การคัดเลือกมิวแทนต์ TnAraOut จาก *Sinorhizobium fredii* S174 ที่ตรึงไนโตรเจนและทนร้อน

นายสมโชค กาลา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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SELECTION OF TnAraOut MUTANTS FROM NITROGEN-FIXING AND HEAT TOLERANT

Sinorhizobium fredii S174

Mr Somchoke Kala

A Thesis Submitted in Partial Fulfillment of the Requirements

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By	Mr Somchoke Kala
Field of Study	Microbiology
Thesis Advisor	Associate Professor Kanjana Chansa-ngavej, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillments of the Requirements for the Master's Degree

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สมโชค กาลา : การคัดเลือกมิวแทนต์ TnAraOut จาก *Sinorhizobium fredii* S174 ที่ตรึงไนโตรเจนและทนร้อน (SELECTION OF TnAraOut MUTANTS FROM NITROGEN-FIXING AND HEAT TOLERANT *Sinorhizobium fredii* S174)
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Sinorhizobium fredii เป็นแบคทีเรียตรึงไนโตรเจนและทนร้อนที่มีอัตราการเพิ่มจำนวนเร็ว (เวลาที่ใช้ในการเพิ่มจำนวนเซลล์เป็นสองเท่า น้อยกว่า 6 ชั่วโมง) พบในปมรากถั่วเหลือง แบคทีเรียตรึงไนโตรเจนในปมรากถั่วเหลืองอีกประเภทหนึ่งได้แก่ *Bradyrhizobium japonicum* มีการเพิ่มจำนวนช้า (เวลาที่ใช้ในการเพิ่มจำนวนเซลล์เป็นสองเท่า มากกว่า 6 ชั่วโมง) วัตถุประสงค์ของการทดลอง เพื่อแยกมิวแทนต์ที่เพิ่มจำนวนช้าจาก *S. fredii* S174 ที่ตรึงไนโตรเจนและทนร้อน โดยทำ biparental mating ระหว่าง *S. fredii* S174 และ *E. coli* S17-1λ*pir* (pNJ17) TnAraOut เป็นทรานสโปซอน บนพลาสมิด pNJ17 ซึ่งประกอบด้วย โพรโมเตอร์ที่ใช้อราบิโนสในการเหนี่ยวนำ (P_{BAD}), ยีน *ara*C และ ยีนระบุการดื้อยากานามัยซิน ทรานสโปซอน TnAraOut จะแทรกเข้าบริเวณ TATA ซึ่งเป็นส่วนหนึ่งของ -10/-35 โพรโมเตอร์ทำให้โพรโมเตอร์นั้นๆ กลายเป็น โพรโมเตอร์ที่ต้องใช้อราบิโนสในการเหนี่ยวนำ (P_{BAD}) ดังนั้นถ้าเลี้ยงมิวแทนต์ในอาหารเลี้ยงเชื้อที่มีอราบิโนส เซลล์จะเพิ่มจำนวนเร็ว ทำให้ได้โคโลนีขนาดใหญ่ แต่เมื่อเลี้ยงมิวแทนต์ในอาหารเลี้ยงเชื้อที่ไม่มีอราบิโนส เซลล์จะเพิ่มจำนวนช้า ทำให้ได้โคโลนีขนาดเล็ก พิสูจน์การมี TnAraOut แทรกในโครโมโซมของมิวแทนต์ โดยตัดดีเอ็นเอของมิวแทนต์ด้วย เรสทริกชั่นเอนไซม์ *Sph*I ทำชิ้นส่วนดีเอ็นเอ ที่ได้ให้เป็นวงกลม หลังจากนั้น อิเลคโตรพอเรต เข้าไปในเซลล์ของ *E. coli* DH5$\alpha\lambda$*pir* แยกโครโมโซมของโคโลนีที่ดื้อยากานามัยซินและใช้เป็น target DNA ในการทำ PCR โดยใช้ไพรเมอร์ซึ่งอยู่บนพลาสมิด pNJ17 ได้แก่ $P_{BADout2}$ ซึ่งเป็นลำดับนิวคลีโอไทด์ส่วนหนึ่งของโพรโมเตอร์ที่ต้องใช้อราบิโนสในการเหนี่ยวนำ ผลการทดลองพบลำดับนิวคลีโอไทด์ (1457 นิวคลีโอไทด์) ซึ่งเป็นรายงานครั้งแรกสำหรับลำดับนิวคลีโอไทด์ของ 16S rDNA ของ *S. fredii* S174 นอกจากนี้ได้แยกมิวแทนต์ 15 ชนิด นำมิวแทนต์ 2 ชนิด ได้แก่ ST49 และ ST60 มาพิสูจน์การมีชิ้นส่วน TnAraOut แทรกในโครโมโซม ผลการทดลองแสดงให้เห็นว่ามีการแทรกของ TnAraOut เข้าไปในบริเวณ TATA ของโพรโมเตอร์ใน TnAraOut มิวแทนต์ นอกจากนี้ TnAraOut มิวแทนต์ ST49 และ ST60 มีสมบัติการทนร้อนเทียบเท่ากับสมบัติการทนร้อนของ wild type *S. fredii* S174 โดยมีโปรตีนโพรไฟด์ของเซลล์ที่เลี้ยง ณ อุณหภูมิสูงคล้ายกัน พบการสร้างพอลิเปปไทด์ 10, 12, 60 และ 62 กิโลดาลตัน เพิ่มขึ้น สมบัติการตรึงไนโตรเจนของ TnAraOut มิวแทนต์ ST60 เทียบเท่ากับสมบัติของ wild type *S. fredii* S174 เมื่อใช้ถั่วเหลือง *Glycine max* พันธุ์ สจ 4 เป็นถั่วเหลืองในการเหนี่ยวนำให้สร้างปม

ภาควิชา จุลชีววิทยา ลายมือชื่อนิสิต..
สาขาวิชา จุลชีววิทยาทางอุตสาหกรรม ลายมือชื่ออาจารย์ที่ปรึกษา..........................
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SOMCHOKE KALA : SELECTION OF TnAraOut MUTANTS FROM NITROGEN-FIXING AND HEAT TOLERANT *Sinorhizobium fredii* S174

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Sinorhizobium fredii is a fast growing nitrogen-fixing heat tolerant bacterium in soybean root nodules with doubling time less than 6 hours. Another category of soybean rhizobia is a slow-growing *Bradyrhizobium japonicum* with doubling time more than 6 hours. The aims of the experiments are to isolate slow-growing TnAraOut mutants from fast-growing nitrogen-fixing and heat tolerant *S. fredii* S174 by biparental mating between *S. fredii* S174 and *E. coli* S17-1λ*pir* (pNJ17). TnAraOut is a transposon on the plasmid pNJ17 with arabinose-inducible promoter (P_{BAD}), *ara*C and kanamycin resistant gene. The transposon inserts in the TATA region of -10/-35 promoters rendering the promoters to be arabinose-inducible. Mutants with cell division gene promoter disruption grew normally and yielded large colonies in the presence of arabinose. They grew slowly resulting in small colonies when arabinose was not in the medium. Confirmation of the presence of TnAraOut in the mutant genomes was carried out by digesting each mutant's DNA with *Sph*I , recircularization, then electroporation into *E. coli* DH5$\alpha\lambda$*pir* and selection for kanamycin resistant colonies. DNA of each kanamycin-resistant colony was isolated and used as target DNA for PCR with $P_{BADout2}$ as the primer. PCR products were sequenced for the presence of an inverted repeat sequence of TnAraOut. Experimental results revealed the first full 16S rDNA sequence of *S. fredii* S174 (1457 nucleotides). In addition, fifteen TnAraOut mutants were obtained. Two TnAraOut mutants (ST49 and ST60) were used to prove the presence of TnAraOut sequence in their genomes. Experimental results revealed the presence of the insertion sequence in the TATA region of the promoters of the mutants. In addition, thermotolerance properties of TnAraOut mutants ST49 and ST60 were found to be comparable to that of the wild type. SDS-PAGE of intracellular proteins of cells grown under high temperatures indicated similar protein profiles with increased synthesis of polypeptides 10, 12, 60, and 62 kDa. Nitrogen fixing potential of TnAraOut mutant ST60 was found to be comparable to that of the wild type when *Glycine max* cultivar SJ4 was used as the soybean host.

Department Microbiology Student's signature……………………

Field of study Industrial Microbiology Advisor's signature…………………….

Academic year 2004

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CHAPTER 1

INTRODUCTION

Soybean rhizobia are nitrogen-fixing bacteria in root nodules of soybeans [*Glycine max* (L.) Merr]. At present there are 2 categories of soybean rhizobia, the fast-growers and the slow-growers. Species of soybean rhizobia which are commonly recognized are shown in Table 1.

Table 1. Commonly recognized species of soybean rhizobia.

Soybean rhizobia	References
Fast-growers	
Sinorhizobium fredii	Chen et al. 1988
Sinorhizobium xinjiangense	Peng et al. 2002
Slow-growers	
Bradyrhizobium elkanii	Kuykendall et al. 1992
Bradyrhizobium japonicum	Jordan, 1982
Bradyrhizobium liaoningense	Xu et al. 1995

At present no information is available regarding genetic differences between the fast-and the slow-growing soybean rhizobia. The aims of the experiments are to isolate TnAraOut mutants from the fast-growing nitrogen-fixing and heat tolerant *S. fredii* S174 in order to obtain some information regarding genes for cell division in *S. fredii* S174 and compare nitrogen fixation and heat tolerance potential between TnAraOut mutants and wild type *S. fredii* S174. One approach to isolate genes for cell division in *S. fredii* is to tag its -10/-35 promoters with the transposon TnAraOut which inserts itself between the TATA sequence of the -10/-35 promoters rendering the natural promoters to be replaced by arabinose - inducible promoters. Figure 1.1 indicated nucleotide sequences at -10 and -35 regions of a-10/-35 promoter.

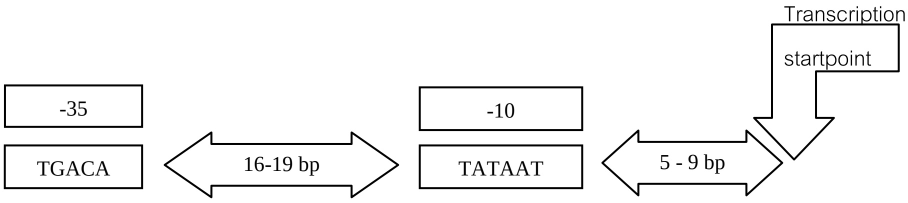


Figure 1.1 Nucleotide sequences at -10 and -35 regions of a -10/-35 promoter (Voet & Voet, 1995).

1.1 TnAraOut

In 2000, Judson & Mekalanos proposed the use of the constructed plasmid pNJ17 containing the transposon TnAraOut to disrupt natural promoters of essential genes and replace them with the arabinose - inducible promoter (P_{BAD}). Transposon TnAraOut is located on pNJ17 plasmid as shown in Figure 1.2

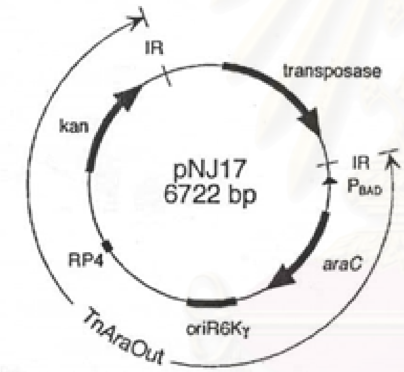


Figure 1.2 Plasmid pNJ17 consists of Inverted Repeats (IR), genes encoding tranposase and kanamycin resistance, Arabinose - inducible promoter (P_{BAD}), *ara*C, *mob* from plamid RP4 and oriR6K$_{\gamma}$ (Judson & Mekalanos, 2000).

Judson & Mekalanos (2000) developed tranposon TnAraOut to tag promoters of essential genes for survival of *Vibrio cholerae*. TnAraOut inserts itself in the TATA regions of -10/-35 promoters rendering the promoters to be replaced by arabinose – inducible promoters. Figure 1.3 showed the mechanism involved in the induction of *araBAD* gene expression.

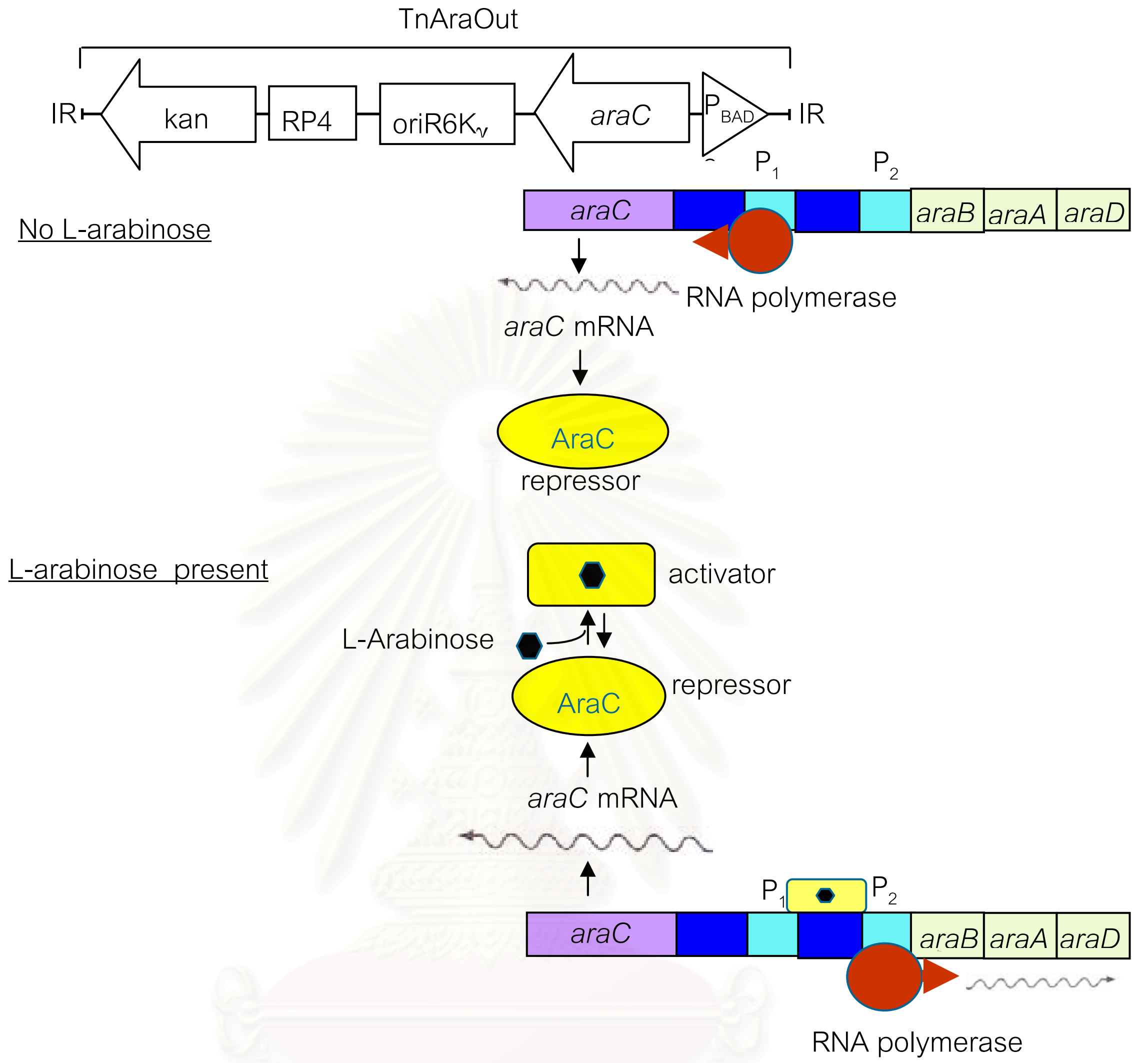


Figure 1.3 Mechanism involved in L-arabinose inducible expression of *araBAD*. There are two promoters (P_1 and P_2) at the P_{BAD} region. When there is no L-arabinose, RNA polymerase will transcribe from P_1 promoter resulting in the transcription of *araC* mRNA which is translated into AraC repressor protein which will bind the operator site of the *araBAD* operon. When L-arabinose is present in the culture medium, L-arabinose will bind with the AraC repressor protein resulting in an activator protein which will activate the expression of the *araBAD* operon from P_2 promoter (modified from Voet & Voet, 1995).

CHAPTER 2

LITERATURE SURVEY

2.1 *Sinorhizobium fredii*

Fast-growing soybean rhizobia were first isolated from root nodules of soybeans in the People's Republic of China (Keyser et al. 1982). Chen et al (1988) reported that Scholla & Elkan (1984) proposed the scientific name *Rhizobium fredii* based mainly on DNA hybridization comparisons of five strains of these bacteria with representatives of the genera *Rhizobium* and *Bradyrhizobium*. In 1988 Chen et al performed 240 biochemical tests on 33 strains of fast-growing soybean rhizobia isolated from soils and soybean nodules collected in the People's Republic of China, and the other 25 strains of representatives of *Rhizobium*, *Bradyrhizobium* and *Agrobacterium*. Results of the biochemical tests were grouped into levels of similarity upon which a dendrogram was constructed. The fast-growing soybean rhizobia were found to form two groups which were clearly separated from *B. japonicum, R. leguminosarum, R. meliloti,* and *Agrobacterium* spp. Chen et al (1988) thus proposed the names *Sinorhizobium xinjiangensis* for the first group of fast-growing soybean rhizobia, and *Sinorhizobium fredii* for the second group of fast - growing soybean rhizobia.

Sinorhizobium fredii S174 was isolated from nodules of soybean (*Glycine max* cv Sor Jor 5) grown in acid soil (average pH 5.25) from Kao Kaw district, Petchaboon province by Suwat Saengkerdsub (1999). The bacterium was deposited at the Bangkok MIRCEN (Microbiological Resources Center) under the code *Sinorhizobium fredii* TISTR 1393. The identification of this bacterium has not been completed. In 2001, Patima Permpoonpattana reported 16SrDNA sequence of this bacterium and compared two partial sequences with those deposited at Genbank. The first partial sequence of 971 bases had 96.9% homology with the 1002 nucleotide sequence of *Rhizobium* sp. K – Ag - 3. The second partial sequence of 272 nucleotides had 100%homology with 272 nucleotide sequence of *Rhizobium* sp. K - Ag - 3. However, since Patima Permpoonpattana (2001) found that this fast - growing rhizobial strain nodulated soybeans *Glycine max* cv. Sor Jor 4, Sor Jor 5 and Sukhothai 2, the bacterium was tentatively identified as *Sinorhizobium fredii* S174. *S. fredii* was reported to nodulate some soybean cultivars such as cultivar Peking.

Another category of soybean rhizobia is the slow - growing *Bradyrhizobium japonicum* and *B. elkanii* with generation time of approximately 2 days. One reason for different cell division rates might be differences in genes for cell division. Discovery of cell division genes which govern the relatively rapid cell division in *S. fredii* will lead to new insight in the understanding of cell division in soybean rhizobia.

Preliminary survey on sequences of promoter of *nifHDK* and *nodABC* showed that TnAraOut will not insert itself into the promoter sequences of the genes which encode nitrogenase and the enzymes in the pathway of Nod factor synthesis respectively (Schofield & Watson, 1985).

Rubin et al (1999) and Judson & Mekalanos (2000) reported on the method for the detection of TnAraOut insertion into the TATA sequences of -10/-35 promoters as follows : isolated chromosomal DNA of TnAraOut mutant was digested with *Sph1* restriction enzyme which had no restriction sites on TnAraOut. The fragments were recircularized and electroporated into *E. coli* DH5$\alpha\lambda$*pir*. Kanamycin-resistant colonies were selected. DNA of each kanamycin-resistant colony was isolated and used as the target DNA in a PCR reaction using $P_{BADout2}$ as the primer. $P_{BADout2}$ with the following sequence : 5'CTGACGCTTTTTATCGCAAC3' annealed to P_{BAD} which allowed sequencing outward from the right end of TnAraOut across the insertion junction. Sequences of an inverted repeat of the transposon TnAraOut and the upstream portions of genes whose promoters were inserted with TnAraOut are shown in Figures 2.1 and 2.2

TA site of promoter | Inverted repeat | Inverted repeat | TA site of promoter

...TTCATCACTA|ACAGGTTG.........CAACCTGT|TACCTCCTGA...

Chromosome **Transposon** **Chromosome**

Figure 2.1 Nucleotide sequence showing insertion of TnAraOut into TATA region of -10/-35 promoter (Rubin et al, 1999).

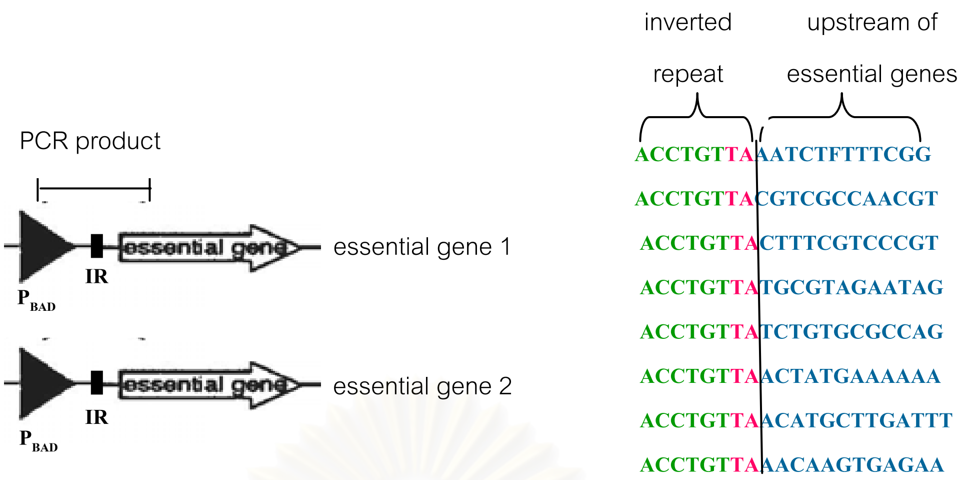


Figure 2.2 Examples of results of nucleotide sequences of PCR products when $P_{BADout2}$ was used as the primer to indicate that TnAraOut had been inserted into the TATA sequences of -10/-35 promoters. Vertical line indicated sequences of the inverted repeats to the left and those to the right indicated upstream sequences of genes whose promoters were distrupted (Judson & Mekalanos, 2000).

The reason the presence of the inverted sequence (ACCTGT) of TnAraOut in the PCR products obtained when $P_{BADout2}$ was used as the primer was used as a proof that TnAraOut had been inserted into the TATA region of promoter -10/-35 was because the method not only provided an evidence for the insertion of TnAraOut between the TATA sequence but also provided identities of tentative genes for cell division. Had the southern blot hybridization been used to provide an evidence that TnAraOut had been inserted into the TATA promoter sequence, tentative genes for cell division would not have straightforwardly been obtained.

2.2 Advantages of using *S. fredii* S174 as the wild type

S. fredii S174 renders itself an excellent subject for research in terms of heat tolerant mechanisms because not much information is known about their heat shock gene expression and regulation. In contrast, more information has been obtained on *B. japonicum* heat shock gene expression and regulation. (Chansa-ngavej, 2005)

Kündig et al (1993) reported that there were five *groESL* operons on the chromosome of *B. japonicum* USDA110 (Figure 2.3).

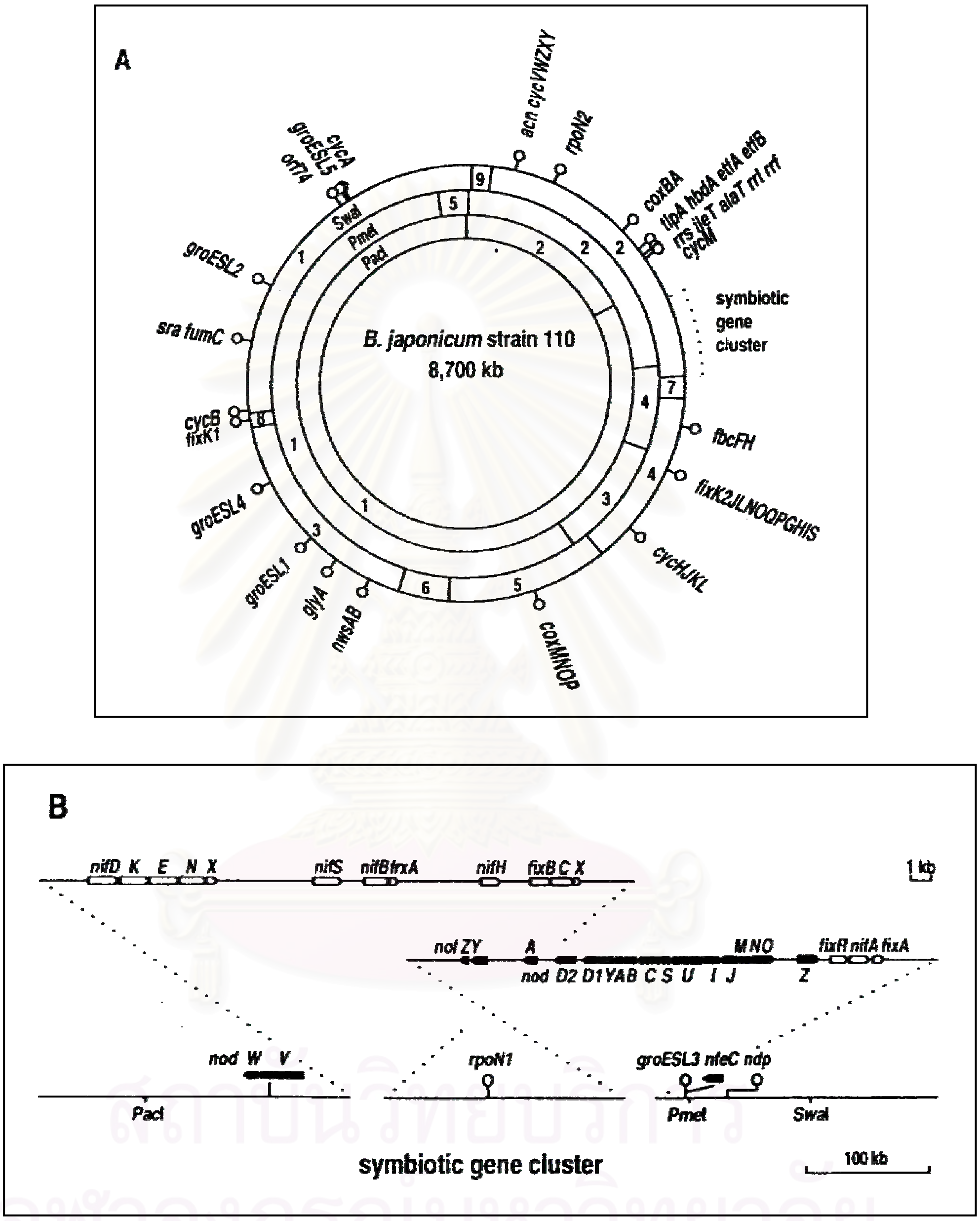


Figure 2.3 (A) Partial genetic map of *Bradyrhizobium japonicum* USDA 110 showing 5 *groESL* operons. (B) Enlarged region of symbiotic gene cluster (Kündig et al.1993).

The expression of *groESL1* is controlled by the availability of σ^{32}. Under normal temperature for growth , σ^{32} binds to DnaK/DnaJ/GrpE complex as indicated in Figure 2.4 During heat shock DnaK/DnaJ/GrpE/σ^{32} complex dissociates for DnaK/DnaJ/GrpE complex to act as molecular chaperones. σ^{32} is then available to form the holoenzyme of RNA polymerase ($\alpha_2\beta\beta'\sigma^{32}$) which binds to the -10/-35 promoter to initiate transcription of *groESL1* as shown in Figure 2.6 (Minder et al. 1997)

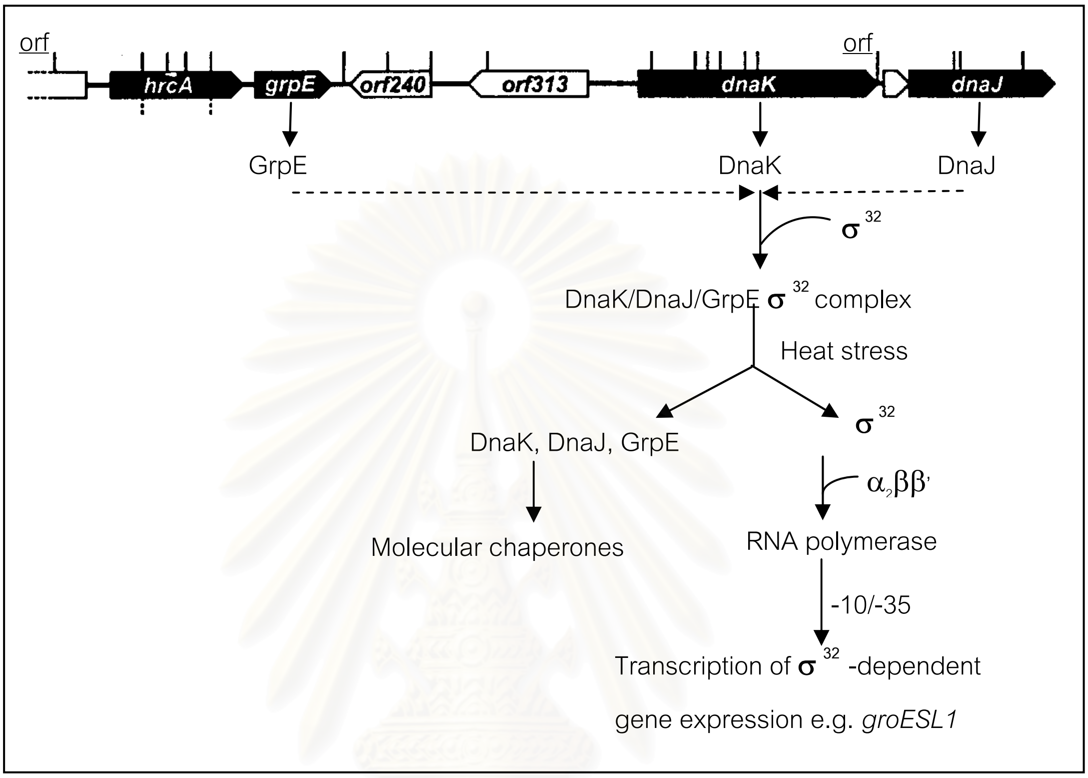


Figure 2.4 Transcriptional control of *groESL1* operon in *Bradyrhizobium japonicum* (Minder et al. 1997).

Babst et al (1996) reported that there was no evidence for up-regulation of *groESL2*, either by heat shock or by any other growth conditions tested. It appeared that *groESL2* was expressed constitutively from a house-keeping promoter which contained -35 and -10 unit.

In 1993 Fischer and co-workers reported that a well-conserved -24/-12 promoter was ~70 bp upstream of the coding region for *groESL3*. A putative binding site for the transcription activator protein NifA was also present at ~120 bp upstream of the -24/-12 promoter. Therefore the expression of *groESL3* in *B. japonicum* appeared to be dependent on NifA and σ^{54} RNA polymerase.

The expression of *groESL4* and *groESL5* was under the control of HrcA repressor protein binding to the operator sequence CIRCE [Babst et al.1996 ; Minder et al. 2000].

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 in bold letters :

-35 -10 +1

5'<u>TTGACA</u>TTTTTCTTGTGGTTTGA<u>TACTTT</u>TGTTAT<u>A</u>GAA**TTAGCACTC**

GCTTATTGA**GAGTGCTAA**CAGAGGTGATGATGATG-3'

CIRCE is abbreviated from <u>C</u>ontrolling <u>I</u>nverted <u>R</u>epeat of <u>C</u>haperone <u>E</u>xpression.

In *B. japonicum* the *hrcA* gene was upstream of the *DnaK* operon. *hrcA* encoded a repressor protein which bound to the operator CIRCE. Upon heat shock, HrcA was released from the operator site enabling the expression of *groESL4* and *groESL5* operons. The control of the five *groESL* operons expression is summarized in Figure 2.5.

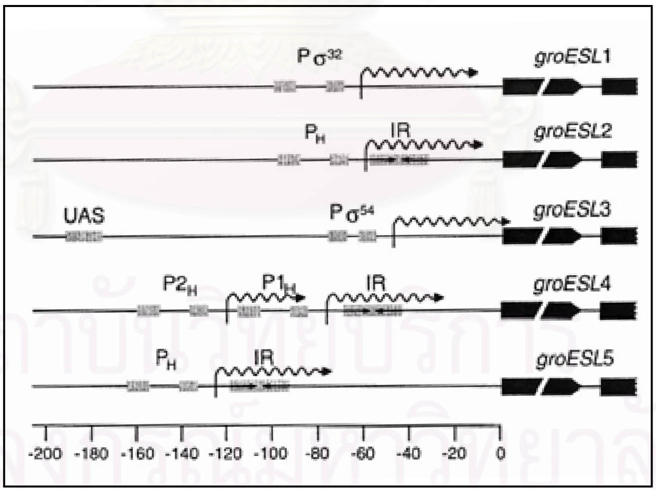


Figure 2.5 : Summary of control of gene expression in the five *groESL* operons of *B. japonicum* (Babst et al. 1996).

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

Transcriptional regulation of the small heat shock gene expression in *B. japonicum* involved the conserved stem and loop structures at the 5' end of the corresponding mRNAs. In 1999 Münchbach et al reported the presence of sequences $ROSE_1$ to $ROSE_5$ (Repression of small heat shock gene expression) upstream of *hspA*, *hspB*, *hspD*, *hspE*, and *hspH* respectively. Nocker et al (2001) reported multiple alignments of ROSE sequences of mRNAs of small heat shock genes of *B. japonicum, Bradyrhizobium* sp. (*Parasponia*) and *Rhizobium* sp. NGR234 yielded several conserved sequences one of which led to the formation of a 5' conserved stem and loop structure which, under normal growth temperature, prevented access of the small subunit ribosome to the Shine Dalgarno (SD) sequences on the mRNAs. Upon heat shock, the stem and loop structures relax to allow binding of the small subunit of the ribosomes to the Shine Dalgarno sequences on the mRNAs (Figure 2.6).

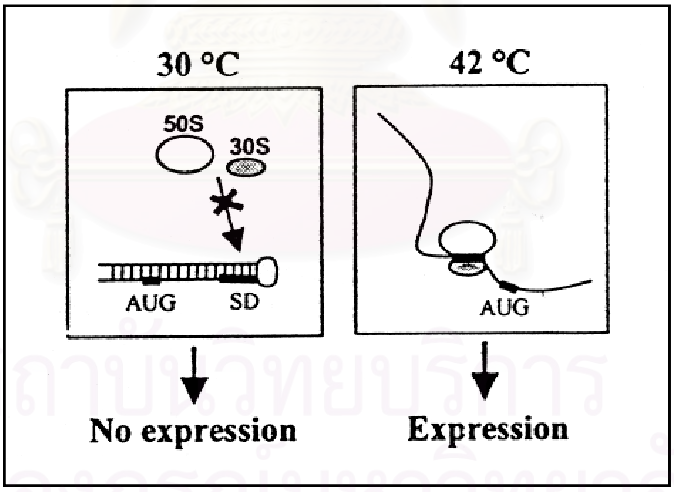


Figure 2.6 Model for temperature-responsive regulation by ROSE. The SD sequence and AUG start codon are indicated in the schematic hairpin structure at the 5'-end of ROSE. Ovals represent large (50S) and small (30S) ribosomal subunits. (Nocker et al. 2001)

The initial nodulation gene expression in *S. fredii* is also less well-known. According to Bellato et al (1997a, 1997b) two isoflavonoids, Daidzein and Coumestrol, induced expression of a *nolX-lacZ* gene fusion at pH values between 5.5 and 8.5, with an optimum at 6.5.

It is well-known that soybean roots secrete flavonoids such as Genistein, Daidzein and their derivatives. *B. japonicum* cells move towards the roots along the concentration gradients of these flavonoids. Meanwhile the flavonoids are absorbed into the periplasm of the bacteria. The flavonoid Genistein or its derivatives secreted by soybean roots forms a complex with $NodD_1$ protein which activates the expression of *nodYABC* resulting in the formation of the enzymes in the pathway of Nod factor synthesis. $NodD_1$-flavonoid complex will bind to *nodY* box to start transcription of *nodYABCSUIJ* operon. *NodA*, *nodB*, and *nodC* encode the following enzymes which catalyse the production of Lipo-chitooligosaccharides (Nod factors) which are essential in the nodulation process but whose function is still not known.

NodC encodes N-acetylglucosaminyl transferase which catalyses the transfer of N-acetylglucosamines.

NodB encodes N-deacetylase which catalyses the removal of acetyl group at the non reducing unit.

NodA encodes N-acyl transferase which catalyses the transfer of an acyl group.

The synthesis of Nod factors is as shown in Figure 2.7. The chemical structures of Nod factors of *B. japonicum* strains USDA 110 and USDA 135 and *B. elkanii* strain USDA 61 are shown in Figure 2.8.

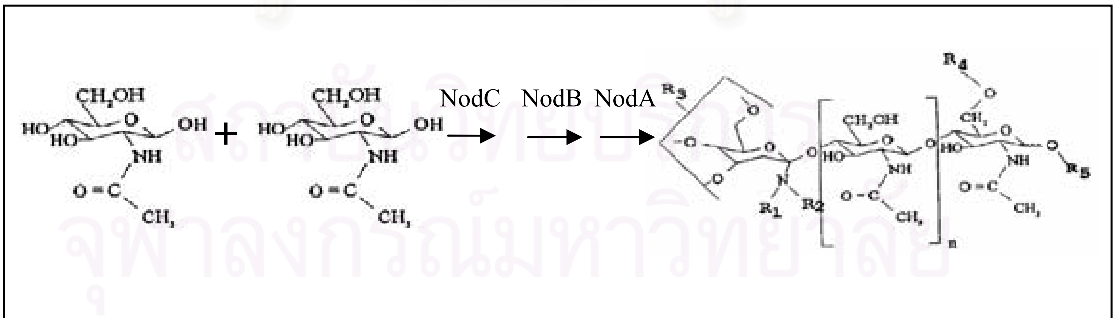
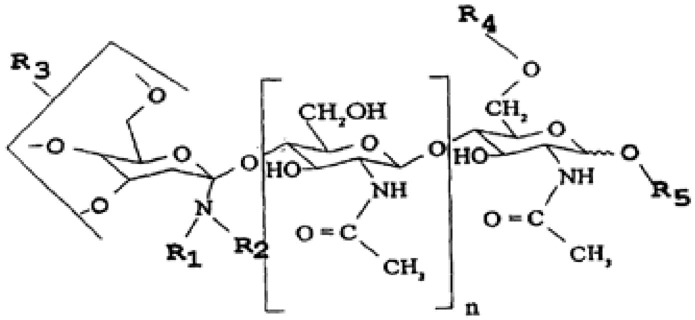


Figure 2.7 Synthesis of Nod factors catalysed by enzymes encoded by *nodC*, *nodB* and *nodA* (Stacey, 1995).



STRAIN	R1	R2	R3	R4	R5	n
B. japonicum USDA110						
NodBj-V(C18:1,MeFuc)	C18:1	H	H	2-O-MeFuc	H	3
B. japonicum USDA135						
NodBj-V(C18:1, MeFuc)	C18:1	H	H	2-O-MeFuc	H	3
NodBj-V(Ac,C18:1,MeFuc)	C18:1	H	Ac	2-O-MeFuc	H	3
NodBj-V(C16:0,MeFuc)	C16:0	H	H	2-O-MeFuc	H	3
NodBj-V(Ac,C16:0,MeFuc)	C16:0	H	Ac	2-O-MeFuc	H	3
NodBj-V(C16:1, MeFuc)	C16:1	H	H	2-O-MeFuc	H	3
B. elkanii USDA61						
NodBe-V(C18:1,MeFuc)	C18:1	H	H	2-O-MeFuc	H	3
NodBe-V(Ac,C18:1,MeFuc)	C18:1	H	Ac	2-O-MeFuc	H	3
NodBe-V(Cb,C18:1,NMe,MeFuc)	C18:1	Me	H	2-O-MeFuc	H	3
NodBe-V(Ac,Cb,C18:1,MeFuc)	C18:1	H	Ac,Cb	2-O-MeFuc	H	3
NodBe-IV(C18:1,MeFuc)	C18:1	H	H	2-O-MeFuc	H	2
NodBe-IV(Cb,C18:1,MeFuc)	C18:1	H	Cb	2-O-MeFuc	H	2
NodBe-IV(C18:1,Fuc,Gro)	C18:1	H	H	Fuc	Gro	2
NodBe-IV(C18:1,NMe,Fuc,Gro)	C18:1	Me	H	Fuc	Gro	2
NodBe-IV(Cb,C18:1,Fuc,Gro)	C18:1	H	Cb	Fuc	Gro	2
NodBe-IV(Cb,C18:1,NMe,Fuc,Gro)	C18:1	Me	Cb	Fuc	Gro	2

Figure 2.8 Summary of the various chito-oligosaccharides nodulation signals produced by *B. japonicum* strains USDA110 and USDA135 and *B. elkanii* strain USDA61. Abbreviations: AC, acetyl; Cb, carbamoyl; 2-0-MeFuc, 2-0-methylfucose; Fuc, fucose; Me, methyl; Gro, glycerol (Stacey et al. 1995).

It is well established that nodulation signals (Lipo-chitooligosaccharides, LCO, Nod factors) produced by *B. japonicum* are pentamers of N-acetylglucosamine with the reducing sugar modified by a 2-*O*-methylfucose and the non reducing end substituted with an 18:1 fatty acid (Stacey, 1995). The presence of specific root exudates flavonoids is essential in initiating the synthesis of Nod factors at the early stages of the nodulation process. Cook et al (1997) reported that many plant responses to Nod factors had been characterized, including alterations in the polar growth of root hairs, induction of cell divisions, and expression of nodulations (Long,1996), but the signal transduction pathway(s) leading to these events had not been discovered. A biochemical search for Nod factor receptors included the characterization of Nod factor binding activities in *Medicago truncatula* (Bono et al. 1995), and Nod factors had been shown to induce the periodic elevation of cytoplasmic calcium in root hair cells (Ehrhardt et al. 1996), a response that might represent a second messenger in the Nod factor signaling cascade. No comparable research findings have been obtained for *S. fredii.*

Zhang & Smith (1996) and Zhang et al (2002) stated that in Canada, low soil temperature was potentially a major factor limiting soybean growth and symbiotic nitrogen fixation because at low root zone temperatures (15-17°C) soybean roots secreted less Genistein resulting in a delay in the onset of nodulation. Therefore, the authors suggested that *B. japonicum* mutants which produced more LCO at low temperature and were independent of the presence of Genistein might lead to better nodulation and eventually better soybean yield under low soil temperatures. In tropical countries there is no comparable research on the effect of high temperatures on the synthesis of the Nod factors.

2.3 Heat shock proteins

Some heat shock proteins are constitutively expressed because they act as molecular chaperones in folding of nescent proteins (Voet & Voet, 1995). An example of constitutively expressed heat shock proteins is GroESL. Synthesis of other heat shock proteins is increased upon heat shock. When micro-organisms are transferred from 37°C to 42°C, synthesis of a set of heat shock proteins increases. These heat shock proteins include the so-called small heat shock proteins with molecular weight 12-43 kDa.

There are two categories of heat shock proteins : The 90-100 kDa ATP-dependent intracellular proteases encoded by the *clp* gene families (*clp* is abbreviated from <u>C</u>aseino<u>l</u>ytic <u>p</u>roteases). The second category constitutes the molecular chaperones which are encoded by *dnaK*, *dnaJ*, *grpE*, *groES*, *groEL*,and small heat shock protein genes.

DnaK/ DnaJ/ GrpE

DnaK protein is 70 kDa with 358 amino acid residues at the N terminal end, 225 amino acid residues forming a peptide binding domain, and 33 amino acid residues at the GC rich region at the C terminal. DnaJ is 40 kDa while GrpE is 20 kDa .

Table 2.1 Heat shock proteins in *B. japonicum*

Operons	Genes	References
1. *dnaK* operon	*hrcA, grpE, dnaK, dnaJ*	Babst et al, 1996
2. *groESL* operons	*groES, groEL*	Fischer et al, 1993
3. ROSE-dependent heat shock operons	*hspA, hspB, hspC, hspD, hspE, hspF, hspH*	Münchbach et al, 1999

GroESL consists of subunits of GroES and GroEL. GroES consists of seven 10 kDa subunits arranged in a hollow sphere, GroEL is made up of seven 60 kDa subunits in two stacks as indicated in Figure 2.9. GroESL assists in protein folding (Voet & Voet, 1995).

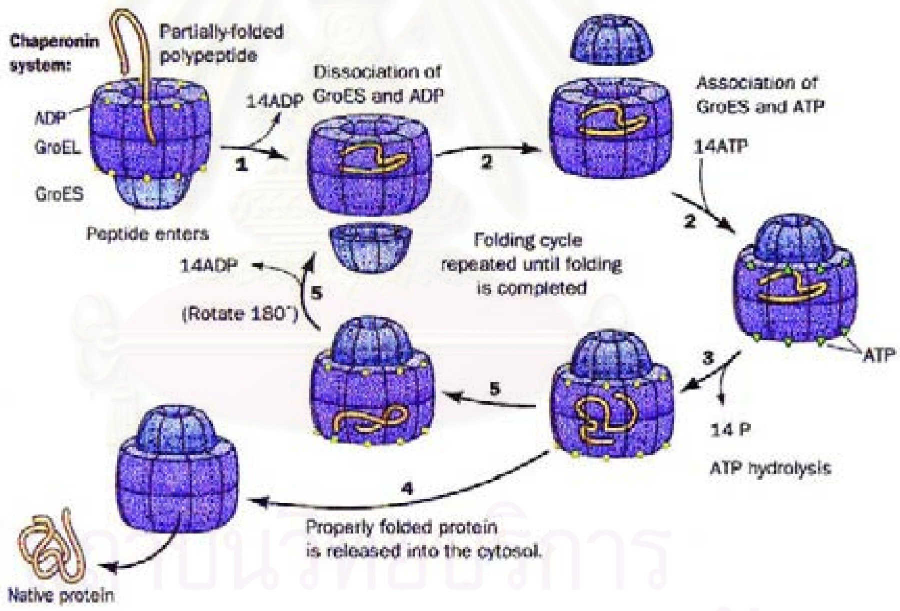


Figure 2.9 Heat shock proteins : GroESL (Voet & Voet, 1995).

Small heat shock proteins (sHSPs)

The first sHSP that was crystallized belonged to *Methanococcus jannaschii* which was a hollow sphere made up of 24 monomers as indicated in Figure 2.10 (Kim et al. 1998). Small heat shock proteins function as multimers.

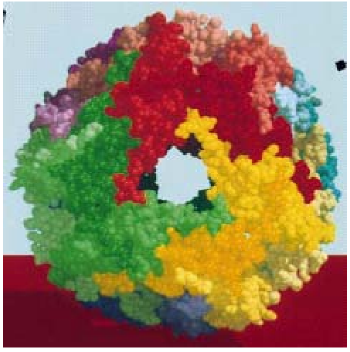


Figure 2.10 Small heat shock protein of *Methanococcus jannaschii* consists of a hollow sphere made up of 24 monomers (Kim et al. 1998).

Small heat shock proteins (sHSPs) bind denatured proteins and maintain them in a folding-competent state. Studer and Narberhaus (2000) report that members of bacterial small heat shock proteins are characterized by a conserved stretch of approximately 100 amino acids called the α–crystallin domain because of the sequence similarity to the vertebrate eye lens protein α–crystallin which prevents protein precipitation and cataract formation in the eye lens. The molecular mass of sHsps monomers ranges between 12 and 43 kDa. These monomers assemble into high molecular weight complexes *in vivo*. In 2002 Studer et al showed that part of the N-terminal of the α-crystallin Hsps was required for complex formation or oligomerization of α–crystallin-type heat shock proteins in *Bradyrhizobium japonicum* and that oligomerization was a prerequisite for the chaperone function.

The rise of atmospheric temperature due to the greenhouse effect indicates that work should be done on the effects of heat stress on the soybean-rhizobium symbiosis in order to maintain soybean yields for consumption. In addition to research or control of heat shock gene regulation future work should be conducted on the effects of high temperatures on soybean rhizobia growth and survival, mechanisms for acquired thermotolerance, as well as increased competitiveness in nodulation by the fast-growing rhizobia and an increased ability to fix nitrogen more efficiently in the field conditions under heat stress (Chansa-ngavej, 2005).

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial strains

E.coli S17-1λ*pir* (pNJ17), *E.coli* DH5α, and *E.coli* DH5αλ*pir* were obtained from Professor Mamoru Yamada, Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Japan. *S. fredii* S174 was a local isolate. (Suwat Saengkerdsub, 1999). 16S rDNA of *S. fredii* was sequenced as described by Patima Permpoonpattana (2001).

3.2 Selection of TnAraOut mutants after biparental mating

3.2.1 Antibiotic sensitivity tests

In order to find out which antibiotics to be used in the selection of mutants obtained after biparental mating, the following experiments were conducted to determine the antibiotic sensitivity of *S. fredii* S174 wild type donor and *E.coli* S17-1λ*pir* (pNJ17) recipient cells.

1 loop of each of *E.coli* S17-1λ*pir* (pNJ17), *E.coli* DH5α, and *S. fredii* S174 was inoculated into 5.0 ml of TY medium. 100 $\mu g.ml^{-1}$ kanamycin was added into *E.coli* S17-1λ*pir* (pNJ17) culture. Each *E.coli* culture was grown at 200 rpm at 37°C while *S. fredii* S174 culture was grown at 30°C until 10^9 $cells.ml^{-1}$ were obtained. Fifty μl of seed medium obtained was put in 5 ml TY medium containing the following antibiotics at 50 $\mu g.ml^{-1}$, 100 $\mu g.ml^{-1}$, 150 $\mu g.ml^{-1}$, and 200 $\mu g.ml^{-1}$: kanamycin, ampicillin, streptomycin, and spectinomycin. The cultures were grown overnight before assessing the extent of growth by visualizing the turbidity.

3.2.2 Biparental mating

Biparental mating between *E.coli* S17-1λ*pir* (pNJ17) and *S. fredii* S174 was carried out as follows : one loop of each of *E.coli* S17-1λ*pir* (pNJ17) or *S. fredii* S174 was inoculated into 5.0 ml TY medium containing 100 $\mu g.ml^{-1}$ kanamycin or 100 $\mu g.ml^{-1}$ ampicillin respectively. The cultures were incubated at 200 rpm at 37°C or 30°C respectively until approximately 10^9 $cells.ml^{-1}$ were obtained (12 h for *E.coli* S17-1λ*pir* (pNJ17) and 15 h for *S. fredii* S174). Cells were harvested by centrifugation at 7,000 rpm, 4°C for 10 minutes. Cells were washed twice with sterilized 0.85% NaCl solution after which they were resuspended in 100 μl sterilized 0.85% NaCl solution. The donor and recipient cells were mixed by brief vortexing. Several of ten μl of mixed cultures were spotted on TY agar plates containing 0.1% Arabinose, incubated at 30°C, 6 h. Colonies from each spot were inoculated into 5.0 ml TY medium containing 100 $\mu g.ml^{-1}$ kanamycin and 100 $\mu g.ml^{-1}$ ampicillin, incubated at 200 rpm , 30°C, for 16 h. Each culture was diluted and 0.1 ml was spread onto TY agar plates containing 100 $\mu g.ml^{-1}$ kanamycin and 100 $\mu g.ml^{-1}$ ampicillin as well as 0.001% Arabinose. The agar plates were incubated at 40°C until small colonies were obtained. One thousand colonies were picked with sterilized tooth-picks to place on two sets of TY agar each with or without 0.1% Arabinose and incubated at 40°C for 5 days. Colonies were picked and grown for 5 days growth at 40°C on TY agar plates with or without 0.1% Arabinose. The colony picking and growth for 5 days at 40 °C were repeated twice. TnAraOut mutants were kept at 4°C on slants containing TY medium with 100 $\mu g.ml^{-1}$ kanamycin and 100 $\mu g.ml^{-1}$ ampicillin.

Biparental mating was carried out twice. A total of 2,000 small colonies were picked and grown repeatedly on TY agar medium with both antibiotics and 0.1% Arabinose at 40°C.

3.2.3 Detection of TnAraOut sequence in the genomes of mutants

DNA of each mutant isolated as described in 3.3.1 was digested with *Sph*I (Promega) according to the manufacturer's instruction. The fragments obtained were recircularized with T_4 DNA ligase (Gibco BRL) according to the manufacturer's instruction. Circularized fragments were electroporated into *E.coli* DH5α*λpir* as follows : one loop of *E.coli* DH5α*λpir* was inoculated in 50 ml LB medium in 250 ml Erhenmeyer flask and grown at 37°C 200 rpm, to mid-log phase. One ml of seed medium was inoculated in 49 ml LB medium and incubated at 200 rpm, 37°C until the optical density reading at 600 nm was 0.35-0.40. The culture was swirled in an ice-water bath for 10-15 min then centrifuged at 7,000 rpm, 4°C for 5 min. Cells were washed with 50 ml ice-cold sterilized distilled water and centrifuged at 7,000 rpm, at 4°C for 5 min three times. The pellets were resuspended in 50 ml ice-cold sterilized 10% glycerol and centrifuged at

7,000 rpm, at 4^{o}C for 10 min twice before resuspending in 0.1 ml ice-cold GYT medium. Forty μl of cell suspension was placed in electroporation cuvette, incubated on ice 1 min before putting in the electroporation chamber (Bio-Rad) which delivered pulses at the following settings : 25 μF electrical pulse, 2.5 kV capacitance, and 200 0hm resistance. The electroporated cells were transferred to 1 ml SOC medium immediately and incubated at 200 rpm, at 37^{o}C for 1 h. Two hundred μl of culture was spreaded on LB medium containing 100 μg.ml^{-1} kanamycin to select for kanamycin resistant colonies.

3.3 RAPD-PCR fingerprinting of *S. fredii* S174 and TnAraOut mutants

3.3.1 Isolation of chromosomal DNA

Cells of each isolate were activated by culturing in tryptone yeast extract agar slants (TY) at 30^{o}C for 2 days. One loop of each activated isolate was inoculated into 50 ml tryptone yeast extract broth (TY). The composition of TY was as described in Appendix A. The culture was grown at 200 rpm, 30^{o}C until mid log phase. Cells were harvested by centrifuging one ml cell suspension at 7,000 rpm, 4^{o}C for 5 minutes. 80 μl 2.5 mg.ml^{-1} lysozyme was added to the cell pellet, mixed thoroughly, and incubated in a 37^{o}C water bath for 1 h before 4 cycles of freezing at –20^{o}C for 5 minutes and thawing at 80^{o}C for 5 minutes. One volume of DNAzol$^{®}$ (Invitrogen) was added to the solution which was gently mixed by inverting the eppendorf tubes. The mixture was centrifuged at 10,000 rpm, 4^{o}C for 5 minutes. The supernatant was transferred to a fresh eppendorf tube. 500 μl ice-cold ethanol was added to the mixture which was gently mixed by inverting the tube before centrifugation at 10,000 rpm, 4^{o}C for 15 minutes. The precipitate was washed with 70% ice-cold ethanol and air dried in a laminar flow hood. Thirty μl high-purity distilled water was added to dissolve the nucleic acid precipitate at room temperature for 1 day. Quantity of isolated DNA was determined by absorbance at 260 nm and quality of the isolated chromosomal DNA was checked by OD_{260}/OD_{280} ratios and 0.8% agarose gel electrophoresis by standard methods (Sambrook et al, 1989).

3.3.2 RAPD-PCR fingerprinting

Sequences of RPO1 and CRL-7 were as reported by Richardson et al (1995) and Mathis & McMillin (1996) as follows :

RPO1 : 5'AATTTTCAAGCGTCGTGCCA3'

CRL-7 : 5'GCCCGCCGCC3'

All primers were synthesized by Invitrogen Life Technologies, USA. To obtain reproducibility all RAPD-PCR fingerprinting experiments were repeated at least twice.

RPO1 primer was used in RAPD-PCR fingerprinting in the following mixture:

Mixture			Program		
10x PCR buffer	2.5	µl	95 °C	15 seconds	5 cycles
50 mM $MgCl_2$	0.8	µl	55 °C	30 seconds	
10 mM dNTPs	0.5	µl	72 °C	90 seconds	
10 µM primer	5.0	µl	95 °C	15 seconds	25 cycles
DNA template (60-100 ng)	1.0	µl	60 °C	30 seconds	
Taq polymerase (5U.µl^{-1})	0.2	µl	72 °C	90 seconds	
High quality double distilled water	15.0	µl	72 °C	10 minutes	
Total	25.0	µl			

CRL-7 primer was used in RAPD-PCR fingerprinting in the following mixture :

Mixture			Program		
10x PCR buffer	5.0	µl	95 °C	15 seconds	5 cycles
50 mM $MgCl_2$	1.5	µl	55 °C	30 seconds	
10 mM dNTPs	1.0	µl	72 °C	90 seconds	
10 µM primer	5.0	µl	95 °C	15 seconds	25 cycles
DNA template (60-100 ng)	2.0	µl	60 °C	30 seconds	
Taq polymerase (5U.µl^{-1})	0.25	µl	72 °C	90 seconds	
High quality double distilled water	35.25	µl	72 °C	10 minutes	
Total	50.00	µl			

PCR products were separated by 1.25 % agarose gel electrophoresis by standard method (Sambrook et al,1989). RAPD-PCR fingerprints were viewed and photographed on a UV transilluminator (Bio-rad).

3.4 Arabinose-dependent growth of *S. fredii* S174 and TnAraOut mutants

Ten loops of activated *S. fredii* S174 or TnAraOut mutants ST49 or ST60 were added into 50 ml Minimum Medium containing 0.1% Arabinose. The composition of the Minimum Medium was as described by Sambrook et al (1989). 100 $\mu g.ml^{-1}$ each of kanamycin and ampicilliin was added to the Minimum Medium for growth of the mutants. The cultures were incubated at 200 rpm, 30°C until mid-log phase was reached. Cells were aseptically harvested at 7,000 rpm, 4 °C, for 10 minutes. These cells were washed with sterilized 0.85% NaCl twice and suspended in 1 ml Minimum Medium for use as the seed culture. One drop of the seed culture was added into each 3 ml Minimum Medium containing 0.001% or 0.005% or 0.01% or 0.05% or 0.1% or no Arabinose. Both antibiotics were added to the medium for the TnAraOut mutants. The cells were grown at 30°C overnight. Growth was determined by Optical Density readings at 570 nm.

3.5 Thermotolerance in *S. fredii* S174 and TnAraOut mutants

One loop of activated *S. fredii* S174 or TnAraOut mutants (ST1-ST7, ST20, ST25, ST 31, ST39, ST40, ST41, and ST60) was added into 50 ml TY medium containing 100 $\mu g.ml^{-1}$ kanamycin and 100 $\mu g.ml^{-1}$ ampicillin as well as 0.1% Arabinose. No antibiotics were added into medium for *S. fredii*. They were incubated at 200 rpm, at 30°C until mid-log phase was reached. Five ml were added into 45 ml TY medium containing 100 $\mu g.ml^{-1}$ kanamycin and 100 $\mu g.ml^{-1}$ ampicillin and 0.1% Arabinose. No antibiotics were added into the medium for wild type. The cultures were incubated at 30°C, 200 rpm and Optical Density readings at 660 nm were measured every 2 h until the stationary phase was reached.

3.6 Extraction of intracellular proteins of *S. fredii* S174 and TnAraOut mutants grown at different temperatures

Seed culture was prepared by inoculating one loop of each of the activated TnAraOut mutants ST49 or ST60 or *S. fredii* S174 into 50 ml of TY medium, pH 6.8 containing 100 $\mu g.ml^{-1}$ kanamycin and 100 $\mu g.ml^{-1}$ ampicillin (in the case of mutants). The cultures were grown at 200 rpm, 30°C until mid log phase. Five ml of each seed culture were inoculated into a set of 45 ml TY medium containing 100 $\mu g.ml^{-1}$ kanamycin and 100 $\mu g.ml^{-1}$ ampicillin (in the case of mutants). Cultures were grown at 200 rpm, 30°C until mid log phase as determined by turbidity measurement at wavelength 660 nanometer. Intracellular proteins were extracted by harvesting cells at 10,000 rpm, 5 min at 25°C. Cell pellet was washed twice with extraction buffer (0.5 M Tris HCl, pH 7.0). Two to three volumes of sterilized glass beads (Sigma G-9143) were added to the cell pellet suspended in 80 μl extraction buffer, vortexed at top speed for 40 seconds, left on ice. Vortexing was repeated 9 more times with tubes on ice after each vortexing. Contents were centrifuged at 12,000 rpm, 40 minutes at 4°C. Concentrations of soluble proteins in the supernatant were determined by the Bradford method (Bradford, 1976) using the protein dye assay (BIO-RAD) with Bovine Serum Albumin as the standard. Soluble proteins were separated by SDS-PAGE as described by Laemmli (1970) with 50 μg protein per well. Proteins were stained by Silver stain kit (BIO-RAD) according to the manufacturer 's instruction.

3.7 Determination of plant and nodule dry weights

3.7.1 Seed surface-sterilization and germination

Soybean seeds cultivars Sor Jor 4, Sor Jor 5, and Sukhothai 2 were surface-sterilized as described by Somasegaran and Hoben (1994). Seeds were placed in an Erlenmeyer flask (wide-mouthed and previously sterilized by autoclaving). The mouth of the flask was covered with a sterilized petri dish. The seeds took up about 25% of the volume of the flask. The petri dish cover was kept in place throughout the operation. The seeds were rinsed in 95% ethanol for 10 seconds to remove waxy materials, and after that ethanol was drained off. 5% hydrogen peroxide solution was added in sufficient volume to immerse the seeds completely. The content was swirled gently to bring the seeds and 5% hydrogen peroxide into contact. After 3-5 minutes, the sterilizing liquid was drained off and the seeds rinsed with six changes of sterilized water. Aseptic procedures were observed throughout the rinsing. After the sixth rinse, the seeds were submerged in water and left in the refrigerator for 4 hours for seed imbibition. After 4 hours, the seeds were rinsed with two changes of water and plated on 0.75% (w/v) water agar in petri dishes. About 20-50 seeds were placed per plate and were incubated at 25°C in the dark for 2 days.

3.7.2 Growth of soybean plants in Leonard jars

Preparation of Leonard jars was as described by Somasegaran & Hoben (1994). Three germinating seeds were placed in each Leonard jar which was placed in a plant growth chamber (EYELA) with continuous 25,000 lux light intensity, 12 h light/12 h dark, 70% relative humidity at 28°C. Nitrogen-free medium, pH 6.8, was used to grow the subsequent seedlings until plants had flowers. Plants were thinned to two plants per Leonard jar after growth for 2 weeks. Plant dry weight was obtained by cutting the shoot portions of the two plants at the cotyledon scars for drying at 70 °C for 72 h. Nodule dry weight was obtained by weighing all dried nodules of the two plants grown in each Leonard jar. Average dry weight per plant and nodule dry weight per plant were reported. Statistical analysis was obtained by Duncan's Multiple Range Test (Steel & Torrie, 1980).

CHAPTER 4

RESULTS

4.1 Identification of *S. fredii* S174

Figure 4.1 indicated 16S rDNA sequence of *S. fredii* S174 after sequencing was performed twice. The first determination of 16S rDNA sequence of *S. fredii* S174 was presented by Patima Permpoonpattana (2001). Sequencing data obtained from the second determination and comparisons of 16S rDNA sequences obtained from the two determinations were presented in the Appendix C. Comparisons of the sequences obtained from the first and the second determinations as well as manual checkup of sequencing peaks in the raw data gave rise to the 16S rDNA sequence of *S.fredii* S174 as shown in Figure 4.1. Pairwise sequence comparisons with 16S rDNA sequences deposited at GenBank indicated that 1432 nucleotides of 16S rDNA of *S. fredii* S174 were similar to those of *Agrobacterium* sp. K-Ag-3 (1432/1454 nucleotides, 98% homology with 12 gaps) as well as those of *Rhizobium tropici* UPRM8033 (1432/1457 nucleotides, 98% homology with 13 gaps) and *Rhizobium* sp. ORS3177 (1433/1458 nucleotides, 98% homology with 14 gaps) as indicated in Figure 4.2.

Comparisons of 16S rDNA sequence of *S. fredii* S174 (Figure 4.1 ; Table 4.1) with those deposited with GenBank indicated 98% homology with 16S rDNA sequences of *Agrobacterium* K-Ag-3, *Rhizobium tropici* UPRM8033, *Rhizobium* sp. ORS3177, *Rhizobium tropici* PRF34. It may be concluded at this stage that the 16S rDNA sequence obtained for *S. fredii* S174 was the first reported complete 16S rDNA sequence of *S. fredii*. The reason is because there is no 16S rDNA sequence deposited at GenBank with at least 99.9% homology with *S. fredii* S174's 16S rDNA sequence. Fox et al (1992) stated that sequences should be at least 99.9% similar in order to conclude that, based on 16S rDNA homology, the microorganisms belonged to the same species.

```
S174  TACGGCTACC TTGTTACGAC TTCACCCCAG TCGCTGACCC TACCGTGGTT AGCTGCCTCC TTGCGGTTAG CGCACTACCT
       1492r
S174  TCGGGTAAAA CCAACTCCCA TGGTGTGACG GGCGGTGTGT ACAAGGCCCG GGAACGTATT CACCGCGGCA TGCTGATCCG
                                        1385r
S174  CGATTACTAG CGATTCCAAC TTCATGCACT CGAGTTGCAG GCAGAGTGCA ATCCGAACTG AGATGGCTTT TGGAGATTAG

S174  CTCACACTCG CGTGCTCGCT GCCCACTGTC ACCACCATTG TAGCACGTGT GTAGCCCAGC CCGTAAGGGC CATGAGGACT
                                            1241f
S174  TGACGTCATC CCCACCTTCC TCTCGGCTTA TCACCGGCAG TCCCCTTAGA GTGCCCAACC AAATGCTGGC AACTAAGGGC

S174  GAGGGTTGCG CTCGTTGCCG GGACTTAACC CAACATCTCT ACGCTCACGA CACGAGCTGA CGACAGCCAT GCAGCACCTG
      1100r
S174  TCTCTGCGCC ACCGAAGTGG ACCCCCTATC TCTAGAGGTA ACACAGGATG TCAAGGGCTG GTAAGGTTCT GCGCGTTGCT

S174  TCGAATTAAA CCACATGCTC CACCGCTTGT GCGGGCCCCC GTCAATTCCT TTGAGTTTTA ATCTTGCGAC CGTACTCCCC
                                                  907r
S174  AGGCGGAATG TTTAATGCGT TAGCTGCGCC ACCGAACAGT ATACTGCCCG ACGGCTAACA TTCATCGTTT ACGGCGTGGA

S174  CTACCAGGGT ATCTAATCCT GTTTGCTCCC CACGCTTTCG CACCTCAGCG TCAGTAATGG ACCAGTGAGC CGCCTTCGCC
          787r
S174  ACTGGTGTTC CTCCGAATAT CTACGAATTT CACCTCTACA CTCGGAATTC CACTCACCTC TTCCATACTC CAGATCGACA

S174  GTATCAAAGG CAGTTCCAGG GTTGAGCCCT GGGATTTCAC CCCTGACTGA TCGATCCGCC TACGTGCGCT TTACGCCCAG

S174  TAATTCCGAA CAACGCTAGC CCCCTTCGTA TTACCGCGGC TGCTGGCACG AAGTTAGCCG GGGCTTCTTC TCCGGATACC
                                        519r
S174  GTCATTATCT TCTCCGGTGA AAGAGCTTTA CAACCCTAGG GCCTTCATCA CTCACGCGGC ATGGCTGGAT CAGGCTTGCG

S174  CCCATTGTCC AATATTCCCC ACTGCTGCCT CCCGTAGGAG TTTGGGCCGT GTCTCAGTCC CAGTGTGGCT GATCATCCTC
                                343r
S174  TCAGACCAGC TATGGATCGT CGCCTTGGTA GGCCTTTACC CCACCAACTA GCTAATCCAA CGCGGGCTCA TCTCTTGCCG

S174  ATAAATCTTT CTCCCGAAGG ACACATACGG TATTAGCACA AGTTTCCCTG CGTTATTCCG TAGCAAAAGG TAGATTCCCA

S174  CGCGTTACTC ACCCGTCTGC CGCTCCCCCT AAAGGGCGCT CGACTTGCAT GTGTTAAGCC TGCCGCCAGG CGTCGTTCTG

S174  AGCCAGGATC AAACTCA
       27f
```

Figure 4.1 16S rDNA sequence of *Sinorhizobium fredii* S174. Sequences of primers are shown in boxes.

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Query= S174COM1st(1457 letters)

                                                                 Score    E
Sequences producing significant alignments:                      (bits) Value

gi|464144|dbj|D14504.1|ATU16SRDJ  Agrobacterium sp. K-Ag-3 g...  2702   0.0
gi|27261750|gb|AY166841.1|  Rhizobium tropici UPRM8033 16S r...  2676   0.0
gi|57867895|gb|AY864736.1|  Rhizobium sp. ORS3177 16S riboso...  2670   0.0
gi|21898743|gb|AY117623.1|  Rhizobium tropici strain PRF34 1...  2662   0.0
gi|1055273|gb|U38469.1|RTU38469  Rhizobium tropici 16S ribos...  2658   0.0
gi|9837365|gb|AF286362.1|  Rhizobium sp. PRY71 16S ribosomal...  2652   0.0
gi|59002183|gb|AY904747.1|  Agrobacterium rhizogenes strain ...  2652   0.0
gi|296479|emb|X67233.1|RL16SRRN  Rhizobium tropici subgroup ...  2650   0.0
gi|61661413|gb|AY945955.1|  Agrobacterium rhizogenes strain ...  2650   0.0
gi|28894114|gb|AY206687.1|  Rhizobium rhizogenes strain 163C...  2650   0.0
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Alignments

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   >gi|464144|dbj|D14504.1|ATU16SRDJ   Agrobacterium sp. K-Ag-3 gene for 16S
ribosomal RNA, complete sequence          Length = 1468

 Score = 2702 bits (1363), Expect = 0.0
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 Strand = Plus / Minus

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Sbjct: 1442 tacggctaccttgttacgacttcaccccagtcgctgaccctaccgtggttagctgcctcc 1383

Query: 61   ttgcggttagcgcactaccttcgggtaaaaccaactcccatggtgtgacgggcggtgtgt 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1382 ttgcggttagcgcactaccttcgggtaaaaccaactcccatggtgtgacgggcggtgtgt 1323

Query: 121  acaaggcccgggaacgtattcaccgcggcatgctgatccgcgattactagcgattccaac 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1322 acaaggcccgggaacgtattcaccgcggcatgctgatccgcgattactagcgattccaac 1263

Query: 181  ttcatgcactcgagttgcaggcagagtgcaatccgaactgagatggcttttggagattag 240
            ||||||||||||||||||||    ||||||||||||||||||||||||||||||||||||
Sbjct: 1262 ttcatgcactcgagttgcag----agtgcaatccgaactgagatggcttttggagattag 1207

Query: 241  ctcacactcgcgtgctcgctgcccactgtcaccaccattgtagcacgtgtgtagcccagc 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1206 ctcacactcgcgtgctcgctgcccactgtcaccaccattgtagcacgtgtgtagcccagc 1147

Query: 301  ccgtaagggccatgaggacttgacgtcatccccaccttcctctcggcttatcaccggcag 360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1146 ccgtaagggccatgaggacttgacgtcatccccaccttcctctcggcttatcaccggcag 1087

Query: 361  tccccttagagtgcccaaccaaatgctggcaactaagggcgagggttgcgctcgttgccg 420
            ||||||||||||||||||| |||||||||||||||||||||||||||||||||||||| |
Sbjct: 1086 tccccttagagtgcccaactaaatgctggcaactaagggcgagggttgcgctcgttgc-g 1028

Query: 421  ggacttaacccaacatctctacgctcacgacacgagctgacgacagccatgcagcacctg 480
            |||||||||||||||||||       ||||||||||||||||||||||||||||||||||
Sbjct: 1027 ggacttaacccaacatctc-------acgacacgagctgacgacagccatgcagcacctg 975

Query: 481  tctctgcgccaccgaagtggacccccctatctctagaggtaacacaggatgtcaagggctg 540
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Sbjct: 974  tctctgcgccaccgaagtggacccccctatctctagaggtaacacaggatgtcaagggctg 915
```

```
Query: 541  gtaaggttctgcgcgttgcttcgaattaaaccacatgctccaccgcttgtgcgggccccc
600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 914  gtaaggttctgcgcgttgcttcgaattaaaccacatgctccaccgcttgtgcgggccccc 855

Query: 601  gtcaattcctttgagttttaatcttgcgaccgtactccccaggcggaatgtttaatgcgt 660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 854  gtcaattcctttgagttttaatcttgcgaccgtactccccaggcggaatgtttaatgcgt 795

Query: 661  tagctgcgccaccgaacagtatactgcccgacggctaacattcatcgtttacggcgtgga 720
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Sbjct: 794  tacgtgcgccaccgaacagtatactgcccgacggctaacattcatcgtttacggcgtgga 735

Query: 721  ctaccagggtatctaatcctgtttgctccccacgctttcgcacctcagcgtcagtaatgg 780
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Sbjct: 734  ctaccagggtatctaatcctgtttgctccccacgctttcgcacctcagcgtcagtaatgg 675

Query: 781  accagtgagccgccttcgccactggtgttcctccgaatatctacgaatttcacctctaca 840
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Sbjct: 674  accagtgagccgccttcgccactggtgttcctccgaatatctacgaatttcacctctaca 615

Query: 841  ctcggaattccactcacctcttccatactccagatcgacagtatcaaaggcagttccagg 900
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 614  ctcggaattccactcacctcttccatactccagatcgacagtatcaaaggcagttccagg 555

Query: 901  gttgagccctgggatttcacccctgactgatcgatccgcctacgtgcgctttacgcccag 960
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 554  gttgagccctgggatttcacccctgactgatcgatccgcctacgtgcgctttacgcccag 495

Query: 961  taattccgaacaacgctagccccccttcgtattaccgcggctgctggcacgaagttagccg 1020
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Sbjct: 494  taattccgaacaacgctagccccccttcgtattaccgcggctgctggcacgaagttagccg 435

Query: 1021 gggcttcttctccggataccgtcattatcttctccggtgaaagagctttacaaccctagg 1080
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 434  gggcttcttctccggataccgtcattatcttctccggtgaaagagctttacaaccctagg 375

Query: 1081 gccttcatcactcacgcggcatggctggatcaggcttgcgcccattgtccaatattcccc 1140
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 374  gccttcatcactcacgcggcatggctggatcaggcttgcgcccattgtccaatattcccc 315

Query: 1141 actgctgcctcccgtaggagtttgggccgtgtctcagtcccaatgtggctgatcatcctc 1200
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 314  actgctgcctcccgtaggagtttgggccgtgtctcagtcccaatgtggctgatcatcctc 255

Query: 1201 tcagaccagctatggatcgtcgccttggtaggcctttaccccaccaactagctaatccaa 1260
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 254  tcagaccagctatggatcgtcgccttggtaggcctttaccccaccaactagctaatccaa 195

Query: 1261 cgcgggctcatctcttgccgataaatctttctcccgaaggacacatacggtattagcaca 1320
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Sbjct: 194  cgcgggctcatctcttgccgataaatctttctcccgaaggacacatacggtattagcaca 135

Query: 1321 agtttccctgcgttattccgtagcaaaaggtagattcccacgcgttactcacccgtctgc 1380
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 134  agtttccctgcgttattccgtagcaaaaggtagattcccacgcgttactcacccgtctgc 75
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>gi|27261750|gb|AY166841.1| Rhizobium tropici UPRM8033 16S ribosomal RNA gene, partial sequence Length = 1477

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 Strand = Plus / Minus

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Sbjct: 1447 tacggctaccttgttacgacttcaccccagtcgctgaccctaccgtggttagctgcctcc 1388

Query: 61   ttgcggttagcgcactaccttcgggtaaaaccaactcccatggtgtgacgggcggtgtgt 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1387 ttgcggttagcgcactaccttcgggtaaaaccaactcccatggtgtgacgggcggtgtgt 1328

Query: 121  acaaggcccgggaacgtattcaccgcggcatgctgatccgcgattactagcgattccaac 180
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Sbjct: 1327 acaaggcccgggaacgtattcaccgcggcatgctgatccgcgattactagcgattccaac 1268

Query: 181  ttcatgcactcgagttgcaggcagagtgcaatccgaactgagatggcttttggagattag 240
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Sbjct: 1267 ttcatgcactcgagttgcag----agtgcaatccgaactgagatggcttttggagattag 1212

Query: 241  ctcacactcgcgtgctcgctgcccactgtcaccaccattgtagcacgtgtgtagcccagc 300
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Query: 301  ccgtaagggccatgaggacttgacgtcatccccaccttcctctcggcttatcaccggcag 360
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Sbjct: 1151 ccgtaagggccatgaggacttgacgtcatccccaccttcctctcggcttatcaccggcag 1092

Query: 361  tccccttagagtgcccaaccaaatgctggcaactaagggcgagggttgcgctcgttgccg 420
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Sbjct: 1091 tccccttagagtgcccaactgaatgctggcaactaagggcgagggttgcgctcgttgc-g 1033

Query: 421  ggacttaacccaacatctctacgctcacgacacgagctgacgacagccatgcagcacctg 480
            |||||||||||||||||||       ||||||||||||||||||||||||||||||||||
Sbjct: 1032 ggacttaacccaacatctc-------acgacacgagctgacgacagccatgcagcacctg 980

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Sbjct: 979  tctctgcgccaccgaagtggacccccctatctctagaggtaacacaggatgtcaagggctg 920

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Sbjct: 919  gtaaggttctgcgcgttgcttcgaattaaaccacatgctccaccgcttgtgcgggccccc 860

Query: 601  gtcaattcctttgagttttaatcttgcgaccgtactccccaggcggaatgtttaatgcgt 660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 859  gtcaattcctttgagttttaatcttgcgaccgtactccccaggcggaatgtttaatgcgt 800

Query: 661  tagctgcgccaccgaacagtatactgcccgacggctaacattcatcgtttacggcgtgga 720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 799  tagctgcgccaccgaacagtatactgcccgacggctaacattcatcgtttacggcgtgga 740

Query: 721  ctaccagggtatctaatcctgtttgctccccacgctttcgcacctcagcgtcagtaatgg 780
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 739  ctaccagggtatctaatcctgtttgctccccacgctttcgcacctcagcgtcagtaatgg 680

Query: 781  accagtgagccgccttcgccactggtgttcctccgaatatctacgaatttcacctctaca 840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 679  accagtgagccgccttcgccactggtgttcctccgaatatctacgaatttcacctctaca 620
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900
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Sbjct: 619  ctcggaattccactcacctcttccatactccagatcgacagtatcaaaggcagttccagg 560

Query: 901  gttgagccctgggatttcaccccctgactgatcgatccgcctacgtgcgctttacgcccag 960
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Sbjct: 559  gttgagccctgggatttcaccccctgactgatcgatccgcctacgtgcgctttacgcccag 500

Query: 961  taattccgaacaacgctagccccttcgtattaccgcggctgctggcacgaagttagccg 1020
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Sbjct: 499  taattccgaacaacgctagccccttcgtattaccgcggctgctggcacgaagttagccg 440

Query: 1021 gggcttcttctccggataccgtcattatcttctccggtgaaagagctttacaaccctagg 1080
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Sbjct: 439  gggcttcttctccggataccgtcattatcttctccggtgaaagagctttacaaccctagg 380

Query: 1081 gccttcatcactcacgcggcatggctggatcaggcttgcgcccattgtccaatattcccc 1140
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Sbjct: 379  gccttcatcactcacgcggcatgggtggatcaggcttgcgcccattgtccaatattcccc 320

Query: 1141 actgctgcctcccgtaggagtttggccgtgtctcagtcccaatgtggctgatcatcctc 1200
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Sbjct: 319  actgctgcctcccgtaggagtttggaccgtgtctcagtcccaatgtgggtgatcatcctc 260

Query: 1201 tcagaccagctatggatcgtcgccttggtaggcctttaccccaccaactagctaatccaa 1260
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Sbjct: 259  tcagaccagctatggatcgtcgccttggtaggcctttaccccaccaactagctaatccaa 200

Query: 1261 cgc-gggctcatctcttgccgataaatctttctcccgaaggacacatacggtattagcac 1319
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Sbjct: 199  cgcggggctcatctcttgccgataaatctttctcccgaaggacacatacggtattagcac 140

Query: 1320 aagtttccctgcgttattccgtagcaaaaggtagattcccacgcgttactcacccgtctg 1379
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Sbjct: 139  aagtttccctgcgttattccgtagcaaaaggtagattcccacgcgttactcacccgtctg 80

Query: 1380 ccgctccccctaaagggcgctcgacttgcatgtgttaagcctgccgccaggcgtcgttct 1439
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Sbjct: 79   ccgctccccttgcggggcgctcgacttgcatgtgttaagcctgccgccagcgttcgttct 20

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Sbjct: 19   gagccaggatcaaactc 3
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☐ >gi|57867895|gb|AY864736.1| Rhizobium sp. ORS3177 16S ribosomal RNA gene, partial sequence Length = 1463

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 Strand = Plus / Minus

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            |||||||||||||||||||||| ||||||||||||| |||||||||||||||||||||||
Sbjct: 1450 tacggctaccttgttacgactttcaccccagtcgcttgaccctaccgtggttagctgcct 1391

Query: 59   ccttgcggttagcgcactaccttcgggtaaaaccaactcccatggtgtgacgggcggtgt 118
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1390 ccttgcggttagcgcactaccttcgggtaaaaccaactcccatggtgtgacgggcggtgt 1331

Query: 119  gtacaaggcccgggaacgtattcaccgcggcatgctgatccgcgattactagcgattcca 178
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Sbjct: 1330 gtacaaggcccgggaacgtattcaccgcggcatgctgatccgcgattactagcgattcca 1271
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Query: 179  acttcatgcactcgagttgcaggcagagtgcaatccgaactgagatggcttttggagatt 238
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Sbjct: 1270 acttcatgcactcgagttgcag----agtgcaatccgaactgagatggcttttggagatt 1215

Query: 239  agctcacactcgcgtgctcgctgcccactgtcaccaccattgtagcacgtgtgtagccca 298
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1214 agctcacactcgcgtgctcgctgcccactgtcaccaccattgtagcacgtgtgtagccca 1155

Query: 299  gcccgtaagggccatgaggacttgacgtcatccccaccttcctctcggcttatcaccggc 358
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Sbjct: 1154 gcccgtaagggccatgaggacttgacgtcatccccaccttcctctcggcttatcaccggc 1095

Query: 359  agtccccttagagtgcccaaccaaatgctggcaactaagggcgagggttgcgctcgttgc 418
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Sbjct: 1094 agtccccttagagtgcccaactgaatgctggcaactaagggcgagggttgcgctcgttgc 1035

Query: 419  cgggacttaacccaacatctctacgctcacgacacgagctgacgacagccatgcagcacc 478
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Sbjct: 1034 -gggacttaacccaacatctc-------acgacacgagctgacgacagccatgcagcacc 983

Query: 479  tgtctctgcgccaccgaagtggacccctatctctagaggtaacacaggatgtcaagggc 538
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Sbjct: 982  tgtctctgcgccaccgaagtggacccctatctctagaggtaacacaggatgtcaagggc 923

Query: 539  tggtaaggttctgcgcgttgcttcgaattaaaccacatgctccaccgcttgtgcgggccc 598
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Sbjct: 922  tggtaaggttctgcgcgttgcttcgaattaaaccacatgctccaccgcttgtgcgggccc 863

Query: 599  ccgtcaattcctttgagttttaatcttgcgaccgtactccccaggcggaatgtttaatgc 658
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Sbjct: 862  ccgtcaattcctttgagttttaatcttgcgaccgtactccccaggcggaatgtttaatgc 803

Query: 659  gttagctgcgccaccgaacagtatactgcccgacggctaacattcatcgtttacggcgtg 718
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 802  gttagctgcgccaccgaacagtatactgcccgacggctaacattcatcgtttacggcgtg 743

Query: 719  gactaccagggtatctaatcctgtttgctccccacgctttcgcacctcagcgtcagtaat 778
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 742  gactaccagggtatctaatcctgtttgctccccacgctttcgcacctcagcgtcagtaat 683

Query: 779  ggaccagtgagccgccttcgccactggtgttcctccgaatatctacgaatttcacctcta 838
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 682  ggaccagtgagccgccttcgccactggtgttcctccgaatatctacgaatttcacctcta 623

Query: 839  cactcggaattccactcacctcttccatactccagatcgacagtatcaaaggcagttcca 898
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 622  cactcggaattccactcacctcttccatactccagatcgacagtatcaaaggcagttcca 563

Query: 899  gggttgagccctgggatttcacccctgactgatcgatccgcctacgtgcgctttacgccc 958
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 562  gggttgagccctgggatttcacccctgactgatcgatccgcctacgtgcgctttacgccc 503

Query: 959  agtaattccgaacaacgctagccccccttcgtattaccgcggctgctggcacgaagttagc 1018
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 502  agtaattccgaacaacgctagccccccttcgtattaccgcggctgctggcacgaagttagc 443

Query: 1019 cggggcttcttctccggataccgtcattatcttctccggtgaaagagctttacaacccta 1078
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 442  cggggcttcttctccggataccgtcattatcttctccggtgaaagagctttacaacccta 383

Query: 1079 gggccttcatcactcacgcggcatggctggatcaggcttgcgcccattgtccaatattcc 1138
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 382  gggccttcatcactcacgcggcatggctggatcaggcttgcgcccattgtccaatattcc 323
```

```
Query: 1139 ccactgctgcctcccgtaggagtttgggccgtgtctcagtcccaatgtggctgatcatcc 1198
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 322  ccactgctgcctcccgtaggagtttgggccgtgtctcagtcccaatgtggctgatcatcc 263

Query: 1199 tctcagaccagctatggatcgtcgccttggtaggcctttaccccaccaactagctaatcc 1258
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 262  tctcagaccagctatggatcgtcgccttggtaggcctttaccccaccaactagctaatcc 203

Query: 1259 aacgcgggctcatctcttgccgataaatctttctcccgaaggacacatacggtattagca 1318
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 202  aacgcgggctcatctcttgccgataaatctttctcccgaaggacacatacggtattagca 143

Query: 1319 caagtttccctgcgttattccgtagcaaaaggtagattcccacgcgttactcacccgtct 1378
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 142  caagtttccctgcgttattccgtagcaaaaggtagattcccacgcgttactcacccgtct 83

Query: 1379 gccgctccccctaaagggcgctcgacttgcatgtgttaagcctgccgccaggcgtcgttc 1438
            |||||||||| |   ||||||||||||||||||||||||||||||||||||   ||||||
Sbjct: 82   gccgctccccttgcggggcgctcgacttgcatgtgttaagcctgccgccagcgttcgttc 23

Query: 1439 tgagccaggatcaaactc 1456
            ||||||| |||| |||||
Sbjct: 22   tgagccaagatctaactc 5
```

Figure 4.2 Pairwise comparisons of 16S rDNA sequence of *Sinorhizobium fredii* S174 with data deposited at GenBank.

4.2 Selection of TnAraOut mutants after biparental mating

4.2.1 Antibiotic sensitivity test

Table 4.1 showed the extent of growth obtained in the antibiotic sensitivity tests. The results indicated that the donor *E.coli* S17-1λ*pir* (pNJ17) was resistant to 50-200 μg.ml^{-1} kanamycin while the recipient *S. fredii* S174 was resistant to 50 μg.ml^{-1} kanamycin and to 50-200 μg.ml^{-1} ampicillin. Therefore 100 μg.ml^{-1} kanamycin and 100 μg.ml^{-1} ampicillin were selected for use in the selection of TnAraOut mutants after biparental mating.

Table 4.1 : Antibiotic sensitivity tests to select antibiotics for use in the selection of TnAraOut mutants.

strains	Kanamycin (µg/ml)				Ampicillin (µg/ml)				Streptomycin (µg/ml)				Spectinomycin (µg/ml)			
	50	100	150	200	50	100	150	200	50	100	150	200	50	100	150	200
E.coli DH5□	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E.coli S17-1λ*pir* (pNJ17)	++++	++++	++++	++++	-	-	-	-	++	+	-	-	++	-	-	-
S. fredii S174	++	-	-	-	++++	++++	++++	++++	++	+	-	-	++	-	-	-

+ = growth ; - = no growth

4.2.2 TnAraOut mutants obtained after biparental mating

Figure 4.3 showed 15 TnAraOut mutants with large colonies in TY agar plates containing 0.1% arabinose and small colonies containing no arabinose. One colony with colony of the same size on TY agar medium was probably a mutant where promoters of genes other than cell division genes were disrupted.

0.1% arabinose no arabinose 0.1% arabinose no arabinose

(a) (b)

Figure 4.3 Fifteen TnAraOut mutants on TY agar plates containing 100 $\mu g.ml^{-1}$ kanamycin and 100 $\mu g.ml^{-1}$ ampicillin. 0.1% arabinose was present in the plates as indicated. Each colony was obtained after replicate-plating twice. TnAraOut mutants obtained from (a) The first biparental mating (b)The second biparental mating.

4.2.3 RAPD-PCR fingerprinting

Figure 4.4 showed RAPD-PCR fingerprints of nine out of the fifteen isolated TnAraOut mutants when either RPO1 or CRL-7 was used as the primer. The results indicated that an approximately 2,800 bp band was obtained when RPO1 was used as the primer for the wild type and all the nine mutant strains. RAPD-PCR fingerprints when CRL-7 was used as the primer indicated there were two groups of mutants.

Group 1 consists of ST6, ST20, ST25, ST39, ST40, ST41, and ST60

Group 2 consists of ST31 and ST49

Group 1 mutants shared the same RAPD-PCR fingerprints as the wild type. Representatives of mutants from both groups (ST49 and ST60) were chosen for further studies.

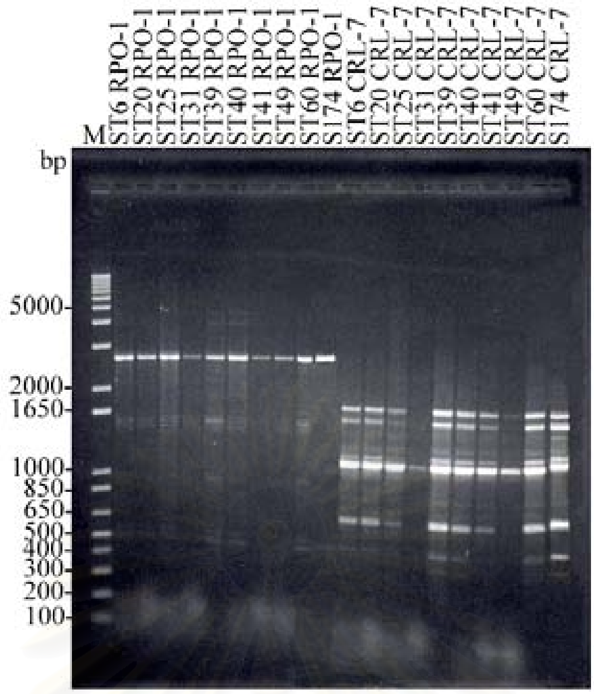


Figure 4.4 RAPD-PCR fingerprints of nine TnAraOut mutants and of wild-type *S. fredii* S174 when either RPO1 or CRL-7 was used as the primer.

4.2.4 Detection of TnAraOut sequence in the genomes of mutants.

Figure 4.5 showed PCR products obtained when $P_{BADout\ 2}$ was used as the primer with target DNA from kanamycin resistant colonies obtained after electroporation of recircularized *Sph*I digested DNA of TnAraOut mutants ST49 and ST60.

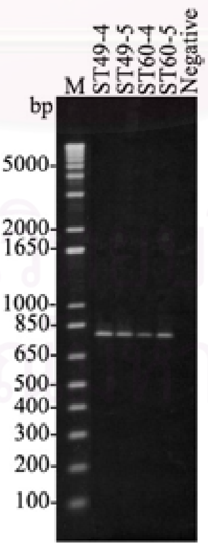


Figure 4.5 PCR products obtained when $P_{BADout\ 2}$ was used as the primer with target DNA from kanamycin resistant colonies after electroporation of recircularized *Sph*I digested DNA of TnAraOut mutants ST49 and ST60.

Sequences of four PCR products (ST49-4, ST49-5, ST60-4, ST60-5) were obtained as follows :

```
                        10         20         30         40         50         60         70
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST49-4_PBA     GCCGAGCTTA GAGGAACCGG CACCGAGGGC ACCCGGATCA AATATATGAT TCGAGCCTCT GTACCTCATA
invertedre     ---------- ---------- ---------- ---------- ---------- ---------- ----------

                        80         90        100        110        120        130        140
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST49-4_PBA     CTAGCATCTC TTGGTTGAAA CGCGTGGCCT TTAGTACACT ACCCACAGGG CTACATCTTA TATTTATCAC
invertedre     ---------- ---------- ---------- ---------- ---------- ---------- --------AC

                       150        160        170        180        190        200        210
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST49-4_PBA     CTAAGTTATA CCTCACTTGT GGGTACTATA GAGCAGTCGT CGCCCTTTTA ATAGTAGGGT GGACCAAGAA
invertedre     CT--GTTA-- ---------- ---------- ---------- ---------- ---------- ----------

                       220        230        240        250        260        270        280
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST49-4_PBA     AGCTTTTCTA CCCGATGTCC GCTACTTACT GATCTTGACC TAGCGACTTA TATACGATTA TTACACTTTT
invertedre     ---------- ---------- ---------- ---------- ---------- ---------- ----------

                       290        300        310        320        330        340        350
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST49-4_PBA     AACGCACGCA CTACTTCGCT AGGTACTACT AGACTGGTCG GACAAACCCG GTTTAATAAG TACGGCCCTA
invertedre     ---------- ---------- ---------- ---------- ---------- ---------- ----------

                       360        370        380        390        400        410        420
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST49-4_PBA     TCCCCTTCCC GCAATAGGCA TCGTACGACC GCTATTTAGT ACGAACTCGC TAATAATGAC GCGGCGTACC
invertedre     ---------- ---------- ---------- ---------- ---------- ---------- ----------

                       430        440        450        460        470        480
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|
ST49-4_PBA     ATTCGTTTTA TTGGTCAAGG GAGGCAGGTG CCTACTTTAT TAGACGGGTT TGTTCCGAAC ATTTA
invertedre     ---------- ---------- ---------- ---------- ---------- ---------- -----
```

```
                        10         20         30         40         50         60         70
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST49-5_PBA     TGACAGACGA TGAAGTAGGT AGTATTTGAT CTGCCTCGCG CTCTTACCAG GATTTCTGAT AGCCCTGTCC
inverted r     ---------- ---------- ---------- ---------- ---------- ---------- ----------

                        80         90        100        110        120        130        140
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST49-5_PBA     ACCATATATT GTGAAACCGG GCCCTACTCA ACTTCCCGTC GCACCTGAGT TACGCTGAAT CGGTGCATCA
inverted r     ---------- ---------- ---------- ---------- --ACCT--GT TA-------- ----------

                       150        160        170        180        190        200        210
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST49-5_PBA     CGAGGAATAC ATACAGCGTG TGGCCATTTG ATAGTGGGTA CCAAAAGCTC CACACCCACT ACCGTCGGAC
inverted r     ---------- ---------- ---------- ---------- ---------- ---------- ----------

                       220        230        240        250        260        270        280
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST49-5_PBA     CGACACAAAA CGACATTCAC AGAACGAGGA CGGTTCACGG TGGGCAACTG GGATAACGTT AAGGGCCCGC
inverted r     ---------- ---------- ---------- ---------- ---------- ---------- ----------

                       290        300        310        320        330        340        350
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST49-5_PBA     CCTCGGTTTT TAGGGGTACA AGATTTTTGT CCAACATAAT AAAAACCCGC AGCAGCCTGA TATGTAAGGA
inverted r     ---------- ---------- ---------- ---------- ---------- ---------- ----------

               ...
ST49-5_PBA     TGA
inverted r     ---
```

```
                    10         20         30         40         50         60         70
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST60-4_PBA  GCCGAGACTA TAAGAGGAGA CGGGCACCAG GGAACCACGT AAAAAAAAGA TGGGAGCCTC TGGACCTCAT
invertedre  ---------- ---------- ---------- ---------- ---------- ---------- ----------

                    80         90        100        110        120        130        140
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST60-4_PBA  ACGAGAAGTT CAGGTGGACA CGGGTGCGCC TGTAGTAAAC TACCCACAGG TGATACATCT TAATTTATTA
invertedre  ---------- ---------- ---------- ---------- ---------- ---------- ----------

                   150        160        170        180        190        200        210
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST60-4_PBA  CCGAAGTTAG ACCTCACTGT GGGTACCATA CAGCAGCGGT GGCCATTTTA ATAGTAGGGA GGACCAGAAA
invertedre  ---------- ---------- ---------- ---------- ---------- ---------- ----------

                   220        230        240        250        260        270        280
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST60-4_PBA  GTAGGCTACA GGATGTCCGT ACTTACTGAT GGACCTACGA CTATAAACGA TTATTACACA AGAAAGCAGG
invertedre  ---------- ---------- ---------- ---------- ---------- ---------- ----------

                   290        300        310        320        330        340        350
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST60-4_PBA  CACTACTTCG CTAGGTACTA CAAACTGGTC GGACAAACCG CGTTTATAAG TAGGGCCTAG CCCGTTGCGG
invertedre  ---------- ---------- ---------- ---------- ---------- ---------- ----------

                   360        370        380        390        400        410        420
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST60-4_PBA  CGATAAGATG TACGACCGCG TATTTAGACG AACTTGTTAA CTAAAGTACG CGGCGTACCA TGCTTAATTG
invertedre  ---------- ---------- ---------- -ACCTGTTA- ---------- ---------- ----------

                   430        440        450        460        470        480
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
ST60-4_PBA  GGAAGGAGAC AGGTCGCTAC CTACTAGACC GGTGTGTCCG AACAGTTTAC GTAGTACCTA GTGAAC
invertedre  ---------- ---------- ---------- ---------- ---------- ---------- ------


                    10         20         30         40         50         60         70
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST60-5_PBA  GGAAGGTACG GCTGGAGTGG AGCCGGCTTT AGTTACGCCG AAAAATTATG ACTGGAGCCC TGGACCTCAT
invertedre  ---------- ---------- ---------- ---------- ---------- ---------- ----------

                    80         90        100        110        120        130        140
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST60-5_PBA  AGGAGATGTC AGGTGGACAC GGGTGCGCCT TTAGTAAACT ACCAACAGGT GATACATCTT AGATTTATCA
invertedre  ---------- ---------- ---------- ---------- ---------- ---------- ----------

                   150        160        170        180        190        200        210
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST60-5_PBA  CTCGAAGTTA ACCTCACCTG CGGTACCATA CAGCAGCGGT GGCAATCTTT AAGAGTAGGG AGGACCGAAC
invertedre  ---------- ---------- ---------- ---------- ---------- ---------- ----------

                   220        230        240        250        260        270        280
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST60-5_PBA  AAAGTATGCT ACCCGATGTC CGTACTTACG ATTTGACCTA CGACTATATA CGATTATTAC ACAAGAAAGC
invertedre  ---------- ---------- ---------- ---------- ---------- ---------- ----------

                   290        300        310        320        330        340        350
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST60-5_PBA  ACGCACTACT TCGCTAGGTA CTACAAAATG GTCTGGACAA ACCGCGTTTA GTAAGTAGCG GACCTACCCG
invertedre  ---------- ---------- ---------- ---------- ---------- ---------- ----------

                   360        370        380        390        400        410        420
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
ST60-5_PBA  TTGCGGCGAT AGAGCAATGT ACGACCGCGT ATTTAGACAC ACTCGTTAAC TAATGTACGC GGCGTACCAT
invertedre  ---------- ---------- ---------- ---------- ACCTGTTA-- ---------- ----------

                   430        440        450        460        470
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|.
ST60-5_PBA  TCGTTAATTG GTAAGGAGAG ACAGGTGGCT AACCTATAGA CCGGGTGGTC CAGAAC
invertedre  ---------- ---------- ---------- ---------- ---------- ------
```

Sequences obtained for the inverted repeat sequences in the PCR products were similar to that reported by Rubin et al. (1999). Therefore TnAraOut might be present in the chromosomes of mutants ST49 and ST60.

4.2.5 Effects of arabinose on growth.

Figure 4.6 showed the effects of arabinose on growth of *S. fredii* S174 wild type and TnAraOut mutants ST49 and ST60. The mutants were found to exhibit the same pattern of arabinose-dependent growth as the wild type. Increase in arabinose concentrations was found to increase growth in both the wild type and the mutants.

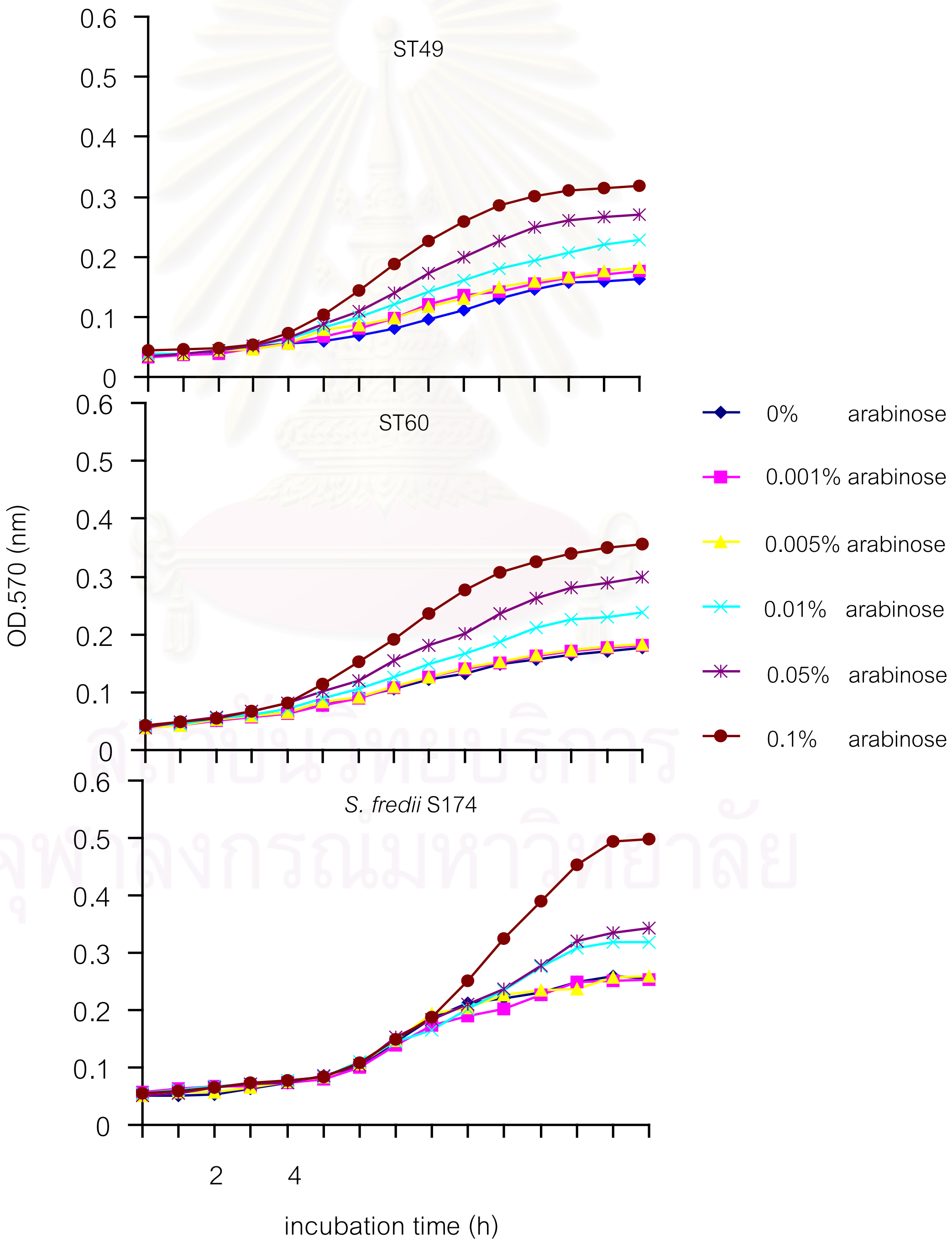


Figure 4.6 Growth curves of *S. fredii* S174 and mutants ST49 and ST60 cultured in Minimal Medium containing 100 μg.ml^{-1} kanamycin, 100 μg.ml^{-1} ampicillin and different arabinose concentrations, pH 6.8 at 200 rpm, 30°C. No antibiotics were added to culture medium of the wild type.

4.3 Themotolorance in wild type *S. fredii* S174 and TnAraOut mutants.

Figures 4.7.1 to 4.7.15 showed growth of wild type *S. fredii* S174 and TnAraOut mutants. The results indicated that both the wild type and TnAraOut mutants were heat-tolerant.

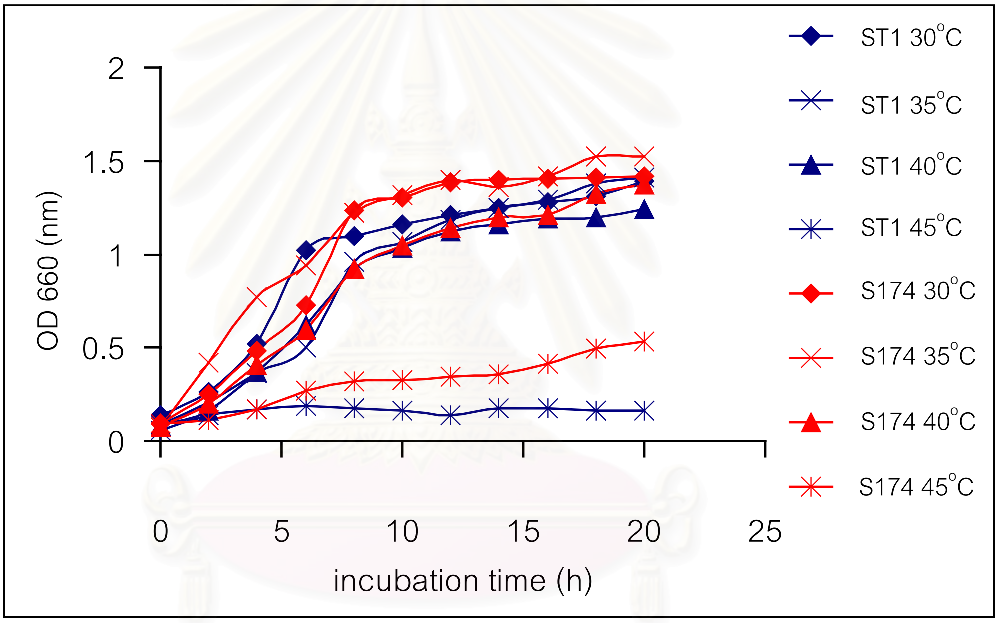


Figure 4.7.1 Growth curves of *S. fredii* S174 and mutant ST1 cultured in tryptone yeast extract broth containing 100 μg.ml^{-1} kanamycin, 100 μg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30°C, 35°C, 40°C and 45°C. No antibiotics were added to the culture medium of wild type.

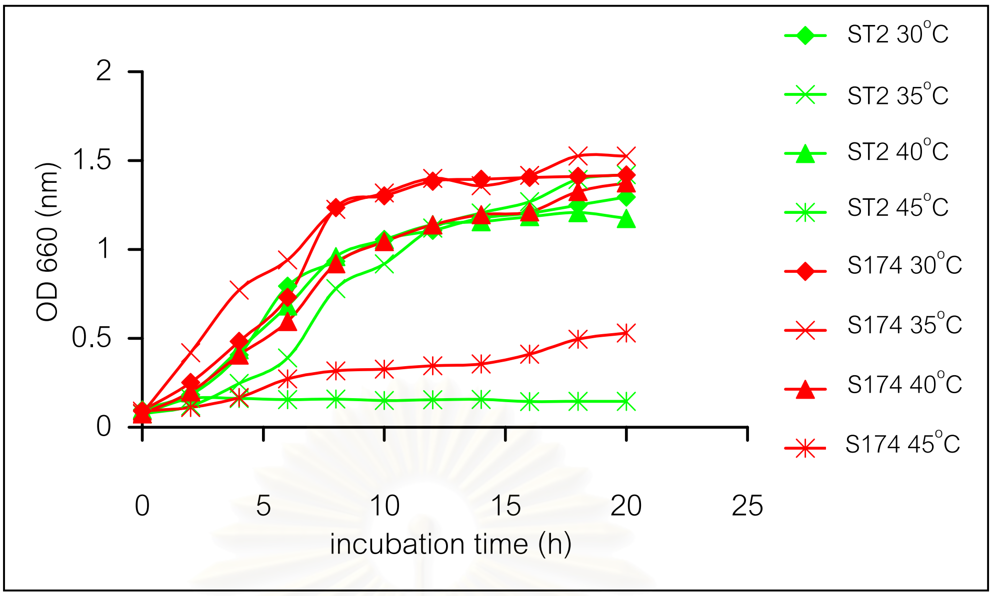


Figure 4.7.2 Growth curves of *S. fredii* S174 and mutant ST2 cultured in tryptone yeast extract broth containing 100 $\mu g.ml^{-1}$ kanamycin, 100 $\mu g.ml^{-1}$ ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30°C, 35°C, 40°C and 45°C. No antibiotics were added to the culture medium of wild type.

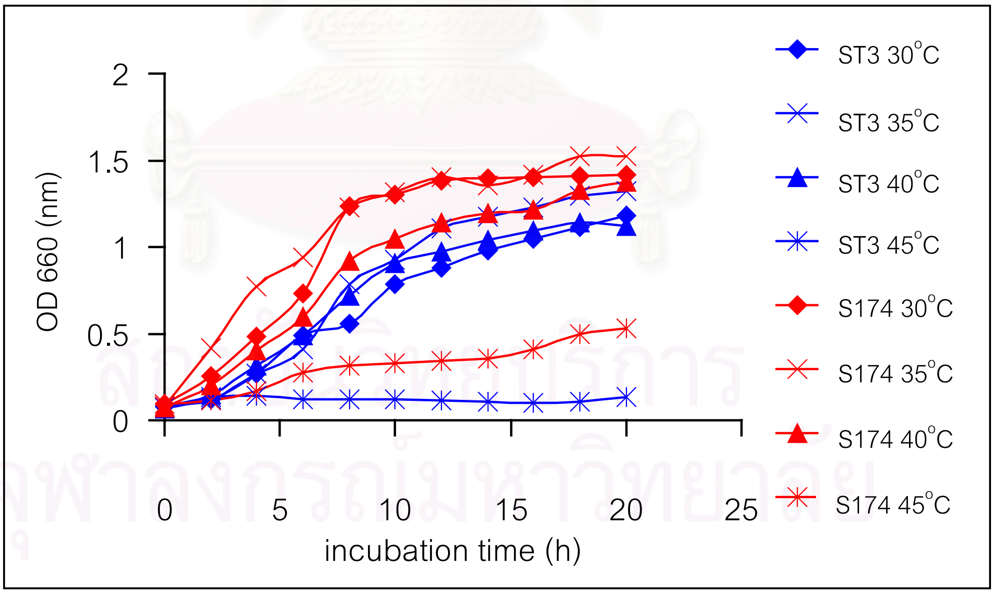


Figure 4.7.3 Growth curves of *S. fredii* S174 and mutant ST3 cultured in tryptone yeast extract broth containing 100 $\mu g.ml^{-1}$ kanamycin, 100 $\mu g.ml^{-1}$ ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30°C, 35°C, 40°C and 45°C. No antibiotics were added to the culture medium of wild type.

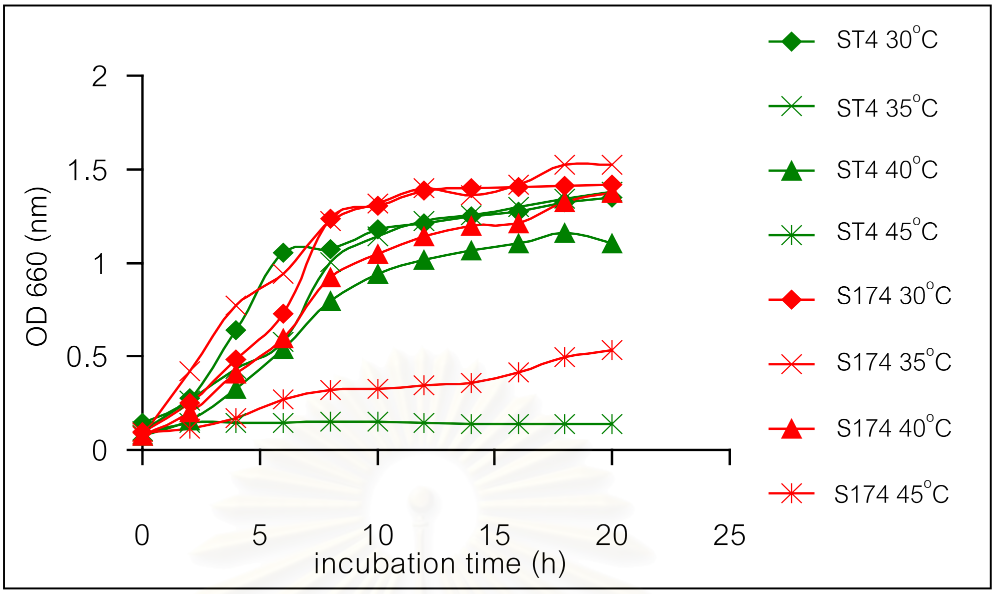


Figure 4.7.4 Growth curves of *S. fredii* S174 and mutant ST4 cultured in tryptone yeast extract broth containing 100 μg.ml^{-1} kanamycin, 100 μg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30°C, 35°C, 40°C and 45°C. No antibiotics were added to the culture medium of wild type.

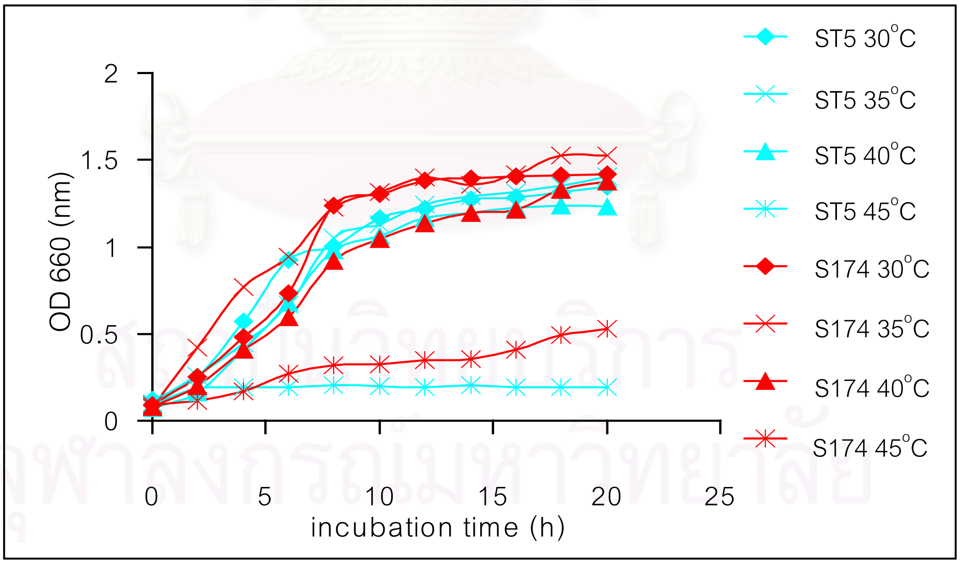


Figure 4.7.5 Growth curves of *S. fredii* S174 and mutant ST5 cultured in tryptone yeast extract broth containing 100 μg.ml^{-1} kanamycin, 100 μg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30°C, 35°C, 40°C and 45°C. No antibiotics were added to the culture medium of wild type.

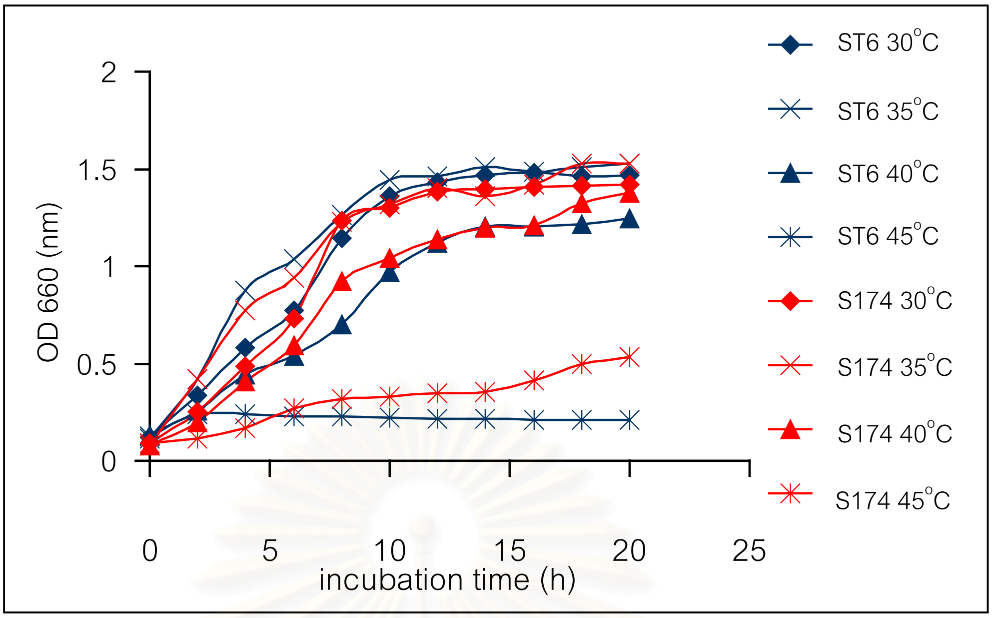


Figure 4.7.6 Growth curves of *S. fredii* S174 and mutant ST6 cultured in tryptone yeast extract broth containing 100 $\mu g.ml^{-1}$ kanamycin, 100 $\mu g.ml^{-1}$ ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, $30^{\circ}C$, $35^{\circ}C$, $40^{\circ}C$ and $45^{\circ}C$. No antibiotics were added to the culture medium of wild type.

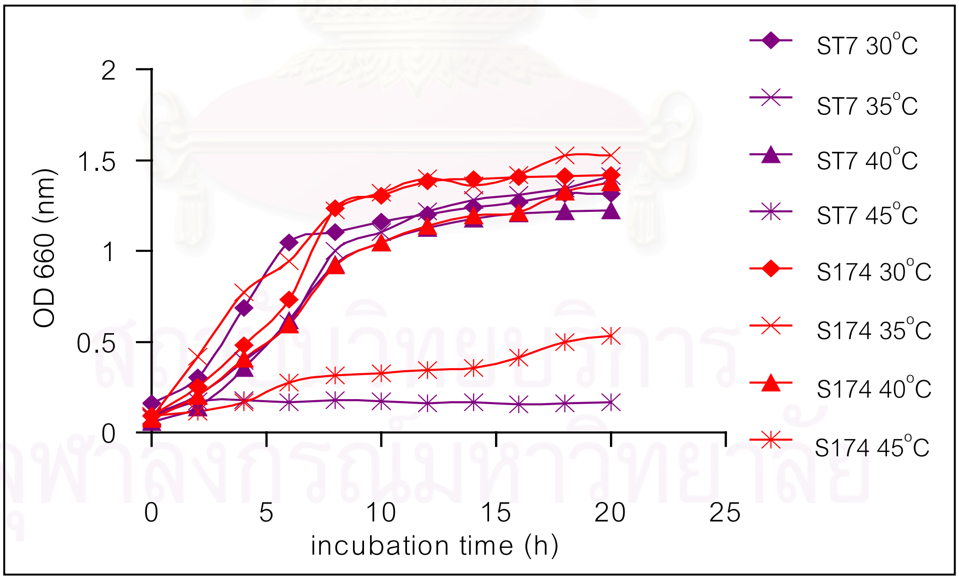


Figure 4.7.7 Growth curves of *S. fredii* S174 and mutant ST7 cultured in tryptone yeast extract broth containing 100 $\mu g.ml^{-1}$ kanamycin, 100 $\mu g.ml^{-1}$ ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, $30^{\circ}C$, $35^{\circ}C$, $40^{\circ}C$ and $45^{\circ}C$. No antibiotics were added to the culture medium of wild type.

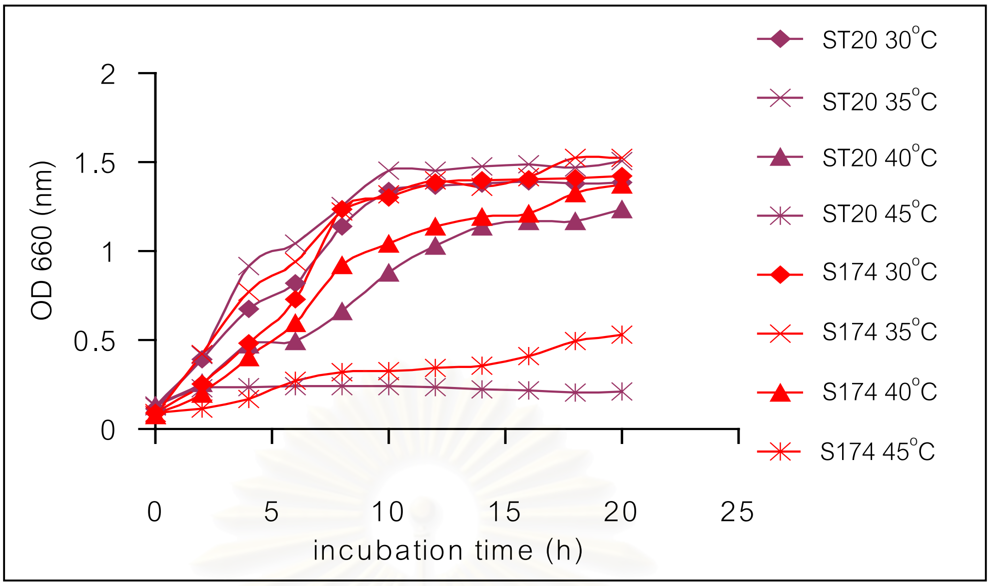


Figure 4.7.8 Growth curves of *S. fredii* S174 and mutant ST20 cultured in tryptone yeast extract broth containing 100 μg.ml^{-1} kanamycin, 100 μg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30°C, 35°C, 40°C and 45°C. No antibiotics were added to the culture medium of wild type.

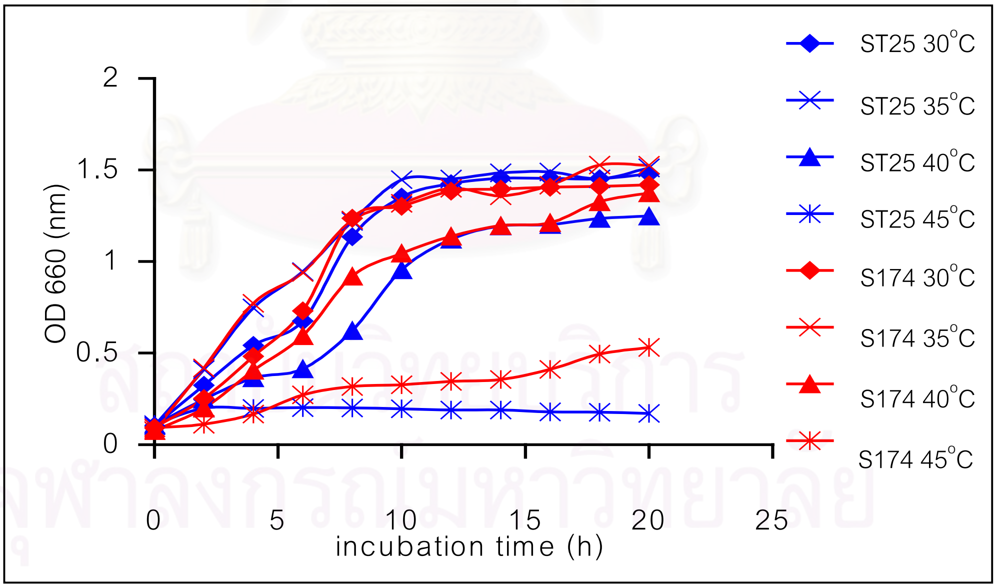


Figure 4.7.9 Growth curves of *S. fredii* S174 and mutant ST25 cultured in tryptone yeast extract broth containing 100 μg.ml^{-1} kanamycin, 100 μg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30°C, 35°C, 40°C and 45°C. No antibiotics were added to the culture medium of wild type.

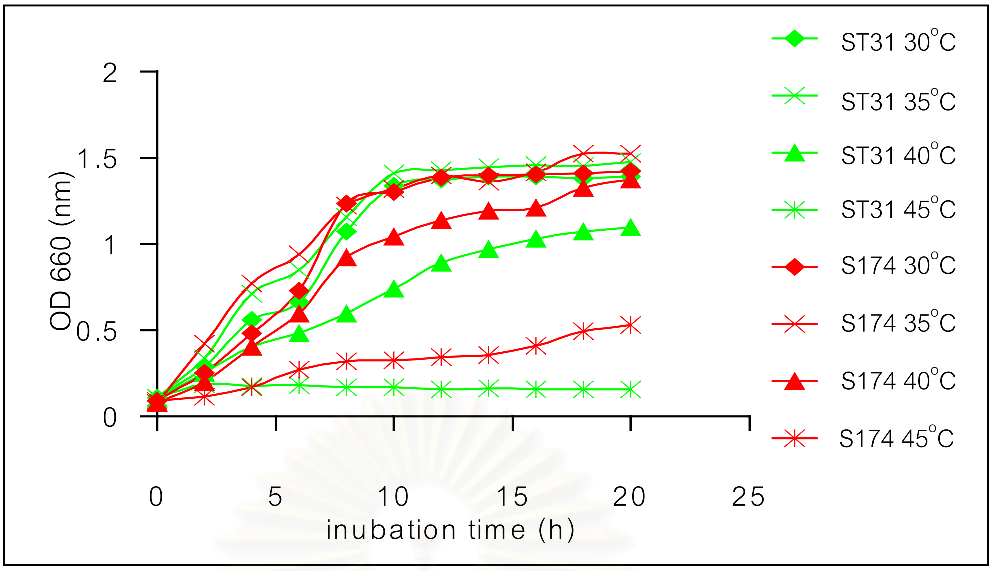


Figure 4.7.10 Growth curves of *S. fredii* S174 and mutant ST31 cultured in tryptone yeast extract broth containing 100 μg.ml^{-1} kanamycin, 100 μg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30°C, 35°C, 40°C and 45°C. No antibiotics were added to the culture medium of wild type.

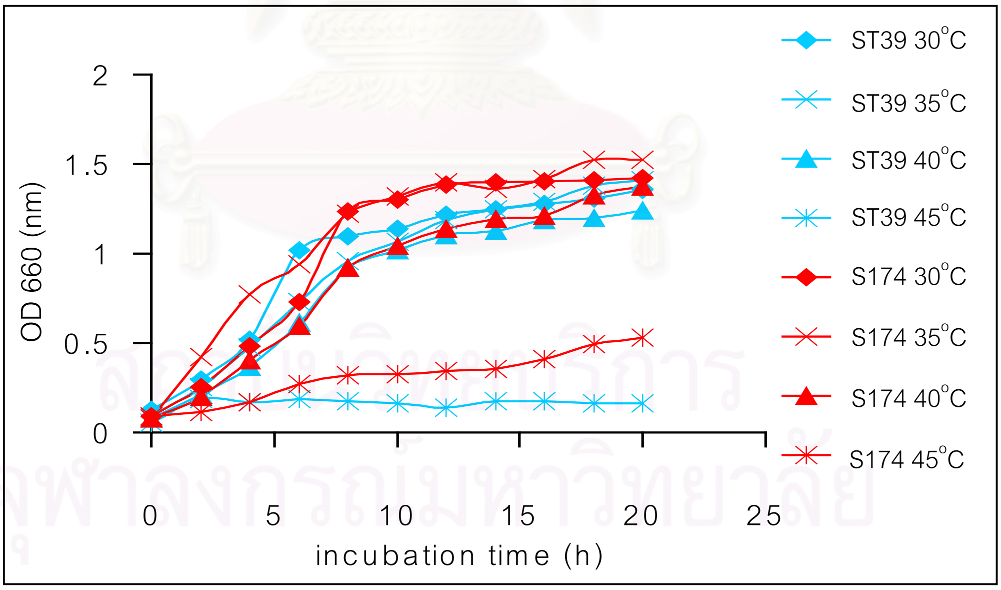


Figure 4.7.11 Growth curves of *S. fredii* S174 and mutant ST39 cultured in tryptone yeast extract broth containing 100 μg.ml^{-1} kanamycin, 100 μg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30°C, 35°C, 40°C and 45°C. No antibiotics were added to the culture medium of wild type.

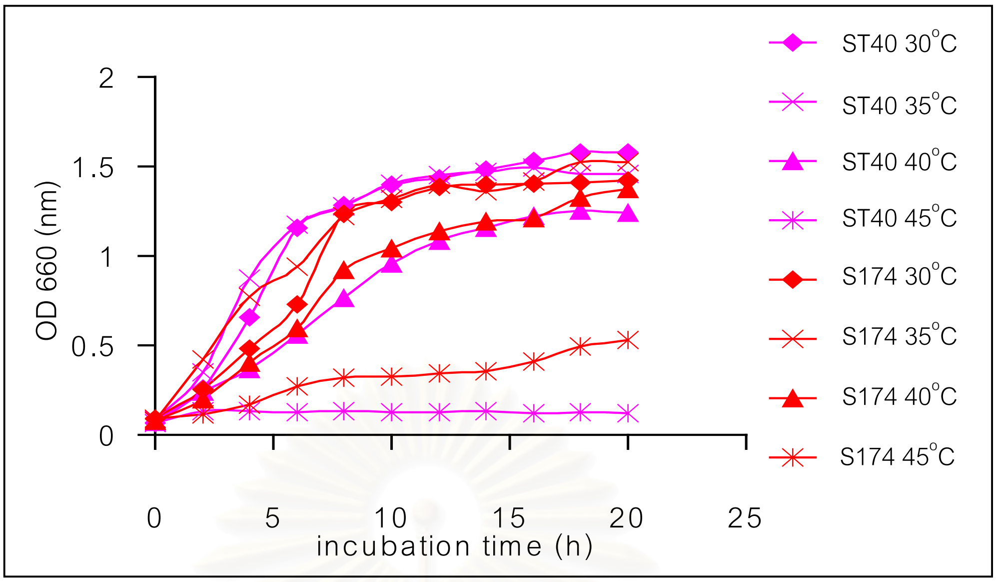


Figure 4.7.12 Growth curves of *S. fredii* S174 and mutant ST40 cultured in tryptone yeast extract broth containing 100 µg.ml^{-1} kanamycin, 100 µg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30°C, 35°C, 40°C and 45°C. No antibiotics were added to the culture medium of wild type.

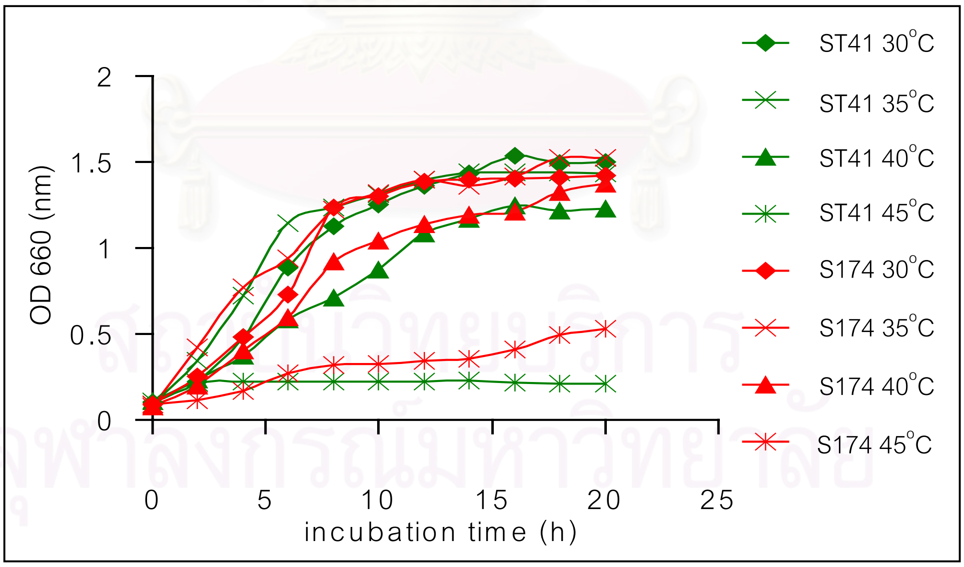


Figure 4.7.13 Growth curves of *S. fredii* S174 and mutant ST41 cultured in tryptone yeast extract broth containing 100 µg.ml^{-1} kanamycin, 100 µg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30°C, 35°C, 40°C and 45°C. No antibiotics were added to the culture medium of wild type.

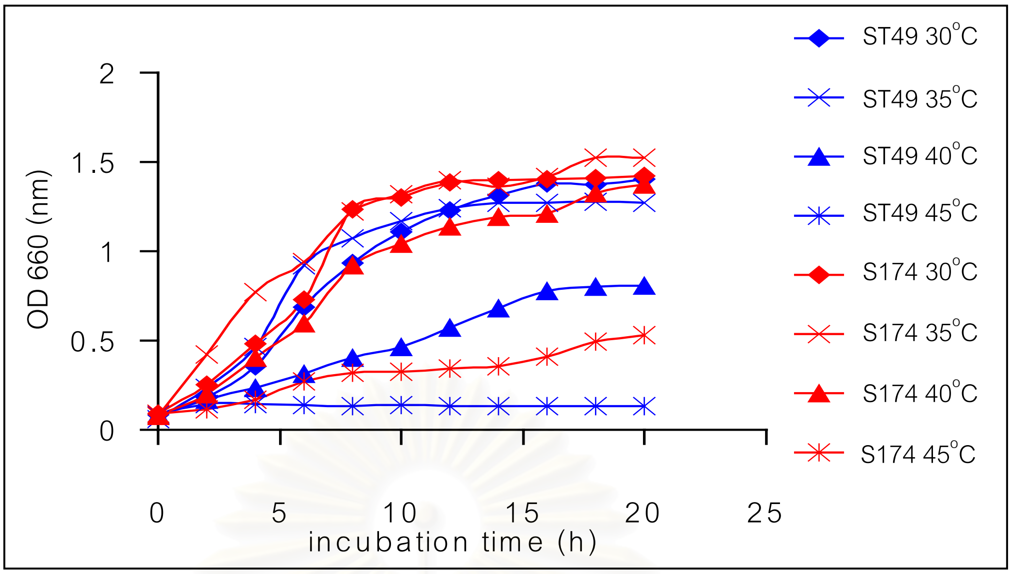


Figure 4.7.14 Growth curves of *S. fredii* S174 and mutant ST49 cultured in tryptone yeast extract broth containing 100 μg.ml^{-1} kanamycin, 100 μg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30°C, 35°C, 40°C and 45°C. No antibiotics were added to the culture medium of wild type.

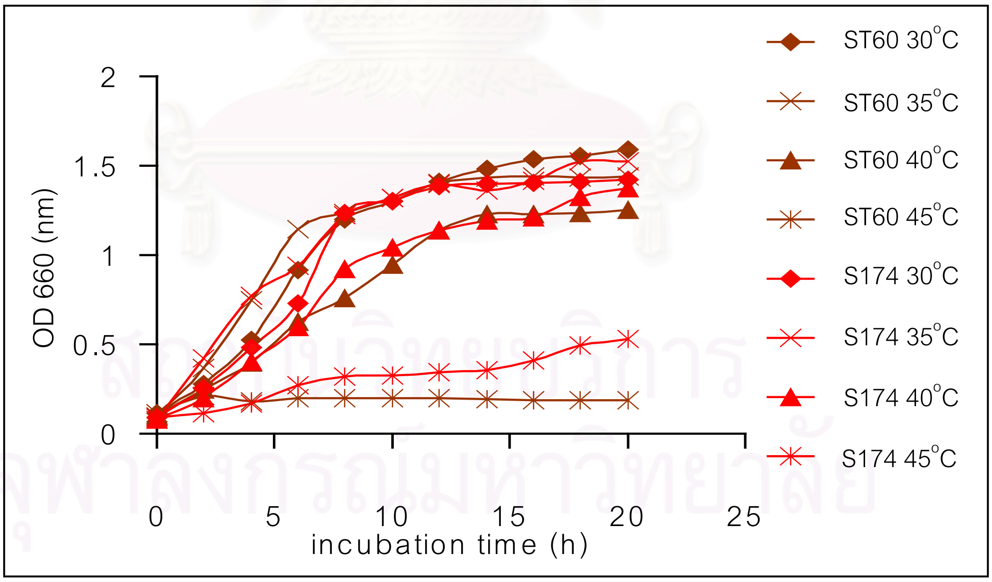


Figure 4.7.15 Growth curves of *S. fredii* S174 and mutant ST60 cultured in tryptone yeast extract broth containing 100 μg.ml^{-1} kanamycin, 100 μg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30°C, 35°C, 40°C and 45°C. No antibiotics were added to the culture medium of wild type.

4.4 Comparisons of intracellular protein profiles of wild type *S. fredii* S174 and TnAraOut mutants.

Figures 4.8.1 to 4.8.8 showed SDS-PAGE intracellular protein profiles of wild type *S. fredii* S174 and TnAraOut mutants grown under different temperatures. The results indicated increased production of 60, 62, 12, 10 kDa polypeptides in all or most of the cells.

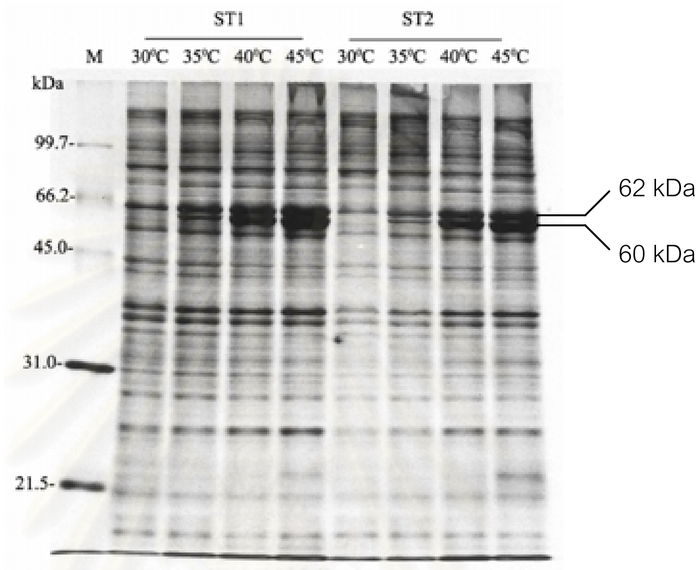


Figure 4.8.1 SDS-PAGE of intracellular protein profiles of mid-log phase cells of mutants ST1 and ST2 when cultured in tryptone yeast extract medium containing 100 μg.ml^{-1} kanamycin, 100 μg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30°C, 35°C, 40°C and 45°C. No antibiotics were added to the culture medium of wild type.

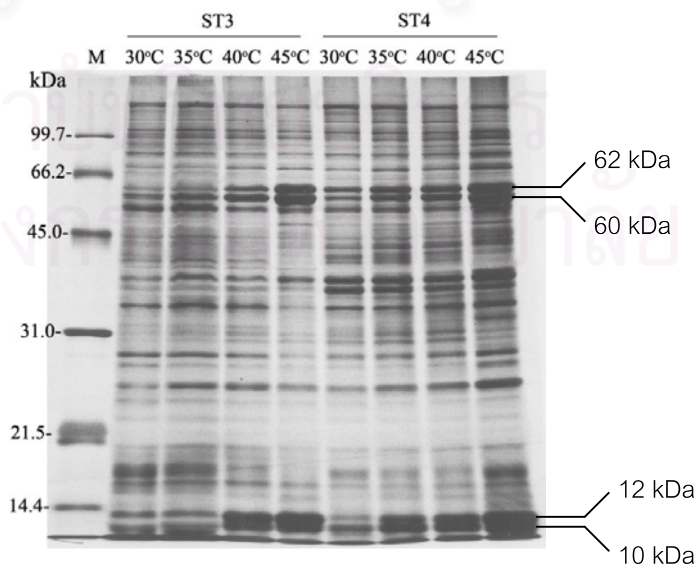


Figure 4.8.2 SDS-PAGE of intracellular protein profiles of mid-log phase cells of mutants ST3 and ST4 when cultured in tryptone yeast extract medium containing 100 μg.ml^{-1} kanamycin, 100 μg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C. No antibiotics were added to the culture medium of wild type.

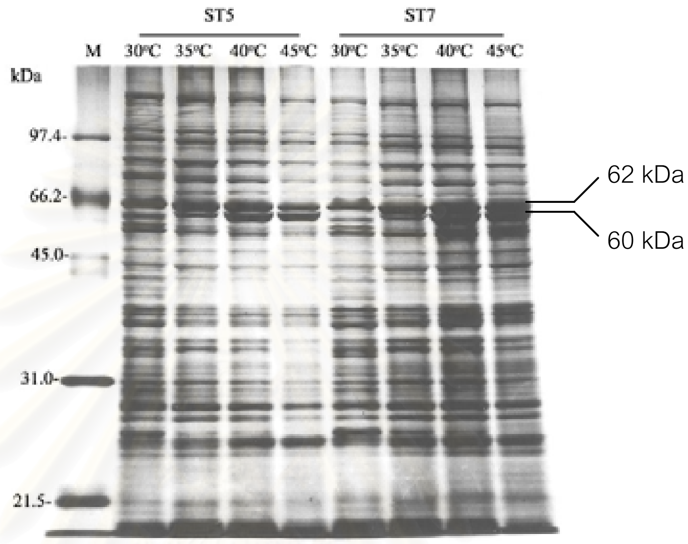


Figure 4.8.3 SDS-PAGE of intracellular protein profiles of mid-log phase cells of mutants ST5 and ST7 when cultured in tryptone yeast extract medium containing 100 μg.ml^{-1} kanamycin ,100 μg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C. No antibiotics were added to the culture medium of wild type.

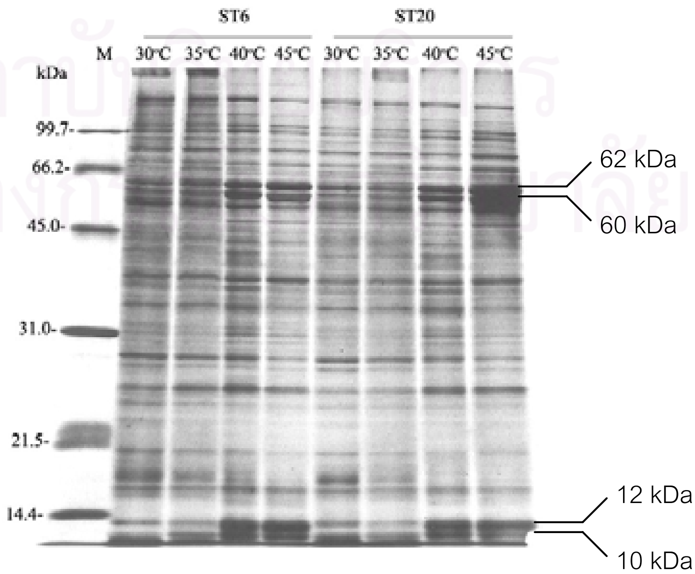


Figure 4.8.4 SDS-PAGE of intracellular protein profiles of mid-log phase cells of mutants ST6 and ST20 when cultured in tryptone yeast extract medium containing 100 μg.ml^{-1} kanamycin, 100 μg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C. No antibiotics were added to the culture medium of wild type.

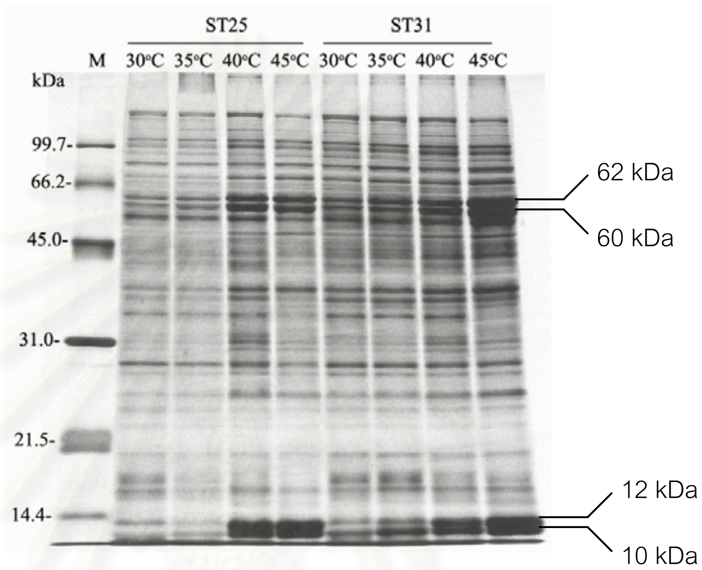


Figure 4.8.5 SDS-PAGE of intracellular protein profiles of mid-log phase cells of mutants ST25 and ST31 when cultured in tryptone yeast extract medium containing 100 μg.ml^{-1} kanamycin, 100 μg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C. No antibiotics were added to the culture medium of wild type.

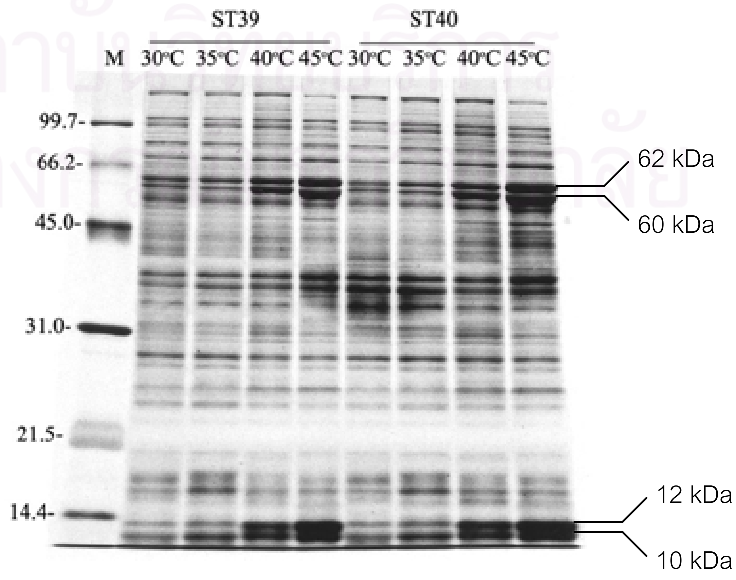


Figure 4.8.6 SDS-PAGE of intracellular protein profiles of mid-log phase cells of mutants ST39 and ST40 when cultured in tryptone yeast extract medium containing 100 μg.ml^{-1} kanamycin, 100 μg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30°C, 35°C, 40°C and 45°C. No antibiotics were added to the culture medium of wild type.

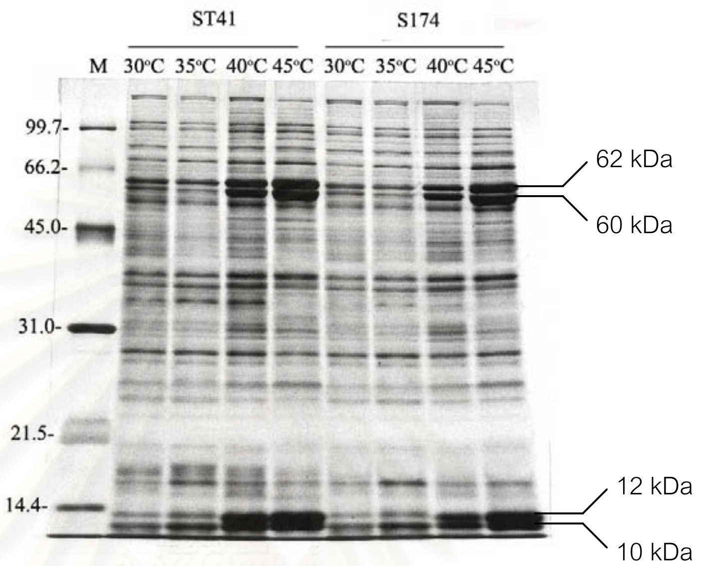


Figure 4.8.7 SDS-PAGE of intracellular protein profiles of mid-log phase cells of wild type *S. fredii* S174 and of mutant ST41 when cultured in tryptone yeast extract medium containing 100 μg.ml^{-1} kanamycin, 100 μg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30°C, 35°C, 40°C and 45°C. No antibiotics were added to the culture medium of wild type.

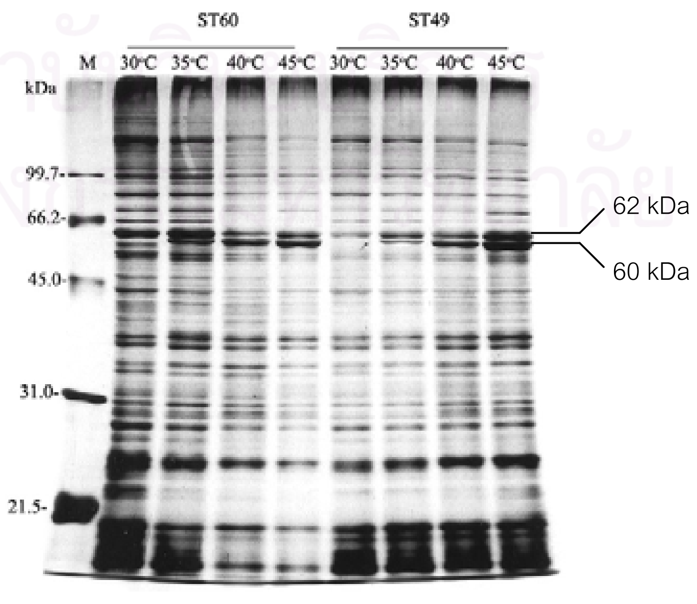
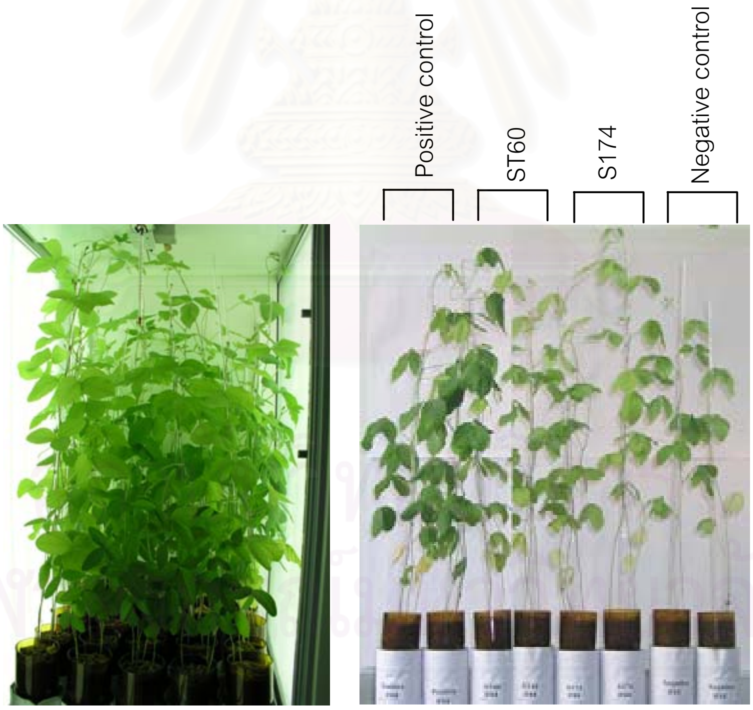


Figure 4.8.8 SDS-PAGE of intracellular protein profiles of mid-log phase cells of mutants ST49 and ST60 when cultured in tryptone yeast extract medium containing 100 μg.ml^{-1} kanamycin,100 μg.ml^{-1} ampicillin and 0.1% arabinose, pH 6.8, at 200 rpm, 30^{o}C, 35^{o}C, 40^{o}C and 45^{o}C. No antibiotics were added to the culture medium of wild type.

4.5 Comparisons of nitrogen fixing potential.

Figure 4.9 showed comparisons of soybean growth in Leonard jars with nitrogen-free medium in plant growth chamber. Plant and nodule dry weights were determined for growth of soybeans (Tables 4.2, 4.3). The results showed that leaves of positive control soybean plants cultivar SJ4 were greener than those of negative controls and the soybean plants inoculated with *S. fredii* S174. Results of plant dry weight as shown in Table 4.2 indicated similar plant dry weight when TnAraOut mutant ST60 or *S. fredii* was used to inoculate the soybeans.



(a) (b)

Figure 4.9 (a) Soybean growth in Leonard jars with nitrogen-free medium in growth chamber. (b) *Glycine max* cultivar SJ4 inoculated with either *S. fredii* S174, or TnAraOut mutant ST60.

Table 4.2 Duncan’s Multiple Range Test for average plant dry weight for *S. fredii* S174 and TnAraOut mutant ST60 and *Glycine max* cv SJ 4 in Leonard jars with nitrogen-free medium pH 6.8 (Level of probability, □ = 0.05).

Strains	Average plant dry weight in grams
	SJ4
ST49	1.29^{c}
ST60	1.42^{c}
S. fredii S174	1.55^{c}
Positive Control	2.55^{e}
Negative Control	0.70^{ab}

Table 4.3 Duncan’s Multiple Range Test for average nodule dry weight for *S. fredii* S174 and TnAraOut mutant ST60 and *Glycine max* cv SJ 4 in Leonard jars with nitrogen-free medium pH 6.8 (Level of probability, □ = 0.05).

Strains	Average nodule dry weight in grams
	SJ4
ST60	0.13^{b}
S. fredii S174	0.15^{b}

CHAPTER 5

DISCUSSION

Fifteen slow-growing TnAraOut mutants were obtained by biparental mating between *S. fredii* S174 and *E. coli* S17-1λ*pir* (pNJ17). Different TnAraOut mutants ST49 and ST60 were chosen for further studies based on their different RAPD-PCR fingerprints obtained when CRL-7 was used as the primer. Welsh & McClelland (1990) and Williams et al (1990) reported some of the first work that utilized random primers to obtain RAPD-PCR fingerprints.

Experiments on sequencing of the PCR products when $P_{BADout2}$ was used as the primer need to be carried out at least twice to obtain the exact sequence of the inverted repeat of TnAraOut. The sequences obtained for the PCR products (ST49-4, ST49-5, ST60-4, ST60-5) showed the presence of sequences which were similar but not identical to that of the reported inverted repeat sequence (ACCTGT). Since the sequencing data were not good due to the presence of overlapping peaks (Appendix C), more sequencing needs to be performed to obtain accurate sequences of the inverted repeat in the PCR products. After reliable sequences of the inverted repeat followed by upstream sequences of putative cell division genes are obtained, tentative identities of the genes will be determined for use in the design of primers for either fast-grower specific or slow-grower specific probes to detect soybean rhizobia in soils. Sequencing of PCR products obtained when $P_{BADout2}$ is used as the primer and each mutant's DNA containing TnAraOut is used as the target DNA will result in the determination of several cell division specific genes. Multiple alignments of sequences of these genes will lead to design of primers specific for the detection of either the fast-growing *S. fredii* or slow-growing *B. japonicum*. These specific primers may be useful in the detection of introduced fast- and slow-growing soybean rhizobia in the inocula and the detection of endogenous soybean rhizobia in the fields (Emampaiwong et al, 2005).

The genes tentatively defined as involved in cell division will also be used in further studies on genes controlling cell division in the fast-growing soybean rhizobia and in comparative studies of cell division genes in fast- and slow-growing soybean rhizobia.

Inoculation with TnAraOut mutant ST60 or *S. fredii* S174 was found to increase plant dry weights although the weights were lower than those of the corresponding positive controls. The leaves of the positive control plants were also greener than those of the experimental plants. These results suggested that *S. fredii* S174 and TnAraOut mutant ST60 were not good nitrogen-fixers under the experimental conditions. Changes in the experimental conditions, for example, adding more inoculants, might result in better growth of inoculated plants.

It is postulated that more PCR products when the arbitrary GC rich primer was used implies more G/C content which would enable organisms to be more thermotolerant. In this research, TnAraOut mutants ST31 and ST49 were found to have less number of PCR products when CRL-7 was used as the primer (Figure 4.4). They were thus less thermotolerant as was confirmed in Figures 4.7.10 and 4.7.14.

In the presence of arabinose, the two TnAraOut mutants were found to exhibit the same extent of thermotolerance when compared with the wild type. Polypeptides 10, 12, 60, and 62 kDa were found to increase upon growth at high temperatures up to 40^{o}C implying that promoters of some but not all of the heat shock genes in *S. fredii* S174 were disrupted by the TnAraOut transposon. Future work will reveal the nature of heat shock genes in the fast-growing *S. fredii* which have not been as extensively studied as those in the slow-growing soybean rhizobia.

CHAPTER 6

CONCLUSION

16S rDNA sequence of *S. fredii* S174 was obtained for the first time in this work. Fifteen TnAraOut mutants which have defects in cell division were obtained from biparental matings between *S. fredii* S174 and *E. coli* S17-1λ*pir* (pNJ17). Detection of the presence of an inverted repeat (ACCTGT) which belonged to TnAraOut sequence was used as an evidence to indicate that TnAraOut had been inserted into the genome of *S. fredii* S174 to give rise to the cell division defective mutants. Two TnAraOut mutants with different RAPD-PCR fingerprints when CRL-7 was used as the primer were chosen for further studies on comparisons of thermotolerance and nitrogen fixation potential with the wild type. The results indicated that thermotolerance properties of the two TnAraOut mutants (ST49 and ST60) were comparable to that of the wild type with the same intracellular protein profiles showing an increase in synthesis of polypeptides 10, 12, 60, and 62 kDa. TnAraOut mutant ST60 was found to have comparable nitrogen fixation potential with the wild type when *Glycine max* cultivar SJ4 was used as the soybean host.

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APPENDICES

APPENDIX A

BACTERIAL GROWTH MEDIA AND PLANT NUTRIENT SOLUTIONS

Preparation of all bacterial growth media and plant nutrient solutions are as described by Somasegaran and Hoben (1994) unless otherwise stated.

Yeast Extract Mannitol Broth (YMB)

Mannitol	10.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Yeast extract	0.5 g
Deionized water	1.0 g

pH of medium was adjusted to 6.8 with 0.1 N NaOH. The medium was autoclaved at 121°C for 15 min.

Yeast Extract Mannitol Agar (YMA)

YMB	1 liter
Agar	15 g

Agar was added to 1 liter of YMB. The solution was shaken to suspend the agar then autoclaved at 121°C for 15 min. After autoclaving, the medium was shaken to ensure even mixing of melted agar with medium before pouring onto petridishes and left to solidify.

YMA with Congo Red

Congo Red stock solution: 250 mg of Congo Red dissolved in 100 ml of deionized water. 10 ml of Congo Red stock solution were added to 1 liter of YMA. The final Congo Red concentration was 25 $\mu g.ml^{-1}$. The medium was autoclaved at 121°C for 15 min.

Tryptone-Yeast (TY) Medium

Tryptone	5.0	g
Yeast extract	3.0	g
$CaCl_2 \cdot H_2O$	0.87	g
Deionized water	1000	ml

pH of medium was adjusted to 6.8 with 0.1 N NaOH. The medium was autoclaved at 121°C for 15 min.

Luria-Bertani (LB) Medium

Tryptone	10.0	g
Yeast extract	5.0	g
NaCl	5.0	g
Deionized water	1000	ml

pH of medium was adjusted to 7.4 with 0.1 N NaOH. The medium was autoclaved at 121°C for 15 min.

Bacto minimal broth Davis's w/o dextrose

Dextrose	1	g
Dipotassium phosphate	7	g
monopotassium phosphate	2	g
Sodium citrate USP	0.5	g
Magnesium sulfate	0.1	g
Ammonium sulfate	1	g
Deionized water	1000	ml

pH of medium was adjusted to 7.0 with 0.1 N NaOH. The medium was autoclaved at 121°C for 15 min.

GYT medium

10% (V/V) glycerol

0.125% (W/V) yeast extract

0.25% (W/V) tryptone

Deionized water	100	ml

pH of medium was adjusted to 7.0 with 0.1 N NaOH. The medium was autoclaved at 121°C for 15 min.

SOC medium

Bacto-tryptone	20	g
Bacto-yeast extract	5	g
NaCl	10	mM
KCl	2.5	mM
$MgCl_2$	10	mM
$MgSO_4$	10	mM
Glucose	20	mM
Deionized water	1000	ml

pH of medium was adjusted to 7.0 with 0.1 N NaOH. The medium was autoclaved at 121°C for 15 min.

N-free Nutrient Solutions

Stock Solutions	Chemicals	g/liter
1	$CaCl_2.2H_2O$	294.1
2	KH_2PO_4	136.1
3	$FeC_6H_5O_7.3H_20$	6.7
	$MgSO_4.7H_2O$	123.3
	K_2SO_4	87.0
	$MnSO_4.H_2O$	0.338
4	H_3BO_3	0.247
	$ZnSO_4.7H_2O$	0.288
	$CuSO_4.5H_2O$	0.100
	$CoSO_4.7H_2O$	0.056
	$Na_2 MoO_2.7\ H_2O$	0.048

Warm water was used to prepare stock solutions to get the ferric-citrate into solution. Ten liters of full-strength plant culture solution were prepared as follows:

- To 5 liters of water, add 5 ml of each stock solution and mix,
- Dilute to 10 liters by adding another 5 liters of water,
- Adjust pH to either 5.0 or 6.8 with 1 N HCl
- For positive control treatment, 0.05% KNO3 was added to give final N concentration of 70 ppm.

APPENDIX B

CHEMICALS AND SOLUTIONS

1. Solutions for DNA extraction (Gibco BRL)

Saline-EDTA solution

15 mM NaCl, 10 mM EDTA, pH 8.0

0.9 g NaCl, 0.29 g EDTA were added to distilled water. The final volume was made to 100 ml. 0.1 N NaOH was used to adjust pH to 8.0 before autoclaving at 121°C for 15 min.

DNAzol

DNAzol solution (Gibco BRL) was used according to manufacturer's instruction.

Restriction enzyme

*Sph*I 200 unit (Promega)

Antibiotic

Kanamycin (Liwinner pharmaceutical LTD, Part)

Ampicillin (Liwinner pharmaceutical LTD, Part)

Streptomycin (Liwinner pharmaceutical LTD, Part)

Spectinomycin (Liwinner pharmaceutical LTD, Part)

Arabinose minimum 99% (Sigma)

2. Solutions for SDS-PAGE (Bio-rad)

Stock solutions

A. Acrylamide/bis (30% T, 2.67%C)

87.6 9 acrylamide (29.2 g/100 ml)

2.4 g N'N'-bis-methylene-acrylamide (0.8 g/100 ml)

Make to 300 ml with deionized water. Filter and store at 4°C in the dark (30 days maximum).

B. 1.5 M Tris-HCl, pH 8.8

27.23 g Tris base (18.15 g/100 ml)

80 ml deionized water

Adjust to pH 8.8 with 6N HCl. Make to 150 ml with deionized water and store at 4°C

C. 0.5 M Tris-HCl, pH 6.8

6 g Tris base

60 ml deionized water

Adjust to pH 6.8 with 6N HCl. Make to 100 ml with deionized water and store at 4°C

D. 10% SDS

Dissolve 10 g SDS in 90 ml water with gentle stirring and bring to 100 ml with ddH_2O

E. Sample buffer (SDS reducing buffer) (store at room temperature)

Deionized water	3.8 ml
0.5 M Tris-HCl, pH 6.8	1.0 ml
Glycerol	0.8 ml
10% (w/v) SDS	1.6 ml
2-mercaptoethanol	0.4 ml
1 % (w/v) bromophenol blue	0.4 ml

Dilute the sample at least 1:4 with sample buffer, and heat at 95°C for 4 minutes

F. 5X electrode (running buffer), pH 8.3

Tris base	9.0 g	(15 g/l)
Glycine	43.2 g	(72 g/l)
SDS	3.0 g	(5 g/l)

Make to 600 ml with deionized water.

Store at 4°C. Warm to room temperature before use if precipitation occurs. Dilute 60 ml 5X stock with 240 ml deionized water for one electrophoretic run.

G. 10% Ammonium persulphate

One milliliter of aqueous 10% (w/v) Ammonium persulphate stock solution was prepared and stored at 4 C. Ammonium persulphate decomposes slowly, and fresh solutions were prepared weekly.

APPENDIX C

SEQUENCES OF PCR PRODUCTS

- Sequences of 16S rDNA of *S. fredii* S174.
- Comparisons of 16S rDNA of *S. fredii* S174 sequences from the first and the second determinations.
- Sequence of PCR products when $P_{BADout2}$ was used as the primer.

ABI PRISM

Model 3100
Version 3.7
Basecaller-3100APOP6SS174_27f
BC 1.5.0.0

30-08-04A_A08_S174_27f_02.ab1
Cap 2

SQ.....25633.....

Signal G:21 T:23 A:32 C:10
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Points 250 to 10106 Pk 1 Loc: 250

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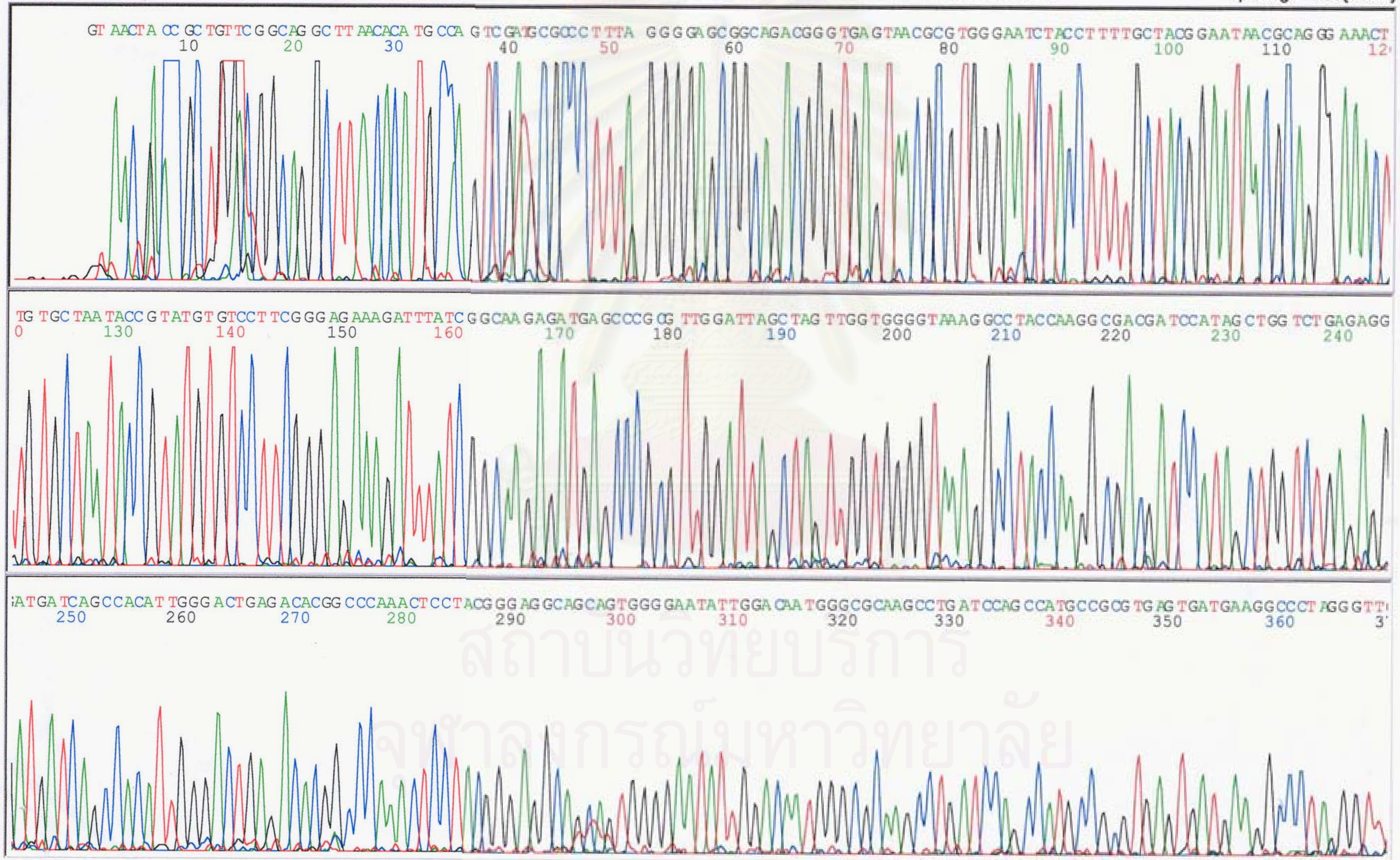

ABI PRISM

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Basecaller-3100APOP6SS174_27f
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Cap 2

SQ...25633...

Signal G:21 T:23 A:32 C:10
DT3100POP6{ET}50cm.mob
demo_3100
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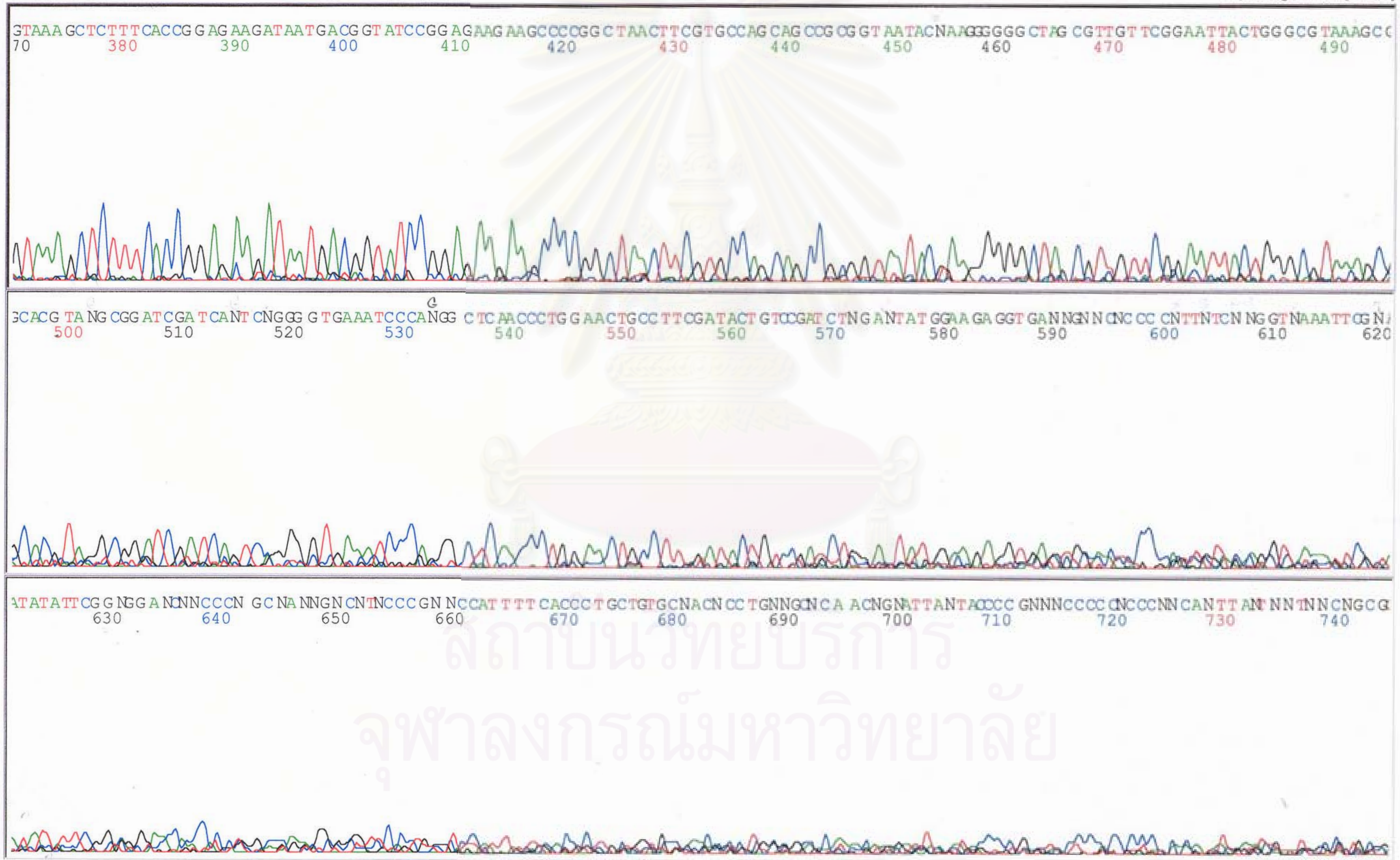

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Cap 5

SQ.....25636.....

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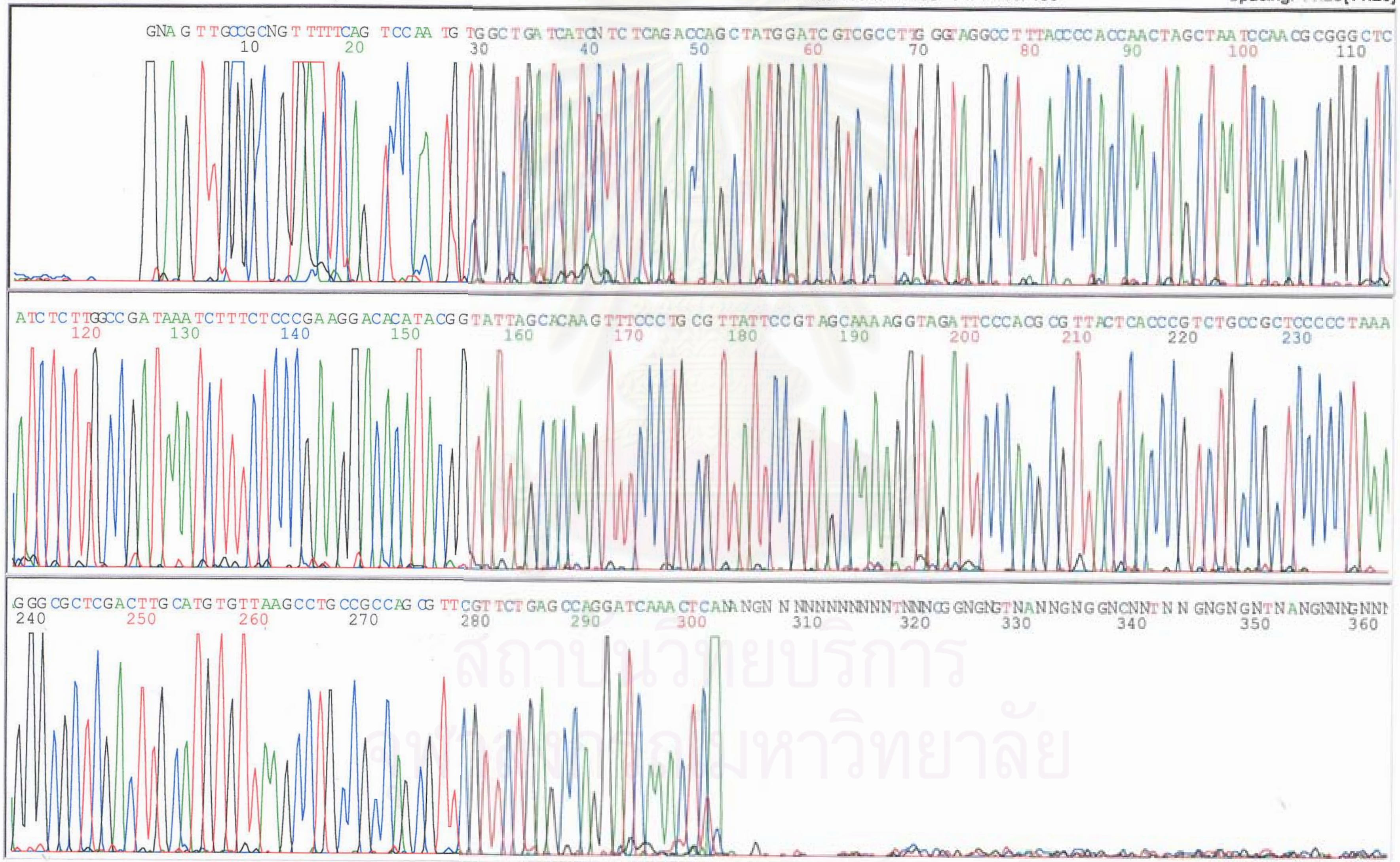

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Cap 9

SQ....26178........

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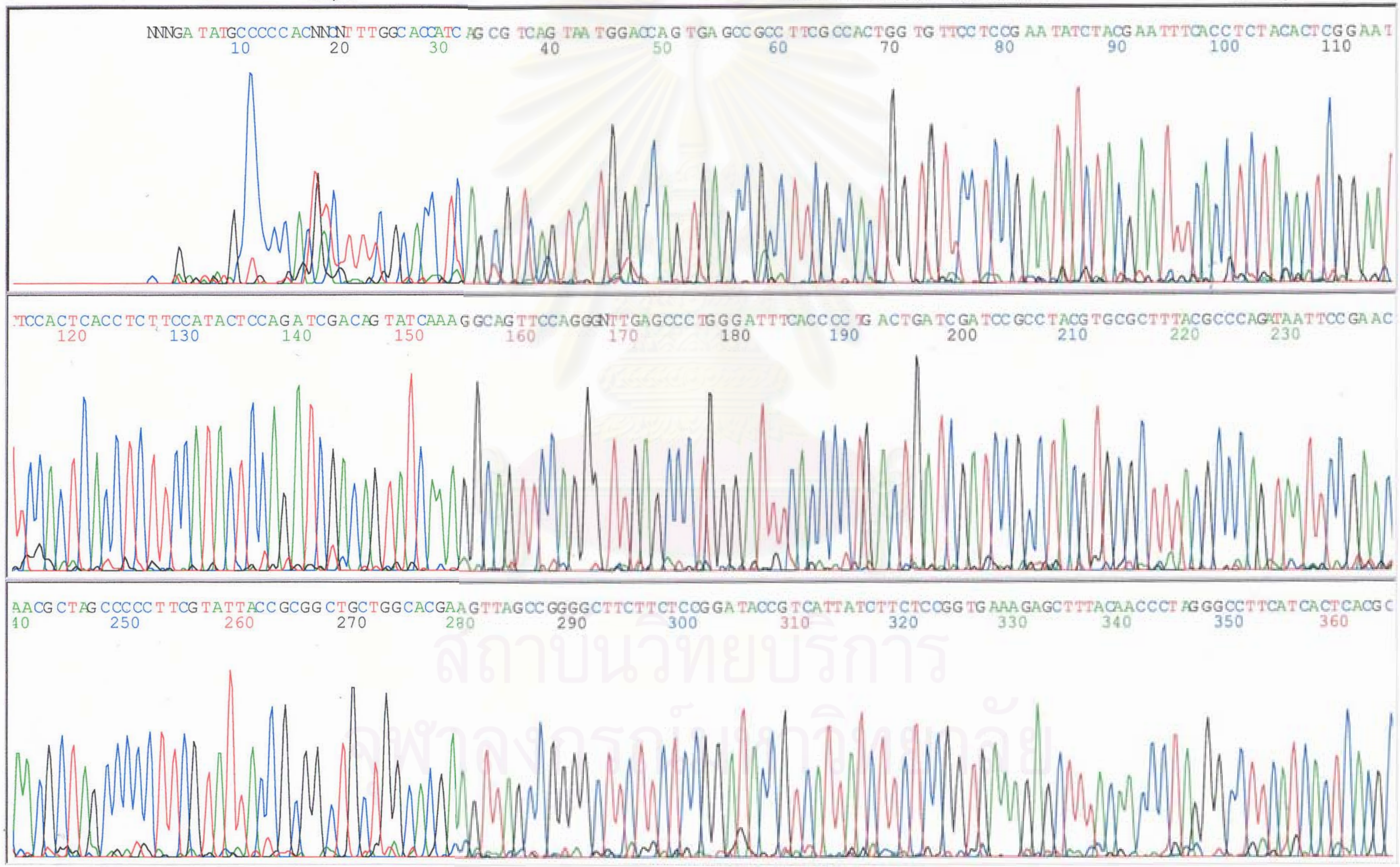

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SQ 26178

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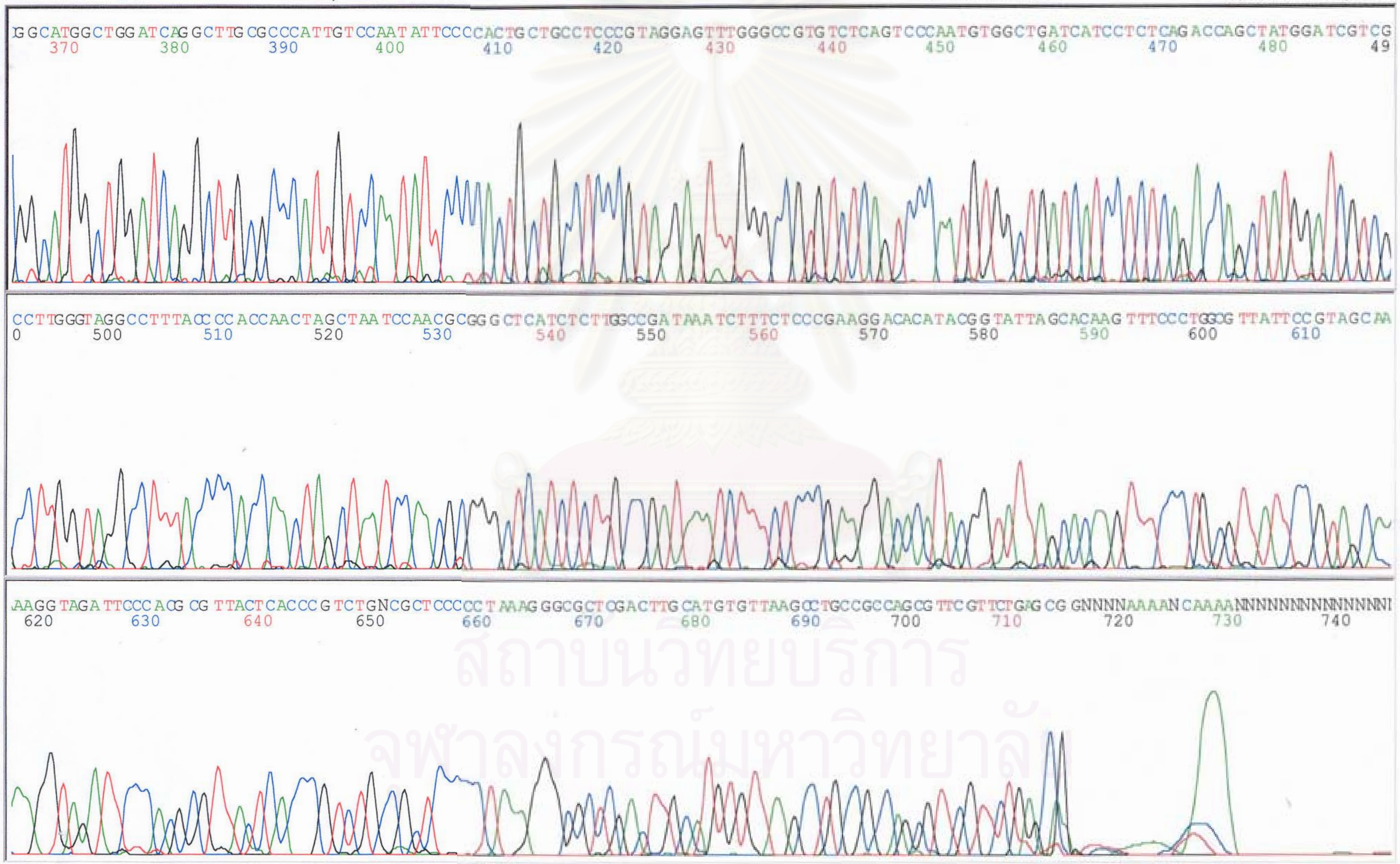

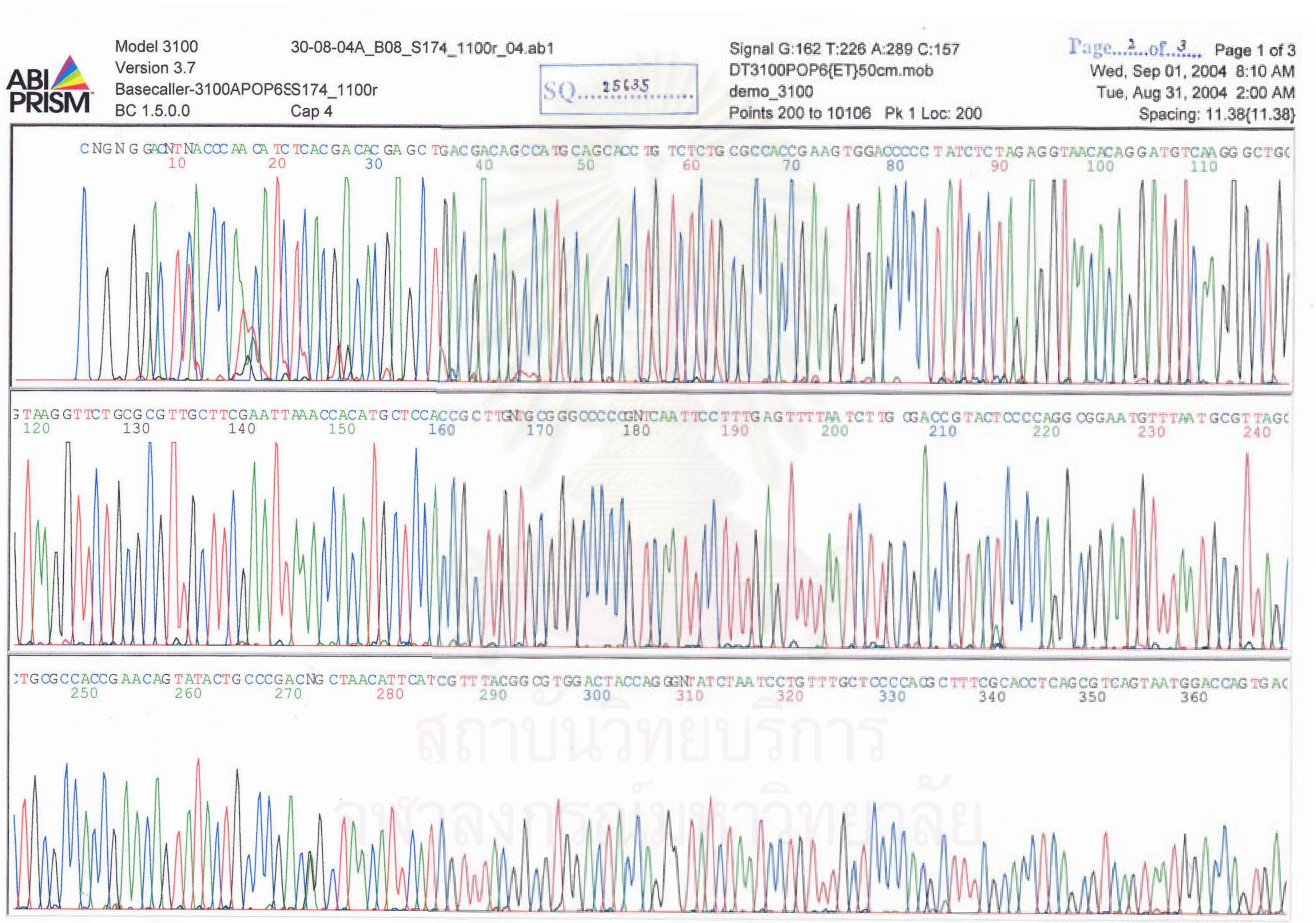
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demo_3100
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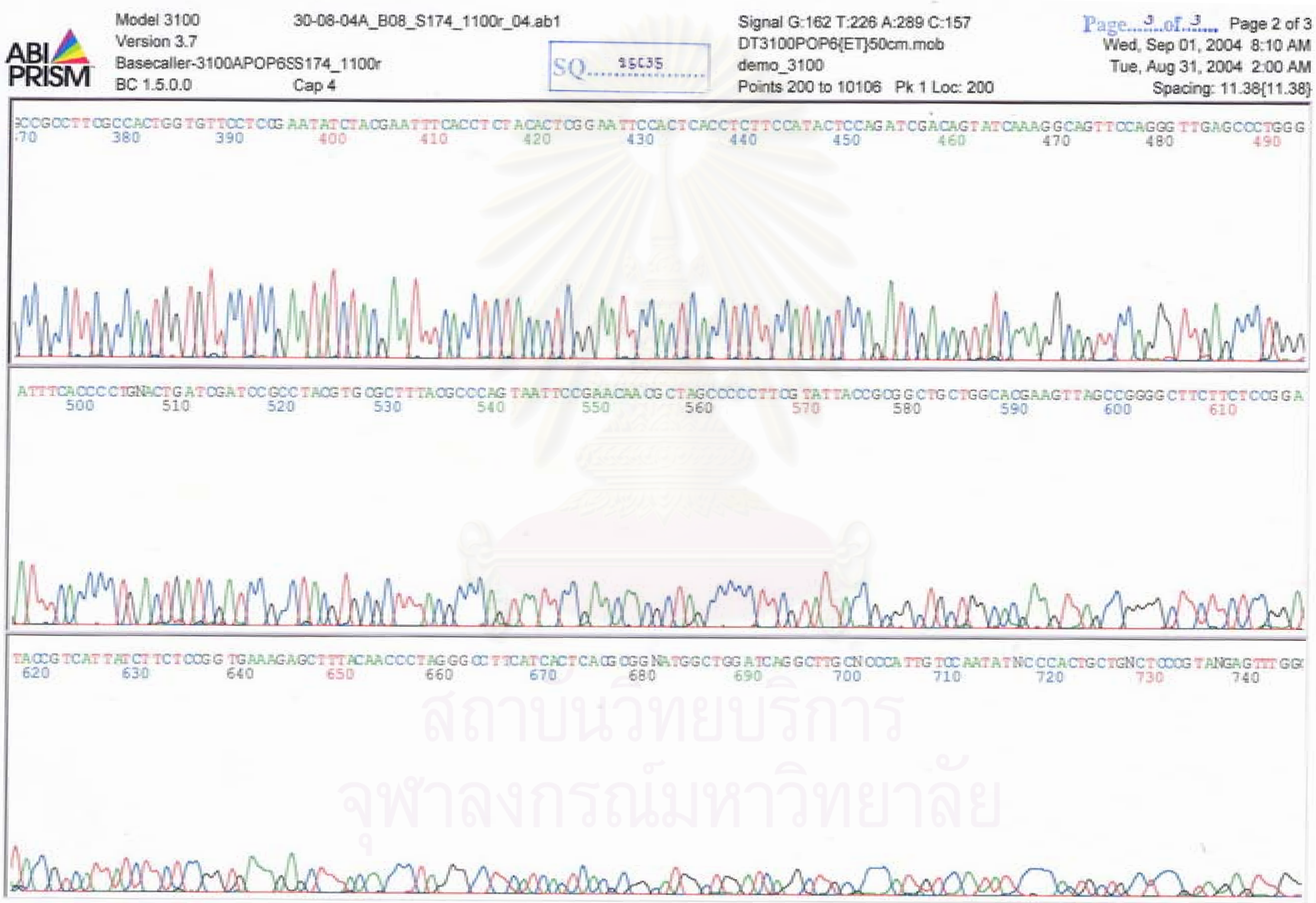
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Cap 4
SQ..... 35C35
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DT3100POP6{ET}50cm.mob
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ABI PRISM

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Cap 6

SQ....25637........

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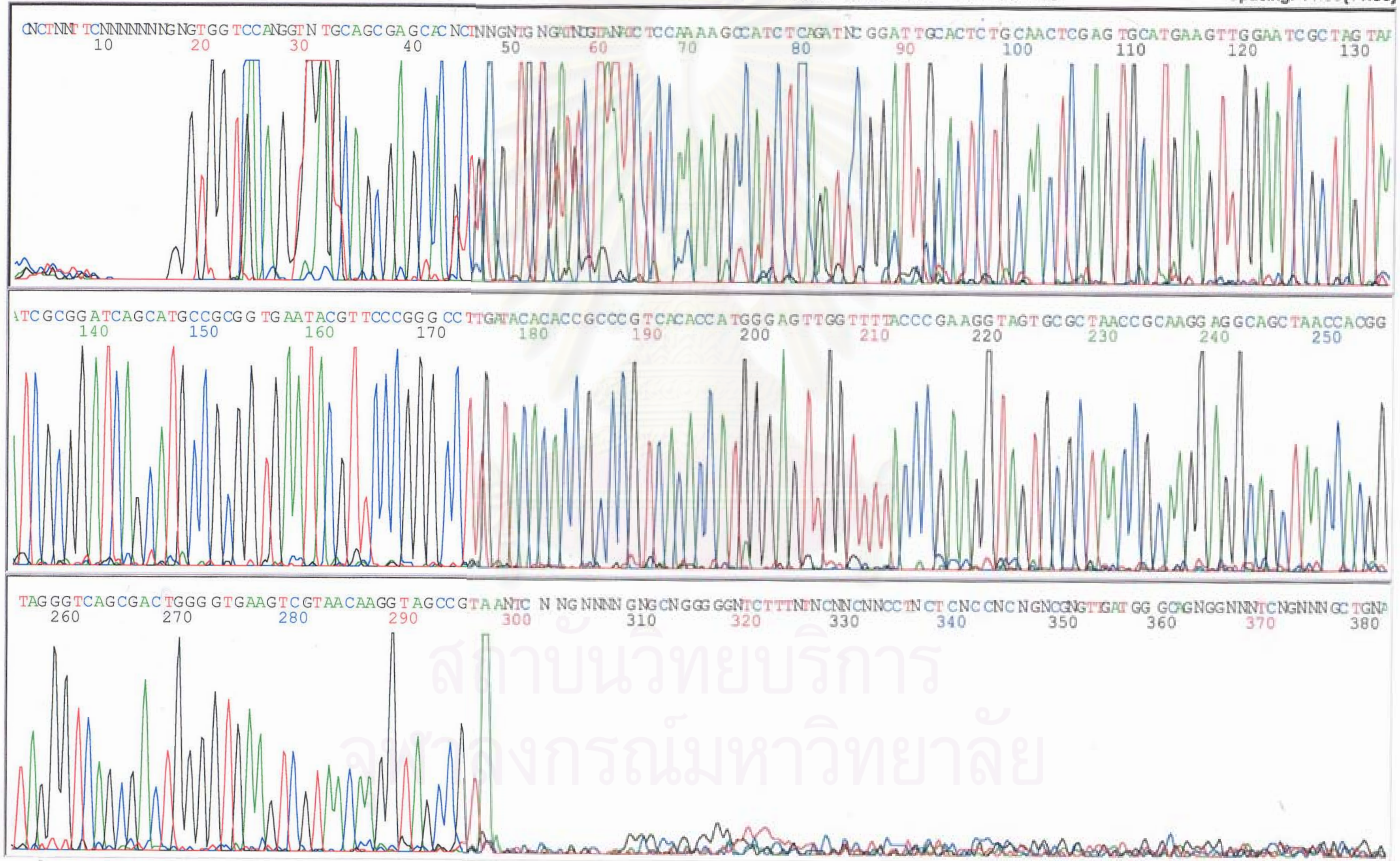

ABI PRISM

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SQ...15634...

Signal G:198 T:260 A:324 C:173
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Points 250 to 10106 Pk 1 Loc: 250

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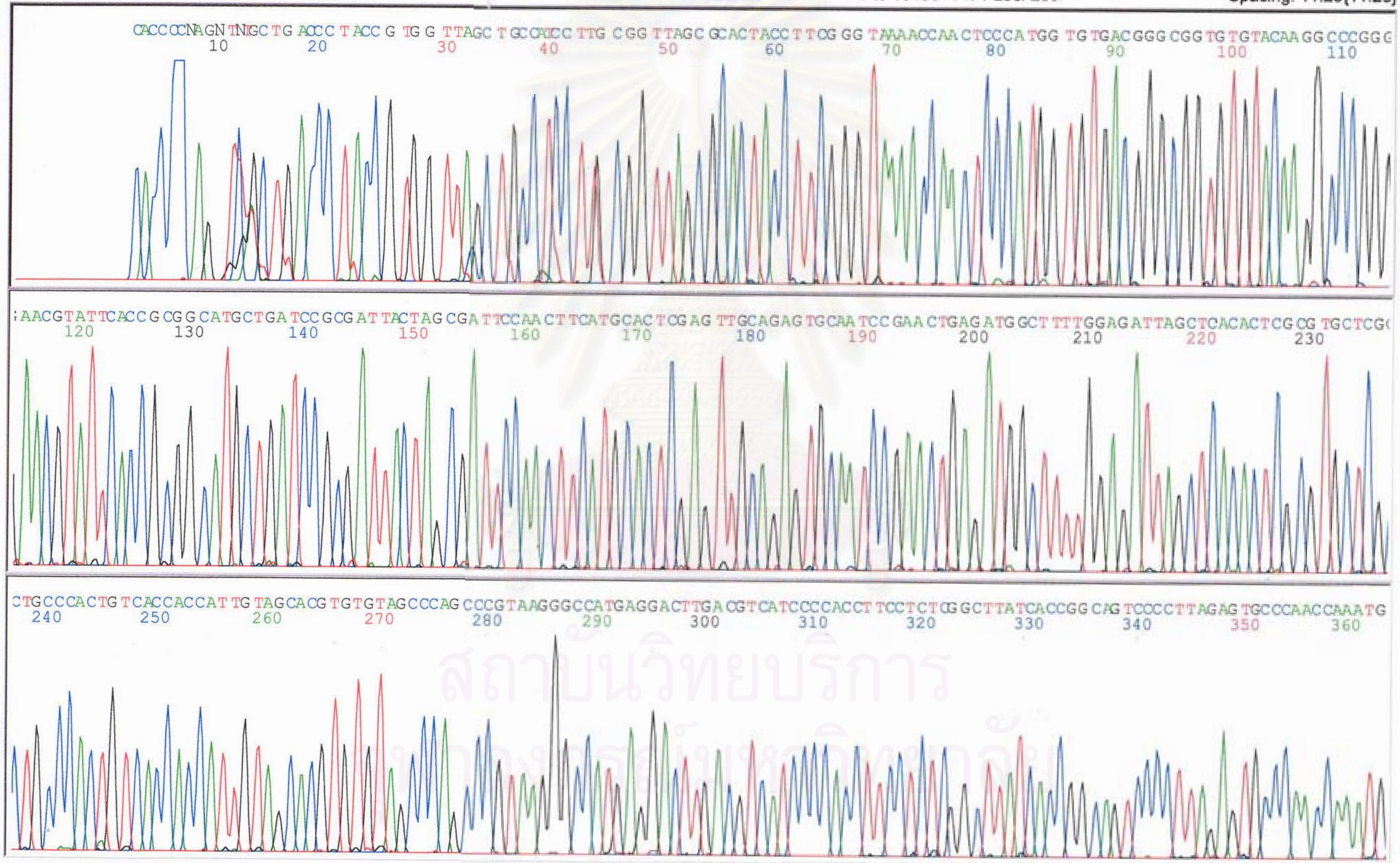

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BC 1.5.0.0	Cap 3	Points 250 to 10106 Pk 1 Loc: 250	Spacing: 11.25{11.25}

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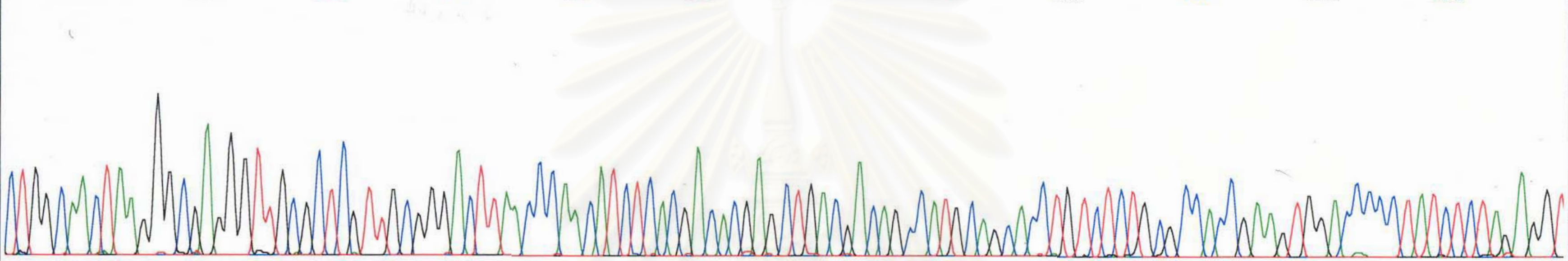
370 380 390 400 410 420 430 440 450 460 470 480



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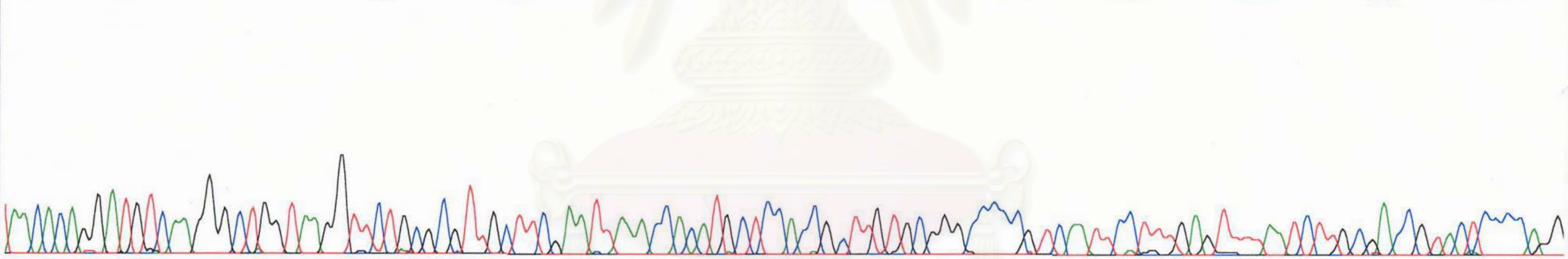
90 500 510 520 530 540 550 560 570 580 590 600 610



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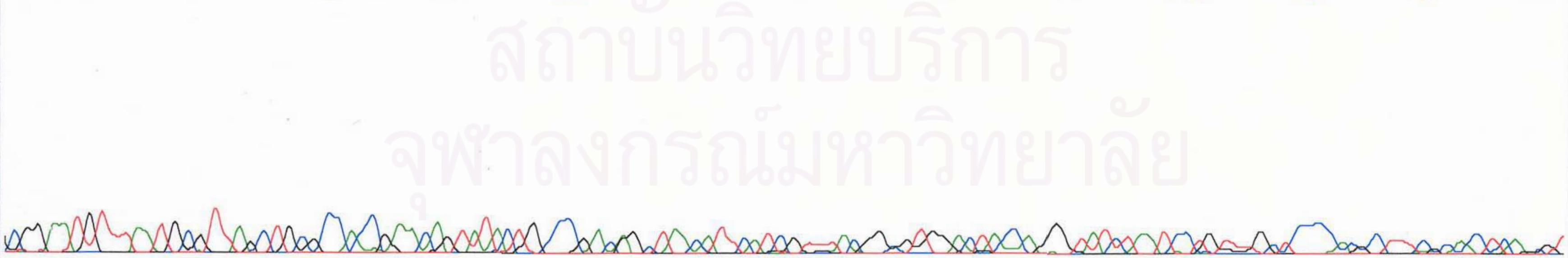

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                     80         90        100        110        120        130        140
S174COM1st  GTTAGCGCAC TACCTTCGGG TAAAACCAAC TCCCATGGTG TGACGGGCGG TGTGTACAAG GCCCGGGAAC
s174com2nd  GTTAGCGCAC TACCTTCGGG TAAAACCAAC TCCCATGGTG TGACGGGCGG TGTGTACAAG GCCCGGGAAC

                    150        160        170        180        190        200        210
S174COM1st  GTATTCACCG CGGCATGCTG ATCCGCGATT ACTAGCGATT CCAACTTCAT GCACTCGAGT T------GCA
s174com2nd  GTATTCACCG CGGCATGCTG ATCCGCGATT ACTAGCGATT CCAACTTCAT GCACTCGAGT TGCAGCCGCA

                    220        230        240        250        260        270        280
S174COM1st  GACTGCAATC CGAACTGAGA TGGCTTTTGG AGATTAGCTC ACACTCGCGT GCTCGCTGCC CACTGTCACC
s174com2nd  GAGTGCAATC CGAACTGAGA TGGCTTTTGG AGATTAGCTC ACACTCGCGT GCTCGCTGCC CACTGTCACC

                    290        300        310        320        330        340        350
S174COM1st  ACCATTGTAG CACGTGTGTA GCCCAGCCCG TAAGGGCCAT GAGGACTTGA CGTCATCCCC ACCTTCCTCT
s174com2nd  ACCATTGTAG CACGTGTGTA GCCCAGCCCG TAAGGGCCAT GAGGACTTGA CGTCATCCCC ACCTTCCTCT

                    360        370        380        390        400        410        420
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s174com2nd  CGGCTTATCA CCGGCAGTCC CCTTAGAGTG CCCAACCAAA TGCTGGCAAC TAAGGGCGAG GGTTGCGCTC

                    430        440        450        460        470        480        490
S174COM1st  GTTGCCGGGT TTAACCCAAC A----T--CT C-ACGACACG AGCTGACGAC AGCCATGCAG CACCTGTCTC
s174com2nd  GTTG-CGGGA CTCACCCAAC ATCTCTACCT CAACGACACG AGCTGACGAC AGCCATGCAG CACCTGTCTC

                    500        510        520        530        540        550        560
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s174com2nd  TGCGCCACCG AAGTGGACCC CCTATCTCTA GAGGTAACAC AGGATGTCAA GGGCTGGTAA GGTTCTGCGC

                    570        580        590        600        610        620        630
S174COM1st  GTTGCTTCGA ATTAAACCAC ATGCTCCACC GCTTGTGCGG GCCCCCGTCA ATTCCTTTGA GTTTTAATCT
s174com2nd  GTTGCTTCGA ATTAAACCAC ATGCTCCACC GCTTGTGCGG GCCCCCGTCA ATTCCTTTGA GTTTTAATCT

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s174com2nd  TGCGACCGTA CTCCCCAGGC GGAATGTTTA ATGCGTTAGC TGCGCCACCG AACAGTATAC TGCCCGAC-G

                    710        720        730        740        750        760        770
S174COM1st  CTAACATTCA TCGTTTACGG CGTGGACTAC CAGGGTATCT AATCCTGTTT GCTCCCCACG CTTTCGCACC
s174com2nd  CTAACATTCA TCGTTTACGG CGTGGACTAC CAGGGTATCT AATCCTGTTT GCTCCCCACG CTTTCGCACC

                    780        790        800        810        820        830        840
S174COM1st  TCAGCGTCAG TAATGGACCA GTGAGCCGCC TTCGCCACTG GTGTTCCTCC GAATATCTAC GAATTTCACT
s174com2nd  TCAGCGTCAG TAATGGACCA GTGAGCCGCC TTCGCCACTG GTGTTCCTCC GAATATCTAC GAATTTCACC
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s174com2nd  TCTACACTCG GAATTCCACT CACCTCTTCC ATACTCCAGA TCGACAGTAT CAAAGGCAGT TCCAGGGTTG

                    920       930       940       950       960       970       980
S174COM1st  AGCCTTGGGA TTTCACCCCT GACTGATCGA TCCGCCTACG TGGCGCTTTA CGCCCAGTAA TTCCGAACAA
s174com2nd  AGCCCTGGGA TTTCACCCCT GACTGATCGA TCCGCCTACG T-GCGCTTTA CGCCCAGTAA TTCCGAACAA

                    990      1000      1010      1020      1030      1040      1050
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s174com2nd  CGCTAGCCCC CTTCGTATTA CCGCGGCTGC TGGCACGAAG TTAGCCGGGG CTTCTTCTCC GGATACCGTC

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s174com2nd  ATTATCTTCT CCGGTGAAAG AGCTTTACAA CCCTAGGGCC TTCATCACTC ACGCGG-ATG GCTGGATCAG

                   1130      1140      1150      1160      1170      1180      1190
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s174com2nd  GCTTGC-CCC ATTGTCCAAT A-T-CCCACT GCTG-CTCCC GTA-GAGTTT GGGCCGTGTC TCAGTCCCAA

                   1200      1210      1220      1230      1240      1250      1260
S174COM1st  TGTGGCTGAT CATCCTCTCA GACCAGCTAT GGATCGTCGC CTTGGTAGGC CTTTACCCCA CCAACTAGCT
s174com2nd  TGTGGCTGAT CATCCTCTCA GACCAGCTAT GGATCGTCGC CTTGGTAGGC CTTTACCCCA CCAACTAGCT

                   1270      1280      1290      1300      1310      1320      1330
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                   1340      1350      1360      1370      1380      1390      1400
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                   1410      1420      1430      1440      1450      1460
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s174com2nd  GGGCGCATCG ACTGGCATGT GTTAAGCCTG CCGAACAGCG GTAGTTACGA GCCAGGATCA AACTCA
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ABI PRISM

Model 3100
Version 3.7
Basecaller-3100/
BC 1.5.0.0

29-3-05B_A10_ST60_pBADout2_02.ab1
P6SST60_pBADout2
Cap 2

Signal G:79 T:131 A:245 C:81
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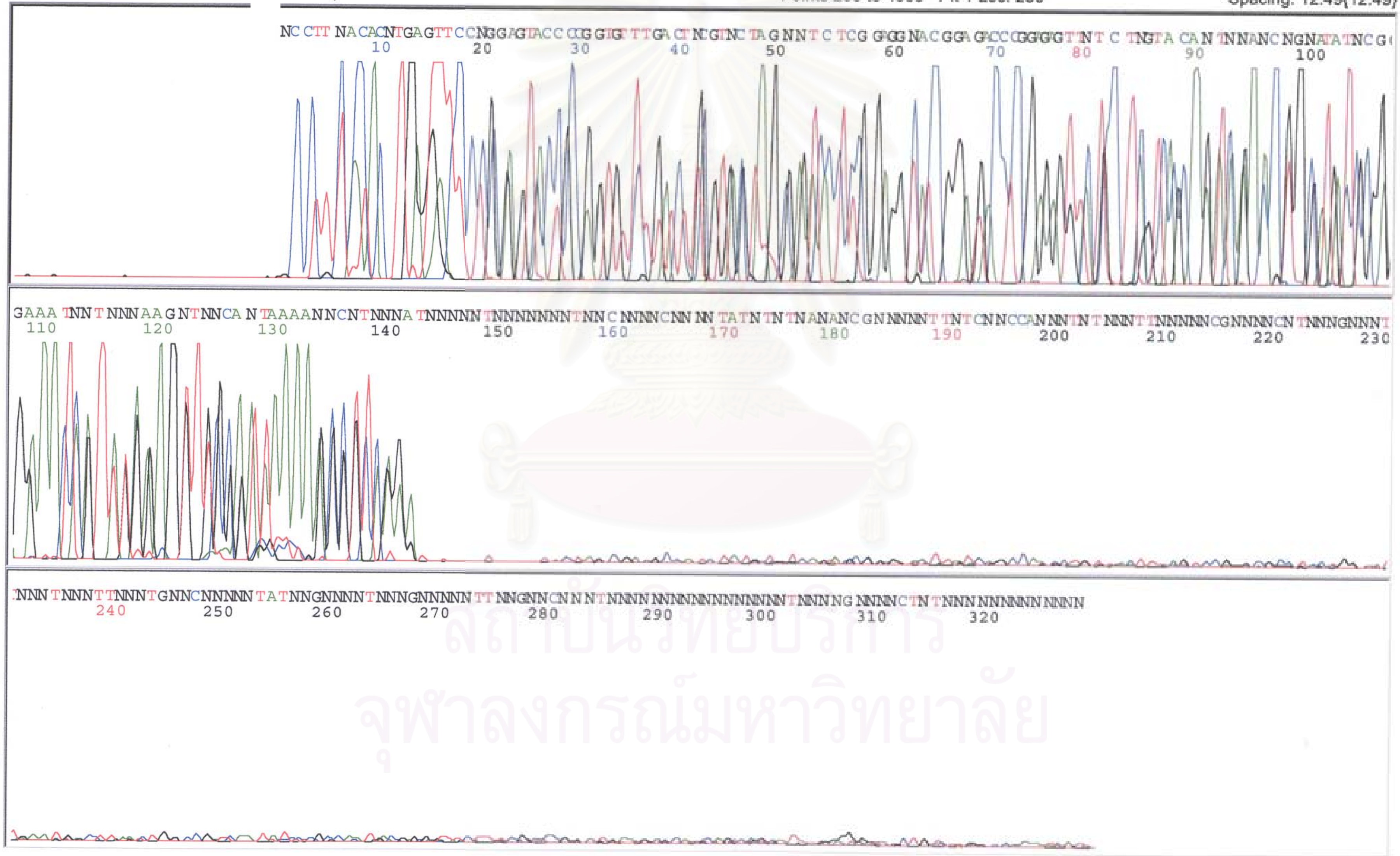

APPENDIX D

DUNCAN'S MULTIPLE RANGE TEST

Duncan's Multiple Range Test has been used to determine multiple groupings. Means which do not differ significantly are grouped into one homogenous range (Steel & Torrie, 1980). The Duncan's Multiple Range Test (SPSS Manual, Chapter 8) is then used to carry out the multiple range groupings. The results are indicated in the following tables.

Table A.1 Duncan's Multiple Range Test for average plant dry weight for *S. fredii* S174 and TnAraOut mutants (ST49 and ST60) and *Glycine max* cv SJ 4, SJ 5, ST 2 in Leonard jars with nitrogen-free medium pH 6.8 (Level of probability, α = 0.05)

Oneway

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9.894	14	.703	29.948	.000
Within Groups	.352	15	2.349E-02		
Total	10.201	29			

Duncan[a]

	N	Subset for alpha = .05				
		1	2	3	4	5
NC,ST2	2	.4450				
ST49,ST2	2	.6950	.6950			
S174,ST2	2	.6950	.6950			
NC,SJ4	2	.7000	.7000			
PC,ST2	2	.7350	.7350			
NC,SJ5	2	.7700	.7700			
ST60,ST2	2		.8900			
S174,SJ5	2			1.2300		
ST49,SJ4	2			1.2900		
ST60,SJ4	2			1.4150		
ST49,SJ5	2			1.4750		
S174,SJ4	2			1.5500		
ST60,SJ5	2			1.5600		
PC,SJ5	2				2.1450	
PC,SJ4	2					2.5500
Sig.		.0760	.2720	.0720	1.0000	1.0000

Means for groups in homogeneous subsets are displayed.

Table A.2 Duncan's Multiple Range Test for average nodule dry weight for *S. fredii* S174 and TnAraOut mutants (ST49 and ST60) and *Glycine max* cv SJ 4, SJ 5, ST 2 in Leonard jars with nitrogen-free medium pH 6.8 (Level of probability, α = 0.05)

Oneway

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.974	8	0.247	40.386	.000
Within Groups	5.500-02	9	6.111E-03		
Total	2.029	17			

Duncan[a]

	N	Subset for alpha = .05	
		1	2
ST49,SJ4	2	.0000	
ST49,SJ5	2	.0000	
S174,SJ5	2	.0000	
ST49,ST2	2	.0000	
S174,ST2	2	.0000	
ST60,ST2	2		.6000
ST60,SJ4	2		.6500
ST60,SJ5	2		.6500
S174,SJ4	2		.7500
Sig		1.0000	.106

Means for groups in homogeneous subsets are displayed.

Table A.6 Values of F

(Steel & Torrie, 1980)

Denominator df	Probability of a larger F	Numerator df: 1	2	3	4	5	6	7	8	9
1	.100	39.86	49.50	53.59	55.83	57.24	58.20	58.91	59.44	59.86
	.050	161.4	199.5	215.7	224.6	230.2	234.0	236.8	238.9	240.5
	.025	647.8	799.5	864.2	899.6	921.8	937.1	948.2	956.7	963.3
	.010	4052	4999.5	5403	5625	5764	5859	5928	5982	6022
	.005	16211	20000	21615	22500	23056	23437	23715	23925	24091
2	.100	8.53	9.00	9.16	9.24	9.29	9.33	9.35	9.37	9.38
	.050	18.51	19.00	19.16	19.25	19.30	19.33	19.35	19.37	19.38
	.025	38.51	39.00	39.17	39.25	39.30	39.33	39.36	39.37	39.39
	.010	98.50	99.00	99.17	99.25	99.30	99.33	99.36	99.37	99.39
	.005	198.5	199.0	199.2	199.2	199.3	199.3	199.4	199.4	199.4
3	.100	5.54	5.46	5.39	5.34	5.31	5.28	5.27	5.25	5.24
	.050	10.13	9.55	9.28	9.12	9.01	8.94	8.89	8.85	8.81
	.025	17.44	16.04	15.44	15.10	14.88	14.73	14.62	14.54	14.47
	.010	34.12	30.82	29.46	28.71	28.24	27.91	27.67	27.49	27.35
	.005	55.55	49.80	47.47	46.19	45.39	44.84	44.43	44.13	43.88
4	.100	4.54	4.32	4.19	4.11	4.05	4.01	3.98	3.95	3.94
	.050	7.71	6.94	6.59	6.39	6.26	6.16	6.09	6.04	6.00
	.025	12.22	10.65	9.98	9.60	9.36	9.20	9.07	8.98	8.90
	.010	21.20	18.00	16.69	15.98	15.52	15.21	14.98	14.80	14.66
	.005	31.33	26.28	24.26	23.15	22.46	21.97	21.62	21.35	21.14
5	.100	4.06	3.78	3.62	3.52	3.45	3.40	3.37	3.34	3.32
	.050	6.61	5.79	5.41	5.19	5.05	4.95	4.88	4.82	4.77
	.025	10.01	8.43	7.76	7.39	7.15	6.98	6.85	6.76	6.68
	.010	16.26	13.27	12.06	11.39	10.97	10.67	10.46	10.29	10.16
	.005	22.78	18.31	16.53	15.56	14.94	14.51	14.20	13.96	13.77
6	.100	3.78	3.46	3.29	3.18	3.11	3.05	3.01	2.98	2.96
	.050	5.99	5.14	4.76	4.53	4.39	4.28	4.21	4.15	4.10
	.025	8.81	7.26	6.60	6.23	5.99	5.82	5.70	5.60	5.52
	.010	13.75	10.92	9.78	9.15	8.75	8.47	8.26	8.10	7.98
	.005	18.63	14.54	12.92	12.03	11.46	11.07	10.79	10.57	10.39
7	.100	3.59	3.26	3.07	2.96	2.88	2.83	2.78	2.75	2.72
	.050	5.59	4.74	4.35	4.12	3.97	3.87	3.79	3.73	3.68
	.025	8.07	6.54	5.89	5.52	5.29	5.12	4.99	4.90	4.82
	.010	12.25	9.55	8.45	7.85	7.46	7.19	6.99	6.84	6.72
	.005	16.24	12.40	10.88	10.05	9.52	9.16	8.89	8.68	8.51
8	.100	3.46	3.11	2.92	2.81	2.73	2.67	2.62	2.59	2.56
	.050	5.32	4.46	4.07	3.84	3.69	3.58	3.50	3.44	3.39
	.025	7.57	6.06	5.42	5.05	4.82	4.65	4.53	4.43	4.36
	.010	11.26	8.65	7.59	7.01	6.63	6.37	6.18	6.03	5.91
	.005	14.69	11.04	9.60	8.81	8.30	7.95	7.69	7.50	7.34
9	.100	3.36	3.01	2.81	2.69	2.61	2.55	2.51	2.47	2.44
	.050	5.12	4.26	3.86	3.63	3.48	3.37	3.29	3.23	3.18
	.025	7.21	5.71	5.08	4.72	4.48	4.32	4.20	4.10	4.03
	.010	10.56	8.02	6.99	6.42	6.06	5.80	5.61	5.47	5.35
	.005	13.61	10.11	8.72	7.96	7.47	7.13	6.88	6.69	6.54
10	.100	3.29	2.92	2.73	2.61	2.52	2.46	2.41	2.38	2.35
	.050	4.96	4.10	3.71	3.48	3.33	3.22	3.14	3.07	3.02
	.025	6.94	5.46	4.83	4.47	4.24	4.07	3.95	3.85	3.78
	.010	10.04	7.56	6.55	5.99	5.64	5.39	5.20	5.06	4.94
	.005	12.83	9.43	8.08	7.34	6.87	6.54	6.30	6.12	5.97
11	.100	3.23	2.86	2.66	2.54	2.45	2.39	2.34	2.30	2.27
	.050	4.84	3.98	3.59	3.36	3.20	3.09	3.01	2.95	2.90
	.025	6.72	5.26	4.63	4.28	4.04	3.88	3.76	3.66	3.59
	.010	9.65	7.21	6.22	5.67	5.32	5.07	4.89	4.74	4.63
	.005	12.23	8.91	7.60	6.88	6.42	6.10	5.86	5.68	5.54
12	.100	3.18	2.81	2.61	2.48	2.39	2.33	2.28	2.24	2.21
	.050	4.75	3.89	3.49	3.26	3.11	3.00	2.91	2.85	2.80
	.025	6.55	5.10	4.47	4.12	3.89	3.73	3.61	3.51	3.44
	.010	9.33	6.93	5.95	5.41	5.06	4.82	4.64	4.50	4.39
	.005	11.75	8.51	7.23	6.52	6.07	5.76	5.52	5.35	5.20
13	.100	3.14	2.76	2.56	2.43	2.35	2.28	2.23	2.20	2.16
	.050	4.67	3.81	3.41	3.18	3.03	2.92	2.83	2.77	2.71
	.025	6.41	4.97	4.35	4.00	3.77	3.60	3.48	3.39	3.31
	.010	9.07	6.70	5.74	5.21	4.86	4.62	4.44	4.30	4.19
	.005	11.37	8.19	6.93	6.23	5.79	5.48	5.25	5.08	4.94
14	.100	3.10	2.73	2.52	2.39	2.31	2.24	2.19	2.15	2.12
	.050	4.60	3.74	3.34	3.11	2.96	2.85	2.76	2.70	2.65
	.025	6.30	4.86	4.24	3.89	3.66	3.50	3.38	3.29	3.21
	.010	8.86	6.51	5.56	5.04	4.69	4.46	4.28	4.14	4.03
	.005	11.06	7.92	6.68	6.00	5.56	5.26	5.03	4.86	4.72

Table A.6 Values of F (*Continued*)

Numerator df: 10	12	15	20	24	30	40	60	120	∞	P	df
60.19	60.71	61.22	61.74	62.00	62.26	62.53	62.79	63.06	63.33	.100	1
241.9	243.9	245.9	248.0	249.1	250.1	251.1	252.2	253.3	254.3	.050	
968.6	976.7	984.9	993.1	997.2	1001	1006	1010	1014	1018	.025	
6056	6106	6157	6209	6235	6261	6287	6313	6339	6366	.010	
24224	24426	24630	24836	24940	25044	25148	25253	25359	25465	.005	
9.39	9.41	9.42	9.44	9.45	9.46	9.47	9.47	9.48	9.49	.100	2
19.40	19.41	19.43	19.45	19.45	19.46	19.47	19.48	19.49	19.50	.050	
39.40	39.41	39.43	39.45	39.46	39.46	39.47	39.48	39.49	39.50	.025	
99.40	99.42	99.43	99.45	99.46	99.47	99.47	99.48	99.49	99.50	.010	
199.4	199.4	199.4	199.4	199.5	199.5	199.5	199.5	199.5	199.5	.005	
5.23	5.22	5.20	5.18	5.18	5.17	5.16	5.15	5.14	5.13	.100	3
8.79	8.74	8.70	8.66	8.64	8.62	8.59	8.57	8.55	8.53	.050	
14.42	14.34	14.25	14.17	14.12	14.08	14.04	13.99	13.95	13.90	.025	
27.23	27.05	26.87	26.69	26.60	26.50	26.41	26.32	26.22	26.13	.010	
43.69	43.39	43.08	42.78	42.62	42.47	42.31	42.15	41.99	41.83	.005	
3.92	3.90	3.87	3.84	3.83	3.82	3.80	3.79	3.78	3.76	.100	4
5.96	5.91	5.86	5.80	5.77	5.75	5.72	5.69	5.66	5.63	.050	
8.84	8.75	8.66	8.56	8.51	8.46	8.41	8.36	8.31	8.26	.025	
14.55	14.37	14.20	14.02	13.93	13.84	13.75	13.65	13.56	13.46	.010	
20.97	20.70	20.44	20.17	20.03	19.89	19.75	19.61	19.47	19.32	.005	
3.30	3.27	3.24	3.21	3.19	3.17	3.16	3.14	3.12	3.10	.100	5
4.74	4.68	4.62	4.56	4.53	4.50	4.46	4.43	4.40	4.36	.050	
6.62	6.52	6.43	6.33	6.28	6.23	6.18	6.12	6.07	6.02	.025	
10.05	9.89	9.72	9.55	9.47	9.38	9.29	9.20	9.11	9.02	.010	
13.62	13.38	13.15	12.90	12.78	12.66	12.53	12.40	12.27	12.14	.005	
2.94	2.90	2.87	2.84	2.82	2.80	2.78	2.76	2.74	2.72	.100	6
4.06	4.00	3.94	3.87	3.84	3.81	3.77	3.74	3.70	3.67	.050	
5.46	5.37	5.27	5.17	5.12	5.07	5.01	4.96	4.90	4.85	.025	
7.87	7.72	7.56	7.40	7.31	7.23	7.14	7.06	6.97	6.88	.010	
10.25	10.03	9.81	9.59	9.47	9.36	9.24	9.12	9.00	8.88	.005	
2.70	2.67	2.63	2.59	2.58	2.56	2.54	2.51	2.49	2.47	.100	7
3.64	3.57	3.51	3.44	3.41	3.38	3.34	3.30	3.27	3.23	.050	
4.76	4.67	4.57	4.47	4.42	4.36	4.31	4.25	4.20	4.14	.025	
6.62	6.47	6.31	6.16	6.07	5.99	5.91	5.82	5.74	5.65	.010	
8.38	8.18	7.97	7.75	7.65	7.53	7.42	7.31	7.19	7.08	.005	
2.54	2.50	2.46	2.42	2.40	2.38	2.36	2.34	2.32	2.29	.100	8
3.35	3.28	3.22	3.15	3.12	3.08	3.04	3.01	2.97	2.93	.050	
4.30	4.20	4.10	4.00	3.95	3.89	3.84	3.78	3.73	3.67	.025	
5.81	5.67	5.52	5.36	5.28	5.20	5.12	5.03	4.95	4.86	.010	
7.21	7.01	6.81	6.61	6.50	6.40	6.29	6.18	6.06	5.95	.005	
2.42	2.38	2.34	2.30	2.28	2.25	2.23	2.21	2.18	2.16	.100	9
3.14	3.07	3.01	2.94	2.90	2.86	2.83	2.79	2.75	2.71	.050	
3.96	3.87	3.77	3.67	3.61	3.56	3.51	3.45	3.39	3.33	.025	
5.26	5.11	4.96	4.81	4.73	4.65	4.57	4.48	4.40	4.31	.010	
6.42	6.23	6.03	5.83	5.73	5.62	5.52	5.41	5.30	5.19	.005	
2.32	2.28	2.24	2.20	2.18	2.16	2.13	2.11	2.08	2.06	.100	10
2.98	2.91	2.85	2.77	2.74	2.70	2.66	2.62	2.58	2.54	.050	
3.72	3.62	3.52	3.42	3.37	3.31	3.26	3.20	3.14	3.08	.025	
4.85	4.71	4.56	4.41	4.33	4.25	4.17	4.08	4.00	3.91	.010	
5.85	5.66	5.47	5.27	5.17	5.07	4.97	4.86	4.75	4.64	.005	
2.25	2.21	2.17	2.12	2.10	2.08	2.05	2.03	2.00	1.97	.100	11
2.85	2.79	2.72	2.65	2.61	2.57	2.53	2.49	2.45	2.40	.050	
3.53	3.43	3.33	3.23	3.17	3.12	3.06	3.00	2.94	2.88	.025	
4.54	4.40	4.25	4.10	4.02	3.94	3.86	3.78	3.69	3.60	.010	
5.42	5.24	5.05	4.86	4.76	4.65	4.55	4.44	4.34	4.23	.005	
2.19	2.15	2.10	2.06	2.04	2.01	1.99	1.96	1.93	1.90	.100	12
2.75	2.69	2.62	2.54	2.51	2.47	2.43	2.38	2.34	2.30	.050	
3.37	3.28	3.18	3.07	3.02	2.96	2.91	2.85	2.79	2.72	.025	
4.30	4.16	4.01	3.86	3.78	3.70	3.62	3.54	3.45	3.36	.010	
5.09	4.91	4.72	4.53	4.43	4.33	4.23	4.12	4.01	3.90	.005	
2.14	2.10	2.05	2.01	1.98	1.96	1.93	1.90	1.88	1.85	.100	13
2.67	2.60	2.53	2.46	2.42	2.38	2.34	2.30	2.25	2.21	.050	
3.25	3.15	3.05	2.95	2.89	2.84	2.78	2.72	2.66	2.60	.025	
4.10	3.96	3.82	3.66	3.59	3.51	3.43	3.34	3.25	3.17	.010	
4.82	4.64	4.46	4.27	4.17	4.07	3.97	3.87	3.76	3.65	.005	
2.10	2.05	2.01	1.96	1.94	1.91	1.89	1.86	1.83	1.80	.100	14
2.60	2.53	2.46	2.39	2.35	2.31	2.27	2.22	2.18	2.13	.050	
3.15	3.05	2.95	2.84	2.79	2.73	2.67	2.61	2.55	2.49	.025	
3.94	3.80	3.66	3.51	3.43	3.35	3.27	3.18	3.09	3.00	.010	
4.60	4.43	4.25	4.06	3.96	3.86	3.76	3.66	3.55	3.44	.005	

Table A.6 Values of *F* (*Continued*)

Denominator *df*	Probability of a larger *F*	1	2	3	4	5	6	7	8	9
		Numerator *df*								
15	.100	3.07	2.70	2.49	2.36	2.27	2.21	2.16	2.12	2.09
	.050	4.54	3.68	3.29	3.06	2.90	2.79	2.71	2.64	2.59
	.025	6.20	4.77	4.15	3.80	3.58	3.41	3.29	3.20	3.12
	.010	8.68	6.36	5.42	4.89	4.56	4.32	4.14	4.00	3.89
	.005	10.80	7.70	6.48	5.80	5.37	5.07	4.85	4.67	4.54
16	.100	3.05	2.67	2.46	2.33	2.24	2.18	2.13	2.09	2.06
	.050	4.49	3.63	3.24	3.01	2.85	2.74	2.66	2.59	2.54
	.025	6.12	4.69	4.08	3.73	3.50	3.34	3.22	3.12	3.05
	.010	8.53	6.23	5.29	4.77	4.44	4.20	4.03	3.89	3.78
	.005	10.58	7.51	6.30	5.64	5.21	4.91	4.69	4.52	4.38
17	.100	3.03	2.64	2.44	2.31	2.22	2.15	2.10	2.06	2.03
	.050	4.45	3.59	3.20	2.96	2.81	2.70	2.61	2.55	2.49
	.025	6.04	4.62	4.01	3.66	3.44	3.28	3.16	3.06	2.98
	.010	8.40	6.11	5.18	4.67	4.34	4.10	3.93	3.79	3.68
	.005	10.38	7.35	6.16	5.50	5.07	4.78	4.56	4.39	4.25
18	.100	3.01	2.62	2.42	2.29	2.20	2.13	2.08	2.04	2.00
	.050	4.41	3.55	3.16	2.93	2.77	2.66	2.58	2.51	2.46
	.025	5.98	4.56	3.95	3.61	3.38	3.22	3.10	3.01	2.93
	.010	8.29	6.01	5.09	4.58	4.25	4.01	3.84	3.71	3.60
	.005	10.22	7.21	6.03	5.37	4.96	4.66	4.44	4.20	4.14
19	.100	2.99	2.61	2.40	2.27	2.18	2.11	2.06	2.02	1.98
	.050	4.38	3.52	3.13	2.90	2.74	2.63	2.54	2.48	2.42
	.025	5.92	4.51	3.90	3.56	3.33	3.17	3.05	2.96	2.88
	.010	8.18	5.93	5.01	4.50	4.17	3.94	3.77	3.63	3.52
	.005	10.07	7.09	5.92	5.27	4.85	4.56	4.34	4.18	4.04
20	.100	2.97	2.59	2.38	2.25	2.16	2.09	2.04	2.00	1.96
	.050	4.35	3.49	3.10	2.87	2.71	2.60	2.51	2.45	2.39
	.025	5.87	4.46	3.86	3.51	3.29	3.13	3.01	2.91	2.84
	.010	8.10	5.85	4.94	4.43	4.10	3.87	3.70	3.56	3.46
	.005	9.94	6.99	5.82	5.17	4.76	4.47	4.26	4.09	3.96
21	.100	2.96	2.57	2.36	2.23	2.14	2.08	2.02	1.98	1.95
	.050	4.32	3.47	3.07	2.84	2.68	2.57	2.49	2.42	2.37
	.025	5.83	4.42	3.82	3.48	3.25	3.09	2.97	2.87	2.80
	.010	8.02	5.78	4.87	4.37	4.04	3.81	3.64	3.51	3.40
	.005	9.83	6.89	5.73	5.09	4.68	4.39	4.18	4.01	3.88
22	.100	2.95	2.56	2.35	2.22	2.13	2.06	2.01	1.97	1.93
	.050	4.30	3.44	3.05	2.82	2.66	2.55	2.46	2.40	2.34
	.025	5.79	4.38	3.78	3.44	3.22	3.05	2.93	2.84	2.76
	.010	7.95	5.72	4.82	4.31	3.99	3.76	3.59	3.45	3.35
	.005	9.73	6.81	5.65	5.02	4.61	4.32	4.11	3.94	3.81
23	.100	2.94	2.55	2.34	2.21	2.11	2.05	1.99	1.95	1.92
	.050	4.28	3.42	3.03	2.80	2.64	2.53	2.44	2.37	2.32
	.025	5.75	4.35	3.75	3.41	3.18	3.02	2.90	2.81	2.73
	.010	7.88	5.66	4.76	4.26	3.94	3.71	3.54	3.41	3.30
	.005	9.63	6.73	5.58	4.95	4.54	4.26	4.05	3.88	3.75
24	.100	2.93	2.54	2.33	2.19	2.10	2.04	1.98	1.94	1.91
	.050	4.26	3.40	3.01	2.78	2.62	2.51	2.42	2.36	2.30
	.025	5.72	4.32	3.72	3.38	3.15	2.99	2.87	2.78	2.70
	.010	7.82	5.61	4.72	4.22	3.90	3.67	3.50	3.36	3.26
	.005	9.55	6.66	5.52	4.89	4.49	4.20	3.99	3.83	3.69
25	.100	2.92	2.53	2.32	2.18	2.09	2.02	1.97	1.93	1.89
	.050	4.24	3.39	2.99	2.76	2.60	2.49	2.40	2.34	2.28
	.025	5.69	4.29	3.69	3.35	3.13	2.97	2.85	2.75	2.68
	.010	7.77	5.57	4.68	4.18	3.85	3.63	3.46	3.32	3.22
	.005	9.48	6.60	5.46	4.84	4.43	4.15	3.94	3.78	3.64
26	.100	2.91	2.52	2.31	2.17	2.08	2.01	1.96	1.92	1.88
	.050	4.23	3.37	2.98	2.74	2.59	2.47	2.39	2.32	2.27
	.025	5.66	4.27	3.67	3.33	3.10	2.94	2.82	2.73	2.65
	.010	7.72	5.53	4.64	4.14	3.82	3.59	3.42	3.29	3.18
	.005	9.41	6.54	5.41	4.79	4.38	4.10	3.89	3.73	3.60
27	.100	2.90	2.51	2.30	2.17	2.07	2.00	1.95	1.91	1.87
	.050	4.21	3.35	2.96	2.73	2.57	2.46	2.37	2.31	2.25
	.025	5.63	4.24	3.65	3.31	3.08	2.92	2.80	2.71	2.63
	.010	7.68	5.49	4.60	4.11	3.78	3.56	3.39	3.26	3.15
	.005	9.34	6.49	5.36	4.74	4.34	4.06	3.85	3.69	3.56
28	.100	2.89	2.50	2.29	2.16	2.06	2.00	1.94	1.90	1.87
	.050	4.20	3.34	2.95	2.71	2.56	2.45	2.36	2.29	2.24
	.025	5.61	4.22	3.63	3.29	3.06	2.90	2.78	2.69	2.61
	.010	7.64	5.45	4.57	4.07	3.75	3.53	3.36	3.23	3.12
	.005	9.28	6.44	5.32	4.70	4.30	4.02	3.81	3.65	3.52

Table A.6 Values of *F* (*Continued*)

10	12	15	20	24	30	40	60	120	∞	*P*	*df*
Numerator *df*											
2.06	2.02	1.97	1.92	1.90	1.87	1.85	1.82	1.79	1.76	.100	15
2.54	2.48	2.40	2.33	2.29	2.25	2.20	2.16	2.11	2.07	.050	
3.06	2.96	2.86	2.76	2.70	2.64	2.59	2.52	2.46	2.40	.025	
3.80	3.67	3.52	3.37	3.29	3.21	3.13	3.05	2.96	2.87	.010	
4.42	4.25	4.07	3.88	3.79	3.69	3.58	3.48	3.37	3.26	.005	
2 03	1.99	1.94	1.89	1.87	1.84	1.81	1.78	1.75	1.72	.100	16
2.49	2.42	2.35	2.28	2.24	2.19	2.15	2.11	2.06	2.01	.050	
2.99	2.89	2.79	2.68	2.63	2.57	2.51	2.45	2.38	2.32	.025	
3.69	3.55	3.41	3.26	3.18	3.10	3.02	2.93	2.84	2.75	.010	
4.27	4.10	3.92	3.73	3.64	3.54	3.44	3.33	3.22	3.11	.005	
2.00	1.96	1.91	1.86	1.84	1.81	1.78	1.75	1.72	1.69	.100	17
2.45	2.38	2.31	2.23	2.19	2.15	2.10	2.06	2.01	1.96	.050	
2.92	2.82	2.72	2.62	2.56	2.50	2.44	2.38	2.32	2.25	.025	
3.59	3.46	3.31	3.16	3.08	3.00	2.92	2.83	2.75	2.65	.010	
4.14	3.97	3.79	3.61	3.51	3.41	3.31	3.21	3.10	2.98	.005	
1.98	1.93	1.89	1.84	1.81	1.78	1.75	1.72	1.69	1.66	.100	18
2.41	2.34	2.27	2.19	2.15	2.11	2.06	2.02	1.97	1.92	.050	
2.87	2.77	2.67	2.56	2.50	2.44	2.38	2.32	2.26	2.19	.025	
3.51	3.37	3.23	3.08	3.00	2.92	2.84	2.75	2.66	2.57	.010	
4.03	3.86	3.68	3.50	3.40	3.30	3.20	3.10	2.99	2.87	.005	
1.96	1.91	1.86	1.81	1.79	1.76	1.73	1.70	1.67	1.63	.100	19
2.38	2.31	2.23	2.16	2.11	2.07	2.03	1.98	1.93	1.88	.050	
2.82	2.72	2.62	2.51	2.45	2.39	2.33	2.27	2.20	2.13	.025	
3.43	3.30	3.15	3.00	2.92	2.84	2.76	2.67	2.58	2.49	.010	
3.93	3.76	3.59	3.40	3.31	3.21	3.11	3.00	2.89	2.78	.005	
1.94	1.89	1.84	1.79	1.77	1.74	1.71	1.68	1.64	1.61	.100	20
2.35	2.28	2.20	2.12	2.08	2.04	1.99	1.95	1.90	1.84	.050	
2.77	2.68	2.57	2.46	2.41	2.35	2.29	2.22	2.16	2.09	.025	
3.37	3.23	3.09	2.94	2.86	2.78	2.69	2.61	2.52	2.42	010	
3.85	3.68	3.50	3.32	3.22	3.12	3.02	2.92	2.81	2.69	.005	
1.92	1.87	1.83	1.78	1.75	1.72	1.69	1.66	1.62	1.59	.100	21
2.32	2.25	2.18	2.10	2.05	2.01	1.96	1.92	1.87	1.81	.050	
2.73	2.64	2.53	2.42	2.37	2.31	2.25	2.18	2.11	2.04	.025	
3.31	3.17	3.03	2.88	2.80	2.72	2.64	2.55	2.46	2.36	.010	
3.77	3.60	3.43	3.24	3.15	3.05	2.95	2.84	2.73	2.61	.005	
1.90	1.86	1.81	1.76	1.73	1.70	1.67	1.64	1.60	1.57	.100	22
2.30	2.23	2.15	2.07	2.03	1.98	1.94	1.89	1.84	1.78	.050	
2.70	2.60	2.50	2.39	2.33	2.27	2.21	2.14	2.08	2.00	.025	
3.26	3.12	2.98	2.83	2.75	2.67	2.58	2.50	2.40	2.31	.010	
3.70	3.54	3.36	3.18	3.08	2.98	2.88	2.77	2.66	2.55	.005	
1.89	1.84	1.80	1.74	1.72	1.69	1.66	1.62	1.59	1.55	.100	23
2.27	2.20	2.13	2.05	2.01	1.96	1.91	1.86	1.81	1.76	.050	
2.67	2.57	2.47	2.36	2.30	2.24	2.18	2.11	2.04	1.97	.025	
3.21	3.07	2.93	2.78	2.70	2.62	2.54	2.45	2.35	2.26	.010	
3.64	3.47	3.30	3.12	3.02	2.92	2.82	2.71	2.60	2.48	.005	
1.88	1.83	1.78	1.73	1.70	1.67	1.64	1.61	1.57	1.53	.100	24
2.25	2.18	2.11	2.03	1.98	1.94	1.89	1.84	1.79	1.73	.050	
2.64	2.54	2.44	2.33	2.27	2.21	2.15	2.08	2.01	1.94	.025	
3.17	3.03	2.89	2.74	2.66	2.58	2.49	2.40	2.31	2.21	.010	
3.59	3.42	3.25	3.06	2.97	2.87	2.77	2.66	2.55	2.43	.005	
1.87	1.82	1.77	1.72	1.69	1.66	1.63	1.59	1.56	1.52	.100	25
2.24	2.16	2.09	2.01	1.96	1.92	1.87	1.82	1.77	1.71	.050	
2.61	2.51	2.41	2.30	2.24	2.18	2.12	2.05	1.98	1.91	.025	
3.13	2.99	2.85	2.70	2.62	2.54	2.45	2.36	2.27	2.17	.010	
3.54	3.37	3.20	3.01	2.92	2.82	2.72	2.61	2.50	2.38	.005	
1.86	1.81	1.76	1.71	1.68	1.65	1.61	1.58	1.54	1.50	.100	26
2.22	2.15	2.07	1.99	1.95	1.90	1.85	1.80	1.75	1.69	.050	
2.59	2.49	2.39	2.28	2.22	2.16	2.09	2.03	1.95	1.88	.025	
3.09	2.96	2.81	2.66	2.58	2.50	2.42	2.33	2.23	2.13	.010	
3.49	3.33	3.15	2.97	2.87	2.77	2.67	2.56	2.45	2.33	.005	
1.85	1.80	1.75	1.70	1.67	1.64	1.60	1.57	1.53	1.49	.100	27
2.20	2.13	2.06	1.97	1.93	1.88	1.84	1.79	1.73	1.67	.050	
2.57	2.47	2.36	2.25	2.19	2.13	2.07	2.00	1.93	1.85	.025	
3.06	2.93	2.78	2.63	2.55	2.47	2.38	2.29	2.20	2.10	.010	
3.45	3.28	3.11	2.93	2.83	2.73	2.63	2.52	2.41	2.29	.005	
1.84	1.79	1.74	1.69	1.66	1.63	1.59	1.56	1.52	1.48	.100	28
2.19	2.12	2.04	1.96	1.91	1.87	1.82	1.77	1.71	1.65	.050	
2.55	2.45	2.34	2.23	2.17	2.11	2.05	1.98	1.91	1.83	.025	
3.03	2.90	2.75	2.60	2.52	2.44	2.35	2.26	2.17	2.06	.010	
3.41	3.25	3.07	2.89	2.79	2.69	2.59	2.48	2.37	2.25	.005	

Table A.6 Values of F (*Continued*)

Denominator *df*	Probability of a larger F	Numerator *df* 1	2	3	4	5	6	7	8	9
29	.100	2.89	2.50	2.28	2.15	2.06	1.99	1.93	1.89	1.86
	.050	4.18	3.33	2.93	2.70	2.55	2.43	2.35	2.28	2.22
	.025	5.59	4.20	3.61	3.27	3.04	2.88	2.76	2.67	2.59
	.010	7.60	5.42	4.54	4.04	3.73	3.50	3.33	3.20	3.09
	.005	9.23	6.40	5.28	4.66	4.26	3.98	3.77	3.61	3.48
30	.100	2.88	2.49	2.28	2.14	2.05	1.98	1.93	1.88	1.85
	.050	4.17	3.32	2.92	2.69	2.53	2.42	2.33	2.27	2.21
	.025	5.57	4.18	3.59	3.25	3.03	2.87	2.75	2.65	2.57
	.010	7.56	5.39	4.51	4.02	3.70	3.47	3.30	3.17	3.07
	.005	9.18	6.35	5.24	4.62	4.23	3.95	3.74	3.58	3.45
40	.100	2.84	2.44	2.23	2.09	2.00	1.93	1.87	1.83	1.79
	.050	4.08	3.23	2.84	2.61	2.45	2.34	2.25	2.18	2.12
	.025	5.42	4.05	3.46	3.13	2.90	2.74	2.62	2.53	2.45
	.010	7.31	5.18	4.31	3.83	3.51	3.29	3.12	2.99	2.89
	.005	8.83	6.07	4.98	4.37	3.99	3.71	3.51	3.35	3.22
60	.100	2.79	2.39	2.18	2.04	1.95	1.87	1.82	1.77	1.74
	.050	4.00	3.15	2.76	2.53	2.37	2.25	2.17	2.10	2.04
	.025	5.29	3.93	3.34	3.01	2.79	2.63	2.51	2.41	2.33
	.010	7.08	4.98	4.13	3.65	3.34	3.12	2.95	2.82	2.72
	.005	8.49	5.79	4.73	4.14	3.76	3.49	3.29	3.13	3.01
120	.100	2.75	2.35	2.13	1.99	1.90	1.82	1.77	1.72	1.68
	.050	3.92	3.07	2.68	2.45	2.29	2.17	2.09	2.02	1.96
	.025	5.15	3.80	3.23	2.89	2.67	2.52	2.39	2.30	2.22
	.010	6.85	4.79	3.95	3.48	3.17	2.96	2.79	2.66	2.56
	.005	8.18	5.54	4.50	3.92	3.55	3.28	3.09	2.93	2.81
∞	.100	2.71	2.30	2.08	1.94	1.85	1.77	1.72	1.67	1.63
	.050	3.84	3.00	2.60	2.37	2.21	2.10	2.01	1.94	1.88
	.025	5.02	3.69	3.12	2.79	2.57	2.41	2.29	2.19	2.11
	.010	6.63	4.61	3.78	3.32	3.02	2.80	2.64	2.51	2.41
	.005	7.88	5.30	4.28	3.72	3.35	3.09	2.90	2.74	2.62

SOURCE: A portion of "Tables of percentage points of the inverted beta (F) distribution," *Biometrika*, vol. 33 (1943) by M. Merrington and C. M. Thompson and from Table 18 of *Biometrika Tables for Statisticians*, vol. 1, Cambridge University Press, 1954, edited by E. S. Pearson and H. O. Hartley. Reproduced with permission of the authors, editors, and *Biometrika* trustees.

Table A.6 Values of F (*Continued*)

Numerator *df* 10	12	15	20	24	30	40	60	120	∞	P	*df*
1.83	1.78	1.73	1.68	1.65	1.62	1.58	1.55	1.51	1.47	.100	29
2.18	2.10	2.03	1.94	1.90	1.85	1.81	1.75	1.70	1.64	.050	
2.53	2.43	2.32	2.21	2.15	2.09	2.03	1.96	1.89	1.81	.025	
3.00	2.87	2.73	2.57	2.49	2.41	2.33	2.23	2.14	2.03	.010	
3.38	3.21	3.04	2.86	2.76	2.66	2.56	2.45	2.33	2.21	.005	
1.82	1.77	1.72	1.67	1.64	1.61	1.57	1.54	1.50	1.46	.100	30
2.16	2.09	2.01	1.93	1.89	1.84	1.79	1.74	1.68	1.62	.050	
2.51	2.41	2.31	2.20	2.14	2.07	2.01	1.94	1.87	1.79	.025	
2.98	2.84	2.70	2.55	2.47	2.39	2.30	2.21	2.11	2.01	.010	
3.34	3.18	3.01	2.82	2.73	2.63	2.52	2.42	2.30	2.18	.005	
1.76	1.71	1.66	1.61	1.57	1.54	1.51	1.47	1.42	1.38	.100	40
2.08	2.00	1.92	1.84	1.79	1.74	1.69	1.64	1.58	1.51	.050	
2.39	2.29	2.18	2.07	2.01	1.94	1.88	1.80	1.72	1.64	.025	
2.80	2.66	2.52	2.37	2.29	2.20	2.11	2.02	1.92	1.80	.010	
3.12	2.95	2.78	2.60	2.50	2.40	2.30	2.18	2.06	1.93	.005	
1.71	1.66	1.60	1.54	1.51	1.48	1.44	1.40	1.35	1.29	.100	60
1.99	1.92	1.84	1.75	1.70	1.65	1.59	1.53	1.47	1.39	.050	
2.27	2.17	2.06	1.94	1.88	1.82	1.74	1.67	1.58	1.48	.025	
2.63	2.50	2.35	2.20	2.12	2.03	1.94	1.84	1.73	1.60	.010	
2.90	2.74	2.57	2.39	2.29	2.19	2.08	1.96	1.83	1.69	.005	
1.65	1.60	1.55	1.48	1.45	1.41	1.37	1.32	1.26	1.19	.100	120
1.91	1.83	1.75	1.66	1.61	1.55	1.50	1.43	1.35	1.25	.050	
2.16	2.05	1.94	1.82	1.76	1.69	1.61	1.53	1.43	1.31	.025	
2.47	2.34	2.19	2.03	1.95	1.86	1.76	1.66	1.53	1.38	.010	
2.71	2.54	2.37	2.19	2.09	1.98	1.87	1.75	1.61	1.43	.005	
1.60	1.55	1.49	1.42	1.38	1.34	1.30	1.24	1.17	1.00	.100	∞
1.83	1.75	1.67	1.57	1.52	1.46	1.39	1.32	1.22	1.00	.050	
2.05	1.94	1.83	1.71	1.64	1.57	1.48	1.39	1.27	1.00	.025	
2.32	2.18	2.04	1.88	1.79	1.70	1.59	1.47	1.32	1.00	.010	
2.52	2.36	2.19	2.00	1.90	1.79	1.67	1.53	1.36	1.00	.005	

BIOGRAPHY

Mr. Somchoke Kala was born on October 16, 1980. He obtained Bachelor of Science degree in Microbiology from Chulalongkorn University, Bangkok, Thailand, in 2002.

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