CHAPTER 2
LITERATURE SURVEY

2.1 Nitrogen-fixing bacteria

Nitrogen-fixing bacteria contain *nifH*, *nifD* and *nifK* encoding nitrogenase which catalyses nitrogen fixation, the reduction of nitrogen to ammonia. The microbes are divided into symbiotic nitrogen-fixing and free-living nitrogen-fixing bacteria. The former which is known as rhizobia consists of *Rhizobium* spp., *Sinorhizobium* spp., *Mesorhizobium* spp., *Bradyrhizobium* spp. and *Azorhizobium* spp.

In 1982 Keyser et al reported the isolation of fast-growing root nodule bacteria which nodulated soybean root nodules (*Glycine max* cv. Peking). The bacteria were named *Rhizobium fredii*. (Scholla & Elkan, 1984) In 1988 Chen et al performed numerical taxonomy on 240 characteristics and found that *Rhizobium fredii* were closely related to but formed a distinct clad from *Rhizobium* spp. and *Bradyrhizobium* spp. Chen et al, (1988) proposed that the fast growing soybean nodulating bacteria be designated a new genus instead of being a species of *Rhizobium*. Thus the bacteria were renamed to *Sinorhizobium fredii*. Differences among *Bradyrhizobium* spp., *Rhizobium* spp. and *Sinorhizobium* spp. are as shown in Table 2.1

Table 2.1 Comparisons among *Bradyrhizobium* spp., *Rhizobium* spp. and *Sinorhizobium* spp. (Modified from Elkan & Bunn, 1992 ; Holt et al, 1994)

Characteristics	*Bradyrhizobium* spp.	*Rhizobium* spp.	*Sinorhizobium* spp.
Doubling time	longer than 6 h	shorter than 6 h	shorter than 6 h
Metabolic pathway	EMP pathway (minor pathway) ED pathway (major pathway) TCA cycle fully active	TCA cycle fully active	TCA cycle fully active
Type of flagella	1 subpolar flagellum	2-6 peritrichous flagella	1 polar flagellum or 2-3 peritrichous flagella
nifHDK	*nifH*, n*ifDK*, are on different operons	*nifHDK* are on the same operon	
nitrogen fixation genes	are on chromosome only	are on plasmids and chromosome	
Intrinsic antibiotic resistance	high	low	

In 1992, Elkan & Bunn did not include *Mesorhizobium* spp. in the taxonomic classification of the rhizobia as shown in Table 2.2 but Haukka et al (1998) reported that in 1997 Jarvis et al proposed the transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum* and *Rhizobium tianshanense* to *Mesorhizobium* gen.nov.

Table 2.2 Taxonomic classification of the rhizobia (Elkan & Bunn, 1992)

Recognized genus	Recognized species
Bradyrhizobium (Jordan, 1982)	*B. japonicum* (Jordan, 1982)
Rhizobium (Jordan, 1982)	*R. leguminosarum* (Jordan, 1982)
	R. meliloti (Jordan, 1982)
	R. loti (Jordan, 1982)
	R. galegae
Azorhizobium	*A. caulinodans*
Sinorhizobium (Chen et al, 1988)	*S. fredii* (Chen et al, 1988)
	S. xinjiangensis (Chen et al, 1988)

Host specificity is indicated in Table 2.3

Table 2.3 Host-microbe specificity in nitrogen fixation (Somasegaran & Hoben, 1994)

Host		Microbe
Scientific name	Common name	
Glycine max	soybean	*Bradyrhizobium japonicum*
Vigna unguiculata (L.)	cowpea	*Bradyrhizobium* (*Vigna*) sp.
Glycine max cv. Peking	soybean	*Sinorhizobium fredii*
Phaseolus vulgaris	black bean	*Rhizobium leguminosarum*
Medicago sativa	alfalfa	*Rhizobium meliloti*
Arachis hypogaea	peanut	*Bradyrhizobium* (*Arachis*) sp.
Trifolium subterranum L.	clover	*Rhizobium trifoli*

In 1995 Gillis et al isolated *Burkholderia vietnamiensis,* a free-nitrogen fixer from rice rhizosphere grown in a phytotron. In 2001 Moulin et al reported *Burkholderia* spp. strains STM 678 and STM 815 as nitrogen fixing symbionts in the leguminous host, *Macroptilium atropurpureum*. The latter findings have caused considerable excitement among workers in the field of symbiotic nitrogen fixation because the results showed, for the first time, that distantly related bacterial genus of the groups nodulated leguminous plants.

2.2 Infection thread formation

Soybean root hair nodulation is said to be host-specific through mechanisms such as the induction of *nod* genes expression by soybean isoflavones excreted by root hair cells and the presence of *Bradyrhizobium* specific polysaccharides and plant specific carbohydrate binding proteins. In 1987 Kosslak et al reported that *nod* genes in *Bradyrhizobium japonicum* were induced by two isoflavones, 4', 7-dihydroxyisoflavone (daidzien) and 4', 5, 7-trihydroxyisoflavone (genistein) isolated from *Glycine max*. It has been speculated that each *B. japonicum*, which possesses one subpolar flagellum, "moves" towards root hairs of *Glycine max* through chemotaxis. Root hair cell wall contained carbohydrate-binding proteins (lectins) which are specific to *B. japonicum* extracellular polysaccharides. Lectins thus act as *B. japonicum*'s receptor proteins. The next phenomenon is root hair curling and in so doing enabling *B. japonicum* to attach to the surface of root hair. (Li & Hubbell, 1969) *B. japonicum* then secreted extracellular enzymes including cellulases and lignin-degrading enzymes (if any) to digest root hair cell wall. The next step is invagination of root hair cell membrane to form invagination sac which elongates towards the root cortex layer in the form of an infection thread within which *B. japonicum* cells are delivered to cortex cells. Upon reaching the cortex cells, the plant cell membrane enclosing *B. japonicum* cells is detached from the cell membrane to form a symbiosome containing *B. japonicum* which have lost flagella and become irregularly-shaped bacteroids which fix nitrogen via symbiosis with soybeans. Table 2.4 showed types of flagella in *Burkholderia* spp., *Sinorhizobium* spp. and *Bradyrhizobium japonicum*.

Table 2.4 Types of flagella in *Burkholderia* spp., *Sinorhizobium fredii*., *Bradyrhizobium japonicum*.

Burkholderia spp.	*Sinorhizobium fredii*	*Bradyrhizobium japonicum*	Reference
monotrichous or multitrichous polar flagella	(formerly *Rhizobium fredii*) 2-6 peritrichous flagella	1 subpolar flagellum	Elkan & Bunn, 1992 ; Holt et al, 1994

In 1999 Lin et al reported that flavonoids : genistein,naringenin,chrysin and apigenin purchased from Sigma (St.Louis,Mo.) promoted growth of *Sinorhizobium fredii* USDA257 in the late log phase . However, only genistein accelerated growth of *S. fredii* TU6 under the same condition. So far there have not been reports on the induction of *S. fredii* common *nod* genes by isoflavones isolated from soybeans .

It is plausible to expect that the mechanism for nodule formation in the case of *Sinorhizobium* spp. and *Bradyrhizobium japonicum* may be the same except the types of inducing isoflavones secreted by different soybean cultivars may be different leading to different host-specificity among *Sinorhizobium fredii*, *Bradyrhizobium japonicum* and different soybean cultivars.

2.3 Strain identification of nitrogen-fixing bacteria

2.3.1 Traditional vs. molecular biology techniques

Apart from the traditional morphology, Gram staining, biochemical tests, and plant inoculation tests employed in strain identification of nitrogen-fixing bacteria, there are several molecular biology techniques being used in strain identification.

With the advent of Polymerase Chain Reaction (PCR) by Mullis & Faloona in 1987, it is possible to obtain DNA fingerprints via a technique called Ramdom Amplification of Polymorphic DNA-PCR (RAPD-PCR) where one primer is used in the amplification of DNA

fragments. The primer may either be arbitrary or sequence-directed. (Young & Cheng, 1998)

2.3.1.1 PCR fingerprints of *Bradyrhizobium japonicum*

In 1992 de Bruijn used two sets of forward and reverse primers the first of which annealed to the Repetitive Extragenic Palindromic (REP) sequences. The second set of primers annealed to the Enterobacterial Repetitive Intergeneric Consensus (ERIC) sequences. The first set of primers was REPIR-1 and REP2-I ; the second set of primers was ERICIR and ERIC2 as indicated in Figure 2.1. de Bruijin (1992) obtained distinct DNA fingerprints for several strains of *Rhizobium meliloti* , *R.leguminosarum*, *R.loti* and *B. japonicum.*

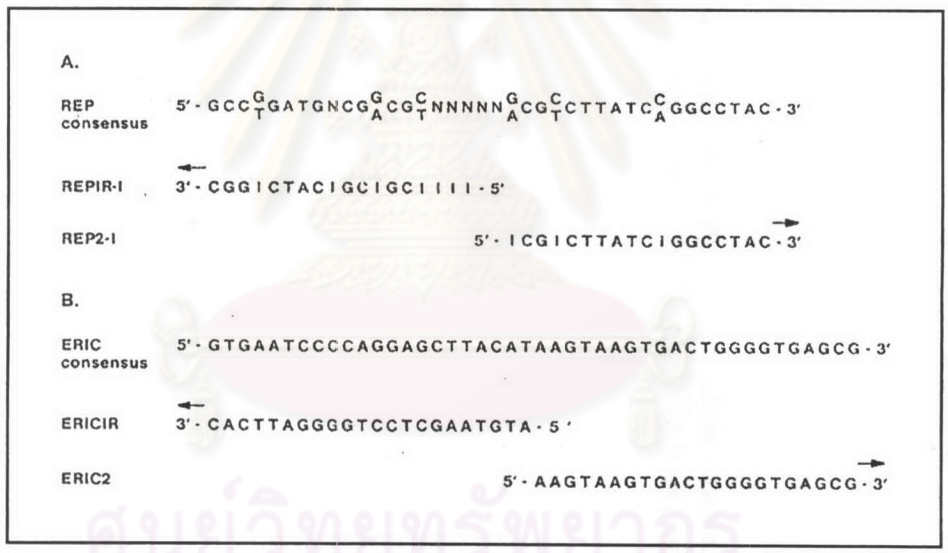


Figure 2.1 Nucleotide sequence of the REP and ERIC primers. Arrows denote the direction of *Taq* polymerase extension (de Bruijn,1992).

In 1993 Judd et al used the two sets of PCR primers REPIR-I and REP2-I ; ERICIR and ERIC2 as indicated in Figure 2.1 to obtain REP and ERIC PCR fingerprints of 11, 12 and 1 strains of *B. japonicum* in serogroups 123, 127 and 129 respectively. The results showed

that REP and ERIC PCR technique could be used to distinguish between genetically and phenotypically near-identical *B. japonicum* strains in serogroups 123, 127, and 129.

In 1995 Richardson et al used PCR in conjunction with two arbitrary primers (RPO4 and RPO5) of 10 nucleotides length and a sequence-specific primer (RPO1) of 20 nucleotides length to obtain RAPD-PCR DNA fingerprints which differentiated strains in a diverse collection of *Rhizobium meliloti* , *R. leguminosarum* bv *trifolii* and *R.l.* bv. *viciae*. The sequence of RPO1 was conserved in the promoter of the *nif HDK* operon of *Rhizobium trifolii* strains Rt329, Rt RS1 and *R. meliloti* RmP1 (Schofield and Watson,1985).

Mathis & McMillin (1996) successfully used CRL-7 which was an arbitrary GC rich primer in the DNA fingerprinting of *B. japonicum.*

2.3.1.2 PCR fingerprints of *Sinorhizobium* spp.

Sanchez-Contreras et al (2000) reported the simultaneous use of four primers : nodbox1, nodbox3, mucRf and mucRr in the PCR amplification of genomic DNA from *Sinorhizobium meliloti* EFB1 ; *S. meliloti* 2011, *S. meliloti* GR4, *Rhizobium etli* CE3 ; *Rhizobium leguminosarum* 3841 ; *Rhizobium leguminosarum* STR6 ; *Sinorhizobium fredii* HH103 ; *Rhizobium* sp. Strain NGR234 ; *Pseudomonas fluorescens* F113. Primers nodbox1 and nodbox3 were used to amplify the nodbox4 region which was located in one of the megaplasmids while the use of primers mucRf and mucRr amplified the 431 bp coding region of a regulatory protein implicated in the regulation of exopolysaccharides in *S. meliloti.* The results as shown in Figure 2.2 indicated that the simultaneous use of the four primers gave rise to DNA fragments specific to *Sinorhizobium meliloti.* Sequences of primers nodbox1, nodbox3, mucRf and mucRr were shown in Table 2.5

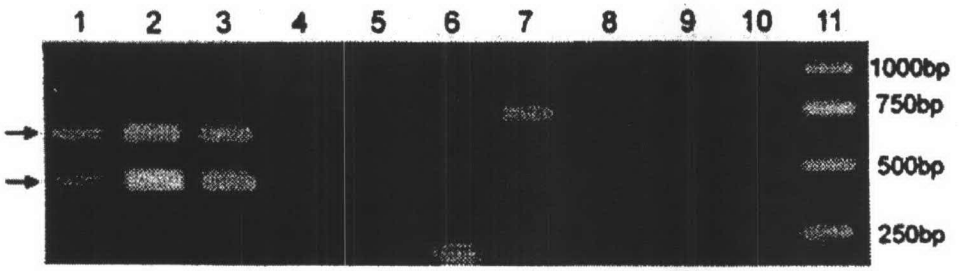


Figure 2.2 PCR amplification of genomic DNA from different soil bacteria when primers nodbox1, nodbox3, mucRf, and mucRr were used simultaneously. The arrows indicate the position of the 431-bp *mucR* amplicon and the 646-bp nodbox4 amplicon. Lane 1, *S. meliloti* EFB1 ; lane 2, *S. meliloti* 2011, ; lane 3, *S. meliloti* GR4, ; lane 4, *Rhizobium etli* CE3 ; lane 5, *Rhizobium leguminosarum* 3841 ; lane 6, *Rhizobium leguminosarum* STR6 ; lane 7, *Sinorhizobium fredii* HH103 ; lane 8, *Rhizobium* sp. Strain NGR234 ; lane 9, *Pseudomonas fluorescens* F113 ; lane 10, negative control without DNA ; lane 11, molecular weight markers.

Table 2.5 Sequences of primers used for PCR amplification of nodbox 4 region and *mucR* region (Sanchez-Contreras et al, 2000).

Primer	Sequence (5'-3')	Melting temp (C^0)
nodbox1	TCTTTTCTTATCCATAGGGTGG	57.28
nodbox3	ACGGATCGTCCTCGAAG	58.4
mucRf	ATGACAGAGACTTCGCTCGGT	57.10
mucRr	TCACTTGCCGCGACGCTT	58.2

2.3.1.3 PCR fingerprints of *Burkholderia* spp.

Estrada-de Los Santos et al (2001) reported that there were 21 species of *Burkholderia* spp. including *Burkholderia vietnamiensis* which was the only known nitrogen-fixing species in this bacterial genus. In 2002, Salles et al reported the use of PCR-DGGE (Denaturing Gradient Gel Electrophoresis) system to detect diversity of *Burkholderia* spp. in bulk soil samples and in rhizosphere samples from two glassland plots in a field located in Wageningen, The Netherlands. The following primers which were specific for the amplification of 16S rDNA of *Burkholderia* spp. were used to obtain amplified fragments of 14 species of referance strains of *Burkholderia* spp.

Forward primer (Burk3) : 5'CTGCGAAAGCCGGAT3'

Reverse primer (BurkR) : 5'TGCCATACTCTAGCYYGC3'

(Y= T or C)

a GC clamp (5'CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG3') was attached to the 5' end of the forward primer so that it could be used in the DGGE system.

Aliquots of the PCR samples (2 to 4 μl) were applied directly to 6 or 8 % (wt/vol) polyacrylamide gels with denaturing gradient of 50 to 60 % of denaturant (100 % denaturant (corresponds to 7 M urea plus 40 % vol/vol of deionized formamide). DGGE was performed in 0.5 x TAE buffer at 60^0C at a constant voltage of 150 V for 4-6 h depending on the PCR products. A routine silver staining protocol was used for detection of DNA in DGGE gels.

Heuer et al (1997) stated that the subsequent eletrophoretic separation of the PCR products in a polyacrylamide matrix over a denaturing gradient was a technique introduced in microbial ecology by Muyzer et al in 1993. DGGE patterns offer the possibility to analyze bands of interest in depth by sequencing or probing.

Rosado et al (1998) stated that DGGE allowed separation of DNA molecules that differed by single bases and hence had the potential to provide information about variations in target genes in bacterial populations in natural systems. Salles et al (2002) reported that DGGE analyses of the PCR products obtained from both pure-culture and soil DNAs

revealed that this technique was useful for evaluating the diversity of *Burkholderia* in soil samples. Figure 2.3 showed DGGE patterns of 16S rDNA fragments of *Burkholderia* spp.

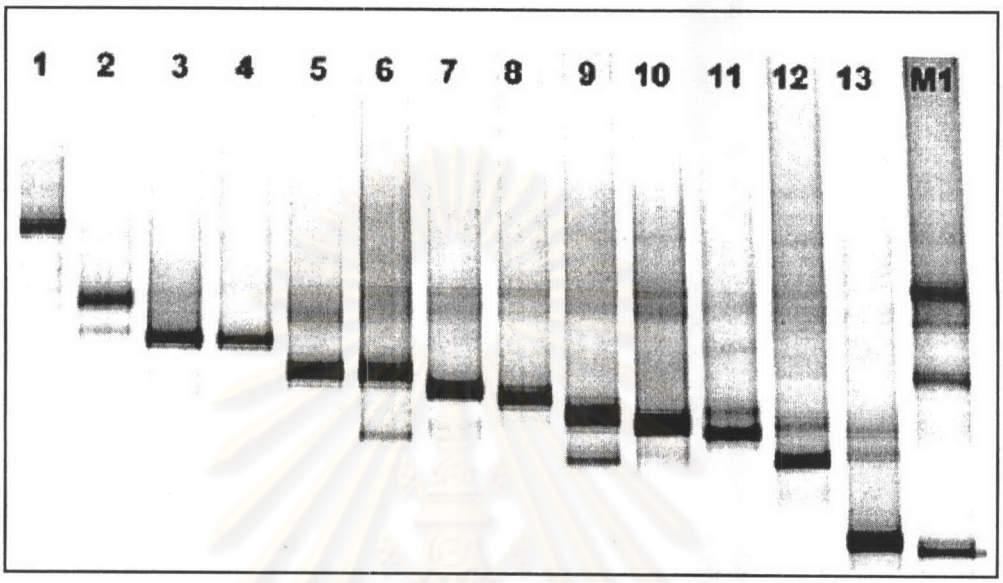


Figure 2.3 DGGE patterns of 16S rDNA fragments of *Burkholderia* species generated by PCR with *Burkholderia*-specific primers (positions 155 to 663 *E. coli* numbering) in a 50 to 60 % denaturing gradient. Lane 1, *B. glathei* WD1 ; lane 2, *B. multivorans* LMG13010 ; lane 3, *B. plantarii* NCPPB3590 ; lane 4, *B. gladioli* ATCC33664 ; lane 5, *B. pyrrocinia* ATCC15958 ; lane 6, *B. stabilis* LMG14294 ; lane 7, *B. vietnamiensis* LMG10929 ; lane 8, *B. phenazinium* LMG2247 ; lane 9, *B. caribensis* WD 3 ; lane 10, *B. glumae* NCPPB3708 ; lane 11, *B. graminis* WD2 ; lane 12, *B. caribensis* LMG18531 ; lane 13, *B. caryophylli* NCPPB353 ; lane M1, *Burkholderia* marker containing (from top to bottom) *B. multivorans* LMG13010, *B. cepacia* ATCC25416, and *B. cepacia* LMG18941 (Salles et al, 2002).

Although the technique PCR-DGGE combined the sensitivity and specificity of the genus-specific PCR with direct screening of the dominant sequences, visualized on the basis of sequence divergence, via DGGE (Salles et al, 2002), the technique has not been used for the detection of *Bradyrhizobium japonicum* and *Sinorhizobium fredii* in both pure culture and soil DNAs.

2.4 Heat shock proteins

Heat shock proteins consist of a set of proteins which increase in quantities upon tranfer of living organisms to high temperatures. Heat shock proteins are conserved throughout evolution from *E. coli* to *Drosophila* to man (Ashburner, 1982). There are 4 major families of heat shock proteins in prokaryotes which function as proteases (Clp, 100 kDa), Chaperones (DnaK, 70 kDa), Chaperonins (GroEL, 60 kDa ; GroES 10 kDa) and regulatory small heat shock proteins (12-30 kDa). Since heat shock proteins function in normal protein folding and protein degradation, they are normally present in the cells but increase in quantities upon heat shock.

2.4.1 Clp (Casein-lytic proteases)

Clp, Casein-lytic proteases are composed of intracellular ATP dependent proteases which degrade either denatured proteins or aggregated proteins resulting from misfolding.

2.4.2 DnaK

dnaK forms an operon consisting of *grpE-dnaK-dnaJ* genes. DnaK is approximately 70 kDa and contains the ATPase-binding site and a polypeptide binding site at the amino terminal and at the carboxy terminal respectively. The ATPase activity of DnaK has been suggested as a major thermodynamic force in the release and/or folding of DnaK-associated proteins. DnaK, DnaJ, and GrpE bind σ^{32} in a complex. Upon heat shock, DnaK, DnaJ and GrpE disassociate from the complex to perform chaperone function leaving σ^{32} available for the σ^{32}-dependent gene expression (Minder et al, 1997).

2.4.3 GroESL

GroEL comprises two stacked hollow rings each of which consists of seven 60 kDa units. GroES is a single ring of seven 10 kDa subunits. The space within the hollow structure of the complex GroESL provides an environment for folding nascent polypeptides preventing undesirable aggregate formation in the cytoplasm. GroEL and GroES form a complex with each other in the presence of ATP. Figure 2.3 is a diagram depicting the folding of a polypeptide in the hollow space of the complex structure GroESL (Voet & Voet, 1995).

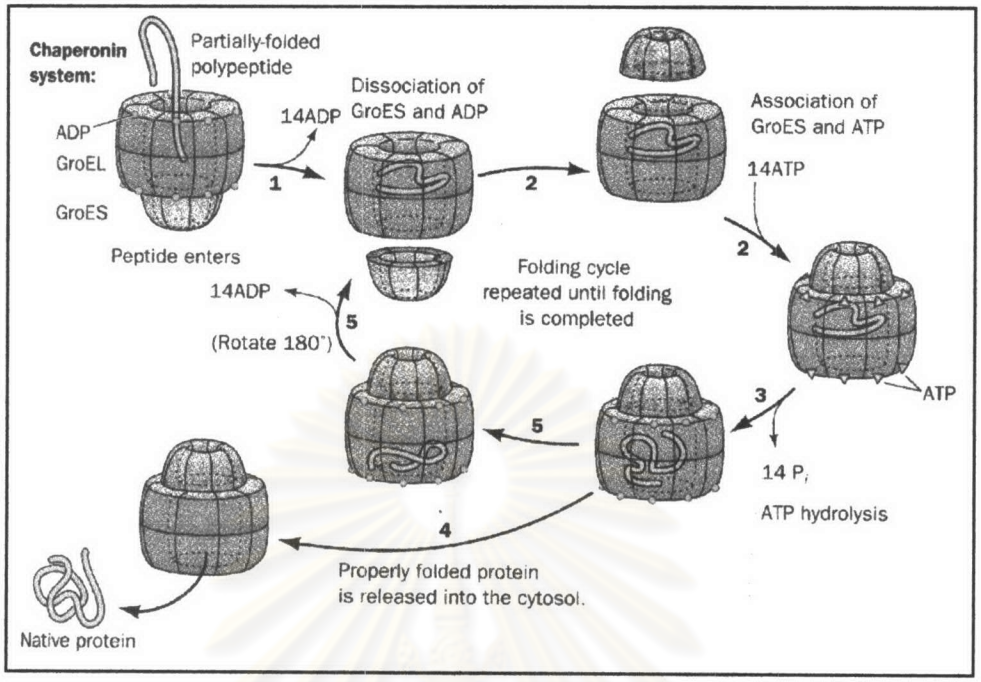


Figure 2.4 The reaction cycle of the *E. coli* chaperonins GroEL and GroES in protein folding (1) GroEL in asymmetric complex with one heptameric ring of GroES and 14 ADP's (one per GroEL subunit) binds an unfolded polypeptide in its centrol cavity in a process that releases all 14 ADP's and the bound GroES. (2) GroEL binds 14 ATPs thereby weakening the interaction between GroESL and the unfolded polypeptide and causing the rebinding of GroES to the opposite face of GroEL. (3) All 14 ATPS are simultaneously hydrolyzed, thereby releasing the bound polypeptide within GroEL. (4) If the polypeptides has folded to its native conformation, it is released from GroEL. (Volt & Voet, 1995).

Bradyrhizobium japonicum contains 5 *groESL* operons as shown in Figure 2.5

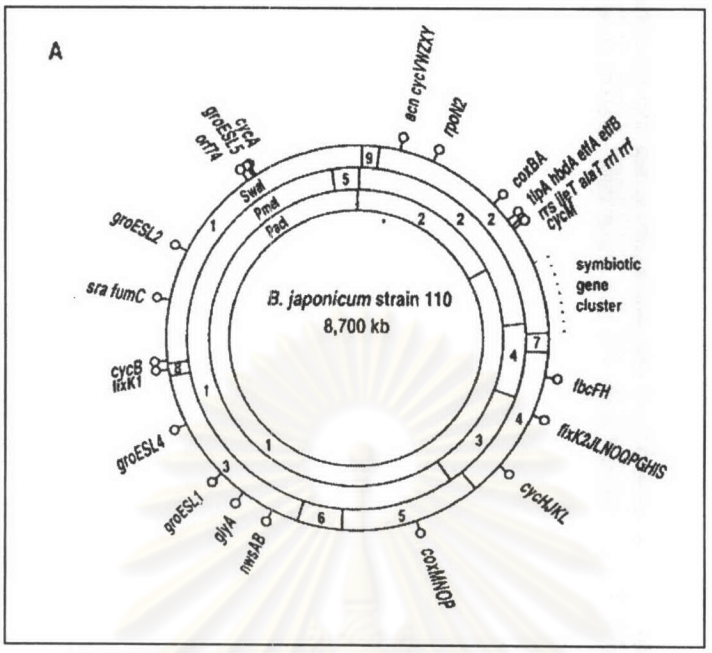


Figure 2.5 (A) Partial genetic map of *Bradyrhizobium japonicum* USDA 110 showing 5 *groESL* operons (B) Enlarged region of symbiotic gene cluster (Kundig et al, 1993).

Fischer et al (1993) determined the transcription start site of *B. japonicum* $groESL_3$ operon by a primer extension experiment using two different 32 mer oligonucleotides complementary to sequence positions 359-390 and 457-488. Template RNA was isolated from anaerobically grown *B. japonicum* strain 7987 and 400 units of Superscript reverse transcriptase were used for the extension reactions for 1 h at 42^0C. A well-conserved –24/-

12 promoter was identified ~70 bp upstream of the coding region for *groES3* (T_{325}GGCCT-N_5 –TTGCT$_{340}$). A putative binding site for the transcription activator protein NifA was present at ~120 bp upstream of the –24/-12 promoter (T_{206}GT-N_{10}-ACA) suggesting that expression of *groESL*$_3$ in *B. japonicum* was dependent on NifA and the σ^{54} RNA polymerase as indicated in Figure 2.6.

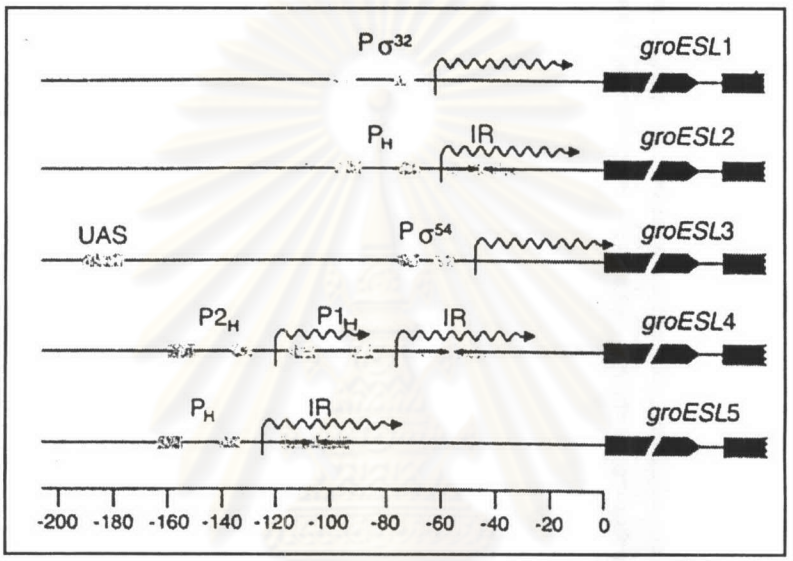


Figure 2.6 Promoter elements in all five *groESL* operons of *B. japonicum*. Wavy arrows schematically indicate *groESL* transcripts. The following elements are marked : P, promoter ; p_H, house-keeping promoter ; IR, inverted repeat ; UAS, upstream activator sequence (binding site for NifA) (Babst et al, 1996).

According to Babst et al (1996) the three heat-shock inducible *groESL* operons (*groESL*$_{1/4/5}$) were not transcribed and regulated by a uniform mechanism. σ^{32} was shown to be required only for *groESL*$_1$ transcription. The promoter regions of *groESL*$_4$ and *groESL*$_5$, both being of the –35/-10 type, were followed by a conserved 9 bp inverted repeat, CIRCE element, which Zuber & Schumann (1994) reported the following sequence

-35 -10 +1

5'TTGACATTTTTCTTGTGGTTTGATACTTTTGTTATAGAA**TTAGCACTC**

GCTTATTGA**GAGTGCTAA**CAGAGGTGATGATGATG-3'

CIRCE is abbreviated from Controlling Inverted Repeat of Chaperone Expression.

Babst et al (1996) reported that there was no evidence for up-regulation of *groESL*$_2$, either by heat shock or by any other growth condition tested. Therefore it appeared that this operon was expressed constitutively from a house-keeping promoter which contained –35 and –10 motifs which was likely to be recognized by by the RNA polymerase holoenzyme containing σ^{96}.

In conclusion other than the NifA activation of *groESL*$_3$ operon, there are three additional two known mechanisms for *groESL* gene regulation in *B. japonicum*. The additional mechanisms are as follows :

1) Transcriptional regulation by σ^{32}

σ^{32} forms a complex with DnaK under normal temperature, However upon heat shock, DnaK associates itself to act as a chaperone in protecting partially denatured proteins leaving σ^{32} available for binding with RNA polymerase core enzyme ($\alpha_2\beta\beta'$ subunits). The holoenzyme formed, $\alpha_2\beta\beta'$ $\sigma,^{32}$ reacts with the –10/-35 promoter of heat shock operon such as the *groESL*$_1$.

2) Repression by HrcA on CIRCE

The operons *groESL*$_4$ and *groESL*$_5$ *B. japonicum* contain an operator called CIRCE (Controlled Inverted Repeat of Chaperone Expression) which consists of 9 inverted base pairs. Under normal temperature for growth, protein HrcA (Heat Regulation at CIRCE) binds at the operotor (CIRCE). Upon heat shock. HrcA loosens and detaches from the operator site enabling RNA polymerase to attach to the promoter site for transcription of heat shock genes (Minder et al, 2000).

Babst et al (1996) concluded that *B. japonicum* was clearly the first example where both the σ^{32}- and the CIRCE-dependent modes of heat-shock regulation were functionally characterized in one and the same bacterium.

2.4.4 ROSE-dependent heat shock operons (ROSE = Repression Of heat Shock gene Expression). (Münchbach, 1999)

Münchbach et al (1999) reported that there were at least 7 small heat shock proteins, 15-30 kDa, in the 5 operons as shown in Figure 2.7

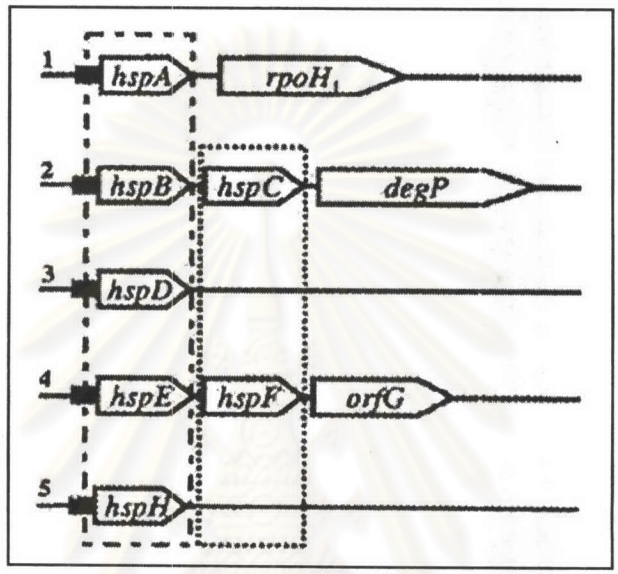
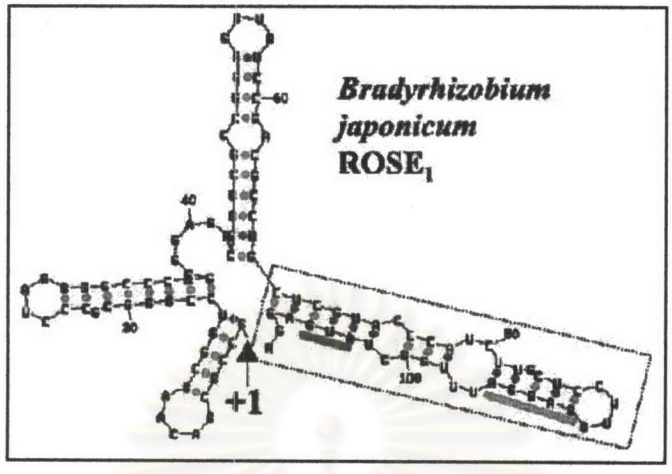


Figure 2.7 Diagram showing ROSE- dependent heat shock operons coding for small heat shock proteins (15-30 kDa) in *B. japonicum*. Black boxes preceding each operon represent $ROSE_1$ to $ROSE_5$ (Münchbach et al,1999).

(A)



(B)

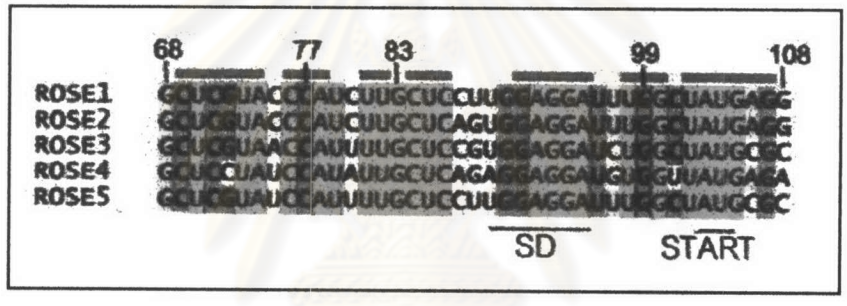


Figure 2.8 (A) Predicted mRNA structure of $ROSE_1$ from *B. japonicum*. Nucleotides are numbered starting from the transcription start site +1. Each sequence comprises the entire ROSE element including the Shine-Dalgarno (SD) sequence, the translation start site (AUG)

(B) Alignment of the 3'ends of ROSE mRNAs from *B. japonicum* ($ROSE_{1-5}$). (Nocker et al, 2001).

When predicted mRNA structures of $ROSE_1$ from *B. japonicum*, $ROSE_{N1}$ from Rhizobium sp. NGR_{234}, $ROSE_{p2}$ *Bradyrhizobium* sp. (Parasponia), and $ROSE_{2387}$ from *Mesorhizobium loti* were analysed, Nocker et al (2001) found that the 3' end of the ROSE sequences starting from nuleotides 68 to 108 form conserved stems and loops.

Therefore the secondary mRNA structure around the ROSE area might play an important role in small heat shock gene regulation. Hence Nocker et al (2001) randomly point mutated the 682 bp *PstI* in translational $ROSE_1$-*hspA-lacZ* fusions as indicated in Figure 2.9 and integrated the mutated fusions into *B. japonicum* chromosome.

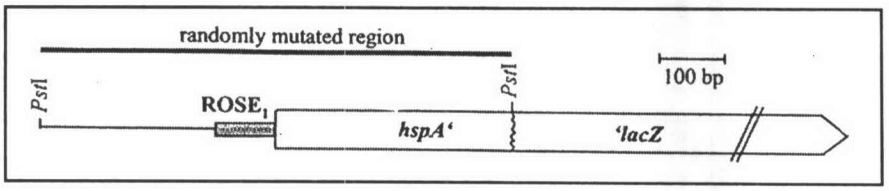


Figure 2.9 Schematic representation of the translational $ROSE_1$-*hspA-lacZ* fusion for site-directed point mutations in ROSE (Nocker et al, 2001).

The results indicated that when nucleotides which formed complementary base-pairing with the Shine-Dalgarno sequence (AGGAGG) were mutated, that is, $T_{81}T_{82}$ and C_{84} were chaped to $G_{81}A_{82}$ and A_{84} there was desepression of the *lacZ* gene. The authers postulated that ROSE-depent gene regulation was at the post-transcriptional level and that at normal temperature the secondary structure of mRNA at $ROSE_1$ prevented the 3' end of 16S rRNA to get access to the Shine-Dalgarno sequence. However, upon heat shock the loosening up of the secondary structure enableed the access to the Shine Dalgarno sequence and hence the translation started as shown in Figure 2.10.

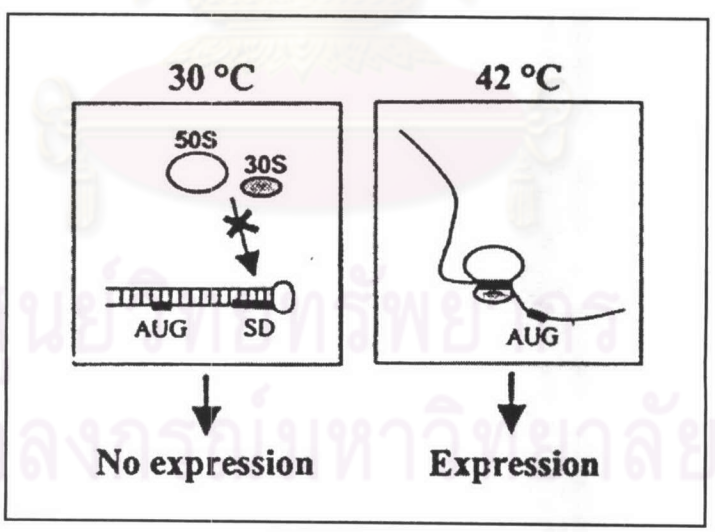


Figure 2.10 Model for temperature-responsive regulation by ROSE. The SD sequence and the AUG start codon are indicated in the schematic hairpin structure at the 3'-end of ROSE. Grey ovals represent large (50S) and small (30S) ribosomal subunits. (Nocker et al, 2001).