell process (E).

(C)

Major Functional Group II Macromolecule metabolism



(D)

Major Functional Group III Cell structure

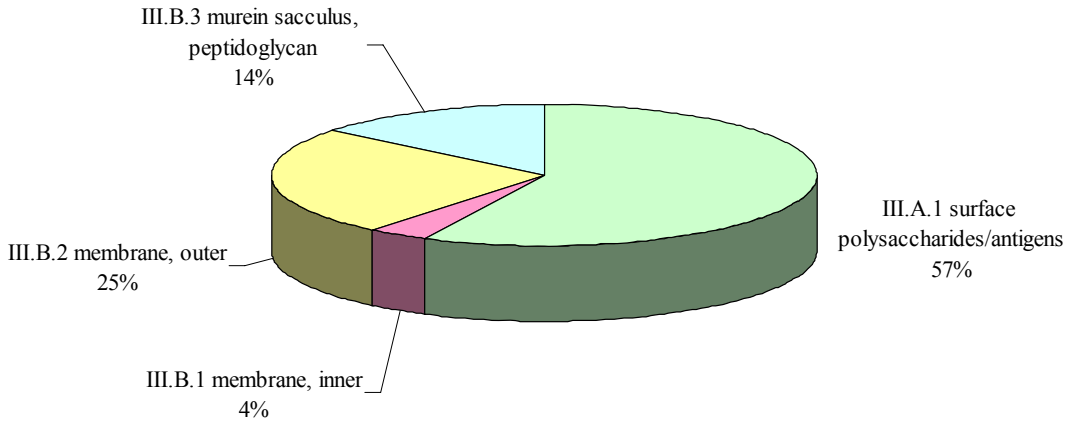


Figure 5.4 (Continued.)

(E) **Major Functional Group IV Cell Process**

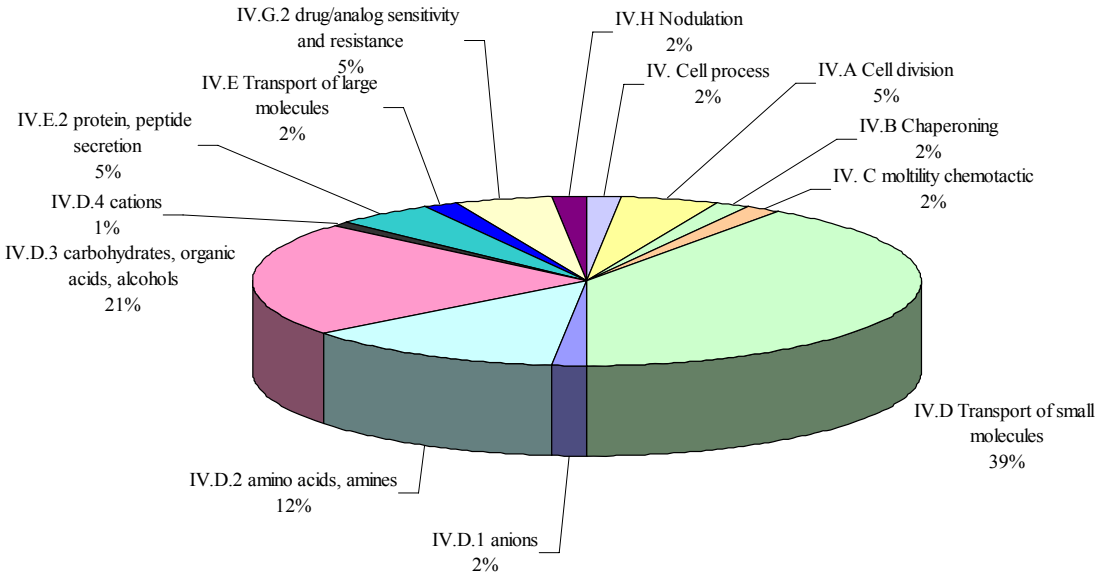


Figure 5.4 (Continued.)

Table 5.1 Membrane proteins of *Sinorhizobium sp.* BL3, which have been experimentally identified in *S. meliloti* and detected as salt response proteins in other organisms

Group	Subgroup	Score	Gene ID	Protein name	Experiment detected	Salt response proteins detected in:				
					S.meliloti membrane proteins	*S.meliloti*	Yeast	*Lactobacillus spp.*	*Bacillus spp.*	Plant
I	I Small Molecule Metabolism	127	SMa0830	nifE NIFE OXIDOREDUCTASE	/				/	
		130	SMb20648	PUTATIVE OXIDOREDUCTASE					/	
		127	SMc00410	OXIDOREDUCTASE (EC 1.-.-.-)					/	
		42	SMc00985	OXIDOREDUCTASE SMALL MOLECULE METABOLISM					/	
		94	SMc04148	AMINOMETHYLTRANSFERASE (EC 2.1.2.-)					/	
	I.A.16 proline	93	SMb20003	PYRROLINE-5-CARBOXYLATE REDUCTASE					/	
	I.A.17 arginine	70	SMc02138	PUTATIVE ACETYLORNITHINE AMINOTRANSFERASE PROTEIN			/			
	I.A.18 glutamine	416	SMc00762	PUTATIVE GLUTAMINE SYNTHETASE PROTEIN	/					
		70	SMc04405	PROBABLE 3-ISOPROPYLMALATE DEHYDROGENASE PROTEIN	/					
		207	SMc01237	RIBONUCLEOTIDE REDUCTASE			/			
		231	SMc00383	PUTATIVE GLUTATHIONE S-TRANSFERASE PROTEIN	/				/	
	I.E.2 ATP-proton motive force interconver	565	SMc00868	atpF TRANSMEMBRANE ATP SYNTHASE B CHAIN (EC 3.6.1.34)			/			
		212	SMc00869	atpF2 TRANSMEMBRANE ATP SYNTHASE SUBUNIT B' (EC 3.6.1.34)			/			
		48	SMc00870	PROBABLE ATP SYNTHASE SUBUNIT C TRANSMEMBRANE PROTEIN			/			
		114	SMc00871	PROBABLE ATP SYNTHASE A CHAIN TRANSMEMBRANE PROTEIN			/			
		451	SMc02499	PROBABLE ATP SYNTHASE ALPHA CHAIN PROTEIN			/			
		145	SMc02500	PROBABLE ATP SYNTHASE GAMMA CHAIN PROTEIN			/			
		248	SMc02501	atpD ATP SYNTHASE BETA CHAIN (EC 3.6.1.34)			/			
		201	SMc02502	atpC ATP SYNTHASE EPSILON CHAIN (EC 3.6.1.34)			/			
		208	SMa0819	FIXB ELECTRON TRANSFER FLAVOPROTEIN ALPHA CHAIN	/		/			
	I.F.13 gluconeogenesis	107	SMc01126	tme NADP-DEPENDENT MALIC ENZYME (EC 1.1.1.40)						/
	I.G.1 electron transport	128	SMa0769	FixP2 cytochrome c oxidase			/			/
		96	SMa1213	FixP1 Di-heme cytochrome c			/			
		112	SMb20174	CYTOCHROME C						/
		211	SMc02897	PUTATIVE CYTOCHROME C TRANSMEMBRANE PROTEIN			/			
		182	SMa1021	PUTATIVE CYTOCHROME C-LIKE PROTEIN			/			/
		101	SMb21368	PUTATIVE CYTOCHROME C OXIDASE CHAIN II (EC 1.9.3.1)			/			/
		209	SMc00010	PUTATIVE CYTOCHROME C OXIDASE POLYPEPTIDE I TRANSMEMBRANE PROTEIN						/
		52	SMc01925	PROBABLE NADH DEHYDROGENASE I CHAIN L TRANSMEMBRANE PROTEIN	/	/				
	I.H.4 oxidative branch, pentose pathway	115	SMc04262	gnd	6-PHOSPHOGLUCONATE DEHYDROGENASE (DECARBOXYLATING) (EC 1.1.1.44)			/			/
	I.H.6 TCA cycle	102	SMc01030	pdhAa PYRUVATE DEHYDROGENASE ALPHA2 SUBUNIT (EC 1.2.4.1)					/	
		114	SMc02087	gltA I CITRATE SYNTHASE (EC 4.1.3.7)					/	
		136	SMc02464	PROBABLE SUCCINATE DEHYDROGENASE MEMBRANE ANCHOR SUBUNIT PROTEIN			/		/	
		324	SMc02479	mdh MALATE DEHYDROGENASE (EC 1.1.1.37)	/		/			
		44	SMc02487	PROBABLE DIHYDROLIPOAMIDE DEHYDROGENASE (E3 COMPONENT OF 2-OXOGLUTA	/					
		117	SMc02162	LONG-CHAIN-FATTY-ACID--COA LIGASE (EC 6.2.1.3)			/			
		53	SMc00857	PUTATIVE PROTEASE PROTEIN					/	
		65	SMc01903	PROBABLE ATP-DEPENDENT CLP PROTEASE PROTEOLYTIC SUBUNIT PROTEIN	/				/	
		144	SMc02819	RIBONUCLEASE	/					
		327	SMc01326	tufB ELONGATION FACTOR TU	/	/				
		650	SMc00556	PUTATIVE DNA REPAIR PROTEIN			/		/	
		147	SMc01090	deaD ATP-DEPENDENT RNA HELICASE			/			
		173	SMc01235	uvrA EXCINUCLEASE ABC SUBUNIT A (DNA REPAIR ATP-BINDING)					/	
		203	SMc00908	ileS ISOLEUCYL-TRNA SYNTHETASE (EC 6.1.1.5)	/	/				
		244	SMc02641	rkpK UDP-GLUCOSE 6-DEHYDROGENASE (EC 1.1.1.22)	/					
III	III.B.2 membrane, outer	1343	SMc02094	omp TRANSMEMBRANE OUTER MEMBRANE PROTEIN	/	/				
		142	SMc02451	OUTER MEMBRANE PROTEIN	/					
		65	SMc01866	PROBABLE UDP-N-ACETYLGLUCOSAMINE--N-ACETYLMURAMYL-PENTAPEPTIDE PYR					/	
IV	IV CELL PROCESSES	323	SMb20181	ABC TRANSPORTER PERIPLASMIC SOLUTE-BINDING PROTEIN					/	
		94	SMb20184	PUTATIVE ABC TRANSPORTER ATP-BINDING PROTEIN					/	
	IV.A Cell division	479	SMc04296	ftsZ2 CELL DIVISION PROTEIN FTSZ	/					
		328	SMc04459	ftsH| TRANSMEMBRANE METALLOPROTEASE (EC 3.4.24.-)			/		/	
	IV.D Transport of small molecules	281	SMc02514	ABC TRANSPORTER PERIPLASMIC BINDING PROTEIN					/	
		239	SMb21206	PUTATIVE ABC TRANSPORTER ATP-BINDING PROTEIN,					/	
		235	SMc02169	ABC TRANSPORTER ATP-BINDING					/	
		232	SMc02259	ABC TRANSPORTER PERIPLASMIC BINDING PROTEIN					/	
		223	SMb21644	PUTATIVE ABC TRANSPORTER ATP-BINDING PROTEIN					/	
		217	SMa0036	PUTATIVE ABC TRANSPORTER ATP-BINDING PROTEIN					/	
		168	SMc01138	ABC TRANSPORTER ATP-BINDING PROTEIN					/	
		158	SMc02518	ABC TRANSPORTER ATP-BINDING PROTEIN					/	
		157	SMa0392	ABC TRANSPORTER, PERIPLASMIC SOLUTE-BINDING PROTEIN (dc=3)				/	/	
		151	SMa1860	PUTATIVE ABC TRANSPORTER, PERIPLASMIC SOLUTE-BINDING PROTEIN					/	
		146	SMa1434	PROBABLE ABC TRANSPORTER, ATP-BINDING PROTEIN					/	
		144	SMa2365	PROBABLE ABC TRANSPORTER, ATP-BINDING PROTEIN					/	

Table 5.1 Continued.

Group	Subgroup	Score	Gene ID	Protein name	Experiment detected	Salt response proteins detected in:				
					S.meliloti membrane proteins	*S.meliloti*	Yeast	*Lactobacillus spp.*	*Bacillus spp.*	Plant
		144	SMa2000	PUTATIVE ABC TRANSPORTER, PERIPLASMIC SOLUTE-BINDING PROTEIN					/	
		144	SMc02726	IRON TRANSPORT PROTEIN	/		/			
		139	SMa0104	PUTATIVE ABC TRANSPORTER, PERIPLASMIC SOLUTE-BINDING PROTEIN					/	
		137	SMa1421	PROBABLE ABC TRANSPORTER ATP BINDING -PROTEIN					/	
		136	SMc02171	ABC TRANSPORTER PERIPLASMIC BINDING PROTEIN					/	
		119	SMa1466	PROBABLE ABC TRANSPORTER ATP BINDING PROTEIN					/	
		114	SMa1370	PROBABLE ABC TRANSPORTER ATP-BINDING PROTEIN					/	
		108	SMa0300	ABC TRANSPORTER, PERMEASE (dc=3)					/	
		95	SMc04127	ABC TRANSPORTER ATP-BINDING PROTEIN					/	
		311	SMc04454	PUTATIVE ATP-BINDING ABC TRANSPORTER PROTEIN					/	
		241	SMc00185	PUTATIVE ABC TRANSPORTER ATP-BINDING TRANSMEMBRANE TRANSMEMBRANE PI	.				/	
		151	SMc00186	PUTATIVE ABC TRANSPORTER ATP-BINDING TRANSMEMBRANE PROTEIN					/	
		147	SMc02869	PUTATIVE ATP-BINDING ABC TRANSPORTER PROTEIN					/	
		102	SMc02829	PUTATIVE ATP-BINDING ABC TRANSPORTER PROTEIN					/	
		94	SMc02474	PUTATIVE ATP-BINDING ABC TRANSPORTER PROTEIN					/	
		65	SMc02831	PUTATIVE PERMEASE ABC TRANSPORTER PROTEIN					/	
		59	SMc00175	PUTATIVE ABC TRANSPORTER ATP-BINDING PROTEIN					/	
		46	SMc00531	PUTATIVE ABC TRANSPORTER ATP-BINDING PROTEIN	/					
		64	SMc00550	PROBABLE ABC TRANSPORTER ATP-BINDING TRANSMEMBRANE PROTEIN					/	
IV	IV.D.1 anions	156	SMb21130	PUTATIVE SULFATE UPTAKE ABC TRANSPORTER ATP-BINDING PROTEIN					/	
		191	SMc03866	ABC TRANSPORTER ATP-BINDING PROTEIN					/	
		167	SMc02121	ABC TRANSPORTER GENERAL L-AMINO ACID TRANSPORT ATP-BINDING PROTEIN			/		/	
		144	SMc01949	ABC TRANSPORTER HIGH-AFFINITY BRANCHED-CHAIN AMINO ACID TRANSPORT ATP			/		/	
		125	SMa0082	PUTATIVE ABC TRANSPORTER, PERIPLASMIC SOLUTE-BINDING PROTEIN					/	
		362	SMc01950	PROBABLE HIGH-AFFINITY BRANCHED-CHAIN AMINO ACID TRANSPORT PERMEASE A			/		/	
		336	SMc01948	PROBABLE HIGH-AFFINITY BRANCHED-CHAIN AMINO ACID TRANSPORT ATP-BINDIN			/		/	
		224	SMc02738	PUTATIVE GLYCINE BETAINE TRANSPORT SYSTEM PERMEASE ABC TRANSPORTER PR	/	/		/	/	
		193	SMc04439	PUTATIVE GLYCINE BETAINE TRANSPORT ATP-BINDING ABC TRANSPORTER PROTEIN	/	/		/	/	
		118	SMc02739	PUTATIVE GLYCINE BETAINE TRANSPORT ATP-BINDING ABC TRANSPORTER PROTEIN	/	/		/	/	
		64	SMc02119	PROBABLE GENERAL L-AMINO ACID TRANSPORT PERMEASE ABC TRANSPORTER PRO			/		/	
	IV.D.3 carbohydrates, organic acids, alcoh	467	SMc01499	ABC TRANSPORTER ATP-BINDING TRANSPORT					/	
		236	SMb21344	PUTATIVE SUGAR UPTAKE ABC TRANSPORTER ATP-BINDING PROTEIN			/			
		228	SMb20713	PUTATIVE SUGAR UPTAKE ABC TRANSPORTER ATP-BINDING PROTEIN			/			
		218	SMb20673	PUTATIVE SUGAR UPTAKE ABC TRANSPORTER ATP-BINDING PROTEIN			/			
		211	SMb20894	gguA PROBABLE SUGAR UPTAKE ABC TRANSPORTER ATP-BINDING PROTEIN			/			
		159	SMa0713	PUTATIVE ABC SUGAR TRANSPORT ATP BINDING PROTEIN, AMINO TERMINUS			/			
		144	SMb20718	PUTATIVE SUGAR UPTAKE ABC TRANSPORTER ATP-BINDING PROTEIN			/			
		125	SMb20317	SUGAR ABC TRANSPORTER ATP-BINDING PROTEIN			/			
		120	SMb20316	ABC TRANSPORTER PERIPLASMIC SUGAR-BINDING PROTEIN			/			
		120	SMb21016	PUTATIVE SUGAR ABC TRANSPORTER PERIPLASMIC SOLUTE-BINDING PROTEIN PREC			/			
		120	SMb21461	PUTATIVE SUGAR UPTAKE ABC TRANSPORTER PERIPLASMIC SOLUTE-BINDING PROTE			/			
		115	SMb20903	PUTATIVE SUGAR UPTAKE ABC TRANSPORTER PERMEASE PROTEIN			/			
		114	SMb20856	PUTATIVE SUGAR UPTAKE ABC TRANSPORTER PERIPLASMIC SOLUTE-BINDING PROTE			/			
		99	SMb20855	PUTATIVE SUGAR UPTAKE ABC TRANSPORTER ATP-BINDING PROTEIN			/			
		99	SMb20630	PUTATIVE SUGAR UPTAKE ABC TRANSPORTER ATP-BINDING PROTEIN			/			
		97	SMb21595	PUTATIVE SUGAR UPTAKE ABC TRANSPORTER PERIPLASMIC SOLUTE-BINDING PROTE			/			
		96	SMb21103	PUTATIVE SUGAR UPTAKE ABC TRANSPORTER PERIPLASMIC SOLUTE-BINDING PROTE			/			
		160	SMb20328	probable trehalosemaltose transporter ATP-binding protein	/					
		147	SMb21152	putative sugar uptake ABC transporter ATP-binding protein			/			
		98	SMb20661	putative sugar uptake ABC transporter ATP-binding protein			/			
		95	SMb21106	putative sugar uptake ABC transporter ATP-binding protein			/			
	IV.D.4 cations	176	SMa1013	ACTP COPPER TRANSPORT ATPASE (EC 3.6.3.4)			/			
	IV.E.2 protein, peptide secretion	735	SMc02082	OUTER MEMBRANE SECRETION PROTEIN	/					
		262	SMc04458	secA PREPROTEIN TRANSLOCASE SECA SUBUNIT	/					
		144	SMc04350	TRANSMEMBRANE MULTIDRUG EFFLUX SYSTEM			/		/	
		63	SMc02867	PUTATIVE MULTIDRUG-EFFLUX SYSTEM TRANSMEMBRANE PROTEIN			/		/	
		152	SMa0853	NODE BETA KETOACYL ACP SYNTHASE			/			
		208	SMc02284	SIGNAL PEPTIDE HYPOTHETICAL	/	/				
		172	SMc02659	GTP PYROPHOSPHOKINASE (ATP:GTP 3'-PYROPHOSPHOTRANSFERASE) (EC 2.7.6.5)					/	
		208	SMa1043	HYPOTHETICAL PROTEIN		/				
		132	SMc01827	HYPOTHETICAL PROTEIN		/				
		166	SMc02634	TRANSMEMBRANE HYPOTHETICAL		/				
		94	SMc03152	HYPOTHETICAL TRANSMEMBRANE PROTEIN	/					
VI	VI MISCELLANEOUS	166	SMb20292	IMMUNOGENIC PROTEIN		/				
				Total	26	12	52	4	62	7

* Yeast (Yale and Bohnert, 2001; Rep et al., 2000); *Lactobacillus spp.* (Angelis and Gobbetti, 2004), *S. meliloti* (Djordjevic et al., 2003; Guerreiro et al., 1999; Ruberg et al., 2003, Wei et al., 2004;and Talbart et al., 1997), *Bacillus spp.* (Steil et al., 2003; Petersohn et al., 2001), Plant (Yan et al., 2004; Shu-bin, 2003)

Quantitative analysis of membrane proteins expression change under salt stress condition

Stable isotope labeling was applied to allow quantitative analysis. There are a number of strategies available varying from *in vitro* labeling (Ong et al., 2002 and Ibarrola et al., 2003) to chemical labeling at the peptide level (Munchbach et al., 2000; Goodlett et al., 2001; Peters et al., 2001; Zhou et al., 2002). The label chosen for quantification was 2-methyoxy-4,5-dihydro-1H-imidazole ('Mass Tag') which is an epsilon-amine specific derivatisation reagent hence only lysine residues are labeled (Peters et al., 2001). The high specificity of the reagent allowed efficient labeling to be performed in the presence of urea and under aqueous conditions. In other words, the reaction could be performed directly on the tryptic digests with no apparent cations of side reactions or significant sample losses. The labeling strategy provides a mass difference of 4 Da between the heavy and light versions of the reagent. Result revealed in Table 5.2 showing that a comprehensive labeling of all lysine residues was accomplished.

Table 5.2 Data set of SMc02501; ATP synthase beta chain; searching from *S. meliloti* data base, showing all peptides containing Lysine were derivertized by mass tag with the total score = 470 E =0.0E0

Score	E	RT	Obs		N	z	theo	Protein position	delta
112++	8.10E-43	50.4472	1067.2634	9746	3	3199.776	-0.0445	260 - 288	VALTGLTVAEQFRDEGQDVLFFVDNIFR missed cleavage
75++	1.80E-26	82.0781	894.5119	8472	3	2681.521	-0.0345	410 - 434	ALQDIIAILGMDELSEEDkIAVAR 19-K_MassTag
53++	7.80E-17	75.9799	1015.572	7038	2	2030.137	-0.0342	440 - 458	FLSQPFFVAEVFTGSPGk 18-K_MassTag+4*Deu
49++	2.20E-15	75.5794	763.4615	9184	2	1525.916	-0.0193	190 - 203	TVLIMELINNVAk 13-K_MassTag
42++	4.40E-12	48.2925	535.328	6908	2	1069.649	-0.0118	458 - 467	LVALEDTIk 9-K_MassTag
40++	2.00E-11	77.598	763.4728	7048	2	1525.938	-0.0306	190 - 203	TVLIMELINNVAk 13-K_MassTag
40++	1.30E-11	96.3248	1067.2649	8969	3	3199.78	-0.046	260 - 288	VALTGLTVAEQFRDEGQDVLFFVDNIFR missed cleavage
37++	5.50E-10	77.598	765.4847	7783	2	1529.962	-0.03	190 - 203	TVLIMELINNVAk 13-K_MassTag+4*Deu
35++	3.40E-09	54.0899	578.8368	8807	2	1156.666	0.0031	166 - 176	VVDLLAPYAk 10-K_MassTag
35++	2.00E-09	52.5577	522.3164	8115	2	1043.626	-0.013	179 - 190	IGLFGGAGVGk 11-K_MassTag
33++	1.80E-08	76.0714	1013.564	7039	2	2026.121	-0.0387	440 - 458	FLSQPFFVAEVFTGSPGk 18-K_MassTag
32++	4.90E-08	44.889	535.3057	9465	2	1069.604	0.0105	458 - 467	LVALEDTIk 9-K_MassTag
32++	5.10E-08	42.6782	787.4144	8071	2	1573.822	-0.0212	243 - 258	AALVYGQMNEPPGAR
31++	1.10E-07	96.9671	1067.2589	7188	3	3199.762	-0.04	260 - 288	VALTGLTVAEQFRDEGQDVLFFVDNIFR missed cleavage
31++	1.50E-07	48.9282	522.2957	9064	2	1043.584	0.0077	179 - 190	IGLFGGAGVGk 11-K_MassTag
30++	4.50E-07	48.0674	537.3355	7372	2	1073.664	-0.0067	458 - 467	LVALEDTIk 9-K_MassTag+4*Deu
28++	3.30E-06	44.889	537.3166	8766	2	1073.626	0.0122	458 - 467	LVALEDTIk 9-K_MassTag+4*Deu
22++	1.30E-03	52.3332	524.3306	7673	2	1047.654	-0.0146	179 - 190	IGLFGGAGVGk 11-K_MassTag+4*Deu
19++	3.60E-02	42.8675	646.8248	8757	2	1292.642	0.0046	87 - 99	TIAMDSTEGLVR

A total of 11,341 MS/MS spectra was obtained from four experimental conditions each analysed in triplicate. The expression profiles for (membrane) proteins were quantitated and validated by VEMS v3.0. The mass spectrometric data was searched against protein sequences based on the complete *S. meliloti* proteome eluting 258 confident matches and allowing quantitation of 138 membrane proteins. Although peptides derivatization had been effective, but not all detected proteins were quantitable due to the identified peptides not containing lysine residues in the sequence. The reproducibility of this system could also be observed in inter-experimental data set. Malate dehydrogenase which exhibited a high expression level under salt stress conditions, was clearly detected in the same peptide of IALIGSGMIGGTLAHLAGLKK in the four different salt stress samples (Figure 5.5). The mass spectra associated to triply charged, which generate similar relative abundance ratio and similar MS/MS spectra. Another interesting protein that is important to maintain cell survival under salt stress condition is metalloprotease. The same peptide from four independent samples could be detected, identified and quantified (Figure 5.6). The spectra for EIPFSQFLK represents one of the lower abundance peptides that have been sequenced and quantitated.

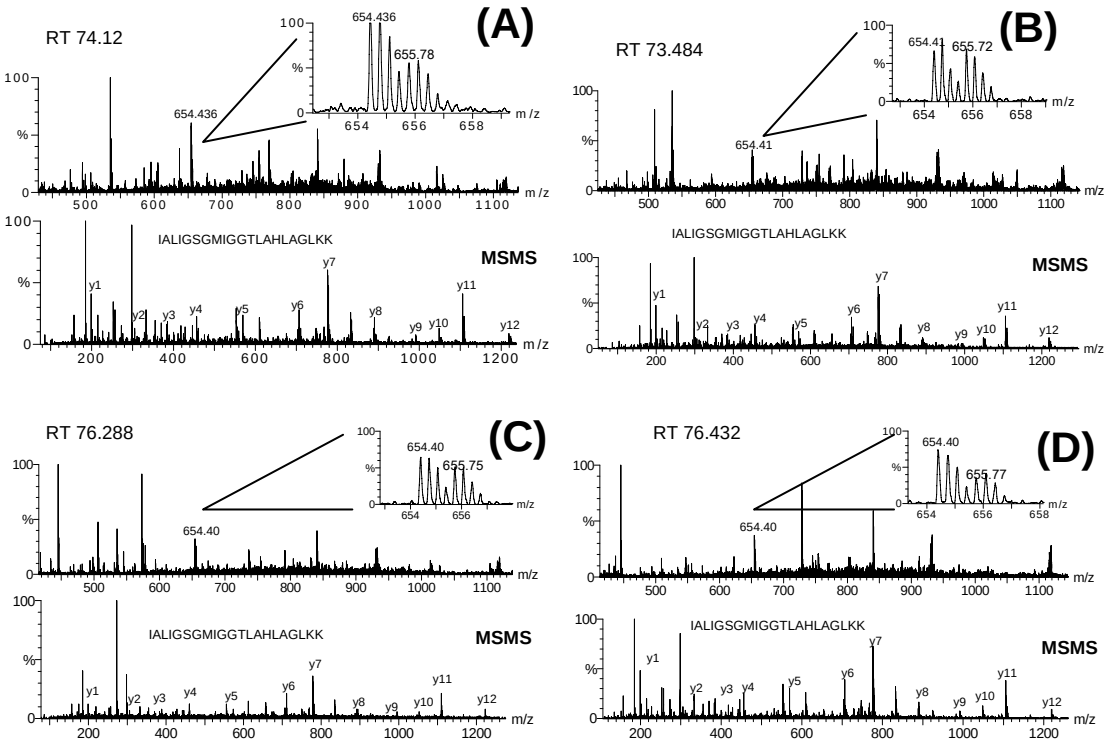


Figure 5.5 Inter-experimental reproducibility of peptide IALIGSGMIGGTLAGLK of malate dehydrogenase under salt stress 0.4 M 1 h (A), 0.4 M 6 h (B), 0.5 M 1 h (C) and 0.5 M 6 h (D)

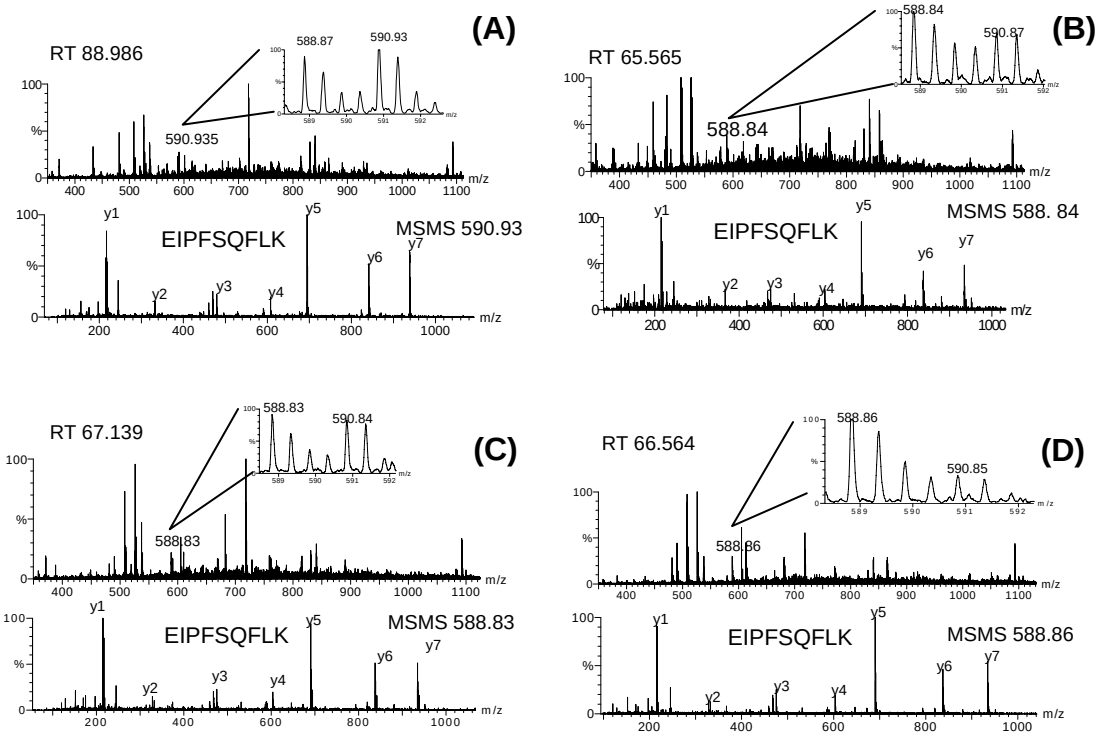


Figure 5.6 Inter-experimental reproducibility of peptide EIPFSQFLK of transmembrane metalloprotease under salt stress 0.4 M 1 h (A), 0.4 M 6 h (B), 0.5 M 1 h (C) and 0.5 M 6 h (D)

Proteomic profiles under salt stress

The membrane proteomic analyses provided profile levels of protein expression for cells exposed to NaCl (0.4 and 0.5 M) at the exposure times of 1 and 6 hours. The expression shift in 1 hour will be termed the immediate stress response and at 6 hours as the late response. Twenty seven membrane proteins exhibited a change in expression level with 22 proteins having their expression levels increased by at least 50% (Table 5.3).

Some functional groups of proteins could be identified by the expression level shift e.g. 10 transportation proteins (Group IV) and 9 small molecule metabolism proteins (group I). The expression pattern under salt stress of membrane proteins observed in this experiment can be categorized into 3 groups. Firstly, the immediate response proteins, these proteins are rapidly produced but transiently over expressed. Secondly, late response proteins, these proteins are expressed after 6 hour induction. Lastly, stress acclimation proteins, which are more or less rapidly induced but still be over expressed several hours after the downshifts (Duché et al., 2002). A selection of proteins that have common expression profile in both salt shift of 0.4 M and 0.5 M NaCl are showed in Figure 5.7. The schematic model of membrane proteins expression of *Sinorhizobium sp*. BL3 under salt stress condition is shown in Figure 5.8.

The common expression in immediate response exhibited 3 proteins. The conserved hypothetic protein SMc02582 was detected a significant increase in expression (2.5x at 0.4 M NaCl and 1.6x at 0.5 M NaCl (Figure 5.7A). It was found that this protein has homology with ErfK_YbiS_YhnG proteins (52% identities) and similarly possesses a region containing a conserved histidine and cysteine, suggesting that this protein has enzymatic activity (NCBI conserved domain search, www). This protein is a good candidate for future characterization of function to provide better understanding to how this protein is related to salt stress. The minor induction of outer membrane secretion protein TolC was observed. TolC family of envelope proteins is ubiquitous throughout Gram-negative bacteria and is central to type I secretion of toxins and proteases (Thanassi and Hultgren, 2000; Andersen et al., 2000; Thanabalu et al., 1998), and to the efflux of small noxious compounds, notably

detergents and a wide range of antibacterial drugs (Nikaido, 1994; Nikaido, 1998). It is therefore important to bacterial survival. However, the function related to salt stress of this protein has not yet been reported. The transmembrane ATP synthase B chain also showed a slightly induced in early expression (Figure 5.7 (A)). This protein family was well documented in related to ion pump to maintain intracellular concentration under salt stress condition (discussion below).

Five acclimation proteins could be observed (Figure 5.7(A)). It is interesting to observe malate dehydrogenase (MDH). Eventhough, the expression pattern of this membrane protein do not exhibited the correlation between at 0.4 M NaCl (1.75x and 1x) and 0.5 M NaCl (1.5x and 2.3x), however, the error bars suggest the protein could be at the same expression level in all four conditions. Due to malate represents a pivotal point in the tricarboxylic acid cycle (Moat and Foster, 1995), MDH might also play an important role in this pathway. MDH catalyze the interconversion of oxaloacetic acid (OAA) and malate and have diversified roles in plant cell metabolism (Gietl, 1992). Activation of NAD^+-MDH has often been associated with adaptation to drought in C_3 plants (Ivanishchev, 1997). A high level of NAD^+-MDH activity in cytosol and mitochondria causes better functioning of TCA cycle as OAA produced in the reaction by mitochondrial MDH is able to react with another molecule of acetyl CoA in order to start another turn of TCA cycle, thus, allow TCA cycle to continue (Salisbury and Ross, 1986). Further, OAA serves as amino acid precursor in plants (Salisbury and Ross, 1986). Increased MDH activity under *in situ* salinization thus appears to be an adaptational feature of salt tolerant rice cultivars in maintaining higher activity of TCA cycle, maintain optimum photosynthesis and respiration rate and possibly helping the plant cells in more synthesis of amino acids (Kumar et al.,

1999; Hare et al., 1998). Some of the key physiological changes that occur during adaptation of plants to salt stress include increased synthesis of certain amino acids and soluble nitrogenous compounds which act as osmolytes (Hare et al., 1998). Therefore, this experiment might be the first insight of MDH detection in relating to salt stress response in bacteria. Eventhough, metabolic pathway of these plants and bacteria are quite different, they might share certain responses to salt stress.

Interestingly, the transmembrane adenylate/guanylate cyclase (cyaD2), which is one protein responsible for catabolite control, was upregulated after 6 hour exposure to salt (Table 5.3). CyaD2 responsible for converting ATP to cAMP, which cAMP is consequently alter the expression of several operons either positively or negatively. In *E. coli* cAMP was increased when cell need to utilized the alternative cabon source, for example, lactose or arabinose. However, this experiment was conducted by minimum medium containing succinate and glutamate as a sole of carbon. Therefore, cAMP might be important substrate for induction of the genes to utilize such carbon source. Subsequently, under severely salt stress, the increase in expression of cAMP could be up higher because of more energy requirement from catabolite metabolism. The pathway of succinate utilization is finally connected to malate production. The results suggested that all metabolic pathways are expressed in the same correlation to maintaining energy balance in cell.

According to Table 5.3 demonstrates the evidence of upregulate in SMc02094 (omp; transmembrane outer membrane protein) of 1.86x and 1.4x under 0.4 M at 1, and 6 h, respectively which could be classified as acclimation protein. However, under 0.5 M NaCl no increase of expression can be detected. These proteins are involved in the passive diffusion of small hydrophilic molecules across the outer

membrane and are preferentially synthesized when increased osmolarity (Mizuno and Mizushima, 1990). It correlates with the expression of outer membrane porin proteins, OmpF and OmpC, which is altered by osmotic changes in *E. coli* (Mizuno and Mizushima, 1990). On the other hand, inactivation of omp10 of *S. meliloti* by a transposon insertion leads to cell wall deformation and sensitivity of the cell to salt stress (Wei et al., 2004). It might be due to at severely high salt concentration, cells have to control transport system tightly to selected only necessary substances in or out of the cell that consequently decrease the passive diffusion from this pathway.

Eight observed proteins correlated to the expression pattern of late response. (Figure 5.7 (B)). One significantly expressed protein, 6-phosphogluconate dehydrogenase (gnd) exhibited down regulation after 1 hour shift to salt, however, after 6 hours, the expression increases dramatically to 7.0 times and 6.65 times higher under 0.4 M and 0.5 M NaCl conditions, respectively. This observation is in agreement with the proteomic observations of yeast salinity response showing 4.1 fold induction, however, a time course had not been applied (Li et al., 2003). In rice, 6-phosphogluconate dehydrogenase gene was up-regulated in shoots under salt stress which suggested that it plays an importance role in cell division (Huang et al., 2003). The function of this protein is to catalyze the oxidative decarboxylation of 6-phosphogluconate to ribulose-5-phosphate in pentose phosphate pathway (PPP). Additionally, ribose-5-phosphate is also the important product of this pathway due to an essential precursor in nucleotide or DNA biosynthesis (Sakai et al., 1971). Moreover, this pathway generates NADPH, which is used primarily for reducing power in biosynthetic reactions (e.g., α-ketoglutarate to glutamate; acetate to fatty acids).

Table 5.3 Membrane proteins expression profile under salt stress condition.

Number	Gene ID	Protein name	VEMS Score	TMDs	Function	Mw.	O.4 M NaCl 1h				O.4 M NaCl 6h				O.5 M NaCl 1h.				O.5 M NaCl 6h.			
							EL	QC	STDV	No.Peptide	EL	QC	STDV	No.Peptide	EL	QC	STDV	No.Peptide	EL	QC	STDV	No.Peptide
1	SMb20181	ABC TRANSPORTER PERIPLASMIC SOLUTE-BINDING PROTEIN	22	1	IV CELL PROCESSES	34955	1.99	66.582	0.2	2	2.91	74.395	0	1	1.24	55.421	5.83	3	1.84	64.827	2.76	3
2	SMc02094	omp TRANSMEMBRANE OUTER MEMBRANE PROTEIN	394	1	III.B.2 membrane, oute	84520	1.92	65.761	3.59	6	1.47	59.489	6.93	5	1.01	50.225	4.99	4	0.59	37.114	1.28	2
3	SMc02582	CONSERVED HYPOTHETICAL PROTEIN	127	1	VI.C Hypothetical/Globa	30573	2.52	71.604	3.14	5	0.82	45.093	1.89	3	1.60	61.554	2.97	4	1.02	50.513	0.31	4
4	SMc04262	gnd 6-PHOSPHOGLUCONATE DEHYDROGENASE (DECARBOXYLATING) (E	15	2	I.H.4 oxidative branch, p	50804	0.48	32.612	0	1	7.04	87.565	2.29	2	0.45	31.187	0	1	6.65	86.935	0	1
5	SMc02479	mdh MALATE DEHYDROGENASE (EC 1.1.1.37)	193	1	I.H.6 TCA cycle	33611	1.76	63.728	2.75	4	1.07	51.741	2.76	2	1.55	60.768	3.28	5	2.38	70.417	5.98	3
6	SMc02305	murA UDP-N-ACETYLGLUCOSAMINE 1-CARBOXYVINYLTRANSFERASE (EC 2	197	1	III.B.3 murein sacculus,	45434	0.72	41.881	3.78	2	1.68	62.647	0	1	1.61	61.682	0	1	1.47	59.491	3.41	3
7	SMc04307	cyaD2 TRANSMEMBRANE ADENYLATE/GUANYLATE CYCLASE (EC 4.6.1.1)	20	4	I.E.1 global regulatory fa	77627	0.92	48.036	2.14	2	1.55	60.82	6.09	2	1.11	52.57	0.8	3	1.27	55.868	7.11	2
8	SMc04459	ftsH TRANSMEMBRANE METALLOPROTEASE (EC 3.4.24.-)	138	2	IV.A Cell division	70543	0.79	44.01	0.03	2	1.72	63.265	0	1	1.05	51.207	1.09	6	2.70	72.992	4.39	7
9	SMc01948	livF ABC TRANSPORTER HIGH-AFFINITY BRANCHED-CHAIN AMINO ACID T	125		IV.D.2 amino acids, ami	26358	0.99	49.703	0	1	1.55	60.717	0	1	1.28	56.056	1.2	5	2.84	73.928	0.98	3
10	SMb21221	PUTATIVE SUGAR UPTAKE ABC TRANSPORTER PERIPLASMIC SOLUT	36		IV.D.3 carbohydrates, o	44433	1.49	59.903	3.56	4	1.41	58.499	3.57	2	1.76	63.819	0	1	1.14	53.168	6.96	2
11	SMc02121	aapP ABC TRANSPORTER GENERAL L-AMINO ACID TRANSPORT ATP-BINDI	146		IV.D.2 amino acids, ami	29266	0.93	48.267	0	1	1.20	54.559	0.68	2	1.57	61.13	0	2	1.88	65.309	2.88	3
12	SMc02502	atpC ATP SYNTHASE EPSILON CHAIN (EC 3.6.1.34)	77		I.E.2 ATP-proton motive	14570	0.89	46.999	1.27	4	0.91	47.766	1	3	1.36	57.547	3.21	4	2.46	71.084	5.04	2
13	SMc02500	atpG ATP SYNTHASE GAMMA CHAIN (EC 3.6.1.34)	136		I.E.2 ATP-proton motive	32002	1.00	50.04	1.21	2	1.15	53.496	0.41	3	1.20	54.582	2.36	2	2.24	69.12	5.8	3
14	SMc03938	pntB TRANSMEMBRANE NAD(P) TRANSHYDROGENASE SUBUNIT BETA (E	157	7	I.F Central intermediary	48322	0.76	43.2	0	1	0.97	49.115	0	1	0.84	45.565	7.03	4	3.17	76.024	3.29	3
15	SMc00556	radA DNA REPAIR PROTEIN	77	1	II MACROMOLECULE	51934	0.64	39.191	2.03	2	1.33	56.99	0.18	2	0.81	44.697	1.22	2	1.84	64.807	2.85	3
16	SMc00009	ctaC CYTOCHROME C OXIDASE SUBUNIT II (EC 1.9.3.1)	152	3	CYTOCHROME C OXI	32224	0.97	49.27	1.08	4	0.87	46.593	1.7	4	1.01	50.248	4.67	7	1.63	62.048	3.57	6
17	SMc02396	OUTER MEMBRANE PROTEIN	66	2	III.B.2 membrane, outer	37294	1.22	55.055	4.19	6	1.30	56.537	1.82	6	1.06	51.57	2.91	5	1.52	60.254	4.58	4
18	SMc01499	smoK ABC TRANSPORTER ATP-BINDING TRANSPORT	111		IV.D.3 carbohydrates, o	36327	0.89	46.957	1.88	2	1.06	51.431	0.14	4	1.18	54.062	2.98	4	1.57	61.095	4.94	3
19	SMc02169	ABC TRANSPORTER ATP-BINDING	186		IV.D Transport of small	28394	0.65	39.514	0	1	0.95	48.617	0	1	1.05	51.159	3.19	2	1.33	57.134	6.22	3
20	SMc00713	chaC CATION TRANSPORT	22		IV.D.4 cations	20409	0.98	49.376	0	1	1.30	56.585	2.49	2	0.66	39.783	0.92	2	1.31	56.734	2.4	2
21	SMb21344	PUTATIVE SUGAR UPTAKE ABC TRANSPORTER ATP-BINDING PROTE	15		IV.D.3 carbohydrates, o	53705	0.66	39.695	1.4	2	1.31	56.76	1.01	2	0.53	34.552	0	1	1.14	53.359	0.64	2
22	SMc02082	tolC OUTER MEMBRANE SECRETION PROTEIN	351	1	IV.E.2 protein, peptide s	48303	1.23	55.211	5.86	3	0.47	31.785	6.3	3	1.37	57.882	6.41	2	1.13	53.069	0	1
23	SMc02400	OUTER MEMBRANE PROTEIN	51	1	III.B.2 membrane, outer	37520	1.31	56.73	2.6	6	1.25	55.481	2.44	8	1.06	51.445	2.56	7	1.43	58.858	4.95	5
24	SMc00868	atpF TRANSMEMBRANE ATP SYNTHASE B CHAIN (EC 3.6.1.34)	164	1	I.E.2 ATP-proton motive	17382	1.03	50.858	2.34	2	0.91	47.652	0	1	1.20	54.603	2.15	2	1.07	51.627	0	1
25	SMc01311	tufA ELONGATION FACTOR TU	371	1	II.B.7 proteins and pept	42748	1.08	52.02	0.01	2	1.05	51.335	6.81	3	0.94	48.351	3.48	3	1.28	56.106	0.18	2
26	SMc02501	atpD ATP SYNTHASE BETA CHAIN (EC 3.6.1.34)	470		I.E.2 ATP-proton motive	53282	0.73	42.223	3.88	12	1.08	51.995	6.02	9	1.07	51.719	3.46	17	1.00	49.896	6.41	6
27	SMc04439	ABC TRANSPORTER GLYCINE BETAINE TRANSPORT ATP-BINDING PR	80	1	IV.D.2 amino acids, ami	32570					1.52	60.357	0	1					2.52	71.578	0.06	2

Remark; EL = expression level; QC = quantified score, TMDs = transmembrane domains

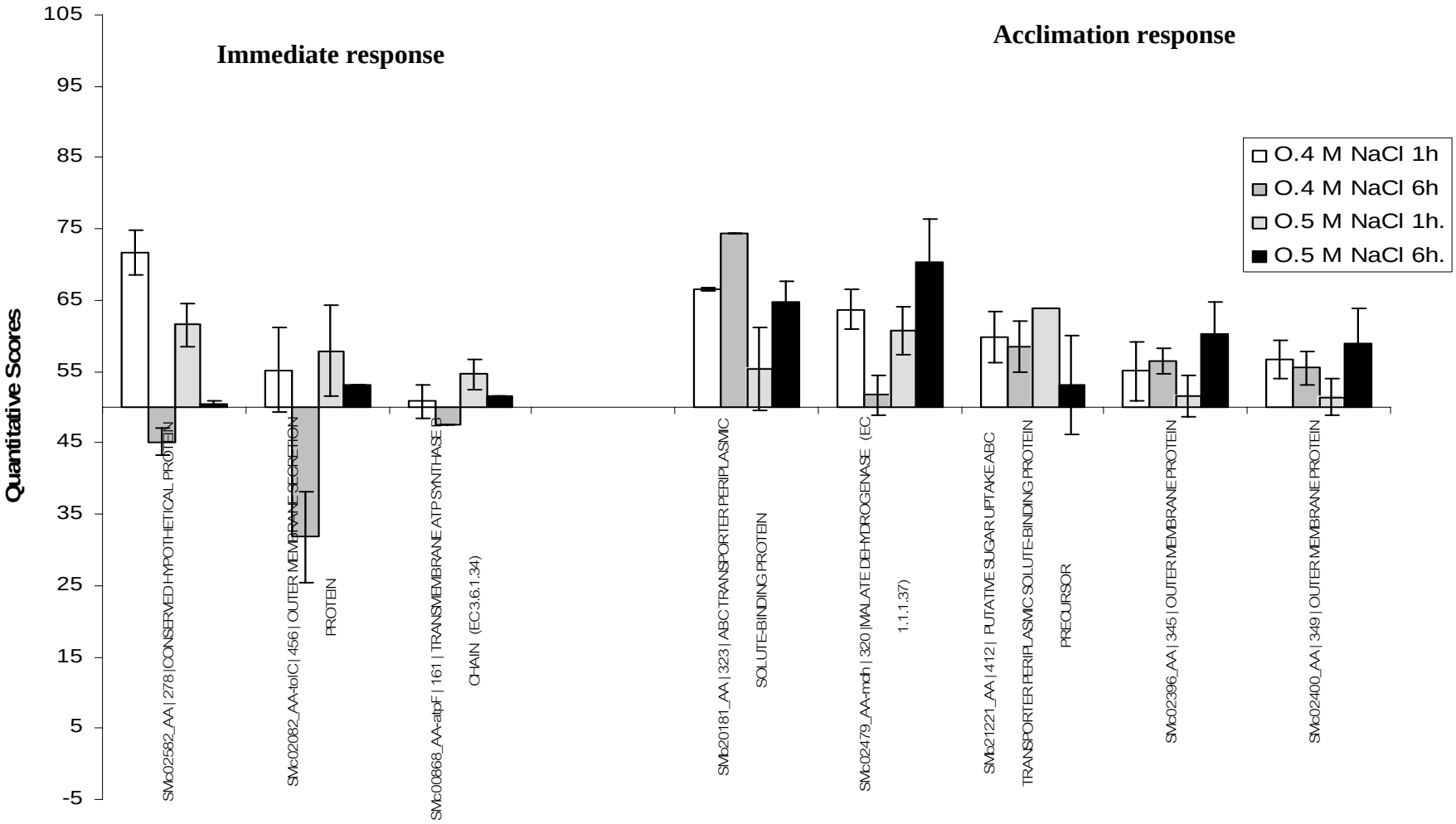


Figure 5.7 (A) Immediate and acclimation response membrane proteins under salt stress.

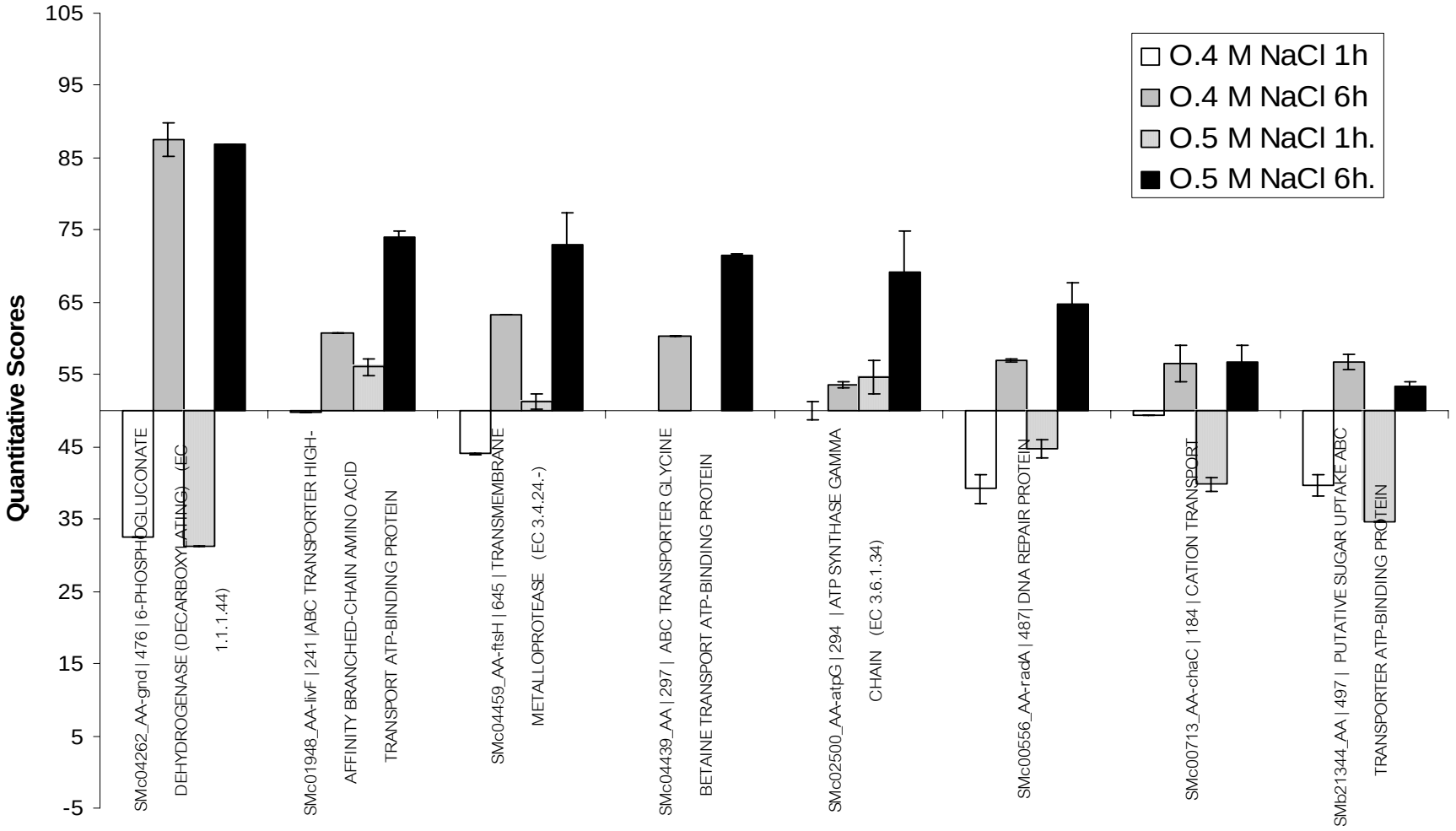


Figure 5.7 (B) Late response membrane proteins expressed under salt stress.

Therefore, the expression of these proteins was hypothesized in relating to DNA and bimolecular synthesis. It was reported that this cycle does not appear to function under normal conditions in bacteria (Moat and Foster, 1995), therefore, the high level induction of this protein appears to be related to the salt stimulus.

Cytochrome C oxidase (ctcC) subunitII was found to be induced only under 0.5 M NaCl for 6 hour. The ctcC is responsible for electron transport system and it is also believed that it is responsible for decreasing reactive oxygen species such as O_2^-, OH and H_2O_2 within cells under salt stress (Ryu et al., 2003). Therefore, it is possibly that at 0.5 M NaCl, the reactive oxygen species are more generated than that 0.4 M NaCl which can affect growth and consequently their stringency. Moreover, DNA repair protein (radA) has been stimulated the expression at level 1.8x under 6 hours 0.5 M NaCl. This protein was categorized in oxidative stress regulon in *E. coli* (Moat and Foster, 1995). Therefore, this finding supports the evidence that under salt stress, the reactive oxygen species are generated that cause the damaging any biological macromolecule. Dmitrieva and Burg (2004) reported the broken of DNA under high NaCl culture in mouse inner medullaly collecting duct cells. In *B. subtilis* and *L. monocytogenes,* showing that GuaB needed for purines synthesis when DNA is being repaired after peroxide shock (Antelmann, 1997). Thus, the expression of DNA repair protein and 6-phospho gluconate dehydrogenase might co-expression for cell recovery pathway.

One interesting finding is that the detection of transmembrane metallo protease; *fts*H; which also has a function related to cell recovery shows strong induction after shift to salt for 6 hours with quantitative scores of 63.26 (1.72x) and 72.99 (2.7x) under 0.4 M, 0.5 M NaCl, respectively. It has been reported that this

protein is required for growth of *E. coli* and is responsible for rapid turnover of key proteins by processively degrading both cytoplasmic and membrane proteins in concert with unfolding, secretion of proteins into and through the membrane, and mRNA decay (Jayasekera, 2000). Salt stress conditions combined with high ionic strength in the cell may cause the aggregation of several proteins, thus this metalloprotease might play an important role in removing these denatured proteins. Various other bacteria including *Bacillus subtilis* and *L. lactis,* (Deuerling, 1995), *Helicobacter pylori* (Ge and Taylor, 1996), *B. japonicum* (Narberhaus et al., 1999) and *Oenococcus oeni* (Bourdineaud, 2000) have beeen reported to show the upregulation of this protein under stress condition.

Therefore, these late response membrane proteins expression suggests that the expression are involved in bimolecular synthesis and repair mechanism which link to function of cell recovery pathway.

A group of transportation proteins were upregulated in either acclimation or late response. Three transportations proteins were acclimated expressed (Figure 5.7(A) and Table 5.3). SMb20181, an ABC transporter periplasmic solute-binding protein was upregulated by 1.99x, 2.90x, 1.24x and 1.84x under 0.4 M NaCl (1 and 6 h) and 0.5 M (1 and 6 h), respectively. SMb21221; putative sugar uptake ABC transporter periplasmic solute-binding protein, was upregulated almost the same level (~1.76x) in all conditions. SMc02121; ABC transporter general L-amino acid transport ATP-binding protein (aapP), was upregulated 1.57x and 1.88x under 0.5 M NaCl at 1 and 6 h. The late response expression could observe 5 transporter proteins (Figure 5.7(B) and Table 5.3). SMc01948; ABC transporter high affinity branched-chain amino acid transport (livF), was significantly upregulated 1.5x and 2.8x after 6

hours exposure to salt 0.4 M and 0.5 M NaCl, respectively. SMc01499; ABC transporter ATP-binding protein (smoK), was increased 1.57x after 6 h exposure to 0.5 M NaCl. SMc02169, ABC transporter ATP-binding was minor upshift (1.3x) under 6 h exposure to 0.5 M NaCl. SMc04439; ABC transporter glycine betaine transport ATP-binding protein, were up regulated 1.52x and 2.52x after 6 h exposure to 0.4 and 0.5 M NaCl, respectively. SMb21344; putative sugar uptake ABC transporter periplasmic solute-binding protein, is slightly express after 6 h. The group of these proteins are well documented in being related to salt stress response (Yale and Bohnert, 2001; Ruberg, 2003 and Steil et al., 2003). Steil and co-workers studied genome wide expression of bacillus under salt stress showing that a distinct group of 41 genes are either involved in the uptake or synthesis of compatible solutes; such as glycine betaine transporter, choline ABC transporter, ABC transporter, exhibited longer-lasting induction (Steil et al., 2003). Recently, BetS could be identified as a major glycine betaine/proline betaine transporter required for early osmotic adjustment in *S. meliloti* (Boscari et al., 2002). Induction of ion transporters were also detected in this examination. ATP-synthase could be observed in both epsilon chain and gamma chain. These proteins exhibited longer-lasting induction (~2.45x) under 0.5 M NaCl (Table 5.3). The upshift by 1.31x of cation transport protein after 6 hours exposure to salt has also been observed. It is relatively similar to several reports which suggested that monovalent cation-proton antiporters are essential for maintaining a neutral cytoplasmic pH. In *B. subtilis*, monovalent cation/proton antiporters also seem to be important since the *mrpA* gene encoding an Na^+/H^+ antiporter is involved in Na^+-dependent pH homeostasis. These transporters probably extrude Na^+ and/ or H^+, perhaps in a nonspecific manner (Vasseur, 2001).

Some proteins do not express in the correlated pattern between 0.4 and 0.5 M NaCl. UDP-N-acetylglucosamine 1-carboxyvinyl transferase was upregulated under 0.4 M NaCl after 6 hours exposure to salt, however under 0.5 M NaCl, this protein was induced after the first hour. It might be responsible for enhancing the rigidity of the cell wall to avoid collapse. Transmembrane NAD(P) transhydrogenase subunit Beta was only expressed under 0.5 M NaCl 6 hours salt conditions. The mechanism related to salt have not yet been reported, however this protein is known to be involved in central metabolism thus it might be needed for biosynthesis of biological molecules or some metabolism pathways.

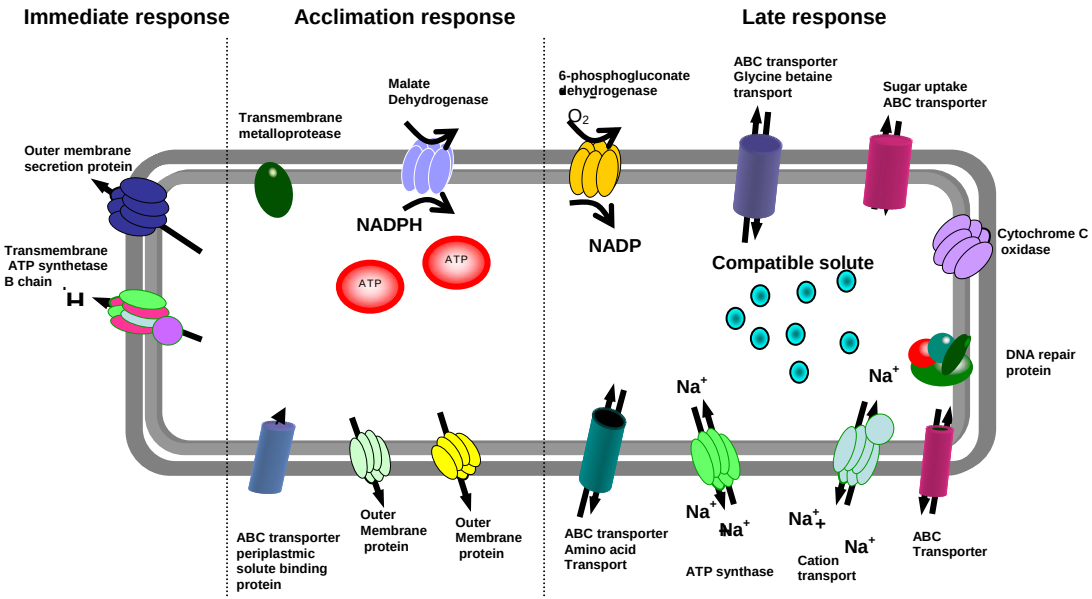


Figure 5.8 Schematic model of membrane proteins expression of *Sinorhizobium sp*. BL3 under salt stress condition.

CONCLUSION

Salt stress contributes simultaneously to both osmolarity and ionic strength, and it may also have ion specific effects on bacterial physiology. Under these conditions the preemptive general stress response system is engaged to ensure survival. However, the global net works of response system is still mysterious. The studying of membrane subproteome is a challenge prospect to explore such mechanism in protein expression level. Multidimentional LC-MS/MS enabled to identify and characterize the membrane proteome of *Sinorhizobium* BL3. Four hundred and twelve membrane proteins were identified, in which the major categories are cell process, small molecule metabolism and hypothetical/global homology protien. Among them, 26 proteins were experimentally identified as membrane proteins in *S. melliloti* and 117 proteins have been reported to be related to salt stress in other organisms. The expression profiles of the proteins under stress could be placed in three groups including immediate response, acclimation response and late response. A group of transportation proteins (10 proteins) were either activated as acclimation or late response. The immediate response exhibited upregulate of SMc02582; conserved hypothetical protein with the underline of new candidate of salt stress protein needed to be more characterized, the tolC and the transmembrane ATP synthase B could be importance to maintain intracellular concentration and cell integrity under salt stress. The acclimation response exhibited the upshift of malate dehydrogenase and transportation proteins. Moreover, a group of late response

proteins demonstrated a function relating to biomolecular synthesis and cell recovery mechanism including 6-phosphogluconate dehydrogenase, DNA repair protein and cytochrome C oxidase (ctcC) subunitII. A group of proteins involving in ion, amino acid and protein transportation are stimulated either on immediate or late response. This identification of large scale membrane proteome and analysis of the expression shift under certain condition gave the first insight to better understanding the complex role of protein and for future investigation of the response protein at molecular level for more understanding the salt stress control membrane complex network.

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CHAPTER VI

OVERALL CONCLUSIONS

This experiment provides the evidence that the nodulation competitive ability of *Bradyrhizobium* strain is influenced by biotic factors. Competitive potential of each *Bradyrhizobium* strain depend upon its intrinsic genotype and phenotype, which each strain exhibit the differences in SCA and GCA. However, increase in amount of inoculum can enhance the competitiveness of *Bradyrhizobium* strain. Moreover, not only *Bradyrhizobium* strain and amount of inoculum but also soybean varieties have an effect on competition. The preferences of soybean varieties on nodulation by *Bradyrhizobium* strain are diverse. Therefore, to achieve the most potent in nodulation, these biotic factors need to be considered.

This work also demonstrated the influence of salt stress; which is one of the abiotic factors; on nodulation competitiveness. The salt tolerant bradyrhizobia strains have been constructed by transferring cosmid contained salt tolerance genes from two different regions of *Sinorhizobium sp.* BL3 genome. Result indicated that cosmid containing ATPase, transcriptional regulator syrB (AraC family), antirestriction protein, xanthine dehydrogenase, DNA methylase, partitioning protein, conserved hypothetical protein or betaine aldehyde dehydrogenase cluster (*bet*A and *bet*B gene) could improve salt tolerant ability of *Bradyrhizobium.* However, the nodulation competitiveness of such recombinant strain under salt stress environment could not be enhanced. In this point, genes using for creating such superior strain might not play

the key role in controlling salt tolerant mechanism, thus these new recombinant *Bradyrhizobium* could not perform the expression of competition in full capacity.

By the gene expression analysis, the two groups of salt tolerant genes could be isolated as described above. These results provide the evidence that salt tolerant mechanism controlled by several genes or proteins. Additionally, the study has identified the global expression change of membrane proteins related to salt tolerant by proteomic approach in order to obtain more information to understand the overall salt tolerant mechanism. Results demonstrated the sequentially expressed of membrane proteins with responsible for several functions. The proteins related to energy metabolism were more or less rapidly induced but still over expressed several hours after the downshifts. The late response proteins demonstrated the expression of proteins involved in DNA synthesis, DNA repair protein and cytochrome C oxidase (ctcC) subunitII. Interestingly, a group of transportation proteins involved in ion transporter, amino acid and proteins transporter were revealed the induction in various stages of response.

Therefore, using gene analyses and proteomic analyses, isolation and identification of candidate genes of the salt tolerant response could be done. This study demonstrated that salt stress response required the coordinated expression of many genes through the altered expression of global network. However, the complete salt tolerant mechanism still largely remained unclear. But, this could be the first insight to understand the basis of salt stress responses in *Rhizobium*. In future work, it will be of great interest to identifying and overexpressing some signaling elements to turn on many down stream effecter genes which might be the successful strategy to construct superior salt tolerant rhizobia strains.

APPENDIX

Identified membrane proteins of *Sinorhizobium sp* . BL3

Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
I	I Small Molecule Metabolism	127	SMa0830	nifE	NIFE OXIDOREDUCTASE	1	/			3	AAEK
											AKMPWLDINQER 3-M_Oxidation/
											ARAAMMVCSTALINLARK 5-M_Oxidation/6-M_Oxidation/
		505	SMc04342		PUTATIVE METHYLTRANSFERASE PROTEIN	1	/			3	AADYGIKPHDIVVDPLVMPIGALGSAGQQVFALLR
											TLEIVQGLVDVPLSIDSSVTAAIEAGLR
											AADYGIKPHDIVVDPLVmPIGALGSAGQQVFALLR 18-M_Oxidation
		251	SMb21107	manR	PUTATIVE MANDELATE RACEMASE OR EVOLUTIONARY RELATED ENZYME OF THE	2	/			3	ANGFSGSKVK
											QKLPLWKLAGGAKESCPLYTTEGGWLHIEK
											ANGFSGSKVKIGKPSGAEDYDR
		142	SMc00270		TRANSKETOLASE ALPHA SUBUNIT	2	/			1	RQDPEWEGR
		130	SMb20648		PUTATIVE OXIDOREDUCTASE	1			/	1	SPLAMGFLTGK 5-M_Oxidation
		127	SMc00410		OXIDOREDUCTASE (EC 1.-.-.-)	1	/			3	AEAAILETLPAAVILRPSIIFGPEDGFFNK
											HVVRALAK
											ECLDIMLKTIDRKR 6-M_Oxidation
		42	SMc00985		OXIDOREDUCTASE SMALL MOLECULE METABOLISM	1	/			6	LFPLSLGSEGSAR
											RIKHAFDPAGIMNPDK
		114	SMb21056		PUTATIVE PROTEIN, SIMILAR TO ESTERASES	1	/			4	DIGK
											ALVIDIDQDGR
											WKRHELGAAGGWGKDIK
											TLQFHWLKIVDAAAHK
		113	SMc00108		ACETYLTRANSFERASE	1	/			3	EQAK
											GTGIGRHLVAK missed clevage
											APMVTIRCAKPR
		106	SMa1406	ttuD3	PUTATIVE TTUD3 HYDROXYPYRUVATE REDUCTASE	2	/			3	DVGK
											KQVSRIK
											GGRLAAACHPAK
		62	SMb20406	hyuA	HYDANTOIN UTILIZATION PROTEIN A	1	/			3	VDAICIMTIFSHVNPVHEK
											VSKYGEAGFSLMELGVIATAEK
											LNVPTLHLDTIGAGAGMILKVDPLTRK
		99	SMc01987		DEHYDROGENASE	2	/			3	GKAK
											GLWYCVAIWVK
											LSSAKAFGTR
		98	SMb21534		PUTATIVE DEHYDROGENASE	1	/			3	LASK
											RSPLYDR missed clevage
											ALAEWIVNGAPTMDLWPVDIRR missed clevage
		94	SMc04148		AMINOMETHYLTRANSFERASE (EC 2.1.2.-)	1	/			1	QPTIGELEWFR
	I.A Amino acid biosynthesis	120	SMa0707		DIHYDRODIPICOLINATE SYNTHASE, PUTATIVE	1	/			2	YVK
											WRR missed clevage
	I.A.1 leucine	169	SMc03823		3-ISOPROPYLMALATE DEHYDRATASE LARGE SUBUNIT (EC 4.2.1.33)	1	/			2	HLVHEVTSPQAFEGLR
											RTSKWRALDYMGLKPGTK 11-M_Oxidation/18-
	I.A.2 isoleucine/valine	115	SMb20890	ilvD5	PUTATIVE DIHYDROXY-ACID DEHYDRATASE (EC 4.2.1.9)	1	/			5	ALTK
											EKALTKSGGIAVLR
											GYPGMAEVGNMGLPPK 11-M_Oxidation
											GNPVDGVVLLGGCDK
											IVEMVKEDLKPSDILTKEAFENAIR
		65	SMb20115		DIHYDROXY-ACID DEHYDRATASE	1	/			4	TTPALVMGAISAGLPMIFLPSGPMLR
											GGPGMPEWGMLPIPKK
											TIDMLVDEEILAMR
											IVEMVHEKLGPEKIITEK
	I.A.4 histidine	153	SMa0398	hisD2	PROBABLE HISD2 HISTIDINOL DEHYDROGENASE (EC 1.1.1.23)	1	/			9	YKDKTMTSVSFYEYSK
											WARTFGPLSVTDFVKR
											TEGDKALARFGRELDK
											TAFSKTVLSGK
											SSIGYVTAPAYPEFAR
											RSETDISGFIEKVAPILEAVR
											LNAEEKAALLR
											KFHEEQKPEAMWLK 11-M_Oxidation/

Continued

Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		144	SMa0306		PUTATIVE HISTIDINE AMMONIA-LYASE (EC 4.3.1.3)	1		/		4	AGGAQAVAAVAYGTETVKPALK DRK AAMAVRLNNILTGGPGVQPHVAEMLLAFLNK 3-M_Oxidation ALVQIQINSSDDNPGIVVGVEPKSDLFQAR
		116	SMb21163	hutU	PUTATIVE UROCANATE HYDRATASE (UROCANASE) (EC 4.2.1.49)	1	/			4	GWTMAEWKAKRESDPK LRTRYLDEKAETLEEAMEMIER 19-M_Oxidation/ LGLAFNEMVRSGELK GTEISAKSWLTEAPLR
	I.A.6 tryptophan	104	SMc02766	trpB	TRYPTOPHAN SYNTHASE BETA CHAIN (EC 4.2.1.20)	1					VEYVPIMDHEALEAFQTLTR IIAETGAGQHGVASATVAAR MGKDEIILMNLSGRGDKDIFTVGK missed clevage LEGIIPALEPSHALAEVIK GDKDIFTVGKILGmGQ 14-M_Oxidation/
	I.A.7 tyrosine	105	SMc00711	tyrC	CYCLOHEXADIENYL DEHYDROGENASE AND ADH PREPHENATE DEHYDROGENASE	2	/			3	HHDK
		85	SMc00387		TYROSINE AMINOTRANSFERASE PROTEIN	1	/			2	GLLPLVDLAYQGFGR SLAEGLRTR missed clevage
	I.A.10 serine	226	SMc00641		D-3-PHOSPHOGLYCERATE DEHYDROGENASE (EC 1.1.1.95)	1				3	SVAGTVFSDGKPR LADVLGAFVGQVTESAIKEIEILYDGSTAAmNTK 31-M_Oxidation/ LAEIIGNYDGLAIR
	I.A.11 glycine	201	SMc01770	glyA1	SERINE HYDROXYMETHYLTRANSFERASE (EC 2.1.2.1)	1	/			2	DYAAQVVkNARTLAETLK NGIPFDPEkPFVTSGVRLGAPAGTTR
	I.A.16 proline	93	SMb20003		PYRROLINE-5-CARBOXYLATE REDUCTASE	2	/			2	LKFRKGQK DKAR missed clevage
	I.A.17 arginine	70	SMc02138		PUTATIVE ACETYLORNITHINE AMINOTRANSFERASE PROTEIN	1	/			2	FHVITFEGAFHGR VPSADLLKAIRAEKLLVVPAGENVLR missed clevage
	I.A.18 glutamine	416	SMc00762		PUTATIVE GLUTAMINE SYNTHETASE PROTEIN	1			/	3	HGDPIELADQVFLFKR missed clevage RGEFETFMQVISPWER missed clevage SILGGQGYSIAGINEFDELIDDIYHFSEK
		70	SMc04405		PROBABLE 3-ISOPROPYLMALATE DEHYDROGENASE PROTEIN	1	/			3	WDAVPYEVRPEAGLLR IAGVAFELAR NVMKSGVLWNQVVTETHK
		144	SMc01124		glnD | PROTEIN-PII URIDYLYLTRANSFERASE (EC 2.7.7.59)	2	/			5	LPEVIASRTRVK KERQAYVR missed clevage NTGPEFIAAKLAER missed clevage FMKHYFLVAK DLHTLFWISKYFYRVKDSADLVK missed clevage
	I.B.3 cobalamin	156	SMc04304	cobW	COBALAMINE BIOSYNTHESIS PROTEIN	1	/			3	IPATVITGFLGAGK ENRPDHIVIETSGLALPQPLVAAFNWPDIR FADDHDKVDALR missed clevage
		101	SMc04281	cobC	COBALAMIN BIOSYNTHESIS PROTEIN PYRIDOXAL-PHOSPHATE-DEPENDENT AMIN(	1	/			1	LAEALNR
		89	SMc04214		PROBABLE NICOTINATE-NUCLEOTIDE--DIMETHYLBENZIMIDAZOLE PHOSPHORIBO	1	/			1	EIAAMAGAILAAR
		58	SMc03193		PROBABLE COBALAMIN BIOSYNTHESIS PROTEIN	1			/	1	GSCPSLAAPMQTGDGLLVR
	I.B.5 menaquinone, ubiquinone	196	SMc01156		UBIQUINONE BIOSYNTHESIS PROTEIN	1	/			2	LAETLIQSFLR MQER 1-M_Oxidation
	I.B.13 lipoate	153	SMa1591		PUTATIVE ADENYLATE CYCLASE	1			/	5	QKK LGDK LFPVLYGDWVFHVVR HLTAEDLIGVGVASVGHRR missed clevage IAGTGAFLRGEIANAR missed clevage
	I.D.1 pyrimidine ribonucleotide biosynth	262	SMc01025		CTP SYNTHASE (EC 6.3.4.2)	1		/		4	ERK EGLDNEVLAAFGIEPAPKPR FTGRSATKTDNITTGRIYK missed clevage DAYKSLIEALYHGGIANRVKVK

Continued

Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		207	SMc01237		RIBONUCLEOTIDE REDUCTASE	2				8	VAPNSPQWFNTGLHWAYGIDGPGQGHFYVDPFTGK YGSGTGSNFSHLR MVVVDIDHPDIEEYINWK RVEENDVPSFLWR missed clevage SAYEHPQPHACFIQSVADDLVNEGGIMDLWVR NATSILDYVFR YLSVESHIR DDFLRAVEADGDWHLTAR missed clevage
		197	SMc01215		CARBAMOYL-PHOSPHATE SYNTHASE LARGE CHAIN (AMMONIA CHAIN ARGININE I	1	/			3	LVVIEmNPRVSRSSALASK 6-M_Oxidation/ LVVIEMNPRVSRSSALASK missed clevage VMATGGTARFLGEQGIVATK missed clevage VECGRLYEGDmmR 11-M_Oxidation/12-M_Oxidation/ EVEIIPLEIVVRNVAAGSLAK missed clevage
		120	SMc00488		PHOSPHORIBOSYLFORMYL GLYCINAMIDINE SYNTHETASE II (EC 6.3.5.3)	1	/			2	WLR SSKKWLRTLPTK
		96	SMc00394		GMP SYNTHASE GLUTAMINE-HYDROLYZING (EC 6.3.5.2)	1	/			2	ELFK HPFPGPGLAIR
	I.E Global functions	152	SMc04292	cyaF3	ADENYLATE/GUANYLATE CYCLASE	1	/			3	WLR VDFPLTEIGCRTFADLPGPLR missed clevage FVRDLHRAGLPD missed clevage
		118	SMc04307	cyaD2	TRANSMEMBRANE ADENYLATE/GUANYLATE CYCLASE (EC 4.6.1.1)	1	/			2	MARTQLVGVIIGLAIVAALTVLRATDPPLLRLAR missed clevage ELTImFVDVRNFTEISERLTPGEVVR 5-M_Oxidation/
		97	SMc01492	suhR	TRANSMEMBRANE SUPPRESSOR OF E.COLI RPOH THERMOSENSITIVE MUTATION	3	/			2	EVLAAVSkkGTAAGEAFAR KGTAAGEAFAR
		231	SMc00383		PUTATIVE GLUTATHIONE S-TRANSFERASE PROTEIN	1	/			1	TAQLTLISHHLCPYVQR
	I.E.2 ATP-proton motive force interconversion	565	SMc00868	atpF	TRANSMEMBRANE ATP SYNTHASE B CHAIN (EC 3.6.1.34)	1	/			6	TALSEQKIKQAESDAINAVR missed clevage RKDAEAEAASIVAAAQR missed clevage RTALSEQKIKQAESDAINAVR missed clevage IKQAESDAINAVR missed clevage ADKISNELAEAKR missed clevage DAEAEAASIVAAAQR
		212	SMc00869	atpF2	TRANSMEMBRANE ATP SYNTHASE SUBUNIT B' (EC 3.6.1.34)	1	/			5	GHSIADTAREAAK missed clevage IGGILETRHDR missed clevage IAQDLDEASR AKADRDGVEAGLAK AKGHSIADTAREAAKAK
		48	SMc00870		PROBABLE ATP SYNTHASE SUBUNIT C TRANSMEMBRANE PROTEIN	1	/			1	YIGAGLACLGMAGTALGLGNIFGSYLSGALR
		114	SMc00871		PROBABLE ATP SYNTHASE A CHAIN TRANSMEMBRANE PROTEIN	6	/			3	mQSVSEMSYEFIASMLR 1-M_Oxidation/ MQSVSEMSYEFIASMLR MQSVSEmSYEFIASMLR 7-M_Oxidation/
		451	SMc02499		PROBABLE ATP SYNTHASE ALPHA CHAIN PROTEIN					5	HALIGYDDLSK VVDALGNPIDGKGPINAK missed clevage EAYPGDVFYLHSR TGAIVDVPVGPELLGRVVDALGNPIDGKGPINAK missed clevage KSVHEPMSTGLK missed clevage
		145	SMc02500		PROBABLE ATP SYNTHASE GAMMA CHAIN PROTEIN					3	AQEAAEAARPYSQR RAQEAAEAARPYSQR missed clevage QRQAQITKELIEIISGAEAL missed clevage
		248	SMc02501	atpD	ATP SYNTHASE BETA CHAIN (EC 3.6.1.34)					5	RSLGSGYAGIDNTLFYKDGTMMLLGDAK missed clevage VIIVPGYGMAVAQAQHALR DGTMMLLGDAK RSLGSGYAGIDNTLFYK missed clevage YAIHPVAGR
		201	SMc02502	atpC	ATP SYNTHASE EPSILON CHAIN (EC 3.6.1.34)					3	TRLEQFIAELTK missed clevage LEQFIAELTK AELEGAGDEKKTRLEQFIAELTK missed clevage

Continued

Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
	I.F Central intermediary metabolism	248	SMc03938	pntB	TRANSMEMBRANE NAD(P) TRANSHYDROGENASE SUBUNIT BETA (EC 1.6.1.1)	7	/			5	RSLGSGYAGIDNTLFYKDGTMMLLGDAK missed clevage
											VIIVPGYGMAVAQAQHALR
											DGTMMLLGDAK
											RSLGSGYAGIDNTLFYK missed clevage
											YAIHPVAGR
		239	SMb20198	cbbL	RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE LARGE SUBUNIT (EC 4.1.1.39)	1	/			5	WLR
											ERYK
											FGKPLLGATTKPK
											LEDMR
											ERLDKFGKPLLGATTKPK
		171	SMa1440		5-DEHYDRO-4-DEOXYGLUCARATE DEHYDRATASE (dc=3) (EC 4.2.1.41)	1				3	DRK
											VDLVRHVTAK
											KAGYPVSIIK
		188	SMc03895		PROBABLE PYRUVATE CARBOXYLASE PROTEIN	1	/			5	KFAEWVK
											FYFIEVNPR
											FIDTTPELFQQVK
											TVAIWAEEDKLALHR 4448
											LAMVREGAPNLLLQMLLR
		75	SMc03950		PROBABLE PROTON-TRANSLOCATING NICOTINAMIDE NUCLEOTIDE TRANSHYDRO	1	/			1	IAASASLLYAK
	I.F.3 sulfur metabolism	265	SMc04049		SULFITE OXIDASE (EC 1.8.3.1)	2		/		2	YVK
											DGWKVEISGVK
		144	SMb20915		PUTATIVE ARYLSULFATASE (EC 3.1.6.1)	1	/			2	VHLDGYNLmPFLSGSSNDAPR 9-M_Oxidation/
	I.F.5 nitrogen metabolism	119	SMc01942		UREASE ACCESSORY PROTEIN	1	/			3	KAmGEEVR /3-M_Oxidation/
											KDASVPKVWTL missed clevage
											VYK
	I.F.6 nitrogen fixation	223	SMc02124		NITRITE REDUCTASE	1				3	AAIQVHDIGLHLK
											DKSETFLEAYRR missed clevage
											SETFLEAYR
		208	SMa0819		FIXB ELECTRON TRANSFER FLAVOPROTEIN ALPHA CHAIN	1	/			3	MKKGLPK
		169	SMa1179	nosR	NOSR REGULATORY PROTEIN FOR N2O REDUCTASE	1	/			5	MYQAAIHK
											REKFmALSTPASRGEAPAK
											EPIVLIGIPEAKVVASVNALIGKDLGR
											MYQAAIHKNLFKRLLR
											TGTSDWETLVGDGSVR
	I.F.13 gluconeogenesis	107	SMc01126	tme	NADP-DEPENDENT MALIC ENZYME (EC 1.1.1.40)	3	/			5	LISAIPMAVAK 7-M_Oxidation
											VREAVK
											VIGVSIALCR
											VAMLAYSTFGHPSGER
											GNMVAVISNGTAILGLGNLGALASKPVMEGKAVLFK
		147	SMc00025	ppdK	PYRUVATE PHOSPHATE DIKINASE (EC 2.7.9.1)	1			/	5	RTAK
											SALK
											SMPEGLREQVR missed clevage
											SMPEGLREQVREGITR 2-M_Oxidation/
											EMILAEDEAGRRTALAK missed clevage
		101	SMc00169	dme	NAD-MALIC ENZYME OXIDOREDUCTASE (EC 1.1.1.39)	2	/			5	YPRPGK
											EALMDEWK
											FVFRSGFIMKPVFAAAKNAAKNR 9-M_Oxidation/
											GALDCGARTINEEMKMAAVR 14-M_Oxidation
											GVVNMAALAVVESSHPV
	I.F.14 amino acids	191	SMc00294		AMINOTRANSFERASE (EC 2.6.1.-)	1				7	RLPPYVFEQVNR missed clevage
											AAGADIIDLGMGNPDLPTPQSIVDKLCEVVQDPR missed clevage
											FRHLGSLEFSK missed clevage
											MGFAVGNER
											HLGSLEFSK
											TFSMPGWR
											HLGSLEFSK

Continued

Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
	I.F.15 polyamine biosynthesis	199	SMc01967	speB2	AGMATINASE (EC 3.5.3.11)	1	/			2	AKK
											VADK
		100	SMc04016	hss	HOMOSPERMIDINE SYNTHASE	1				3	NAKKHK
											YDKNRLIVVEPR
											ALLNLADDLGLK
	I.F.18 pool of unassigned individual reversibles reactions	251	SMc04028	gltB	GLUTAMATE SYNTHASE NADPH LARGE CHAIN (EC 1.4.1.13)	1			/	6	SAIK
											SSLAGKHPYR
											IISDEEVKSSLAGK
											MIEHWKAR 1-M_Oxidation
											MNTIRGLFTIK
											EVDEKEVVYRYIKAVGK
		172	SMc02728	fhs	FORMATE--TETRAHYDROFOLATE LIGASE (EC 6.3.4.3)	1	/			3	VMDMNDRALR missed clevage
											REDLGRESVEAVRK missed clevage
											TQYSFSTDPNLR
	I.G Energy transfer	199	SMa1391	etfB2	PROBABLE ETFB2 ELECTRON TRANSPORT FLAVOPROTEIN, BETA SUBUNIT	1				5	AKK
											LKEAGK
											VKADGSGVELANVK
											LKEAGKASEVVVVSIGPGKAEETLR
											ILVTVKRVVDYNVK
		283	SMa1389		probable EtfA2 electron-transport flavoprotein, alpha-subunit	1	/			2	VVAPDLYIACGISGAIQHLAGMK
											VVAPDLYIACGISGAIQHLAGMKDSK missed clevage
		283	SMc00728		PUTATIVE ELECTRON TRANSFER FLAVOPROTEIN ALPHA-SUBUNIT ALPHA-ETF FLA	1				2	VVAPDLYIACGISGAIQHLAGMK
											VVAPDLYIACGISGAIQHLAGMKDSK missed clevage
	I.G.1 electron transport	128	SMa0769	FixP2	cytochrome c oxidase	1				1	IGDPVVKELAVFVHSLGGGE missed clevage
		96	SMa1213	FixP1	Di-heme cytochrome c	1				1	HVDEVSGVETTGHEWDGIR
		112	SMb20174		CYTOCHROME C	1		/		3	KEAK
											GADALPRGRPAKK
											EAKSDAIK
		211	SMc02897		PUTATIVE CYTOCHROME C TRANSMEMBRANE PROTEIN	1		/		4	ANLIAWLR
											HVPGTAMGFAGIK
											KHVPGTAMGFAGIK
											HVPGTAMGFAGIKK
	I.H Energy metabolism, carbon	482	SMb20984		nirB | PUTATIVE NITRITE REDUCTASE [NAD(P)H], LARGE SUBUNIT (EC 1.6.6.4)	1	/			9	TEDEALEHIVALTQMYR
											FMWGSWTPAK
											MLEELFEK
											GMDVTVLHVMPTLMER
											QlmDDVEKR 3-M_Oxidation/
											VVTKANTKRILGEEK missed clevage
											GMDVTVLHVMPTLMER
											AIADVVDKFNVPLVK missed clevage
											QlmDDVEKR 3-M_Oxidation/
		182	SMa1021		PUTATIVE CYTOCHROME C-LIKE PROTEIN	1	/			3	AKADRDVR
											QDGMKAMAAAAK 4-M_Oxidation/7-M_Oxidation
											MQSGDAMIGGPLAR 1-M_Oxidation/
	I.H.2 anaerobic respiration	148	SMc00609	dmsA	ANAEROBIC DIMETHYL SULFOXIDE REDUCTASE CHAIN A (EC 1.8.99.-)	1				4	IVVIDVYDNPTIK
											LMVPQRRVGAKGEGR 2-M_Oxidation
											LMVPQRRVGAK
											GEIRLHAKIGGGTRR
	I.H.3 aerobic respiration	112	SMa0045	cah	PROBABLE CARBONIC ANHYDRASE, CAH (EC 4.2.1.1)	2		/		6	RGDK
											KFTALYSMNARPALAGNR
											KFTALYSMNARPALAGNRR
											KFTALYSMNARPALAGNRRYVLSSS
											ADIPELTADWK
											GAVKADIPELTADWK

Continued

Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		101	SMb21368		PUTATIVE CYTOCHROME C OXIDASE CHAIN II (EC 1.9.3.1)	3	/	/		1	LPDRHVEEAAR
		599	SMc00009	ctaC	CYTOCHROME C OXIDASE SUBUNIT II (EC 1.9.3.1)	3	/			3	IDAVPGRLNETWFK missed clevage
											LNETWFK
											LLAVDNEVVVPVGKTVR missed clevage
		209	SMc00010		PUTATIVE CYTOCHROME C OXIDASE POLYPEPTIDE I TRANSMEMBRANE PROTEIN	11	/			2	AELQEPGIQIFHGLAQMVYGFEGDAAIDGGK
											WFLSTNHK
		586	SMc00188		PROBABLE CYTOCHROME B TRANSMEMBRANE PROTEIN	9	/			4	RIPNSITEAVLEK missed clevage
											WVDSRLPLPR missed clevage
											TPAHIVPEWYYLPFYAMLR
											TPAHIVPEWYYLPFYAmLR 17-M_Oxidation/
		135	SMc01923		PROBABLE NADH DEHYDROGENASE I CHAIN J TRANSMEMBRANE PROTEIN	5	/			1	GIAMPIPAPSER
		118	SMc01913		NADH-UBIQIONONE OXIDOREDUCTASE SUBUNIT K TRANSMEMBRANE PROTEIN	1			/	1	YDAERFGFAPR missed clevage
		52	SMc01925		PROBABLE NADH DEHYDROGENASE I CHAIN L TRANSMEMBRANE PROTEIN	15	/			2	RLGTFLWKEGDGRVIDGYGPNGIAAR missed clevage
											GLYQFLLNK
	I.H.4 oxidative branch, pentose pathway	115	SMc04262	gnd	6-PHOSPHOGLUCONATE DEHYDROGENASE (DECARBOXYLATING) (EC 1.1.1.44)	2	/			2	DGLK
											DGFIKDLENALLAAK
	I.H.6 TCA cycle	102	SMc01030	pdh	PYRUVATE DEHYDROGENASE ALPHA2 SUBUNIT (EC 1.2.4.1)	1				7	SGKGPIILEMLTYR missed clevage
											DFAGGTIAEFSKEDDLK missed clevage
											DFAGGTIAEFSKEDDLKAYREMLLIR missed clevage
											SGKGPIILEMLTYR missed clevage
											GPIILEMLTYR
											MRSEHDPIEQVK missed clevage
											DFAGGTIAEFSKEDDLKAYR missed clevage
		114	SMc02087	gltA	CITRATE SYNTHASE (EC 4.1.3.7)	2			/	4	IFILHADHEQNASTSTVR
											ITYIDGDEGVLLHR
											YHIGQPFVYPK
											mVASLRmIAK 1-M_Oxidation/7-M_Oxidation/
		136	SMc02464		PROBABLE SUCCINATE DEHYDROGENASE MEMBRANE ANCHOR SUBUNIT PROTEIN	3	/			2	GLGSAKEGTDHFWR missed clevage
											EGTDHFWR
		324	SMc02479	mdh	MALATE DEHYDROGENASE (EC 1.1.1.37)	1	/			9	TGSAYYAPAASAIEMAEAYLKDK missed clevage
											DITAFVLGGHGDTMVPLAR
											IALIGSGMIGGTLAHLAGLK
											DGGAEIVGLLK
											EKLDQIIQR missed clevage
											VMEQVGAGIK
											IALIGSGMIGGTLAHLAGLK
											MARNKIALIGSGMIGGTLAHLAGLK missed clevage
											YAPNAFVICITNPLDAMVWALQK
		96	SMc02481	sucD	SUCCINYL-COA SYNTHETASE ALPHA CHAIN (EC 6.2.1.5)	1	/			3	ARLEKSSSR
											IGIMPGNIFRK
											KPmVGFIAGRTAPPGRTMGHAGAVISGGK 3-M_Oxidation
		44	SMc02487		PROBABLE DIHYDROLIPOAMIDE DEHYDROGENASE (E3 COMPONENT OF 2-OXOGLU	1	/			1	VTVVEFLDTILGGMDGEVAK
	I.H.7 pyruvate dehydrogenase	44	SMc01035		PROBABLE DIHYDROLIPOAMIDE DEHYDROGENASE (E3 COMPONENT OF PYRUVATI	1				2	SAEILDHANHAK
											EHLGGICLNWGCIPTK
	I.H.8 glycolysis	66	SMc01852		PROBABLE PYROPHOSPHATE--FRUCTOSE 6-PHOSPHATE 1-PHOSPHOTRANSFERASE	1	/			3	EKAHVLHR
											GLVKEGENPLR
											VKLTNTADCVKR
	I.I.1 fatty acid	203	SMc02227	fadB	TRANSMEMBRANE FATTY Oxidation COMPLEX ALPHA SUBUNIT(EC 4.2.1.17)(EC 1.1.1.	1	/			8	AMGLVHEVVDPDKLIEAAKAMIK 21-M_Oxidation/
											MTGLFRK 1-M_Oxidation
											MMLTEVILGKETGDR 1-M_Oxidation/2-M_Oxidation/
											IALPEVKVGIFPGAGGTQRVPR
											SLFVSMQELGKGARRPAGVPK
											RPAGVPKTELK
											AKAMGLVHEVVDPDKLIEAAKAMIK 23-M_Oxidation
											THSEGLVKDAIGK

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Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		156	SMc04399		ACYL-COA TRANSFERASE (EC 2.8.3.-)				/	2	AKK IVFSGMFNAGAKLAVADGRLVIEK
		117	SMc02162		LONG-CHAIN-FATTY-ACID--COA LIGASE (EC 6.2.1.3)	1	/			4	DPNLTEEEVK TGDVGFMNAEGLTKIVDRK TELPKSNVGKILRK VKKLVPAWSIPGHLSFKTVLAK
	I.I.3 amino acids	209	SMc02049	gcvP	GLYCINE DEHYDROGENASE DECARBOXYLATING (EC 1.4.4.2)	2	/			6	REK MDK 1-M_Oxidation VDKYWSPVNRVDNVYGDR missed clevage AIAQSVHQKTVRLAMGLEK 15-M_Oxidation EEARAIEDGRMDKVNNPLK missed clevage SKAKAFFIDENCHPQTIALLKTR 2-21-+4*/
		99	SMb20277		AMINOTRANSFERASE (EC 2.6.1.-)	1	/			3	LNARIKKEAMAR 10-M_Oxidation/ KEAMARGLMVYPMGGTIDGQR /4-M_Oxidation/9-M_Oxidation/13-M_Oxidation SPFDPGLKLNARIKK
	I.I.4 carbon compounds	190	SMb20173		METHANOL DEHYDROGENASE PROTEIN, LARGE SUBUNIT (EC 1.1.99.8)	1	/			3	ELYRFKTPSGVIGNVmTYAREGK 16-M_Oxidation AVDAATGKELYR NGDATKGETNTATVMPVKDK
		163	SMb20924	abfA	PUTATIVE ALPHA-L-ARABINOFURANOSIDASE (EC 3.2.1.55)	1	/			3	SDRVKIACMAQLVNVIAPILTK RDVKICFDEWNVWYHDRK missed clevage YIVTIGGVIDYIKAK
		144	SMb20500		ALDO/KETO REDUCTASE	2	/			1	AEEIVGK
		104	SMa1961		PUTATIVE POLYHYDROXYALKANOATE DEPOLYMERASE	2	/			1	LARFRKDK
II	II.A.1 degradation of DNA	122	SMc02292	hsdR	TYPE I RESTRICTION ENZYME R PROTEIN (EC 3.1.21.3)	1	/			4	ISAAIEVIAER GKAMFVAIDKATAVRMYDK AGSKEHLKTLLRGNER missed clevage KISAAIEVIAERIR missed clevage
	II.A.2 degradation of RNA	53	SMc00867		PROBABLE RIBONUCLEASE HII PROTEIN	1	/			1	SVSIAAASIVAK
	II.A.4 degradation of proteins, peptides, glycopeptides	249	SMb20757	bhbA	METHYLMALONYL-COA MUTASE (EC 5.4.99.2)	1	/			6	TLMPQLAEALKK LGHKPKIMVAK TLMPQLAEALKKR LGHKPK LGHKPKIMVAK LGHKPKIMVAKLGQDGHDRGAK
		98	SMc00114	ptrB	PROTEASE II OLIGOPEPTIDASE B HYDROLASE SERINE PROTEASE (EC 3.4.21.83)	1	/			4	IKEDNSSVPmK 10-M_Oxidation/ AENWQAMFKDPSILDPEIR missed clevage ImEAPVEAPQK 2-M_Oxidation/ IKEDNSSVPmK 10-M_Oxidation/
		557	SMc00585	pepA	AMINOPEPTIDASE A/I (EC 3.4.11.1)	1	/			2	EYDKMIDSKFADmKNTGGR 13-M_Oxidation/ LVVMQWK
		53	SMc00857		PUTATIVE PROTEASE PROTEIN	2	/			2	VVLDPFQPEKER missed clevage IRDLAQEKR missed clevage
		60	SMc01135		PUTATIVE PROTEASE IV TRANSMEMBRANE PROTEIN	1	/			2	SRPLKAEPSPFHPPSDEAR missed clevage LVDELGGDDEIR
		171	SMc01440	hflC	HYDROLASE SERINE PROTEASE TRANSMEMBRANE (EC 3.4.-.-)	1	/			5	VYGLRGFEAALSDER missed clevage LAEAELIR ARGNEEGQRRR missed clevage RVYGLRGFEAALSDER missed clevage RVYGLRGFEAALSDERASMMR missed clevage
		555	SMc01441		PUTATIVE MEMBRANE BOUND PROTEASE PROTEIN	1	/			7	EVADAFDEVQRAEQDEDRFVEEANQYANQVLGR missed clevage AEQDEDRFVEEANQYANQVLGR missed clevage GGPPDLEEIIR RPAQDIFRDNR missed clevage GGPPDLEEIIRR missed clevage RPAQDIFR GGRGGPPDLEEIIR missed clevage

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Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		65	SMc01903		PROBABLE ATP-DEPENDENT CLP PROTEASE PROTEOLYTIC SUBUNIT PROTEIN	1	/			3	IMVHQPSGGFQGQASDIER TYEEVEQTLDRDHFMSADEALDWGLIDK missed clevage LNEVYVKHCGR missed clevage
		442	SMc01905	lon	ATP-DEPENDENT PROTEASE LA (EC 3.4.21.53)	1	/			8	DIHVHVPEGATPK TSPATESATYPVLPLRDIVVFPHMIVPLFVGR missed clevage DIVVFPHMIVPLFVGR SNPLFLLDEIDKMGQDFR missed clevage AIDFGIEPPLFDKR missed clevage DIVVFPHmIVPLFVGR 8-M_Oxidation/ SNPLFLLDEIDKMGQDFR missed clevage VLDTDHFGLDKVK missed clevage
		96	SMc02606	soxA1	TRANSMEMBRANE SARCOSINE OXIDASE ALPHA SUBUNIT (EC 1.5.3.1)	1	/			2	NAGLCDVSmLGK 9-M_Oxidation/ ATMQELYEGLEAR
		144	SMc02819		RIBONUCLEASE	2		/		2	DRK ISHFGLLEMSR
		103	SMc02825		AMINOPEPTIDASE (EC 3.4.-.-)	1				2	DLAEASLTVDDPVWRmPLYR 16-M_Oxidation/ LLEMRWGR missed clevage
		179	SMc03931		soxA2| TRANSMEMBRANE SARCOSINE OXIDASE ALPHA SUBUNIT (EC 1.5.3.1)	2	/			2	HMVFANNDRPGIMLASAGR mTGVNRISGAGRLTPARTAR 1-M_Oxidation/
		92	SMc04012		PUTATIVE OLIGOENDOPEPTIDASE F PROTEIN	1				1	DKPYQLDDRLEQLFLEK missed clevage
	II.B Macromolecule synthesis, modification	650	SMc00296		POLY3-HYDROXYBUTYRATE POLYMERASE PROTEIN	1	/			2	MPAANHSFYLR GmQmLAEDIAAGRGELRLRQTDTSK 2-M_Oxidation/4-M_Oxidation/
	II.B.5 polysaccharides	198	SMb21446	glgX2	PROBABLE GLYCOSYL HYDROLASE (EC 3.2.1.-)	1	/			4	VLDTAADEPFGmR 12-M_Oxidation/ EETARmPLPK 6-M_Oxidation/ FTEFKTMVKR VLDTAADEPFGMR
		121	SMc00231	glmS	GLUCOSAMINE--FRUCTOSE-6-PHOSPHATE AMINOTRANSFERASE (NODM PARALOG	2	/			3	YCK AFTCQLAVLAALAVGAGK GTSFPLAMEGALKLK
	II.B.6 RNA synthesis, modification , DNA transcription	94	SMc00324	pnp	POLYRIBONUCLEOTIDE NUCLEOTIDYLTRANSFERASE (EC 2.7.7.8)	1	/			3	FAVLSDILGDEDHLGDMDFK IEGITEEImGVALNQAKGGR 9-M_Oxidation/ MFETHKVEIEWAGRPLKLETGKIAR missed clevage
	II.B.7 proteins and peptides - translation and modification	280	SMc00427	prfC	PEPTIDE CHAIN RELEASE FACTOR RF-3	1	/			4	VRLEDAMK 7-M_Oxidation YPAVKMVAIK TLTAVDAAVMVIDAAKGIEPRTLK 10-M_Oxidation/ IAFVRVCSGK
		327	SMc01311	tufA	ELONGATION FACTOR TU	2	/			9	TTCTGVEMFRK missed clevage GSALAALEDSDKK missed clevage LLDQGQAGDNIGALLR QVGVPAIVVFLNK KLLDQGQAGDNIGALLR missed clevage FAIREGGRTVGAGIVASIVE missed clevage LRFAIR missed clevage GITISTAHVEYETPNR FKAEAYILTK missed clevage
		55	SMb20049	fusA1	ELONGATION FACTOR G	1	/			8	QIPIRDGDRIIGSCDLISER missed cleavage VIDVGVLLTDGQHHSVDSSEYAFR
		327	SMc01326	tufB	ELONGATION FACTOR TU	2	/			9	TTCTGVEMFRK missed clevage GSALAALEDSDKK missed clevage LLDQGQAGDNIGALLR KLLDQGQAGDNIGALLR missed clevage FKAEAYILTK missed clevage GITISTAHVEYETPNR QVGVPAIVVFLNK FAIREGGRTVGAGIVASIVE missed clevage LRFAIR missed clevage
		94	SMc02368	glnE	GLUTAMATE-AMMONIA-LIGASE ADENYLYLTRANSFERASE (EC 2.7.7.42)	1	/			2	LLRRLIRILQER missed clevage YLDYAAIADIHSIKRQIHAHK missed clevage

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Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
	II.B.8 DNA - replication, repair, restr./modif.	101	SMc00292	recJ 3	SINGLE-STRANDED-DNA-SPECIFIC EXONUCLEASE (EC 3.1.-.-)	1	/			2	REK GKLGQLRQFFEER
		70	SMc02586	helO	ATP-DEPENDENT HELICASE	1					AAAGRMAELLGEKVGETVGYRMR VQELFGLK IRDLALPVRLAAmAVAAAEEGR
		650	SMc00556		PUTATIVE DNA REPAIR PROTEIN	1	/			6	KGGICGFVDAEHALDPVYAR missed clevage KLGVDLENLLISQPDTGEQALEITDTLVR missed clevage ALEAALSQIER IIEIYGPESSGK TGELIDLGVK SGAWFSYNSQRLGQGRENAK missed clevage
		312	SMc00760	recA	DNA STRAND EXCHANGE AND RECOMBINATION PROTEIN	1	/			6	KGGICGFVDAEHALDPVYAR missed clevage KLGVDLENLLISQPDTGEQALEITDTLVR missed clevage ALEAALSQIER IIEIYGPESSGK TGELIDLGVK SGAWFSYNSQRLGQGRENAK missed clevage
		153	SMc00932	mutL	DNA MISMATCH REPAIR PROTEIN	1	/			3	RmAIAFPRVR 2-M_Oxidation/ QMEATPGSGQCNHGRPTYIELK LRTEEMNALLR missed clevage
		116	SMc01018	parE	TOPOISOMERASE IV SUBUNIT B (EC 5.99.1.-)	1				6	STLEVVMTVLHAGGK 7-M_Oxidation/ VELGRFKGLGEMLPAQLK 12-M_Oxidation DYLAATLGKEFTVTREIFAGK GIAQGGLEDLGDVHNRRGTRVR missed clevage GLGEMLPAQLKETTMDPAKR 5-M_Oxidation /15-M_Oxidation/ DKLATVEAQRIVENALR
		147	SMc01090	deaD	ATP-DEPENDENT RNA HELICASE	1				7	EHK FDK MLEK 1-M_Oxidation/ LLAAIGPNRMLEKGIRVK 10-M_Oxidation/ AGQKGVSALIVPVNAR missed clevage GDKPWSKKKGKPEAQK WLIPMLCRYGK 5-M_Oxidation/
		156	SMc01167	dnaA	CHROMOSOMAL REPLICATION INITIATOR PROTEIN	1	/			6	VDELLGHLVNAGEPR AQVGPDVFASWFGR SFEPQLSIERVDELLGHLVNAGEPR missed clevage QVVVAADRAPWELESLDSR missed clevage LKAQVGPDVFASWFGR missed clevage SFPEIGRR missed clevage
		173	SMc01235	uvrA	EXCINUCLEASE ABC SUBUNIT A (DNA REPAIR ATP-BINDING)	1				2	LGSRWSELSEEAQEAILHGTK KPKKKK
		153	SMc02760		ATP-DEPENDENT NUCLEASE/HELICASE	1	/			5	RIEAIEGK FLEK DEVRVMTVHAAK AALVLAERLNRDYEALKK AEVSVMGTLKLGVR missed clevage
		146	SMc02782	gyrB	DNA GYRASE SUBUNIT B (EC 5.99.1.3)	1				3	FDK GKSVQYLKDEK GVKEAAVLDmALIGSSDARHIDQLK /10-M_Oxidation
	II.D.1 amino acyl tRNA synthesis; tRNA modification	92	SMa0015	selB	SELB SELENOCYSTEINE-SPECIFIC ELONGATION FACTOR	1	/			6	RILK MIVGTAGHIDHGK ILKLCARLGRVDQIR missed clevage LVLEGAFVRLPGHEVRLSEK KVAIEILEFFDRQGVTIR DFAEALGVDEREIRRILK

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Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		380	SMc00659		PUTATIVE TRNA 5-METHYLAMINOMETHYL-2-THIOURIDYLATE-METHYLTRANSFER	1	/			5	ELGADALATGHYIR
											EGYDVLGITLQLYDHGAAVHR
											LKPNAALAGEIVHLDGR
											RSEREPAAEAALK missed clevage
											AGSCCAGQDIDDAR
		203	SMc00908	ileS	ISOLEUCYL-TRNA SYNTHETASE (EC 6.1.1.5)	1				4	YREK
											YREKGK
											EKGK
											AGLPQKEPETVARWQKMELYK
		135	SMc01100	fmt	METHIONYL-TRNA FORMYLTRANSFERASE (EC 2.1.2.9)	1	/			2	AGELHDRLmQVGAVLmTEAMAR 9-M_Oxidation/16-M_Oxidation/
											GAAPIQRAIAGDRETGMMVMKMDK
		141	SMc02804	leuS	LEUCYL-TRNA SYNTHETASE (EC 6.1.1.4)	1	/			4	AVHIETGEEVAIGSIEKMSKSKK
											TLKAVEADYDKLAFNKAVAR
											VFETKNDDPR missed clevage
											TLKAVEADYDKLAFNKAVAR
III	III.A.1 surface polysaccharides /antigens	156	SMc02273	rkpA	TRANSMEMBRANE FATTY ACID SYNTHASE	6	/			8	VTANPIELLK
											FWHPVMGTPGK
											VTANPIELLKGKRAR
											VSKDTVTRGKILK
											IAAVPHWQKCLADLDLGDVVVTDR missed clevage
											VIATAGTREKR
											AKEAFLSDIPDIAPR
		43	SMb21250		PUTATIVE GLYCOSYLTRANSFERASE					2	RETVAAAAATTPVRDAR missed cleavage
											WVQRRLYPRAFGLVTM
		308	SMc00195	cgmA	TRANSMEMBRANE CYCLIC BETA-1,2-GLUCAN MODIFICATION PROTEIN	4	/			6	NTIK
											FAWQNFPK
											DQMKAEHETPLVVWSNKTGPKK
											KGPKDQMK
											YAKNTIKVEGDLPEADRQVLATYAQGVK
											KGPK
		284	SMc04382	ndvB	TRANSMEMBRANE BETA-(1,2)-GLUCAN PRODUCTION ASSOCIATED PROTEIN	5	/			5	EWFHADPVIEAAELLLQEK
											GLYHVDAFEAALK
											IGELWALPSILR
											GQADLLRPEVR
											SQVQMRHVGITSKEAASFQmLGR 20-M_Oxidation/
		244	SMc02641	rkpK	UDP-GLUCOSE 6-DEHYDROGENASE (EC 1.1.1.22)	1		/		5	RGDGHADLSYVYAAAR missed clevage
											FLHAGPGYGGSCFPK
											IAVLGLTFKPNTDDMR
											EGAAIEDFKRPDR missed clevage
											TPLLVDLR
		190	SMb20813		PUTATIVE LIPID A + LPS CORE EXPORTING ABC TRANSPORTER PROTEIN,	3	/			2	DRK
											KER
		173	SMb21582		PUTATIVE EXOD-LIKE MEMBRANE PROTEIN	3	/			1	LVEGTGRTmKKWSR 9-M_Oxidation
		57	SMc02270		TRANSMEMBRANE CAPSULAR POLYSACCHARIDE BIOSYNTHESIS\EXPORT	5	/			1	AINVKMNTVR missed clevage
		153	SMc01861		UDP-N-ACETYLMURAMOYLALANYL-D-GLUTAMATE--2, 6-DIAMINOPIMELATE LIGA	2				2	SEVPEVIRAEIMAAAK
											AEAIRAAVGMLK
		130	SMb20843		PUTATIVE MEMBRANE PROTEIN, PROBABLY INVOLVED IN ACETYLATING A CELL S	5	/			2	KFISFDPLNIAVGLAFISIGMFK
											YKRmCKSTSPFFR 4-M_Oxidation/
		94	SMb20961		PROTEIN TYROSINE KINASE, MPA1 FAMILY, INVOLVED IN SUCCINOGLYCAN CHAIN	3				5	TLLIDADIR
											VIAIASALPDEGKSIIAANFAALLAASGKR
											AAALKTLYESYLGR
											YEQATQQQSFPIAKAR missed clevage
											SLGYVPLLGTR
		220	SMc02272		ACYL-TRANSFERASE TRANSFERASE PROTEIN	2	/			2	HNDTADLEHVLR
											DLVIHDEFIHNSALAGIK
		110	SMc01794		PUTATIVE POLYSACCHARIDE EXPORT SYSTEM PERIPLASMIC TRANSMEMBRANE PI	1	/	/		1	VPITDPVLAGDTVYIR
		79	SMc00894		PUTATIVE 3-DEOXY-D-MANNO-OCTULOSONIC-ACID TRANSFERASE PROTEIN	1				1	LGSAVVHQYVPLDFKPAVSR

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Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		67	SMc04111		PUTATIVE PILUS ASSEMBLY TRANSMEMBRANE PROTEIN	1		/		2	DVAGLEANLRR missed clevage
											IPILGTLFR
		64	SMb20958		glucosyltransferase protein	1	/			1	RPELAETLR
	III.B.1 membrane, inner	101	SMc01721		PUTATIVE INNER-MEMBRANE TRANSMEMBRANE PROTEIN	5	/			1	MGLQQAMMQLYKEEK missed clevage
	III.B.2 membrane, outer	1343	SMc02094	omp	TRANSMEMBRANE OUTER MEMBRANE PROTEIN	1		/		18	KDVYNPDKLRADEELLR missed clevage
											DVYNPDKLRADEELLR missed clevage
											DVYNPDKLRADEELLRQFYYNR missed clevage
											VDYAVPVAKEDFDEVQNFK missed clevage
											ADEELLRQFYYNR missed clevage
											LRADEELLR missed clevage
											VDYAVPVAKEDFDEVQNFK missed clevage
											TKITQINFVGNEVYSDGRLQSVIATK missed clevage
											GEDSQTYNVSFTEPYFLGYR
											DVYNPDKLRADEELLR missed clevage
											VNLAFVINEGER
											ESGIFSFLTRK missed clevage
											ASVGVSLIWASPFGPLR
											VIDGSPWTR
											ASVGVSLIWASPFGPLR
											ESGIFSFLTR
											KDVYNPDKLRADEELLR missed clevage
											REFDVGEGDAFNQEmVAR 15-M_Oxidation/
		323	SMc02400		OUTER MEMBRANE PROTEIN	1		/		2	AGFFYSWWDK
											AYISFDAK
		290	SMc02396		OUTER MEMBRANE PROTEIN	2		/		1	AGFFYSWWDK
		172	SMb20815		PUTATIVE PROTEIN, SIMILAR TO PROTEIN INVOLVED IN ASSEMBLY OF OUTER MEM			/		1	SIDATIKVNAKK
		142	SMc02451		OUTER MEMBRANE PROTEIN	1		/		1	DKYLELLNSAK
		1832	SMc00604		PROBABLE OUTER MEMBRANE PROTEIN	1		/		3	TLVGWTAGVGAETFVTDNITAR
											AYISFDAK
											AGFFYSWWDK
		97	SMc02475		PUTATIVE OUTER MEMBRANE LIPOPROTEIN PRECURSOR	1		/		1	VSSAGSSCDMFLTLTNLGSGSR
	III.B.3 murein sacculus,	105	SMc01871		D-ALANINE--D-ALANINE LIGASE B (EC 6.3.2.4)	1	/	/		1	HVAVLLGGFSSERPVSLSSGTACADALEAEGYR
	peptidoglycan	98	SMc02305		UDP-N-ACETYLGLUCOSAMINE 1-CARBOXYVINYLTRANSFERASE (EC 2.5.1.7)	1				5	IVGGNELHGVIPISGAK
											NAALPLmIASLLTDDTLTLENVPHLADVEQLIR 7-M_Oxidation/
											NAALPLMIASLLTDDTLTLENVPHLADVEQLIR
											FMHVQELAR
											ASFWVIGPLLAR
		87	SMc01867		PROBABLE UDP-N-ACETYLMURAMATE--ALANINE LIGASE PROTEIN	2	/			3	RAEMLAELMR missed clevage
											RAEMLAELMR missed clevage
											KVVTYGENPQADVRFHNIR missed clevage
		65	SMc01866		PROBABLE UDP-N-ACETYLGLUCOSAMINE--N-ACETYLMURAMYL-PENTAPEPTIDE P	1		/		2	LKVTQQARPEDR missed clevage
										4	MLASRVKAVAGGFLPEGTGAFAAK missed clevage
											AKGmHELVSVSDAAAALK 4-M_Oxidation/
											AAmLASGTVTWEISTIK 3-M_Oxidation/
											MRAKGmHELVSVSDAAAALK 6-M_Oxidation/
IV	IV CELL PROCESSES	323	SMb20181		ABC TRANSPORTER PERIPLASMIC SOLUTE-BINDING PROTEIN			/		3	AAmLASGTVTWEISTIK 3-M_Oxidation/
											AKGmHELVSVSDAAAALK 4-M_Oxidation/
											MRAKGmHELVSVSDAAAALK 6-M_Oxidation/
		94	SMb20184		PUTATIVE ABC TRANSPORTER ATP-BINDING PROTEIN					2	AFLSMTAEGREVPA missed clevage
											AGTIGVAGFDIAREPR missed clevage
	IV.A Cell division	479	SMc04296	ftsZ2	CELL DIVISION PROTEIN FTSZ	1				6	EGLMNLDFADVK
											KPIITEMRPK
											AmLAAEAAIANPLLDEVSMRGAK 2-M_Oxidation/
											IREEVYDEADIVVGAIFDR missed clevage
											AmLAAEAAIANPLLDEVSMRGAK 2-M_Oxidation/
											VLYSGVSCITDLIVKEGLMNLDFADVK missed clevage

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Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		414	SMb21523	minD	PUTATIVE CELL DIVISION INHIBITOR	1	/			16	RVVYDLVNVIQGDAKLPQALIR missed clevage DSDRIIGLLDAK missed clevage RVVYDLVNVIQGDAK missed clevage RLDTLFLLPASQTR missed clevage HFDWIICDSPAGIER HADLAVIVTNPEVSSVR HADLAVIVTNPEVSSVRDSDRIIGLLDAK missed clevage KHFDWIICDSPAGIER missed clevage TTSTAALGAALAQR VVYDLVNVIQGDAKLPQALIRDK missed clevage LDTLFLLPASQTR NLDLVMGAER DKRLDTLFLLPASQTR missed clevage TVVVDFDVGLR RVVYDLVNVIQGDAKLPQALIR missed clevage DSLTPEGVERVmEELR 12-M_Oxidation/
		328	SMc04459	ftsH	TRANSMEMBRANE METALLOPROTEASE (EC 3.4.24.-)	2	/			9	VTFDDVAGVDEAKQDLEEIVEFLRDPQK missed clevage RLVTMQEFEDAKDK missed clevage LVTMQEFEDAKDK missed clevage NHEFVALAEGLLEYETLTGDEIK VTFDDVAGVDEAKQDLEEIVEFLRDPQK missed clevage NHEFVALAEGLLEYETLTGDEIK KNAPCIIFIDEIDAVGR missed clevage EIPFSQFLK ALGmVmQLPEGDRYSmSYK 4-M_Oxidation/6-M_Oxidation/16-M_Oxidation/
		195	SMc01874	ftsZ1	CELL DIVISION PROTEIN	1				6	RPLSPEASLYAPR SHEDDQLEIPAFLRR missed clevage IREEVDPDANIILGATFDEELEGLIR missed clevage EGLINLDFADVR SHEDDQLEIPAFLR SVDTLIVIPNQNLFR
		70	SMc01873		CELL DIVISION PROTEIN	1	/			4	AILHSLPTGFSLDGER LKAAETNLER LPELRENEAKFAAGLQRLQIAR GVERELERLKIER
		94	SMc00024	smc	CHROMOSOME PARTITION PROTEIN	1	/			2	EAQRMLARQLSEARDALAVAER missed clevage REAADRLAEAENHQREADR missed clevage
	IV.B Chaperoning	133	SMc01758	groEL4	60 KD CHAPERONIN B (GROEL)	1	/			5	AVASGmNPmDLKRGIDLAVEAIVK 6-M_Oxidation/9-M_Oxidation VGNEGVITVEEAK AKRImVDkETTTIVDGAGSK 5-M_Oxidation EKKDRVDDALHATR YLAEAMEKVGNEGVITVEEAK
		79	SMc00234		PUTATIVE PEPTIDYL-PROLYL CIS-TRANS ISOMERASE PROTEIN	1		/		4	TLDEVRDEVAADWTAEQQR missed clevage LTFSSVLR VLVDAIGKYR missed clevage LAYERQVALLSRQLGTPLSR missed clevage
	IV. C moltility chemotactic	138	SMc03007	cheA	CHEMOTAXIS PROTEIN (SENSORY TRANSDUCTION HISTIDINE KINASE) (EC 2.7.3.-)	1	/			4	LTAK EKVRQK DSFCPLVDVGR VAAGKNPEGTVRLTAK
		122	SMc00975	mcpU	TRANSMEMBRANE CHEMORECEPTOR (METHYL-ACCEPTING CHEMOTAXIS PROTEI	2	/			1	AGEAGKGFAVVAQEVR missed clevage
	IV.D Transport of small molecules	281	SMc02514		ABC TRANSPORTER PERIPLASMIC BINDING PROTEIN	1		/		7	NKADFK SHVGLTFIR VVSETITTHEYESK EGLPVVNEDGTPK EIDGKKVYGHMDYGKK 11-M_Oxidation RHLLTTTAAMLLAFTGSAFAGMDEAKQFLDK KVYGHMDYGK

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Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		239	SMb21206		PUTATIVE ABC TRANSPORTER ATP-BINDING PROTEIN,					4	FTTRLAGK
											DLPDPTMEDAFIALIQGSEAK
											FTTRLAGK
											LSGGMkQkLGLACALLRkPR
		235	SMc02169		ABC TRANSPORTER ATP-BINDING					7	GLPIVLISHNMPHVFEVADR
											AKLSELGLMTIQNINQAVETLSGGQR missed clevage
											GLPIVLISHNmPHVFEVADR 11-M_Oxidation/
											RGLPIVLISHNMPHVFEVADR missed clevage
											LSELGLMTIQNINQAVETLSGGQR
											GLPIVLISHNMPHVFEVADR
											RVLELILDVR missed clevage
		232	SMc02259		ABC TRANSPORTER PERIPLASMIC BINDING PROTEIN	1		/		2	MKKTLK
											GETELKEKVNAAIKAIR missed clevage
		223	SMb21644		PUTATIVE ABC TRANSPORTER ATP-BINDING PROTEIN					2	GMRPLRSKIQIVFQDPFSSLNPR missed clevage
											KVAVSFKVENGTVQAVK
		217	SMa0036		PUTATIVE ABC TRANSPORTER ATP-BINDING PROTEIN					9	AREVLAGLSFSQEMMDGDVAKLSGGWK missed clevage
											TIYDGLDFMVR
											RFLSALSNR missed clevage
											LSGGWKmRVALAR 7-M_Oxidation/
											FLSALSNR
											LSGGWKmRVALAR 7-M_Oxidation/
											EIKFIER missed clevage
											IENISKSNSHR missed clevage
											LEKIDRVEPPR missed clevage
		186	SMc04396		PERIPLASMIC BINDING PROTEIN	1		/		1	MKEAFDR
		177	SMb20842		PUTATIVE TONB-DEPENDENT RECEPTOR PROTEIN	1	/			3	LRNSYLER missed clevage
											LTRNDVPRDVVVRWVDVVSK missed clevage
											MVDLKSLR
		168	SMc01138		ABC TRANSPORTER ATP-BINDING PROTEIN					5	ALATDPTFMLLDEPFAGVDPISVADIQALVR
											GIGVLITDHNVRETLGLIDR missed clevage
											AYIIHAGEVLTHGR
											KSPAIALSGGERR missed clevage
											LGVGYLPQEASIFR
		160	SMc02832		PERIPLASMIC BINDING PROTEIN	1		/		1	TMAMMVR
		158	SMc02518		ABC TRANSPORTER ATP-BINDING PROTEIN					3	SQIK
											INLEHIRHAYGARPK
											IALNFQPQIK
		157	SMa0392		ABC TRANSPORTER, PERIPLASMIC SOLUTE-BINDING PROTEIN (dc=3)					3	AGVSDSFTKMTGIAVR missed clevage
											EAKKNGIEIAWVVPEEGAK
											GEIDAACTISTNAREAK
		157	SMa2075		PROBABLE EXTRACELLULAR SOLUTE-BINDING PROTEIN, FAMILY 5	1	/			2	SEKLLDEAGYAR missed clevage
											ENHPRGRATFAHVK missed clevage
		151	SMa1860		PUTATIVE ABC TRANSPORTER, PERIPLASMIC SOLUTE-BINDING PROTEIN	1	/			5	LRKGVTFHDGKPLAADDVIFSLKR
											GGTPQMELAEAIETKDAKTWTVK
											KGVTFHDGKPLAADDVIFSLK
											EVFEPGVRSVGIK
											VPADGYWDNYWLK
		146	SMa1434		PROBABLE ABC TRANSPORTER, ATP-BINDING PROTEIN	1	/			4	HYKLGSGPFSPK
											GKALDGFRRR
											GKALDGFRRR
											QIAEVFESHGEGEGGAVRDKVVR
		144	SMa2365		PROBABLE ABC TRANSPORTER, ATP-BINDING PROTEIN	1				4	AGEVLGIIGVSGNGQTTLAHLLSGTLR
											ELPPGKPR
											GLDEGAVAAVHER
											LAKETNKAELAELmVGR 14-M_Oxidation/
		144	SMa2000		PUTATIVE ABC TRANSPORTER, PERIPLASMIC SOLUTE-BINDING PROTEIN			/		2	VKKYYR
											KYYR
		144	SMc02726		IRON TRANSPORT PROTEIN	1		/		1	YKIGDYTGFEDRDR missed clevage

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Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		139	SMa0104		PUTATIVE ABC TRANSPORTER, PERIPLASMIC SOLUTE-BINDING PROTEIN			/		3	NKAYPASGWLPISSPAFDKDK
											LVKNKAYPASGWLPISSPAFDKDK
											KNAVFHNGKPLTADDIIYSYKR
		137	SMa1421		PROBABLE ABC TRANSPORTER ATP BINDING -PROTEIN					7	NVDKQYYR
											SAGADIMLLDHPTRGLDIGAK
											LGVINWKEMAKAAKAQLAK
											GEIVGLVGENGAGKSTLMK
											VLVEMSGIDLPGRTK
											KFPGVHALKAIDFHIKR
											AMSDAGVGIVLVADTLEEAIGLSHTIVVVKDGRIQK missed clevage
		136	SMc02171		ABC TRANSPORTER PERIPLASMIC BINDING PROTEIN			/		6	FKAKASK
											VAQMGLATEKMDTAAK
											DGTVANYDGDLEDYR
											GLIVGGPKPKDDKPR
											VNGSDEALSKADQRK
											MGTVAAVIEDHVQGFTFPDPEK
		132	SMc00590		TRANSPORTER ATP-BINDING PROTEIN		/			6	FKAKASK
											VAQMGLATEKMDTAAK
											DGTVANYDGDLEDYR
											GLIVGGPKPKDDKPR
											VNGSDEALSKADQRK
											MGTVAAVIEDHVQGFTFPDPEK
		119	SMa1466		PROBABLE ABC TRANSPORTER ATP BINDING PROTEIN					5	MADKIAIFR
											LIQPTSGKVFINGK
											VGVLRALAADPPVMLMDEPFGAIDPINR
											TIIFVSHDLDEAVKMADKIAIFR
											MINRLIQPTSGKVFINGK
		114	SMa1370		PROBABLE ABC TRANSPORTER ATP-BINDING PROTEIN					3	RSVNAVSDVSFDLAPGETLGLVGESGSGKTTVGR
											HISHRVAIMYAGR missed clevage
											RSVNAVSDVSFDLAPGETLGLVGESGSGK
		110	SMc04205		IRON/HEME TRANSPORT PROTEIN	1	/			2	VFDIYGSYSFSDSAK
											QSGIQISQAAAGRTSNVTTRAVRGR missed clevage
		108	SMa0300		ABC TRANSPORTER, PERMEASE (dc=3)		/			1	VLRDSLIEVLRSDYVRLAELK
		102	SMc01828		TRANSMEMBRANE TRANSPORT PROTEIN			/		2	LAVGQTATVKLAGSTTTIEGK
											LAVGQTATVK
		101	SMa2067		PROBABLE SULFATE/THIOSULFATE BINDING PROTEIN	1	/			1	NIGFVFQHYALFR
		101	SMc01606		PERMEASE	5	/			1	LVDKDVLEAADAFGSSNWQK
		101	SMc01829		TRANSMEMBRANE TRANSPORT PROTEIN	12	/			3	ALEDEKRR missed clevage
											VELDEDRLNSFGISAADVNAQLRR missed clevage
											DEEALPLDQEALSK
		98	SMb20365		ABC TRANSPORTER IRON-BINDING PROTEIN	1		/		3	TGISVNMVRLSSGETYAK
											TVGVYAGALGWGYNTEIFKQK
											GTKNLDNAKKWYDWALSADVQSSMK
		95	SMc04127		ABC TRANSPORTER ATP-BINDING PROTEIN	1				3	RIIEMRDQGK
											EmER 2-M_Oxidation/
											TIILITHKLR
		343	SMc02653		PROBABLE SIGNAL PEPTIDASE I TRANSMEMBRANE PROTEIN	1		/		6	EFIVPEGHYFMMGDNRDNSADSR missed clevage
											EFIVPEGHYFMMGDNR
											YSLPFSPDLFSGR
											RLVGLPGDR missed clevage
											FPPNPDIDYIKR missed clevage
											FDVGFVPAENLVGR
		311	SMc04454		PUTATIVE ATP-BINDING ABC TRANSPORTER PROTEIN					2	YFLDNVTGWILELDR

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Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		241	SMc00185		PUTATIVE ABC TRANSPORTER ATP-BINDING TRANSMEMBRANE TRANSMEMBRANE PROTEI		/			6	SQIGVVTQDTSLLHR
											RGIYADLWSR missed clevage
											MFGWFESR
											TTLMNLLLR
											GIYADLWSR
											ISKEQADARSMMTGR missed clevage
		152	SMc02379		PUTATIVE PERMEASE PROTEIN	6	/			2	LVDKDVLEAADAFGSSNWQK missed clevage
											IIDEGFR
		151	SMc00186		PUTATIVE ABC TRANSPORTER ATP-BINDING TRANSMEMBRANE PROTEIN		/			2	DAPILVLDEATSALDSEVEAAIQSNLER
											QSLAFFQNDFSGR
		147	SMc02869		PUTATIVE ATP-BINDING ABC TRANSPORTER PROTEIN					1	LFLFDEPLSNLDAALR
		102	SMc02829		PUTATIVE ATP-BINDING ABC TRANSPORTER PROTEIN					3	DMKVWFPIKAGFLRR missed clevage
											DLLKASDDELRHVR missed clevage
											RLGAYPHQLSGGQR missed clevage
		94	SMc02474		PUTATIVE ATP-BINDING ABC TRANSPORTER PROTEIN					2	LRVQmRTEIKALHQK 5-M_Oxidation/
											AAmDRSQVYVFDR 3-M_Oxidation/
		80	SMc03957		PUTATIVE TRANSPORT TRANSMEMBRANE PROTEIN	1	/			1	LEAIATTGYNER
		65	SMc02831		PUTATIVE PERMEASE ABC TRANSPORTER PROTEIN	6	/			2	FDFGESFFR
											NSFIDEIKK missed clevage
		61	SMc02142		PROBABLE PHOSPHATE TRANSPORT ATP-BINDING ABC TRANSPORTER PROTEIN					2	VGMVFQKPNPFPK
											AELDEVVETSLQK
		61	SMc01261		PUTATIVE TRANSPORTER TRANSMEMBRANE PROTEIN	5	/			1	LVGVLTIDDVVDVIQEEAEEDFLR
		59	SMc01965		PUTATIVE SPERMIDINE/PUTRESCINE TRANSPORT ATP-BINDING ABC TRANSPORTER PROTEIN					1	LFLLDEPLSALDAK
		59	SMc00175		PUTATIVE ABC TRANSPORTER ATP-BINDING PROTEIN					2	LGVLFQHGALFSALTVR
											GEILGFVGASGTGKSVLMRTVLR missed clevage
		46	SMc00531		PUTATIVE ABC TRANSPORTER ATP-BINDING PROTEIN					2	RPLNVGFSGGEKK missed clevage
											AGEVAAImGPNGSGK 8-M_Oxidation/
		64	SMc00550		PROBABLE ABC TRANSPORTER ATP-BINDING TRANSMEMBRANE PROTEIN		/			1	TTLMNLLLR
		70	SMc02836		PUTATIVE ATP-BINDING ABC TRANSPORTER PROTEIN		/			1	GLPIVLISHNMPHVFEVADR
	IV.D.1 anions	156	SMb21130		PUTATIVE SULFATE UPTAKE ABC TRANSPORTER ATP-BINDING PROTEIN					1	NIGFVFQHYALFR
		119	SMc02862		PIT ACCESSORY PROTEIN (dc=3)	1	/			2	MLGLFRKLLPREDR
											GDIKDLIQSMDDAIDMMHK
	IV.D.2 amino acids, amines	258	SMc00771		PUTRESCINE TRANSPORT ATP-BINDING					6	MASPEKPGNGAKAAVAIRPEK
											MASPEKPGNGAK
											VAQMLKLVKLEK
											RPKVLLLDEPLGALDKKLR
											VAQMLKLVKLEKFAK
											MASPEKPGNGAKAAVAIRPEKIR
		191	SMc03866		ABC TRANSPORTER ATP-BINDING PROTEIN	1	/			2	LVIEAEKITKAYGDR missed clevage
											RNMRRLGELQDmR 12-M_Oxidation/
		167	SMc02121		ABC TRANSPORTER GENERAL L-AMINO ACID TRANSPORT ATP-BINDING PROTEIN					9	GKIVVDGIELTNDLKK missed clevage
											TKLFLSQILH missed clevage
											EVGmVFQHFNLFPHLTILENCTLAPIWVR 4-M_Oxidation/
											IVVDGIELTNDLKK missed clevage
											WYGDFHVLR
											YPGQLSGGQQQR
											EVGmVFQHFNLFPHLTILENCTLAPIWVR 4-M_Oxidation/
											LFLSQILH
											EVGMVFQHFNLFPHLTILENCTLAPIWVR
		85	SMc01529		ABC TRANSPORTER DIPEPTIDE TRANSPORT ATP-BINDING PROTEIN		/			1	GKRIAMILQDPK
		144	SMc01949		ABC TRANSPORTER HIGH-AFFINITY BRANCHED-CHAIN AMINO ACID TRANSPORT A					3	mFSGLTVLENLLVAQHNK 1-M_Oxidation/
											TTVFNCITGFYKPTMGMITMR
											MFSGLTVLENLLVAQHNK
		144	SMc03269		ABC TRANSPORTER PEPTIDE-BINDING PERIPLASMIC PROTEIN	1		/		2	DGLK
											ARTK
		125	SMa0082		PUTATIVE ABC TRANSPORTER, PERIPLASMIC SOLUTE-BINDING PROTEIN	1		/		3	REGLMNISRR 5-M_Oxidation/
											VKADGQLNAIHEKWLGSPLPEFVTEAK
											FNQK

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Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		116	SMb20477		DIDPEPTIDE ABC TRANSPORTER PERMEASE PROTEIN	6	/			3	GDLK ILPASGMYAVYGRGDLK TARAKGLPER missed clevage
		92	SMb21527		PUTATIVE TAURIN UPTAKE ABC TRANSPORTER ATP-BINDING PROTEIN					4	GVDK HALLPWLNVIDNTEFGLK DLATRNLALVGLQDFHR missed clevage METLNVSNVSLTYPGLYSDQAVIALK 1-M_Oxidation
		362	SMc01950		PROBABLE HIGH-AFFINITY BRANCHED-CHAIN AMINO ACID TRANSPORT PERMEAS	10	/			2	AWEALREDEIACR missed clevage mPIGRAWEALR 1-M_Oxidation/
		336	SMc01948		PROBABLE HIGH-AFFINITY BRANCHED-CHAIN AMINO ACID TRANSPORT ATP-BIND					3	VTMSGSGKDLLANPEVR missed clevage SVETYYGNIR LLLLDEPSLGLAPLIVK
		224	SMc02738		PUTATIVE GLYCINE BETAINE TRANSPORT SYSTEM PERMEASE ABC TRANSPORTER	7	/			1	LGIISTPPALVEAAESFGATPWQVLR
		193	SMc04439		PUTATIVE GLYCINE BETAINE TRANSPORT ATP-BINDING ABC TRANSPORTER PROTI	1	/			4	VVNVETIMRPLSGNPEGLPLAAGTVLEAAAR TVVFITHDLDEALR FALLPHR ALTNDADILLmDEAYSALDPLIR 11-M_Oxidation/
		118	SMc02739		PUTATIVE GLYCINE BETAINE TRANSPORT ATP-BINDING ABC TRANSPORTER PROTI		/			2	TIIFVSHDLDEAFR AFATGAPILLMDEPFSALDPLIR
		64	SMc02119		PROBABLE GENERAL L-AMINO ACID TRANSPORT PERMEASE ABC TRANSPORTER P	7	/			1	IIIPPLTSQYLNLTK
		49	SMc00789		PROBABLE DIPEPTIDE TRANSPORT ATP-BINDING ABC TRANSPORTER PROTEIN	1	/			1	KIEEADVLSLFESPK missed clevage
	IV.D.3 carbohydrates, organic acids, alcohols	467	SMc01499		ABC TRANSPORTER ATP-BINDING TRANSPORT					4	VNMRLEISQLHQQLK missed clevage RGLSMVFQSYALYPHMSVR missed clevage GLSMVFQSYALYPHMSVR LEISQLHQQLK
		43	SMc02437	ptsP	PHOSPHOENOLPYRUVATE PHOSPHOTRANSFERASE PTSP	1	/				MMLPMVTEVAELKSAR NVMKQTAGkPVTFRTLDIGGDK ATAGAELKMMLPMVTEVAELK
		236	SMb21344		PUTATIVE SUGAR UPTAKE ABC TRANSPORTER ATP-BINDING PROTEIN					4	LALVRHMLGK GIDIGAKSEILRLLR missed clevage DGKTVATAAMADMPKLALVRHmLGK 22-M_Oxidation/ TVATAAMADMPKLALVR
		228	SMb20713		PUTATIVE SUGAR UPTAKE ABC TRANSPORTER ATP-BINDING PROTEIN	1	/			4	LRGAGIRLK missed clevage ImVmHEGRVTGILDR 2-M_Oxidation/4-M_Oxidation/ EVAHLFEIIR IMVmHEGR 4-M_Oxidation/
		218	SMb20673		PUTATIVE SUGAR UPTAKE ABC TRANSPORTER ATP-BINDING PROTEIN		/			4	DRGK AGEIHALMGENGAGK AGEIHALmGENGAGK 8-M_Oxidation HGWR
		211	SMb20894	gguA	PROBABLE SUGAR UPTAKE ABC TRANSPORTER ATP-BINDING PROTEIN	1	/			4	ALSKKVR QQLVEIAK STRLSGKTAPAK missed clevage VVLSKWLFTNPEVLILDEPTR
		159	SMa0713		PUTATIVE ABC SUGAR TRANSPORT ATP BINDING PROTEIN, AMINO TERMINUS					5	RSMSRTIR missed clevage APRSARFTSASASR missed clevage GmPKAEIDR 2-M_Oxidation RSMSRTIR missed clevage STMLKILAGLEPASGGKImIGDR 19-M_Oxidation
		144	SMb20718		PUTATIVE SUGAR UPTAKE ABC TRANSPORTER ATP-BINDING PROTEIN					2	VVLEARR missed clevage AGEIHALMGENGAGK
		126	SMb20538		SOLUTE-BINDING PROTEIN	1		/		4	AIADIFQLEPSTISDYSKR LVDEGKATVR SKLGIGmLPR AGITPPGVDTTWEEYADIAIEmTK 22-M_Oxidation

Continued

Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		125	SMb20317		SUGAR ABC TRANSPORTER ATP-BINDING PROTEIN		/			3	LRGAFGLLDSRR missed clevage
											ADLSSPASAIEAGIGYVPEDR
											STLIKIISGAQPADGGELTINGK
		120	SMb20316		ABC TRANSPORTER PERIPLASMIC SUGAR-BINDING PROTEIN			/		3	RAMNRGIK
											KYPEMELVSEKNESFNDANK
											FNEKLAECMGK
		120	SMb21016		PUTATIVE SUGAR ABC TRANSPORTER PERIPLASMIC SOLUTE-BINDING PROTEIN PR	1		/		3	EKAK
											VGEK
											LVGVGFFTSGGAGAVKAGEEVGAK
		120	SMb21461		PUTATIVE SUGAR UPTAKE ABC TRANSPORTER PERIPLASMIC SOLUTE-BINDING PRO			/		3	RLERDVK
											YSKKPELAAELLRYMVGAEDQK
											VGVSALPVGAEGQKSSGALGTAYLGVSKYSK
		115	SMa0203		PUTATIVE ABC TRANSPORTER		/			4	EVKKDLVVPFLR
											DMTIRK
											WWKEQK
											SQVEYLSNKLPDGGNLLEIR missed clevage
		115	SMb20903		PUTATIVE SUGAR UPTAKE ABC TRANSPORTER PERMEASE PROTEIN		/			4	ADQKPGAQAGR
											MAMADITK
											MAMADITKANQTRADQKPGAQAGRAR
											LMGGGTEGSIGATASWIVGSLACIAIVGAILNSRK
		114	SMb20856		PUTATIVE SUGAR UPTAKE ABC TRANSPORTER PERIPLASMIC SOLUTE-BINDING PRO		/			5	SSNDQNGVLVGQWLANAMKGKPMKIILLSGDK
											EALALIKEGK
											RLGVFKGLVEGQLVNDGK
											TAVDVGLKAVNGELPADFPK
											AVNGELPADFPKLNLTTPAVITKENVDK
		106	SMc02773		ABC TRANSPORTER ATP-BINDING PROTEIN					3	SLQK
											VLASAMSGGNQQK
											KAMDLGIALAPESR
		99	SMb20855		PUTATIVE SUGAR UPTAKE ABC TRANSPORTER ATP-BINDING PROTEIN					2	SLRLKASSIDAPVSSLSGGNQQKVVLAK
											AIFGADPLDSGTISLKGKALKLK
		99	SMb20630		PUTATIVE SUGAR UPTAKE ABC TRANSPORTER ATP-BINDING PROTEIN					4	TDPAEIAR
											ELGNTMVYVTHDQTEAMTLADQIVVLR 6-M_Oxidation/
											MANGISNKSVVLSDIRK 1-M_Oxidation/
											AIVRKPDVFLFDEPLSNLDAELR missed clevage
		97	SMb21595		PUTATIVE SUGAR UPTAKE ABC TRANSPORTER PERIPLASMIC SOLUTE-BINDING PRO			/		4	ALDVWKTIIDQK
											MFKDFGQLPPR
											YAKPRGPHPAWPKISKAIQDAIQAALTGQMSSK
											SSVVKPENYFK
		96	SMb21103		PUTATIVE SUGAR UPTAKE ABC TRANSPORTER PERIPLASMIC SOLUTE-BINDING PRO			/		3	IGEWEKATGAKVNILSKK
											LVMLPRAQFDVSALYFQK
											AQFDVSALYFQK
		399	SMc01498		PROBABLE SORBITOL/MANNITOL TRANSPORT INNER MEMBRANE PROTEIN	1	/			3	TKDVLMWMLSTK missed clevage
											LSAASTmAIAPILILGWFSQK 7-M_Oxidation/
											MMPPVGVLVPMYLIFR
		160	SMc03065		ALPHA-GLUCOSIDES TRANSPORT ATP-BINDING ABC TRANSPORTER PROTEIN					2	VFLFDEPLSNLDAALR
											GAADMLQLTPYLDR
		160	SMb20328		probable trehalosemaltose transporter ATP-binding protein					1	VFLFDEPLSNLDAALR
		147	SMb21152		putative sugar uptake ABC transporter ATP-binding protein					1	IFLFDEPLSNLDAALR
		98	SMb20661		putative sugar uptake ABC transporter ATP-binding protein					1	AIVRDPQVFLFDEPLSNLDAK missed clevage
		95	SMb21106		putative sugar uptake ABC transporter ATP-binding protein					1	AIVRQPDVFLFDEPLSNLDAK 8587
	IV.D.4 cations	176	SMa1013		ACTP COPPER TRANSPORT ATPASE (EC 3.6.3.4)	7	/			2	LREGGR missed clevage
											AKGRTSQAIK missed clevage
	IV.E Transport of large molecules	269	SMc02786		PUTATIVE TRANSLOCASE TRANSMEMBRANE PROTEIN	1				3	TGSERPPFDPYSPR
											MAYEMIVMAFADGDRK missed clevage
											IISQLISATYDK
		246	SMc03958		PUTATIVE TRANSPORT TRANSMEMBRANE PROTEIN	3	/			2	RQLDNFEQVFWSGQSLEELYR missed clevage
											MEAFADEFSAILSR

Continued

Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
	IV.E.2 protein, peptide secretion	735	SMc02082		OUTER MEMBRANE SECRETION PROTEIN	1	/			6	AGLRATDEGVPIAK missed clevage
											ASVQQTVVSAHAQLESALAR
											LEVPIYQGGAEYGQIR
											RQNLAFLR missed clevage
											DKWFGLR missed clevage
											QNLAFLR
		141	SMc02265		PROTEIN-EXPORT MEMBRANE PROTEIN	10	/	/			NRVDQVGVAEPLIQR missed clevage
											VLTAPVINEPILGGR
											ILVQLPGLQDPTR
		117	SMb21315	expD2	PUTATIVE PROTEIN SECRETION PROTEIN, HLYD FAMILY MEMBRANE FUSION PROT	1	/			5	MNKEKILPVR
											LSELAATRGELAGDKAQAEK
											DIDSITIGSSTQIR
											LAIQRKTIEEYSEKAK
											LDLQPPVKTESPER
		262	SMc04458	secA	PREPROTEIN TRANSLOCASE SECA SUBUNIT	1	/			6	NLLKYDDVLNDQRK missed clevage
											SVVLQTLDHLWR
											YERGQmVQR 6-M_Oxidation/
											EHIVNLDHLR
											TLDDILVPAFAVVR
											VLGLRPFDVQLIGGMILHER
		279	SMc02058		PROBABLE YAJC PROTEIN	1	/			2	RGDQVVTGGGIVGK missed clevage
											RGDQVVTGGGIVGKVTK missed clevage
		99	SMc01289		PROBABLE PREPROTEIN TRANSLOCASE TRANSMEMBRANE	9	/			2	MFQGDTSHLPLK
											KVINQYTR missed clevage
	IV.G.2 drug/analog sensitivity and resistance	208	SMa1662		PUTATIVE DRUG RESISTANCE PROTEIN	11	/			3	EQAK
											SSSMLMGVAIYSPEGTR
											NWYVAmLDRmSR 6-M_Oxidation/10-M_Oxidation/
		144	SMc04350		TRANSMEMBRANE MULTIDRUG EFFLUX SYSTEM	1	/			1	ATLAGDR
		127	SMb21497		PUTATIVE ACRIFLAVIN RESISTANCE PROTEIN	1	/			2	SSRGQAVVAKGLKEGENVITEGVGK
											ESVDLTGKVVAVQKVDIR
		94	SMc02856		PENICILLIN-BINDING PROTEIN	1	/			2	REKR missed clevage
											VDASDRVAGGGSKPRR missed clevage
		97	SMc01334		PROBABLE PENICILLIN-BINDING 1A TRANSMEMBRANE PROTEIN	1		/		1	KALQDGLLSYDERR missed clevage
		63	SMc02867		PUTATIVE MULTIDRUG-EFFLUX SYSTEM TRANSMEMBRANE PROTEIN	12	/			2	GFFGWFNR
											TTEVIEQTETIFGQEK
	IV.H Nodulation	121	SMa0878		NODM GLUTAMINE AMINOTRANSFERASE	1	/			2	AFTCQLAVLAALAVGAGK
											GTSFPLAMEGALKLK
		152	SMa0853		NODE BETA KETOACYL ACP SYNTHASE	1	/			5	KVRVAMSNAFAMGGTNAVLAFK
											SAIGPLLNTELHGLK
											VRVAMSNAFAMGGTNAVLAFKQV missed clevage
											EPDPDCDLDVTPNVPRERK missed clevage
											KGVVLGEGAGMAVLESYEHATAR 11-M_Oxidation/
VI	VI.A Not classified regulator	245	SMc01630		TRANSCRIPTION REGULATOR NOT CLASSIFIED REGULATOR	1	/			2	FDTALAKK
											GLmSTLmTFDYVVVFAYRGK 3-M_Oxidation/7-M_Oxidation
		211	SMc04212		TRANSMEMBRANE SENSOR HISTIDINE KINASE	4	/			3	VLQSLPPVEDVPSLTR
											DDLIAELEVAKSMSDEAR
											KAKREDVVTEK 3
		208	SMc02284		SIGNAL PEPTIDE HYPOTHETICAL	1		/		2	AKK
											VDAK
		173	SMc00074		SIGNAL PEPTIDE TRANSMEMBRANE	3	/			2	ELDMR
											AmERKELSLVYQPIVR 2-M_Oxidation
		172	SMc03186		TRANSCRIPTION REGULATOR NOT CLASSIFIED REGULATOR	1	/			3	EGIDVAIR
											SDITLERMR missed clevage
											SDITLERMRTFARVAER missed clevage
		172	SMc02659	relA	GTP PYROPHOSPHOKINASE (ATP:GTP 3'-PYROPHOSPHOTRANSFERASE) (EC 2.7.6.5)	1	/			1	KLDLVTKKAK
		163	SMb20356		SENSORY HISTIDINE KINASE	2	/			3	QNLFNLLSNAAK
											DLSASELKWLRDR
											GANSRSQLVAALER missed clevage

Continued

Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		160	SMc00994		TRANSCRIPTION REGULATOR NOT CLASSIFIED REGULATOR	1	/			1	mPDADHFRSLEWIEKANAPVLAIGR 1-M_Oxidation
		153	SMc00888		CONTAINS A 2-COMPONENT RECEIVER DOMAIN	1	/			2	SLDmQQLASAGSK 4-M_Oxidation
											TSGmDDHIMKPVSPDMIEmLLR 4-M_Oxidation/19-M_Oxidation/
		150	SMc01049	hflX	GTP-BINDING PROTEIN	1	/			4	NITK
											NITKRDSK
											EDLEDGRVSLDLR missed clevage
											LTEGEATELER
		145	SMc01595		TRANSMEMBRANE SENSOR HISTIDINE KINASE	2				2	EGTTPADGGWKR
							/				mASFDGLkREGTTPADGGWK 1-M_Oxidation/
		136	SMb21209		PUTATIVE TWO-COMPONENT SENSOR HISTIDINE KINASE (EC 2.7.3.-)	2	/			1	DLDALTHILEQLTSLAR
		123	SMc00098		TRANSCRIPTION REGULATOR	1	/			1	mTRkAAK 1-M_Oxidation
		122	SMa0849	syrM	SYRM TRANSCRIPTIONAL REGULATOR	1	/			3	mDQPTWKRPHR 1-M_Oxidation/
											MDQPTWKRPHR
											QMPNLASIDLNLLVDLEALLQYR
		118	SMa1956		PUTATIVE LYSR-FAMILY TRANSCRIPTIONAL REGULATOR	1	/			2	AEHPTAARKLTLER
											ALFSDALLVDGPGGYLLTSR
		111	SMc02721		OUTER MEMBRANE RECEPTOR PROTEIN		/			3	RGDTSRPRSGATSEKAAKPGAWR missed clevage
											SEPAQVDHAR
											ADILGGSYGGRR missed clevage
		101	SMc02893		TRANSCRIPTION REGULATOR NOT CLASSIFIED REGULATOR	1		/		1	SASEILKEIDNLADLVR missed clevage
		97	SMc00861		SIGNAL PEPTIDE HYPOTHETICAL/PARTIAL HOMOLOGY	1		/		3	QKLWEK
											EKLPKK
											EMGELYDRAVAAIGPVPKR
		94	SMc00620		SIGNAL PEPTIDE HYPOTHETICAL	1		/		2	AVRKIGYVDLLK
											VYKIAMDDENVGKAVRK
		186	SMc00471		PUTATIVE SENSOR HISTIDINE KINASE TRANSMEMBRANE PROTEIN	2	/			6	ELQNAIDYLDHAPAGFFSAGR
											QAGDYQMPVVDR
											TDLGQFEQVLLNLAVNAR
											ILDLDLR
											EGDDGDQSASNAAmR 14-M_Oxidation/
											RGGKRmLETR 6-M_Oxidation/
		152	SMc02585		SENSOR HISTIDINE KINASE TRANSMEMBRANE PROTEIN	4	/			2	RLTTLSSESEEHMR missed clevage
											LLPLSSLIEEVMAPHR
		151	SMc04446		HISTIDINE KINASE SENSORY TRANSMEMBRANE PROTEIN	3	/			2	IAAIENFAADVSHELKNPLTSLR missed clevage
											HLYSGGQVLR
		88	SMc02756		PUTATIVE SENSOR HISTIDINE KINASE PROTEIN	1	/			3	FAHIITSFDDERPSSR
											ELLATTARERIR missed clevage
											FESYGQR
		76	SMc02984		PUTATIVE TRANSCRIPTION REGULATOR PROTEIN	1	/			2	SASEILKEIDNLADLVR missed clevage
											DRGIYAIYPHRR missed clevage
		75	SMc00820		PUTATIVE TRANSCRIPTIONAL REGULATOR PROTEIN	1	/			1	TEPLCWVSSINHPLPENAPIPLAVGR
		74	SMc03857		PROBABLE SIGNAL RECOGNITION PARTICLE PROTEIN (FIFTY-FOUR HOMOLOG)	1	/			4	AERANPDILK missed clevage
											mHRQMADMmK 1-M_Oxidation/9-M_Oxidation/
											mHRQMADmMK 1-M_Oxidation/8-M_Oxidation/
											mHRQmADMMK 1-M_Oxidation/5-M_Oxidation/
		73	SMc00016		PROBABLE LYTB PROTEIN	1	/			3	RLVEVALR missed clevage
											KQPVVFSAHGVPK missed clevage
											MPTSGFVRSTGVFMMAPSAAAK missed clevage
		64	SMb20450		putative regulatory protein	1	/			1	LGAELAVAR
	VI.C Hypothetical/ Global homology	225	SMa0209		HYPOTHETICAL PROTEIN	1	/				FTPLTVKQRR
											TVVLHKSGIPVTVPDPSRYAVHNLIVASRR
											FLDFLIRDPVRTVVLHK
											SIDLMYQAMLAELR
											AGAAMLPDEGRK
		95	SMc02490		CONSERVED HYPOTHETICAL PROTEIN	1	/			2	SKLK
											KGDRFDFR

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Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		96	SMa0280		HYPOTHETICAL PROTEIN	4	/				TKDLLQR
											AAEWFIAFSMVVLAMMK
											AVLYRMVMPGHTCPYGLKTKDLLQR
											AVLYRMVMPGHTCPYGLKTK
		130	SMa0364		HYPOTHETICAL PROTEIN	1				9	QPAmVEREAmQALGGER 4-M_Oxidation/10-M_Oxidation/
											ELGARKGRTLETR missed clevage
											DMDSLQAKVAGAIRSK missed clevage
											NGAQMVASEREEPLR missed clevage
											SGSHDSPVTIVEPR
											SSVEALLRDLAAAR missed clevage
											HLAAERQRAAK missed clevage
											EAEAAREEERAAHQAAR missed clevage
		197	SMa0601		CONSERVED HYPOTHETICAL PROTEIN	1	/			2	VSACLESAVAR
											GGYGPTFSWKEAVR
		193	SMa0607		HYPOTHETICAL PROTEIN	1		/		7	TNFFYMATVNTPAMAAK
											FVIDMGAPGPDQGK
											LFREGLKVYPLAKK
											GGKYLIVPADYKGDLPKDK missed clevage
											TNFFYMATVNTPAMAAKLIGK
											EPIDLFDPELRGLAAGIGIR missed clevage
											DKLVANADGSVDLYFGPK
		104	SMa0665		CONSERVED HYPOTHETICAL PROTEIN	8	/			1	LANSAQGFASR
		164	SMa0690		CONSERVED HYPOTHETICAL PROTEIN	2	/			7	KKPDLKPK
											KPDLKPK
											SSANRPAGKKK
											KMASKAQNRPK
											LKSGPGGGASQLK
											AGVNRPKGKKSSANRPAGK
											SKLKFDR
		149	SMa1037		HYPOTHETICAL PROTEIN	1	/	/		4	LAKEELTRLMGLWGSGIDYQVPNRLPQLPK missed clevage
											TKIEEESLLTYNAMITNTFELLADSREK 14-M_Oxidation
											VVSDRVKTLMAKK
											LAKEELTRLMGLWGSGIDYQVPNR
		208	SMa1043		HYPOTHETICAL PROTEIN	1		/		2	VDAK
											ATVK
		104	SMa1092		HYPOTHETICAL PROTEIN	1				2	STSFSSDLmGSLLAGSAMAGVLLQSGGSR 9-M_Oxidation/
											GRKPVWPTSTGASPPAATPDEIIK
		141	SMa1252		CONSERVED HYPOTHETICAL PROTEIN	12	/			2	KWKDLK
											MSGSKVAKR
		96	SMa1397		CONSERVED HYPOTHETICAL PROTEIN	1	/			3	LGEGDVSAFR
											RAYLDQVEAVQK
											DKEIVMRRLLPAGVK
		139	SMa1564		HYPOTHETICAL PROTEIN	3	/			3	KKMKGK
											SVEKTLREIEEKR
											GKIKAMSSEAK
		120	SMa1657		HYPOTHETICAL PROTEIN	2	/			1	SPPNSRTVPHGR missed clevage
		117	SMa1666		HYPOTHETICAL PROTEIN	4	/			1	WMILNTVAEmK 10-M_Oxidation/
		212	SMa1674		HYPOTHETICAL PROTEIN	1		/		2	LLVALLCSEKR
											AISVARRRDK
		128	SMa2131		HYPOTHETICAL PROTEIN	1	/			3	RWLRER missed clevage
											WLRERGLR missed clevage
											SFHQTILGQDKPILENQMPKR
		42	SMc00073		HYPOTHETICAL PROTEIN	1				2	DKNIAGKPITLTLR
											TFGEGPGRYTGYDVPVAFPLTLKR missed cleavage

Continued

Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		179	SMa2235		HYPOTHETICAL PROTEIN	1	/			4	GTKALAALMKR
											MRPPTPVRFSKNPMDMYPKGTK 1-M_Oxidation/16-M_Oxidation/
											NPMDMYPKGTKALAALMKR
		95	SMb20194		CONSERVED HYPOTHETICAL PROTEIN	1					LQYRGR missed clevage
		133	SMb20252		HYPOTHETICAL PROTEIN	2	/			4	VIAEMKRQPGRYAVVGIPCFIK missed clevage
											STRFVESFAWQMDTEMNAVAGVDYR missed clevage
											LVDAIARPGRK
											TADDLRKGAKSR missed clevage
		101	SMb20291		TRANSMEMBRANE HYPOTHETICAL PROTEIN	16	/			2	WLR
											ERWLR
		144	SMb20355		HYPOTHETICAL PROTEIN	1		/		2	GFVPAIFAR
											KLLQAQVEAMR 10-M_Oxidation/
		127	SMb20464		HYPOTHETICAL PROTEIN	1	/			3	MKVKTK
											TKALFGLFEARK
											TKALFGLFEAR
		101	SMb20968		HYPOTHETICAL PROTEIN	1	/			1	ERWLR
		259	SMb20982		HYPOTHETCAL PROTEIN	1	/			2	ALSCASAVILGVTLVK
											ALSCASAVILGVTLVKDDARR
		242	SMb21264		PUTATIVE MEMBRANE-ANCHORED PROTEIN,				/	3	APPSLRANGLR missed clevage
											EPMGLRGEVEDTLAYLKTHGTRLVLGLR missed clevage
											ILmYSHDTFGLGHLRRCR 3-M_Oxidation/
		199	SMc00020		HYPOTHETICAL PROTEIN	1	/	/		2	VVAGPAGGPGK
											MSGKVSRRLLAVSLLLAAAGCNK 1-M_Oxidation/
		144	SMc00071		CONSERVED HYPOTHETICAL PROTEIN	1		/		2	KAK
											KAKILR
		70	SMc00176		CONSERVED HYPOTHETICAL PROTEIN	1		/		2	RVDTLVAAVDAQK missed clevage
											RTVQSLQNTISDFDKNPQR missed clevage
		140	SMc00190		TRANSMEMBRANE HYPOTHETICAL	2	/			5	QIADRLRSGVETSFADANK missed clevage
											VSETARQASDMLSTSTRLIEGK missed clevage
											SSADDAIMDLDLR
											SRQIADRLR missed clevage
											MRSTTGEIAER missed clevage
		423	SMc00354		TRANSMEMBRANE HYPOTHETICAL/GLOBAL HOMOLOGY	3	/	/		5	TLIDVDGHTDSTGSASYNQGLSER
											mSNTAGGALIGAGLGAATGLLVGGSAAGRR 1-M_Oxidation/
											TLIDVDGHTDSTGSASYNQGLSER
											RVEISIAPIK missed clevage
											AQLQGTGVSVTR
		104	SMc00477		CONSERVED HYPOTHETICAL PROTEIN	1	/			2	SWSLPDIEKVK
											DRFMMAQLKFLGIEK
		116	SMc00478		CONSERVED HYPOTHETICAL PROTEIN	1		/		4	EGNWLATVPGR
											VGAEEAFGTGYNVMPIWTKRLDAK
											GGKFLLLPPGYK
											VTGFEKGIAGADFRVYEDR missed clevage
		98	SMc00492		CONSERVED HYPOTHETICAL TRANSMEMBRANE PROTEIN	1	/	/		1	LAPELNSFSGRPR
		111	SMc00497		TRAMSMEMBRANE PROTEIN	1	/			2	IMGSPDPRQIDGIGGADPLTSK missed clevage
											EELEANESVK
		102	SMc00535		CONSERVED HYPOTHETICAL PROTEIN	1	/			4	VRMATSYR 3-M_Oxidation/
											IVMGNMAK 3-M_Oxidation/6-M_Oxidation
											IFAGGHLHFLMYQK 11-M_Oxidation
											MKSEAIDLVTEGDEAAER
		120	SMc00575		CONSERVED HYPOTHETICAL PROTEIN	1	/			4	AHASMQEIMELLK
											GLPPTPIANPGR
											AGEYEIKAHASMQEIMELLK missed clevage
											GMRLQSDPTIIYGIFGGDGKPADRAILRSDLDK missed clevage
		287	SMc00582		HYPOTHETICAL PROTEIN	1		/		4	GNVQIEYGGYK
											RNYFDLR missed clevage
											RTFIAPGGLVLTPLLAAR missed clevage
											DTDSSLANDWSIGAR

Continued

Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		49	SMc00583		HYPOTHETICAL, TRANSMEMBRANE PROTEIN	6	/			1	ADSIQFFDLPR
		138	SMc00592		HYPOTHETICAL, TRANSMEMBRANE	10	/			2	IISSADQPGELVYRGPNVMmGYASSR 20-M_Oxidation/
											LEGDAIAPHPELAVLLSTSGSTGHGK
		51	SMc00648		CONSERVED HYPOTHETICAL TRANSMEMBRANE PROTEIN	2		/		1	IVADVTVR 166
		148	SMc00697		HYPOTHETICAL TRANSMEMBRANE PROTEIN	1	/			1	LGGVLDLGELEVSDVMIHR
		113	SMc00722		TRANSMEMBRANE HYPOTHETICAL/PARTIAL HOMOLOGY	3	/			3	LMDELR 2-M_Oxidation/
											GLENLGIKPGK
											EAKGTTSRNLSEHPLAGK
		40	SMc00897		HYPOTHETICAL PMBA PROTEIN	1	/			3	HWFLSTSAAR
											AAELVDQARR missed clevage
											RVATQKNVTVVFDPR missed clevage
		93	SMc00907		TRANSMEMBRANE HYPOTHETICAL	1	/	/		1	LSAKEQQEAYRQAR missed clevage
		156	SMc00996		HYPOTHETICAL PROTEIN	1		/		3	KLVAKYmPSASRR 7-M_Oxidation/
											ISKSTPVPFSKKASAEPPSK
											YAGIVVDAKTGK
		41	SMc01118		CONSERVED HYPOTHETICAL PROTEIN	1				1	ELGEIDR
		101	SMc01136		TRANSMEMBRANE HYPOTHETICAL	1	/			1	HPIAISMK
		264	SMc01200		HYPOTHETICAL TRANSMEMBRANE PROTEIN	1	/	/		2	LHGTNEPWTIGQAVSSGCIR
											YGIGVGEEGR
		97	SMc01491		CONSERVED HYPOTHETICAL PROTEIN	1	/			2	VMHDRALEVR missed clevage
		212	SMc01515		CONSERVED HYPOTHETICAL PROTEIN	1		/			EWCMLRESR missed clevage
		114	SMc01552		TRANSMEMBRANE HYPOTHETICAL/GLOBAL HOMOLOGY	1	/			3	LRNSYLER missed clevage
											MVDLkSLR
											WGAR
		121	SMc01708		HYPOTHETICAL PROTEIN	2	/			2	VTNVANGTVAKDSK
											SKGTLTLEGANGTK
		103	SMc01710		HYPOTHETICAL PROTEIN	3		/		3	LVGGVGGTGDNFLR
											SGRPSAVHGAL
											AGGGGGGGGAAGASSGSTAPDPK
		132	SMc01827		HYPOTHETICAL PROTEIN	1		/		3	KLTSLLAAGVFSLAAFHANAAEK
											MVKFVRASMKGWK
											MNKK 1-M_Oxidation
		144	SMc01859		TRANSMEMBRANE HYPOTHETICAL	1		/		2	KAK
											GASLVK
		126	SMc01876		HYPOTHETICAL TRANSMEMBRANE PROTEIN	1	/	/		2	KEYLAAISR missed clevage
											QHPFSEYAR
		144	SMc01929		CONSERVED HYPOTHETICAL PROTEIN	1				1	GASLVK
		201	SMc02059		CONSERVED HYPOTHETICAL PROTEIN	1	/			1	GMGKSSLVKAVHAK
		144	SMc02102		TRANSMEMBRANE CONSERVED HYPOTHETICAL PROTEIN	1		/		2	AKK
											NGNFR
		45	SMc02543		HYPOTHETICAL TRANSMEMBRANE PROTEIN	1		/		2	YTTIYLANR
											ALFADGKQPSmEQLAAAR 11-M_Oxidation/
		66	SMc02580		HYPOTHETICAL PROTEIN	1	/			1	LRPIETILSGPAASLVGAR
		166	SMc02634		TRANSMEMBRANE HYPOTHETICAL	1	/			1	VDGK
											YDYVYK
		154	SMc02729		HYPOTHETICAL TRANSMEMBRANE PROTEIN	2	/			4	LGFFGIDWDDTPWLDNKASFQR missed clevage
											LGFFGIDWDDTPWLDNK
											TPTPSFYR
											KFNVSLGK missed clevage
		203	SMc02821		TRANSMEMBRANE HYPOTHETICAL	4	/			4	ERRKMK
											RKMKAK
											KAEGRLRR
											VSASETDRTKIK
		63	SMc03097		CONSERVED HYPOTHETICAL PROTEIN	1	/			1	SYVSGSLEAR
		94	SMc03152		HYPOTHETICAL TRANSMEMBRANE PROTEIN	1	/			1	AIVRDPQVFLFDEPLSNLDAK 8587
		101	SMc03174		TRANSMEMBRANE UNKNOWN	1		/		2	WIR
											GQRWIR
		121	SMc03233		HYPOTHETICAL TRANSMEMBRANE PROTEIN	1	/			1	VRPVGSVEGDTPEAVIAR

Continued

Group	Subgroup	Score	Gene ID	Gene name	Protein name	TMDs	Localization			No. Peptide	Peptides
							IMP	OMP	LPAS		
		158	SMc03234		TRANSMEMBRANE HYPOTHETICAL	2	/			2	SPRDPAWTADGVTSPVWLPVSPVSGR missed clevage
											HWMAQALKSPR missed clevage
		230	SMc03238		HYPOTHETICAL TRANSMEMBRANE PROTEIN	2	/			1	SLYQLTEPLYRPIRR missed clevage
		179	SMc03872		HYPOTHETICAL PROTEIN	2	/	/		1	GRDYYLAGIDGILYGDSPQEGYVR missed clevage
		226	SMc03941		CONSERVED HYPOTHETICAL TRANSMEMBRANE PROTEIN	1	/			1	AEHHSGPVETGAPMDYSEHEK
		93	SMc03964		TRANSMEMBRANE HYPOTHETICAL/GLOBAL HOMOLOGY	1		/		2	KWKR
											VPSRVALTLSGHTHGGQVR missed clevage
		99	SMc03995		HYPOTHETICAL PROTEIN	1		/		1	ADELMKK
		305	SMc04085		CONSERVED HYPOTHETICAL PROTEIN	2	/			3	ASEGSVAEAVARLRDGLQK missed clevage
											NPVLAWQDRDRQR missed clevage
											VATVGDQLRLLTEKGETTLAK missed clevage
		114	SMc04182		TRANSMEMBRANE HYPOTHETICAL/GLOBAL HOMOLOGY	1	/			1	MIDAGVHQQLGTLVAEVKNLR missed clevage
		98	SMc04289		CONSERVED HYPOTHETICAL PROTEIN	1	/			1	RmAFDHYFGR 2-M_Oxidation/
VI	VI MISCELLANEOUS	166	SMb20292		IMMUNOGENIC PROTEIN	2		/		3	LLNKDRK
											FNVGNPGSGTR
											MIKDGLSAPLHPGAEKYYKEK

Remark: IMP inermembrane protein, OMP outermembrane protein; LPAS Prokaryotic membrane lipoprotein lipid attachment site

BIBLIOGRAPHY

Miss Waraporn Payakapong was born on 31 July, 1974 in Nakhon-Ratchasima, Thailand. She studied in primary school at Muang Prachinburi School and in high school at Prachin Kulayanee School. She graduated with the Bachelor's degree of Science in Microbiology, Burapha University in 1996. She worked at Thai President Food Company, Chonburi, in position microbiologist for 3 years. In 1999, she went to study at School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima and received a scholarship from the Royal Golden Julibee (RGJ) grant of the Thailand Research Fund in 1997. She had presented posters in the topic of "Soybean root nodule preservation for typing by FA technique and evaluation of competitive ability of *Bradyrhizobium* strains" in World Congress of Soil Science, 14^{th}-24^{th} August 2002 at Bangkok and "Isolation of genes for salt tolerance from *Rhizobium* LT11" in CTAHR student research symposium 25^{th} April 2003 at University of Hawaii, Hawaii. Her works have been published in World Journal of Microbiology & Biotechnology in the topic of "Strain-specific antisera to identify Thai *Bradyrhizobium japonicum* strains in preserved soybean nodules" of volume 19, page 981-983 in 2003 and "Soybean cultivar affect nodulation competition of *Bradyrhizobium japonicum* strains" of volume 20 page 311-315 in 2004.