

CLONING, EXPRESSION AND PURIFICATION OF RECOMBINANT ELONGATION
FACTOR Tu FROM HYPERTHERMOPHILIC BACTERIA
GEOBACILLUS ANATOLICUS

by

Barış Akalın

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GEOBACILLUS ANATOLICUS

APPROVED BY:

Prof. Neş'e Bilgin
(Thesis Supervisor)

Assoc. Prof. Esra Battaloğlu

Prof. Zehra Sayers

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Dedicated to humanity

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ABSTRACT

CLONING, EXPRESSION AND PURIFICATION OF RECOMBINANT ELONGATION FACTOR Tu FROM HYPERTHERMOPHILIC BACTERIA *GEOBACILLUS ANATOLICUS*

In this study, elongation factor Tu (EF-Tu) from *Geobacillus anatolicus* was cloned, expressed and purified as a His-tagged recombinant protein in *Escherichia coli*. At first, the nucleic acid sequence of *Geobacillus anatolicus tuf* gene was determined by using sequence data obtained from the bacteria phylogenetically related to *Geobacillus anatolicus*. The *tuf* gene and its neighbouring genes of the *tuf* gene sequences were used to design appropriate oligonucleotide primers and these primers were used to amplify the chromosomal region carrying the *tuf* gene of *Geobacillus anatolicus*. The sequence information from this fragment was then used to design primers to sequence and clone the complete *tuf* gene into an appropriate expression vector. This vector was used to express and purify the protein in high quantities in *Escherichia coli*. Six additional histidine residues were inserted into the recombinant protein of *Geobacillus anatolicus* at its C-terminal region to purify the protein using Ni-affinity chromatography to homogeneity. It was found that recombinant *Geobacillus anatolicus* EF-Tu is fully competent in forming a binary complex with *Geobacillus anatolicus* EF-Ts. It was determined that it has a significantly higher mobility rate in non-denaturing PAGE as compared to *Escherichia coli* EF-Tu, not explainable with molecular mass difference between these proteins. The mobility of the *Geobacillus anatolicus* EF-Tu.EF-Ts complex does not differ from the mobility of the *Geobacillus anatolicus* EF-Ts, indicating a large conformational change of EF-Tu upon binding to EF-Ts. The recombinant *Geobacillus anatolicus* EF-Tu can form a ternary complex with GTP and Phe-tRNA^{Phe}. *Geobacillus anatolicus* retained its GDP binding activity fully after incubation at 60°C for 10 min, and it still has approximately %20 of its activity after incubating for 10 min at 80°C.

ÖZET

HİPERTERMOFİLİK *GEOBACILLUS ANATOLICUS* BAKTERİLERİNDEN REKOMBİNANT ELONGASYON FAKTÖRÜ Tu’NUN KLONLANMASI, EKSPRESYONU VE SAFLAŞTIRILMASI

Bu çalışmada, *Geobacillus anatolicus* elongasyon faktörü Tu klonlanmış, *Escherichia coli*’de ekspresyonu yapılmış ve His-kuyruklu bir rekombinant protein olarak saflaştırılmıştır. İlk olarak, *Geobacillus anatolicus tuf* geninin dizisi belirlenmiştir. *Geobacillus anatolicus tuf* geninin dizisinin belirlenmesi için, *Geobacillus anatolicus*’a filogenetik olarak yakın bakterilerin *tuf* geni ve *tuf* geninin etrafındaki genler için belirlenmiş dizi bilgileri kullanılarak uygun oligonukleotit primerler tasarlanmıştır ve bu oligonükleotit primerler kullanılarak *tuf* genini taşıyan kromozomal bölge çoğaltılmıştır. Elde edilen dizi bilgisi daha sonra *tuf* geninin uygun bir ekspresyon vektörüne klonlanması için kullanılmıştır. Rekombinant proteinin daha sonra Ni-afinite kromatografisi ile saflaştırılabilmesi için vektör üzerinde klonlanan proteinin C-terminal bölgesine 6 His aminoasidi eklenmiştir. Bu vektör aracılığı ile *Geobacillus anatolicus* EF-Tu’sunun *Escherichia coli* içinde yüksek miktarda ekspresyonu sağlanmış ve Ni-afinite kromatografisi kullanılarak saflaştırılmıştır. *Geobacillus anatolicus*’tan elde edilen EF-Tu’nun *Geobacillus anatolicus* EF-Ts proteini ile etkileşime girebildiği gösterilmiştir. *Geobacillus anatolicus* EF-Tu’su, *Escherichia coli* EF-Tu’suna göre denatüre etmeyen poliakrilamid elektroforezinde anlamlı derecede hızlı bir ilerleme hızına sahiptir. *Geobacillus anatolicus* EF-Tu.EF-Ts bileşiği ile *Geobacillus anatolicus* EF-Ts’sinin denatüre etmeyen poliakrilamid elektroforezinde aynı ilerleme hızına sahip olması, bileşiğin oluşması sırasında EF-Tu proteininin önemli yapısal değişime uğradığına işaret etmektedir. Rekombinant *Geobacillus anatolicus* EF-Tu’sunun GTP ve Phe-tRNA^{Phe} ile üçlü bileşik oluşturabildiği de yine denatüre etmeyen poliakrilamid elektroforezi ile gösterilmiştir. *Geobacillus anatolicus* EF-Tu’su 60°C’de 10 dakika inkübe edildiğinde GDP bağlama aktivitesini korumaktadır. Bu değer 80°C’de yaklaşık olarak %20’ye inmektedir.

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LIST OF ABBREVIATIONS

aa	Aminoacid
A	Adenine
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
APS	Ammonium persulphate
Arg	Arginine
Asp	Aspartic acid
ATP	Adenosine 5'-triphosphate
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
Ca	Calcium
Cys	Cysteine
C-terminal	Carboxyl terminal
D	Aspartic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotides
dsDNA	Double stranded deoxyribonucleic acid
DTE	1,4-Dithioerythritol
E	Glutamic acid
EDTA	Ethylenediaminetetraacetate
EF-G	Elongation factor G
EF-Ts	Elongation factor Ts

EF-Tu	Elongation factor Tu
EtBr	Ethidium bromide
F	Phenylalanine
G	Guanine
GDP	Guanosine diphosphate
Gla	γ -carboxy glutamic acid
Gln	Glutamine
Glu	Glutamate
GTP	Guanosine triphosphate
h	Hour
H	Histidine
His	Histidine
I	Isoleucine
Ile	Isoleucine
IPTG	Iopropyl-1-thio- β -D-galactoside
K	Lysine
kb	Kilo base
kDa	Kilo Dalton
L	Leucine
LB	Luria-Bertani broth
Leu	Leucine
Lys	Lysine
Met	Methionine
Mg	Miligram

Mg	Magnesium
MgCl ₂	Magnesium chloride
min	Minute
mg	Milligram
mM	Millimolar
mRNA	Messenger RNA
N	Asparagine
ng	Nano gram
Ni	Nickel
NTA	Nitrilotriacetic acid
N-terminal	Amino terminal
Nres	Nucleotide residues
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
pmol	picomole
PMSF	Phenylmethyl ulfonylfluoride
Q	Histidine
R	Arginine
RNA	Ribonucleic acid
<i>rpsB</i>	ribosomal protein S2 gene
rRNA	Ribosomal RNA
S	Serine
sec	Seconds
Ser	Serine
SDS	Sodiumdodecylsulphate

SDS-PAGE	SDS-polyacrylamide gel electrophoresis
<i>smbA</i>	uridylate kinase gene
T	Thymine
<i>Taq</i>	<i>Thermus aquaticus</i>
TE	Tris-EDTA
TEMED	N,N',N',N'-Tetramethylethylenediamine
Thr	Threonine
T _m	Melting temperature
Tris	Tris(hydroxymethyl)aminomethane
tRNA	Transfer RNA
Trp	Tryptophan
<i>tsf</i>	elongation factor Ts gene
Tyr	Tyrosine
u	Unit
UV	Ultra Violet
V	Valine
Val	Valine
W	Tryptophan
Y	Tyrosine

1. INTRODUCTION

1.1. Protein Synthesis

Life is dependent on protein synthesis. By using this process, living cells produce structural and functional proteins and reproduce themselves. Given this dependency, it can be said that protein synthesis is a central biological process in all living cells. Protein synthesis takes place on ribosomes where the genetic information transcribed into mRNA is translated into protein. (Clark *et al.*, 1999).

Translational machinery is composed of ribosomes, mRNAs, tRNAs and various protein factors known as translation factors. Peptidyl-transferase activity is intrinsic to the ribosomes and resides in the 23 S ribosomal RNA (rRNA) in ribosomes (Marintchev and Wagner, 2005).

In prokaryotes, the ribosome is made up of a large subunit, referred to as 50S, and a small subunit, referred to as 30S. The 30S subunit binds mRNA and controls whether the codon–anticodon interaction between tRNA and mRNA is correct or not. The 50S subunit performs peptide bond formation and interacts with the acceptor end of the aminoacyl-tRNAs. The two subunits therefore, based on rRNA and ribosomal protein content, perform different tasks and contact with the L-shaped tRNA at the opposite ends. On the ribosome there are three different tRNA-binding sites named A-, P- and E- sites (Figure 1.1) (Nilsson and Nissen, 2005).

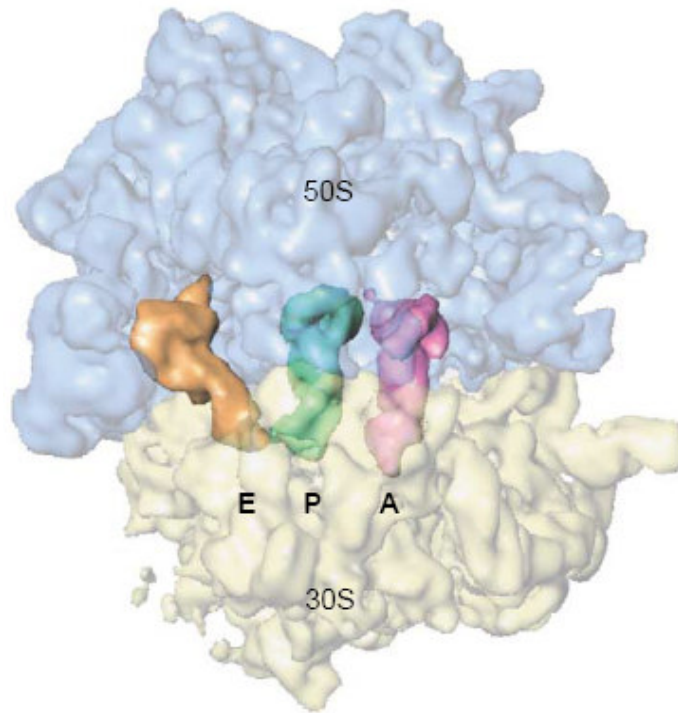


Figure 1.1. *E.coli* ribosome visualized by cryo-EM. There are three tRNAs on the A-, P- and E-sites (Nilsson and Nissen, 2005) (PDB: 1QZB)

In the aminoacyl (A) site, aminoacyl-tRNA (aa-tRNA) is accommodated; at the A site of the small subunit - the decoding center -, it was checked whether interaction of the anticodon of tRNA with the codon on mRNA is correct or not and after that tRNA is rejected or accepted.

In the peptidyl (P) site, peptidyl-tRNA is accommodated through codon-anticodon interactions on the small subunit. Another functional center, the peptidyltransferase center is present as a pocket-like structure on the large subunit. Following peptidyl transfer the elongated peptidyl-tRNA in the A-site is translocated to the P-site while deacylated tRNA in the P-site moves to the E-site.

In the exit (E) site, deacylated tRNA exits ribosome (Frank, 2003).

There are four major steps in the protein synthesis process: initiation, elongation, termination and recycling.

Translation elongation factors can be considered molecular motors in the protein synthesis on the ribosome. In the presence of them, amino acids are added to the nascent polypeptide chain and the polypeptide chain elongates. Because translation is a crucial process in terms of liveliness, general biochemical structure of the translation is well preserved in all biological kingdoms (Andersen *et al.*, 2003).

Among bacterial translational elongation factors, elongation factor thermo unstable (EF-Tu) has special significance. In translation elongation cycle, its major role is the binding and transporting of the appropriate codon-specified aminoacyl-tRNA to the aminoacyl site of the ribosome. In performing its function, it forms a ternary complex with GTP and aminoacyl-tRNA and it interacts with ribosomes. A second elongation factor, EF-Ts is responsible for GDP/GTP exchange on EF-Tu (Richarme, 1998).

In *E.coli*, EF-Tu is a monomeric protein of 43.1 kD (Young and Bernlohr, 1991). It is one of the most abundant proteins in the cell such that approximately 10% of the total protein content is EF-Tu. It was determined that there is about one EF-Tu molecule for every tRNA molecule under different growth conditions (Gouy and Grantham, 1980).

In addition of its crucial role in polypeptide elongation, EF-Tu can have other functions. It was determined that EF-Tu is an essential host-donated subunit of the $Q\beta$ replicative complex, the transcriptional apparatus and membranes; it has a role in the degradation of N-terminally blocked proteins by 26S proteasome; in the presence of stress factors, EF-Tu can display chaperone-like properties in protein folding and renaturation and it has protein disulfide isomerase activity (Richarme, 1998).

As a crucial part of an ancient and common biological process and as a protein highly conserved across the three major kingdoms of life, EF-Tu can be used as a tool for construction of a universal tree in phylogeny studies (Baldauf *et al.*, 1996).

Because it has a crucial role in translation elongation cycle and it may have diverse possible functions, to gain insight into fundamental principals of protein structure and function, EF-Tu is being extensively studied in a wide range of species.

1.1.1. Initiation

Initiation is a process where start codon on the mRNA is correctly located and placed on the P-site of the ribosomes with the help of initiation factors and initiator tRNA. Prokaryotic protein synthesis is the most studied protein synthesis system. The initiation phase of prokaryotic protein synthesis is controlled by initiation factors (IFs), IF-1, IF-2 and IF-3. Among them, IF-2 forms a ternary complex with GTP and initiator tRNA^{fMet} (methionyl-tRNA). All three factors and the additional components form an initiation complex; this complex is composed of initiation factors, initiator tRNA^{fMet}, the ribosomal subunits and mRNA. Eukaryotic initiation is much more complex and involves a large number of eukaryotic initiation factors (eIFs) (Clark *et al.*, 1999) (Figure 1.2).

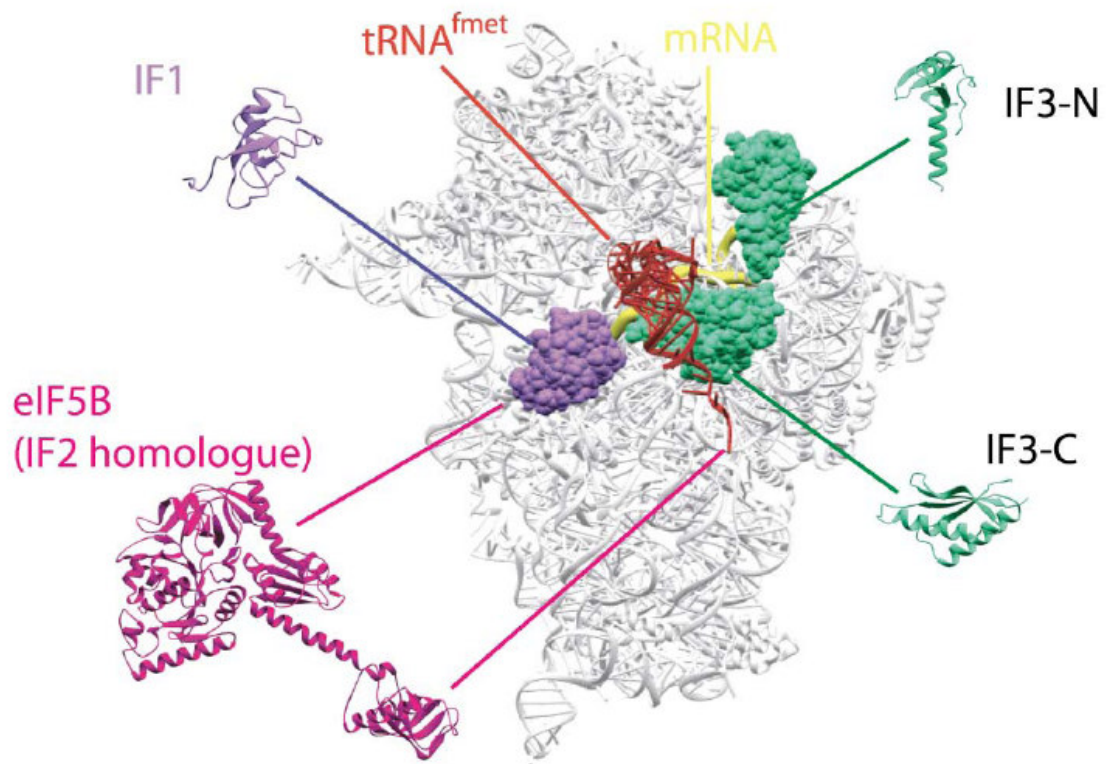


Figure 1.2. IF-1, IF-2 and IF-3 are shown with their corresponding locations on the ribosome 30S subunit (Ramakrishnan, 2002) (PDB: 1HR0 for 30S-IF1)

1.1.2. Elongation

The most intensively studied phase of the translation process has been the elongation phase and it has been examined both functionally and structurally over the last 30 years. In the elongation phase ribosome selects the correct aminoacyl-tRNAs through codon-anticodon interactions following the sequence on the mRNA and catalyzes peptide bond formation. There are three elongation factors (EFs) that speed up this process (Krab and Parmeggiani, 1998).

Elongation factor EF-Tu forms a ternary complex with GTP and aminoacylated tRNAs (aa-tRNAs) in procaryotes. Formation of the ternary complex protects the amino acid ester bond against hydrolysis. EF-Tu carries the aa-tRNA to the ribosomal A-site at which the decoding of mRNA by codon-anticodon interaction is performed. If correct

codon-anticodon interaction is present, ribosome stimulates GTP hydrolysis on EF-Tu and EF-Tu:GDP is released (Clark *et al.*, 1999).

The nucleotide exchange factor EF-Ts converts EF-Tu:GDP into active EF-Tu:GTP (Figure 1.3).

The accuracy of the aa-tRNA in the A-site is further checked in a proofreading step (Hopfield, 1974). Proofreading continues until the acceptor end of the aa-tRNA fully enter the A-site of the 50S subunit during which incorrectly accepted aa-tRNA's can be released (Valle *et al.*, 2003). When docking of the aa-tRNA is complete, the aa-tRNA contacts with the peptidyl-tRNA in the ribosomal P-site. Then peptide bond formation occurs and the nascent polypeptide chain is elongated by one amino acid (Clark *et al.*, 1999).

The active site for peptide bond formation, the peptidyl transferase (PT) center, is located on the large ribosomal subunit. By using high-resolution crystal structures of the large ribosomal subunits, it has been determined that the PT center, as localized by an intermediate-state analog, is composed of RNA (23S rRNA). This suggests that the PT reaction is catalyzed by RNA (Nissen *et al.*, 2000).

Translocation occurs at different times in the 30S and 50S subunits. This situation is called hybrid-states model. For example, after peptide bond formation, peptidyl-tRNA is at a hybrid site, in which the anticodon end is at the A-site on the 30S subunit, whereas the acceptor end is at the P-site on the 50S subunit (Moazed and Noller, 1989).

Elongation factor, EF-G, forms a complex with GTP and is responsible for the translocation of tRNAs on the ribosome (Clark *et al.*, 1999) (Figure 1.3.).

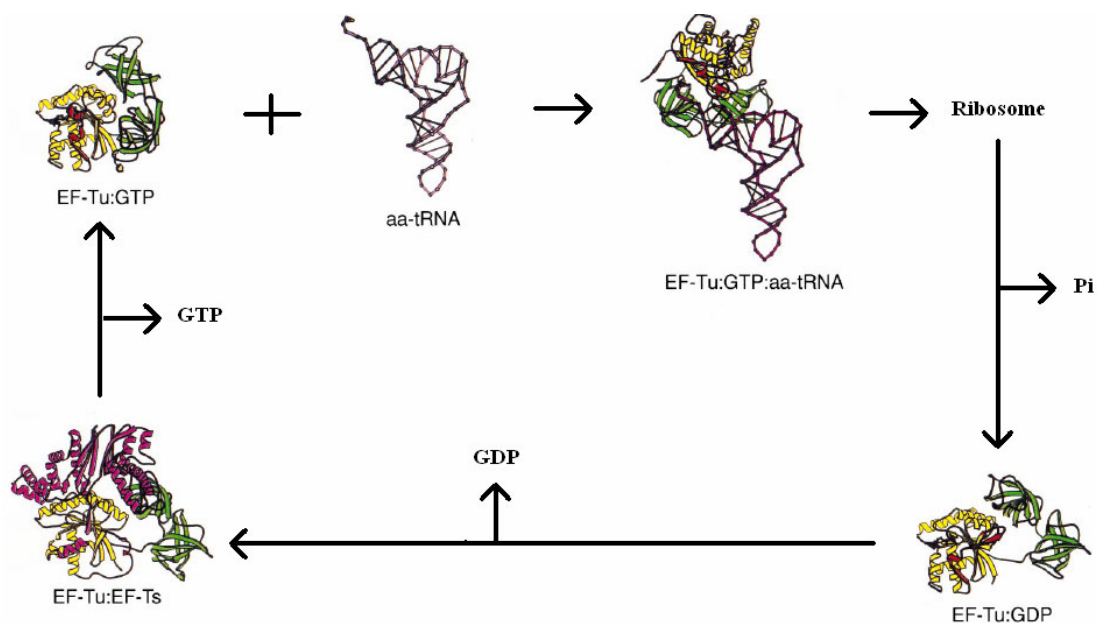


Figure 1.3. The functional cycle of EF-Tu (adapted from Clark *et al.*, 1999)

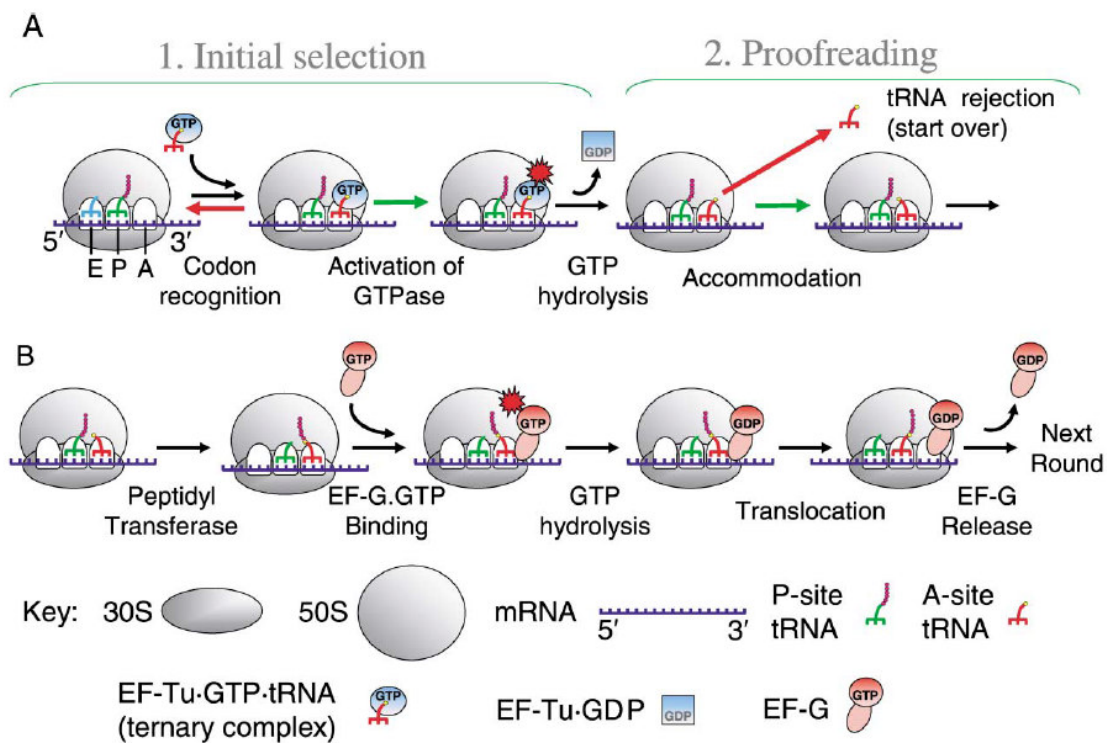


Figure 1.4. Overview of elongation cycle (Ramakrishnan, 2002)

1.1.3. Termination and Recycling

Termination phase of the protein synthesis begins when any one of the stop codons (UAA, UAG, UGA) on the mRNA enters to the A site on the ribosome. In bacteria, recognition of the stop codon is performed by two “class I” release factors, RF1 and RF2. Both factors recognize UAA codon; RF1 additionally recognize UAG and RF2 additionally recognize UGA codons (Kisselev and Buckingham, 2000). A “class II” release factor, RF3, which is a GTPase protein, binds to the complex of RF1/2 with the ribosome (Ramakrishnan, 2002).

When the appropriate stop codon is present in the A site of the ribosome and the RF1/2 complex binds to the ribosome, the hydrolysis reaction occurs on the tRNA and the peptide chain is released from the tRNA in the P site. Dissociation of RF1 and RF2 is facilitated by RF3. GTP doesn't bind to the RF3 on the ribosome until the hydrolysis of peptidyl tRNA by RF1/2 is completed. After binding of GTP to the RF3, RF3 switches into a new conformation; this conformation has a high affinity for ribosomes and facilitates the dissociation of RF1/2 from the ribosome. The hydrolysis of GTP is required for subsequent dissociation of RF3 (Ramakrishnan, 2002).

After the polypeptide chain is released, mRNA and the deacylated tRNA are still on the ribosome. For the ribosome to begin a new cycle of protein synthesis, this complex should be disassembled. Ribosome recycling factor (RRF) along with EF-G is required for this process. By using GTP hydrolysis, RRF and EF-G lead to the dissociation of ribosomes into its subunits (Gao *et al.*, 2005) (Figure 1.5).

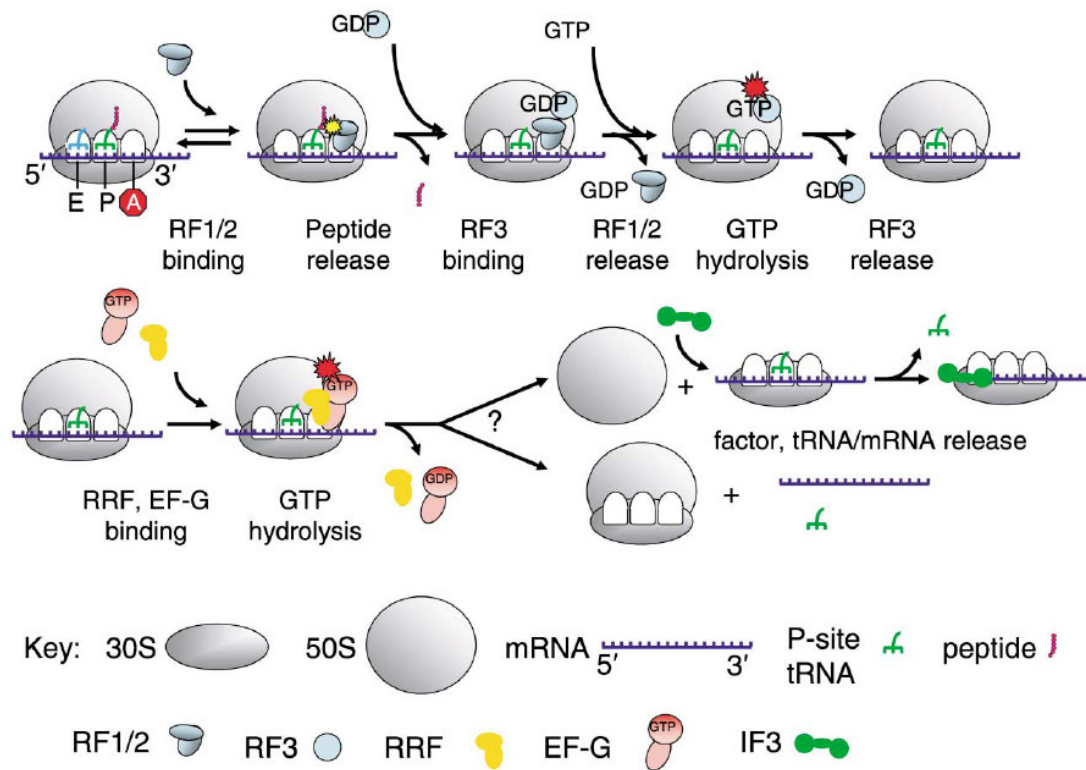


Figure 1.5. Overview of termination and recycling (Ramakrishnan, 2002)

1.2. Elongation Factor Tu (EF-Tu)

The elongation factor that is responsible for carrying aa-tRNA's to ribosomes is called EF-Tu. In the recently changed nomenclature it is called EF-1A. EF-Tu is composed of three structural domains. Domain 1 (referred to as G-domain) is about 200 amino acid long and responsible for binding of GDP or GTP; this domain is a typical nucleotide-binding domain; it has a central β -sheet surrounded by α -helices. It is structurally highly conserved as in the other G-proteins (Kjeldgaard *et al.*, 1996). Domains 2 and 3 are about 100 residues long. Each one of them is β -barrels and based on structure studies, it was determined that these domains are kept together by strong interdomain interactions as one structural unit. EF-Tu has an extraordinary ability; its two structural units (domain 1 and domains 2 and 3 together) may have large variations in their relative orientations. The structures of EF-Tu:GDP and EF-Tu:GTP are very different from each other (Kjeldgaard *et al.*, 1996). This difference was provided by structural changes in two regions on the G-domain of EF-Tu called switch I and switch II. Switch I changes from a β -hairpin in EF-

Tu:GDP to a short α -helix in EF-Tu:GTP. In switch II, the β -helix moves on the sequence by approximately four residues and the axes of the helix are rotated by about 45° . Because this helix (called helix B) is part of the interface between domains 1 and 3, after its activation, EF-Tu can show large conformational changes. The change in helix B correlates with the introduction of a γ -phosphate in the nucleotide-binding site; this phosphate induces an 180° peptide flip at a conserved Gly just prior to the α -helix (Clark *et al.*, 1999).

Crystal structures of EF-Tu-GDPNP (where GDPNP is a non-hydrolyzable analog of GTP) have been determined to high resolution from both *Thermus thermophilus* (Berchtold *et al.*, 1993) and *Thermus aquaticus* (Kjeldgaard *et al.*, 1993). Similarly, crystal structures of EF-Tu-GDP have been determined to high resolution from *Escherichia coli* (Abel *et al.*, 1996) and *T. aquaticus* (Polekhina *et al.*, 1996) (Figure 1.6).

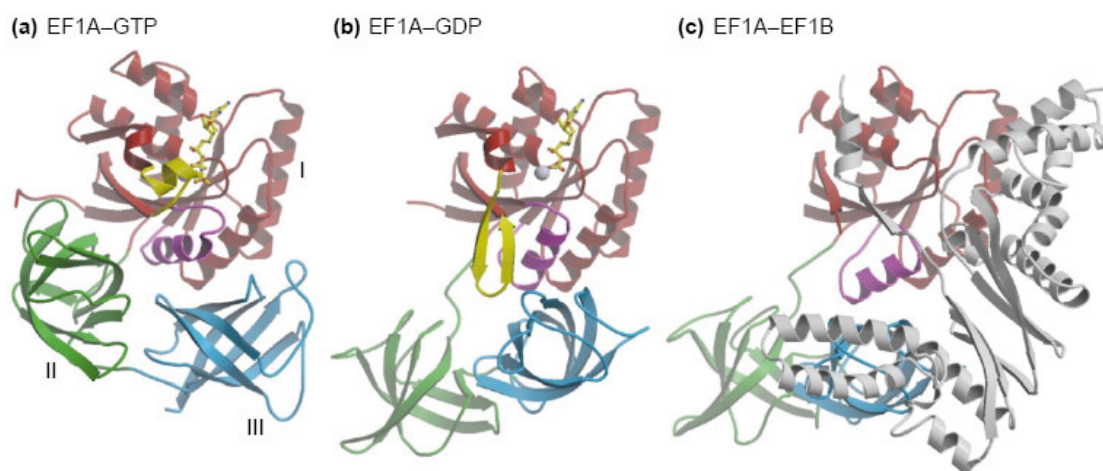


Figure 1.6. Structures of bacterial elongation factor EF-Tu. (a) EF-Tu-GDPNP from *Thermus aquaticus* (PDB: 1TTT), (b) EF-Tu-GDP from *Escherichia coli* (PDB: 1EFC), and (c) the EF-Tu-EF-Ts complex from *E. coli* (PDB: 1EFU). Domain I of EF-Tu is red, domain II is green and domain III is blue. EF-Ts is gray. The switch 1 region of EF-Tu is yellow and switch 2 is magenta. Nucleotides are shown in ball-and-stick models. Mg²⁺ ions are shown as gray balls (Andersen *et al.*, 2003)

1.3. Location of EF-Tu Gene on the Bacterial Chromosome

In many organisms, the EF-Tu gene (*tuf*) is the most distal part of the *streptomycin* (*str*) operon which is composed of the following four successive genes: 5'-*rpsL*(S12)-*rpsG*(S7)-*fus*(EF-G)-*tuf*(EF-Tu)-3'. In *B. stearrowthermophilus* it was determined that *tuf* gene is in the same place on the *str* operon and is between *fus* gene and *rplC* gene of the S10 operon which follows the *str* operon (Figure 1.7) (Krasny *et al.*, 1998).

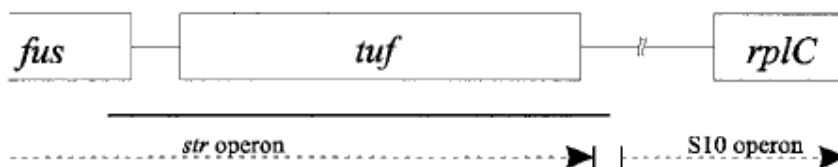


Figure 1.7. Location of *tuf* gene on the bacterial chromosome (Krasny *et al.*, 1998)

1.4. *Geobacillus anatolicus*

Bacillaceae is a large and diverse family of bacteria including various aerobic and facultatively anaerobic, rod-shaped, gram-positive to gram variable, endospore-forming species. This family includes various thermophilic, psychrophilic, acidophilic, alkalophilic, freshwater and halophilic bacteria. *Geobacillus* is recently identified within this family (Nazina *et al.*, 2001).

23 *Geobacillus* species have so far been defined and registered into the Bacterial GenBank database. In this study, as a source of EF-Tu, a hyperthermophilic *Geobacillus* species *Geobacillus anatolicus* was used. *Geobacillus anatolicus* was isolated from a thermal vent in Balıkesir, at 98°C, and based on 16S rRNA sequence comparisons, identified as a new hyperthermophilic bacteria in 2001 (Uysal *et al.*, 2001). No sequence information has been available for the translational components of the *Geobacillus* species when this work had started. Only recently the genome sequence of *Geobacillus kaustophilus* has been completed (Takami *et al.*, 2004).

A phylogenetic relationship between all known *Geobacillus* species including *G. anatolicus* in this genus was drawn by using ClustalW multiple alignment technique based on the 16S rRNA sequence data available for these species in the GeneBank (Figure 1.8.). In Table 1.1., gene IDs of the 16S sequences used for construction of the phylogenetic tree were shown.

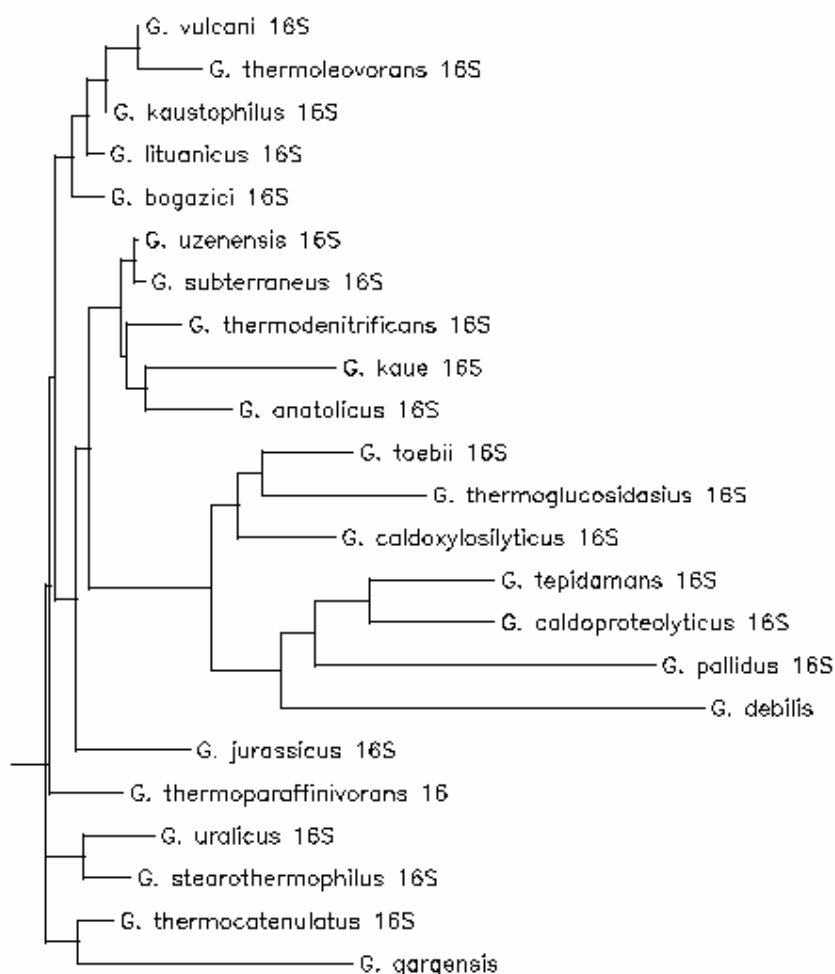


Figure 1.8. Phylogenetic relationship between *Geobacillus* species based on 16S rRNA sequences

Table 1.1. Gene IDs of the 16S sequences used for construction of the phylogenetic tree in Figure 1.8

Organism name	GeneBank ID	Organism name	GeneBank ID
<i>G. vulcani</i>	11877135	<i>G. caldoxylosilyticus</i>	54261298
<i>G. thermoleovorans</i>	6406842	<i>G. tepidamans</i>	45272587
<i>G. kaustophilus</i>	49482179	<i>G. caldoproteolyticus</i>	37538487
<i>G. lituanicus</i>	49482190	<i>G. pallidus</i>	57753943
<i>G. bogazici</i>	32815087	<i>G. debilis</i>	31414367
<i>G. uzenensis</i>	49482204	<i>G. jurassicus</i>	32442349
<i>G. subterraneus</i>	49482203	<i>G. thermoparaffinivorans</i>	83637833
<i>G. thermodenitrificans</i>	54261341	<i>G. uralicus</i>	22651495
<i>G. kaue</i>	16118568	<i>G. stearothermophilus</i>	82655168
<i>G. anatolicus</i>	16118566	<i>G. thermocatenulatus</i>	49482180
<i>G. toebii</i>	77819901	<i>G. gargensis</i>	28395524
<i>G. thermoglucosidasius</i>	49688769		

1.5. Hyperthermophiles

Hyperthermophilic organisms represent the organisms at the upper temperature border of life. Hyperthermophiles belong to phylogenetically distant groups and may represent rather ancient adaptations to heat. As a rule, they grow fastest (optimally) between 80°C and 110°C. Due to their heat tolerance there is an increasing interest for applications in biotechnological processes (Huber and Stetter, 1998). Growth temperatures of several *Geobacillus* species were shown in Table 1.2.

Table 1.2. Growth temperatures of several *Geobacillus* species (adapted from Zeigler, 2005).

Organism name	Growth temperature (°C)
<i>Geobacillus stearothermophilus</i>	37°C - 65°C
<i>Geobacillus kaustophilus</i>	37°C - 68°C
<i>Geobacillus subterraneus</i>	60°C - 75°C
<i>Geobacillus uzenensis</i>	60°C - 70°C
<i>Geobacillus thermodenitrificans</i>	45°C - 70°C
<i>Geobacillus thermoglucosidasius</i>	37°C - 68°C
<i>Geobacillus thermoleovorans</i>	42°C - 75°C
<i>Geobacillus anatolicus</i>	65°C - 98°C

1.6. Thermostability of Proteins in Hyperthermophiles

Normally, proteins from mesophilic organisms quickly become inactivated above the growth temperatures of these organisms (10-45°C). Accordingly, proteins produced by thermophilic microorganisms are more resistant to thermal and chemical denaturation than their mesophilic counterparts (Scandurra *et al.*, 1998). Proteins from hyperthermophilic organisms show extraordinary stability even at 100°C (Table 1.3.).

Table 1.3. Stability of some enzymes at 100°C (adapted from Daniel and Cowan, 2000 and Huber and Stetter, 1998)

Enzyme	Source Organism	Growth temperature	T _{1/2} at 100°C
Cellobiohydrolase	<i>Thermotoga</i>	90 °C	> 200 min
β-Glucosidase	<i>Thermotoga</i>	90 °C	90 min
Xylanase	<i>Thermotoga</i>	90 °C	20 min
Xylosidase	<i>Thermotoga</i>	90 °C	150 min
Esterase	<i>Sulfolobus</i>	85 °C	60 min
Hydrogenase	<i>Pyrococcus</i>	96 °C	120 min
Amylase	<i>Pyrococcus</i>	96 °C	360 min
DNA-dependent RNA polymerase	<i>Thermoproteus</i>	97 °C	>120 min

In the course of evolution, organisms have succeeded to survive under extreme conditions. Generally it can be said that there are three strategies for adaptation: escaping or compensating the stress or enhancing the stability of cellular structure and content. In the case of temperature, there is no alternative to mutative adaptation for survival. Proteins can have extremely high stabilities by using relatively small number of weak intermolecular interactions. In this respect, proteins from hyperthermophiles don't differ strongly from their mesophilic counterparts. Although there are no universal rules for thermostability, based on previous studies, several principles could be determined (Jaenicke and Böhm, 1998).

Charge clusters, networks of hydrogen bonds, optimization of packing density and hydrophobic interactions are examples of strategies used by thermophilic organism for protein stability (Jaenicke and Böhm, 1998).

For example, in thermophiles, while number of charged residues such as Glu, Asp, Lys and Arg increases, number of polar residues such as Ser, Thr, Asn and Gln decreases (Fukuchi and Nishikawa, 2001). This observation is called *CvP* bias and was very clearly shown in a study by Sanderova *et al.* in which *E. coli* and *B. stearothermophilus* elongation

factor Tu's were compared (Sanderova *et al.*, 2004). Similarly, in Table 1.4. this bias is presented explicitly.

Table 1.4. The relative amino acid compositions of proteins from mesophiles and thermophiles (adapted from Jaenicke and Böhm, 1998)

Amino acid	Mesophiles	Thermophiles
Charged residues (DEKRH)	24.11 %	29.84 %
Polar/uncharged residues (GSTNQYC)	31.15 %	26.79 %
Hydrophobic residues (LMIVWPAF)	44.74 %	43.36 %

As mentioned above, there are several methods for the establishing of thermostability. In Table 1.5., for particular proteins some of them were shown.

Table 1.5. Strategies of thermal stabilization of particular thermophilic enzymes (partially adapted from Jaenicke and Böhm, 1998)

Protein (Organism)	Major cause(s) of thermostability
CS (<i>P. furiosus</i>)	Increased compactness, enhanced subunit interactions, increased number of intersubunit ion pairs, shortening of loops
GAPDH (<i>T. maritima</i>)	Large number of additional salt bridges
IPMDH (<i>T. thermophilus</i>)	Increased number of ion pairs and hydrogen bonds, extended hydrophobic subunit interactions and improved packing of the hydrophobic core, shortened chain termini
LDH (<i>T. maritima</i>)	Increased number of ion pairs, decreased hydrophobic surface area, increased helicity, less cavity volume
Ferredoxin (<i>S. elongatus</i>)	Extension of hydrophobic core, a unique hydrophobic patch on the surface of a β sheet, two unique ion-pair networks

1.7. Biotechnological Implications for Hyperthermophilic Enzymes

As a consequence of the growth temperatures of hyperthermophiles, their enzymes are highly temperature stable, making them attractive for new biotechnological applications. There is an unanticipated diversity of species of hyperthermophiles within high temperature environments. Based on their unique properties, hyperthermophiles proteins may play an important role in technical processes (Huber and Stetter, 1998).

Until now, various thermophilic proteins have been commercialized. In molecular biotechnology, *T. aquaticus* (*Taq*) DNA polymerase (essential for PCR technology), *P. furiosus* (*Pfu*) DNA polymerase and DNA ligase and *Thermus* Rt41A serin protease PRETAQ are only a few examples which are widely in use. In industry, especially in starch processing, hyperthermophilic β -amylases, α -glucosidases and pullulanases have great potential. Using hyperthermophilic enzymes in cellulose degradation and ethanol production may support important advancements in these areas. In addition to the direct industrial applications, complete understanding of thermostability in hyperthermophiles is going to make it possible to design enzymes which have specific properties via protein engineering techniques (Vieille and Zeikus, 2001).

2. PURPOSE

Up to now, the question “how hyperthermophilic bacteria perform translation process properly at such high temperatures at which Watson-Crick base-pairing is very weakened?” has not been fully answered. The ultimate aim of this study is to understand translation process at high temperatures. One of the essential enzymes of protein synthesis is EF-Tu. Therefore, the structure and function of EF-Tu which will be purified from *Geobacillus anatolicus*, a newly described hyperthermophilic bacteria isolated from a hot-spring at 98 °C in Turkey, will be studied. For this purpose, the *tuf* gene coding EF-Tu will be cloned, expressed and purified in order to study it functionally in its interactions with EF-Ts, aminoacyl-tRNAs and ribosomes. As a future prospect, the data which will be obtained from this study is going to be used to construct an *in vitro* thermophilic protein synthesis machinery for further analysis of protein synthesis at high temperatures.

3. MATERIALS

3.1. Bacterial Strains

3.1.1. *Geobacillus anatolicus*

Geobacillus anatolicus was used as the source for Elongation factor Tu gene (*tuf*) in molecular cloning studies. *Geobacillus anatolicus* was previously collected from a hydrothermal vent at a water temperature of 98°C in Hisaralan, Balıkesir, Turkey and it was identified as a new species in our laboratory (GenBank Accession No: AF411064) (Uysal *et al.*, 2001).

3.1.2. *Escherichia coli*

Escherichia coli strain JM109(DE3) was used as the plasmid host for cloning and for expression of the *tuf* gene from *Geobacillus anatolicus*. The genotype of the JM109(DE3) is given in Table 3.1.

Table 3.1. Genotype of the *Escherichia coli* strain used throughout this study

JM109(DE3)	<i>endA1, recA1, gyrA96, thi, hsdR17</i> (rk ⁻ ,mk ⁺), <i>relA1, supE44, mcrAΔ(lac-proAB)</i> , [F', <i>traD36, proAB, laq</i> [^q ZΔM15], λDE3
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3.2. Oligonucleotide Primers

Oligonucleotide primers used throughout this study were shown in Table 3.2., 3.3. and 3.4.

Table 3.2. Oligonucleotide primers used for the amplification of *tuf* gene and for DNA sequencing of *tuf* gene

(V: any base except T, R: A or G, N: a base, M: C or A, H: any base except G, Y: C or T, W: T or A, D: any base except C)

TuF413	5'- GCC VCA RAC NMG NGA RCA -3'
TuR1174	5'- TCN CCN GGC ATN ACC AT - 3'
TuF888	5'- AGA AAT GTT CCG TAA GCT GC - 3'
TuR766	5'- ATC GAG AAA ACG TCC TCA AC - 3'
FusF	5'- CHG ARA TGT TYG GWT AYG CDA C - 3'
RplC	5'- CCR ATY TTW CKW CCT AAG ATT CCT TT - 3'

Table 3.3. Oligonucleotide primers used for cloning of *tuf* gene

TuF2	5'- CGG GGG GCA TAT GGC TAA AGC GAA ATT TGA GCG TAC GAA A - 3'
TuR2	5'- CAA AAA ATT CGA ATT AAT GAT GAT GAT GAT GCT CAA TGA TTT CCG ATA CGG AAC CAG C - 3'

Table 3.4. Oligonucleotide primers used for plasmid DNA sequencing

T7 Forward	5'-TAA TAC GAC TCA CTA TAG GG
pRSET Reverse	5'-TAG TTA TTG CTC AGC GGT GG

3.3. Enzymes Used Throughout This Study

Taq DNA polymerase (EC# 2.7.7.7) : Promega, USA

Nde I (EC# 3.1.21.4) : Promega, USA

Csp45 I (EC# 3.1.21.4) : Promega, USA

T4 DNA Ligase (EC # 6.5.1.1) : Promega, USA

Lysozyme (EC # 3.2.1.17) : Appligene, USA

RNase-A (EC # 3.1.27.5) : Promega, USA

Proteinase K (EC # 3.4.21.64) : Promega, USA

3.4. Chemicals

Agar : Oxoid, United Kingdom

Agarose : Sigma, USA

Ampicillin : Mustafa Nevzat, Turkey

Ammoniumpersulfate (APS) : Saveen, Sweden

Bromophenol Blue : Sigma, USA

Chloroform : Ambresco, USA

Coomassie Brilliant Blue R-250 : Sigma, USA

Crystal Violet : Merck, Germany

D-Glucose : Riedel de-Häen, Germany

DNA molecular size markers : 100 bp (Promega, USA)
1 kb (Promega, USA)

Dithioerythritol (DTE) : Fluka BioChemika, USA

Ethidium Bromide : Sigma, USA

Glacial Acetic Acid	:	Merck, Germany
Glass beads (0.1 mm diameter)	:	Biospec Products INC., USA
Glycerol	:	Merck, Germany
Guanosine 5'-diphosphate (GDP)	:	Sigma, USA
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	:	Sigma, USA
Imidazole	:	Sigma, USA
Isopropyl- β -D-thiogalactopyranoside (IPTG)	:	Saveen, Sweden
β -Mercaptoethanol	:	Merck, Germany
Nitrilotriacetic acid (NTA)	:	Fluka, Switzerland
N,N,N',N'-Tetramethylethylenediamine (TEMED)	:	Sigma, USA
Phenylmethylsulfonylfluoride (PMSF)	:	Sigma, USA
Phenol	:	Riedel de-Häen, Germany
Phenol Red	:	Sigma, USA
Polyethylene glycol (PEG 6000)	:	Merck, Germany
Protein Molecular Weight Markers	:	PageRuler Prestained Protein Ladder (Fermentas, USA)

PageRuler Protein Ladder
(Fermentas, USA)

Sodium Dodecyl Sulfate (SDS)	:	Kebolab, USA
Sucrose	:	Merck, Germany
Tryptone	:	Oxoid, United Kingdom
Yeast Extract	:	Oxoid, United Kingdom

All other laboratory salts are analytical grade and obtained from Merck and Fluka, Germany.

3.5. Buffers and Solutions

3.5.1. Bacterial Growth

3.5.1.1. Stock Solutions for Growth Medium for *Geobacillus anatolicus*

5X Solution A	:	20 g/l yeast extract 40 g/l tryptone 15 g/l NaCl
10X Solution B	:	1 g/l NTA 0.6 g/l $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ 1 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1 g/l KNO_3 6.9 g/l NaNO_3 1 g/l Na_2HPO_4
100X Solution C	:	28 mg/l FeCl_3

100X Solution D	:	220 mg/l $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 50 mg/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 1.6 mg/l CuSO_4 2.5 mg/l $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 4.6 mg/l $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.5 ml/l concentrated H_2SO_4
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Final liquid culture medium (1X) contained appropriate dilutions of the concentrated stock solutions of A, B, C, and D. After autoclaving, D-glucose is added to a final concentration of 2%.

3.5.1.2. Growth Medium for *Escherichia coli* JM109 (DE3) Strain

1X LB-ampicillin Medium (pH 7.0)	:	5 g/l NaCl 10 g/l Tryptone 5 g/l Yeast Extract 150 $\mu\text{g/ml}$ ampicillin
LB–Ampicillin Agar	:	5 g/l NaCl 10 g/l Tryptone 5 g/l Yeast Extract 14 g/l agar 150 $\mu\text{g/ml}$ ampicillin

3.5.2. Stock Solutions for Genomic DNA Isolation

Homogenization Buffer	:	50 mM Tris-HCl (pH 8.0) 20 mM EDTA
20 % SDS	:	200 g/l SDS in H_2O
TE Buffer	:	10 mM Tris-HCl (pH 8.0) 1 mM EDTA

3.5.3. Stock Solutions for Polymerase Chain Reaction (PCR)

10 X PCR Buffer	:	10 mM Tris-HCl (pH 9) 50 mM KCl 0.1 % Triton-X 100 (Promega, USA)
MgCl ₂	:	25 mM MgCl ₂ (Promega, USA)
Deoxyribonucleotide mixture	:	100 mM of each dATP, dGTP, dCTP, dTTP (Promega, USA) in H ₂ O

3.5.4. Stock Solutions for Agarose Gel Electrophoresis

25 X TAE Buffer	:	121 g/l Tris (Base) 28.55 ml/l Glacial acetic acid 25 mM EDTA (pH 8.0)
1 or 2 % Agarose Gel	:	1 or 2 % (w/v) Agarose in 1 X TAE Buffer, containing 0.5 µg/ml Ethidium Bromide
6 X Loading Buffer	:	10mM Tris-HCl (pH 7.6) 0.03% bromophenol blue 0.03% xylene cyanol FF 60% glycerol 60mM EDTA

3.5.5. Stock Solutions for Enzyme Digestions

10X concentrated buffers for enzymes below were purchased together with the enzymes.

10X Buffer D for Nde I digestion	:	60 mM Tris-HCl (pH 7.9) 1.5 M NaCl 60 mM MgCl ₂ 10 mM Dithiotheitol (DTT)
10X Buffer B for Csp45 I digestion	:	60 mM Tris-HCl (pH 7.5) 500 mM NaCl 60 mM MgCl ₂ 100 mM DTT
10X T4 DNA Ligase Buffer	:	300 mM Tris-HCl (pH 7.8) 100 mM MgCl ₂ 100 mM DTT 10 mM ATP

3.5.6. Stock Solutions for Competent Cell Preparation

TSS Buffer	:	10 % PEG 6000 5 % DMSO 20 mM MgSO ₄ 5 g/l NaCl 10 g/l Tryptone 5 g/l Yeast extract
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3.5.7. Stock Solutions for Transformation

SOC Medium	:	2 % Tryptone 0.5 % Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM MgCl ₂ 10 mM MgSO ₄
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20 mM D-Glucose

3.5.8. Stock Solutions for Plasmid DNA Isolation

TEL Solution	:	10 mM Tris-HCl (pH 8) 1 mM EDTA 5 mg/ml lysozyme
NAS Solution	:	0.2 M NaOH 1 % SDS
K-acetate Solution (pH 4.8)	:	60 ml/l 5M K-acetate 28.5 ml/l Glacial acetic acid
Resuspension Buffer	:	10 mM Tris-HCl (pH 8.0) 1mM EDTA 0.3 M NaCl

3.5.9. Stock Solutions for SDS-Polyacrylamide Gel Electrophoresis

Protein Lysis Solution	:	167 mM Tris-HCl (pH 6.8) 0.33 M SDS 10 % (w/v) sucrose 25 µl/ml β-mercaptoethanol 0.01 % (w/v) bromophenol blue
Solution I	:	20 % acrylamide 1 % N,N'-methylene- bisacrylamide (37.5/1)
Solution II A	:	3 M Tris (pH 8.9)
Solution II B	:	60 g/l Tris (base)

		68.6 g/l $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (pH 7.8)
Gel Buffer (1:1)	:	200 ml Solution II A 8 ml 20 % SDS 1 ml TEMED 600 ml H_2O
Spacer	:	41.5 ml Solution I 31.25 ml Solution II B 1.25 ml 20 % SDS 0.25 ml TEMED 207 ml H_2O
5X SDS Sample Buffer	:	5 ml 20 % SDS 4 ml Solution II B 1 ml β -Mercaptoethanol 10 ml glycerol
10 % Ammoniumpersulfate	:	0.1 g/ml APS in H_2O
40% Acrylamide Stock (37.5:1)	:	37.5 % Acrylamide 1 % N,N'- methylenebisacrylamide (purchased as a ready-made solution from Biorad, USA)
7X Solution 2C	:	24 g/l Tris (base) 115.2 g/l Glycine
SDS-PAGE Electrophoresis running buffer	:	167 ml/l 7X Solution 2C 5.83 ml/l 20 % SDS
Staining Solution	:	40 % Methanol

10 % Glacial Acetic acid
 1 g/l Coomassie Brilliant Blue
 (R-250)

Destaining Solution : 30 % Isopropanol
 10 % Glacial acetic acid

3.5.10. Stock Solutions for Non-denaturing Polyacrylamide Gel Electrophoresis for Ternary Complex Analysis

10 X PAGE Buffer for Ternary
 Complex Analysis : 100 mM MOPS
 50 mM Mg Acetate
 650 mM NH₄ Acetate
 10 mM EDTA
 pH = 6.65 (adjusted with KOH)

Gel Buffer for Ternary Complex Analysis : 5 ml acryl:bis 40 % (19:1)
 4 ml 10 x PAGE buffer
 400 µl APS 10 %
 4 µl 100 mM GTP
 200 µl 50 mM DTE

Running Buffer for Ternary
 Complex Analysis : 100 ml 10 x PAGE Buffer
 100 µl 100 mM GTP
 154 mg DTE
 Add up to 1 lt with dH₂O
 (prepared ice-cold)

3.5.11. Buffer for Cell Lysis and Homogenization

10X Homogenization buffer : 500 mM Hepes-KOH (pH 7.5)
1.5 M KCl
100 mM MgCl₂

For 1X homogenization buffer the following components are added freshly:

10 % Glycerol
7 mM β -Mercaptoethanol
10 μ M GDP
0.1 mM PMSF

3.5.12. Stock Solutions for Non-denaturing Polyacrylamide Gel Electrophoresis for EF-Tu.EF-Ts Binding

10 X Gel Buffer for EF-Tu.EF-Ts

Binding : 80 mM Tricine (pH = 8.2)

Gel Buffer for EF-Tu.EF-Ts

Binding : 3 ml 10 x Gel Buffer
7.5 ml acryl:bis 40 % (37.5:1)
19.5 ml dH₂O

Running Buffer for EF-Tu.EF-Ts

Binding : 100 ml 10 x Gel Buffer
900 ml dH₂O
(prepared ice-cold)

3.5.13. Buffers for Ni-NTA Affinity Chromotography

Buffer A : 50 mM Hepes KOH (pH 7.5)
0.15 M KCl

		10 mM MgCl ₂
		10 % Glycerol
		7 mM β-Mercaptoethanol
		10 μM GDP
		0.1 mM PMSF
Buffer C	:	50 mM Hepes KOH (pH 7.5)
		0.15 M KCl
		10 mM MgCl ₂
		10 % Glycerol
		7 mM β-Mercaptoethanol
		10 μM GDP
		0.1 mM PMSF
		1 M NH ₄ Cl
		10 mM imidazole

3.5.14. Polymix Buffer for Dialysis, Storage and Assay

1X Polymix Buffer	:	95 mM KCl
		5 mM NH ₄ Cl
		5 mM MgAc ₂ ·4H ₂ O
		0.5 mM CaCl ₂ ·2H ₂ O
		8 mM Putrescine
		1 mM Spermidine
		5 mM K-phosphate pH 7.5
		1 mM DTE

10 X Polymix buffer is prepared without K-phosphate to avoid precipitation and DTE. In assays, correct working strength of 1X Polymix buffer is obtained by mixing 10X Polymix buffer appropriately with 100 mM K-phosphate and 50 mM DTE. For dialysis DTE is freshly added as solid.

3.6. Expression Vector

In figure 3.1. the original vector used in this study was shown. The other two vectors mentioned below was obtained from this vector.

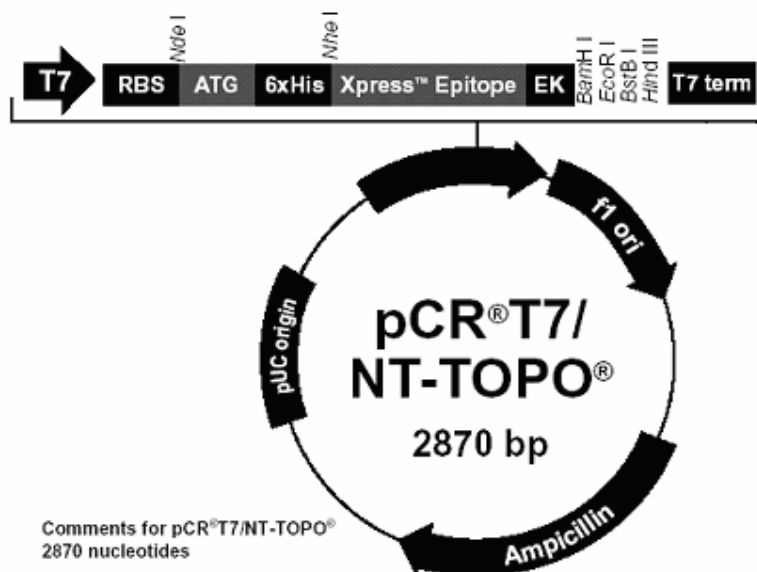


Figure 3.1. Map of pCR® T7/NT-TOPO® vector

(From http://www.invitrogen.com/content/sfs/vectors/p crt7nttopo_map.pdf)

pCR® T7/NT-TOPO®	:	INVITROGEN, USA
pT7D3	:	Derived from pCR® T7/NT-TOPO® after Nde I and Csp45 I restriction endonuclease reactions (this work)
pT7D3Tu	:	Derived from pT7D3 after ligation of PCR fragment of 6XHis-tag containing <i>Geobacillus</i>

anatolicus tuf gene (this work)

3.7. PCR Product Purification

Wizard SV Gel and
PCR Clean-Up System : Promega, USA

3.8. Equipments

Autoclave : Model MAC-601 (Eyela, Japan)

Balance : MonoBloc Model PB602, USA

Centrifuges : Beckman Microfuge E, Germany
Beckman L7-55 Ultracentrifuge, USA
Beckman J2-MC Centrifuge, USA
Beckman J2-21 Centrifuge, USA
Centurion K2R, United Kingdom

Rotors : Beckman JS-7.5A, JA-14, Germany
Beckman 70 Ti, USA

Deep Freezers (-20°C) : Arçelik 2031D and 2020D, Turkey

Deep Freezer (-80°C) : Sanyo Ultra Low, UK

Refrigerator (4°C) : Arçelik 1071D, Turkey

Documentation System : BioRAD, USA

Gel Drier : Honeywell, UK

Magnetic Stirrer	:	Jankey δ Kunkel KM02, USA
Ovens	:	Microwave Oven (Vestel, Turkey) 110°C (Gallenkamp 300, UK)
Incubator	:	65°C (LEEK, USA)
Power Supplies	:	Betherde Research Model 200 (BMC, Uppsala)
Spectrophotometer	:	Agilent 8453, UV-Visible, USA
Thermocyclers	:	Techne (Progene, UK) Techne Gradient (BioRAD, USA)
UV Transilluminator	:	Chromato-Vue Transilluminator, Model 1TM-20UVP (USA)
Water Baths	:	RE100B, Grant, UK and Innova 3100, USA
Heat-block	:	Grant FE10, Cambridge, UK
pH Meter	:	Jenway 3010, USA
Vortex	:	MS2 Minishaker IKA, USA
Beat Beater	:	Model 1107900, Biospec Products,USA
Liquid Chromotography system	:	Pharmacia, Sweden

4. METHODS

4.1. Extraction of the Genomic DNA from *Geobacillus anatolicus*

Geobacillus anatolicus glycerol stock was taken from -80°C freezer and cells were streaked out on agar plates containing the growth medium at pH 8.5. Agar plates were incubated at 65°C overnight. A toothpick of cells was taken from agar plates and resuspended in a 1.5 ml sterile eppendorf tube containing 750 µl TE buffer. Then 23 µl of 20 % SDS and 9.3 µl of 10 mg/ml proteinase K were added to the tube. The solution was vortexed well and incubated at 37°C for 1 h. For the deproteinization of DNA, approximately one volume of TE-saturated phenol/chloroform mixture (1:1) was added to the tube. The tube was vortexed and centrifuged at RT for 2 min. The upper phase was transferred into another tube carefully avoiding denaturated proteins that are found at the aqueous/phenol interface. Extraction step was repeated. The upper phase from the last extraction step was transferred into another tube and one volume of chloroform was added. After vortexing, the tube was centrifuged at RT for 2 min. The upper phase was transferred into another tube and 0.1 volume sodium acetate (pH = 5.2) was added. After vortexing 0.6 volume of isopropanol was added. Then the tube was placed on ice for 15 min. After that the tube was centrifuged at 4 °C for 5 min. The upper phase was discarded and DNA pellet was washed with 1 ml of ice-cold %70 ethanol in order to remove salts. Then the tube was centrifuged at 4 °C for 5 min. The upper phase was discarded. DNA pellet was dried under N₂ and after drying 750 µl TE buffer was added into the tube. RNase-A was then added to a final concentration of 0.1 mg/ml and the tube was incubated at 37°C for 30 min in order to remove RNA. After that one volume of phenol was added to the tube. The tube was vortexed and centrifuged at RT for 2 min. After phenol extraction the upper phase was transferred into another tube. To remove phenol from the sample, the extraction step was repeated with one volume of chloroform two times. Then the upper phase was transferred into another tube and 0.1 volume sodium acetate (pH = 5.2) was added. After vortexing 0.6 volume of isopropanol was added. Then the tube was placed on ice for 15 min. After that the tube was centrifuged at 4 °C for 5 min. The supernatant was discarded and DNA pellet was washed with 1 ml of ice-cold %70 ethanol in order to remove salts. Then the tube was centrifuged at 4 °C for 5 min. The upper phase was discarded. DNA pellet was dried under N₂. DNA pellet was dissolved in 100 µl TE buffer at RT overnight. DNA was stored in

-20°C freezer. The concentration of the genomic DNA was determined spectrophotometrically. The calculation was based on the fact that 50 µg of double stranded DNA has an absorbance of 1.0 at OD_{260 nm}. 260 nm/280 nm ratio of the absorbances were used for DNA quality estimations.

4.2. Analysis of Extracted DNA by Agarose Gel Electrophoresis

Based on the DNA ladder types used, 1-2 % agarose gels in 1X TAE buffer were used to visualize and analyze the extracted genomic DNA. 50 ml TAE buffer containing %1-2 agarose was melted in microwave oven, cooled down below 55 °C and ethidium bromide was added to a final concentration of 0.5 µg/ml under a hood. The solution was poured into the electrophoresis plate. A comb was placed onto the electrophoresis plate. Gel was polymerized at RT under a hood. Then gel was placed into an electrophoresis chamber containing 1X TAE buffer. The comb was removed. 1 µl 6X loading dye and 5 µl of samples were mixed and applied to the wells. A DNA ladder was applied to one of the wells also. The electrophoresis was at 150 Volt for 15-20 min. The gels were then analyzed under UV_{254nm} light and the images were recorded digitally.

4.3. Analysis of *Geobacillus anatolicus* Elongation Factor Gene (*tuf*)

4.3.1. Degenerate Primer Design for Unknown *G. anatolicus tuf* Gene

Because there were no nucleotide or amino acid sequence data for *G. anatolicus tuf* gene at the beginning of this study, to obtain partial sequence of *tuf* gene, two degenerate primers were designed based on nucleotide sequences of EF-Tu genes which come from various bacteria that are closely related to *G. anatolicus*. By using CLUSTALW multiple alignment technique, the sequences were aligned and two conserved regions were determined. Based on these conserved regions, two degenerate primers called TuF413 and TuR1174 were designed (See Table 3.2.).

4.3.2. Primer Design for Partially Known *G. anatolicus tuf* Gene

After sequencing of PCR product obtained with TuF413 and TuR1174, partial sequence of *tuf* gene was determined. Based on these data, within this region two new primers, TuF888 and TuR766 were designed (See Table 3.2.). These primers were used directly in sequencing reactions to confirm the obtained sequence data and reveal flanking regions of *tuf* gene.

4.3.3. Degenerate Primer Design for Adjacent Genes *fus* and *rplC*

To confirm sequence data obtained with, TuF413, TuF888, TuR766 and TuR1174, two primers were designed for adjacent genes *fus* and *rplC* (See Figure 1.7.). Because there were no nucleotide or amino acid sequence data for *G. anatolicus fus* and *rplC* genes at the beginning of this study, two degenerate primers were designed based on nucleotide sequences of *fus* and *rplC* genes which come from various bacteria that are closely related to *G. anatolicus*. By using CLUSTALW multiple alignment software, the sequences were aligned and one conserved region was determined for each gene. Based on these conserved regions, two degenerate primers called FusF and RplC were designed (See Table 3.2.).

4.3.4. PCR Amplifications of *tuf* and Adjacent Genes *fus* and *rplC*

Three PCR amplifications were performed on the *G. anatolicus* DNA. First of all, *tuf* gene was partially amplified using TuF413 and TuR1174 degenerate primers. PCR reactions were performed in 25 µl volume in PCR buffer containing 2.5 mM of MgCl₂, 0.2 mM of each dNTP, 1 pmol of each primer, 50 ng of genomic DNA and 0.125 units of *Taq* DNA polymerase. PCR cycles were as follows:

94°C for 2 minutes 30 seconds	} X 30
95°C for 1 minutes 30 seconds (denaturation)	
50-60°C gradient for 1 minute 30 seconds (annealing)	
72°C for 2 minutes 20 minutes (extension)	
72°C for 7 minutes	

Secondly, by using degenerate FusF and RplC primers, the fragment that contains complete *tuf* was amplified. PCR reactions were performed in 25 µl volume in PCR buffer containing 2.5 mM of MgCl₂, 0.2 mM of each dNTP, 1 pmol of each primer, 50 ng of genomic DNA and 0.125 units of *Taq* DNA polymerase. PCR cycles were as follows:

94°C for 2 minutes 30 seconds	
95°C for 1 minutes 30 seconds (denaturation)	
50-60°C gradient for 1 minute 30 seconds (annealing)	} X 35
72°C for 4 minutes (extension)	
72°C for 7 minutes	

Thirdly, in order to clone *tuf* gene, by using TuF2 and TuR2 primers, complete *tuf* gene was amplified. PCR reactions were performed in 25 µl volume in PCR buffer containing 1.5 mM of MgCl₂, 0.2 mM of each dNTP, 1 pmol of each primer, 50 ng of genomic DNA and 0.125 units of *Taq* DNA polymerase. PCR cycles were as follows:

94°C for 2 minutes 30 seconds	
95°C for 1 minutes 30 seconds (denaturation)	
50-65°C gradient for 1 minute 30 seconds (annealing)	} X 35
72°C for 2 minutes (extension)	
72°C for 7 minutes	

After amplification, 5 µl of each PCR product was mixed with 1 µl 6 X loading dye and electrophoretically analyzed on 1 % agarose gel.

4.3.5. Purification of PCR Products

Purification of PCR products was performed by using Wizard SV PCR Clean-Up system (Promega) as follows: An equal volume of membrane binding solution was added to the PCR reaction mix. After vortexing, the mixture was applied onto a SV minicolumn assembly (a minicolumn which was placed on a 2 ml collection tube) and incubated at RT for 1 min. Then the tube was centrifuged at 5000xg for 1 min, the flowthrough was discarded and the minicolumn was reinserted into a new 2 ml collection tube. To wash the

column, 700 µl membrane washing solution was added onto the column. After centrifugation for 1 min at 5000xg, the flowthrough was discarded and the column was inserted into a new 2 ml collection tube. The column was washed with 500 µl membrane washing solution and the tube was centrifuged for 5 min at 5000xg. The flowthrough was discarded and a new sterile 2 ml collection tube was placed under the minicolumn. After adding 50 µl nuclease-free water to the minicolumn, the column was left at room temperature for 1 min then centrifuged for 5 min at 5000xg to collect the purified DNA. Purified PCR products were electrophoretically analyzed on 1 % agarose gel.

4.3.6. DNA sequencing

The fragment, which was amplified by using TuF413 and TuR1174 was sequenced with the same primers. The fragment, which was amplified by using FusF and RplC was sequenced with FusF, RplC, TuF888, TuR776. The six sequence data that comes from sequencing reactions was aligned and used to construct complete *tuf* sequence. DNA sequencing was carried out by cycle sequencing method using DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, USA) using ABI 9700 Thermocycler (Applied Biosystems) at Acıbadem Hospital, Istanbul. Purified PCR products (or plasmids) were mixed with the sequencing mixture containing 5 pmol primer, 8 µl of sequencing reagent premix and water to a total reaction volume of 20 µl. The sequencing reaction was performed for both forward and reverse primers separately. The reaction conditions are 25 cycles of 95°C for 20 s, 50°C for 15 s and 60°C for 60 s. After cycle sequencing, the unbound dyes were removed by using DyeEx 2.0 Dye removal Kit (Qiagen). The purified products were analysed on the ABI 3100 Genetic Analyzer (Applied Biosystems). The sequencing results were analyzed by using CHROMAS software.

4.4. Cloning of *G. anatolicus tuf* Gene

4.4.1. Restriction Enzyme Digestions of Plasmid pCR® T7/NT-TOPO® and PCR Fragment Obtained Using TuF2 and TuR2 Primers

Restriction enzyme digestions were performed as a two-step procedure. Firstly, 20 µl of reaction mixture that contains Buffer D, acetylated BSA, 2 µl of either PCR fragment

(obtained using TuF2 and TuR2) or plasmid and 20u of NdeI restriction enzyme was incubated at 37 °C for 4 hours. Secondly, Buffer B, acetylated BSA and 40u of Csp45 I restriction enzyme were added into reaction mixture and reaction volume was adjusted to 40 µl. The reaction mixture was incubated at 37 °C for 4 hours again. As a control, 2µg of plasmid pCR[®] T7/NT-TOPO[®] was digested using the same procedure. 5 µl of samples were taken from reaction mixtures for analyzing in agarose gel electrophoresis to confirm digestion. pCR[®] T7/NT-TOPO[®] was named as pT7D3 after successful double-digestion.

4.4.2. Purification of the Digested PCR Fragment and Plasmid pT7D3

To obtain purified double digested PCR fragment, Wizard SV Gel and PCR Clean-Up System (Promega, USA) was used. Because DNA fragments more than approximately 100 bp aren't retained on the column in this system, PCR fragment could be separated from the digested parts of the fragment (digested parts were 7-9 bp long).

To obtain purified plasmid, 40 µl reaction mixture was loaded on 1% agarose gel. After the run, the longer of the two bands appearing in the gel was cut out of the gel. This gel slice was applied to Wizard SV Gel and PCR Clean-Up System (Promega, USA) for the extraction of the digested DNA from gel.

4.4.3. Ligation of the Digested PCR fragment and Plasmid pT7D3

Content of 10 µl of ligation mixture to ligate the digested PCR fragment and pT7D3 was as follows: T4 DNA Ligase Buffer, 100ng of digested plasmid pT7D3, 1u of T4 DNA Ligase 41,2 ng of digested PCR fragments. The ligation reaction were carried out at 11°C, overnight. Plasmid containing the PCR fragment was named pT7D3Tu.

As a ligation control reaction, Csp45I-digested plasmid was used. At this same 30 µl of reaction volume, 7u T4 DNA Ligase and 700 ng plasmid were used. The rest of the procedure was same as in the ligation experiment mentioned above.

4.5. Transformation of the *Escherichia coli* JM109 (DE3) Cells

4.5.1. Preparation of *Escherichia coli* JM109 (DE3) Competent Cells

Escherichia coli JM109(DE3) cells were streak out on LB plates and incubated at 37 °C overnight. Then cells were subcultured in 20 ml liquid LB medium at 37 °C to OD₆₀₀ = 0.1. Cells were grown until OD₆₀₀ = 0.5 at 200 rpm; the cell growth was monitored spectrophotometrically by taking 1 ml samples and measuring the OD₆₀₀ values. When the absorbance reached to OD₆₀₀ = 0.5, cells were subcultured 1:100 in 20 ml LB liquid medium and grown until OD₆₀₀ = 0.4 at 200 rpm. Then cells were collected by centrifugation using Beckman JA-14 rotor at 4 °C at 5000 rpm for 10 min. The supernatant was discarded and 2 ml of ice cold TSS buffer was added onto cell pellet. Cells were resuspended on ice gently stirring cells. Then cells were dispensed into ice cold eppendorf tubes in 100 µl aliquots in cold room. The tubes were stored in -80 °C freezer.

4.5.2. Transformation

JM109(DE3) competent cells were thawed on ice and 2 µl of ligation reaction containing 50 ng of pT7D3Tu, or (as a positive control) 2 µl of solution containing 50 ng of pCR[®]T7/NT-TOPO[®] plasmid or (as a negative control) 2 µl of water were added onto cells (See results section 5.2.4). The cells were incubated on ice for 30 min, then heat shocked at 42°C for 1 min 30 sec, then immediately transferred onto ice and kept on ice for 2 min. 1 ml of ice cold SOC buffer was added onto the cells. The cells were incubated at 37 °C for 50 min for recovery and for the expression of the plasmid encoded genes. For each transformation 1 ml of each sample were layered on LB-agar plates containing 150 µg/ml ampicillin, and incubated overnight at 37°C. Colonies that had been grown on the plates were re-streaked on new plates twice in order to obtain homogeneous populations of transformants.

4.6. Verification of Existence of *tuf* Gene in the Plasmid pT7D3Tu

4.6.1. Plasmid Purification

After obtaining homogeneous populations of transformants, a single colony streaked out on an agar LB-ampicillin plate and incubated overnight at 37°C. A tooth-pick of cells were taken from the plate and resuspended in ice cold 200 µl of TEL buffer by vortexing vigorously. Then 400 µl of NAS solution was added into the suspension. Cells were lysed by gently inverting the microtube for about ten times. The mixture was stored on ice for 5 min. 300 µl of KAC solution was added gently mixed and the mixture was kept on ice for 5 min to precipitate the chromosomal DNA. The mixture was centrifuged for 5 min at 20000xg. Approximately 750 µl of the supernatant was transferred into a new tube and the plasmid DNA was precipitated by adding two volume of ice-cold 100% ethanol. The mixture was stored on ice for 5 min and then centrifuged for 5 min at 20000xg. Supernatant was discarded and the plasmid DNA pellet was washed with ice cold 70 % ethanol. Then the solution was centrifuged again for 2 min at 20000xg. DNA pellet was air-dried for 15 min, dissolved in 50 µl TE buffer and stored at -20°C.

4.6.2. Confirmation of *tuf* Fragment in the Plasmid pT7D3Tu by Using PCR

To confirm that *tuf* gene is present in the pT7D3Tu, a PCR reaction was performed using two primers: T7 which complements to a region located on the plasmid and TuR1174 which complements to a region located on the *tuf* sequence. PCR reaction was performed in 25 µl volume in PCR buffer containing 1.5 mM of MgCl₂, 0.2 mM of each dNTP, 1 pmol of each primer, 50 ng of plasmid DNA and 0.25 units of *Taq* DNA polymerase. PCR cycles were as follows:

94°C for 2 minutes 30 seconds	} X 30
95°C for 1 minutes 30 seconds (denaturation)	
52°C for 1 minute 30 seconds (annealing)	
72°C for 2 minutes 30 seconds (extension)	
72°C for 7 minutes	

After amplification, 5 µl of each PCR product was mixed with 1 µl 6 X loading dye and electrophoretically analyzed on a 1 % agarose gel.

4.6.3. PCR Amplification of the Plasmid pT7D3Tu for DNA Sequencing

After confirmation of the presence of the *tuf* gene in the pT7D3Tu, a fragment that contains the complete *tuf* gene was amplified by using T7 and pRSET primer pair. PCR reaction was performed in 100 µl volume in PCR buffer containing 1.5 mM of MgCl₂, 0.2 mM of each dNTP, 1 pmol of each primer, 50 ng of plasmid DNA and 1 units of *Taq* DNA polymerase. PCR cycles were as follows:

94°C for 2 minutes 30 seconds	
95°C for 1 minutes 30 seconds (denaturation)	
52°C for 1 minute 30 seconds (annealing)	} X 30
72°C for 2 minutes 30 seconds (extension)	
72°C for 7 minutes	

After amplification, 5 µl of the PCR product was mixed with 1 µl 6 X loading dye and electrophoretically analyzed on 1 % agarose gel. The rest of the PCR product was purified and prepared for sequencing.

4.6.4. Storage of JM109(DE3) Cells Containing pT7D3Tu.

The JM109(DE3) cells containing pT7D3Tu plasmid were grown overnight on agar LB plate containing ampicillin. A toothpick of cells were resuspended in 1 ml liquid LB medium containing 50 % glycerol. Cells were resuspended until a homogeneous suspension was obtained and stored at -80°C freezer.

4.7. Expression of *G. Anatolicus tuf* Gene in JM109(DE3) Cells

JM109(DE3) cells containing pT7D3Tu carrying the *tuf* gene and original plasmid without *tuf* gene (as a control) were grown on agar plates overnight. 20 ml of LB liquid medium containing 100 µg/ml ampicillin were inoculated from these plates. Cells were

grown until OD₆₀₀: 0.6 at 37 °C at 200 rpm. At this point, IPTG was added to the flasks to the final concentration of 2 mM and from this point on 500 µl of samples were taken to eppendorf tubes at the beginning and after 2 and 5 hours. Each sample is processed immediately: samples were centrifuged at 20000xg, supernatants were discarded and the pellets were resuspended in 150 µl of protein lysis solution. Samples were placed on ice until use in the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

To facilitate denaturation of proteins, samples were boiled at 90 °C for 5 min before applying to SDS-PAGE. 22,5 µl of each samples, or 15 µl of *E.coli* Tu or 15 µl of protein molecular weight marker were loaded onto 10% SDS-PAGE. Electrophoresis was performed at 10 mA overnight. After electrophoresis, the gel was stained with Coomassie Brilliant Blue staining solution for minimum 2 h then destained until the protein bands become visible.

4.8. Isolation of *G. anaticus* EF-Tu

For the isolation of the recombinant protein, JM109(DE3) cells containing pT7D3Tu were grown in 800 ml of LB liquid medium containing 150 µg/ml ampicilin at 37°C. Cells were grown until OD₆₀₀: 0.6 at 200 rpm. At this point, IPTG was added to the final concentration of 2 mM. Cells were grown for additional 4 hours. The cells were harvested by centrifugation at 10000 rpm at 4°C for 15 min in Beckman JS-14 rotor.

Total 16 g of harvested cells were resuspended in 16 ml of ice cold Buffer A (homogenization buffer). Then equal volume of glass-beads were added to this suspension. Mixture was placed into the chamber of the bead-beater. Ice was placed around the chamber to prevent warming. Cells were disrupted in the Bead-beater for three times 30 second runs with minimum of 2 min waiting intervals in between to prevent warming. The cell lysate was centrifuged at 16000 rpm at 4°C for 30 min using Beckman JA-20 fixed-angle rotor. The supernatant was transferred to a clean centrifuge tube and centrifuged at 45000 rpm at 4°C for 1 h 30 min. The supernatant was saved and then applied directly onto Ni²⁺-affinity column.

4.9. Ni-Affinity Column Chromatography

4.9.1. Ni-Affinity Column Regeneration

Ni-NTA chromatography matrix (Pharmacia) was packed into a glass column (column dimensions: 0.9x6 cm). Column was washed with 50 ml of Buffer A containing 250 mM imidazole. Washing of the column was repeated with 50 ml of dH₂O, 50 ml of 100 mM NiSO₄, 50 ml of dH₂O and 50 ml of buffer A, respectively.

4.9.2. Purification Step

Before applying the samples to the column, the column was pre-equilibrated with 50 ml of equilibration buffer containing 10 mM imidazole. Then, the supernatant was applied to the column with a speed of 0.7 ml/min. To wash the unbound proteins, the column was washed with approximately 100 ml of buffer A containing 1 M NH₄Cl and 10 mM imidazole until OD_{260nm} reaches the base-line. His-tagged recombinant *Geobacillus anatolicus* elongation Factor Tu was eluted with a 100 ml of linear gradient from 25 mM to 100 mM imidazole. Fractions were collected in 7 ml of samples.

After purification step, from each fraction tube 20 µl of samples were taken, mixed with 5 µl 5X SDS-PAGE sample buffer and applied to the 10 % SDS-PAGE for analysis. Electrophoresis was run for 4 h at 30 mA.

4.10. Dialysis and Storage of *G. anatolicus* EF-Tu protein

Fractions containing pure *G.anatolicus* EF-Tu were pooled and placed on ice. 0,5 gr ammonium sulphate per ml of this sample was added slowly under continuous magnetic stirring. After 30 min, the sample was centrifuged at 16.000 rpm for 20 min using Beckman JA-20 fixed-angle rotor. Precipitated protein was dissolved in approximately 3 ml of 1x polymix buffer and dialyzed overnight at 4°C against 1 lt 1x polymix buffer. Dialysis buffer was changed after 4 h with a fresh buffer and dialysis was continued overnight. After dialysis, to discard the particles, sample was centrifuged at 12.000 at 4 °C

for 5 min. Then, sample was divided into 50 and 100 μ l aliquots in microcentrifuge tubes, freezed and stored at -80 °C until use.

4.11. Determination of Protein Concentration

Protein concentration was determined using Bradford assay (Bradford, 1976). Bovine Serum Albumin (BSA) was used as the protein standard. Using 1 mg/ml stock solution of BSA, several dilutions were prepared containing from 1 μ g to 8 μ g of BSA in 0.15 M NaCl in each tube. Final volume of dilutions were 100 μ l. Aproprate dilutions of EF-Tu in 0.15M NaCl was also prepared. Then, 1 ml of Bradford's reagent was added into each tube. Tubes were vortexed and incubated at RT for 10 min. The absorbance of each sample at OD_{595nm} was measured. A plot of the absorbance at 595nm versus the volume of the protein sample was obtained after exactly 10 min. A plot of the absorbance at 595nm versus the known amount (from 1 μ g to 8 μ g) of the BSA protein was obtained. By using BSA plot as a standard, protein concentration of the EF-Tu was calculated.

4.12. Mobility-Shift Assay on Non-Denaturing PAGE for EF-Tu·EF-Ts complex formation

A 10% non-denaturing polyacrylamide gel (37.5:1) was prepared in 8 mM Tricine buffer at pH 8.2 and cooled in a cold room after polymerization . From 0 pmol to 400 pmol of *G. anatolicus* EF-Tu was prepared in 20 μ l of the reaction mix in the presence of 300 pmol *G.anatolicus* EF-Ts. As a control experiment, same titrations were done for *G.anatolicus* EF-Ts and *E.coli* EF-Tu. Samples were prepared on ice and incubated at 37 °C for 10 min then transferred onto ice for at least 10 min before applying to the gel. 20 μ l samples were mixed with 5 μ l loading dye (phenol red in 50% glycerol) from which 20 μ l were applied to 10% non-denaturing PAGE gel. Electrophoresis was done in a cold room at 4°C in the same buffer. Before applying the samples, the gel was prerun for 1 hour at 250 V. Samples were then applied and electrophorized for 3 hrs at 250V. Protein bands were visualized by Commasie staining and destaining.

4.13. Mobility-Shift Assay on Non-Denaturing PAGE for Ternary Complex Formation between EF-Tu·GTP·Phe-tRNA^{Phe}

A non-denaturing 5% polyacrylamide gel (19:1) in ternary complex buffer which was given in Materials section (10 mM MOPS, 10 mM Mg-acetate, pH 6.5, containing freshly added 100 μ M GTP and 1 mM DTE) was prepared. Gel plate was 0.5 mm thick and 20 cm long. After polymerization, gel was transferred to cold room at 4°C. The gel was prerun for 90 min at 40 mA (about 100 V) before applying the samples. 200 pmol of *G.anatolicus* EF-Tu or, as a control, 200 pmol of *E.coli* EF-Tu in 80 μ l of polymix buffer containing 30 nmol of [¹⁴C]-Phe, 0.1 μ mol of ATP and 1 μ mol of PEP, 0.1 μ mol of GTP, 5 μ g of PK, 0.3 μ g of MK and 1 u of Phe-tRNA synthetase was mixed with varying amounts of tRNA^{Phe} in 20 μ l (from 50 pmol to 400 pmol) and incubated at 37 °C for 15 min. After incubation, samples were cooled on ice for at least 15 min. Then 20 μ l of samples were mixed with 2 μ l of sample buffer containing 50 % glycerol and traces of Bromophenolblue as a marker. Samples were applied onto pre-electrophoresed 5 % non-denaturing PAGE gel. The electrophoresis was performed at 60 mA (about 150 V) for 3 hours. After electrophoresis, protein bands were visualized by Commassie staining and destaining. Duplicate samples were used to calculate the amount of the Phe-tRNA. For this purpose, after the incubations, 10% ice-cold TCA was added onto samples and kept on ice until filtering. Samples were filtered through glass fiber filters (Whatman, GF/C), washed with approximately 5 ml of each ice-cold 10% TCA and isopropanol. Filters were dried at 110 °C for 15 min. 4 ml of scintillation cocktail was added onto the samples. The radioactivity retained on the filters were counted using a liquid scintillation counter.

4.14. Thermostability Test

At first, a binding mix, 90 μ l for each sample, in polymix buffer containing 0.5 mM ³H-GDP was prepared (specific activity 6.6 cpm/pmol). The binding mixes were stored on ice until use. Then 10 μ l of samples containing 68 pmol/ μ l of EF-Tu in polymix buffer were incubated at between 20-90 °C in 10 °C intervals for 10 min. After incubation the samples were cooled on ice for at least 30 min. Then the samples were mixed with 90 μ l of the binding mix and incubated at 37 °C for 15 min. After incubation the samples were placed on ice for 10 min. To each sample approximately 3 ml of ice-cold polymix buffer

was added and the samples were immediately filtered through nitrocellulose filters (Schleicher and Schüll, BA85). Nitrocellulose filters were washed twice, each time with 3 ml of polymix buffer and dried at 110 °C for 15 min. 4 ml of scintillation cocktail was added onto the samples. The radioactivity retained on the filters were counted using a liquid scintillation counter.

5. RESULTS

5.1. Determination of *Geobacillus anatolicus* Elongation Factor Tu Gene (*tuf*) Sequence

5.1.1. Extraction of DNA from *Geobacillus anatolicus*

DNA extraction (see section 4.1) from *Geobacillus anatolicus* appears as a single band when analyzed on agarose gels (Figure 5.1).

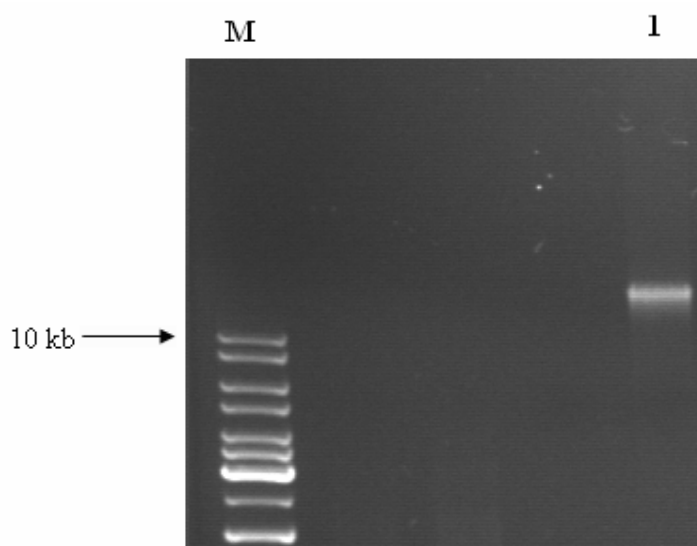


Figure 5.1. Genomic DNA of *Geobacillus anatolicus*. M, DNA Molecular marker; 1, *G. anatolicus* genomic DNA

5.1.2. Primer design

All primers that were used in this study were checked for hairpin structure, homodimer and heterodimer by using OligoAnalyzer software (Integrated DNA Technologies <http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>). ClustalW multiple alignment results were shown in Table 5.1 and Figure 5.2. for *tuf* gene, in Table 5.2. and Figure 5.3. for *fus* gene and in Table 5.3. and Figure 5.4. for *rplC* gene respectively.

Table 5.1. Bacterial *tuf* genes used for degenerate primer design

Organism name	GeneBank ID
<i>Geobacillus stearothermophilus</i>	2661391
<i>Bacillus licheniformis</i> DSM 13	52346471
<i>Oceanobacillus iheyensis</i> HTE831	1015206
<i>Streptococcus pneumoniae</i> TIGR4	14972978
<i>Clostridium acetobutylicum</i> ATCC 824	15026203
<i>Mycoplasma genitalium</i> G-37	3845045
<i>Escherichia coli</i> CFT073	26110363
<i>Aquifex pyrophilus</i>	2687828
<i>Thermus thermophilus</i> HB27	46199632
<i>Thermotoga maritima</i> MSB8	4982068

					
	5	15	25	35	45
Geobacillu	AAGCATCCTA	TACTTACCTA	ACTATACAAA	TTCATATTTT	ACTTTTAAGG
Bacillus l	-----	-----	-----	-----	-----
Oceanobaci	-----	-----	-----	-----	-----
Streptococ	-----	-----	-----	-----	-----
Clostridiu	-----	-----	-----	-----	-----
Mycoplasma	-----	-----	-----	-----	-----
Escherichi	-----	-----GTG	CTCTCTCCTG	AAGGGGAGAG	CACTATAGTA
Aquifex py	-----	-----	-----	-----	-----
Thermus th	-----	-----	-----	-----	-----
Thermotoga	-----	-----	-----	-----	-----
Consensus	AAGCATCCTA	TACTTACSTR	MYWYW CM WR	WWSRKRWKWK	MMY TWT ARKR
					
	55	65	75	85	95
Geobacillu	AGGATCTTTC	TCATGGCTAA	AGCGAAATTT	GAGCGCACGA	AACCGCACGT
Bacillus l	-----	--ATGGCTAA	AGAAAAATTC	GACCGTTCCA	AATCACATGC
Oceanobaci	-----	--ATGGCTAA	AGAAAAATTC	GATCGCTCCA	AAAGTCACGT
Streptococ	-----	--ATGGCAAA	AGAAAAATAC	GATCGTAGTA	AACCACACGT
Clostridiu	-----	--ATGGCAAA	GGAAAAATTT	GAAAGAACGA	AACCACATGT
Mycoplasma	-----	--ATGGCAAG	AGAGAAATTT	GACCGTTCCA	AACCACATGT
Escherichi	AGGAATATAG	CCGTGTCTAA	AGAAAAATTT	GAACGTACAA	AACCGCACGT
Aquifex py	-----	--ATGGCCAA	GGAGAAGTTT	GAGAGGACAA	AAGAACACGT
Thermus th	-----	--ATGGCGAA	GGGCGAGTTT	ATTCGGACGA	AGCCTCACGT
Thermotoga	-----	--ATGGCGAA	GGAAAAATTT	GTGAGAACAA	AACCGCATGT
Consensus	AGGA WYW T WS	YCRTGK C N AR	R GVV R ART WY	RWN MGN WS NA	ARNVD CAYG

Figure 5.2. ClustalW multiple alignment result of nucleotide sequences indicated in Table 5.1. Primer sites (TuF413 and TuR1174) were shown as in blue and consensus sequence was shown in red (Continued).

					
	105	115	125	135	145
Geobacillu	CAACATTGGC	ACGATCGGCC	ACGTTGACCA	TGGGAAAACG	ACGCTGACAG
Bacillus l	CAACATTGGT	ACAATTGGAC	ACGTTGACCA	TGGTAAAACG	ACATTAACAG
Oceanobaci	TAACGTTGGT	ACTTTAGGAC	ACGTTGACCA	TGGTAAAACG	ACATTAACAG
Streptococ	TAACATTGGT	ACTATCGGAC	ACGTTGACCA	CGGTAAAACG	ACCCTAACTG
Clostridiu	AAACATAGGA	ACAATAGGAC	ACGTAGATCA	CGGTAAAACA	ACATTAACAG
Mycoplasma	CAATGTTGGT	ACCATTGGTC	ACATTGACCA	TGGTAAAACC	ACTTTAACAG
Escherichi	TAACGTTGGT	ACTATCGGCC	ACGTTGACCA	CGGTAAAACG	ACTCTGACCG
Aquifex py	AAACGTGGGT	ACCATAGGTC	ACGTTGACCA	CGGAAAATCT	ACGCTTACAT
Thermus th	GAACGTGGGG	ACGATTGGGC	ACGTGGACCA	CGGGAAGACG	ACGCTGACGG
Thermotoga	TAACGTTGGA	ACGATTGGAC	ATATCGACCA	CGGAAAATCC	ACACTGACAG
Consensus	NAAYRTDGGN	ACNWTGGNC	AYRTNGAYCA	YGGDAARWCN	ACNYTDACNK
					
	155	165	175	185	195
Geobacillu	CTGCGATCAC	GACGGTTCTT	---GCGAAAC	AAGGG-----	----AAAGCC
Bacillus l	CTGCTATCAC	TACTGTGCTT	CATAAGAAAT	CTGGT-----	----AAAGGT
Oceanobaci	CTGCTATCAC	TACTGTACTT	---GCAAAAC	ACGGC-----	----GGTGGA
Streptococ	CAGCTATCAC	AACTGTTTTG	-GCACGTCGC	TTGCCTTCA-	-TCAGTTAAC
Clostridiu	CAGCAATAAC	AACAATATTA	---GCAAAAG	AAGGA-----	----AAAGCA
Mycoplasma	CTGCTATCTG	TACAGTTTTA	---GCAAAAG	AAGGA-----	----AAATCA
Escherichi	CTGCAATCAC	CACCGTACTG	---GCTAAAA	CCTAC-----	----GGCGGT
Aquifex py	CAGCTATAAC	ATGCGTTCTC	GCAGCAGGAC	TCGTTGAAGG	TGGTAAAGCT
Thermus th	CGGCGTTGAC	GTA-----	--TGTGGCGG	CGGCGGAGAA	TCCGAATGTA
Thermotoga	CCGCTATAAC	AA-----	--AGTACCTT	TCTCTCAAGG	GACTT--GCC
Consensus	CNGCDWTVWS	NWVNRTDYTN	SVHRHDNVDN	HNKNNBWVRR	KNSDDDDHNDH
					
	205	215	225	235	245
Geobacillu	GAAGCAAAAG	CGTACGACCA	AATCGACGCA	GCTCCGGAAG	AGCGTGAACG
Bacillus l	ACTGCTATGG	CATACGACCA	AATCGACGGT	GCTCCAGAAG	AGCGCGAGCG
Oceanobaci	GAAGCACGTG	CGTATGACCA	AATTGATGGT	GCGCCTGAAG	AAAGAGAACG
Streptococ	CAACCTAAAG	ACTATGCGTC	TATCGATGCT	GCTCCAGAAG	AACGCGAACG
Clostridiu	AAAGCATTCA	ATTACGAAGA	GATTGATAAA	GCACCAGAGG	AAAAAGAAAG
Mycoplasma	GCTGCAACGC	GTTATGATGA	AATTGATAAA	GCCCCTGAAG	AAAAAGCAAG
Escherichi	GCTGCTCGTG	CATTGACCA	GATCGATAAC	GCGCCGGAAG	AAAAAGCTCG
Aquifex py	AAGTGCTTCA	AATACGAAGA	AATTGACAAA	GCTCCAGAAG	AAAAAGAAAG
Thermus th	GAGGTAAAG	ACTACGGGGA	GATTGACAAAG	GCGCCGGAAG	AGCGTGCGCG
Thermotoga	CAGTATATTC	CTTACGACCA	GATCGACAAG	GCCCCTGAAG	AAAAGGCAAG
Consensus	VMDBNHNNV	VNTWYGVNBM	DATYGA YRVN	GCNCCDGARG	ARMRNGMDMG

Figure 5.2. ClustalW multiple alignment result of nucleotide sequences indicated in Table 5.1. Primer sites (TuF413 and TuR1174) were shown as in blue and consensus sequence was shown in red (Continued).

	
	255 265 275 285 295
Geobacillu	CGGGATCACG ATTTTCGACGG CCCACGTCGA GTATGAAACA GAGGCTCGTC
Bacillus l	CGGTATCACA ATTTCTACTG CACACGTTGA GTACGAAACT GACAACCGTC
Oceanobaci	TGGTATCACA ATCTCAACTG CACACGTAGA GTATGAAACT GAAACTCGTC
Streptococ	CGGTATCACT ATCAACACTG CGCACGTTGA GTACGAAACT GAAAAACGTC
Clostridiu	AGGAATAACA ATCAACACAG CACACGTTGA GTATGAAACA GAGAATAGAC
Mycoplasma	GGGAATCACA ATTAACCTCTG CACACGTAGA ATATTCTTCT GACAAACGTC
Escherichi	TGGTATCACC ATCAACACTT CTCACGTTGA ATACGACACC CCGACCCGTC
Aquifex py	AGGTATAACC ATAAACATCA CTCACGTTGA GTACGAAACC GCTAAGAGAC
Thermus th	GGGGATTACG ATCAACACGG CGCACGTGGA GTACGAGACG GCGAAGCGGC
Thermotoga	AGGAATCACC ATCAACATCA CACACGTTGA GTATGAGACC GAAAAGAGAC
Consensus	NGGDATHACN ATHWMN WYND CNCACGTNGA RTAYKMNW CN SMNRMNM GD C
	
	305 315 325 335 345
Geobacillu	ACTACGCGCA CGTTGACTGC CCGGGCCACG CTGACTACGT CAAAAACATG
Bacillus l	ACTATGCACA CGTTGACTGC CCAGGACACG CTGACTATGT TAAAAACATG
Oceanobaci	ACTATGCACA CGTTGACTGC CCAGGTCACG CTGACTATGT TAAAAACATG
Streptococ	ACTACGCTCA CATCGACGCT CCAGGACACG CGGACTACGT TAAAAACATG
Clostridiu	ACTATGCTCA CGTTGACTGC CCAGGACATG CTGACTATGT AAAGAACATG
Mycoplasma	ACTATGCCCA TGTGACTGT CCTGGACATG CTGACTACAT TAAAAATATG
Escherichi	ACTACGCGCA CGTAGACTGC CCGGGGCACG CCGACTATGT TAAAAACATG
Aquifex py	ACTACGCACA CGTTGACTGC CCCGGACACG CGGACTACAT CAAGAACATG
Thermus th	ACTATTCCCA CGTGGATTGC CCTGGGCACG CGGACTACAT CAAGAACATG
Thermotoga	ACTACGCTCA TATTGACTGT CCCGGTCACG CGGACTACAT CAAGAACATG
Consensus	ACTAYKCNCA YR TNGAY KSY CCNGGNCAYG CBGACTAYRT H AAAA YATG
	
	355 365 375 385 395
Geobacillu	ATCACGGGCG CAGCGCAAAT GGACGGTGCA ATCCTTGTTG TATCGGCAGC
Bacillus l	ATCACTGGTG CTGCGCAAAT GGACGGTGCG ATCCTTGTTG TTTCTGCTGC
Oceanobaci	ATCACTGGTG CTGCACAAAT GGACGGAGCT ATCCTTGTA G TATCTGCTGC
Streptococ	ATCACTGGTG CTGCTCAAAT GGACGGAGCT ATCCTTGTA G TAGCTTCAAC
Clostridiu	ATTACAGGAG CAGCGCAAAT GGATGGAGCA ATCCTAGTAG TAAGTGCAGC
Mycoplasma	ATCACAGGTG CTGCACAAAT GGATGGAGCT ATTCTAGTTG TTTCAGCAAC
Escherichi	ATCACCGGTG CTGCTCAGAT GGACGGCGCG ATCCTGGTAG TTGCTGCGAC
Aquifex py	ATTACCGGTG CGGCCAGAT GGACGGGGCT ATACTCGTGG TTTCCGCAGC
Thermus th	ATCACGGGTG CCGCGCAGAT GGACGGGGCG ATCCTTGTTG TGTCCGGCGC
Thermotoga	ATCACAGGAG CAGCTCAGAT GGACGGAGCC ATCCTTGTTG TTGCCGCAAC
Consensus	ATYACNGGHG CNGCNCARAT GGAYGGNGCN ATHCTNGTDG TDDSNKCDRC

Figure 5.2. ClustalW multiple alignment result of nucleotide sequences indicated in Table 5.1. Primer sites (TuF413 and TuR1174) were shown as in blue and consensus sequence was shown in red (Continued).

					
	405	415	425	435	445
Geobacillu	TGACGGTCCG	ATGCCGCAAA	CGCGCGAACA	CATTCTTCTC	TCCCGCCAAG
Bacillus l	TGACGGCCCA	ATGCCACAAA	CTCGTGAGCA	CATCCTTCTT	TCTCGTAACG
Oceanobaci	TGATGGCCCA	ATGCCACAAA	CTCGTGAGCA	CATCCTTCTA	TCTCGTAACG
Streptococ	TGACGGACCA	ATGCCACAAA	CTCGTGAGCA	CATCCTTCTT	TCACGTCAGG
Clostridiu	AGATGGTCCA	ATGCCACAAA	CAAGAGAGCA	CATACTATTG	GCATCAAGAG
Mycoplasma	TGATAGTGTG	ATGCCCCAAA	CCCGCGAGCA	CATCTTACTT	GCCCGCCAAG
Escherichi	TGACGGCCCG	ATGCCGCAGA	CTCGTGAGCA	CATCCTGCTG	GGTCGTCAGG
Aquifex py	TGACGGCCCG	ATGCCCCAAA	CCAGAGAACA	CGTCCTTCTC	GCAAGACAGG
Thermus th	GGACGGGCCG	ATGCCGCAGA	CGCGGGAGCA	CATTTTGCTG	GCGCGGCAGG
Thermotoga	CGATGGTCCC	ATGCCCCAGA	CAAGAGAGCA	CGTGCTTCTC	GCAAGACAGG
Consensus	NGAYR GS YV	ATGCCVCARA	CNMGN GARCA	CR TNY TDY TN	KSNHNSNMRV G
					
	455	465	475	485	495
Geobacillu	TCGGCGTTCC	GTACATCGTT	GTTTTCTTGA	ACAAATGCGA	CATGGTGGAC
Bacillus l	TAGGTGTGCC	TTACATCGTA	GTATTCTTGA	ACAAATGCGA	CATGGTAGAC
Oceanobaci	TTGGGGTACC	TGCTTTCGTA	GTATTCCTTA	ACAAAACAGA	TATGGTTGAC
Streptococ	TTGGTGTAA	ACACCTTATC	GTCTTCATGA	ACAAAGTTGA	CTTGGTTGAC
Clostridiu	TTGGTGTGA	ATATATAGTA	GTATTCTTAA	ATAAAGCAGA	CCAAGTAGAT
Mycoplasma	TAGGGGTTC	TAAAATGGTA	GTTTTCTTAA	ACAAGTGTGA	TATTGTAGT
Escherichi	TAGGCGTTCC	GTACATCATC	GTGTTCCCTGA	ACAAATGCGA	CATGGTTGAT
Aquifex py	TTAACGTTCC	CTACATAGTC	GTTTTCATGA	ACAAGTGTGA	TATGGTTGAC
Thermus th	TGGGGGTGCC	GTACATTGTG	GTGTTTCATGA	ACAAGGTGGA	CATGGTGGAC
Thermotoga	TTGAGGTTCC	CTACATGATC	GTCTTCATAA	ACAAGACAGA	CATGGTTGAC
Consensus	TN RRBG TD VM	NNMHHT NR TN	GTNTT Y HTDA	AYA ARDBN GA	YHWD G YDRRY
					
	505	515	525	535	545
Geobacillu	GACGAAGAAT	TGCTTGAACT	CGTTGAAATG	GAAGTTCGCG	ACCTTCTCTC
Bacillus l	GACGAAGAGC	TTCTTGAGCT	AGTTGAAATG	GAAGTTCGCG	ATCTTCTTTC
Oceanobaci	GATGAAGAGC	TACTTGAATT	AGTAGAAATG	GAAGTTCGTG	ACCTATTAAC
Streptococ	GACGAAGAAT	TGCTTGAATT	GGTTGAAATG	GAAATCCGTG	ACCTATTGTC
Clostridiu	GATCCAGAAT	TAATCGACTT	AGTAGAAATG	GAAGTAAGAG	AGTTATTAAA
Mycoplasma	GATGAAGAGG	TACAAGAACT	TGTTGCTGAA	GAAGTACGTG	ATCTGTTAAC
Escherichi	GACGAAGAGC	TGCTTGAACT	GGTTGAAATG	GAAGTTCGTG	AACTTCTGTC
Aquifex py	GACGAAGAGC	TCCTTGAGCT	CGTTGAGCTT	GAAGTGAGAG	AGCTTCTCAG
Thermus th	GACCCCGAGT	TGCTGGACCT	GGTGGAGATG	GAGGTGCGGG	ACCTTTTGAA
Thermotoga	GATCCTGAGC	TCATCGACCT	CGTCGAGATG	GAAGTGAGAG	ACCTTCTGAG
Consensus	GA YSMH GA RB	TN MWNG AVYT	NGT NGMDVWD	GARR TNM GNG	ANY T DY TNWV

Figure 5.2. ClustalW multiple alignment result of nucleotide sequences indicated in Table 5.1. Primer sites (TuF413 and TuR1174) were shown as in blue and consensus sequence was shown in red (Continued).

					
	555	565	575	585	595
Geobacillu	TGAATACGAC	TTCCCGGGCG	ATGAAGTGCC	GGTTATCAAA	GGTTCGGCAT
Bacillus l	TGAGTATGAG	TTCCCTGGTG	ACGATGTACC	AGTTATCAAA	GGTTCGTCTC
Oceanobaci	TGAGTACGAT	TTCCCTGGTG	ACGATCTACC	AGTAATCAAA	GGTTCGTGAC
Streptococ	AGAATACGAC	TTCCAGGTG	ACGATCTTCC	AGTTATCCAA	GGTTCAGCAC
Clostridiu	CGAATATGGA	TTCCAGGGCG	ATGATACACC	AATCGTAGTA	GGAAGTGCCT
Mycoplasma	TTCCTATGGT	TTTGATGGTA	AGAACACTCC	TATTATTTAT	GGCTCAGCTT
Escherichi	TCAGTACGAC	TTCCCGGGCG	ACGACACTCC	GATCGTTCGT	GGTTCGTCTC
Aquifex py	CAAATACGAG	TATCCCGGAG	ACGAAGTTCC	CGTAATAAGA	GGTTCCGCTC
Thermus th	CCAGTACGAG	TTTCCTGGGG	ACGAGGTTCC	GGTGATTCCG	GGGAGTGCTC
Thermotoga	CCAGTACGGT	TACCCTGGAG	ACGAAGTGCC	AGTCATAAGA	GGTTCGTCTC
Consensus	HNMV TAYGRN	TWYSMNGG NR	ABRANVYDCC	NR TNR THNDD	GGNWSNGCDY
					
	605	615	625	635	645
Geobacillu	TAAAAGCGCT	C--GAAGGCG	-----ATCC	GAAATGGGAA	----GAAAAA
Bacillus l	TTAAAGCTCT	T--GAAGGAG	-----ATGC	TCAGTACGAA	----GAAAAA
Oceanobaci	TTAAAGCTCT	A--GAAGGCG	-----TTGC	AGAATACGAA	----GAAAGA
Streptococ	TTAAAGCTCT	T--GAAGGTG	-----ACTC	TAAATACGAA	----GACATC
Clostridiu	TAAAAGCATT	A--CAGAATC	C-----AGAT	GATGCAGAAG	C---AATAAA
Mycoplasma	TAAAAGCATT	G--GAAGGTG	-----ATCC	AAAGTGGGAG	----GCTAAG
Escherichi	TGAAAGCGCT	G--GAAGGCG	-----ACGC	AGAGTGGGAA	----GCGAAA
Aquifex py	TGGGAGCCCT	TCAGGAGCTT	GAACAAAAC	CTCCCGGAAA	GTGGGTAGGA
Thermus th	TTTTGGCGCT	TGAGCAGATG	CACAGGAACC	CGAAGACGAG	GCGTGGGGAG
Thermotoga	TGAAAGCCGT	C--GAAGCTC	CTAACGATCC	GAATCACGAA	GCTTACAA-A
Consensus	TDDDRGCNBT	NS ASVRRV HB	SWMMVRWNNY	NNHNBR SRAR	SYKKRNNR DV
					
	655	665	675	685	695
Geobacillu	ATCA-----	-----TT	----GAATTG	ATGAACGCGG	TTGATGAGTA
Bacillus l	ATCT-----	-----TT	----GAAGT	ATGGCTGCTG	TTGACGAGTA
Oceanobaci	ATTC-----	-----TT	----GAGTTA	ATGGCTGCAG	TTGATGAGTA
Streptococ	GTTA-----	-----TG	----GAATTG	ATGAACACAG	TTGATGAGTA
Clostridiu	ACCA-----	-----AT	AAAAGACTTA	ATGGCAGAAG	TAGATGCATA
Mycoplasma	ATCC-----	-----AT	----GATTTG	ATTAAAGCAG	TTGATGAATG
Escherichi	ATCC-----	-----TG	----GAAGT	GCTGGCTTCC	TGGATTCTTA
Aquifex py	AGC-----	-----AT	AAAGGAACTT	CTGAACGCGA	TGGACGAGTA
Thermus th	AACGAGTGGG	TGGACAAGAT	TTGGGAGCTG	TTGGACGCGA	TTGACGAGTA
Thermotoga	CCC-----	-----AT	CCAGGAGCTC	CTCGACGCTA	TGGATAACTA
Consensus	VNYN AGTGGG	TGGACAAG WK	HHRR GANYTN	NYBRVHDHNV	TDGAYDMNTR

Figure 5.2. ClustalW multiple alignment result of nucleotide sequences indicated in Table 5.1. Primer sites (TuF413 and TuR1174) were shown as in blue and consensus sequence was shown in red (Continued).

					
	705	715	725	735	745
Geobacillu	CATCCCGACT	CCGCAACGTG	AAGTTGACAA	ACCGTTCATG	ATGCCGATTG
Bacillus l	CATCCCAACT	CCAGAGCGTG	AAACTGACAA	GCCATTCATG	ATGCCTGTTG
Oceanobaci	TATCCCAACT	CCAGAACGTG	ACAAAGAAAA	ACCATTTCATG	ATGCCAGTTG
Streptococ	TATCCCAGAA	CCAGAACGTG	ACACTGACAA	ACCATTGCTT	CTTCCAGTCG
Clostridiu	CATCCCAACA	CCAGAAAGAC	CAACAGATAA	AGCATTCTTA	ATGCCAATCG
Mycoplasma	GATTCCAACT	CCTACACGTG	AAGTAGATAA	ACCTTTCTTA	TTAGCAATTG
Escherichi	CATTCCGGAA	CCAGAGCGTG	CGATTGACAA	GCCGTTCCCTG	CTGCCGATCG
Aquifex py	CATACCGACA	CCTGAGAGAG	AGGTTGACAA	GCCCTTCCTG	ATGCCCATAG
Thermus th	CATTCCCACG	CCGGTGCGGG	ACGTGGACAA	GCCGTTCTTG	ATGCCGGTGG
Thermotoga	CATTCTTGAT	CCTCAGAGAG	ACGTCGATAA	GCCGTTCCCTC	ATGCCCATCG
Consensus	BATHCC NRMD	CCD VHRM GD S	MVRHN GAHAA	RS CN TT SH TN	H TD S CN RT NG
					
	755	765	775	785	795
Geobacillu	AGGACGTTTT	CTCGATCACA	GGCCGTGGTA	CGGTTGCGAC	GGGCCGTGTT
Bacillus l	AGGACGTATT	CTCAATCACA	GGTCGTGGAA	CAGTTGCTAC	TGGACGCGTA
Oceanobaci	AGGATGTATT	CTCAATCACT	GGTCGTGGAA	CAGTTGCAAC	TGGACGTGTT
Streptococ	AGGACGTATT	CTCAATCACT	GGACGTGGTA	CAGTTGCTTC	AGGACGTATC
Clostridiu	AAGATGTCTT	CACAATAACA	GGAAGAGGAA	CAGTTGCAAC	AGGAAGAGTT
Mycoplasma	AAGATACGAT	GACCACTACT	GGTAGAGGTA	CAGTTGTTAC	AGGAAGAGTT
Escherichi	AAGACGTATT	CTCCATCTCC	GGTCGTGGTA	CCGTTGTTAC	CGGTCTGTGA
Aquifex py	AAGACGTGTT	CAGCATATCG	GGACGTGGAA	CGGTTGTGAC	CGGAAGAGTG
Thermus th	AGGACGTGTT	TACGATCACG	GGTCGTGGGA	CGGTGGCCAC	GGGTCTGGATT
Thermotoga	AAGACGTGTT	CTCCATCACA	GGAAGAGGAA	CGGTTGTTAC	AGGAAGAATA
Consensus	ARGA YRYNW T	BWSV ATH WC N	GGH MG WGGDA	CVGT K GY NW C	NGGH MG NRT N
					
	805	815	825	835	845
Geobacillu	GAGCGTGGTA	CGCTCAAAGT	CGGTGACCCG	GTTGAAATCA	TCGGTCTTTC
Bacillus l	GAGCGCGGAC	AAGTTAAAGT	CGGTGACGAA	GTTGAAATCA	TCGGTCTTCA
Oceanobaci	GAGCGTGGAG	AAGTTAAAGT	TGGAGATGAA	GTTGAAATCA	TCGGTCTTGC
Streptococ	GACCGTGGTA	TCGTAAAGT	CAACGACGAA	ATCGAAATCG	TTGGTATCAA
Clostridiu	GAAACTGGAA	CATTAAAAGT	TGGAGACGAA	GTAGAAATCG	TTGGAATGAA
Mycoplasma	GAAAGAGGTG	AACTCAAAGT	AGGTCAAGAA	GTTGAAATTG	TTGGTTTTAA
Escherichi	GAACGCGGTA	TCATCAAAGT	TGGTGAAGAA	GTTGAAATCG	TTGGTATC--
Aquifex py	GAAAGGGGCG	TGTTGAGACC	CGGAGATGAG	GTAGAGATAG	TAGGACTGAG
Thermus th	GAGCGGGGCA	AGGTGAAGGT	TGGGACGAG	GTGGAGATTG	TGGGCCTTGC
Thermotoga	GAAAGAGGAA	GAATCAGACC	CGGTGATGAA	GTTGAGATCA	TAGGTCTCAG
Consensus	GA VM SN GG HV	NVN TNA RRSY	HRRNS AH SMR	RTNGA RA THR	TNGG H HT NNV

Figure 5.2. ClustalW multiple alignment result of nucleotide sequences indicated in Table 5.1. Primer sites (TuF413 and TuR1174) were shown as in blue and consensus sequence was shown in red (Continued).

					
	855	865	875	885	895
Geobacillu	TGACGAGCCG	AAAGCCACGA	CGGTTACGGG	TGTTGAAATG	TTCCGTAAAC
Bacillus l	AGAAGAAAAC	AAGAAAACAA	CTGTTACAGG	TGTTGAAATG	TTCCGCAAGC
Oceanobaci	TGAAGATGCA	AGCAAAACAA	CTGTAACCTGG	TGTTGAAATG	TTCCGTAAGC
Streptococ	AGAAGAAACT	CAAAAAGCAG	TTGTTACTGG	TGTTGAAATG	TTCCGTAAAC
Clostridiu	AGATGAAATA	ACAAAAGTAG	TAGTAACAGG	CGTAGAAATG	TTCAGAAAAA
Mycoplasma	ACC--AATT	AGAAAAGCAG	TTGTTACTGG	AATTGAAATG	TTCAAAAAGG
Escherichi	-AAAGAGACT	CAGAAGTCTA	CCTGTACTGG	CGTTGAAATG	TTCCGCAAAC
Aquifex py	GGAAGAGCCC	TTAAAGACTG	TGGCAACGTC	CATAGAGATG	TTCAGGAAGG
Thermus th	TCCGGAGACG	CGGAAGACGG	TGGTGACGGG	TGTGGAGATG	CACCGGAAGA
Thermotoga	CTACGAGATC	AAGAAGACCG	TTGTGACGAG	TGTGGAAATG	TTCAGAAAAGG
Consensus	NNMN GA DVHN	HNVRMVDYNR	YNKBD AC DDS	HR TDGARATG	YW CMR NAARV
					
	905	915	925	935	945
Geobacillu	TTCTTGACCA	AGCAGAAGCT	GGAGACAACA	TCGGTGCGCT	TCTCCGCGGT
Bacillus l	TTCTTGACTA	TGCAGAAGCT	GGAGACAACA	TCGGTGCACT	TCTTCGCGGT
Oceanobaci	TTCTAGATTA	TGCTGAAGCT	GGAGATAACA	TTGGTGCACT	TCTTCGTGGG
Streptococ	AACTTGACGA	AGGTCTTGCT	GGAGATAACG	TAGGTGTCCT	TCTTCGTGGT
Clostridiu	TACTTGATAG	TGCATTAGCA	GGAGATAACA	TCGGAGCATT	ATTAAGAGGA
Mycoplasma	AACTTGATTC	AGCAATGGCT	GGTGACAATG	CTGGGGTATT	ATTACGTGGT
Escherichi	TGCTGGACGA	AGGCCGTGCT	GGTGAGAACG	TAGGTGTTCT	GCTGCGTGCT
Aquifex py	TGCTTGATGA	GGCGCTTCCC	GGAGACAATA	TTGGAGTTCT	TCTGAGGGGA
Thermus th	CCCTGCAGGA	GGGGATTGCT	GGGGACAATG	TGGGGGTGCT	CCTGCGGGGT
Thermotoga	AACTCGATGA	AGGAATCGCA	GGAGACAACG	TTGGATGTCT	GCTCAGAGGA
Consensus	HN CTNSA BNV	DGSNNDNSCH	GGDGABAA YR	YN GGD KBNY T	NY TNMGN GGD
					
	955	965	975	985	995
Geobacillu	GTATCGCGCG	ACGAAGTTGA	GCGTGGCCAA	GTATTGGCAA	AACCGGGCTC
Bacillus l	GTATCTCGTG	AAGAAATCCA	ACGCGGTCAA	GTAATTGCTC	AACCAGGTAC
Oceanobaci	GTTTCTCGTG	AAGACATCAA	CCGTGGTCAA	GTATTAGCTA	AACCAGGTTT
Streptococ	GTTCAACGTG	ATGAAATCGA	ACGTGGACAA	GTTATCGCTA	AACCAGGTTT
Clostridiu	GTACAGAGAG	AAGACATCGA	AAGAGGTCAG	GTATTAGCAA	AACCAGGTTT
Mycoplasma	GTTGAACGTA	AAGAAGTTGA	AAGAGGTCAA	GTTTTAGCAA	AACCAGGCTC
Escherichi	ATCAAACGTG	AAGAAATCGA	ACGTGGTCAG	GTAATTGCTA	AGCCGGGCAC
Aquifex py	GTAGGAAAGG	ACGACGTAGA	GAGGGGACAG	GTGCTTGCCC	AGCCCGGGAG
Thermus th	GTGAGCCGGG	AGGAGGTGGA	GCGGGGGCAG	GTGCTGGCGA	AGCCTGGGAG
Thermotoga	ATCGACAAGG	ATGAAGTTGA	AAGAGGACAG	GTTCTCGCAG	CTCCCGGAAG
Consensus	R TNNVN MRNR	ANGAVR T NTVA	VM GNGGN CAR	GT DH TNGC NV	MD CCNGGN WS

Figure 5.2. ClustalW multiple alignment result of nucleotide sequences indicated in Table 5.1. Primer sites (TuF413 and TuR1174) were shown as in blue and consensus sequence was shown in red (Continued).

					
	1005	1015	1025	1035	1045
Geobacillu	AATCACGCCG	CATACGAAAT	TTAAAGCACA	AGTTTACGTT	CTGACGAAAG
Bacillus l	AATCACTCCG	CACAAAAAAT	TCAAAGCTGA	AGTTTACGTT	CTTCTAAAAG
Oceanobaci	AATCACTCCA	CATACTAACT	TCAAAGCTGA	AGTTTATGTA	TTATCAAAAAG
Streptococ	AATCAACCCA	CACACTAAAT	TCAAAGGTGA	AGTCTACATC	CTTACTAAAAG
Clostridiu	AATAACTCCA	CATAATAAAT	TCGTAGGTCA	AGTATACGTA	TTAAAGAAAAG
Mycoplasma	TATTAAACCG	CACAAGAAAT	TTAAAGCTGA	GATCTATGCT	TTAAAGAAAAG
Escherichi	CATCAAGCCG	CACACCAAGT	TCGAATCTGA	AGTGTACATT	CTGTCCAAAAG
Aquifex py	CGTGAAGGCA	CACAGAAAGT	TCAGGGCACA	GGTATACGTT	CTTAGCAAGG
Thermus th	CATTACGCCG	CACACGAAGT	TTGAGGCCTC	GGTGTACGTG	TTGAAGAAGG
Thermotoga	CATCAAACCT	CACAAGAGGT	TCAAGGCTCA	GATCTACGTT	TTGAAGAAGG
Consensus	HR TNA MNS CD	CAYAVNARVT	TYRDRKSHBM	RR TNTA YRYN	Y TDWVNAA RG
					
	1055	1065	1075	1085	1095
Geobacillu	AGGAAGGCGG	ACGCCATACT	CCGTTCTTCT	CGAACTATCG	TCCGCAATTC
Bacillus l	AAGAGGGTGG	ACGTCACACT	CCATTCTTCT	CTAACTACCG	CCCTCAGTTC
Oceanobaci	AAGAAGGTGG	ACGTCATACT	CCATTCTTCT	CTAACTACCG	TCCACAGTTC
Streptococ	AAGAAGGTGG	ACGTCACACT	CCATTCTTCA	ACAACTACCG	TCCACAATTC
Clostridiu	AAGAAGGCGG	AAGACATACA	CCATTCTTCA	ATGGATATAG	ACCACAATTC
Mycoplasma	AAGAAGGTGG	TAGACACACT	GGTTTTTTAA	ACGGTTACCG	TCCTCAATTC
Escherichi	ATGAAGGCGG	TCGTCATACT	CCGTTCTTCA	AAGGCTACCG	TCCGCAGTTC
Aquifex py	AGGAAGGAGG	AAGGCACACG	CCTTCTTTTG	TGAACTACAG	GCCACAGTTT
Thermus th	AGGAGGGTGG	ACGGCACACG	GGGTTTTTTT	CGGGGTACCG	TCCGCAGTTT
Thermotoga	AAGAGGGAGG	AAGACATACA	CCGTTCACAA	AAGGCTACAA	GCCTCAGTTC
Consensus	ADGA RG GHGG	WM GNCA YACD	SSD TTY WYHD	HNRRN TA YMR	N CCDCA RTTY
					
	1105	1115	1125	1135	1145
Geobacillu	TACTTCCGCA	CAACGGACGT	AACAGGC---	ATCATCACGC	TTCCGGAAGG
Bacillus l	TACTTCCGTA	CAACTGACGT	AACTGGT---	ATCATTACGC	TTCCAGAAGG
Oceanobaci	TACTTCCGTA	CTACGGACGT	AACTGGT---	GTTATTGAAT	TACCAGAAGG
Streptococ	TACTTCCGTA	CTACTGACGT	TACAGGT---	TCAATCGAAC	TTCCAGCAGG
Clostridiu	TATTTTCAGAA	CAACAGACGT	AACAGGA---	TCAATCCAGT	TACCAGATGG
Mycoplasma	TATTTCCGTA	CCACTGATGT	AACTGGT---	TCTATTGCTT	TAGCTGAAAA
Escherichi	TACTTCCGTA	CTACTGACGT	GACTGGT---	ACCATCGAAC	TGCCGGAAGG
Aquifex py	TACTTCAGGA	CCGCTGATGT	AACGGGAAC	GTAGTGAAGC	TACCTGAAGG
Thermus th	TACTTTCGGA	CGACGGACGT	GACGGGG---	GTGGTGCAGT	TGCCTCCGGG
Thermotoga	TACATAAGAA	CCGCTGACGT	TACAGGAGAA	ATCGTAGGAC	TTCTGAAGG
Consensus	TA Y WTHM GN A	CNRCD GAYGT	DACD GG NRMW	DYNR T NVVDY	TDS CD SMDDR

Figure 5.2. ClustalW multiple alignment result of nucleotide sequences indicated in Table 5.1. Primer sites (TuF413 and TuR1174) were shown as in blue and consensus sequence was shown in red (Continued).

					
	1155	1165	1175	1185	1195
Geobacillu	CGTTGAAATG	GTCATGCCTG	GCGACAACGT	TGAGATGACG	GTTGAGCTGA
Bacillus l	CGTAGAAATG	GTTATGCCTG	GAGACAACAT	CGAAATGACT	GTTGAATTGA
Oceanobaci	AACTGAAATG	GTAATGCCTG	GTGATAACAT	TGAAATGACA	GTAGAACTTA
Streptococ	TACTGAAATG	GTAATGCCTG	GTGATAACGT	GACAATCGAC	GTTGAGTTGA
Clostridiu	AGTAGAAATG	GTAATGCCAG	GAGACCACAT	AGATATGACA	GTTGAATTAA
Mycoplasma	TACTGAAATG	GTTCTACCTG	GTGATAATGC	TTCTATTACT	GTTGAGTTAA
Escherichi	CGTAGAGATG	GTAATGCCCG	GCGACAACAT	CAAAATGGTT	GTTACCCTGA
Aquifex py	CGTTGAGATG	GTGATGCCCG	GGGATAACGT	TGAGCTTGAG	GTAGAGCTTA
Thermus th	CGTGGAGATG	GTGATGCCTG	GGGACAACGT	GACGTTTACG	GTGGAGCTGA
Thermotoga	TGTCGAAATG	GTCATGCCTG	GAGACCACGT	CGAAATGGAA	ATAGAACTCA
Consensus	HRYN GA ATG	GTN MTRC NC G	GNGA YMA Y RY	NDMDHTBRHN	R TD RMVYT NA
					
	1205	1215	1225	1235	1245
Geobacillu	TCGCGCCGAT	CGCGATCGAG	GAAGGAACGA	AATTCTCGAT	CCGTGAAGGC
Bacillus l	TTTCAACAAT	CGCGATCGAA	GACGGAACATC	GCTTCTCTAT	CCGTGAAGGC
Oceanobaci	TTTCTCCAAT	CGCGATTGAA	GACGGAACATC	GTTTTTCAAT	CCGTGAAGGT
Streptococ	TTCACCCAAT	CGCCGTAGAA	CAAGGTACTA	CATTCTCTAT	CCGTGAGGGT
Clostridiu	TAACAAAAGT	AGCAATGGGA	GACAACTTAA	GATTCTGCTAT	CAGAGAAGGC
Mycoplasma	TTGCTCCTAT	CGCTTGTGAA	AAAGGTAGTA	AGTTCTCAAT	TCGTGAAGGT
Escherichi	TCCACCCGAT	CGCGATGGAC	GACGGTCTGC	GTTTCGCAAT	CCGTGAAGGC
Aquifex py	TAGCGCCTGT	AGCACTTGAG	GAAGGACTGA	GGTTTGCGAT	AAGGGAAGGT
Thermus th	TCAAGCCGGT	GGCGCTGGAG	GAGGGTTTGC	GGTTTGCCAT	CCGTGAGGGT
Thermotoga	TCTACCCTGT	CGCTATCGAA	AAGGGACAGA	GATTCTGCTGT	AAGGGAAGGC
Consensus	T HNMMMD R T	V GCNNK N GRV	V AVRRHHNDM	VN TT YK C N R T	HM GD G AR GGY
					
	1255	1265	1275	1285	1295
Geobacillu	GGCCGTACAG	TTGGCGCTGG	TTCCGTATCG	GAAATCATCG	AGTAATCAAG
Bacillus l	GGACGTACAG	TTGGTTCTGG	CGTTGTTTCT	TCAATCATCG	AATAA-----
Oceanobaci	GGACGTACTG	TAGGTTCTGG	CGTTGTTTCT	TCTATCCAAA	AATAA-----
Streptococ	GGACGTACTG	TTGGTTCAGG	TATGGTTACA	GAAATCGAAG	CTTAA-----
Clostridiu	GGAAGAACAG	TTGGATCAGG	AGTTGTTACT	AGTATAATAG	AGTAA-----
Mycoplasma	GGTAGAACTG	TAGGGGCAGG	CACGTGAACA	GAAGTTCTAG	AATAG-----
Escherichi	GGCCGTACCG	TTGGCGCGGG	CGTTGTTGCT	AAAGTTCTGG	GCTAA-----
Aquifex py	GGAAGGACTG	TAGGTGCTGG	TGTCGTTACC	AAAATCCTTG	ACTGA-----
Thermus th	GGGCGGACCG	TGGGCGCCGG	CGTCGTCACC	AAGATCCTGG	AGTAA-----
Thermotoga	GGAAGAACAG	TTGGAGCTGG	TGTGGTTACA	GAAGTCATCG	AGTGA-----
Consensus	GG N MG D A C H G	T D GG NK C NGG	HDYB G T H D C N	DVDR T HVWNR	VNTRR T CAAG

Figure 5.2. ClustalW multiple alignment result of nucleotide sequences indicated in Table 5.1. Primer sites (TuF413 and TuR1174) were shown as in blue and consensus sequence was shown in red (Continued).

					
	1305	1315	1325	1335	1345
Geobacillu	AAAAAAGGAT	GTCCAATCGT	TGGACATCCT	TTTTCTTTAT	CCTGGCT
Bacillus l	-----	-----	-----	-----	-----
Oceanobaci	-----	-----	-----	-----	-----
Streptococ	-----	-----	-----	-----	-----
Clostridiu	-----	-----	-----	-----	-----
Mycoplasma	-----	-----	-----	-----	-----
Escherichi	-----	-----	-----	-----	-----
Aquifex py	-----	-----	-----	-----	-----
Thermus th	-----	-----	-----	-----	-----
Thermotoga	-----	-----	-----	-----	-----
Consensus	AAAAAAGGAT	GTCCAATCGT	TGGACATCCT	TTTTCTTTAT	CCTGGCT

Figure 5.2. ClustalW multiple alignment result of nucleotide sequences indicated in Table 5.1. Primer sites (TuF413 and TuR1174) were shown as in blue and consensus sequence was shown in red.

Table 5.2. Bacterial *fus* genes used for degenerate primer design.

Organism name	GeneBank ID
<i>Bacillus anthracis str. Sterne</i>	2851108
<i>Bacillus subtilis subsp. subtilis str. 168</i>	936826
<i>B. thuringiensis serovar konkukian str. 97-27</i>	2853707
<i>Bacillus licheniformis ATCC 14580</i>	3029537
<i>Bacillus halodurans C-125</i>	892222
<i>Geobacillus kaustophilus HTA426</i>	3184849
<i>Oceanobacillus iheyensis HTE831</i>	1015203
<i>Aquifex aeolicus VF5</i>	1192533

					
	5	15	25	35	45
Bacillus a	ATGGCAAGAG	AGTTCTCTTT	AGAAAAACACT	CGTAATATTG	GTATCATGGC
Bacillus s	ATGGCAAGAG	AGTTCTCTTT	AGAAAAAACT	CGTAATATCG	GTATCATGGC
Bacillus t	ATGGCAAGAG	AGTTCTCTTT	AGAAAAACACT	CGTAATATTG	GTATCATGGC
Bacillus l	ATGGCAAGAG	AGTTCTCTTT	AGAAAAAACT	CGTAATATCG	GAATCATGGC
Bacillus h	ATGGCAAGAG	AGTTCTCTTT	AGAAAAATACG	CGTAATATCG	GGATCATGGC
Geobacillu	ATGGCAAGAG	AGTTCTCTTT	GGAAAAAGACT	CGCAACATAG	GGATTATGGC
Oceanobaci	ATGGCTAGAG	AGTTCTCTTT	GGAAAAGACG	CGTAATATTG	GTATTATGGC
Aquifex ae	ATGGCGAGAG	AGGTGCCTAT	AGAGAAAATTG	AGAAACATAG	GTATAGTTGC
Consensus	ATGGCDAGAG	AGKTSYCYWT	RGARAA NWYK	MGHAAATHG	GDATHRTKGC
					
	55	65	75	85	95
Bacillus a	TCACATCGAT	GCTGGTAAAA	CAACAGCTAC	TGAGCGTATT	CTGTACTACA
Bacillus s	TCACATCGAT	GCCGGTAAAA	CGACTACTAC	AGAACGTATC	TTGTTCTATA
Bacillus t	TCACATCGAT	GCTGGTAAAA	CAACAGCTAC	TGAGCGTATT	CTGTACTACA
Bacillus l	TCACATCGAT	GCCGGTAAAA	CGACAACAAC	TGAACGTATC	TTGTTCTACA
Bacillus h	TCACATCGAT	GCCGGTAAAG	CAACAACAAC	AGAGCGGATT	TTGTTCTACA
Geobacillu	GCACATTGAC	GCCGGGAAAA	CGACGACGAC	TGAACGGATC	TTGTTCTATA
Oceanobaci	CCACATTGAT	GCGGGTAAAA	CAACCACTAC	TGAGCGTATT	CTTTTCTATA
Aquifex ae	TCACATTGAC	GCGGGTAAAA	CTACGACTAC	CGAGAGAATT	CTCTATTACA
Consensus	BACATYGA	GCBGGKAARA	CDACNRC DAC	HGARMG DATY	YTB TWY TAYA
					
	105	115	125	135	145
Bacillus a	CAGGACGTAT	TCACAAAATC	GGTGAAACTC	ACGAAGGTGC	ATCTCAGATG
Bacillus s	CTGGTCGTAT	CCACAAAATT	GGTGAAACTC	ATGAAGGAGC	TTACAAAATG
Bacillus t	CAGGACGTAT	TCACAAAATC	GGTGAAACTC	ACGAAGGTGC	ATCTCAGATG
Bacillus l	CAGGCCGTAT	CCATAAAATT	GGTGAAACTC	ACGAAGGGGC	TTCCCAAATG
Bacillus h	CAGGACGTAT	CCATAAAATT	GGTGAGACTC	ACGAAGGTGC	TTCTCAAATG
Geobacillu	CCGGCCGCGT	TCATAAAATC	GGGGAAGTGC	ATGAAGGTGC	GGCAACGATG
Oceanobaci	CAGGACGCAT	TCACAAAATT	GGTGAAACTC	ACGAAGGTGC	TTCTCAAATG
Aquifex ae	CGGGTAAGAC	TTACAAGATA	GGTGAAAGTTC	ACGAAGGTGC	TGCAACGATG
Consensus	CNGGHMRBRY	YYA AA RATH	GGK GARRY KC	AYGAAGG DGC	DKCHMMR ATG
					
	155	165	175	185	195
Bacillus a	GACTGGATGG	AGCAAGAGCA	AGAGCGTGGT	ATCACAATTA	CTTCTGCTGC
Bacillus s	GACTGGATGG	AGCAGGAACA	AGAACGTGGT	ATCACAATCA	CTTCCGCTGC
Bacillus t	GACTGGATGG	AGCAAGAGCA	AGAGCGTGGT	ATCACAATTA	CTTCTGCTGC
Bacillus l	GACTGGATGG	AGCAGGAACA	AGAACGCGGT	ATCACAATCA	CATCTGCTGC
Bacillus h	GACTGGATGG	AGCAGGAGCA	AGAGCGTGGA	ATCACGATCA	CATCTGCTGC
Geobacillu	GACTGGATGG	AACAAGAACA	AGAGCGCGGA	ATTACGATCA	CGTCGGCGGC
Oceanobaci	GACTGGATGG	AGCAAGAGCA	AGAACGTGGT	ATTACAATCA	CTTCTGCTGC
Aquifex ae	GACTGGATGC	CCCAGGAAAA	GGAAAGAGGT	ATAACCATAA	CCGTTGCAAC
Consensus	GACTGGATGS	MV CAR GARMA	R GA RM G HGGW	ATHACV ATHA	CNKYB G CDRC

Figure 5.3. ClustalW multiple alignment result of nucleotide sequences indicated in Table

5.2. Primer site (FusF) was shown in blue and consensus sequence was shown in red

(Continued).

					
	205	215	225	235	245
Bacillus a	AACTACAGCA	CAATGGA---	-----A	AGGTCACCGT	GTAAACATCA
Bacillus s	GACTACTGCA	CAGTGGA---	-----A	AGGATACCGC	GTAAACATCA
Bacillus t	AACTACAGCA	CAATGGA---	-----A	AGGTCACCGT	GTAAACATCA
Bacillus l	AACAACAGCT	CAGTGGA---	-----A	AGGCTACCGC	GTAAACATCA
Bacillus h	AACAACAGCT	CAATGGA---	-----A	GAACAATCGT	ATCAACATCA
Geobacillu	GACAACGGCG	CAATGGA---	-----A	AGGCCATCGC	ATCAACATCA
Oceanobaci	GACAACAGCT	GCTTGGA---	-----G	AGACCACCGT	ATTAACATCA
Aquifex ae	GACCGCATGT	TATTGGACGA	GAAACGGGGA	GAGGTATCAA	ATAAACATAA
Consensus	RACH RCDKSD	BMD TGGACGA	GAAACGGGGR	RRRNH AYCRH	R THAACATMA
					
	255	265	275	285	295
Bacillus a	TTGACACTCC	AGGTCACGTA	GATTTCACAG	TAGAAGTAGA	ACGTTCTTTA
Bacillus s	TCGATACTCC	AGGACACGTA	GACTTCACAG	TTGAAGTTGA	ACGTTCTCTT
Bacillus t	TTGACACTCC	AGGTCACGTA	GATTTCACAG	TAGAAGTAGA	ACGTTCTTTA
Bacillus l	TCGACACACC	AGGACACGTA	GACTTCACAG	TTGAGGTTGA	ACGTTCCCTG
Bacillus h	TCGATACTCC	TGGACACGTA	GACTTTACCG	TTGAGGTTGA	ACGTTCTTTA
Geobacillu	TCGACACGCC	GGGGCACGTC	GACTTTACGG	TCGAGGTTGA	GCGTTCGTTG
Oceanobaci	TCGATACTCC	GGGACACGTA	GACTTCACTG	TAGAAGTTGA	GCGTTCCTTG
Aquifex ae	TTGACACACC	CGGACACGTT	GACTTCTCCG	TTGAAGTTGT	ACGTTCCATG
Consensus	TYGAYACDCC	NGGD CACGTH	GAYTT YWCNG	THGAR GTWGW	RCGTT CBHTD
					
	305	315	325	335	345
Bacillus a	CGCGTACTTG	ATGGCGCAGT	AGCAGTACTT	GATGCACAAT	CTGGTGTAGA
Bacillus s	CGTGTACTTG	ATGGTGCTGT	TGCTGTACTT	GACGCGCAAT	CAGGCGTTGA
Bacillus t	CGCGTACTTG	ATGGCGCAGT	AGCAGTACTT	GATGCACAAT	CTGGTGTAGA
Bacillus l	CGCGTACTTG	ATGGTGCCGT	TGCCGTCTT	GACGCACAAT	CAGGCGTTGA
Bacillus h	CGTGTATTAG	ATGGAGCTGT	TGCGGTACTT	GATGCTCAAT	CAGGTGTTGA
Geobacillu	CGCGTATTGG	ACGGGGCCAT	CACAGTCTTA	GACGCGCAAT	CGGGTGTGTA
Oceanobaci	CGTGTACTTG	ATGGTGCGGT	GACTGTACTA	GATGCACAAT	CTGGGGTGGGA
Aquifex ae	AAAGTTCTCG	ACGGAATAGT	TTTCATATTC	TCCGCGGTTG	AAGGTGTGCA
Consensus	MRH GTWY TNG	AYGGN RYNR T	NDYNR THY TH	KMY GC DSWWK	MD GGB GTDS A
					
	355	365	375	385	395
Bacillus a	ACCACAAACA	GAAACTGTTT	GGCGTCAGGC	TACTACTTAC	GGCGTACCTC
Bacillus s	GCCTCAAACA	GAAACTGTTT	GGCGTCAGGC	AACAAC TTAT	GGAGTACCGC
Bacillus t	ACCACAAACA	GAAACTGTTT	GGCGTCAGGC	TACTACTTAC	GGCGTACCTC
Bacillus l	GCCGCAAAC	GAAACAGTTT	GGCGTCAGGC	GACAAC TTAC	GGAGTACCTC
Bacillus h	GCCGCAAACA	GAGACAGTTT	GGCGTCAAGC	AACAAC TTAC	GGTGTTCCTC
Geobacillu	ACCGCAAACG	GAAACCGTTT	GGCGCCAAGC	GACGACATAT	GGCGTCCAC
Oceanobaci	GCCACAAACT	GAAACAGTAT	GGCGCCAAGC	AACAAC TTAT	GGTGTTCAC
Aquifex ae	ACCTCAGTCC	GAAGCAAAC	GGAGATGGGC	GGACAGGTTC	CAAGTTCGGA
Consensus	RCDC ARWCN	GA RRCHR WHT	GG M G HYRR GC	DRMN A SD TWY	SRH GT HCC DM

Figure 5.3. ClustalW multiple alignment result of nucleotide sequences indicated in Table

5.2. Primer site (FusF) was shown in blue and consensus sequence was shown in red

(Continued).

					
	405	415	425	435	445
Bacillus a	GTATCGTATT	CGTTAACAAA	ATGGATAAAA	TCGGTGCAGA	TTTCT-TATA
Bacillus s	GTATCGTTTT	TGTTAACAAA	ATGGACAAAA	TCGGTGCAGA	CTTCC-TTTA
Bacillus t	GTATCGTATT	CGTTAACAAA	ATGGATAAAA	TCGGTGCAGA	TTTCT-TATA
Bacillus l	GTATCGTATT	TGTCAACAAG	ATGGACAAAA	CGGGTGCAGA	CTTCC-TTTA
Bacillus h	GTGTCGTGTT	CGTTAACAAA	ATGGACAAAA	CAGGAGCAGA	CTTCT-TATA
Geobacillu	GGATCGTGTT	CGTCAACAAA	ATGGATAAAA	TCGGCGCCGA	TTTCT-TGTA
Oceanobaci	GTATCGTATT	CATCAATAAA	ATGGATAAAA	CAGGTGCGGA	CTTCT-TGTA
Aquifex ae	GGATAGCCTT	CATAACAAG	ATGGACCGTC	TGGGTGCGGA	TTTTTACAGA
Consensus	GKR TM GYNTT	YR TH AA YAA R	AT GG A YMR WM	YV GG H GC V G A	Y TT Y Y A Y D K A
					
	455	465	475	485	495
Bacillus a	CTCTGTAGGA	ACAATCCACG	ATCGTTTACA	AGCAAACGCA	CACCCAATTTC
Bacillus s	CTCTGTAGGA	ACTTTAAGAG	ACCGTCTTCA	AGCAAACGCT	CATGCAATTTC
Bacillus t	CTCTGTAGGA	ACAATCCACG	ATCGTTTACA	AGCAAACGCA	CACCCAATTTC
Bacillus l	CTCTGTAGGA	ACTCTTAGAG	ACCGTCTTGA	GGCGAACGCT	CACGCGATCC
Bacillus h	TTCCGTAAGC	ACTCTTCATG	ACCGCCTTCA	AGCAAACGCA	CATCCGATCC
Geobacillu	TTCCGTTGAA	ACGCTCCATG	ACCGCCTGCA	AGCGAACGCC	CATCCGGTGC
Oceanobaci	TTCTACAGGT	ACATTAAAA	AGCGCCTAGG	TGCTAATGCC	CATCCGATTTC
Aquifex ae	GTGTTTAAGG	A-AATAGAAG	AAAAGCTAAC	CATAAAGGCC	GTTGCCATTTC
Consensus	B T S B D Y R R R N	A C D H T H V R H G	A N M R B Y T D V V	N R Y D A A B S C H	S W Y S C V R T B C
					
	505	515	525	535	545
Bacillus a	AGTTACCAAT	CGGTGCTGAA	GATGAGTTCA	ATGGTATCAT	TGACCTTGTT
Bacillus s	AATTGCCGAT	CGGTGCTGAA	GATAACTTCG	AAGGTATCAT	TGACCTTGTA
Bacillus t	AGTTACCAAT	CGGTGCTGAA	GATGAGTTCA	ATGGTATCAT	TGACCTTGTT
Bacillus l	AATTACCGAT	TGGCGCCGAA	GACAATTTTG	AAGGGATCAT	CGACCTTGTA
Bacillus h	AACTACCAAT	TGGTGCGGAA	GACAATTTTG	AAGGAATTAT	CGACTTAGTA
Geobacillu	AACTGCCGAT	CGGCGCTGAA	GATCAATTCT	CCGGCATTAT	CGACTTGTTT
Oceanobaci	AGATGCCAAT	TGGTGCTGAA	GATGAGTTTC	ACGGAATCAT	TGACTTAATC
Aquifex ae	AAATACCCCT	GGGAGCGGAG	GACCAGTTTG	AAGGTGTTAT	AGATCTAATG
Consensus	A R H T R C C V M T	B G G H G C B G A R	G A Y V A N T T Y N	M H G G N R T Y A T	H G A Y Y T D R T N
					
	555	565	575	585	595
Bacillus a	GAAGAATGTG	CTT---ACAT	GTACGGTAAC	GATTTAGGAA	CAGACATTCA
Bacillus s	GAAAACGTTG	CGT---ATTT	CTACGAAGAT	GACCTCGGAA	CTCGTTCTGA
Bacillus t	GAAGAATGTG	CTT---ACAT	GTACGGTAAC	GATTTAGGAA	CAGACATTCA
Bacillus l	GAAAACGTTG	CGT---ACTA	CTATGAAGAT	GACCTTGGAA	CACGCTCTGA
Bacillus h	GATATGGTCG	CTT---ATTT	CTATGAAGAT	GATCTCGGAA	CAAGAACAGA
Geobacillu	GAAATGTGCG	CGT---ACCA	TTACCATGAT	GAGCTTGGCA	AAAACATTGA
Oceanobaci	ACAATGGATG	CTT---ACTT	CTATTTAGAT	GATTTAGGTC	AACGTTCCGA
Aquifex ae	GAAATGAAGG	CAATAAGGTG	GCTCGAAGAA	ACCCTCGGAG	CTAAATACGA
Consensus	R M W R W V D D B G	C D W T A A R B H D	B Y W Y B D W R A H	R M B Y T H G G H V	M W V R H W H H S A

Figure 5.3. ClustalW multiple alignment result of nucleotide sequences indicated in Table

5.2. Primer site (FusF) was shown in blue and consensus sequence was shown in red

(Continued).

					
	605	615	625	635	645
Bacillus a	ACGTGTTGAA	ATTCCTGAAG	AGCATAAAGA	ACTAGCTGAA	GAATACCGTG
Bacillus s	TGCAAAAGAA	ATCCCTGAAG	AGTACAAAGA	GCAAGCTGAA	GAGCTTCGCA
Bacillus t	ACGTGTTGAA	ATTCCTGAAG	AGCATAAAGA	ACTAGCTGAA	GAATACCGTG
Bacillus l	AGCGCGTGAA	ATCCCTGCTG	AGTACAAAGA	CAAAGCTGAA	GAGCTTCGCG
Bacillus h	AGCGAAGGAA	ATTCCTGACG	AGTATAAAGA	GCAAGCGCAA	GAGTATCATG
Geobacillu	GCGCATTGAC	ATTCCGGAAG	AATACCGCGA	TATGGCAGAA	GAGTACCATA
Oceanobaci	AACACGTGAA	ATTCCTGCTG	AGTACAAAGA	CGAAGCAGAA	GAGTTACGTG
Aquifex ae	AGTAGTAGAC	ATTCCTCCAG	AATACCAGGA	AAAGGCTCAA	GAATGGCGCG
Consensus	DVBNVDD GAM	ATY CK SMHG	ARY AYMRV GA	NVWR GC DS AA	GARY DN CR YR
					
	655	665	675	685	695
Bacillus a	GAAAACCTTAT	TGAAGCGGTA	GCTGAGCTTG	ATGAAGAAAT	GATGATGAAG
Bacillus s	ACAGCCTTAT	TGAAGCTGTA	TGTGAGCTTG	ATGAAGAGCT	TATGGATAAG
Bacillus t	GAAAACCTTAT	TGAAGCGGTA	GCTGAGCTTG	ATGAAGAAAT	GATGATGAAG
Bacillus l	CTAGCTTGAT	TGAAGCTGTT	GCTGAGCTTG	ATGAAGAGCT	TATGATGAAG
Bacillus h	AGAAACTAGT	GGAAGCTGCT	GCTGAACTAG	ATGAAGAACT	CATGATGAAA
Geobacillu	ACAAGCTGAT	TGAGGCGGTC	GCTGAGCTGG	ACGAAGAATT	AATGATGAAA
Oceanobaci	CAAGCTTAAT	CGAAGCTGTT	GCAGAACTG	ACGAAGAATT	TATGATGAAA
Aquifex ae	AAAAGATGAT	AGAAACCATC	GTAGAAACCG	ACGACGAGTT	AATGGAAAAG
Consensus	VN AR VH TD R T	NGA RR C BRYH	KBW GA RM Y NG	AYGA M GA R HT	NATG RWD AA R
					
	705	715	725	735	745
Bacillus a	TACCTAGAAG	GTGAAGAAAT	CACTGTAGAA	GAGCTTAAAG	CTGGTATCCG
Bacillus s	TACCTTGAGG	GTGAAGAACT	TACAATTGAC	GAGTTAAAG	CAGGAATTCCG
Bacillus t	TACCTAGAAG	GTGAAGAAAT	CACTGTAGAA	GAGCTTAAAG	CTGGTATCCG
Bacillus l	TACTTGGAAG	GTGAAGAAAT	CACAGTTGAC	GAGCTGAAAG	CAGCAATCCG
Bacillus h	TACTTAGAAG	GAGAAGAACT	TACAAAGGAC	GAATTAAAA	GTCGATCCG
Geobacillu	TATTTGGAAG	GGGAAGAAAT	CACGACGGAA	GAGCTGAAGG	CCGCGATCCG
Oceanobaci	TATCTTGAAG	GAGAAGAAAT	CTCCAACGAT	GAATTGAAAA	CTGCTATTCCG
Aquifex ae	TACTTAGAAG	GACAGGAAAT	ATCTATAGAT	GAACTAAGAA	AAGCTTTAAG
Consensus	TAYY TD GAR G	GDS AR GA AM T	HW C NRHN GA H	GARY TD ARRR	MH GSD W TH M G
					
	755	765	775	785	795
Bacillus a	TAAGGCTACA	ACTTCTGTAG	AATTCTTCCC	AGTAATCTGT	GGTTCTGCAT
Bacillus s	TAAAGGTACA	TTAAATGTTG	AATTCTACCC	TGTTCTTGTT	GGTTCTGCCT
Bacillus t	TAAGGCTACA	ACTTCTGTAG	AATTCTTCCC	AGTAATCTGT	GGTTCTGCAT
Bacillus l	CAAAGGAACT	TGTAACGTTG	AGTTCTATCC	GGTTCTATGT	GGTTCAGCTT
Bacillus h	TAAAGGAACG	TGTAATGTAG	AATTCTATCC	AGTCCTTTGT	GGTTCGGCAT
Geobacillu	CAAAGCGACG	ATCAGCGTTG	AATTCTTCCC	GGTCTTCTGC	GGTTCGGCAT
Oceanobaci	TCAAGCAACT	CTTAATGTTG	ATTTTATCC	AGTACTTTGC	GGTTCAGCAT
Aquifex ae	AAAGGCAACA	ATAGAGAGAA	AGCTCGTTCC	CGTTCTTTGC	GGTTCTGCAT
Consensus	HM ARG SD ACD	HBHDVBRKWR	ADY T YKWY CC	NGT HH TH KKY	GGTTC D GCH T

Figure 5.3. ClustalW multiple alignment result of nucleotide sequences indicated in Table

5.2. Primer site (FusF) was shown in blue and consensus sequence was shown in red

(Continued).

					
	805	815	825	835	845
Bacillus a	TCAAAAACAA	AGGTGTTCAA	ATTCTGTTAG	ACGCAGTTAT	CGACTACCTA
Bacillus s	TCAAAAACAA	AGGTGTTCAA	CTAGTACTTG	ATGCTGTTCT	TGACTACCTT
Bacillus t	TCAAAAACAA	AGGTGTTCAA	ATTCTGTTAG	ACGCAGTTAT	CGACTACCTA
Bacillus l	TCAAAAACAA	AGGTGTTCAA	TTAGTGCTTG	ACGCAGTTCT	TGACTACCTG
Bacillus h	TTAAGAACAA	AGGTGTTTCA	CTTATGCTAG	ACGCTGTTCT	TGATTACCTC
Geobacillu	TCAAAAACAA	AGGTGTTTCA	CTGCTTCTTG	ACGGCGTTGT	CGACTACTTG
Oceanobaci	TTAAGAACAA	AGGTGTTTCA	TTAATGCTAG	ACGCAGTACT	TGATTATCTA
Aquifex ae	TCAAGAACAA	AGGTGTTCAA	CCCCTTCTTG	ACGCAGTTAT	AGATTACCTG
Consensus	TYAARAACAA	AGGTGTTCAR	HYNVTDYTWG	AYGSHGTWVT	HGAYTAYYTN
					
	855	865	875	885	895
Bacillus a	CCATCTCCAT	TAGACGTACC	TGCTATTAAA	GGTATCGTTC	CTGATACAGA
Bacillus s	CCTGCACCAA	CTGACGTTGC	TGCAATAAAA	GGTACACGTC	CTGATACAAA
Bacillus t	CCATCTCCAT	TAGACGTACC	TGCTATTAAA	GGTATCGTTC	CTGATACAGA
Bacillus l	CCAGCTCCAA	CTGACGTACC	AGCAATCAAA	GGTACTCTGC	CTGATTCTGA
Bacillus h	CCTTCTCCAC	TTGATGTACC	TGCAATAAAA	GGACATGTTC	CAGATACAGA
Geobacillu	CCGTCTCCGG	TTGACATCCC	GGCGATTTCG	GGCGTCGTTC	CGGATACCGA
Oceanobaci	CCTGCACCAA	CAGATGTACC	TCCAATCGAA	GGTATCATCC	CTGGTACAGA
Aquifex ae	CCTTCTCCTA	TAGACCTTCC	TCCCGTTAAG	GGGACAAATC	CCAAGACCGG
Consensus	CCDKCWC CDN	YWGAYVTHSC	DS CNR TH VRV	GGNVHHVDB C	CNRRKW CHRR
					
	905	915	925	935	945
Bacillus a	TGAAGAAGTA	GAACGTAAGT	CTAGCGATGA	AGAACCATTG	GCAGCTCTAG
Bacillus s	TGAAGAGATT	GAGCGTCATT	CTTCTGACGA	AGAGCCATTG	TCTGCATTAG
Bacillus t	TGAAGAAGTA	GAACGTAAGT	CTAGCGATGA	AGAACCATTG	GCAGCTCTAG
Bacillus l	TGAAGAGGTT	ACTCGTGAGT	CTTCTGACGA	TGCACCTTTC	TCAGCGCTTG
Bacillus h	AGAAGAAGCT	GTTCTGTAAAC	CTGGTGACGA	TCAGCCGTTT	GCGGCGCTTG
Geobacillu	AGAAGAAGTG	ACACGTGAAG	CAAGCGATGA	CGCTCCGTTT	GCCGCTTTGG
Oceanobaci	GGAAAAAGTA	ACTCGTCCAT	CTTCTGATGA	TGAGCCTTTC	TCTGCATTAG
Aquifex ae	GGAAGAAGAG	GTCAGACACC	CCTCTGACGA	CGAACCCTTC	TGCGCTTACG
Consensus	DGAARA RRHD	RHNM GWV MNB	CHDSY GAY GA	HSMD CC NTTY	KSN GCDY WNG
					
	955	965	975	985	995
Bacillus a	CATTCAAAAT	CATGACTGAC	CCTTATGTTG	GTAAGTTAAC	ATTCTTCCGT
Bacillus s	CATTTAAAGT	TATGACTGAC	CCTTACGTTG	GTAAGTTGAC	GTTCTTCCGT
Bacillus t	CATTCAAAAT	CATGACTGAC	CCTTATGTTG	GTAAGTTAAC	ATTCTTCCGT
Bacillus l	CATTTAAAGT	TATGACTGAC	CCTTACGTTG	GTAAGTTGAC	GTTCTTCCGT
Bacillus h	CGTTTAAGGT	TATGACAGAC	CCTTATGTTG	GTAAGCTTAC	GTTCTTCCGT
Geobacillu	CATTTAAAAAT	CATGACTGAC	CCGTATGTCG	GGAAATTGAC	GTTTATTTCGC
Oceanobaci	CTTTCAAAGT	AATGACAGAC	CCTTATGTAG	GTAAACTTAC	ATTCTTCCGT
Aquifex ae	CCTTTAAGGT	TATGTCCGAC	CCGTATGCCG	GACAACCTAC	CTACATCAGA
Consensus	CNTTYA ARR T	HATG W CHGAC	CC K TAY G YHG	GDM ARY TDAC	V TWY WTYMGH

Figure 5.3. ClustalW multiple alignment result of nucleotide sequences indicated in Table

5.2. Primer site (FusF) was shown in blue and consensus sequence was shown in red

(Continued).

					
	1005	1015	1025	1035	1045
Bacillus a	GTGTACTCTG	GTGTGTTAAA	CTCTGGATCA	TACGTGAAAA	ACTCAACTAA
Bacillus s	GTGTACTCTG	GAACACTTGA	TTCTGGTTCA	TACGTGAAAA	ACTCTACTAA
Bacillus t	GTGTACTCTG	GTGTGTTAAA	CTCTGGATCA	TACGTGAAAA	ACTCAACTAA
Bacillus l	GTGTACTCAG	GAACACTTGA	TTCCGGTTCA	TACGTAAGAA	ACTCAACAAA
Bacillus h	GTATATTCTG	GAACGCTAGA	TTCAGGATCA	TACGTTAAAA	ACTCGACAAA
Geobacillu	GTCTACTCTG	GAACGCTTGA	TTCCGGTTCA	TATGTGATGA	ACACGACGAA
Oceanobaci	GTGTACTCTG	GAACATTAGA	TTCTGGTTCA	TACGTGAAAA	ACTCAGTAAA
Aquifex ae	GTGTTCTCAG	GAACGCTAAA	AGCGGGTTCT	TACGTCTACA	ACGCAACCAA
Consensus	GT V T W Y T C W G	G W R Y R Y T W R A	H K C N G G W T C W	T A Y G T N W D V A	A C D C D R Y N A A
					
	1055	1065	1075	1085	1095
Bacillus a	AGGTAAGCGT	GAGCGTGTAG	GTCGTATCCT	ACAAATGCAC	GCTAACAGCC
Bacillus s	AGGCAAACGT	GAGCGTGTTG	GACGTATTCT	TCAAATGCAC	GCTAACAGCC
Bacillus t	AGGTAAGCGT	GAGCGTGTAG	GTCGTATCCT	ACAAATGCAC	GCTAACAGCC
Bacillus l	AGGCAAACGT	GAGCGCGTCG	GCCGTATCCT	GCAAATGCAC	GCCAACAGCC
Bacillus h	AGATAAGCGT	GAGCGCGTCG	GTCGTATCCT	ACAAATGCAC	GCGAACCACC
Geobacillu	AGGGAAGCGC	GAACGGATCG	GCCGCTTGCT	GCAAATGCAC	GCGAACCACC
Oceanobaci	AGATAAGCGT	GAGCGTGTAG	GACGTATTCT	ACAAATGCAC	GCTAACTCTC
Aquifex ae	GGACGAAAAG	CAAAGGGCTG	GAAGACTTCT	TCTCATGCAC	GCGAACTCCA
Consensus	R G R B R A R M R B	S A R M G B R Y H G	G H M G H H T B C T	D C W M A T G C A C	G C B A A C H V Y M
					
	1105	1115	1125	1135	1145
Bacillus a	GTGAAGAGAT	CTCAACAGTT	TACGCTGGTG	ATATCGCTGC	TGCTGTAGGT
Bacillus s	GTGAAGAAAT	CTCTACTGTA	TACGCAGGGG	ATATCGCGGC	TGCTGTAGGT
Bacillus t	GTGAAGAGAT	CTCAACAGTT	TACGCTGGTG	ATATCGCTGC	TGCTGTAGGT
Bacillus l	GTGAAGAAAT	CTCTACTGTG	TACGCAGGGG	ATATCGCTGC	TGCTGTAGGT
Bacillus h	GTGAGGAAAT	TTTCGACAGT	TACTCTGGTG	ACATCGCAGC	AGCTGTAGGT
Geobacillu	GTCAAGAAAT	TTTCGAAAGTC	TATGCCGGTG	ATATTGCCGC	AGCGGTAGGT
Oceanobaci	GAGAAGAAAT	CTCTAGAGCA	TATTCAGGTG	AAATTGCTGC	AGCAGTTGGT
Aquifex ae	GAGAGGAAAT	ACAGCAGGTT	TCCGCGGGTG	AAATTTGTGC	AGTTGTAGGA
Consensus	G W S A R G A R A T	H Y M D M V D G Y N	T M Y K C N G G K G	A H A T Y K S N G C	W G Y D G T W G G W
					
	1155	1165	1175	1185	1195
Bacillus a	TTAAAAGATA	CTACTACTGG	TGATACTCTT	TGTGACGAGA	AGAGCCTTGT
Bacillus s	CTTAAAGATA	CAACTACTGG	AGATACTCTA	TGTGATGAGA	AGGATCTTGT
Bacillus t	TTAAAAGATA	CTACTACTGG	TGATACTCTT	TGTGACGAGA	AGAGCCTTGT
Bacillus l	TTGAAAGACA	CGACTACAGG	GGACACGCTT	TGCGACGAGA	AAAACCTTGT
Bacillus h	TTAAAAGATA	CGTCTACAGG	GGATACACTT	TGTGACGAAA	AGAATCTTGT
Geobacillu	TTAAAAGATA	CGACGACCGG	TGATACTCTA	TGTGATGAGA	AGCATCCTGT
Oceanobaci	TTGAAAGATA	CTTCTACAGG	GGATACTCTT	TGTGATGAGA	AGAACCTTGT
Aquifex ae	CT---AGACG	CCGCAACGGG	TGATACTCTC	TGTGATGAAA	AGCACCCCAT
Consensus	Y T D A A A G A Y R	C N D C D A C N G G	D G A Y A C D C T H	T G Y G A Y G A R A	A R V R Y C Y Y R T

Figure 5.3. ClustalW multiple alignment result of nucleotide sequences indicated in Table

5.2. Primer site (FusF) was shown in blue and consensus sequence was shown in red

(Continued).

					
	1205	1215	1225	1235	1245
Bacillus a	TATCCTTGAG	TCTATGGAAT	TCCCAGAGCC	AGTTATCTCT	GTAGCTATCG
Bacillus s	TATCCTTGAG	TCAATGGAAT	TCCCAGAACC	AGTTATCGAC	GTTGCTATTG
Bacillus t	TATCCTTGAG	TCTATGGAAT	TCCCAGAGCC	AGTTATCTCT	GTAGCTATCG
Bacillus l	TATCCTGGAA	TCAATGGAAT	TCCCTGAGCC	TGTTATCCAC	GTTGCAATCG
Bacillus h	TATTCTTGAG	TCTATGGAGT	TCCCAGAGCC	TGTTATCCAC	TTGTCCGTTG
Geobacillu	CATTTTAGAG	TCGATGCAAT	TCCCAGAACC	GGTCATTTTCG	GTGGCAATCG
Oceanobaci	TATCTTAGAG	TCTATGGACT	TCCCAGAACC	AGTTATCTCT	GTTGCGATTG
Aquifex ae	AATCCTTGAA	AAGCTTGAAT	TCCCTGACCC	CGTTATATCT	ATGGCTATAG
Consensus	HATYYT DGAR	WMDMT TKSAVT	TCCCW GAV CC	NGTY ATHBMB	D TDK CNR THG
					
	1255	1265	1275	1285	1295
Bacillus a	AACCAAAATC	AAAAGCTGAC	CAAGATAAAA	TGGGTACAGC	ATTATCTAAG
Bacillus s	AGCCTAAATC	AAAAGCTGAC	CAAGATAAAA	TGGGTATCGC	TTAGCGGAAA
Bacillus t	AACCAAAATC	AAAAGCTGAC	CAAGATAAAA	TGGGTACAGC	ATTATCTAAG
Bacillus l	AGCCTAAATC	TAAGGCTGAC	CAAGACAAAA	TGTCTACTGC	ACTGGCTAAA
Bacillus h	AGCCTAAATC	AAAAGCGGAC	CAAGATAAAA	TGGGCTTAGC	GCTTGCGAAG
Geobacillu	AGCCGAAGTC	GAAAGCCGAT	CAAGACAAAA	TGAGCCAAGC	GCTGCAAAAA
Oceanobaci	AACCAGAAAC	TAAAGCTGAC	CAAGATAAAA	TGGCAATTGC	TTTATCTAAG
Aquifex ae	AGCCAAAGAC	CAAGAAGGAC	CAAGAAAAAC	TCTCACAAGT	TCTCAACAAG
Consensus	ARCCDR A RW C	NAARRMB GAY	CAAGA H AAAM	TSDSHHHH G Y	D Y TNNMN AA R
					
	1305	1315	1325	1335	1345
Bacillus a	CTTTCTGAAG	AAGATCCAAC	ATTCCGTGCT	CACACTGACC	AAGAAACTGG
Bacillus s	CTTGCTGAAG	AAGATCCTAC	ATTCCGTACT	CAAACAAACC	CAGAAACTGG
Bacillus t	CTTTCTGAAG	AAGATCCAAC	ATTCCGTGCT	CACACTGACC	AAGAAACTGG
Bacillus l	TTGGCTGAAG	AGGATCCAAC	ATTCCGTGCG	CATACTGACC	CTGAAACAGG
Bacillus h	CTTGCTGAAG	AGGATCCAAC	ATTCAAAACA	CACACAGATG	AAGAAACTGG
Geobacillu	TTGCAAGAAG	AAGACCCGAC	TTTCCGCGCT	CACACCGACC	CGGAAACGGG
Oceanobaci	TTAGCTGAAG	AGGATCCAAC	TTTCCGTACT	GAAACTAACC	CAGAAACTGG
Aquifex ae	TTCATGAAAG	AGGATCCAAC	CTTCAGGGCA	ACAACCGATC	CCGAAACTGG
Consensus	Y TNNH DR AAG	ARGAY CCDAC	H TTC MRNR CD	VMH AC HR A YS	MN GAAACDGG
					
	1355	1365	1375	1385	1395
Bacillus a	CCAAACAATC	ATCGCTGGTA	TGGGTGAACT	TCACCTTGAT	ATCATCGTTG
Bacillus s	CCAAACGATC	ATCTCTGGTA	TGGGTGAACT	TCACCTTGAT	ATCATTGTTG
Bacillus t	CCAAACAATC	ATCGCTGGTA	TGGGTGAACT	TCACCTTGAT	ATCATCGTTG
Bacillus l	TCAAACGATC	ATCGGCGGTA	TGGGTGAGCT	TCACCTTGAT	ATCATCGTTG
Bacillus h	GCAAACGATC	ATTGCTGGAA	TGGGTGAGCT	TCACCTAGAC	ATTATCGTTG
Geobacillu	ACAAACGATC	ATCTCCGGAA	TGGGCGAGTT	GCATCTTGAC	ATCATCGTCG
Oceanobaci	TCAAACAATT	ATCTCAGGTA	TGGGTGAACT	TCACCTTGAT	ATCATTGTTG
Aquifex ae	TCAGATACTC	ATACACGGAA	TGGGTGAGCT	CCACCTCGAA	ATAATGGTTG
Consensus	N C A A YRM T Y	A THBVH GGWA	TGGG Y G A RY T	B C A Y CTH G A H	A TH A T B G T Y G

Figure 5.3. ClustalW multiple alignment result of nucleotide sequences indicated in Table

5.2. Primer site (FusF) was shown in blue and consensus sequence was shown in red

(Continued).

					
	1405	1415	1425	1435	1445
Bacillus a	ACCGTATGCG	CCGTGAATTC	AAAGTTGAAG	CAAACGTTGG	TGCTCCTCAG
Bacillus s	ACCGTATGAA	ACGCGAATTT	AAAGTAGAAG	CCAATGTTGG	TGCTCCTCAA
Bacillus t	ACCGTATGCG	CCGTGAATTC	AAAGTTGAAG	CAAACGTTGG	TGCTCCTCAG
Bacillus l	ACCGTATGAA	ACGCGAATTT	AAAGTTGAAG	CCAACGTCGG	TGCTCCTCAA
Bacillus h	ACCGTCTTCG	CCGTGAGTTT	AAAGTGGAAG	CCAATGTTGG	TGCACCTCAA
Geobacillu	ACCGGATGCG	CCGCGAGTTC	AAAGTCGAAG	CGAACGTCGG	GGCGCCGCAA
Oceanobaci	ACCGTTTGAA	GCGTGAGTTT	AAAGTAGGAG	CTCAAGTAGG	GGCACCTCAA
Aquifex ae	ACAGAATGAA	GAGGGAATAC	GGAATTGAAG	TGAACGTCGG	TAAACCGCAG
Consensus	ACMGDH T KMR	VM GBGART WY	RR ARTNGRAG	YNMA HGTHGG	KRMD CCCKAR
					
	1455	1465	1475	1485	1495
Bacillus a	GTAGCATACC	GTGAGACTTT	CCGCGCTGCT	GCGAAAGTTG	AAGGTAAGTT
Bacillus s	GTTGCGTACC	GTGAAACATT	CCGTACCGGT	GCAAAAGTTG	AAGGTAAATT
Bacillus t	GTAGCATACC	GTGAGACTTT	CCGCGCTGCT	GCGAAAGTTG	AAGGTAAGTT
Bacillus l	GTTGCGTATC	GTGAAACATT	CCGTGCGAGC	GCGCAAGTTG	AAGGTAAATT
Bacillus h	GTTTCTTACC	GTGAAACGAT	TCGTCAAGCA	GCTCAAGTCG	AAGGTAAGTT
Geobacillu	GTTGCGTACC	GCGAAACGTT	CCGCAAAATCG	GCGCAAGTCG	AAGGCAAATT
Oceanobaci	GTTTCTTATC	GTGAAACATT	CCGTGGTTCT	GCTGAAGTCG	AAGGTAAATT
Aquifex ae	GTTGCTTACA	AGGAAACCAT	CAGGAAAAAG	GCAATTGGTG	AGGGTAAGTT
Consensus	GT WK CNTA YM	RB GARACNWT	YM GBV VNDVN	GCDV WW GKYG	ARGGYAARTT
					
	1505	1515	1525	1535	1545
Bacillus a	CGCTCGTCAA	TCTGGTGGAC	GTGGACAATT	CGGTCACGTT	TGGATTGAGT
Bacillus s	CGTACGTCAG	TCTGGTGGAC	GCGGTCAGTT	CGGACACGTT	TGGATCGAAT
Bacillus t	CGCTCGTCAA	TCTGGTGGAC	GTGGACAATT	CGGTCACGTT	TGGATTGAGT
Bacillus l	CGTACGTCAG	TCTGGTGGAC	GCGGACAGTT	CGGACACGTT	TGGATCGAAT
Bacillus h	CGTACGTCAA	TCTGGTGGTC	GTGGTCAATA	CGGTCACGTG	TGGATTGAAT
Geobacillu	CATCCGTCAA	TCCGGCGGCC	GCGGTCAATA	CGGCCACGTT	TGGATCGAAT
Oceanobaci	CGTACGTCAG	TCTGGTGGAC	GCGGACAATT	CGGTCACGTT	TGGGTAAAT
Aquifex ae	CATCAAGCAA	ACTGGTGGTA	GAGGGCAGTA	CGGTCACGCG	ATAATCGAAA
Consensus	CRYHMRKCAR	W CYGGYGGHM	GHGGDCARTW	CGGH CACGYK	WKRRTY RARW
					
	1555	1565	1575	1585	1595
Bacillus a	TTGAACCTAA	TGAAGAAGGT	AAAGGATTCG	AATTCGAAAA	CAAGATCGTC
Bacillus s	TGAACCAAAA	CGAAGAAGGC	GCTGGCTTTG	AATTTGAAAA	TGCGATTGTC
Bacillus t	TTGAACCTAA	TGAAGAAGGT	AAAGGATTCG	AATTCGAAAA	CAAGATCGTC
Bacillus l	TCTCTCCAAA	CGAAGAAGGA	AAAGGCTTTG	AGTTCGAAAA	CGCAATCGTC
Bacillus h	TCTCTCCTAA	CGAAGAAGGT	GCCGGTTTCG	AATTTGTGAA	TGGTATCGTC
Geobacillu	TCTCGCCGAA	CGAGCGCGGC	AAAGGCTTCG	AATTCGAGAA	CGCCATCGTC
Oceanobaci	TGAACCAAAA	CGAAGAAGGC	GCTGGATTTC	AATTTGAAAA	CAAAATTGTT
Aquifex ae	TGAACCCCT	CCCCAGAGGT	GCGGGATTTC	AATTCATAGA	CGACATTCAC
Consensus	TYKMDCC NMW	YSMVVR MGGH	RMN GGH TTYG	ARTTYR WRR A	YRVN ATYSWY

Figure 5.3. ClustalW multiple alignment result of nucleotide sequences indicated in Table

5.2. Primer site (FusF) was shown in blue and consensus sequence was shown in red

(Continued).

					
	1605	1615	1625	1635	1645
Bacillus a	GGTGGTGTG	TTCCACGTGA	ATACATCCCA	GCTGTAGGCG	CAGGTCTTGA
Bacillus s	GGTGGGGTCG	TTCTCTCGTGA	ATACATCCCA	GCTGTTCAAG	CAGGTCTGGA
Bacillus t	GGTGGTGTG	TTCCACGTGA	ATACATCCCA	GCTGTAGGCG	CAGGTCTTGA
Bacillus l	GGTGGTGTG	TTCTCTCGTGA	ATACATTCCG	GCTGTTCAAG	CAGGACTTGA
Bacillus h	GGAGGGGTAG	TACCAAGAGA	ATACATCCCA	TCTGTTCAAG	CTGGTCTTGA
Geobacillu	GGTGGGGTTG	TTCCGAAAGA	GTATGTGCCG	GCCGTCCAAG	CTGGATTGGA
Oceanobaci	GGTGGTGTAG	TTCTCTCGTGA	ATACATTCCG	GCAGTTCAAC	AAGGTATCCA
Aquifex ae	GGAGGAGTTA	TCCCCAAAGA	ATTCATACCC	TCCGTTGAGA	AGGGTGATAA
Consensus	GGWGGD GT HR	TH CC NMRW GA	RTWYR TN CCV	K CHGT HSRVV	MD GGWN TNV A
					
	1655	1665	1675	1685	1695
Bacillus a	AGATGCACTT	AAAAATGGTG	TACTTGCTGG	ATATCCAGTA	GTAGACATTA
Bacillus s	AGATGCACTC	GAAAATGGTG	TACTAGCAGG	CTTCCCGCTT	ATCGACATTA
Bacillus t	AGATGCACTT	AAAAATGGTG	TACTTGCTGG	ATATCCAGTA	GTAGACATTA
Bacillus l	AGATGCACTT	CAAAACGGTG	TAGTCGCAGG	TTACCCTGTT	ATCGACATCA
Bacillus h	AGAAGCGCTT	GAAAATGGTC	TTCTTGCAAG	TTACCCAGTG	ATTGACATTA
Geobacillu	AGAAGCGATG	CAAAACGGCG	TCTTAGCCGG	CTATCCGGTT	GTCGACATTA
Oceanobaci	AGAGTCTATG	GAAAATGGGG	TTCTTGCAAG	ATATCCGTTG	ATCGATATCA
Aquifex ae	GGAAGCTATG	CAAAACGGAA	TTCTGCAGG	ATACCCCGTT	GTTGACGTTA
Consensus	R GA DK CD MT B	V AAAA Y GG NV	T HB T H GCH GG	HTWY CC N B T D	R TH GA YR TY A
					
	1705	1715	1725	1735	1745
Bacillus a	AAGCTGCATT	AGTTGACGGA	TCTTACCATG	ATGTCGATTG	ATCTGAGATG
Bacillus s	AAGCTAAACT	TTTCGACGGT	TCTTACCATG	ATGTTGACTC	AAACGAAATG
Bacillus t	AAGCTGCATT	AGTTGACGGA	TCTTACCATG	ATGTCGATTG	ATCTGAGATG
Bacillus l	AAGCGAAACT	TTTTGACGGT	TCTTACCACG	ATGTCGACTC	TAACGAAATG
Bacillus h	AGGCGAAGCT	TTTTGACGGT	TCTTACCATG	ATGTCGACTC	AAGTGAGATG
Geobacillu	AAGCGAAGCT	GTTTGACGGA	TCGTACCACG	ATGTCGACTC	GAGCGAAGATG
Oceanobaci	AAGCAACACT	TTATGATGGT	AGCTACCATG	ATGTCGACTC	AAACGAGATG
Aquifex ae	GAGTTAGACT	CTTTGACGGT	TCTTACCACG	AAGTTGACTC	TTCGGACATA
Consensus	RR GY DRVRY T	NKWY GAY GGW	WSB TACCA Y G	AWG TY GAY TC	DWVB GAV ATR
					
	1755	1765	1775	1785	1795
Bacillus a	GCGTTCAAAA	TCGCTGCATC	TATGGCACTT	AAAGCTGCGG	TTTCTAAATG
Bacillus s	GCATTTAAAG	TCGCTGCTTC	TATGGCATTG	AAAAATGCAG	TCAGCAAATG
Bacillus t	GCGTTCAAAA	TCGCTGCATC	TATGGCACTT	AAAGCTGCGG	TTTCTAAATG
Bacillus l	GCGTTTAAAA	TCGCTGCTTC	CATGGCGCTT	AAAAACGCGG	CAAGCAAGTG
Bacillus h	GCGTTTAAAG	TTGCTGCTTC	TATGGCACTT	AAGAACGCAA	AATCAAAATG
Geobacillu	GCATTTCAAAA	TCGCTGCTTC	CTTGGCATTG	AAAAACGCCG	CGACGAAGTG
Oceanobaci	GCATTTAAAG	TTGCAGCATC	TATGGCGCTA	AAAGCTGCTA	AAAACAAATG
Aquifex ae	GCATTCCAGG	TTGCGGGTTC	CTTGGCATTG	AAAGATGCAG	CCAAAAAGGC
Consensus	GCR TT Y MA R	TY GCD GSW T	YW TGGC RY T N	AA RRMY GC N R	HNWVN AA RKS

Figure 5.3. ClustalW multiple alignment result of nucleotide sequences indicated in Table

5.2. Primer site (FusF) was shown in blue and consensus sequence was shown in red

(Continued).

					
	1805	1815	1825	1835	1845
Bacillus a	TAACCCAGTA	ATTCTTGAGC	CAATGATGAA	AGTTGAAGTT	GTAATTCCTG
Bacillus s	TAACCCAGTT	CTTCTTGAAC	CAATCATGAA	AGTGGAAGTG	GTTATTCCAG
Bacillus t	TAACCCAGTA	ATTCTTGAGC	CAATGATGAA	AGTTGAAGTT	GTAATTCCTG
Bacillus l	TAACCCAGTT	ATTCTTGAGC	CGATCTCCAA	AGTTGAAGTC	GTTATCCAG
Bacillus h	TAACCCAGTT	CTTCTAGAAC	CAATGATGAA	AGTGGAAGTC	GTTGTACCTG
Geobacillu	CGATCCGGTT	CTTTTGGAAC	CGATCATGAA	AGTCGAAGTC	GTCATCCCTG
Oceanobaci	TAAACCAGTT	CTTCTTGAAC	CAATGATGAG	AGTTGAAGTT	GTTGTTCCCTG
Aquifex ae	AGATCCCGTT	CTTCTGGAAC	CCATAATGGA	AGTTGAAGTG	GAAACTCCCG
Consensus	HR AHCCVGTW	M TTYTDGARC	C VAT VWYSRR	AGTBGAAGTB	G WHRYHCCHG
					
	1855	1865	1875	1885	1895
Bacillus a	AAGAGTACAT	GGGTGACATT	ATGGGTGACG	TAACATCTCG	TCGTGGACGT
Bacillus s	AAGAATATAT	GGGAGATATC	ATGGGTGATA	TCACCTTCACG	TCGCGGACGC
Bacillus t	AAGAGTACAT	GGGTGACATT	ATGGGTGACG	TAACATCTCG	TCGTGGACGT
Bacillus l	AAGAATACAT	GGGAGACATC	ATGGGCGACA	TTACGTCACG	CCGCGGTCGC
Bacillus h	AAGAGTACAT	GGGTGACGTT	ATGGGTGACA	TCACCTTCACG	CCGTGGTCGC
Geobacillu	AAGAGTACCT	CGGCGACATT	ATGGGTGACA	TCACGTCCCG	CCGCGGCCGC
Oceanobaci	AAGAATACAT	GGGAGACATT	ATGGGTGACG	TAACATCCCG	TCGTGGACGC
Aquifex ae	AAAAGTACGT	GGGTGACGTT	ATAGGTGACC	TTAACTCCAG	AAGAGGAAAG
Consensus	AAAR TA Y VT	S GGHGA Y RT	A TRGGYGA YV	THAMN TC H MG	HMGGH GHMRB
					
	1905	1915	1925	1935	1945
Bacillus a	GTAGAAGGTA	TGGAAGCTCG	CGGTAACGCT	CAAGTTGTTC	GCGCTATGGT
Bacillus s	GTAGAAGGTA	TGGAAGCACG	CGGTAACGCT	CAAGTTGTTC	GTGCTATGGT
Bacillus t	GTAGAAGGTA	TGGAAGCTCG	CGGTAACGCT	CAAGTTGTTC	GCGCTATGGT
Bacillus l	GTAGAAGGTA	TGGAAGGACG	CGGAAACGCT	CAGGTCGTTT	GCGCGATGGT
Bacillus h	GTAGAAGGTA	TGGAAGCTCG	CGGTAATGCT	CAAGTGTTTA	AAGCATTCGT
Geobacillu	ATCGAGGGGA	TGGAAGCGCG	CGGCAACGCC	CAAGTTGTTC	GTGCAATGGT
Oceanobaci	GTAGAAGGTA	TGGAAGCACG	CGGTACAGCA	CAAGTTGTTA	AAGCGTTCGT
Aquifex ae	ATTATGGGAA	TGGAAAACAA	GGGAGTTATA	ACAGTCATAA	AGGCTCACGT
Consensus	R THRWR GGDA	TGGAA RVNMR	S GGH RHHRYH	MMR GTBR TWM	RN GCD HWS GT
					
	1955	1965	1975	1985	1995
Bacillus a	TCCACTTTCT	GAAATGTTTCG	GTTATGCAAC	GTCATTACGT	TCTAACACTC
Bacillus s	TCCTCTTGCT	GAAATGTTTCG	GTTACGCTAC	TGCACTTCGT	TCTAATACGC
Bacillus t	TCCACTTTCT	GAAATGTTTCG	GTTATGCAAC	GTCATTACGT	TCTAACACTC
Bacillus l	TCCACTTTCT	GAAATGTTTCG	GTTACGCTAC	GGCTCTCCGT	TCAAACACAC
Bacillus h	TCCTCTTGCT	GAAATGTTTCG	GTTACGCAAC	CTCTTTGCGT	TCTCGTACAC
Geobacillu	GCCGATGGCC	GAAATGTTTCG	GTTATGCGAC	ATCGCTCCGT	TCGAACACGC
Oceanobaci	ACCACTTTCA	GAAATGTTTCG	GTTATGCAAC	TGCCCTACGT	TCGAACACAC
Aquifex ae	TCCCCTCGCA	GAGATGTTTCG	GATACGCTAC	GACGCTCAGG	AGCTTGACAC
Consensus	D CCNM TBK CH	G ARATG TTYG	G WTAYG CDAC	ND CNY T N MGK	WSNHDB ACDC

Figure 5.3. ClustalW multiple alignment result of nucleotide sequences indicated in Table

5.2. Primer site (FusF) was shown in blue and consensus sequence was shown in red

(Continued).

					
	2005	2015	2025	2035	2045
Bacillus a	AAGGACGCGG	AACATTCTCT	ATGGTGTTTG	ACCACTATGA	AGAAGTACCA
Bacillus s	AAGGTCGCGG	TACGTTCACT	ATGCACATGG	ATCACTACGG	AGAAGTGCCG
Bacillus t	AAGGACGCGG	AACATTCTCT	ATGGTGTTTG	ACCACTATGA	AGAAGTACCA
Bacillus l	AAGGACGCGG	TACGTTTACA	ATGGTCTTTG	ACCACTATGA	AGAAGTGCCG
Bacillus h	AAGGACGTGG	AACTTACACA	ATGTTCTTCG	ATCACTACGA	GGAAGTACCT
Geobacillu	AAGGACGCGG	AACGTTCTCG	ATGGTGTTTG	ACCACTATGA	AGAAGTTCCG
Oceanobaci	AAGGTCGCGG	TGTTTACACA	ATGCACTTTG	ATCACTACGA	AGAAACACCG
Aquifex ae	AAGGTAGGGG	AACCTTTATA	ATGAAATTTT	CCCACTACGA	CGAAGTTCCG
Consensus	AAGGWMGBGG	WRYNTWYWYD	ATGNWVWTBK	MY CACTAYGR	V GAA RYD CCD
					
	2055	2065	2075	2085	2095
Bacillus a	AAGTCTGTTT	CTGAAGAAAT	TATCAAAAAA	AATAAAGGTG	AATAA-----
Bacillus s	AAGAGCGTCG	CAGAAGAAAT	TATCAAAAAA	AATAAAGGCG	AATAA-----
Bacillus t	AAGTCTGTTT	CTGAAGAAAT	TATCAAAAAA	AATAAAGGTG	AATAA-----
Bacillus l	AAGAGCATCG	CTGATGAGAT	CATCAAAAAA	AATCAAGGTG	AATAA-----
Bacillus h	AAGAGCATTT	CTGAAGAAAT	TATTAAGAAA	AACTCTGGTG	AATAA-----
Geobacillu	AAAAACATCG	CCGATGAAAT	TATCAAAAAA	AATAAAGGCG	AATAA-----
Oceanobaci	AAAAGCATTG	CTGAAGAAAT	TATTAAGAAA	AATGCTGGTG	AATAA-----
Aquifex ae	CAGCAAATTG	CGGAAAAGAT	TATCGGCGAA	AG-AATGGCC	GGTAAGAGCT
Consensus	MARHVHRTYD	CNGAWRARAT	YATYRRVRAA	ARYNMWGGYS	RR TAAGAGCT
					
	2105				
Bacillus a	-----				
Bacillus s	-----				
Bacillus t	-----				
Bacillus l	-----				
Bacillus h	-----				
Geobacillu	-----				
Oceanobaci	-----				
Aquifex ae	CTTAA				
Consensus	CTTAA				

Figure 5.3. ClustalW multiple alignment result of nucleotide sequences indicated in Table 5.2. Primer site (FusF) was shown in blue and consensus sequence was shown in red.

Table 5.3. Bacterial *rplC* genes used for degenerate primer design.

Organism name	GeneBank ID
<i>Bacillus licheniformis</i> ATCC 14580	3027551
<i>Geobacillus kaustophilus</i> HTA426	3183452
<i>Oceanobacillus iheyensis</i> HTE831	1015209
<i>Bacillus cereus</i> ZK	3022952
<i>Bacillus anthracis</i> str. 'Ames Ancestor'	2818190
<i>Bacillus clausii</i> KSM-K16	3201528

					
	5	15	25	35	45
Bacillus l	ATGACCAAAG	GAATCTTAGG	AAGAAAAATT	GGTATGACGC	AAGTTTTTCGC
Geobacillu	ATGACGAAAAG	GAATCTTAGG	AAGAAAGATC	GGTATGACGC	AAATATTTTGC
Oceanobaci	ATGACGAAAAG	GAATCTTAGG	TCGTAAAAATC	GGCATGACTC	AGCTATTCTC
Bacillus c	ATGACCAAAG	GAATCTTAGG	AAGAAAGATC	GGTATGACTC	AAGTATTTTGC
Bacillus a	ATGACCAAAG	GAATCTTAGG	AAGAAAGATC	GGTATGACTC	AAGTATTTTGC
Bacillus c	ATGACCAAAG	GAATCTTAGG	TAGAAAAATC	GGTATGACTC	AAGTGTTTTGC
Consensus	ATGAC AAAAG	GAATCTTAGG	WMGWAARATY	GGYATGAC KC	ARVTD TTYKC
					
	55	65	75	85	95
Bacillus l	AGAAAACGGT	GATCTTATTC	CGGTAACGTG	TATCGAAGCT	GCTCCTAACG
Geobacillu	GGAAAATGGC	GATTTGATTC	CGGTAACCGT	CATCCATGCG	ACGCCGAACG
Oceanobaci	TGATAATGGA	GAGCTAATTC	CAGTAACAGT	AATTCAAGCT	GAGCCAAATG
Bacillus c	TGAGAACGGT	GAGTTAATCC	CAGTAACAGT	TATCGCTGCT	AATCCAAACG
Bacillus a	TGAGAACGGT	GAGTTAATCC	CAGTAACAGT	TATCGCTGCT	AATCCAAACG
Bacillus c	AGAAAACGGA	GAAATCATTC	CAGTAACAGT	AATTGAAGCA	GAACCAAATA
Consensus	DGADAA YGGH	GADBNAT YC	CRGTAACHGT	HATYS MW GCD	RMDCCD AA YR
					
	105	115	125	135	145
Bacillus l	TTGTGCTTCA	GAAGAAAAC	TCTGAAAACG	ACGGCTATGA	AGCGATTTCAG
Geobacillu	TCGTCTTGCA	AAAGAAAACG	ATCGAAAACG	ACGGTTACGA	AGCGATCCAA
Oceanobaci	TTGTATTGCA	AAAGAAAACA	TTAGAAAATG	ATGGTTACGA	AGCGTTACAA
Bacillus c	TTGTTCTTCA	AAAGAAAACA	ACTGAAAACG	ATGGCTACAA	CGCAATTCAG
Bacillus a	TTGTTCTTCA	AAAGAAAACA	ACTGAAAACG	ATGGCTACAA	CGCAATCCAG
Bacillus c	TTGTCTTCA	AAAGAAGACA	GTGGAATCTG	ATGGCTATGA	GGCGATTTCAG
Consensus	TYGTNY TKCA	RAAGAA RACD	DYNGAA W MYG	AYGGY TA YRA	VGCRW TH CAR
					
	155	165	175	185	195
Bacillus l	ATCGGTTTTG	ACGACAAGCG	TGAAAAGCTT	GCCAACAAAC	CAGAAAAAGG
Geobacillu	TTAGGTTTTG	AAGATATTAG	CGAAAAACGC	GCCAATAAAC	CGCAAATTGG
Oceanobaci	ATCGGTTTTG	CTGATAAAAA	GGAATCACGT	ACTAATAAAG	CGGAAAAAGG
Bacillus c	TTAGGATTTG	AAGATAAACG	TGAAAAGTTA	ACTAACAAAC	CTGAACAAGG
Bacillus a	TTAGGATTTG	AAGATAAACG	TGAAAAGTTA	ACTAACAAAC	CTGAACAAGG
Bacillus c	ATCGGTTTTG	CTGATGCGAA	AAAGCCGAAC	AAACCAGCTA	CTGGCCATGC
Consensus	WTMGGW TTTG	MHGAYR HDMR	NRAR HMRHDH	RMHMMH RMWV	CDSRMM WWGS
					
	205	215	225	235	245
Bacillus l	ACACGTTGCA	AAAGCGGAAA	CTGCTCCTAA	GCGCTTCGTT	AAGGAATTAC
Geobacillu	CCATGCTGCC	AAAGCGAACA	CGGCACCTAA	GCGCTTCATT	CGTGAAATCC
Oceanobaci	TCATGCTGAG	AAAGCAGGCA	CTGCCCTAA	GCGCTACGTT	CGTGAAATCC
Bacillus c	CCACACTGCT	AAAGCATCTA	CAACTCCTAA	GCGCTTCATT	CGCGAAATCC
Bacillus a	CCACACTGCT	AAAGCATCTA	CAACTCCTAA	GCGCTTCATT	CGCGAAATCC
Bacillus c	CGCTAAAGCT	GAAACGGCTC	CTAAGCGCTT	CATTAAGGAA	ATCCGTGGCG
Consensus	HSMYRHW GMN	RAAR CRD VHM	CDR MNC SYWW	SMKYW WSRWW	MDBSR WDKMS

Figure 5.4. ClustalW multiple alignment result of nucleotide sequences indicated in Table 5.3. Primer site (RplC) was shown in blue and consensus sequence was shown in red (Continued).

					
	255	265	275	285	295
Bacillus l	GCGGAGTGGG	TATGGATGCG	TATGAAGTTG	GTCAGGAAGT	CAAAGTTGAT
Geobacillu	GCGGCGCCAA	CATCGATGAA	TATGAAGTTG	GCCAAGAAGT	GAAAGTTGAC
Oceanobaci	GTGGCGCAGA	GGTTAATAGC	TTTGAAGTTG	GGCAAGAGAT	CAAAGTCGAC
Bacillus c	GCGATGCAGA	CGTGGACGGA	TTAGAGGTTG	GTCAAGAGGT	AAAAGTTGAC
Bacillus a	GCGATGCAGA	CGTGGACGGA	TTAGAGGTTG	GTCAAGAGGT	AAAAGTTGAC
Bacillus c	TTAATCTTGA	CGAAGTTGAA	GTAGGTCAAG	CGATTAACGT	AACAACATTT
Consensus	KYRRHSYNRA	BRWNRWYRVV	KWWGRDSWWG	SBMWDRAVRT	VAMARYHKWY
					
	305	315	325	335	345
Bacillus l	ATTTTCTCTA	ACGGAGAAAT	CGTAGATGTA	ACAGGAACAT	CGAAAGGTAA
Geobacillu	ATTTTCAGCG	AAGGCGAAAT	TGTCGATGTC	ACGGGCATTT	CGAAAGGGAA
Oceanobaci	ATCTTTGAGT	CTGGAGAAAA	AATCGATGTA	ACTGGTACAT	CTAAAGGGAA
Bacillus c	GTATTCGCTA	CAGGTGAAAT	CGTTGATGTA	ACAGGAATTT	CTAAAGGTAA
Bacillus a	GTATTCGCTA	CAGGTGAAAT	CGTTGATGTA	ACAGGAATTT	CTAAAGGTAA
Bacillus c	GCAGCAGGAG	ATCTTGTTGA	CGTAACAGGA	ACATCTAAAG	GGAAAGGGTT
Consensus	RYHKYHDVND	MHSKHGWWRW	HRTHRMWGKM	ACDKSHAHWK	SKAAAGGKWW
					
	355	365	375	385	395
Bacillus l	AGGTTTCCAA	GGCGCTATCA	AACGCCACGG	ACAATCTCGC	GGACCAATGT
Geobacillu	AGGTTTCCAA	GGGGCGATCA	AACGCCACGG	TCAATCACGC	GGACCAATGG
Oceanobaci	AGGTTTCCAG	GGTGTAATTA	AGCGTCATGG	TCAACAACGT	GGACCAACTA
Bacillus c	AGGTTTCCAA	GGTGTTATCA	AACGCCACGG	ACAATCTCGC	GGACCTATGT
Bacillus a	AGGTTTCCAA	GGTGTTATCA	AACGCCACGG	ACAATCTCGC	GGACCTATGT
Bacillus c	CCAAGGCGCG	ATCAAACGCC	ATAACCAATC	CCGTGGACCA	ATGTCACACG
Consensus	MSRWKKCSMR	RKBRHDMKYM	ADMRYCAHKS	HCRWBVWCSH	RKRYCWMHBD
					
	405	415	425	435	445
Bacillus l	CTCACGGTTC	ACGCTACCAC	CGCCGTCCTG	GTTCAATGGG	ACCGGTAGAC
Geobacillu	CTCACGGTTC	CCGTTATCAT	CGCCGTCCGG	GTTTCGATGGG	GTCCATCGCG
Oceanobaci	CACACGGTTC	TCATTTCCAT	AGAGCTCCAG	GTGCAATGGG	TGTAATTGAT
Bacillus c	CTCATGGTTC	TCGCTATCAC	CGTCGTCCAG	GTTCAATGGG	CCCAGTTGCT
Bacillus a	CTCATGGTTC	TCGCTATCAC	CGTCGTCCAG	GTTCAATGGG	CCCAGTTGCT
Bacillus c	GCTCTCGTTA	CCACCGTCGT	CCAGGTTCAT	TGGGCCCTGT	AGCGCCAAAC
Consensus	SHYMYSGTTM	HCRYDYDCRY	MSHSSTYCDR	KKKSVMYK GK	NBYVVYHRMB
					
	455	465	475	485	495
Bacillus l	CCTAACCGTG	TATTCAAAGG	AAAGCTGTTG	CCTGGACGTA	TGGGCGGAGA
Geobacillu	CCAAACCGCG	TTTTCAAAAC	GAAAAACTTG	CCGGGCCGCA	TGGGCGGTGA
Oceanobaci	CCAATGCGTG	TATTCAAAGG	TAAAAAATA	CCAGGTCACA	TGGGTTCTGA
Bacillus c	CCGAACCGTG	TATTCAAAGG	CAAAAACTT	GCTGGACGTA	TGGGTGGAGA
Bacillus a	CCGAACCGTG	TATTCAAAGG	CAAAAACTT	GCTGGACGTA	TGGGTGGAGA
Bacillus c	CGTGTATTTA	AAGGAAAAGC	GCTTCCTGGC	CGTATGGGCG	GAGAACAAAT
Consensus	CSDRWVYKYR	WWKKMAAARS	NMWDMHNBNK	SSDRKNSRYR	KRGHRBVRWR

Figure 5.4. ClustalW multiple alignment result of nucleotide sequences indicated in Table 5.3. Primer site (RplC) was shown in blue and consensus sequence was shown in red (Continued).

					
	505	515	525	535	545
Bacillus l	GCAAATCACT	GTTCAAAACC	TTGAAATCGT	AAAAGTAGAT	GCAGAGCGCA
Geobacillu	GCGCGTGACG	ATCCAAAAC	TGAAAATCGT	GAAAGTGGAT	CCGGAGCGCA
Oceanobaci	ACAAGTAACG	ATCCAAAACC	TTGAAGTTGT	AAGTGTAGAT	ACGGATAAGA
Bacillus c	CCAAGTTACT	ATCCAAAAC	TAGAAAATCGT	TCAAGTTGAC	ACTGAGCGCA
Bacillus a	CCAAGTTACT	ATCCAAAAC	TAGAAAATCGT	TCAAGTTGAC	ACTGAGCGCA
Bacillus c	TACAATGCAA	AACCTTGAGA	TCGTTGCGGT	TGACGAAGAG	CGCAACTTGC
Consensus	NMVMR T NMMD	RWYCWW R ASH	T NRWWVKY G T	DVRH G WD G AB	VSNR A BHDSM
					
	555	565	575	585	595
Bacillus l	ACCTTCTTTT	AGTTAAAGGT	AACGTTCCCTG	GTGCCAAAAA	ATCACTAGTC
Geobacillu	ACTTGCTGCT	CATTAAAGGC	AATGTGCCCG	GTCCGAGAAA	AGGCTTAGTC
Oceanobaci	ATCTACTACT	AATTAAAGGT	AACGTGCCTG	GCGCGAAAAA	ATCTTACGTT
Bacillus c	ACTTATTATT	AGTAAAAGGT	AACGTTCCAG	GTGCTAAGAA	ATCTCTTGTA
Bacillus a	ACTTATTATT	AGTAAAAGGT	AACGTTCCAG	GTGCTAAGAA	ATCTCTTGTA
Bacillus c	TTCTCGTTAA	AGGCAACGTA	CCAGGGGCTA	AGAAAAGCTA	TGTAAGTGTA
Consensus	WYY T NB T DHW	MRKH A AMGKH	MMH G KK S CDR	RBVMN A RVW A	WKBHHHH G TH
					..
	605	615	625	635	
Bacillus l	ACTGTTAAAA	GTGCTGTTAA	ATCTAAATAA		..
Geobacillu	ATCGTCAAAA	GCGCCGTGAA	AGCGGCGAAG	AAGGCGAAAT	AA
Oceanobaci	AAAATCACAA	GTGCAGTAAA	AGGTAATAA		..
Bacillus c	GTTGTTCAAG	GCGCTGTGAA	GGTAGCAAA	TAA.....	..
Bacillus a	GTTGTTCAAG	GCGCTGTGAA	GGTAGCAAA	TAA.....	..
Bacillus c	CAATCTGCAG	TTAAGGCGTA	A.....		..
Consensus	VHHDYYVM A R	KYRMN G YDW A	RKBKR V W A R	W A R G CGAAAT	AA

Figure 5.4. ClustalW multiple alignment result of nucleotide sequences indicated in Table 5.3. Primer site (RplC) was shown in blue and consensus sequence was shown in red.

5.1.3. Partial PCR Amplification of *tuf* Gene

By using degenerate TuF413 and TuR1174 primers, *tuf* gene was partially amplified in a gradient PCR in the range of 50-60 °C annealing temperature. As an optimal degree, 56 °C annealing temperature was chosen. According to the ClustalW multiple alignment results of the species related to *G. anatolicus*, expected PCR product size was 761 bp. At 56 °C, PCR product appeared as a single band at the expected size when analyzed electrophoretically on 1 % agarose gels (Figure 5.5.).

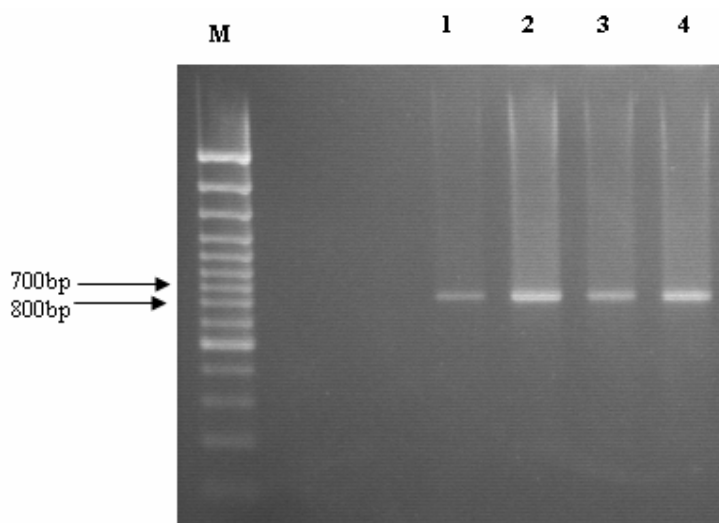


Figure 5.5. Partial PCR amplification of *G.anatolicus tuf* gene. M, molecular size marker; 1, 2, 3, 4, PCR product containing 761 bp partial *tuf* gene at 56 °C annealing temperature at 1,5 mM, 2 mM, 3 mM and 4 mM of magnesium concentrations

5.1.4. Confirmation of Partial *tuf* Sequence

After obtaining of the DNA fragment that was supposed to contain a part of a *tuf* sequence, this fragment was sequenced by using using TuF413 and TuR1174 primers,. The sequence data obtained was compared with other EF-Tu genes in GeneBank and confirmed that this fragment indeed contains part of a *tuf* gene. This sequence will later be used to design sequencing primers for the complete *Geobacillus anatolicus tuf* gene.

5.1.5. Amplification of the Fragment Containing Complete *tuf* Sequence

To amplify the fragment that contains the complete *tuf* gene, FusF and RplC primers located in the adjacent genes of *tuf* were used. Expected product size was determined as approximately 2100 bp by analyzing genomic sequences of various phylogenetically related species indicated in Table 5.2. and Table 5.3. PCR results were shown in Figure 5.6. confirming that the PCR products were at the expected size. Before sequencing of this fragment, it was confirmed, by an additional PCR amplification using TuF413 and TuR1174 primers that *tuf* gene is indeed present in this fragment (Figure 5.7.)

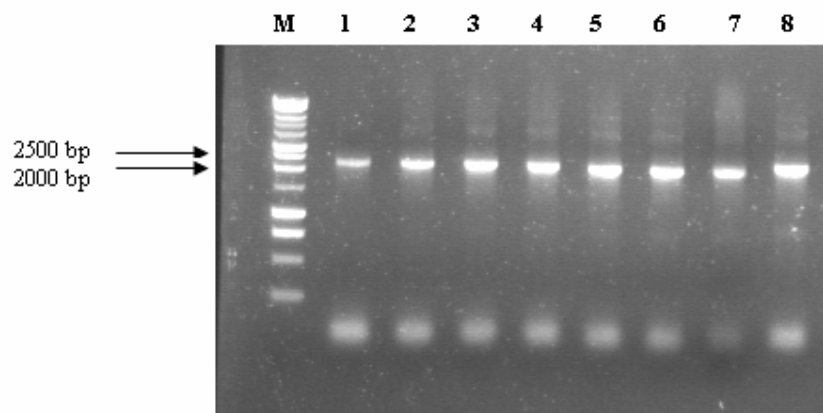


Figure 5.6. PCR amplification using FusF and RplC primers. M, molecular weight marker, 1, 2, 3, 4, 5, 6, 7, 8, PCR results in the range of 50-60 °C annealing temperature

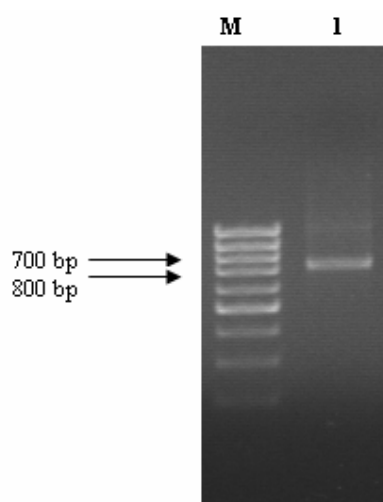


Figure 5.7. Confirmation of the presence of the *tuf* gene in the fragment amplified by using FusF and RplC primers. M, molecular weight marker; 1, PCR product by using TuF413 and TuR1174 primers

5.1.6. Complete *G.anatolicus tuf* Sequence

At first, the fragment amplified by using TuF413 and TuR1174 primers was sequenced with the same primers. After sequencing of this fragment with TuF413 and TuR1174, obtained sequence data was used to design new non-degenerate primers TuF888

and TuR776. The fragment amplified with FusF and RplC primers carrying the complete *tuf* gene was then sequenced with TuF888, TuR776, FusF and RplC primers. Obtained six sequence data was aligned and used to construct the complete *tuf* sequence. 1188 bp long complete *tuf* gene was shown in the Figure 5.8. Nucleic Acid Statistics (NASTATS) were given in Table 5.4.

					
	5	15	25	35	45
EF-Tu G. a	ATG GCTAAAG	CGAAATTTGA	GCGTACGAAA	CCGCACGTCA	ACATTGGCAC
					
	55	65	75	85	95
EF-Tu G. a	GATCGGCCAC	GTTGACCATG	GGAAAACGAC	GTTGACAGCT	GCGATCACAA
					
	105	115	125	135	145
EF-Tu G. a	CAGTTCTTGC	GAAACAAGGG	AAAGCGGAAG	CAAGAGCGTA	CGACCAAATC
					
	155	165	175	185	195
EF-Tu G. a	GACGCAGCTC	CGGAAGAGCG	TGAACGCGGA	ATCACGATT	CGACAGCGCA
					
	205	215	225	235	245
EF-Tu G. a	CGTTGAGTAT	GAAACGGACG	CTCGTCACTA	TGCGCACGTT	GACTGCCCAG
					
	255	265	275	285	295
EF-Tu G. a	GCCACGCTGA	CTACGTGAAA	AACATGATCA	CGGGCGCAGC	GCAAATGGAC
					
	305	315	325	335	345
EF-Tu G. a	GGCGCGATCC	TTGTTGTATC	GGCTGCTGAC	GGTCCGATGC	CGCAAACGCG
					
	355	365	375	385	395
EF-Tu G. a	CGAACACATT	CTTCTCTCCC	GCCAAGTCGG	TGTACCGTAC	ATCGTTGTTT
					
	405	415	425	435	445
EF-Tu G. a	TCTTGAACAA	ATGCGACATG	GTGGACGACG	AAGAATTGCT	TGAACTCGTT
					
	455	465	475	485	495
EF-Tu G. a	GAAATGGAAG	TTCGCGATCT	TCTCTCTGAA	TACGACTTCC	CGGGCGACGA

Figure 5.8. Complete *G. anatolicus tuf* sequence. Start and stop codons were shown as bold
(Continued).

```

      ....|....| ....|....| ....|....| ....|....| ....|....|
      505      515      525      535      545
EF-Tu G. a AGTGCCGGTC ATCAAAGGTT CGGCATTAAA AGCGCTCGAA GGCGACCCGC

      ....|....| ....|....| ....|....| ....|....| ....|....|
      555      565      575      585      595
EF-Tu G. a AATGGGAAGA AAAAATCATT GAACTGATGA ACGCGGTTGA CGAGTACATC

      ....|....| ....|....| ....|....| ....|....| ....|....|
      605      615      625      635      645
EF-Tu G. a CCGACTCCGC AACGTGAAGT AGACAAACCG TTCATGATGC CGGTTGAGGA

      ....|....| ....|....| ....|....| ....|....| ....|....|
      655      665      675      685      695
EF-Tu G. a CGTTTTCTCG ATCACAGGCC GCGGTACGGT TGCGACGGGC CGTGTGAGC

      ....|....| ....|....| ....|....| ....|....| ....|....|
      705      715      725      735      745
EF-Tu G. a GCGGTACGTT AAAAGTCGGC GACCCGGTTG AAATTATCGG TCTTTCGGAC

      ....|....| ....|....| ....|....| ....|....| ....|....|
      755      765      775      785      795
EF-Tu G. a GAGCCGAAAA CGACGACGGT TACGGGTGTA GAAATGTTCC GTAAGCTGCT

      ....|....| ....|....| ....|....| ....|....| ....|....|
      805      815      825      835      845
EF-Tu G. a TGACCAAGCG GAAGCTGGCG ACAACATCGG TGCGCTTCTC CGCGGTGTAT

      ....|....| ....|....| ....|....| ....|....| ....|....|
      855      865      875      885      895
EF-Tu G. a CGCGTGACGA AGTTGAGCGC GGCCAAGTAT TGGCGAAACC GGGCTCGATC

      ....|....| ....|....| ....|....| ....|....| ....|....|
      905      915      925      935      945
EF-Tu G. a ACGCCGCACA CGAAATTTAA AGCGCAAGTT TACGTTCTGA CGAAAGAAGA

      ....|....| ....|....| ....|....| ....|....| ....|....|
      955      965      975      985      995
EF-Tu G. a AGGCGGACGC CATACTCCGT TCTTCTCGAA CTACCGTCCG CAATTCTACT

      ....|....| ....|....| ....|....| ....|....| ....|....|
      1005     1015     1025     1035     1045
EF-Tu G. a TCCGTACAAC GGACGTAACG GGCATCATCA CGCTTCCGGA AGGCGTAGAA

      ....|....| ....|....| ....|....| ....|....| ....|....|
      1055     1065     1075     1085     1095
EF-Tu G. a ATGGTTATGC CTGGCGACAA CGTTGAAATG ACGGTTGAAC TGATCGCTCC

      ....|....| ....|....| ....|....| ....|....| ....|....|
      1105     1115     1125     1135     1145
EF-Tu G. a GATCGCGATC GAGGAAGGAA CAAAATTCTC GATCCGTGAA GGCGGCCGCA

      ....|....| ....|....| ....|....| ....|....
      1155     1165     1175     1185
EF-Tu G. a CGGTTGGTGC TGGTTCCGTA TCGGAAATCA TTGAGTAA

```

Figure 5.8. Complete *G. anatolicus tuf* sequence. Start and stop codons were shown as bold (Continued).

Table 5.4. NATSTATS result *G. anatolicus tuf* sequence

	Total #	Percentage
All nucleotides	1188	
A	312	26.3 %
T	240	20.2 %
G	299	25.2 %
C	337	28.4 %
A and T	552	46.5 %
C and G	636	53.5 %

By using complete *tuf* sequence obtained, *G. anatolicus* complete EF-Tu amino acid sequence was determined. This sequence was shown in Figure 5.9. The amino acid sequence of *G. anatolicus* EF-Tu was aligned and compared with other thermophilic EF-Tu sequences using ClustalW multiple alignment. The species used in this alignment were as follows: *Geobacillus stearothermophilus*, *Aquifex pyrophilus*, *Aquifex aeolicus*, *Thermus aquaticus*, *Thermus thermophilus* HB27, *Thermotoga maritima*, *Thermoanaerobacter tengcongensis*. The alignment result and conserved regions were shown in Figure 5.10.

```

MAKAKFERTK PHVNIGTIGH VDHGKTTLTA AITTVLAKQG KAEARAYDQI
DAAPEERERG ITISTAHVEY ETDARHYAHV DCPGHADYVK NMITGAAQMD
GAILVVSAAAD GPMPQTREHI LLSRQVGVPY IVVFLNKCDM VDDEELLELV
EMEVRDLLSE YDFPGDEV PV IKGSALKALE GDPQWEEKII ELMNAVDEYI
PTPQREVDKP FMMPVEDVFS ITGRGT VATG RVERGTLKVG DPVEIIGLSD
EPKTTTVTGV EMFRKLLDQA EAGDNIGALL RGVSRDEVER GQVLAKPGSI
TPHTKFKAQV YVLTKEEGGR HTPFFSNYRP QFYFRTTDVT GIITLPEGVE
MVMPGDNVEM TVELIAPIAI EEGTKFSIRE GGRTVGAGSV SEIIE

```

Figure 5.9. Complete *G. anatolicus* EF-Tu amino acid sequence.

					
	5	15	25	35	45
Geobacillu	MAKAKFERTK	PHVNIGTIGH	VDHGKTTLTA	AITTVLAK--	-QGK-AEAKA
Aquifex py	MAKEKFERTK	EHVNVGTIGH	VDHGKSTLTS	AITCVLAAGL	VEGGKAKCFK
Aquifex ae	MAKEKFERTK	EHVNVGTIGH	VDHGKSTLTS	AITCVLAAGL	VEGGKAKCFK
Thermus aq	MAKGEFIRTK	PHVNVGTIGH	VDHGKTTLTA	ALTYVAAAE-	--NPNVEVKD
Thermus th	MAKGEFIRTK	PHVNVGTIGH	VDHGKTTLTA	ALTYVAAAE-	--NPNVEVKD
Thermotoga	MAKEKFVRTK	PHVNVGTIGH	IDHGKSTLTA	AITKYLSL--	--KGLAQYIP
Thermoanae	MAKQKFERTK	PHVNVGTIGH	VDHGKTTLTA	AITLILSKA-	---GLAQAKG
G. anatoli	MAKAKFERTK	PHVNIGTIGH	VDHGKTTLTA	AITTVLAK--	-QGK-AEARA
Consensus	MAK F RTK	HVN GTIGH	DHGK TLT	A T	
					
	55	65	75	85	95
Geobacillu	YDQIDAAPEE	RERGITISTA	HVEYETEARH	YAHVDCPGHA	DYVKNMITGA
Aquifex py	YEEIDKAPEE	KERGITINIT	HVEYETAKRH	YAHVDCPGHA	DYIKNMITGA
Aquifex ae	YEEIDKAPEE	KERGITINIT	HVEYETAKRH	YAHVDCPGHA	DYIKNMITGA
Thermus aq	YGDIDKAPEE	RARGITINTA	HVEYETAKRH	YSHVDCPGHA	DYIKNMITGA
Thermus th	YGEIDKAPEE	RARGITINTA	HVEYETAKRH	YSHVDCPGHA	DYIKNMITGA
Thermotoga	YDQIDKAPEE	KARGITINIT	HVEYETEKRH	YAHIDCPGHA	DYIKNMITGA
Thermoanae	YDEIDKAPEE	KARGITINTT	HVEYETAKRH	YAHVDCPGHA	DYVKNMITGA
G. anatoli	YDQIDAAPEE	RERGITISTA	HVEYETDARH	YAHVDCPGHA	DYVKNMITGA
Consensus	Y ID APEE	RGITI	HVEYET RH	Y H DCPGHA	DY KNMITGA
					
	105	115	125	135	145
Geobacillu	AQMDGAILVV	SAADGMPMQT	REHILLSRQV	GVPIYIVVFLN	KCDMVDDEEL
Aquifex py	AQMDGAILVV	SAADGMPMQT	REHVLLARQV	NVPYIVVFMN	KCDMVDDEEL
Aquifex ae	AQMDGAILVV	SAADGMPMQT	REHVLLARQV	NVPYIVVFMN	KCDMVDDEEL
Thermus aq	AQMDGAILVV	SAADGMPMQT	REHILLARQV	GVPIYIVVFMN	KVDMVDDPEL
Thermus th	AQMDGAILVV	SAADGMPMQT	REHILLARQV	GVPIYIVVFMN	KVDMVDDPEL
Thermotoga	AQMDGAILVV	AATDGPMPQT	REHVLLARQV	EVPMIVVFMN	KTDMVDDPEL
Thermoanae	AQMDGAILVV	SAADGMPMQT	REHILLARQV	GVPIYIVVFLN	KADMVDDPEL
G. anatoli	AQMDGAILVV	SAADGMPMQT	REHILLSRQV	GVPIYIVVFLN	KCDMVDDEEL
Consensus	AQMDGAILVV	A DGPMPQT	REH LL RQV	VPY VF N	K DMVDD EL
					
	155	165	175	185	195
Geobacillu	LELVEMEVRD	LLSEYDFPGD	EVPVIKGSAL	KALEGDP---	-----KWE
Aquifex py	LELVELEVRE	LLSKYEYPGD	EVPVIRGSAL	GALQELEQNS	PG-----KWV
Aquifex ae	LELVELEVRE	LLSKYEYPGD	EVPVIRGSAL	GALQELEQNS	PG-----KWV
Thermus aq	LDLVEMEVRD	LLNQYEFPGD	EVPVIRGSAL	LALEEMHKNP	KTKRGENEWV
Thermus th	LDLVEMEVRD	LLNQYEFPGD	EVPVIRGSAL	LALQMHRNP	KTRRGENEWV
Thermotoga	IDLVEMEVRD	LLSQYGYPGD	EVPVIRGSAL	KAVEAP-NDP	NH-----EAY
Thermoanae	IELVEMEVRD	LLNQYEFPGD	DTPIVVGSAL	KALECGCGKR	ECQ-----WC
G. anatoli	LELVEMEVRD	LLSEYDFPGD	EVPVIKGSAL	KALEGDP---	-----QWE
Consensus	LVE EVR	LL Y PGD	P	GSAL	A

Figure 5.10. Sequence alignment of thermophilic EF-Tu's and G.anatolicus EF-Tu amino acid sequences. Conserved regions were shown as bold (Continued).

					
	205	215	225	235	245
Geobacillu	EKIIELMNAV	DEYIPTPQRE	VDKPFMMPIE	DVFSITGRGT	VATGRVERGT
Aquifex py	GSIKELLNAM	DEYIPTPERE	VDKPFLMPIE	DVFSISGRGT	VVTGRVERGV
Aquifex ae	ESIKELLNAM	DEYIPTPQRE	VDKPFLMPIE	DVFSISGRGT	VVTGRVERGV
Thermus aq	DKIWELLDAL	DEYIPTPVRD	VDKPFLMPVE	DVFTITGRGT	VATGRIERGK
Thermus th	DKIWELLDAL	DEYIPTPVRD	VDKPFLMPVE	DVFTITGRGT	VATGRIERGK
Thermotoga	KPIQELLDAM	DNYIPDPQRD	VDKPFLMPIE	DVFSITGRGT	VVTGRIERGR
Thermoanae	GKIWELMDVV	DEYIPTPERD	IDKPFLMPVE	DVFTITGRGT	VATGRVERGK
G. anatoli	EKIIELMNAV	DEYIPTPQRE	VDKPFMMMPVE	DVFSITGRGT	VATGRVERGT
Consensus	I EL	D YIP P R	DKPF MP E	DVF I GRGT	V TGR ERG
					
	255	265	275	285	295
Geobacillu	LKVGDPVEII	GLSDEPKATT	VTGVEMFRKL	LDQAEAGDNI	GALLRGVSRD
Aquifex py	LRPGDEVEIV	GLREEPLKTV	ATSIEMFRKV	LDEALPGDNI	GVLLRGVGKD
Aquifex ae	LRPGDEVEIV	GLREEPLKTV	ATSIEMFRKV	LDEALPGDNI	GVLLRGVGKD
Thermus aq	VKVGDEVEIV	GLAPETRKTIV	VTGVEMHRKT	LQEGIAGDNV	GLLLRGVSR
Thermus th	VKVGDEVEIV	GLAPETRKTIV	VTGVEMHRKT	LQEGIAGDNV	GVLLRGVSR
Thermotoga	IRPGDEVEII	GLSYEIKKTV	VTSVEMFRKE	LDEGIAGDNI	GCLLRGIDKD
Thermoanae	VKVGDEVEII	GLTTESRKTIV	VTGVEMFRKT	LDEAQAGDNI	GVLLRGIQRD
G. anatoli	LKVGDPVEII	GLSDEPKTTT	VTGVEMFRKL	LDQAEAGDNI	GALLRGVSRD
Consensus	GD VEI	GL E T	T EM RK	L GDN	G LLRG
					
	305	315	325	335	345
Geobacillu	EVERGQVLAK	PGSITPHTKF	KAQVYVLTKE	EGGRHTPFFS	NYRPQFYFRT
Aquifex py	DVERGQVLAQ	PGSVKAHRKF	RAQVYVLSKE	EGGRHTPFFV	NYRPQFYFRT
Aquifex ae	DVERGQVLAQ	PGSVKAHRKF	RAQVYVLSKE	EGGRHTPFFV	NYRPQFYFRT
Thermus aq	EVERGQVLAK	PGSITPHTKF	EASVYILKKE	EGGRHTGFFT	GYRPQFYFRT
Thermus th	EVERGQVLAK	PGSITPHTKF	EASVYVLKKE	EGGRHTGFFS	GYRPQFYFRT
Thermotoga	EVERGQVLAA	PGSIKPHKRF	KAQIYVLKKE	EGGRHTPFTK	GYPKQFYIRT
Thermoanae	EVERGQVLAK	PGTIKPHTKF	EAQVYVLTKE	EGGRHTPFFN	GYRPQFYFRT
G. anatoli	EVERGQVLAK	PGSITPHTKF	KAQVYVLTKE	EGGRHTPFFS	NYRPQFYFRT
Consensus	VERGQVLA	PG H F	A Y L KE	EGGRHT F	Y PQFY RT
					
	355	365	375	385	395
Geobacillu	TDVTG-IITL	PEGVEMVMPG	DNVEMTVELI	APIAIEEGTK	FSIREGGRTV
Aquifex py	ADVTGTIVKL	PEGVEMVMPG	DNVELEVELI	APVALEEGLR	FAIREGGRTV
Aquifex ae	ADVTGTIVKL	PEGVEMVMPG	DNVELEVELI	APVALEEGLR	FAIREGGRTV
Thermus aq	TDVTG-VVRL	PQGVEMVMPG	DNVTFTVELI	KPVALEEGLR	FAIREGGRTV
Thermus th	TDVTG-VVQL	PPGVEMVMPG	DNVTFTVELI	KPVALEEGLR	FAIREGGRTV
Thermotoga	ADVTGEIVGL	PEGVEMVMPG	DHVEMEIELI	YPVAIEKGQR	FAVREGGRTV
Thermoanae	TDVTG-TIQL	PEGVEMVMPG	DHVTLRVELI	TPIAMEEGLK	FAIREGGRTV
G. anatoli	TDVTG-IITL	PEGVEMVMPG	DNVEMTVELI	APIAIEEGTK	FSIREGGRTV
Consensus	DVTG	L P GVEMVMPG	D V	ELI P A E G	F REGGRTV

Figure 5.10. Sequence alignment of thermophilic EF-Tu's and G.anatolicus EF-Tu amino acid sequences. Conserved regions were shown as bold (Continued).

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          ....|....|
          405
Geobacillu  GAGSVSEIIE
Aquifex py  GAGVVTKILD
Aquifex ae  GAGVVTKILD
Thermus aq  GAGVVTKILE
Thermus th  GAGVVTKILE
Thermotoga  GAGVVTEVIE
Thermoanae  GAGVSAIIE
G. anatoli  GAGSVSEIIE
Consensus  GAG V

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Figure 5.10. Sequence alignment of thermophilic EF-Tu's and *G. anatolicus* EF-Tu amino acid sequences. Conserved regions were shown as bold.

5.2. Cloning of *tuf* Gene of *G. anatolicus*

5.2.1. Cloning Method

In this study, pT7D3 vector was used as the cloning vector. pT7D3 was derived from a commercially available pCR[®]T7/NT-TOPO[®] vector. pCR[®]T7/NT-TOPO[®] contains the bacterial phage T7 promoter site for high-level protein expression in *Escherichia coli*, N-terminal Xpress[™] epitope for detection with an Anti-Xpress[™] Antibody, N-terminal polyhistidine (6xHis) tag for purification using Ni-affinity chromatography, detection site with an Anti-HisG Antibody, enterokinase cleavage site for efficient removal of the N-terminal fusion tag and ampicillin resistance site for detection of transformants carrying plasmid. In this study, original pCR[®]T7/NT-TOPO[®] vector wasn't used because of enterokinase encountered non-specific cleavage problems in previous experiments in our laboratory. Instead of this, NdeI and Csp45I double digested pCR[®]T7/NT-TOPO[®] plasmid was used as a cloning vector (Figure 5.11.). This new vector was called pT7D3.



Figure 5.11. pCR[®]T7/NT-TOPO[®] cloning sites (BstBI cleavage site is identical to Csp45I)

5.2.2. Constructing Primers for Cloning of *tsf* Gene

To ligate *tuf* gene into pT7D3 vector, two appropriate forward and reverse primers named TuF2 and TuR2 were designed. These primers contained additional sites for appropriate digestion and for ligation steps necessary during the cloning of the *tuf* gene. Primers and additional sites were shown in Figure 5.12.

TuF2: 5'- **CGG GGG GCA TAT GGC** TAA AGC GAA ATT TGA GCG TAC GAA A -
3'

1 **2**

TuR2: 5'- **CAA AAA ATT CGA ATT AAT GAT GAT GAT GAT GAT GCT** CAA
TGA

1 **2** **3** **4**

TTT CCG ATA CGG AAC CAG C - 3'

Figure 5.12. TuF2: 1, 7 bp long overhang for efficient digestion; 2, NdeI cut site (it contains translation start codon ATG); the rest of primer corresponds to the beginning of *tuf* gene

TuR2: 1, 7 bp long overhang for efficient digestion; 2, Csp45I cut site; 3, stop codon; 4, 6xHis tag coding region; the rest of primer corresponds to the end of *tuf* gene

After construction of TuF2 and TuR2 primers, these primers were used to amplify the complete *G. anaticus tuf* gene. The expected product size was approximately 1200

bp. The results of a gradient PCR in the range of 50-65 °C. as analyzed electrophoretically on a 1% agarose gel is shown in Figure 5.13.

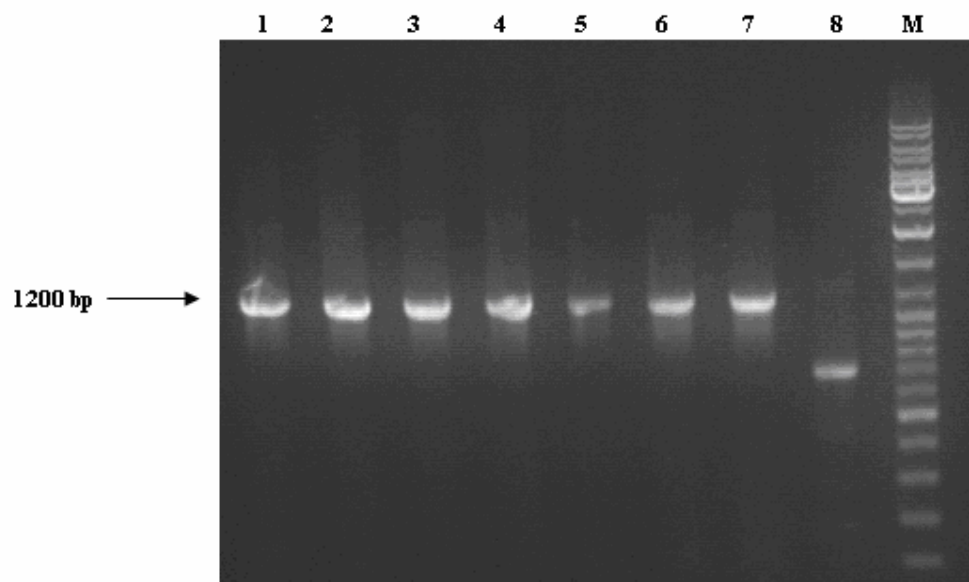


Figure 5.13. Result of the PCR amplification using TuF2 and TuR2 primers; 1, 2, 3, 4, 5, 6, 7, the fragment containing *tuf* gene with annealing temperatures in the range of 50-65°C, 8, positive control with the primers TuF413 and TuR1174, M, DNA molecular weight marker

5.2.3. Construction of pT7D3Tu

pCR[®]T7/NT-TOPO[®] vector was digested with Nde I and Csp45 I restriction enzymes. The resulting linear vector was named as pT7D3. The PCR fragment that was amplified using TuF2 and TuR2 pair was ligated into pT7D3. The resulting circular plasmid was named as pT7D3Tu. pT7D3Tu was used to transform *Escherichia coli* JM109 (DE3) cells. After transformation, plasmids were isolated from transformants. Analysis of purified plasmids on 1% agarose gel confirmed the insertion of the fragments into the vector (Figure 5.14.).

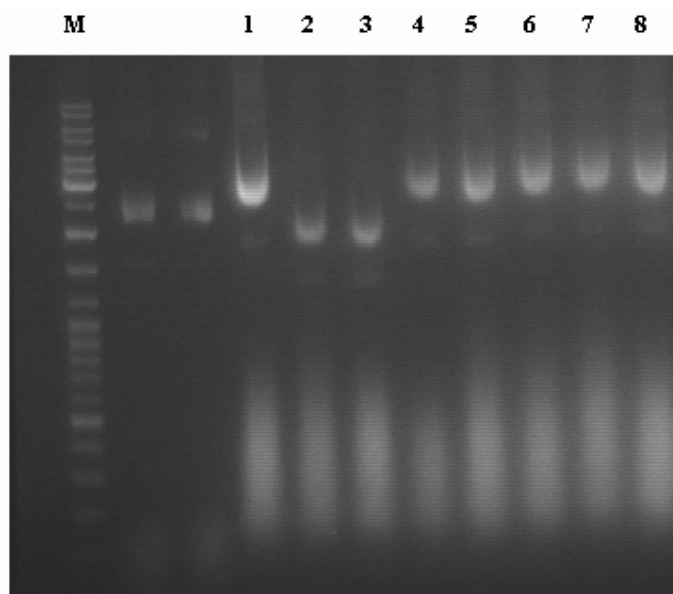


Figure 5.14. Result of plasmid isolation from transformants; M, molecular size marker; 1, 4, 5, 6, 7, 8, plasmids with *tuf* gene; 2, 3, plasmids without *tuf* gene

5.2.4. Confirmation of the presence of the *tuf* Gene in the pT7D3Tu vector

To confirm that the *tuf* gene is actually present in the pT7D3Tu vector, a PCR amplification was performed using two primers: T7 which is located on the plasmid and TuR1174 which is located on the *tuf* sequence. Expected product size of this reaction was approximately 1200 bp. Result of this PCR reaction confirmed that *tuf* gene was present in the pT7D3Tu (Figure 5.12.). At the same time, another PCR amplification was performed using TuF2 and TuR2 primer pair, for the confirmation of the correct insertion of the *tuf* gene by sequencing (data not shown). Expected product size in this reaction was approximately 1200 bp. Result of this PCR amplification also confirmed that *tuf* gene was present in the pT7D3Tu (Figure 5.15.).

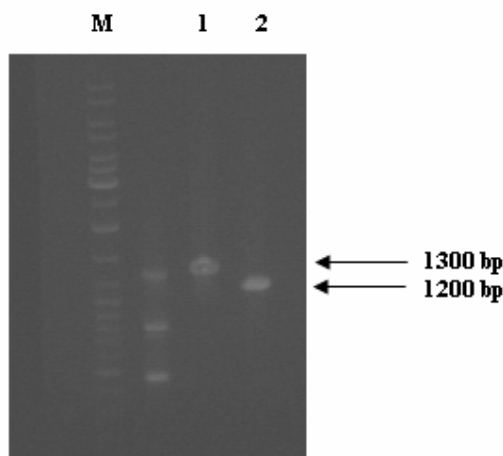


Figure 5.15. PCR results on the pT7D3Tu plasmid; M, molecular size marker; 1, PCR result with T7-pRSET primer pair; 2 PCR result with T7-TuR1174 primer pair.

5.3. Expression of *G. Anatolicus tuf* Gene in JM109(DE3) Cells

In this study, for the expression of *tuf* gene, JM109(DE3) cells were used as the plasmid host. The plasmid pCR[®]T7/NT-TOPO[®] and thus, its derivative pT7D3Tu contains T7 RNA polymerase promoter site in front of the Shine-Dalgarno sequence of the inserted gene. In the presence of IPTG, pT7D3Tu can be induced to express the recombinant *G. anatolicus tuf* protein in *E.coli* JM109(DE3) cells because this DE3 derivative of the JM109 contain T7 RNA polymerase gene. The JM109(DE3) cells were induced by IPTG to express the *tuf* gene. As a control the JM109(DE3) cells transformed with pCR[®]T7/NT-TOPO[®] (without *tuf* gene) were used. Size of the protein band appeared for the cells carrying pT7D3Tu but absent for pCR[®]T7/NT-TOPO[®] was very close to the calculated molecular weight of the protein (44.1 kDa; *G. anatolicus* EF-Tu [43.3 kDa] + 6xHis-tag) when analyzed electrophoretically by 10% SDS-PAGE. The amount of recombinant protein increased as time passed. Results of the experiment were shown in Figure 5.16.

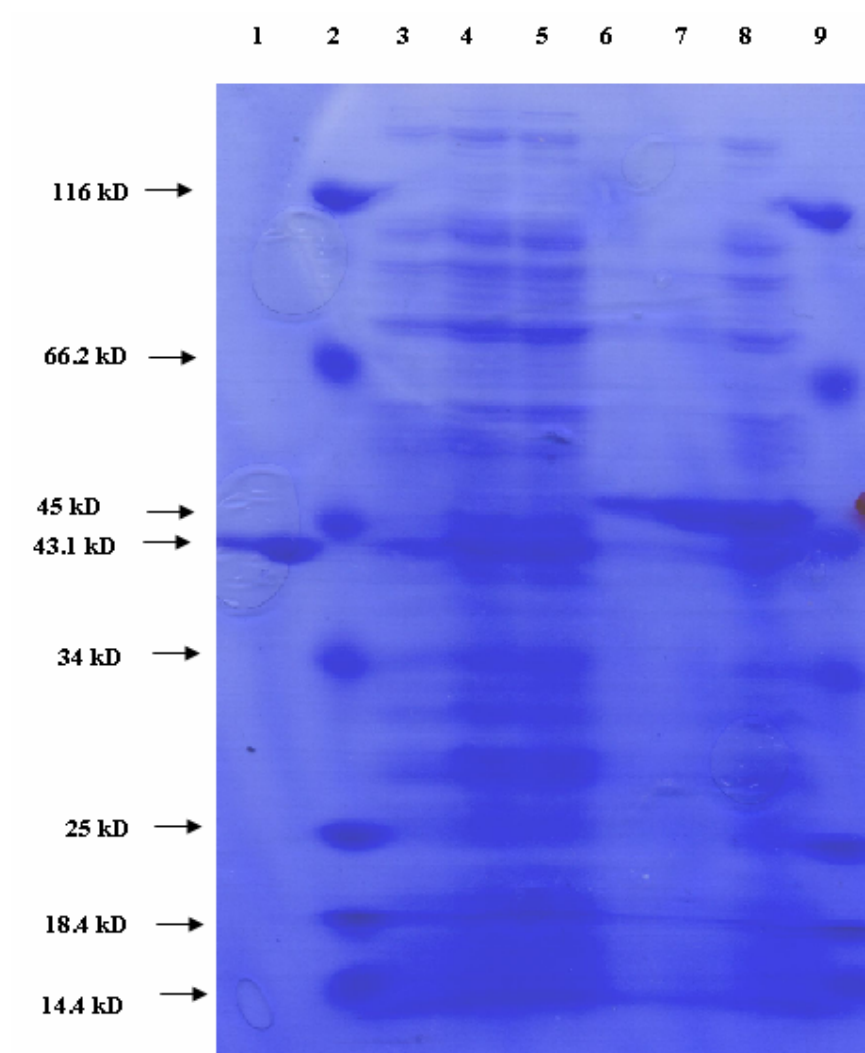


Figure 5.16. Results of expression experiment. Lane 1, *E.coli* EF-Tu (43.1 kD); Lanes 2 and 9, protein molecular weight markers; Lanes 3,4 and 5, JM109(DE3) cells carrying pCR[®]T7/NT-TOPO[®] (-*tuf*) at 0, 2, 5 hours respectively; Lanes 6,7 and 8, JM109(DE3) cells carrying pT7D3Tu at 0, 2, 5 hours, respectively

5.4. Ni-Affinity Column Chromatography

Because the recombinant *G. anaticus* EF-Tu includes an additional 6 Histidine residues at its C-terminal, it is purified by using Ni-Affinity Column Chromatography. *G. anaticus* Elongation Factor Tu was isolated from JM109(DE3) carrying the pT7D3Tu vector. For preparative purpose 800 ml of culture was induced with IPTG and 6 hrs after

induction cells were harvested, lyzed and centrifuged and applied to Ni-affinity column as explained in section 4.9. Elution profile from the Ni-Affinity column is analyzed by SDS-PAGE (Figure 5.17). *G. anaticus* EF-Tu elutes from the column at about 87.5 mM imidazole as a single protein band. Pooled and concentrated fractions after dialysis of the recombinant *Geobacillus anaticus* EF-Tu is shown in Figure 5.18. By using Bradford assay, total protein yield was determined to be 6,47 mg (from a 800 ml cell culture) and the protein concentration to be 3,41 mg/ml.

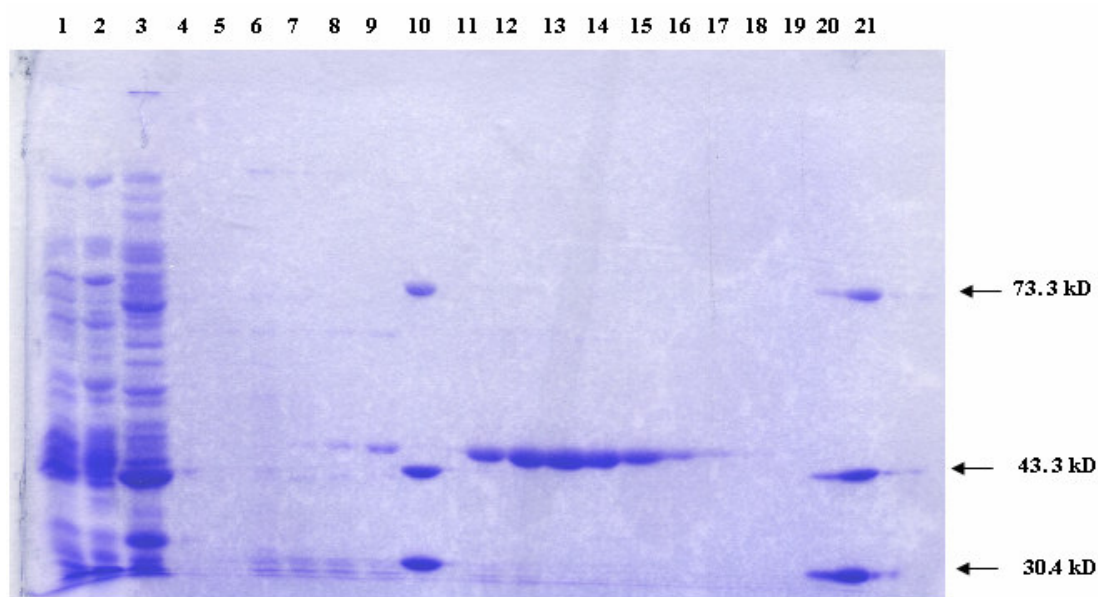


Figure 5.17. SDS analysis of Ni-Affinity chromatography; Lanes 1 and 2: samples before applying the column; lane 3: flowthrough; lanes 4 to 9: NH_4Cl wash; lane 10: protein molecular weight marker (from the top to the bottom, *E. coli* EF-G, EF-Tu and EF-Ts respectively); lanes 11 to 18: eluates with imidazole gradient, lane 20-21, protein molecular weight marker (from the top to the bottom, *E. coli* EF-G, EF-Tu and EF-Ts respectively)

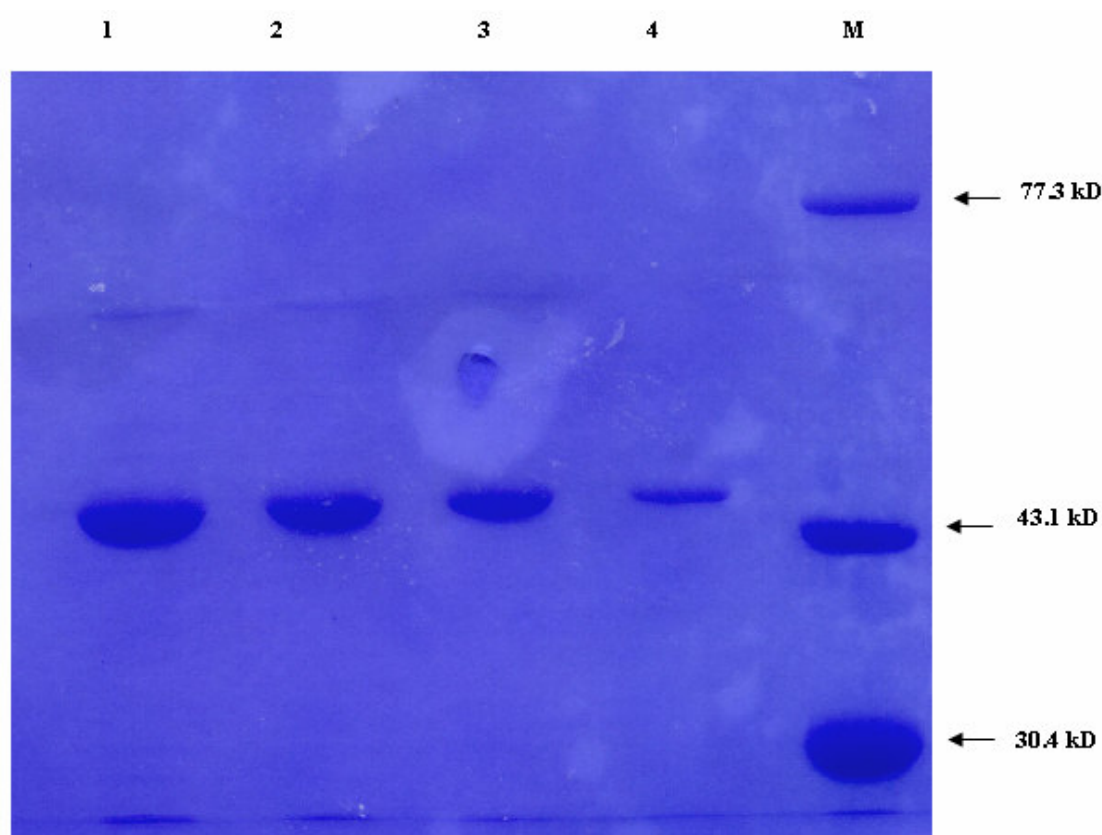


Figure 5.18. *G. Anatolicus* EF-Tu (lanes 1-4); M, protein molecular weight marker (from the top to the bottom, *E. coli* EF-G, EF-Tu and EF-Ts respectively).

5.5. Interactions of *G. anatolicus* EF-Tu with EF-Ts

In a previous study *G. anatolicus* EF-Ts has been cloned and purified (Sahin, 2006, MSci. Thesis). This recombinant *G. anatolicus* EF-Ts as well as *E. coli* EF-Ts is used in a mobility-shift assay on non-denaturing PAGE for complex formation with the purified recombinant *G. anatolicus* EF-Tu. Recombinant *G. anatolicus* EF-Tu is fully competent in complex formation with *G. anatolicus* EF-Ts (Figure 5.16., lanes 2-5) as well as with *E. coli* EF-Ts (Figure 5.19., lanes 8-11). From lane 2 to 5, intensity of the complex band increased. It suggests that with the increasing amount of EF-Tu, EF-Ts binds to more EF-Tu molecules at the subsequent lanes. Mobility of EF-Tu molecule and position of the complex band will be discussed in the Discussion section.

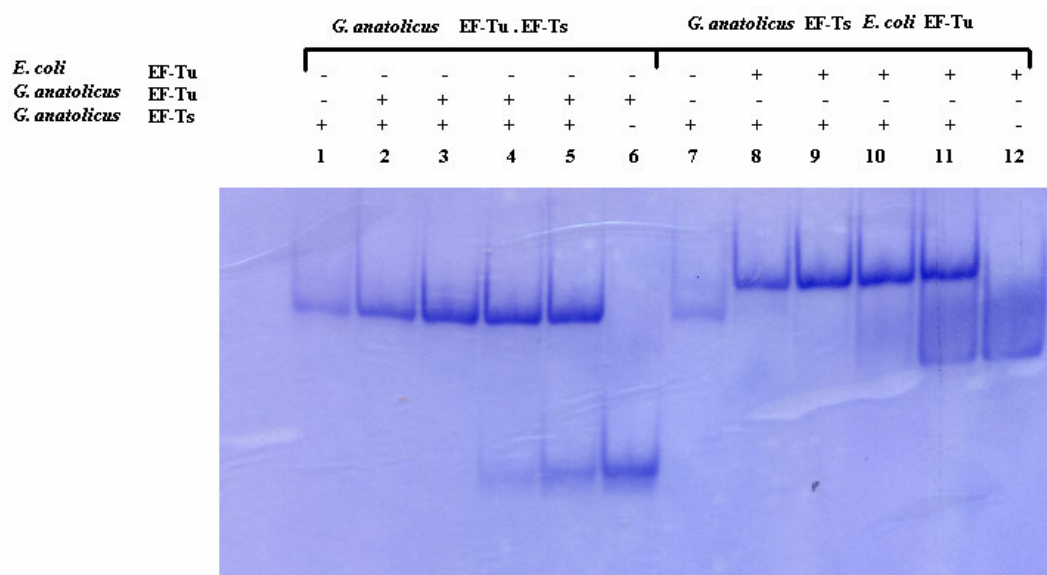


Figure 5.19. Mobility-shift assay for complex formation between EF-Tu and EF-Ts. All lanes contain 300 pmol of *G. anatolicus* EF-Ts except lane 6 and 12. *G. anatolicus* EF-Tu (lanes 1-6) and *E. coli* EF-Tu (lanes 7-12) were titrated (0 pmol, 100 pmol, 200 pmol, 300 pmol, 400 pmol, 400 pmol) , respectively.

5.6. Ternary Complex Formation between *G. anatolicus* EF-Tu, GTP and Phe-tRNA^{Phe}

Mobility shift on a non-denaturing PAGE is used in order to analyze the complex formation between *G. anatolicus* EF-Tu, GTP and Phe-tRNA^{Phe}. In this experiment it was shown that *G. anatolicus* EF-Tu can form a ternary complex with GTP and Phe-tRNA^{Phe} (Figure 5.20.). With the increasing amounts of tRNA^{Phe}, negatively charged tRNA^{Phe} binding shifts the upper EF-Tu band to a high mobility (lower) band corresponding to the ternary complex. Thus results suggest that ternary complex was formed at the bottom band. In the case of *E. coli* EF-Tu, all EF-Tu molecules could participate in ternary complex formation; however it wasn't the case for *G. anatolicus* EF-Tu, some Tu remain free (see Discussion section).

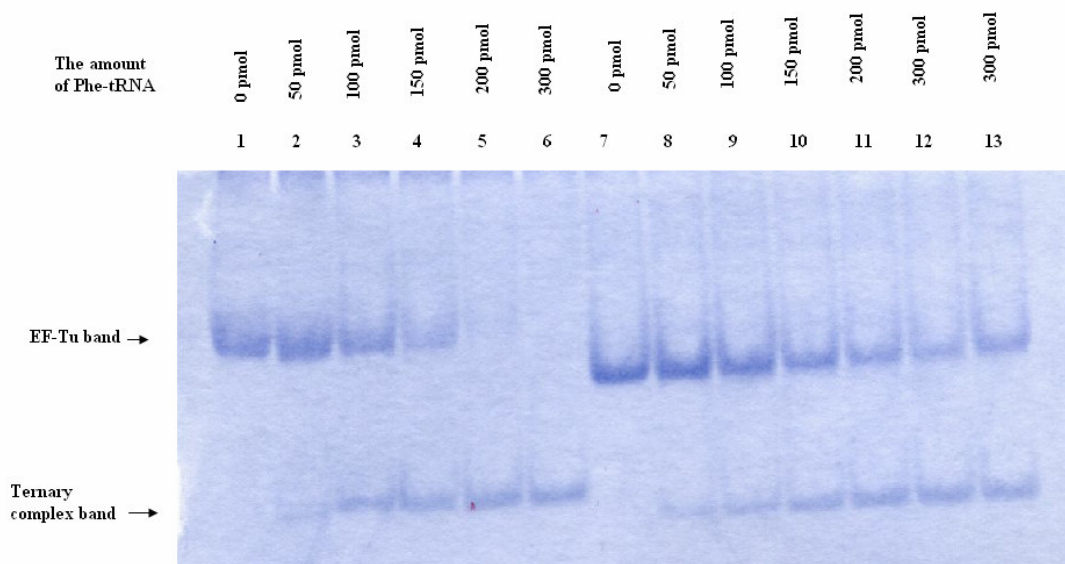


Figure 5.20. Ternary complex formation experiment. In lanes 1-6 there are 200 pmol of *E.coli* EF-Tu and in lanes 7-13 there are 200 pmol of *G.anatolicus* EF-Tu. In lanes 1-6, 0, 50, 100, 150, 200, 300 pmol of tRNA^{Phe} are present respectively. In lanes 7-13, 0, 50, 100, 150, 200, 300 and 300 pmol of tRNA^{Phe} are present respectively. In lane 13, incubation time was increased by 20 min.

5.7. Thermostability Test

Because *G. anatolicus* EF-Tu is a hyperthermophilic protein, it was expected that EF-Tu would be active above mesophilic temperatures. To determine thermostability profile of *G. anatolicus* EF-Tu, a thermostability test was performed based on EF-Tu's ability to bind GDP. *E. coli* EF-Tu can be inactivated significantly when subjected to 60 °C for 10 min, *G. anatolicus* EF-Tu retained 98.7 % of its activity for GDP binding at this temperature. Thermostability profile based on GDP binding ability of *G.anatolicus* EF-Tu was shown in Figure 5.21.

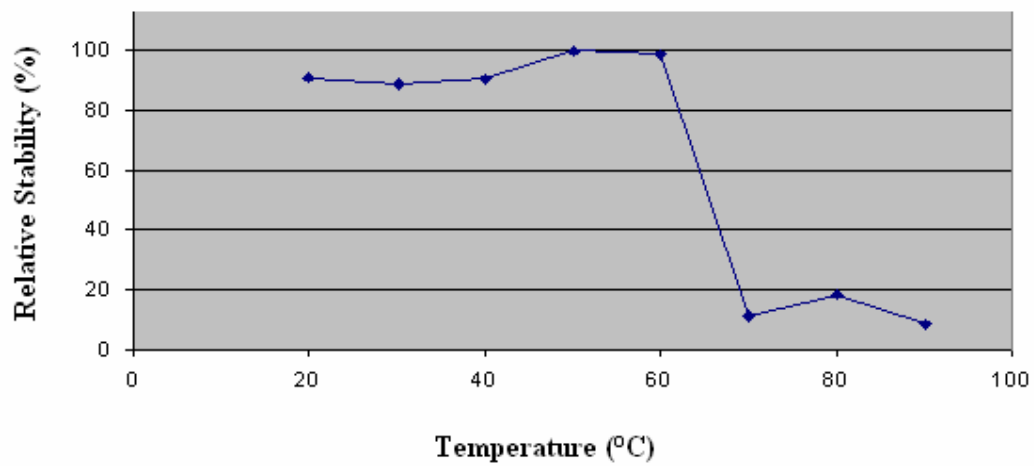


Figure 5.21. Thermostability of *G. anatolicus* EF-Tu as judged by GDP binding. GDP binding ability after incubating for 10 min at temperatures between 20-90 °C in 10 °C intervals. Because it retained the maximum amount of ^3H -GDP at 50 °C, this value was taken as 100%.

6. DISCUSSION

6.1. Determination of *Geobacillus anatolicus tuf* Gene Sequence

Because there is no sequence information about *Geobacillus anatolicus* EF-Tu gene (*tuf*) before this study, the species phylogenetically related to *Geobacillus anatolicus* were chosen for the sequence determination. For this purpose, the known 16S rRNA sequence that was previously obtained in our laboratory was used in BLAST search to look up related organisms. Those from which *tuf* genes were previously sequenced were chosen. By using ClustalW multiple alignment technique, the *tuf* gene sequences from these species were aligned and the conserved regions were determined. Based on this information, degenerate primers were designed to amplify a part of the *tuf* gene. After DNA sequencing of this region, partial *tuf* sequence of *Geobacillus anatolicus* was obtained. By using this sequence information, now specific for the *Geobacillus anatolicus*, non-degenerate primers were designed for *tuf* gene sequencing. In addition to this, to doublecheck the flanking sequences of the *tuf* gene, two additional degenerate primers were designed within the genes expected to be upstream and downstream of the *tuf* gene. This was possible, because there is a high degree of conservation at and around the *tuf* gene in the organisms that were chosen from the BLAST search. Using these primers, a fragment of *Geobacillus anatolicus* DNA containing the full *tuf* gene was obtained and sequenced by using six primers. *tuf* gene of *Geobacillus anatolicus* is found to be 1182 bp long. The nucleotide sequence of the *Geobacillus anatolicus tuf* gene was used to determine the EF-Tu amino acid sequence. Based on BLAST results of both the nucleotide and the amino acid sequences in the bacterial GenBank databases, it was confirmed that the amplified fragment, in fact, contained the *tuf* gene, corresponded to *Geobacillus anatolicus* EF-Tu protein.

By the course of this study, the complete genome of *Geobacillus kaustophilus* has been published. The nucleotide sequence comparisons between *Geobacillus anatolicus* and its closest relative *Geobacillus kaustophilus* revealed 93% sequence identity. This result confirmed that *Geobacillus anatolicus* is a new species based on its 16S ribosomal DNA comparisons previously reported (Uysal *et al.*, 2001) (Figure 6.1.). In a previous study in our laboratory (Sahin, 2006, MSci. Thesis) on EF-Ts from *Geobacillus anatolicus*,

it was shown that there is a 95,5% amino acid sequence identity between *Geobacillus anatolicus* and *Geobacillus kaustophilus* EF-Ts. But in the case of EF-Tu amino acid sequences, the identity was 99%. Only two amino acids were found to be different (Figure 6.2.) when EF-Tu sequences compared. EF-Tu has a more conserved amino acid sequence. This may be because EF-Tu is a more ancient protein. In addition to this, given that *Geobacillus kaustophilus* is a thermophilic organism, this may suggest that overall amino acid sequence of EF-Tu is essential for the thermoadaptation.

	
	5 15 25 35 45
G. kaustop	ATGGCTAAAG CGAAATTTGA GCGCACGAAA CCGCACGTCA ACATTGGCAC
G. anatoli	ATGGCTAAAG CGAAATTTGA GCGTACGAAA CCGCACGTCA ACATTGGCAC
Consensus	ATGGCTAAAG CGAAATTTGA GCGYACGAAA CCGCACGTCA ACATTGGCAC
	
	55 65 75 85 95
G. kaustop	GATCGGCCAC GTTGACCACG GGAAAACGAC GTTGACAGCT GCGATCACGA
G. anatoli	GATCGGCCAC GTTGACCATG GGAAAACGAC GTTGACAGCT GCGATCACAA
Consensus	GATCGGCCAC GTTGACCAYG GGAAAACGAC GTTGACAGCT GCGATCACRA
	
	105 115 125 135 145
G. kaustop	CGGTTTTTGGC GAAACAAGGG AAAGCCGAAG CAAAAGCGTA CGACCAAATC
G. anatoli	CAGTTCTTGC GAAACAAGGG AAAGCGGAAG CAAGAGCGTA CGACCAAATC
Consensus	RGTTTKGC GAAACAAGGG AAAGCSGAAG CAARAGCGTA CGACCAAATC
	
	155 165 175 185 195
G. kaustop	GACGCTGCTC CGGAAGAGCG TGAACGCGGG ATTACGATT CGACGGCGCA
G. anatoli	GACGCAGCTC CGGAAGAGCG TGAACGCGGA ATCAGCATT CGACAGCGCA
Consensus	GACGCWGCTC CGGAAGAGCG TGAACGCGGR ATYACGATT CGACRGCGCA
	
	205 215 225 235 245
G. kaustop	CGTCGAGTAT GAAACAGATG CTCGCCACTA TGCGCACGTT GACTGCCCCG
G. anatoli	CGTTGAGTAT GAAACGGACG CTCGTCCTA TGCGCACGTT GACTGCCCCG
Consensus	CGTYGAGTAT GAAACRGAYG CTCGYCACTA TGCGCACGTT GACTGCCCCRG
	
	255 265 275 285 295
G. kaustop	GCCACGCTGA CTACGTAAAA AACATGATCA CGGGCGCGGC GCAAATGGAC
G. anatoli	GCCACGCTGA CTACGTGAAA AACATGATCA CGGGCGCAGC GCAAATGGAC
Consensus	GCCACGCTGA CTACGTAAAA AACATGATCA CGGGCGCRGC GCAAATGGAC
	
	305 315 325 335 345
G. kaustop	GGCGCGATCC TTGTTGTATC GGCGGCTGAC GGTCCGATGC CGCAAACGCG
G. anatoli	GGCGCGATCC TTGTTGTATC GGCTGCTGAC GGTCCGATGC CGCAAACGCG
Consensus	GGCGCGATCC TTGTTGTATC GGCKGCTGAC GGTCCGATGC CGCAAACGCG

Figure 6.1. Nucleotide sequence comparison between *G. kaustophilus* and *G. anatolicus*.

Different bases were shown in red.

	
	355 365 375 385 395
G. kaustop	CGAACACATT CTTCTCTCCC GCCAAGTCGG TGTTCCTGAC ATCGTTGTAT
G. anatoli	CGAACACATT CTTCTCTCCC GCCAAGTCGG TGTACCGTAC ATCGTTGTTT
Consensus	CGAACACATT CTTCTCTCCC GCCAAGTCGG TGT W CCGTAC ATCGTTGT W T
	
	405 415 425 435 445
G. kaustop	TCTTGAACAA ATGCGACATG GTGGACGACG AAGAATTGCT TGAAC TTGTT
G. anatoli	TCTTGAACAA ATGCGACATG GTGGACGACG AAGAATTGCT TGAAC TCGTT
Consensus	TCTTGAACAA ATGCGACATG GTGGACGACG AAGAATTGCT TGAAC TYGTT
	
	455 465 475 485 495
G. kaustop	GAAATGGAAG TTCGCGATCT TCTCTCTGAA TACGACTTCC CAGGCGATGA
G. anatoli	GAAATGGAAG TTCGCGATCT TCTCTCTGAA TACGACTTCC CGGGCGACGA
Consensus	GAAATGGAAG TTCGCGATCT TCTCTCTGAA TACGACTTCC C R GGCGA Y GA
	
	505 515 525 535 545
G. kaustop	AGTGCCGGTC ATCAAAGGTT CGGCATTAAA AGCGCTTGAA GGCGACCCGC
G. anatoli	AGTGCCGGTC ATCAAAGGTT CGGCATTAAA AGCGCTCGAA GGCGACCCGC
Consensus	AGTGCCGGTC ATCAAAGGTT CGGCATTAAA AGCGCT Y GAA GGCGACCCGC
	
	555 565 575 585 595
G. kaustop	AATGGGAAGA AAAAATCATT GAACTGATGA ATGCGGTTGA CGAATACATC
G. anatoli	AATGGGAAGA AAAAATCATT GAACTGATGA ACGCGGTTGA TCGAGTACATC
Consensus	AATGGGAAGA AAAAATCATT GAACTGATGA A Y GCGGTTGA CGA R TACATC
	
	605 615 625 635 645
G. kaustop	CCGACGCCGC AACGTGAAGT GGACAAACCG TTCATGATGC CAATCGAGGA
G. anatoli	CCGACTCCGC AACGTGAAGT AGACAAACCG TTCATGATGC CGGTTGAGGA
Consensus	CCGAC K CCGC AACGTGAAGT R GACAAACCG TTCATGATGC C RR T Y GAGGA
	
	655 665 675 685 695
G. kaustop	CGTCTTCTCG ATCACGGGCC GCGGTACGGT TGCAACAGGC CGTGTGTAAC
G. anatoli	CGTTTTCTCG ATCACAGGCC GCGGTACGGT TGCGACGGGC CGTGTGAGC
Consensus	CGT Y TTCTCG ATCAC R GGCC GCGGTACGGT TGC RACR GGC CGTGTG ARC
	
	705 715 725 735 745
G. kaustop	GCGGTACGTT GAAAGTTGGC GACCCGGTTG AAATCATCGG TTTGTCGGAT
G. anatoli	GCGGTACGTT AAAAGTCGGC GACCCGGTTG AAATTATCGG TCTTTCGGAC
Consensus	GCGGTACGTT R AAAGT Y GGC GACCCGGTTG AAAT Y ATCGG T Y T K TCGG AY
	
	755 765 775 785 795
G. kaustop	GAGCCGAAAA CGACGACCGT TACGGGTGTA GAAATGTTCC GCAAAC TTCT
G. anatoli	GAGCCGAAAA CGACGACCGT TACGGGTGTA GAAATGTTCC GTAAGCTGCT
Consensus	GAGCCGAAAA CGACGAC S GT TACGGGTGTA GAAATGTTCC G Y A R CT K CT

Figure 6.1. Nucleotide sequence comparison between *G. kaustophilus* and *G. anaticus*.

Different bases were shown in red.

	
	805 815 825 835 845
G. kaustop	CGATCAAGCG GAAGCCGGCG ACAACATCGG TCGCTTCTC CGCGGCGTAT
G. anatoli	TGACCAAGCG GAAGCTGGCG ACAACATCGG TCGCTTCTC CGCGGTGTAT
Consensus	Y G A YCAAGCG GAAGC Y GGCG ACAACATCGG TCGCTTCTC CGCGG Y GTAT
	
	855 865 875 885 895
G. kaustop	CTCGTGATGA AGTCGAGCGC GGCCAAGTAT TGGCGAAACC TGGTTCGATC
G. anatoli	CGCGTGACGA AGTTGAGCGC GGCCAAGTAT TGGCGAAACC GGGCTCGATC
Consensus	K CGTG A Y GA AGT Y GAGCGC GGCCAAGTAT TGGCGAAACC K GG Y TCGATC
	
	905 915 925 935 945
G. kaustop	ACGCCGCACA CGAAATTCAA AGCGCAAGTA TACGTCCTGA CGAAAGAAGA
G. anatoli	ACGCCGCACA CGAAATTTAA AGCGCAAGTT TACGTTCTGA CGAAAGAAGA
Consensus	ACGCCGCACA CGAAATT Y AA AGCGCAAGT W TACGT Y CTGA CGAAAGAAGA
	
	955 965 975 985 995
G. kaustop	AGGCGGACGC CATACTCCGT TCTTCTCGAA CTACCGTCCG CAATTCTACT
G. anatoli	AGGCGGACGC CATACTCCGT TCTTCTCGAA CTACCGTCCG CAATTCTACT
Consensus	AGGCGGACGC CATACTCCGT TCTTCTCGAA CTACCGTCCG CAATTCTACT
	
	1005 1015 1025 1035 1045
G. kaustop	TCCGTACGAC GGACGTAACG GGCATTATCA CGCTTCCGGA AGGCGTCGAA
G. anatoli	TCCGTACAAC GGACGTAACG GGCATCATCA CGCTTCCGGA AGGCGTAGAA
Consensus	TCCGTAC R AC GGACGTAACG GGCAT Y ATCA CGCTTCCGGA AGGCGT M GAA
	
	1055 1065 1075 1085 1095
G. kaustop	ATGGTTATGC CTGGCGACAA CGTTGAAATG ACGGTGGAAC TGATCGCCCC
G. anatoli	ATGGTTATGC CTGGCGACAA CGTTGAAATG ACGGTGGAAC TGATCGCTCC
Consensus	ATGGTTATGC CTGGCGACAA CGTTGAAATG ACGGT K GAAAC TGATCGC Y CC
	
	1105 1115 1125 1135 1145
G. kaustop	GATCGCGATC GAAGAAGGTA CGAAATTCTC GATCCGCGAA GGCGGCCGTA
G. anatoli	GATCGCGATC GAGGAAGGAA CAAAATTCTC GATCCGTGAA GGCGGCCGCA
Consensus	GATCGCGATC GA R GAAGG W A C RAAATTCTC GATCCG Y GAA GGCGGCCG Y A
	
	1155 1165 1175 1185
G. kaustop	CGGTCGGCGC TGGCTCTGTA TCGGAAATCA TCGAGTAA
G. anatoli	CGGTTGGTGC TGGTTCCGTA TCGGAAATCA TTGAGTAA
Consensus	CGGT Y GG Y GC TGG Y TC Y GTA TCGGAAATCA Y GAGTAA

Figure 6.1. Nucleotide sequence comparison between *G. kaustophilus* and *G. anatolicus*.

Different bases were shown in red.

	
	5 15 25 35 45
G. anatoli	MAKAKFERTK PHVNIGTIGH VDHGKTTLTA AITTVLAKQG KAEARAYDQI
G. kaustop	MAKAKFERTK PHVNIGTIGH VDHGKTTLTA AITTVLAKQG KAEAKAYDQI
Consensus	MAKAKFERTK PHVNIGTIGH VDHGKTTLTA AITTVLAKQG KAEA AYDQI
	
	55 65 75 85 95
G. anatoli	DAAPEERERG ITISTAHVEY ETDARHYAHV DCPGHADYVK NMITGAAQMD
G. kaustop	DAAPEERERG ITISTAHVEY ETDARHYAHV DCPGHADYVK NMITGAAQMD
Consensus	DAAPEERERG ITISTAHVEY ETDARHYAHV DCPGHADYVK NMITGAAQMD
	
	105 115 125 135 145
G. anatoli	GAILVVSaad GPMPQTREHI LLSRQVGVPY IVVFLNKCDM VDDEELLELV
G. kaustop	GAILVVSaad GPMPQTREHI LLSRQVGVPY IVVFLNKCDM VDDEELLELV
Consensus	GAILVVSaad GPMPQTREHI LLSRQVGVPY IVVFLNKCDM VDDEELLELV
	
	155 165 175 185 195
G. anatoli	EMEVRDLLSE YDFPGDEVFV IKGSALKALE GDPQWEEKII ELMNAVDEYI
G. kaustop	EMEVRDLLSE YDFPGDEVFV IKGSALKALE GDPQWEEKII ELMNAVDEYI
Consensus	EMEVRDLLSE YDFPGDEVFV IKGSALKALE GDPQWEEKII ELMNAVDEYI
	
	205 215 225 235 245
G. anatoli	PTPQREVDKP FMMPVEDVFS ITGRGTVATG RVERGTLKVG DPVEIIGLSD
G. kaustop	PTPQREVDKP FMMPEDVFS ITGRGTVATG RVERGTLKVG DPVEIIGLSD
Consensus	PTPQREVDKP FMMP EDVFS ITGRGTVATG RVERGTLKVG DPVEIIGLSD
	
	255 265 275 285 295
G. anatoli	EPKTTTGTGV EMFRKLLDQA EAGDNIGALL RGVSRDEVER GQVLAKPGSI
G. kaustop	EPKTTTGTGV EMFRKLLDQA EAGDNIGALL RGVSRDEVER GQVLAKPGSI
Consensus	EPKTTTGTGV EMFRKLLDQA EAGDNIGALL RGVSRDEVER GQVLAKPGSI
	
	305 315 325 335 345
G. anatoli	TPHTKFKAQV YVLTKEEGGR HTPFFSNYRP QFYFRITDVT GIITLPEGVE
G. kaustop	TPHTKFKAQV YVLTKEEGGR HTPFFSNYRP QFYFRITDVT GIITLPEGVE
Consensus	TPHTKFKAQV YVLTKEEGGR HTPFFSNYRP QFYFRITDVT GIITLPEGVE
	
	355 365 375 385 395
G. anatoli	MVMPGDNVEM TVELIAPIAI EEGTKFSIRE GGRTVGAGSV SEIIE
G. kaustop	MVMPGDNVEM TVELIAPIAI EEGTKFSIRE GGRTVGAGSV SEIIE
Consensus	MVMPGDNVEM TVELIAPIAI EEGTKFSIRE GGRTVGAGSV SEIIE

Figure 6.2. Amino acid sequence comparison between *G. kaustophilus* and *G. anaticolicus*.

Different amino acids were shown in red.

Escherichia coli EF-Tu·EF-Ts crystal structure was determined to a high resolution (Kawashima *et al.*, 1996). According to this structure there are 27 amino acid residues on the EF-Tu interacting with EF-Ts. In the *G. anaticolicus* EF-Tu, except one amino acid (In nucleotide binding domain of EF-Tu interacting with N-terminal domain of EF-Ts,

threonine was changed into alanine), 26 amino acids were conserved (Figure 6.3.). It can be inferred that dramatic increase in the growth temperature doesn't cause any effect on the interaction mechanism of EF-Tu and EF-Ts, even though EF-Ts sequence outside this domain is drastically different.

Some aminoacid residues in EF-Tu were shown to have regulatory functions, as determined in the previous studies on the *E. coli* EF-Tu. These residues are subjected to post-translational modifications. For example, during exponential growth, EF-Tu becomes monomethylated at Lys56; this is converted to Me2Lys (dimethyl-Lysine) when the bacteria enter the stationary phase (Kraal *et al.*, 1999). This highly conserved residue is not conserved in the *G. anatolicus* EF-Tu, neither in *G. kaustophilus*. However, the change is from a positively charged Lys to another positively charged residue, Arg. Another site of post-translational modification, the site of phosphorylation, Thr382 was also conserved in *G. anatolicus*. In addition to this, as *E. coli*, *T. thermophilus* EF-Tu was found to be phosphorylated in vivo (Kraal *et al.*, 1999). Phosphorylation of EF-Tu in Thr382 abolishes the binding of EF-Tu to aminoacyl tRNA (Alexander *et al.*, 1995). High degree of conservation of this residue may indicate its essential role in protein synthesis.

					
	5	15	25	35	45
G. anatoli	MAKAKFERTK	PHVNIGT IGH	VDHGK TTLTA	AITTVLAKQG	KAEARAYDQI
E. coli	MSKEKFERTK	PHVNVGT IGH	VDHGK TTLTA	AITTVLAKTY	GGAARAFDQI
Consensus	M K K FERTK	PHVN GTIGH	VDHGK TTLTA	AITTVLAK	ARA DQI
					
	55	65	75	85	95
G. anatoli	DAAPEERERG	ITISTAH VEY	ETDAR H YAHV	DCPG HAD YVK	NMITGAAQMD
E. coli	DNAPEEKARG	ITINTSH VEY	DTPTR H YAHV	DCPG HAD YVK	NMITGAAQMD
Consensus	D APEE RG	ITI T HVEY	T R YAHV	DCPG HAD YVK	NMITGAAQMD
					
	105	115	125	135	145
G. anatoli	GAILVVS AD	GPMP Q TREHI	LLSRQVGVPY	IVVFLNKCDM	VDDEE LLELV
E. coli	GAILVVAA TD	GPMP Q TREHI	LLGRQVGVPY	IIVFLNKCDM	VDDEE LLELV
Consensus	GAILVV A D	GPMP Q TREHI	LL RQ VGVPY	I V FLNKCDM	VDDEELLELV
					
	155	165	175	185	195
G. anatoli	EMEVRDLLSE	YDFPGDEVVP	IKGSALKALE	GDPQWEEKII	ELMNAVDEYI
E. coli	EMEVR ELLSQ	YDFPGDDTPI	VRGSALKALE	GDAEWEAKIL	ELAGFLDSYI
Consensus	EMEVR LLS	YDFPGD P	GSALKALE	GD WE KI	EL D YI

Figure 6.3. Amino acid sequence comparison and EF-Tu interactions sites. Conserved residues were shown in blue. Non-conserved residues were shown in red.

	
	205 215 225 235 245
G. anatoli	PTPQREVDKP FMMPVEDVFS ITGRGTVATG RVERGTLKVG DPVEIIGLSD
E. coli	PEPERAIDKP FLLPIEDVFS ISGRGTVVTG RVERGIIKVG EEVEIVGIK-
Consensus	P P R DKP F P EDVFS I GRGTV TG RVERG KVG VEI G
	
	255 265 275 285 295
G. anatoli	EPKTTTGTGV EMFRKLLDQA EAGDNIGALL RGVSRDEVER GQVLAKPGSI
E. coli	ETQKSTCTGV EMFRKLLDEG RAGENVGVL RGIKREEIER GQVLAKPGTI
Consensus	E T TGV EMFRKLLD AG N G LL RG R E ER GQVLAKPG I
	
	305 315 325 335 345
G. anatoli	TPHTKFKAQV YVLTKEEGGR HTP FF SNYRP QFYFRITDVT GIITLPEGVE
E. coli	KPHTKFESEV YILSKDEGGR HTP FF KGYRP QFYFRITDVT GTIELPEGVE
Consensus	PHTKF V Y L K EGGR HTPFF YRP QFYFRITDVT G I LPEGVE
	
	355 365 375 385 395
G. anatoli	MVMPGD NVEM TVELIAPIAI EEGTKFSIRE GGRTVGAGSV SEIIE
E. coli	MVMPGD NIKM VVTLIHPIAM DDGLRFAIRE GGRTVGAGVV AKVLS
Consensus	MVMPGDN M V LI PIA G F IRE GGRTVGAG V

Figure 6.3. Amino acid sequence comparison and EF-Tu interactions sites. Conserved residues were shown in blue. Non-conserved residues were shown in red.

6.2. Number of *tuf* genes

Most gram-positive bacteria carry one *tuf* gene on their chromosomes, whereas gram-negative bacteria contain usually two almost identical genes, *tufA* and *tufB* for EF-Tu (Krasny *et al.*, 1998). For example, in *E. coli*, it was found that there are two *tuf* genes and they are almost identical but for 13 bases (Jaskunas *et al.*, 1975). Also in *Thermus thermophilus* HB8 it was determined that there are two *tuf* genes; these genes are different from each other in 10 nucleotide positions (Satoh *et al.*, 1991). Given that *Geobacillus anatolicus* is a gram-negative bacterium (Caglayan, 2005, MSci. Thesis), and that the complete genome sequence of *G. kaustophilus* reveals a single copy of *tuf* gene, it may be hypothesized that *G. anatolicus* may contain one *tuf* gene. However, it remains to be investigated whether there is one *tuf* gene in *Geobacillus anatolicus* or not.

6.3. Cloning of the *tuf* Gene

In this study, the vector pCR[®] T7/NT-TOPO[®] was used to clone *tuf* gene. This vector contains multiple restriction enzyme sites which could be used for PCR cloning reactions. Also there is an enterokinase cleavage site in the vector to discard the 6xHis-tag. However, during this study, it was found that the commercially available enterokinases cleaves *E.coli* EF-Tu protein unspecifically. This made it impossible to use this vector as it is commercially supplied. According to the literature, 6xHis-tag at the C-terminal position of EF-Tu doesn't interfere with the EF-Tu function in *E. coli* (Boon *et al.*, 1992) Because of this, enterokinase cleavage site was removed from the vector and a new vector was constructed named pT7D3 which expresses EF-Tu with a His-tag.

6.4. Expression of the Recombinant Elongation Factor Tu

The expression vector, pT7D3Tu contains a strong T7 RNA polymerase promoter site. The vector was cloned into a special *E. coli* strain, DE3 which expresses T7 RNA polymerase. DE3 is the designation for the lambda DE3 lysogen carrying the gene for T7 RNA polymerase under the control of the *lacUV5* promoter, inducible by IPTG. Because there is a similar *tuf* gene in *E.coli*, to avoid recombination, a recombination deficient *Escherichia coli* JM109 (DE3) strain was used in the expression experiments.

6.5. Purification of the Recombinant His-tagged Elongation Factor Tu

Metal-ion chelating chromatography was used for the purification of the recombinant 6xHis-tagged elongation factor Tu. By adding 18 nucleotides to the TuR2 primer, 6xHis-tag was inserted into the recombinant *Geobacillus anatolicus* elongation factor Tu. This insertion allowed EF-Tu to be purified by Ni²⁺-affinity chromatography.

6.6. Mobility-Shift Assay for EF-Tu.EF-Ts complex formation

Because *Geobacillus anatolicus* EF-Ts was available from a previous study (Sahin, 2006, MSci. Thesis), interaction of EF-Tu with EF-Ts could be examined. There is a significant difference between the mobilities of *Geobacillus anatolicus* EF-Tu and *E. coli*

EF-Tu, not explainable with the presence of 6 additional His residues in the recombinant EF-Tu from *Geobacillus anatolicus*. In fact, *Geobacillus anatolicus* EF-Tu has a higher mobility towards the positive electrode.

The mobilities of free *Geobacillus anatolicus* EF-Tu and *Geobacillus anatolicus* EF-Tu.EF-Ts complex were almost identical, in contrast to heterologous complex between *E. coli* EF-Tu and *Geobacillus anatolicus* EF-Ts. This may indicate a significant structural change due to complex formation. In addition to this, titrations suggests that there is a 1:1 stoichiometry between *Geobacillus anatolicus* EF-Tu and EF-Ts.

6.7. Ternary Complex Formation between *G. anatolicus* EF-Tu, GTP and Phe-tRNA^{Phe}

G. anatolicus EF-Tu forms a complex with GTP and Phe-tRNA^{Phe} as indicated by mobility shift assay. However, some EF-Tu remained free. During complex formation, samples were incubated at 37 °C for GDP exchange to GTP. Because *Geobacillus anatolicus* Tu is thermophilic, some GDP may not be exchanged to GTP because of lower exchange rate at 37 °C. To overcome this, the last sample was incubated at 37 °C for additional 20 min. No significant change was observed. Some of the recombinant Tu may become inactivated for tRNA binding during the purification process even though fully active in EF-Ts binding. This observation remains to be investigated.

6.8. Thermostability

G. anatolicus EF-Tu was fully stable up to 60 °C. After 10 min at 70°C, most of the EF-Tu becomes inactive. Although *G. anatolicus* is taken from a spring at 98°C, this is unexpected. However, during the purification of the bacteria to homogenous cultures, agar plates were used, because of its convenience for selecting colonies. Because agar melts above 65 °C, *G. anatolicus* was grown at 65 °C throughout this study. Although *G. anatolicus* is a hyperthermophilic organism originally living at 98 °C, a possible adaptation to this temperature might have occurred.

6.9. Conclusion and Future Perspective

Because *G. anatolicus* EF-Ts is also available, *G. anatolicus* EF-Tu can further be studied functionally in its interactions with EF-Ts. Kinetic parameters of the EF-Tu.EF-Ts interaction should be measured.

As a future perspective, this study can be considered as a part of construction of a *in vitro* thermophilic translation system. If such a system can be constructed, this may provide information about thermophilic protein translational machinery. EF-G and ribosomes remain to be purified for this purpose. A thermophilic *in vitro* translation system may have biotechnological applications as well.

Because *G. anatolicus* is a hyperthermophilic organism, structural and functional investigation of EF-Tu will gain insight into thermophilic proteins.

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