

The Pennsylvania State University
The Graduate School
The Huck Institutes of the Life Sciences

ROLES OF
ARCHAEAL GENERAL TRANSCRIPTION FACTORS
TFB1 AND TFB2 IN *THERMOCOCCUS KODAKARENSIS*
DURING HEAT STRESS RESPONSE

A Dissertation in
Integrative Biosciences

by

Momoko Tajiri

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2008

The dissertation of Momoko Tajiri was reviewed and approved* by the following:

Katsuhiko Murakami
Assistant Professor of Biochemistry and Molecular Biology
Dissertation Adviser
Chair of Committee

James G. Ferry
Stanley Person Professor of Biochemistry and Molecular Biology

B. Tracy Nixon
Professor of Biochemistry and Molecular Biology

Christopher H. House
Associate Professor of Geosciences

Peter J. Hudson
William Professor of Biology
Director of Huck Institutes of the Life Sciences

*Signatures are on file in the Graduate School.

Abstract

This dissertation summarizes two projects concerning roles for homologues of a general transcription factor (GTF), called transcription factor B (TFB) during transcription regulation in *Archaea*. Since the discovery of the third domain of life, *Archaea*, numerous efforts have been made to understand life cycle of multiple archaeal species. The discovery of archaea with multiple homologues of GTFs in their genomes lead to the hypothesis that these archaea may use the multiplicity to control gene expression. However, in what extent the roles of these factors differ has not been fully investigated.

First part of the dissertation research uses both biochemical and genetic approaches to investigate roles of two TFB homologues from hyperthermophilic archaeon *Thermococcus kodakarensis*. The overall goal of this study is to provide insights into how the multiple TFB homologues are involved in transcription regulation. *T. kodakarensis* was chosen as a study model because of the the availability of well-established genetic systems, which is not true for many other archaea. *T. kodakarensis* also possess two homologues of TFB (annotated as TFB1 and TFB2) with one TBP, providing simplified system for investigation. Present study provided evidence that each TFB homologues has different characteristics in DNA binding and abortive transcription activities. TFB1 appeared to have stronger association with DNA-TBP complex than TFB2. It was also found that TFB1-containing pre-initiation complex produced more abortive products than TFB2 suggesting the former spend more time for abortive transcription prior to promoter escape than the latter. Because both TFB homologues are transcribed at the same level in an optimum condition, more promoters possibly associate with TFB1 than TFB2 under that condition. Under heat stress, I found that transcription of *tfb2* is induced more than *tfb1*. The population increase will potentially overcome less affinity of TFB2 to DNA and more

promoter may be recognized by TFB2 than TFB1 under heat stress. Since TFB2 spend less time and resources for abortive transcription, it may be an ideal TFB homologue to be used to express stress response proteins. Possibly, *T. kodakarensis* manipulate population of the two TFB homologues in the cell to adjust for transcription activity. I did not observe clear differences in gene expression patterns between *tfb1* or *tfb2* disrupted strains under heat stress. However, for multiple genes, larger fold changes in *tfb1* disrupted strain were observed than *tfb2* disrupted strain, suggesting these genes could be transcribed efficiently by TFB2 under heat stress. Overall, observations suggest that a unique mechanism to utilize the two TFB homologues for fine adjustment of intracellular gene expression may exist in *T. kodakarensis*.

Second part of the research is the development of a new genetic methodologies to control gene expression in the cells. Using the inducible promoters from a *T. kodakarensis* enzyme, 1,6 bis-phosphatase, conditional gene expression system was established. This is the system to control gene expression in archaea and will provide various applications in genetic studies. The system was applied to over-express TFB homologues in cells and consequences of additional expression were observed. Interestingly, over-expression of *tfb1* resulted in ineffective cell growth, whereas increased synthesis of *tfb2* did not affect cell growth properties. Both deletion and over-expression of *tfb1* resulted in ineffective cell growth suggesting TFB1 population need to be kept within certain range in *T. kodakarensis* for optimal growth.

Table of Contents

List of Tables	vii
List of Figures	viii
List of Abbreviation	x
Acknowledgments	xi
Chapter 1. Introduction	1
1.1 <i>Archaea</i>	1
1.2 Archaeal transcription initiation	3
1.3 Structure and function of archaeal TFB	4
1.4 Transcription regulation in <i>Archaea</i>	5
Chapter 2. Roles of archaeal TFB homologues from <i>Thermococcus kodakarensis</i> in gene regulation	13
2.1 Introduction	13
2.2 Results	16
2.2.1 Basic characterization of TFB homologues in <i>T. kodakarensis</i>	16
2.2.1.1 TFB sequence alignment and phylogenetic analysis	16
2.2.1.2 Verifications of <i>tfb1</i> and <i>tfb2</i> operon configurations	17
2.2.1.3 Transcript abundance of <i>tfb1</i> and <i>tfb2</i> in <i>T. kodakarensis</i>	18
2.2.2 Comparisons of transcription activities in the presence of TFB1 and TFB2	19
2.2.2.1 Transcription assay using heat shock promoter	19
2.2.2.2 Transcription from strong archaeal promoter	20
2.2.3 Study of TFB association with DNA-TBP complex	21
2.2.4 Evaluation of QCM data	23
2.2.5 Gene expression profiles in <i>tfb</i> disrupted strains	26
2.2.5.1 Transcript quantification by quantitative real-time PCR	26
2.2.5.2 Global analysis of gene expression in <i>tfb</i> deletion strains	28
2.2.6 Temperature effects on growth properties	28
2.3 Discussion	29
2.4 Materials and methods	30
2.4.1 Protein purification	30
2.4.2 Transcription assay	32
2.4.2.1 Transcription assay from heat shock gene promoter	32
2.4.2.2 Transcription assay from strong archaeal promoter	34
2.4.3 Electrophoretic mobility shift assay (EMSA)	34
2.4.4 Quartz crystal microbalance (QCM)	35
2.4.5 Growth media for <i>T. kodakarensis</i>	36
2.4.5.1 Cell growth experiment	37
2.4.5.2 Preparation of heat shock and cold shock cells	38
2.4.6 Quantitative real-time PCR (qRT-PCR)	38
2.4.7 DNA microarray	39
2.5 Acknowledgements	40

Chapter 3.	Development of a conditional gene expression system	61
3.1	Introduction	61
3.2	Results	62
3.2.1	Design and construction of conditional gene expression strains	62
3.2.2	Synthesis of RNA Polymerase subunits using conditional gene expres- sion system	62
3.2.3	A study of transcription factor B conditional expression strains	64
3.2.3.1	Quantification of transcripts and proteins	64
3.2.3.2	Growth properties of TFB conditional gene expression strain	64
3.2.3.3	Verification of TFB1 conditional expression constructs	65
3.3	Discussion	66
3.4	Materials and methods	66
3.4.1	Media used for <i>T. kodakarensis</i> transformation	66
3.4.2	Construction of conditional gene expression strains	67
3.4.3	Westernblotting	69
3.5	Acknowledgements	70
Chapter 4.	Future directions	77
4.1	Introduction	77
4.2	Identifying factors associating with TFB homologues	77
4.2.1	Introduction	77
4.2.2	Materials and methods	79
4.2.2.1	Pull-down assay	79
4.2.2.2	DNA-protein interaction assay	80
4.2.3	Preliminary results	81
4.2.3.1	Pull-down assay	81
4.2.3.2	DNA-protein interaction assay	82
4.2.4	Future works	82
4.3	Structural study of TFB-RNAP complex	83
4.3.1	Introduction	83
4.3.2	Materials and methods	83
4.3.3	Future works	84
4.4	Study of TFE functions	84
4.4.1	Introduction	84
4.4.2	Experimental approaches	85
4.4.3	Future work	86
4.5	Acknowledgements	87
Appendix A.	Media compositions for <i>T. kodakarensis</i> studies	96
Appendix B.	Phylogenetic tree of TFBs from euryarchaeal species	98
Appendix C.	Amino acid sequences for antibody preparation	101
Appendix D.	Protein purification profiles	102
Appendix E.	QCM output	106
Appendix F.	Attempts to construct <i>tfb</i> deletion strain with other parental strains	107
Bibliography		111

List of Tables

2.1 Summary of archaeal species encoding multiple GTFs in their genome. 42

2.2 *T. kodakarensis* strains used for this study. 43

2.3 DNA oligonucleotide sequences used for qRT-PCR. 43

3.1 *T. kodakarensis* strains and plasmids used for this study. 71

4.1 *T. kodakarensis* strains and plasmids used for this study. 88

A.1 Artificial Sea Water (ASW) 96

A.2 Mineral 96

A.3 Vitamin 97

A.4 Amino Acid (AA) 97

List of Figures

1.1	Phylogenetic tree of organisms based on small subunit rRNA sequences.	9
1.2	Comparison of the PIC formation in the three domains of life.	10
1.3	Comparison of RNAP structures from three domains of life.	11
1.4	X-ray crystal structure of the DNA-TBP-TFB(C-terminal domain) complex from <i>P. woesei</i>	12
2.1	Structure and an amino acid sequence alignment of TFB homologues from <i>T. kodakarensis</i>	44
2.2	Sequence analyses of TFBs from <i>T. kodakarensis</i> and <i>Pyrococcus</i>	45
2.3	Gene arrangements of operons encoding <i>T. kodakarensis</i> <i>tfb1</i> and <i>tfb2</i>	46
2.4	Quantification of <i>tfb1</i> and <i>tfb2</i> transcripts by qRT-PCR in wildtype <i>T. kodakarensis</i> KOD1.	47
2.5	Transcript abundance of <i>tfb1</i> and <i>tfb2</i> in wildtype <i>T. kodakarensis</i> KOD1 following temperature shift.	48
2.6	Run-off transcription assay using <i>cpkB</i> promoter.	49
2.7	Accumulation of transcripts from <i>cpkB</i> promoter.	50
2.8	Temperature effect of transcription from <i>cpkB</i> promoter.	51
2.9	Summary of <i>in vitro</i> transcription assays with strong archaeal promoter.	52
2.10	EMSA analysis of DNA-TBP-TFB complex.	53
2.11	Schematic representation of QCM assay.	54
2.12	QCM analyses of TFB dissociation constants.	55
2.13	Model of three molecule interactions.	56
2.14	Change in transcript relative abundances of GTFs in response to heat shock quantified by qRT-PCR.	57
2.15	Change in transcript relative abundances of thermosome subunits in response to heat shock quantified by qRT-PCR.	58
2.16	DNA microarray analysis.	59
2.17	Growth properties of <i>tfb1</i> or <i>tfb2</i> deletion strains.	60
3.1	Construction of conditional expression strains.	72
3.2	Construction of <i>tfb</i> conditional expression strains.	73
3.3	Fold changes of transcripts and proteins in <i>tfb</i> conditional expression strains.	74
3.4	Cell growth experiments with <i>tfb1</i> or <i>tfb2</i> conditional expression strains.	75
3.5	Verification of <i>tfb1</i> conditional expression strains.	76
4.1	Sequence alignment of TFB C-terminal helical repeats.	89
4.2	Similarity of TFB homologues from <i>T. kodakarensis</i>	90
4.3	Preliminary results from pull-down assay.	91
4.4	Preliminary results from <i>cpkB</i> promoter binding assay.	92
4.5	Construction of a <i>tfe</i> conditional expression strain.	93
4.6	Deletion of <i>tfe</i> from its original locus.	94
4.7	Growth properties of <i>tfe</i> conditional expression strain.	95
B.1	Phylogenetic tree of TFBs from euryarchaeal species.	99
C.1	Peptide sequences used for antibody preparation.	101
D.1	Elution profiles and SDS-PAGE of <i>T. kodakarensis</i> TBP and TFE.	103

D.2	Elution profiles and SDS-PAGE of <i>T. kodakarensis</i> TFB1 and TFB2.	104
D.3	Elution profiles and SDS-PAGE of histidine-tagged <i>T. kodakarensis</i> TFB1 and TFB2.	105
E.1	An example of QCM output.	106
F.1	Construction of <i>tfb2</i> deletion strain.	109
F.2	Phenotypes of <i>tfb2</i> deletion strains.	110

List of Abbreviation

ATP	adenosine triphosphate
CTP	cytidine triphosphate
GTP	guanosine triphosphate
UTP	uridine triphosphate
bp	basepair(s)
nt	nucleotide(s)
mRNA	messenger RNA
tRNA	transfer RNA
rRNA	ribosomal RNA
N-terminal	amino-terminal
C-terminal	carboxyl-terminal
BSA	bovine serum albumin
DTT	dithiothreitol; (2S,3S)-1,4-Bis-sulfanylbuthane-2,3-diol
EDTA	ethylenediaminetetraacetic acid; 2-[2-(Bis(carboxymethyl)amino)ethyl-(carboxymethyl)amino]acetic acid
Hepes	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	isopropyl β -D-1-thiogalactopyranoside
PMSF	phenylmethanesulfonyl fluoride
SDS	sodium dodecyl sulfate
Tris	tris(hydroxymethyl)aminomethane; 2-Amino-2-hydroxymethyl-propane-1,3-diol
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase polymerase chain reaction

Acknowledgments

I would like to thank my dissertation advisor Dr. Katsuhiko Murakami for giving me an exciting topic for my doctoral research. Archaeal genetics research was very interesting because it is relatively new field and lots to explorer. However, it also was a quite challenging from the same reason. I am grateful for Katsu's courage to throw something this new and challenging to a student coming from engineering field with no background in molecular biology. I appreciate his patience and understandings throughout my years here and I learned a lot from the research in his group.

All former and current Murakami laboratory members have been assisting me in various aspects. Among them, my special thanks goes to Dr. Akira Hirata, a former postdoctoral fellow in our group. He spent time at Kyoto University to learn manipulation of the archaeon used in this study and guide me through the projects. He also kindly allows me to use figures he prepared in my dissertation. My colleague, Brianna Klein, was always willing to take her time to discuss issues on experiments and also to proofread my writings. Matthew Richlen gave me suggestions on experiments and critically read this dissertation. Dr. Rieko Yajima and Dr. Michael Greghorn have gave me constructive suggestions on experiments, writings and presentations.

I also thank for doctoral committee members who spent time to discuss my projects. Many researchers who initiated archaeal genetic studies helped me throughout the project, including support from overseas. Dr. Tadayuki Imanaka and Dr. Tamotsu Kanai at Kyoto University helped us setting up an environment to start archaeal research and gave me guidance on handling of the organism. Dr. John Reeve and Dr. Thomas Santangelo at Ohio State University

provided us with *T. kodakarensis* strains and RNA polymerase.

Last but most important to me is my family both in Japan and here. They always encourage me to continue research and helped me a lot in various ways. Without their support, this work has not been completed.

Nothing in life is to be feared,
it is only to be understood.
Now is the time to understand more,
so that we may fear less.

—Maria Skłodowska-Curie

Chapter 1

Introduction

1.1 *Archaea*

The domain *Archaea* is one of the three domains of life along with *Eukarya* and *Bacteria*. In 1977, Carl Woese and his colleagues analyzed 16S rRNA sequences from 10 methanogenic bacteria, and discovered there are two distinct groups in *Prokaryotes* comprising *Bacteria* and *Archaea* [32]. *Archaea* is categorized in four phyla: Euryarchaeota, Crenarchaeota, Korarchaeota and Nanoarchaeota (Figure 1.1) [1][19][42]. The last two phyla are represented by only one species each and their exact locations on the phylogenetic tree remain obscured. Euryarchaeota consists of methanogenic, halophilic and some thermophilic archaea. Another major archaeal phylum, Crenarchaeota contains *Sulfolobus* species. In addition to the common habitats like soils and freshwater, a number of archaeal species have been isolated from environments, with extremes in pH, salt concentration and/or temperature. For instance, *Pyrolobus fumarii* is capable of living above 113°C. Archaea have also been isolated from very acidic environments, where pH is lower than 1.0 [59][64]. Since the discovery of *Archaea*, numerous studies have been conducted to investigate the physiology and life cycles in various archaeal species. Currently, over thirty archaeal genomes have been deposited in the public database (<http://www.ncbi.nlm.nih.gov/>; <http://www.cmr.jcvi.org/>). The phylogenic tree based on small subunit rRNA (16S and 18S rRNA) clearly illustrates that *Archaea* and *Eukarya* have branched after the separation from *Bacteria* (Figure 1.1) [1][19][42]. Thus, it is not surprising to discover that *Archaea* possesses a mixture of features characteristic of both *Bacteria* and *Eukarya* domains. In some aspects, *Archaea* resemble *Bacteria*, such as lack of a membrane bound nucleus and common metabolic

pathways. However, when it comes to genetic information processing, such as gene replication, transcription and translation, archaeal machineries are found to be more similar to their eukaryal counterparts [8][27][36][47]. Archaeal transcription machinery contains fewer general transcription factors (GTFs) and has less complexity than its eukaryal counterpart. Thus archaea are often more amenable for investigating protein functions. Interestingly, numerous regulatory proteins, which are homologous to bacterial transcription factors have been identified in archaeal genomes, consistent with a similar mechanism of transcription regulation between *Archaea* and *Bacteria* [5][11][13][34].

Capabilities of many archaeal species to live in extreme environments have several implications relevant to biological research. For structural studies, hyperthermophilic proteins often produce better quality crystals, which could reveal detailed protein functions [1]. Thermostable enzymes have been used in molecular biology researches (*e.g.* *Pyrococcus* and *Thermococcus* DNA polymerases for PCR). Despite various positive aspects archaea can offer, fewer researchers are involved in the field of archaeal studies compared with other domains. In addition to difficulties of reproducing extreme environments in which many archaea live, a lack of well-established genetic systems could have deterred researchers from the field. Although, recent efforts of various laboratories lead to the establishments of genetic methodologies for several archaeal species (reviewed in [1][63]), development of new assays is still important, because investigations with genetics complement findings from biochemical, structural and bioinformatics studies. Our group recently contributed to archaeal genetics by developing a conditional gene expression system in the hyperthermophilic archaeon, *Thermococcus kodakarensis*. The development of the system and its application were summarized in this dissertation (Chapter 3).

1.2 Archaeal transcription initiation

Transcription is the first step of gene expression. The central enzyme in transcription is RNA polymerase (RNAP), which synthesizes RNA using DNA as a template [14]. In order to initiate transcription from promoters, RNAP requires aid of promoter recognition factors. Archaeal and eukaryal RNAP utilize multiple factors, so called GTFs, which assist various activities during transcription, such as DNA melting [62].

Archaeal transcription machinery consists of a minimum three protein factors: an RNAP and two GTFs including TATA-binding protein (TBP) and transcription factor B (TFB) [38][60]. Two other GTFs, TFE and TFS, have been identified in archaeal genomes, although they are not essential for transcription initiation *in vitro*. Archaeal pre-initiation complex (PIC) formation is initiated by TBP association with DNA. The DNA-TBP complex is stabilized by binding of TFB to the complex. TFB recruits RNAP to the DNA-TBP-TFB ternary complex to form the PIC, and transcription is initiated (Figure 1.2). This PIC assembly model has been proposed based on eukaryal RNA polymerase II (RNAPII), however, it has not been tested experimentally in archaea. Based on the homologies between archaeal and eukaryal factors involved in transcription (Figure 1.2), it is expected that the understandings of archaeal transcription mechanisms will also provide insights into transcription in eukaryotes.

Archaeal genomes encode a single type of RNAP, which consists of 11 to 12 subunits. The subunit composition of archaeal RNAP is similar to eukaryal RNAPII, and a recently reported archaeal RNAP X-ray crystal structure [41] showed striking structure similarity with eukaryal RNAPII (Figure 1.3).

TBP is an essential protein for transcription initiation [80]. TBP recognizes and binds to the DNA *cis*-acting element called TATA-box, which is an 8-bp AT-rich sequence located approximately 25 bp upstream of the transcription initiation site [11]. TBP binds TATA-box and the binding introduces a sharp kink in the DNA promoter.

TFB is a eukaryal TFIIB ortholog and recruits RNAP to the promoter [10]. It also facilitates RNAP transcription start site determination [83]. The goal of this dissertation project is to address the roles of TFB in transcription regulation. The next section summarizes the structure and functions of archaeal TFB.

Two other GTFs, TFE and TFS, orthologs of the eukaryal N-terminal half of TFIIIE α and TFIIS, respectively, have been identified in all archaeal genomes, although they are not essential for transcription initiation *in vitro*. Currently, no orthologs of eukaryal TFIIA, TFIIF and TFIIH have been identified in any available archaeal genomic sequences [37].

1.3 Structure and function of archaeal TFB

Archaeal TFB is highly conserved among different species (50 - 60% similarities), but sequence similarity between archaeal TFBs and eukaryal TFIIBs is approximately 20 to 30% [75]. TFB interacts with a DNA sequences known as the B-factor Recognition Element (BRE), which is a *cis*-acting DNA element in archaeal promoters. BRE is a purine rich (within non-template strand) sequence found immediately upstream of the TATA-box. The interaction between TFB and BRE appears to be necessary to determine direction of transcription [12][49].

The C-terminal of TFB has two domains, each consisting of 5 or 6 α -helices. The X-ray crystal structure of DNA-TBP-TFB complex (Figure 1.4) showed that both domains are involved

in interaction with DNA and TBP [49]. The last two helices in the second repeat form a helix-turn-helix motif, which recognizes the BRE [49].

The N-terminal domain of TFB contains two motifs including Zn-ribbon and B-finger. Zn-ribbon is found in various transcription-related proteins and contains a sequence motif, Cys-X₂-Cys/His-X₁₅₋₁₇-Cys-X₂-Cys, to coordinate Zn²⁺ [23]. One of the identified functions of Zn-ribbon is to interact with RNAP [10]. The B-finger in eukaryal TFIIB is shown to be proximal to the RNAPII active site in the PIC by X-ray crystal structure of RNAPII-TFIIB complex [20] and biochemical analysis [21][22] and it interacts with RNA-DNA hybrid to stabilize the transcription initiation complex [20]. It is expected that archaeal TFB B-finger has a similar function in the PIC. Previous *in vitro* transcription analyses showed that once 9-12 bases of the nascent transcripts are formed, TFB is released from the transcription initiation complex and recycled for the next round of transcription [76][85]. TFB release from RNAP is necessary to clear the exit channel for the nascent RNA [20]. The TFIIB B-finger has been proposed to have roles in determining transcription start site [83] and timing of TFIIB liberation [81], although similar activities have not been observed for archaeal TFB.

1.4 Transcription regulation in *Archaea*

In order to effectively survive in a competitive environment, organisms need to balance production of gene required for cell activities, while avoiding extraneous production of unnecessary proteins. The regulation of gene expression could potentially take place at any stage from transcription initiation to protein degradation. Regulation of transcription initiation is one of the most important steps governing gene expression. Therefore, organisms have developed a variety of methods to achieve effective outcomes. Some archaeal transcription regulation mechanisms have been shown to be more closely related to bacterial than eukaryal systems [5][34], whereas

others are more closely related to those in eukaryotes. Three examples of currently proposed models of transcription regulation in archaea are described below.

The first example is the utilization of bacterial-type transcription activators and repressors for transcription regulation. Although archaeal transcription machinery consists of a eukaryal-type RNAP and GTFs, most archaeal genomes encode homologues to bacterial-type transcription regulators [5][11][34][56][82]. The mechanism of repressing transcription in these cases entails binding to DNA in the promoter by this transcription factor leading the blocking TFB and/or TBP binding to DNA (*e.g.* *Sulfolobus solfataricus* Lrs-14 [31]) or inhibiting RNAP recruitment to the promoter (*e.g.* *Pyrococcus furiosus* LrpA [17][26], *Archaeoglobus fulgidus* MDR1 [7]). Some bacterial Lrp (Leusine responsive regulator protein)-like factors in *Archaea* are proposed to function as transcription activators, which enhance the formation of the PIC [56]. Examples of currently identified activators are *S. solfataricus* LysM [16] and *Methanocaldococcus janaschii* Ptr2 [18].

The second example involves DNA packaging proteins, which is analogous to transcription regulation in eukaryotes. In eukaryotes, nucleosome structure and histone modification play key roles in gene regulation, in addition to the coordinated work of transcription factors. Eukaryal histone proteins possess positively charged N-terminal tails, and specific residues within the tails are covalently modified by acetylation, phosphorylation, methylation and ubiquitination (reviewed in [84]). Acetylation of histone tails, for example, allows the access of transcription factors to DNA to promote transcription initiation [84].

Archaeal species from Euryarchaeota phylum possess one to six different histones forming both heterodimers and homodimers. For example, two histone proteins in *Methanothermus fervidus*, HMfA and HMfB, exist as either a heterodimer (HMfA+HMfB) or a homodimer ((HMfA)₂

or (HMfB)₂) *in vivo* [58]. Interestingly, alternative dimer forms have different affinities to DNA sequences. Therefore, combinations of histone proteins could potentially control accessibility of transcription factors to DNA promoters in *Archaea* (reviewed in [58][61]). Identification of archaeal histones raises the possibility of nucleosome structure presence in Euryarchaeota and a potential transcription regulation mechanism via its modification [61]. However, it has been found that the archaeal histones lack N and C-terminal tails, which are the targets for modifications in eukaryal histones. In addition, homologues to histone modification factors found in eukaryotes have not been identified in archaeal genomes [61].

Alba (Acetylation lowers binding affinity) is another double-stranded DNA binding protein identified in *Archaea*, which can be reversibly acetylated and deacetylated [6]. It has been proposed that acetylation and deacetylation of Alba could influence transcription activity due to the lower DNA binding affinity exhibited by acetylated Alba compared with deacetylated form [6]. Also, repressed transcription activity *in vitro* on DNA templates in the presence of acetylated Alba had been reported [6].

The third proposed model involves GTFs. Among currently available 31 whole archaeal genome sequences, 18 species are found to possess multiple homologues of GTFs, TBP and/or TFB. The multiplicity suggests that they could be used in transcription regulation [3][9][56]. Certain combination of TBP and TFB may lead RNAP to different promoters. Recent reports for *Halobacterium* sp. NRC-1 suggest a role for multiple TBP or TFB homologues in governing expression of different genes in response to environmental factor change, such as nutrient availabilities or growth temperatures [25][28]. I have conducted several experiments to further investigate this model for TFB in *T. kodakarensis* (Chapter 2).

It is noteworthy that use of multiple GTFs is not specific in archaeal transcription. In addition to possess three distinct RNAPs, eukaryotes also utilize multiple GTFs for transcription initiation. In *Homo sapiens*, RNA polymerase III (RNAPIII) is found to carry out transcription using two homologues of GTFs, known as TFIIB-related factor (Brf), Brf1 and Brf2. The Brf is one of the components of TFIIB, an RNAPIII GTF, along with TBP and Bdp (B double prime) [30][57]. Although both Brfs in *H. sapiens* contain the same characteristic domains found in TFIIB, such as the Zn-ribbon motif and two imperfect helical repeats, they each recruit RNAPIII to a distinct set of promoters. The TFIIB with Brf1 is found associated with transcription from tRNA or 5S rRNA promoters while TFIIB containing Brf2 recruits RNAPIII to a different set of promoters [70]. This example illustrates how different GTF homologues can be utilized to recruit an RNAP to different sets of promoters.

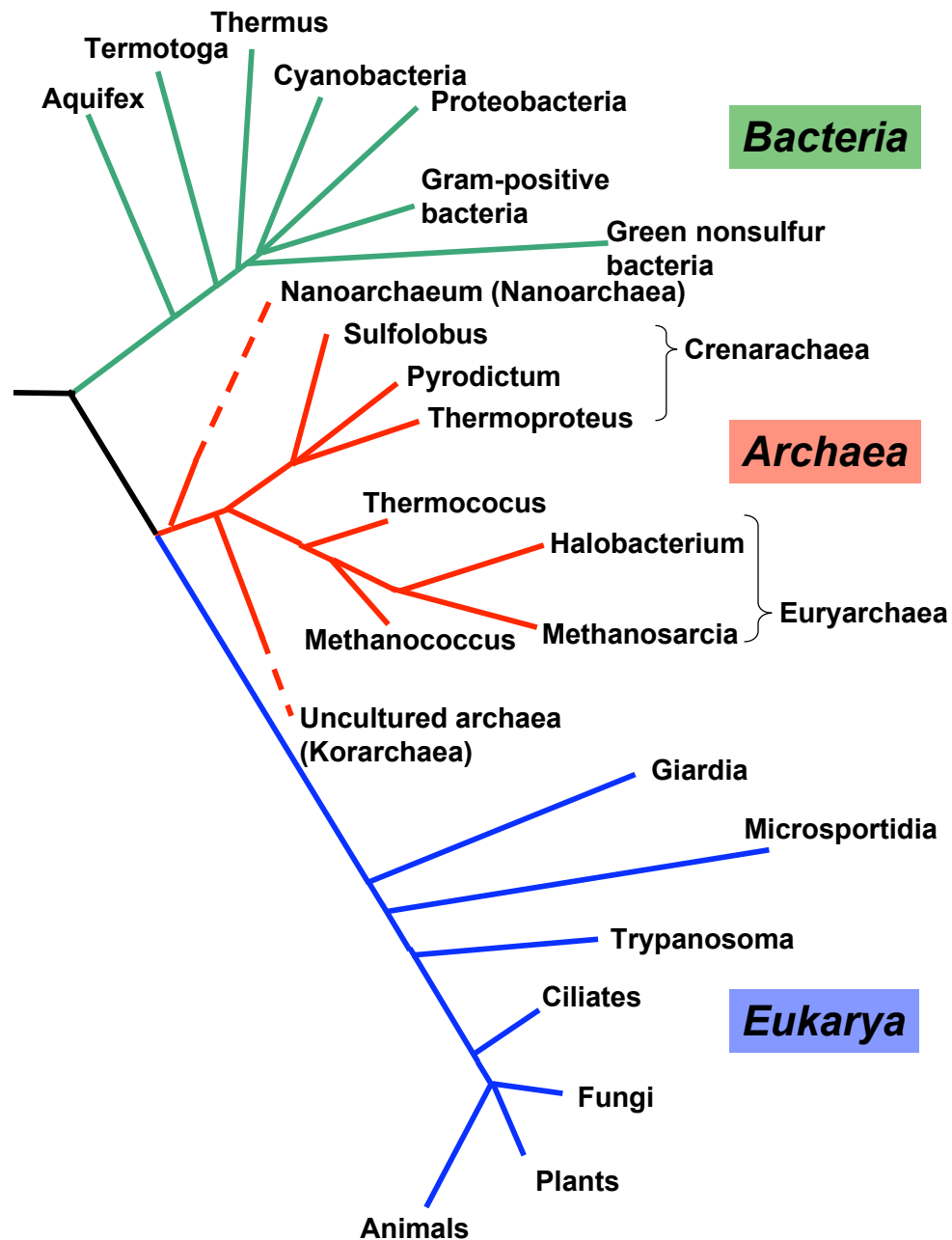


Fig. 1.1: Phylogenetic tree of organisms based on small subunit rRNA sequences. Phylogenetic tree of organisms based on small subunit ribosomal RNA sequences prepared based on [1][19][42]. Branches of each domain are shown in different colors and length of lines connecting two species is approximately proportional to the evolutionary distances between them. Dotted lines indicate locations of two species on the tree are not clear at this point.

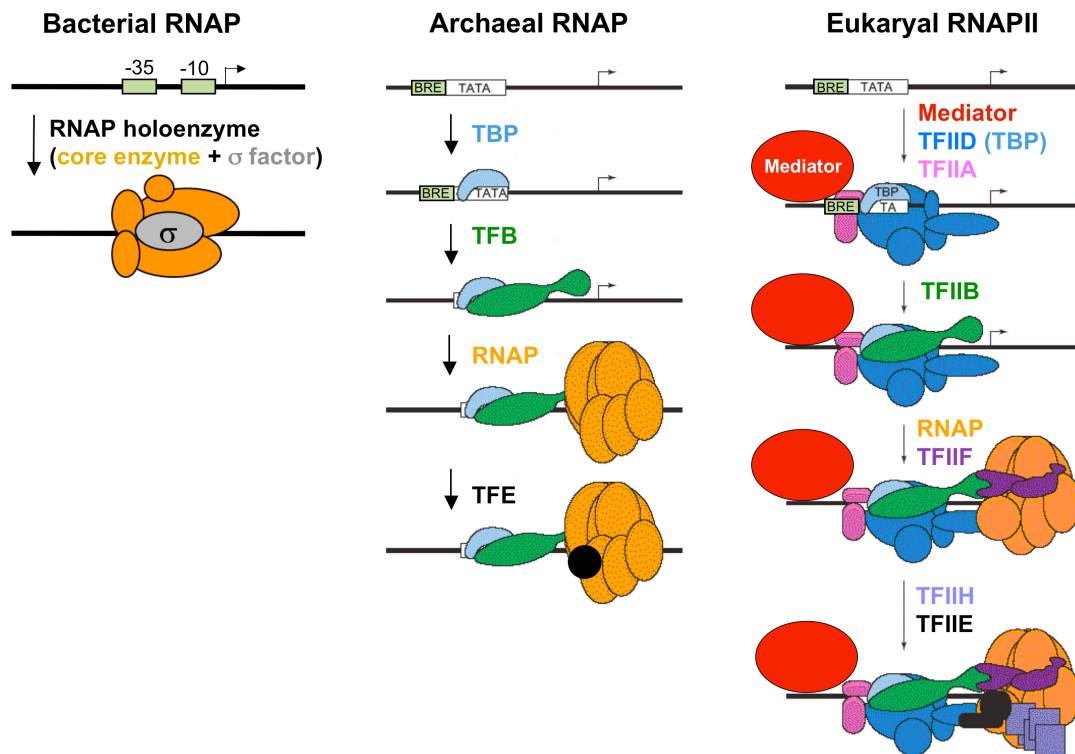


Fig. 1.2: Comparison of the PIC formation in the three domains of life. Schematic representation of the PIC assembly and GTFs involved in these processes. Figure modified from [8].

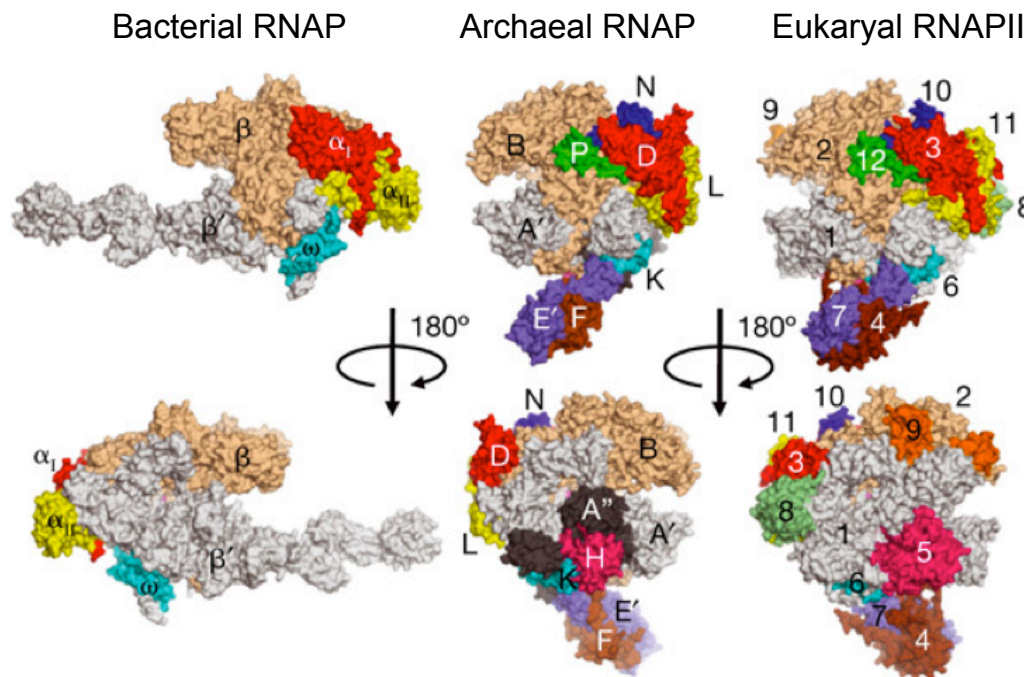


Fig. 1.3: Comparison of RNAP structures from three domains of life [41]. Surface representations of multi-subunit cellular RNAP from *Bacteria* (left, *Thermus aquaticus*), *Archaea* (center, *S. solfataricus*) and *Eukarya* (right, *Saccharomyces cerevisiae* RNAPII). Each subunit is denoted by a unique color and labelled. Orthologous subunits are depicted by the same color.

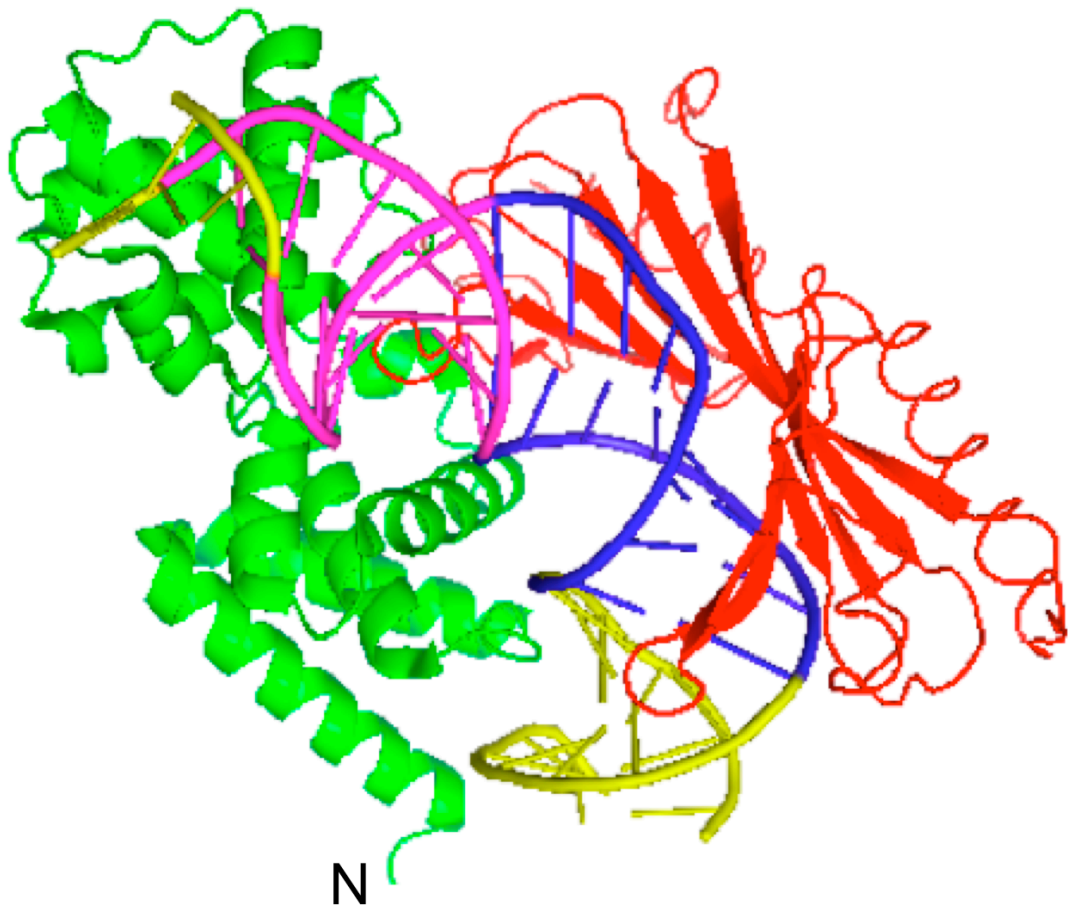


Fig. 1.4: X-ray crystal structure of the DNA-TBP-TFB(C-terminal domain) complex from *P. woesei* (PDB:1D3U) [49].

DNA contains consensus archaeal promoter (both TATA-box and BRE) and shown as a cartoon representation with TATA-box is colored blue, BRE is colored magenta and other nucleotides are colored yellow. TBP and C-terminal of TFB are shown as a ribbon diagram and colored red and green respectively. The figure was prepared by PyMol.

Chapter 2

Roles of archaeal TFB homologues from *Thermococcus kodakarensis* in gene regulation

2.1 Introduction

The archaeal transcription machinery consists of a minimum three protein factors: a multi-subunit RNA polymerase (RNAP) and two general transcription factors (GTFs) including TATA-binding protein (TBP) and transcription factor B (TFB) [38][60]. Interestingly, more than half of archaeal species, whose whole genome sequences are available, possess multiple homologues of GTFs, TBP and/or TFB (Table 2.1) [28]. However, it remains unclear whether these homologues have redundant functions or have specialized roles in transcription regulation. It has been hypothesized that alternative TBP-TFB combination could direct RNAP to a specific set of promoters for gene regulation at different stages of growth or environments [3][9][56]. This chapter summarizes investigation to assess the involvement of multiple TFB homologues in gene regulation.

In support of the model that each homologue participates in transcription regulation at different stages of growth or environments, mRNA quantification studies have shown differences in expression levels of TBP and/or TFB homologues in various growing conditions. It has been reported that expressions of one of the two TFB homologues in *Pyrococcus furiosus* and one of the six TFB-related genes in *Haloferax volcanii* were induced upon heat shock [74][79]. It also has been found that TFB homologues in *Sulfolobus* showed changes in their expressions during

cell cycle [51].

In addition to the mRNA quantification studies, a recent gene knockout study with *Halobacterium* sp. NRC-1 showed deletion of either *tbpD* or *tfbA* resulted in heat sensitive phenotype. And a change in expression for $\sim 10\%$ of genes in NRC-1 with $\sim 90\%$ correlation between the $\Delta tbpD$ and $\Delta tfbA$ strains consistent with co-regulation of those genes by TBP_D and TFB_A [25]. Also, for *Halobacterium* sp. NRC-1, several possible TBP-TFB combinations used for environmental stress response were proposed based on DNA microarray analyses [28].

In contrast, the hypothesis that alternative TBP-TFB pair directs RNAP to specific sets of promoters, was not supported by *in vitro* studies with hyperthermophilic euryarchaea, *Thermococcus kodakarensis* and *P. furiosus*. In the case of *T. kodakarensis*, each of the two TFB homologues showed almost equivalent transcriptional activity from various promoters [66]. In *P. furiosus*, TFB2 lacks a B-finger motif and has only a third of the transcription activities compared with TFB1 for all tested promoters [54]. Although the trend remain unchanged for both heat shock and non-heat shock promoters indicating each TFB has no promoter specificities.

Using *T. kodakarensis* genetic system, deletion strains of each *tfb* homologue were constructed and their growth was monitored. The deletion strains did not exhibit any growth defects under an optimal condition. This observation indicates that either TFB1 or TFB2 is sufficient for cell viability [66]. However, the consequences of the *tfb1* or *tfb2* deletion at other conditions have not been reported.

In order to study roles of TFB homologues closely, investigations using both genetic and biochemical approaches are necessary. There are several reported genetic studies using haloarchaea, however, they were not suitable for this study for the following reason. Proteins from

halophilic species require high salt concentration to maintain their biological activities, therefore very few proteins from haloarchaeal species have been purified and biochemically characterized (*e.g.* Ferredoxin in *Halobacterium salinarum* [4]). For this study, *T. kodakarensis* was chosen as the model organism. One of the greatest advantages to use *T. kodakarensis* as a study model, is the availability of a powerful genetic system, which is not true for many other archaea. Dr. Imanakas group at Kyoto University developed methods to effectively disrupt targeted genes [67][68]. *T. kodakarensis* was found to possess natural competency for foreign DNA uptake into cells, thus no special treatment of the cells (*e.g.* CaCl_2 treatment) was required for transformation. Additional methodologies, such as a reporter system [65], gene pop-out method [68], DNA microarray (*e.g.* [46]) and conditional gene expression system [40] are available for further investigations.

T. kodakarensis is a hyperthermophilic archaeon, isolated from solfatara on the shore of Kodakara island, Kagoshima, Japan and belongs to the euryarcheota phylum [2][33]. The genome of *T. kodakarensis* is annotated with 2,306 protein coding genes and more than half of them have been assigned protein functions. *Thermococcus* is one of the two major genera composing the order, Thermococcales, along with *Pyrococcus* [43]. Species from *Thermococcus* and *Pyrococcus* genera are closely related and share common energy metabolisms. Members of both genera grow on organic substrates and require the presence of elemental sulfur as an electron acceptor. Due to its sulfur-reducing activity and high growth temperature, *T. kodakarensis* was originally reported as a *Pyrococcus* species. However, its 16S rRNA sequence was found to be sufficiently divergent from *Pyrococcus* and reclassified as a *Thermococcus* species [2]. *T. kodakarensis* is capable of utilizing various carbon sources including free amino acids, starch and maltooligosaccharides [2]. When grown on yeast extract, tryptone or free amino acids, it requires the presence of elemental sulfur as a terminal electron acceptor. It also has the capacity to grow via fermentation when supplemented with soluble starch or pyruvate. *T. kodakarensis* is strictly anaerobic, and the

reported growth temperature range is between 60 °C and 100 °C with optimum growth observed at 85 °C [2].

T. kodakarensis has two TFB homologues and one TBP. Thus, it is an ideal organism to investigate the function of each TFB homologue independent of the effect of multiple TBPs. In the present study, I established an *in vitro* transcription system using native and recombinant proteins and enzymes from *T. kodakarensis* and investigated transcription activities in the presence of either TFB1 or TFB2. In addition, gene expression profiles were investigated in *tfb* deletion strains to observe consequences of *tfb* disruption.

2.2 Results

2.2.1 Basic characterization of TFB homologues in *T. kodakarensis*

2.2.1.1 TFB sequence alignment and phylogenetic analysis

Two TFB homologues, TFB1 and TFB2, are encoded by the genome of *T. kodakarensis*. TK1280 encodes for TFB1 (300 amino acids) and TK1287 encodes for TFB2 (306 amino acids). Amino acid sequences of two homologues are 67% identical (Figure 2.1). The N-terminal domains have a higher identity (81% between TFB1 amino acids 7 to 66 and TFB2 amino acids 10 to 69) than C-terminal domains (66% between TFB1 amino acids 86 to 300 and TFB2 amino acids 95 to 306). The linker region between N and C-terminal domains has less identity (50% identity) than either the N-terminal or C-terminal domains.

In order to investigate the evolutionary relationships of TFB homologues, I conducted an amino acid sequence alignment of archaeal TFBs in *T. kodakarensis* and the closely related

Pyrococcus genus. Notable differences were identified in two N-terminal motifs, Zn-ribbon and B-finger (Figure 2.2A). While these two characteristic motifs are present in both TFB homologues in *T. kodakarensis*, one of the TFBs from *Pyrococcus* species lack one or both motifs. Overall, TFB homologues have higher N-terminal sequence identity in *T. kodakarensis* than *Pyrococcus* TFB homologues. The close relationship of the two *T. kodakarensis* TFB homologues is also shown in the phylogenetic tree generated from a sequence alignment of eight TFBs (Figure 2.2B). TFB1 was found to be cluster with the solo TFBs found in *P. woesei* and *P. abyssi* and with one of the TFBs from *P. furiosus* and *P. horikoshii*. The second TFB from *P. furiosus* and *P. horikoshii* is located away from the TFB1 cluster in each case. This is primarily due to the variation in N-terminal sequences. Alternatively, *T. kodakarensis* TFB2 shows a very close relationship to TFB1 cluster.

2.2.1.2 Verifications of *tfb1* and *tfb2* operon configurations

Both *tfb1* and *tfb2* are expected to be co-transcribed with other genes in their vicinities (Figure 2.3A and B). RT-PCR results support this prediction (Figure 2.3C), however, functions of other genes in both operons have not been experimentally determined, and their functional relevance to each TFB is unknown. RT-PCR products were detected between *tfb1* and other two genes immediately upstream consistent with co-transcription of all three as a single operon. The first gene (TK1278) encodes a protein containing a double-stranded DNA binding domain, and the second gene (TK1279) has no assigned function currently. RT-PCR products were detected between *tfb2* and the gene located immediately upstream and downstream consistent with co-transcription of all three as a single operon. The gene immediately upstream of *tfb2* (TK2286) is annotated as a component of Gar1 (subunit of ribonucleoprotein complex that is required for pre-mRNA processing [48]), and the gene downstream of *tfb2* (TK2288) is annotated as a metallophosphoesterase. The configuration of operons may explain the outcome of efforts made for

tfb1 or *tfb2* deletion strain constructions. The *tfb1* deletion strain was readily constructed. Due to the observation it is the last gene of an apparent operon, no polar effects on neighboring genes were anticipated by *tfb1* deletion. Attempts to delete the *tfb2* gene alone were unsuccessful. Only when *tfb2* and TK2288 were both deleted, *tfb2* deletion strain was successfully constructed [66].

2.2.1.3 Transcript abundance of *tfb1* and *tfb2* in *T. kodakarensis*

In order to investigate the relative contributions of each TFB to transcription during cell growth, I measured transcript abundance of *tfb1* and *tfb2* by quantitative real-time PCR (qRT-PCR). For *tfb1* transcripts, no more than ~ 2.5 -fold increases were observed during growth between mid-log to stationary phase (Figure 2.4A). Transcription of *tfb2* was initially 4-fold higher than the reference, which was taken at cell turbidity at ~ 50 KLETT units (indicated by an arrow). In the following hours, no more than ~ 2.5 -fold changes were observed compared with the reference. The expression of *tfb2* may be higher at early stage of cell growth (~ 30 KLETT units), although conclusive results was not obtained from this assay due to the uncertainty associating with *tfb2* quantification within the RNA sample. Absolute quantification showed that both *tfb1* and *tfb2* transcripts are present at equal abundances (Figure 2.4B) at a stationary phase. Conversely, in *P. furiosus*, transcript abundances of *tfb2* are much less than that of *tfb1* under an optimal growth condition [74].

I next asked whether environmental stress could influence the abundance of *tfb1* and *tfb2* transcripts. I shifted incubation temperature of wildtype *T. kodakarensis* KOD1 cells from its optimum growth temperature (85°C) to 100°C or 60°C at mid-log phase (~ 50 KLETT units) and monitored changes in transcript abundance during the shifts. After the temperature upshift, cells continue to increase in turbidity for an additional two hours. The previous result showed ~ 2.5 -fold increase with constant temperature during mid-log (~ 50 KLETT units) to entry into the

stationary phase (~ 110 KLETT units) for both *tfb1* and *tfb2* transcript abundances. However, during the same growth period, shifting the temperature to 100°C resulted in 6-fold increase in transcript abundance of *tfb2* (Figure 2.5). The observation suggesting a temperature-dependent induction of *tfb2*. A 3-fold increase in *tfb1* transcript abundance was observed with upshifted temperature (Figure 2.5), which is slightly higher than ~ 2.5 -fold induction without temperature shift. Because the differences between the two induction levels were very small, I was not able to conclude that *tfb1* is induced under heat stress from these assays. In the case of temperature downshift, no increase in cell turbidity was observed indicating that *T. kodakarensis* cells were not vital at 60°C . Therefore, decrease in transcripts under cold stress is likely resulted from mRNA decay (Figure 2.5).

2.2.2 Comparisons of transcription activities in the presence of TFB1 and TFB2

2.2.2.1 Transcription assay using heat shock promoter

It has been hypothesized that alternative TBP-TFB combination could direct RNAP to a specific set of promoters. Because qRT-PCR analysis described above showed two *tfb* homologues were differentially expressed under heat stress, heat shock promoter could be a potential target for gene regulation using two TFB homologues. A previous study showed no significant difference in TFB1 and TFB2 transcription activities from various promoters [66]. However, no promoters for gene involved in stress response were included in the study. I used a promoter from *cpkB* to investigate whether any differences in transcription with either TFB were observed. The *cpkB* encode for one of the two subunits of thermosome, CpkB, in *T. kodakarensis* and it has been reported the expression of the subunit was induced following heat stress [44]. If TFB2 is required to induce heat shock genes, transcription activities from *cpkB* promoter would exhibit differences in the presence of TFB2 versus TFB1. The *in vitro* transcription assay showed no differences in transcription activities in the presence of either two TFB homologue from *cpkB*

promoter (Figure 2.6). I also conducted transcription assays at various temperatures and incubation times, however, none of the conditions used showed differences between the two TFB homologues (Figure 2.7 and 2.8).

In a previous *in vitro* transcription study with *P. furiosus* system, it was shown that TFE could enhance transcription activity of TFB2 that lacks B-finger motif [54]. The report suggests TFE has some influence on TFB activities. To test whether addition of TFE had any effect on transcription activities of *T. kodakarensis*, I conducted transcription assays with recombinant TFE. These assays demonstrated that the addition of TFE did not affect transcription activities of *T. kodakarensis* in the presence of TFB1 or TFB2 (Figure 2.7 and 2.8). In these assays, endogenous RNAP from *T. kodakarensis* was used, which is known to co-purify with TFE [40]. Therefore, in order to observe TFE effect, addition of excess amount of TFE may be required.

2.2.2.2 Transcription from strong archaeal promoter

To compare transcription activities in the presence of either TFB1 or TFB2, initial stages of transcription was observed using abortive transcription assays. For these assays, DNA template containing strong archaeal T6 promoter from *Sulfolobus shibatae* virus SSV1 (Figure 2.9A) [12] was used. For the abortive product formation, dinucleotides (UpA) and [α - 32 P]GTP were added as substrates to produce the trinucleotides products (UAG). It showed considerable differences in abortive transcript accumulation for the two TFB homologues in *T. kodakarensis* (Figure 2.9B, compare lanes 1 and 4). TFB1 dependent transcription produced much more abortive products than TFB2 dependent transcription. The differences in trinucleotide accumulation were also observed when all four nucleotides were added to chase the transcription to the full-length (Figure 2.9B, compare lanes 3 and 6). While putative run-off products were found at approximately same level for pre-initiation complexes (PICs) with either TFB1 or TFB2, more than

10-fold trinucleotides accumulation was observed for transcription with TFB1 versus TFB2 (Figure 2.9C and D).

2.2.3 Study of TFB association with DNA-TBP complex

The differences in abortive transcription is consistent with the two TFB homologues have different effects on PIC during early stages of transcription. There are two interactions, which may explain the differences. The first is the interaction of TFB to DNA-TBP complex. A stronger association between TFB and DNA-TBP could lead to more abortive transcripts as dissociation of TFB from DNA requires time and energy. The second is the interaction between TFB and RNAP during promoter escape. TFB liberation is necessary to promoter escape and transition to the elongation phase. Therefore, the stronger the interaction between TFB and RNAP, the more abortive transcription is likely to occur. Because TFB1 containing PIC synthesized more abortive transcripts, I speculated that TFB1 has a stronger association with other components in PIC: TBP, DNA, and/or RNAP, than TFB2.

In order to test this hypothesis, Electrophoretic mobility shift assay (EMSA) was carried out and binding of the two TFB homologues to DNA-TBP complex were compared. The result indicated that TFB1 had a stronger association with the DNA-TBP complex than TFB2 (Figure 2.10A). However, the effort to determine the dissociation constants was not successful by EMSA. When less than 250 nM TFB was used, the ratio of bound DNA to free-DNA did not change in response to the TFB concentration (Figure 2.10B). At higher concentrations of TFB, aggregations of DNA-TBP-TFB complexes were observed and they were trapped in the wells. A previous study used a high concentration of proteins (*e.g.* 0.9 μ M TFB1 in [52]) for EMSA to observe DNA shift. I attempted to reproduce this experimental condition, but I was

not successful.

To avoid this aggregation problem, I selected an alternative method, quartz crystal microbalance (QCM) for the determination of binding constant between DNA-TBP binary complex and TFB. QCM is a balance with ultrahigh sensitivity and is a useful tool for investigating association of molecules [39]. The QCM sensor contains quartz crystals, which are oscillated by the application of voltage. When molecules bind on the crystal surface, changes in oscillation are recorded as frequency changes. The change in frequency is proportional to the mass deviation on the sensor surface, so that interactions of original molecule (bait) on the surface and incoming molecule (prey) can be calculated from the QCM record (Figure 2.11).

In this QCM assay, biotinylated DNA template with strong archaeal promoter was immobilized on sensor surfaces. TBP was added to saturation followed by titration of TFB1 or TFB2. The changes in frequency of the sensor was recorded during the titration. From the record, changes in frequency were plotted as a function of TFB concentration (Figure 2.12). To fit the data points, bimolecular binding reaction model between bait (DNA-TBP) and prey (TFB1 or TFB2) was applied. If the model is adequate, dissociation constants between the two molecules were determined as 1 μ M and 2 μ M for TFB1 and TFB2, respectively. This indicates TFB1 binds more tighter to DNA-TBP complex, consistent with EMSA finding. However, the residual plots show large discrepancy between the model and observed value, particularly at the low concentration of TFB (Figure 2.12). This suggests there are some systematic errors (either model and/or measurement) associating with QCM analyses. In order to consider experimental approaches for future work, QCM data and procedures were evaluated as summarized in the following section.

Attempts to determine dissociation constants of TBP to DNA, and RNAP to TFB using QCM were also made. It has been shown that EMSA was not suitable to observe binding of TBP to DNA, because the TBP-DNA complex did not resolve far enough to be distinguished from free-DNA [52]. Dissociation constant determination of the TBP to DNA was not successful using QCM. It appears association of TBP and DNA is a slow process, thus TBP may diffuse away from DNA before binding reaction takes place. A similar problem was pointed out for surface plasmon resonance assay [72] and further adjustment in experimental conditions and data analyses were required to overcome the issue. The dissociation constants of RNAP to TFB1 or TFB2 were determined as 12.5 nM and 14.5 nM, respectively. However, negative control experiments with BSA (in place of TFB) resulted in the similar reading. This suggests the dissociation constants obtained for TFB determined by QCM were possibly influenced by the non-specific interaction of RNAP and DNA or sensor surface.

2.2.4 Evaluation of QCM data

QCM experiments were carried out as described above (see also materials and methods) to obtain dissociation constants between DNA-TBP complex and TFB1 or TFB2 (K_{d2} in Figure 2.13). In order to fit data points, bimolecular reaction model was applied using a following equation [35]:

$$Y = Y_{max} \times ([TFB] / ([TFB] + K_{d2}))$$

Here, Y corresponds to the changes in frequency. However, it was found that the curve does not fit data points appropriately, especially at the low TFB concentrations (Figure 2.12), suggesting the fitting model needs to be reconsidered.

Both TFB1 and TFB2 plots (Figure 2.12) appeared to be sigmoidal shapes suggesting there are two different stages during the DNA-TBP-TFB complex formation. At low TFB concentration ($[TFB]$ is less than $0.2 \mu\text{M}$), the addition of TFB contributes large in sensor frequency change. This indicates that the presence of small amount of TFB in the system promotes TFB binding to DNA-TBP complex, thus large increase in frequency changes were observed in response to the small TFB addition. When TFB concentration becomes higher, TFB addition has less effect in frequency change, possibly due to the saturation of TFB binding sites. In the present study, Y_{max} was not reached during the TFB titration between 0 to $1.6 \mu\text{M}$. In the assay, TFB was diluted and added to the $500 \mu\text{l}$ buffer in increments of either 1 or $5 \mu\text{l}$. Because addition of protein changes total buffer volume, thus protein concentration, total amount of protein added was limited up to $50 \mu\text{l}$ (10%), which corresponds final concentration of $1.6 \mu\text{M}$. Therefore, the QCM responses to the higher TFB titration were not observed. In future experiments, it is suggested that TFB dilution to be adjusted in order to obtain data from wider range of TFB concentrations.

Since the experiment includes three biomolecules, other possible reaction within the system may influence the interaction of TFB to DNA-TBP complex. There are several possible interactions among three molecules as illustrated in Figure 2.13. Multiple possible interactions, which potentially provide sigmoidal plots are discussed below. Experimental approaches would be taken in order to address the issue are also summarized.

When considering the binding model, it was assumed the equilibrium between DNA-TBP binding reaction (boxed in blue in Figure 2.13) had already reached when TFB was added. However, a review of QCM output suggests this assumption may not be adequate. What happens if TFB is added before DNA-TBP interaction was equilibrated? Because addition of TFB stabilizes TBP-DNA interaction, it would potentially increase k_1 and decrease K_{d1} , and the equilibrium of

TBP-DNA binding reaction would be shifted. Therefore, it is provable even after TFB titration was started, further TBP binding to DNA could take place. In that case, increase of frequency changes may result from not only the association of TFB, but also the binding of free TBP to DNA. In order to address this possibility, experiment can be repeated and buffer will be added in place of TFB under the same condition. If frequency change is still observed, it suggests equilibrium had not been reached. In addition, alternative method, such as DNase I footprinting assay can be employed to estimate TBP concentration required to saturate DNA.

The analyses also consider that DNA-TBP complex formation takes place prior to the interaction with TFB. For the modeling, only the interaction between DNA-TBP and TFB (boxed in red in Figure 2.13) was considered. However, it is possible that alternative pathway of ternary complex formation may occur in the system. For instance, TFB and TBP may form complex in solution and the complex could have different affinity to DNA from TFB or TBP alone. In order to determine dissociation constant between TBP and TFB, labeled protein (fluorescence or radioisotope) can be prepared and the complex formation can be detected via various methods (*e.g.* fluorescence resonance energy transfer, fluorescence anisotropy or EMSA). If the reaction between two proteins is very weak, the K_{d5} can be ignored by conducting experiment with protein concentrations less than the dissociation constant. Another possible model is that TFB may be capable of binding to DNA without TBP. Because previous EMSAs did not show any bands for TFB-DNA complex (Figure 2.10 and [52]), alternative approaches are necessary in order to observe DNA-TFB interaction (K_{d3}). One of the approaches is DNase I footprinting assay, which could provide information of whether TFB bind to DNA in absence of TBP. The result can be compared with assays with TBP to obtain K_{d2} , and comparison of the two K_d s would provide information if TBP and TFB cooperatively bind to DNA. For instance, if K_{d2} is smaller than K_{d3} , they have positive cooperativity [35].

There also be a possibility that interaction between TFB and QCM apparatus affect the binding reaction. Because only molecules binds to the sensor surface (located at the bottom of sensor cell, Figure 2.11A) will be recognized, TFB bound to other portion of the sensor cell, such as wall, will not be detected. If TFB has higher affinity to the other parts than DNA-TBP complex, free TFB available for interaction with DNA-TBP in the buffer could become less than I estimated. As TFB concentration increases, favored binding site on non-sensor surface started to be occupied and additional TFB may have started to react with TBP-DNA complex. This model also explains sigmoidal shape of the plots, however, finding conditions to eliminate interaction between TFB and non-sensor surfaces can be challenging. Potentially, QCM assay with TFB alone provide some information on interaction between sensor cell components and TFB, although it could be difficult to monitor as frequency changes for non-specific binding between TFB and sensor can be very small.

Numerous biological system involves three or more types of biomolecules to control cell activities (*e.g.* regulatory proteins and DNA). In order to understand binding reaction of each step, how the binding of one molecule affect the binding of others should be carefully assayed. Although, current QCM data provided evidence that TBP and TFB binding to DNA involve multiple reactions and can not be modeled as a simple bimolecular binding, further assays will be required to fully understand PIC formation in *Archaea* and compare them between the two TFB homologues.

2.2.5 Gene expression profiles in *tfb* disrupted strains

2.2.5.1 Transcript quantification by quantitative real-time PCR

To observe effect of *tfb1* or *tfb2* deletions, gene expression profiles within *tfb* deletion strains were observed by qRT-PCR. Three *T. kodakarensis* strains (KW128, KW128 Δ *tfb1* and

KW128 $\Delta tfb2$) were grown at 85 °C until mid-log phase and incubation temperature was upshifted to 100 °C. Cells were harvested at various time points during the temperature shift and fold changes from pre-heat shock level were monitored in each strain. As in *T. kodakarensis* KOD1 strain, the *tfb2* transcript abundance increased 6 to 8-fold upon the temperature shift in KW128 and KW128 $\Delta tfb1$ strains. No more than $\sim$ 2-fold increase in *tfb1* transcript abundance was observed (Figure 2.14A and B). Since TFB must pair with TBP for transcription, I also checked the abundance for *tbp* transcripts (Figure 2.14C). In accordance with total *tfb* transcript population, *tbp* transcript abundance increased in KW128 $\Delta tfb1$ strain ($\sim$ 10-fold). However, no increase of *tbp* transcript abundance was observed for both KW128 and KW128 $\Delta tfb2$ strains.

In addition to the GTFs, I also monitored the change in transcript population of heat shock genes. The most well-studied heat shock protein in *T. kodakarensis* is thermosome that comprises subunits CpkA and CpkB. A previous Western blotting analysis showed no increase in CpkA at high temperature while CpkB is induced upon heat shock [44], suggesting the ratio of CpkA to CpkB in the thermosome changes in response to the growth temperature in *T. kodakarensis*. Consistent with this report, the parental strain KW128 showed no increase in *cpkA* transcript abundance following temperature upshift to 100 °C. However, in both *tfb* deletion strains, *cpkA* transcript abundance increased 10-fold and 4-fold for KW128 $\Delta tfb1$ and KW128 $\Delta tfb2$, respectively (Figure 2.15A). In KW128 $\Delta tfb1$, the *cpkB* transcript abundance increased substantially ($\sim$ 40-fold) compared to KW128 ($\sim$ 10-fold) and KW128 $\Delta tfb2$ strains ($\sim$ 5-fold)(Figure 2.15B).

Since some genes were substantially upregulated in KW128 $\Delta tfb1$ in samples isolated after 60 minutes after the temperature upshift, I was concerned possibility that RNA quality of the particular sample was skewing qRT-PCR quantification. To verify this was not the case, the heat shock experiment was repeated for *cpkB* and similar induction patterns were observed. Thus, I

concluded sample quality was not the cause of transcript increase.

2.2.5.2 Global analysis of gene expression in *tfb* deletion strains

To observe global changes in gene expression in *tfb1* and *tfb2* deletion strains, DNA microarray was carried out. Single experiment was conducted to obtain an overview of gene expression patterns in *tfb1* and *tfb2* deletion strains, and also to complement the observations of qRT-PCR profiles. In this experiment, gene expression patterns of before and after heat shock were compared for three strains of *T. kodakarensis*, KW128, KW128 Δ *tfb1* and KW128 Δ *tfb2*. The overall expression profile showed little differences among three strains (Figure 2.16A). While majority of genes showed no fold changes by heat stress, several genes exhibited considerable downregulation or upregulation under heat stress (Figure 2.16B). Multiple genes including *cpkB* (TK2303) showed significant induction in *tfb1* deletion strains. Some genes were uniquely down-regulated in KW128 Δ *tfb1* or KW128 Δ *tfb2* strains, which may be regulated by TFB1 or TFB2.

2.2.6 Temperature effects on growth properties

In light of the qRT-PCR and DNA microarray results, I investigated either the *tfb1* or *tfb2* deletion strain exhibited any differences in growth phenotypes. It was reported that growth under an optimum condition was unaffected when either TFB was deleted [66]. However, the growth properties of *tfb* deletion strains under sub-optimum conditions had not been examined. *T. kodakaraensis*, KW128, KW128 Δ *tfb1* and KW128 Δ *tfb2*, were grown in nutrient-rich ASW-YT-S^o-pyruvate medium at 70 °C, 85 °C and 90 °C (Figure 2.17). At 70 °C and 90 °C, the KW128 Δ *tfb1* strain grow inefficiently compared to the other two strains. At 90 °C, the maximum turbidity reached for KW128 Δ *tfb1* was 82 KLETT units (~19.3 hours), while both KW128 (148 KLETT units, ~14.7 hours) and KW128 Δ *tfb2* (121 KLETT units, ~14.7 hours) reached higher

cell densities.

2.3 Discussion

It has been hypothesized the purpose for additional copies of the GTFs, TBP and/or TFB in some archaea is to coordinate changes in gene expression by utilizing different TBP and/or TFB homologues to govern the expression of different gene sets. Previous studies with several archaeal species showed the explanation for this model may differ depending on the species. Expression profiles of each homologues were shown to differ depending on the growth condition [74][79] and the stage in the cell cycle [51]. However, *in vitro* transcription assays did not support the model that different TFB homologues direct RNAP to alternate promoters [54][66]. Using *T. kodakarensis*, I investigated the roles of different TFB homologues in controlling the expression of gene required for the heat shock responses.

The question addressed by this study was whether the two TFB homologues have distinct roles in transcription regulations. TFB1 is found to produce more abortive products than TFB2, suggesting TFB1 associate stronger to other components of PIC and spend longer time at this early stage of transcription. To support the model, binding assays showed stronger association of TFB1 to DNA-TBP complex than TFB2. Because TFB1 and TFB2 are equally present in optimally grown cells, if two TFB homologues compete for the same promoter, TFB1 will more likely be used in transcription. Under heat stress, however, *tfb2* transcription was induced. Thus, more TFB2 could bind to DNA than TFB1 to initiate transcription. Because TFB2 spend less time and resources for abortive transcription, it could be an ideal homologue to be used to respond to environmental stress. *T. kodakarensis* may be manipulating the ratio of the two TFB homologues to adjust transcript population in the cell for optimizing cell activity. By having such a coordinated regulation mechanism, cells probably could effectively use limited resources,

thus advantageous for survival in competitive environments.

Data from studies using *Halobacterium* sp. NRC-1 suggest that this organism utilizes different TBP and TFB homologues to coordinate genome-wide changes in gene expression [25][28]. In contrast to *Halobacterium* sp. NRC-1, which has 6 TBPs and 7 TFBs presenting as many as 42 possible TBP-TFB combinations, *T. kodakarensis* and *Pyrococcus* species encode only two TFB homologues along with a single TBP. Therefore, the usage of multiple TFBs in these species is likely to be much less complex than it is in haloarcheal species. The study with *P. furiosus* showed only one of the TFB genes was expressed in optimally grown cells [74], whereas both TFB homologues were transcribed in *T. kodakarensis* under an optimal condition. Thus, even for closely related these two species, the role of each TFB homologue could be different.

Archaeal GTFs probably have evolved in various directions so that different lineages have acquired unique ways to utilize these proteins to control gene expression. Thus, it may be impossible to develop an all encompassing model to explain the multiplicity of GTFs for all archaea. Perhaps, the diversity of GTF usage has provided fitness to archaea to survive in various environments.

2.4 Materials and methods

2.4.1 Protein purification

The encoding genes of TBP (TK0132), TFE (TK2024), TFB1 (TK1280), and TFB2 (TK2287) were amplified from *T. kodakarensis* KOD1 genomic DNA with *Nde*I and *Bam*HI restriction sites by PCR. These sites were used to clone target genes into pET21a vector (Novagen, EMD Chemicals, Inc., Gibbstown, NJ). For TFB1 and TFB2, PCR fragments were also

introduced into the pET15b vector to construct proteins with an N-terminal 6 histidine-tag. The resulting plasmids were transformed into *Escherichia coli* BL21(DE3) (for non histidine-tagged TFB) or BL21(DE3) codon-plus RIPL (for others) (Invitrogen, Co., Carlsbad, CA). The transformant was then grown in 1.5 L Lucia-Bertani media with 100 $\mu\text{g}/\text{ml}$ ampicillin at 37 °C until OD_{600} reached approximately 0.5. Protein expressions were induced by the addition of 0.5 mM IPTG and cells were further incubated for an additional 3 hours at 37 °C. Cells were, then, harvested by centrifugation at 4 °C (5,000 rpm, 10 minutes). The cell pellets were resuspended in 20 ml TGED buffer (10 mM Tris-HCl, pH 8 at room temperature, 0.1 mM EDTA, 1 mM DTT and 5% glycerol) with 0.1M NaCl and 1mM PMSF and disrupted by sonication. Sonication was conducted for 5 sets of 30-second program (on; 2 seconds, off; 1 second, power level 5.0) with microtip (Model S3000, Misonix Inc., Farmingdale, NY). The lysed cells were centrifuged (16,000 rpm, 15 minutes) at 4 °C to remove cell debris and supernatant was used for following purifications.

The supernatants from TBP and TFE lysate were heat treated at 65 °C for 30 minutes and centrifuged (16,000 rpm, 15 minutes) at 4 °C. The supernatants were diluted with TGED buffer, so that it contains less than 50 mM NaCl, then applied to a HiTrap Q-FF column (5 ml, GE Healthcare, Piscataway, NJ). Unbound proteins were washed out with TGED buffer with 50 mM NaCl until OD_{280} reading was back to baseline. Following the wash, bound proteins were eluted by 50 ml TGED buffer with 50 mM to 0.8 M NaCl gradient (5 ml/minute flow rate) (See Appendix D, Figure D.1).

Supernatants from TFB1 and TFB2 without histidine tag expression were diluted to contain less than 50 mM NaCl by TGED buffer, then applied to a Hi-Trap Q-FF column (5ml, GE Healthcare) to trap proteins other than the target proteins. The column was washed with 20 ml TGED buffer with 0.1 M NaCl and both flowthrough and wash were directly applied to Heparin

column (5ml, GE Healthcare). Bound proteins were eluted by 100 ml TGED buffer containing salt gradient of 0.1 M to 1 M NaCl with 5 ml/minute flow rate (Appendix D, Figure D.2). Heat treatment was not used for TFB1 and TFB2, as previous experiments showed fragmentation of the proteins by the treatment. Non histidine-tagged TFB1 and TFB2 were used for transcription assays from *cpkB* promoter and quartz crystal microbalance studies.

Lysate of histidine-tagged TFB1 and TFB2 were diluted with TGED buffer to contain less than 50 mM NaCl, then applied to a Heparin column (5ml, GE Healthcare) without heat treatment. The loaded column was washed by TGED buffer with 50 mM NaCl until OD₂₈₀ reading became baseline level. Then, bound proteins were eluted by 50 ml TGED buffer with a salt gradient of 50 mM to 1 M NaCl (5ml/minute flow rate) (Appendix D, Figure D.3). TFB2 used additional purification step (Ni-affinity column) prior to application to the Heparin column, however, this step was not used for TFB1 purification as I observed fragmentation of protein through the column. Purified histidine-tagged TFB1 and TFB2 were used for abortive transcription and electrophoretic mobility shift assays.

The fractions containing target proteins were identified by SDS-PAGE and each protein was quantified by Bradford method using BSA as a standard [15]. *T. kodakarensis* RNAP was provided by Dr. Thomas Santangelo (Ohio State University) [66].

2.4.2 Transcription assay

2.4.2.1 Transcription assay from heat shock gene promoter

DNA 299-bp fragment containing *cpkB* promoter was prepared by PCR with *T. kodakarensis* genomic DNA and primers CpkB_F (5'-AGGTGCTTGAATTGGGAGAGAAGAAGCTCG) and CpkB_R (5'-CCTGGCAGCAAGAATGTTGAGCC) (Figure 2.6A). The amplified DNA was

then cloned into the pUC18 *Hinc*II restriction site and resulting plasmid was used as a PCR template for DNA preparations for transcription assays. Transcription reaction contained 20 mM Tris-HCl (pH 8 at room temperature), 250 mM KCl, 5% glycerol, 5 mM MgCl₂, 10 mM DTT, 40 ng/ μ l heparin, 200 μ M each of ATP, GTP and CTP, 20 μ M UTP, 250 nCi/ μ l [α -³²P]UTP, 40 nM DNA, 40 nM RNAP, 80 nM TBP and 80 nM TFB1 or TFB2. The reaction mix was prepared on ice and incubated at 85 °C (or gradient for *cpkB* temperature effect assay) in thermocycler (TGradient, Biometra, Goettingen, Germany) with heated-lid (100 °C) for 10 minutes unless otherwise noted. The transcription reaction was stopped by chilling the reaction mix to 4 °C on ice block and addition of equal amount of loading buffer containing urea (10 M), EDTA (200 mM) and trace amount of bromophenol blue.

Marker RNA was prepared using Perfect RNA marker template mix (Novegen). For preparation of the marker, 0.5 μ g of the template, transcription buffer (50 mM Hepes, pH 7.5 at room temperature, 12 mM MgCl₂ and 10 mM NaCl), 2 mM each of ATP, GTP and CTP, 1 mM UTP, 20 μ Ci of [α -³²P]UTP were mixed and RNA were synthesized by T7 RNAP by incubating the tube at 37 °C for one hour.

The transcripts were separated on 15% acrylamide gel (1/20th the concentration of acrylamide is N,N-methylenebisacrylamide) containing 6.7 M urea with TBE (0.5x) running buffer. Electrophoresis was conducted at 25W for 2.5 hours. The transcripts were visualized by storage phosphor autoradiography [45] and quantification of transcripts were done by the typhoon imaging system and ImageQuant software (GE Healthcare).

2.4.2.2 Transcription assay from strong archaeal promoter

DNA template for this assay contains strong archaeal promoter from *Sulfolobus shibatae* virus (Figure 2.9A) [12] and was purchased from IDT (Coralville, IA). Each transcription reaction mix contained 20 mM Tris-HCl (pH 8 at room temperature), 100 mM KCl, 5% glycerol, 10 mM MgCl₂, 2 mM DTT, 25 µg/ml of BSA, 40 ng/µl heparin, 100 µM each of ATP, CTP and UTP, 250 nCi/µl [α -³²P]GTP, 100 M of UpA, 40 nM DNA, 40 nM RNAP, 80 nM TBP and 80 nM TFB1 or TFB2. Addition of cold GTP resulted in an ineffective incorporation of radioactive GTP and difficulties with visualizing products (Figure 2.9B, lanes 2 and 5), thus I omitted cold GTP from this assay. All the components are mixed on ice and incubated at 70 °C in thermocycler (TGradient, Biometra) with heated-lid (100 °C) for 30 minutes. The transcription reaction was stopped by chilling the reaction mix to 4 °C in thermocycler and the addition of equal amount of loading buffer containing urea (10 M), EDTA (200 mM) and trace amount of bromophenol blue. The transcripts were separated on 24% acrylamide gel (1/30th the concentration of acrylamide is N,Nmethylenebisacrylamide) containing 8 M urea with TBE (0.5x) running buffer (90 minutes at 400V). Separated transcripts were visualized by storage phosphor autoradiography [45] . Quantification and analyses of transcripts were done by the typhoon imaging system and ImageQuant software (GE Healthcare).

2.4.3 Electrophoretic mobility shift assay (EMSA)

For EMSA assays, DNA template was labeled as follows. The kinase labeling reaction contains 25 pmol template DNA, 20 units of T4 polynucleotide kinase (New England Biolabs, Ipswich MA), 50 µCi of [γ -³²P]ATP, buffer from manufacturer in 50 µl reaction. The kinase reaction was carried out at 37 °C for 30 minutes, and the enzyme was inactivated by additional 30 minutes incubation at 65 °C. Each binding reaction contained 20 mM Tris-HCl (pH 8 at room

temperature), 100 mM KCl, 5% glycerol, 10 mM MgCl₂, 2 mM DTT, 25 μ g/ml of BSA, and varied amount of TBP, TFB1 or TFB2 and DNA. Following the incubation at 70 °C for 30 minutes, 4 μ l of 6x bromophenol blue dye (30% glycerol, 60 mM EDTA and 0.06% bromophenol blue) was added to 20 μ l reaction mix for gel loading. The mixtures were, then, loaded on non-denaturing 8% acrylamide (1/30th the concentration of acrylamide is N,Nmethylenebisacrylamide) gel with 20% glycerol. Electrophoresis was conducted for 1 hour at 200V. The free DNA and protein-DNA complex were visualized by storage phosphor autoradiography [45] and the typhoon imaging system (GE Healthcare).

2.4.4 Quartz crystal microbalance (QCM)

The QCM (Model AFFINIX Q4, initium Inc., Tokyo, Japan) used in this study has four sensor cells to analyze up to four conditions simultaneously. Thus, one cell was used to obtain background reading from non-specific protein binding to sensor (has no DNA) and the other three were used for data collection for TFB interaction with DNA-TBP complex. The DNA template used in this assay was a 54-bp long oligonucleotide, which contains the strong archaeal promoter used in abortive transcription assays (Figure 2.9A). Template DNA was biotin conjugated at the 5' end to immobilize DNA on avidin coated sensor surfaces. Each QCM sensor was washed and treated to adhere molecule bait (DNA-TBP complex). Approximately 10 μ l of NeutrAvidin (Pierce Biotechnology Inc., Rockford, IL) (1 mg/ml in 20 mM Hepes, pH 7.5 at room temperature and 200 mM NaCl) was placed on each sensor for 15 minutes and washed. Then, the sensors were immersed in buffer (10 mM Tris-HCl, pH 7.8 at room temperature, 200 mM NaCl and 1 mM EDTA) and biotinylated DNA (25 μ M) was added. Biotin was added to prevent further binding of DNA once frequency reading reached defined level, so that all sensors will have approximately same amount of bound DNA. After washing out unbound DNA, TBP was added until saturation. TFB (prey) was titrated to each sensor cell to observe changes in frequency.

For the protein binding reaction, buffer containing 10 mM Hepes (pH 8 at room temperature) and 50 mM NaCl was used. The data was recorded in the form of frequency change over time (see Appendix E for an example of QCM data). Data was analyzed following the manufacturers guidance and Kaleidagraph software (Synergy Software, Reading, PA).

2.4.5 Growth media for *T. kodakarensis*

All strains of *T. kodakarensis* were grown under anaerobic conditions in ASW (Artificial Sea Water)-YT-S^o medium supplemented with sodium pyruvate (ASW-YT-S^o-pyruvate) [67]. The ASW-YT-S^o-pyruvate medium (1 L) consisted of 0.8x artificial seawater (see Appendix A for media compositions), 10 g of sodium pyruvate (Alpa Aezar, Ward Hill, MA), 5 g each of yeast extract and tryptone (Nacalai Tesque, Inc., Kyoto, Japan), and 2 g of elemental sulfur. All media were autoclaved prior to use and transferred to an anaerobic environment (CO₂:H₂:N₂ (5:5:90)) for inoculation in Gloveless anaerobic chamber (Coy laboratory product Inc., Grass Lake, MI). As an indicator of oxygen presence, 0.8 mg of resazurin salt per liter was added to media. The media color was red when oxygen is present, so that 5% Na₂S · 9H₂O was added to the medium until it restored the original color prior to the inoculation. Cultures were incubated in mechanical convection oven (DKN600, Yamato Scientific America, Inc., Santa Clara, CA) at the temperatures indicated in the text. *T. kodakarensis* strains used for the experiments were provided by Dr. Imanaka at Kyoto University and Dr. Santangelo at Ohio State University (Table 2.2) [66][67].

2.4.5.1 Cell growth experiment

Cell growth experiments were performed using nutrient-rich ASW-YT-S[°]-pyruvate medium to eliminate possible growth defects due to nutrient limitations. *T. kodakarensis* cells were pre-cultured in ASW-YT-S[°]-pyruvate medium overnight (85 °C). Then, the cultures were transferred into fresh ASW-YT-S[°]-pyruvate medium in the ratio of 1 to 100. The pre-culture was further incubated until late-log phase (~110 KLETT units for 85 °C pre-culture, ~70 KLETT units for 90 °C pre-culture) at the temperature used for cell growth observation. At the beginning of growth curve measurements, pre-culture was inoculated in pre-warmed fresh ASW-YT-S[°]-pyruvate medium in the ratio of about 1 to 100, and incubated at the various temperatures to monitor the cell growth. Amounts of inocula were adjusted based on turbidity readings to ensure all tubes start with approximately the same number of cells. Three experimental replicates were prepared for each measurement and each condition was tested at least two independent times. Because cell cultures required to be incubated in anaerobic conditions, KLETT-SUMMERSON Photoelectric Colorimeter (800-3, KLETT Manufacturing Co., NY, with a green filter) was used for turbidity measurement. KLETT is the arbitrary unit and 100 KLETT units was equivalent to OD₆₀₀ of 0.65 (Ultraspec 3100Pro, Amersham Biosciences). The use of KLETT photoelectrometer allowed turbidity measurement without opening culture container during growth, however, it also introduced a limitation for the observation. For the assay, each culture was grown in a glass tube which fits to the KLETT apparatus. Because the volume of each culture (4 ml in 9-ml glass tube) was small, transferring tubes from oven (70 °C to 90 °C) to the apparatus (room temperature) changes cell growing conditions. In order to minimize changes in culture temperature, frequency of measurement was limited to every two hours especially at the initial stages of growth.

2.4.5.2 Preparation of heat shock and cold shock cells

T. kodakarensis culture was prepared as described in the previous section, and 4 ml each culture was incubated in 9-ml glass tube at 85 °C in mechanical convectional oven until it reached mid-log phase (~50 KLETT units). Cell cultures were then transferred to another convectional oven to upshift (100 °C) or downshift (60 °C) growth temperature. Cultures were collected at 0, 30, 60, 90, 120, and 240 minutes time points after the temperature shift. Cultures were immediately aliquoted, frozen in liquid nitrogen and stored at -80 °C until RNAs were purified.

2.4.6 Quantitative real-time PCR (qRT-PCR)

Primers and probe sequences used in qRT-PCR were selected from the regions unique to each gene (Table 2.3 for sequences). The DNA sequence of *tfb1* and *tfb2* are 64% identical. Therefore, probes and primer sets for *tfb1*, *tfb2* were tested for cross-reactivity. Plasmids containing either *tfb1* or *tfb2* gene were prepared and used as templates for qRT-PCR assays. The primer sets for *tfb1* did not amplify from *tfb2* templates, and *vice versa*. Likewise, probes and primers of *cpkA* and *cpkB* (82% sequence identity) were tested for cross-reactivity using same protocol, and their cross-reactivity was also negligible.

Total cellular RNA from heat shock or cold shock cells were prepared by Qiagen RNeasy Mini kits (Qiagen, Inc., Valencia, CA) following manufacturers protocols. Reference RNA for absolute quantification were prepared and purified using MEGA script kit (Ambion, Foster City, CA). The quantities of RNA were determined either by nanodrop spectrophotometer (ND-1000, Nanodrop Technologies, Wilmington, DE) or UV-VIS spectrophotometer (Ultraspec 3100Pro, Amersham Biosciences) and quality was checked by agarose gel electrophoresis. For both spectrophotometers, $OD_{260} = 1$ corresponds to 40 $\mu\text{g/ml}$ single stranded RNA.

The qRT-PCR assay described below was carried out by the Penn State Nucleic Acid Facility. The total RNA was further treated with DNase I as follows. In 20 μ l reaction, total RNA (200 ng), 10x reaction buffer (Ambion, 2 μ l), DNase I (Ambion, 4U), yeast tRNA (~660 ng, Invitrogen Co., Carlsbad, CA) were mixed and incubated at 37 °C for 30 minutes followed by additional incubation at 65 °C for 5 minutes. After the DNase I treatment, cDNA was generated using High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) following protocols provided by the manufacturer. Taqman probes (Biosearch Technologies, Inc., Novato, CA) were used to monitor amplification of target genes. PCR reaction and fluorescence monitoring were carried out with 7300 Real-Time PCR system (Applied Biosystems) following standard protocol provided by the manufacturer. For each primer set, standard curve was prepared to ensure the threshold was set within exponential phase of amplification. Relative abundances of target mRNA were calculated by $\Delta\Delta$ Ct method [50]. Variation among samples were adjusted using 16S rRNA as an internal standard. Previous studies on internal control selection suggested small subunit rRNA could be an suitable internal control because of its stability under various environmental stress [71][77]. Preliminary experiment with wildtype *T. kodakarensis* KOD1 strain verify this is applicable for present heat shock experiment, thus 16S rRNA was used as an internal control throughout this study. For each measurement, three experimental replicates were prepared and averages of the three readings were used for data analyses.

2.4.7 DNA microarray

The DNA microarray analysis was conducted by Imanaka laboratory at Kyoto University. The microarray plate used in this study was manufactured at Takara Bio (Otsu, Japan), that covers 2,226 genes among the total predicted 2,306 genes of *T. kodakarensis* KOD1 (96.5% coverage). DNA fragments of about 300 bp, corresponding to the 3'-terminal regions of each coding region, were spotted on the glass plate. Two identical sets (left and right) were loaded on each

plate, allowing for two sets of data to be obtained from a single experiment. *T. kodakarensis* cells were cultivated at 85 °C in ASW-YT-S^o-pyruvate medium and incubation temperature was up-shifted to 100 °C at mid-log phase ($OD_{600} = 0.6 - 0.8$). Cells were harvested at 0 and 30 minutes after the temperature shift and total RNA was extracted using the RNeasy Midi kit (Qiagen Inc.). Fluorescently labeled cDNA used for hybridization was prepared using the RNA Fluorescence Labeling Core Kit ver. 2.0 (Takara Bio). Total RNA (10 μ g) was annealed with random hexamers, and reverse transcription was performed in solutions containing Cy dye-labeled dUTP (Cy3-dUTP or Cy5-dUTP) (GE Healthcare). RNA was subsequently degraded with RNase H and the labeled cDNA purified using a column supplied in the kit according to the manufacturers instructions. The labeled cDNA was dissolved in hybridization buffer (30 μ l) containing 6x SSC (1x SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.2% SDS, 5x Denhardt's solution (Sigma-Aldrich) and 0.1 mg/ml denatured salmon sperm DNA. Hybridization was performed under a coverslip (Spaced Cover Glass XL, Takara Bio) in a humidity chamber at 65 °C for 12 to 15 hours. After hybridization, the microarray plates were washed four times with 2x SSC and 0.2% SDS at 55 °C for 5 min, rinsed in 0.05x SSC and dried by centrifugation. The intensities of the Cy3 and Cy5 dyes were measured using an Affymetrix 428 Array Scanner (Affymetrix, Inc., Santa Clara, CA). The microarray images were analyzed using ImaGene ver. 5.5 (BioDiscovery, Inc., El Segundo, CA).

2.5 Acknowledgements

I thank Dr. Tadayuki Imanaka and his group (Kyoto University) for *T. kodakarensis* strains used in this study and also for guidance in manipulating the organism. Dr. Tamotsu Kanai (Kyoto University) performed DNA microarray analyses and discussed result with our group. I also thank J. E. Brenchley for providing us a KLETT-SUMMERSON photoelectric colorimeter. I would like to thank initium Inc. for the use and assistance of the Affinix Q4

system for dissociation constant studies. Probes and primers for qRT-PCR were designed by Dr. Deborah Grove (Penn State Nucleic Acid Facility). Finally, I thank Dr. Thomas Santangelo (Ohio State University) for providing us with TFB deletion strains and *T. kodakarensis* RNAP.

Table 2.1: Summary of archaeal species encoding multiple GTFs in their genome [28].

Phylum	Organism name	Number of TBP homologues	Number of TFB homologues
Crenarchaeota	<i>Aeropyrum pernix</i> K1	1	3
	<i>Sulfolobus acidocaldarius</i>	1	3
	<i>Sulfolobus tokodaii</i> strain7	1	3
Euryarchaeota	<i>Haloarcula marismortui</i>	1	8
	<i>Halobacterium</i> sp. NRC-1	6	7
	<i>Haloquadratum walsbyi</i> DSM 16790	2	9
	<i>Methanopyrus kandleri</i> AV19	1	2
	<i>Methanosarcina acetivorans</i> C2A	3	1
	<i>Methanosarcina barkeri</i> fusaro	2	1
	<i>Methanosarcina mazei</i> Goe1	1	3
	<i>Methanospirillum hungatei</i> JF-1	2	1
	<i>Natronomonas pharaonis</i> sp.	1	7
	<i>Picrophilus torridus</i> DSM9790	1	2
	<i>Pyrococcus furiosus</i> DSM 3638	1	2
	<i>Pyrococcus horikoshii</i> shinkaj OT3	1	2
	<i>Thermococcus kodakarensis</i> KOD1	1	2
	<i>Thermoplasma acidophilum</i> DSM 1728	1	3
	<i>Thermoplasma volcanium</i> GSS1	1	3

Table 2.2: *T. kodakarensis* strains used for this study.

Strain	Relevant characteristics	References
KOD1	Wildtype	[2][33]
KW128	$\Delta pyrF; \Delta trpE::pyrF$	[68]
KW128 $\Delta tfb1$	$\Delta pyrF; \Delta trpE::\Delta tfb1::trpE$	[66]
KW128 $\Delta tfb2$	$\Delta pyrF; \Delta trpE::\Delta tfb2::trpE$	[66]

Table 2.3: DNA oligonucleotide sequences used for qRT-PCR.

Target gene		Sequence (5' to 3')
<i>tfb1</i>	Forward primer	AATAGACAGGTCGCTAACTGGCTT
	Taqman probe	ATGCGCGAGAAGATGTACAGGCTGAGG
	Reverse primer	GCTGACCCTGAGACGGGAC
<i>tfb2</i>	Forward primer	GACTGGCGCGACAAGGATA
	Taqman probe	CACGGGAACCAGATAACCGGCATG
	Reverse primer	GGAGCCTTCTAAGCTTGTTCC
<i>tbp</i>	Forward primer	CGGTGACATCGGTATGGAGTT
	Taqman probe	CCTCGATGCCGTTGCTCTGATCCT
	Reverse primer	TCGGGCTCGTATTCACAGTTC
<i>cpkA</i>	Forward primer	ATTCACCCGAGCATAATCATCA
	Taqman probe	TTACGCCCTCGCCGCCGA
	Reverse primer	CGTCAAGTATTTCTTGGGCCTT
<i>cpkB</i>	Forward primer	GGTCGGCGCGAATGTC
	Taqman probe	TGACGACCTCGCCCAGCACTACCTT
	Reverse primer	GGCCATTATGCCGTACTTGG
16S rRNA	Forward primer	CCGAGAGGAGGTGCATGG
	Taqman probe	CGTCAGCTCGTACCGTGAGGCGT
	Reverse primer	TCTCGCTCGTTACCACACTTAAGT

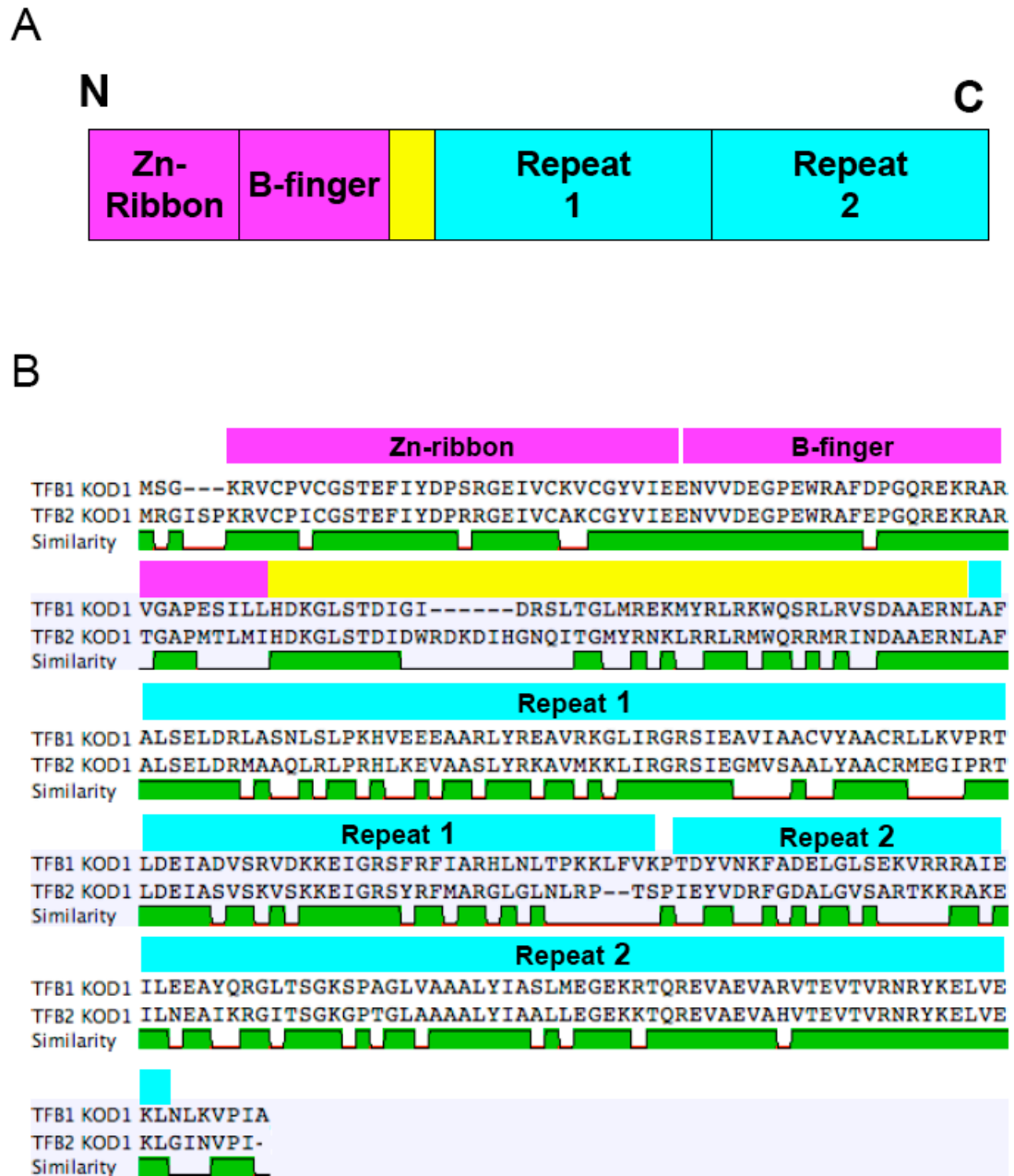


Fig. 2.1: Structure and an amino acid sequence alignment of TFB homologues from *T. kodakarensis*.

(A) Structure of *T. kodakarensis* TFB. N-terminal motifs (Zn ribbon and B-finger) and C-terminal motifs (two set of helical repeats) are shown in purple and light blue, respectively. Yellow indicates linker between the N and C-terminal domains. (B) An amino acid sequence alignment of the two TFB homologues from *T. kodakarensis*. Residues composing N- and C-terminal motifs are indicated in the figure using same color schematics as in (A). The sequence alignment was prepared by geneious software (Biomatters Ltd., Auckland, New Zealand, <http://www.geneious.com/>).

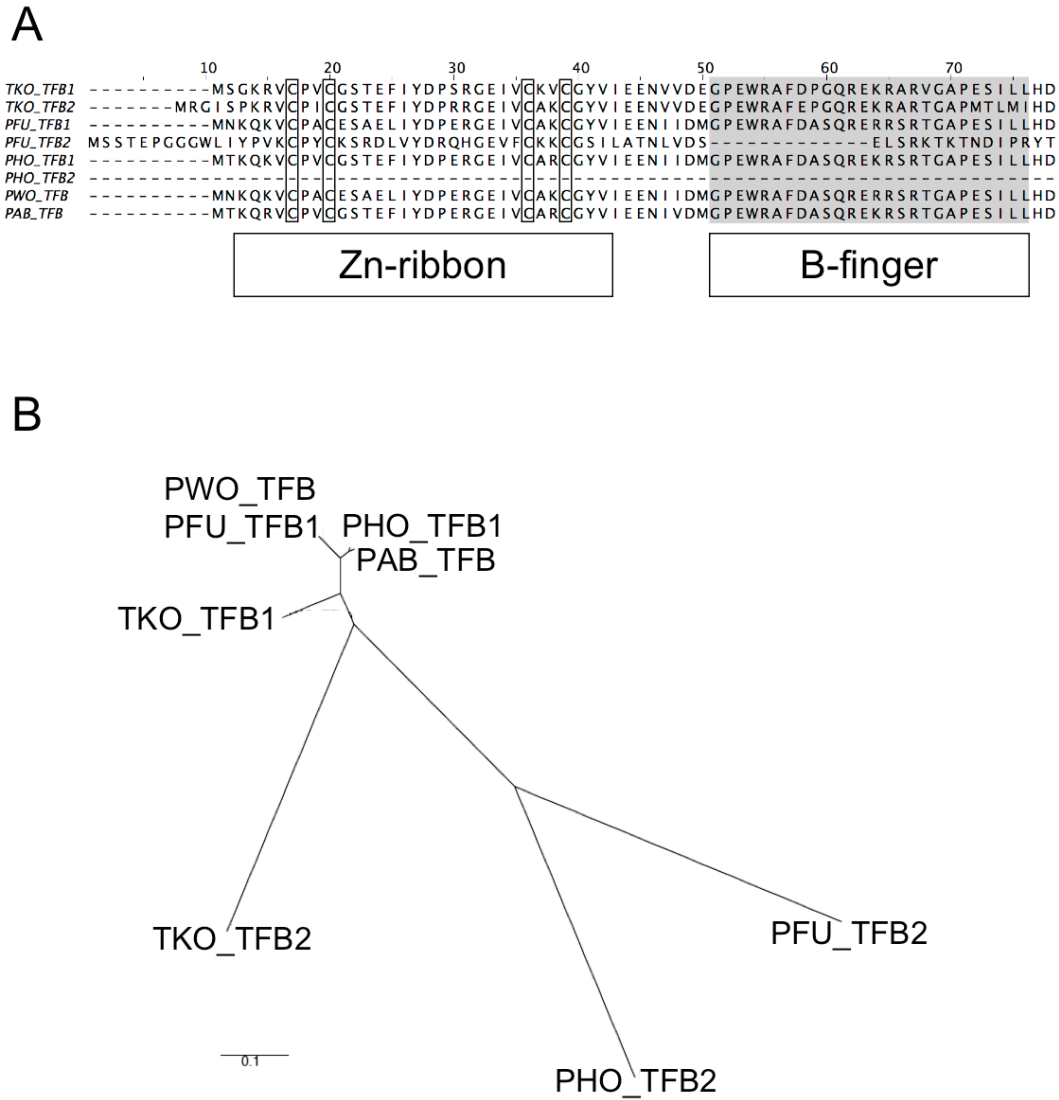


Fig. 2.2: Sequence analyses of TFBs from *T. kodakarensis* and *Pyrococcus*.

(A) Multiple sequence alignment for Zn-ribbon and B-finger motifs of TFB homologues from *T. kodakarensis* and four *Pyrococcus* species. (See Figure 2.1B for corresponding sequences within *T. kodakarensis*). Amino acid sequences were aligned by ClustalW [24]. Zn binding sites found in Zn-finger motif (4 cysteines) are boxed and B-finger motifs are shaded. (B) Illustration of evolutionary relationships of TFB homologues of *T. kodakarensis* and *Pyrococcus* species. The unrooted tree was generated using Phylip [29] version 3.5c and Pylo dendron version 0.8d (<http://iubio.bio.indiana.edu/treeapp/treeprint-form.html>) programs to illustrate evolutionary relationship of TFB homologues. Scale bar represents 0.1 substitutions per site. TKO: *T. kodakarensis*, TFB1 (TK1280), TFB2 (TK2287); PFU: *P. furiosus*, TFB1 (PF1377), TFB2 (PF0687); PHO: *P. horikoshii*, TFB1 (PH1482), TFB2 (PH0864); PWO: *P. woesei*, TFB (GenBank Accession No. X70668.1); PAB: *P. abyssi*, TFB (PAB1912).

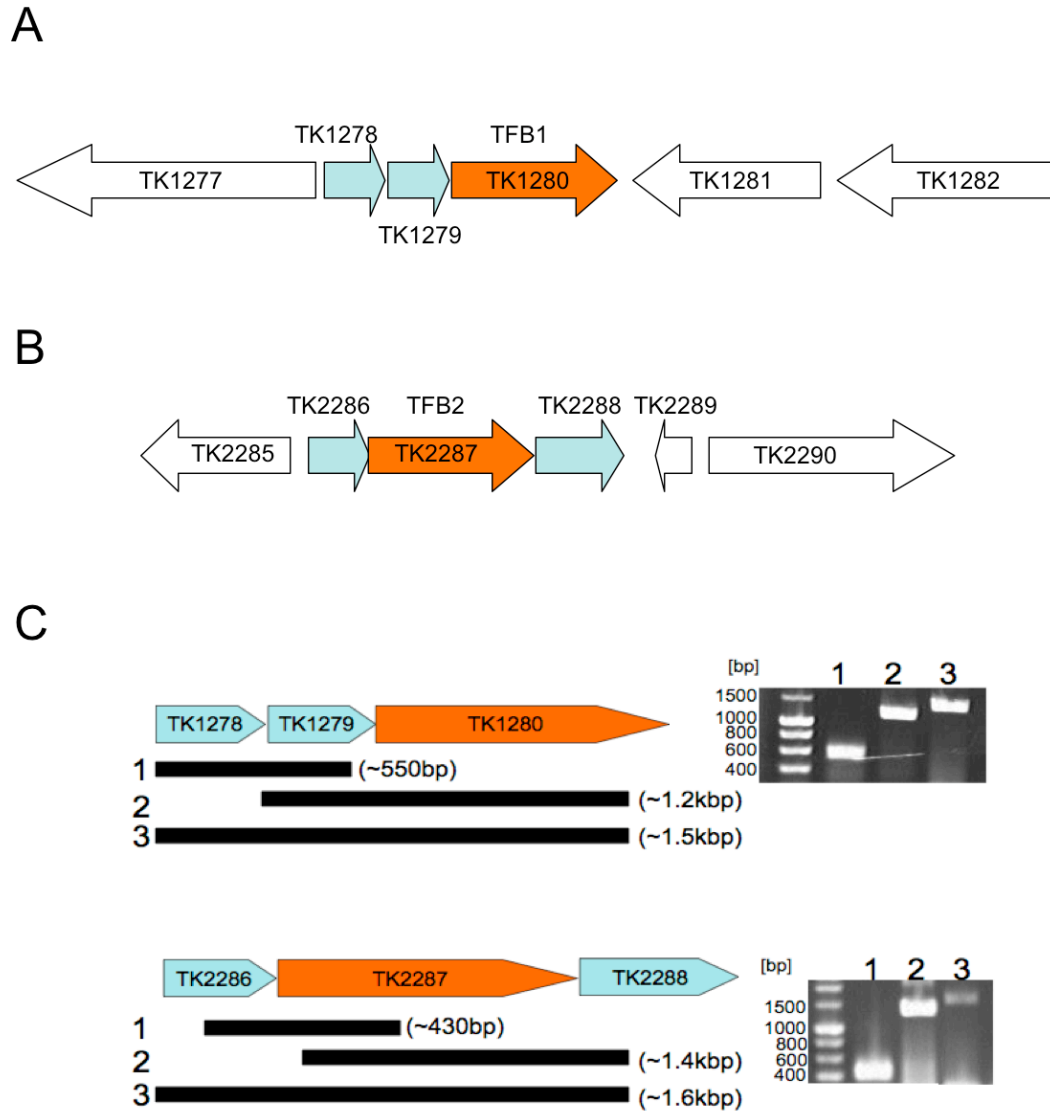


Fig. 2.3: Gene arrangements of operons encoding *T. kodakarensis* *tfb1* and *tfb2*. Schematic representation of genes forming (A) *tfb1* and (B) *tfb2* operons (shown in light blue and orange). Genes found in their vicinities are also shown as white arrows. Details of each gene within operons are described in text. (C) RT-PCR verification of operon configurations. Location and sizes of each amplicons are shown by black bars.

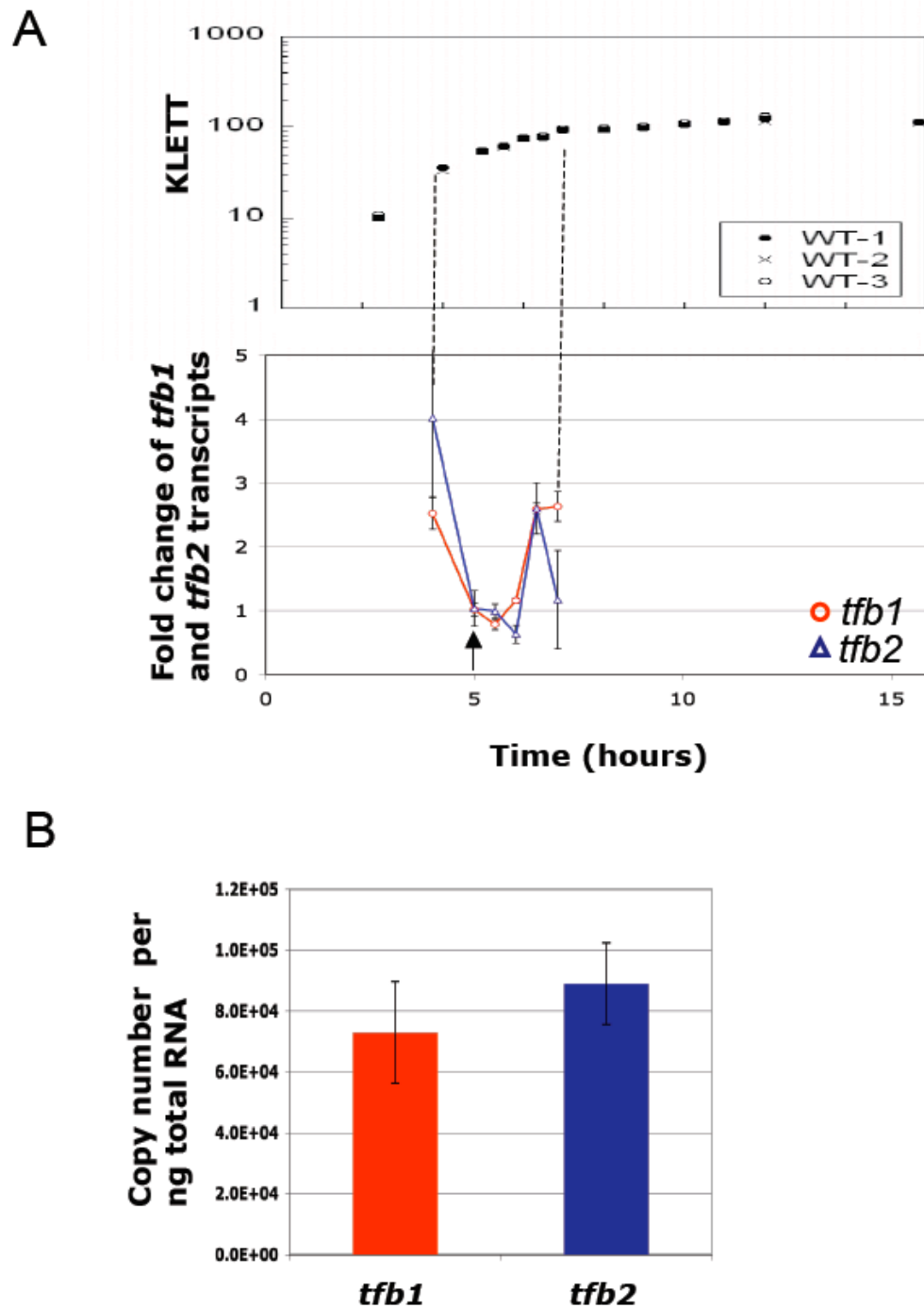


Fig. 2.4: Quantification of *tfb1* and *tfb2* transcripts by qRT-PCR in wildtype *T. kodakarensis* KOD1.

(A) The top panel shows turbidity measured in KLETT units during growth of wildtype *T. kodakarensis* KOD1 in nutrient-rich media. The bottom panel shows fold changes of transcript abundances of *tfb1* and *tfb2* during growth between mid-log to stationary phases. The y-axis corresponds to the fold changes from the reference (indicated by an arrow). (B) Absolute quantification of transcripts of two TFB homologues from *T. kodakarensis* KOD1. Copy number was normalized per ng of total RNA extracted from cells grown in nutrient-rich media.

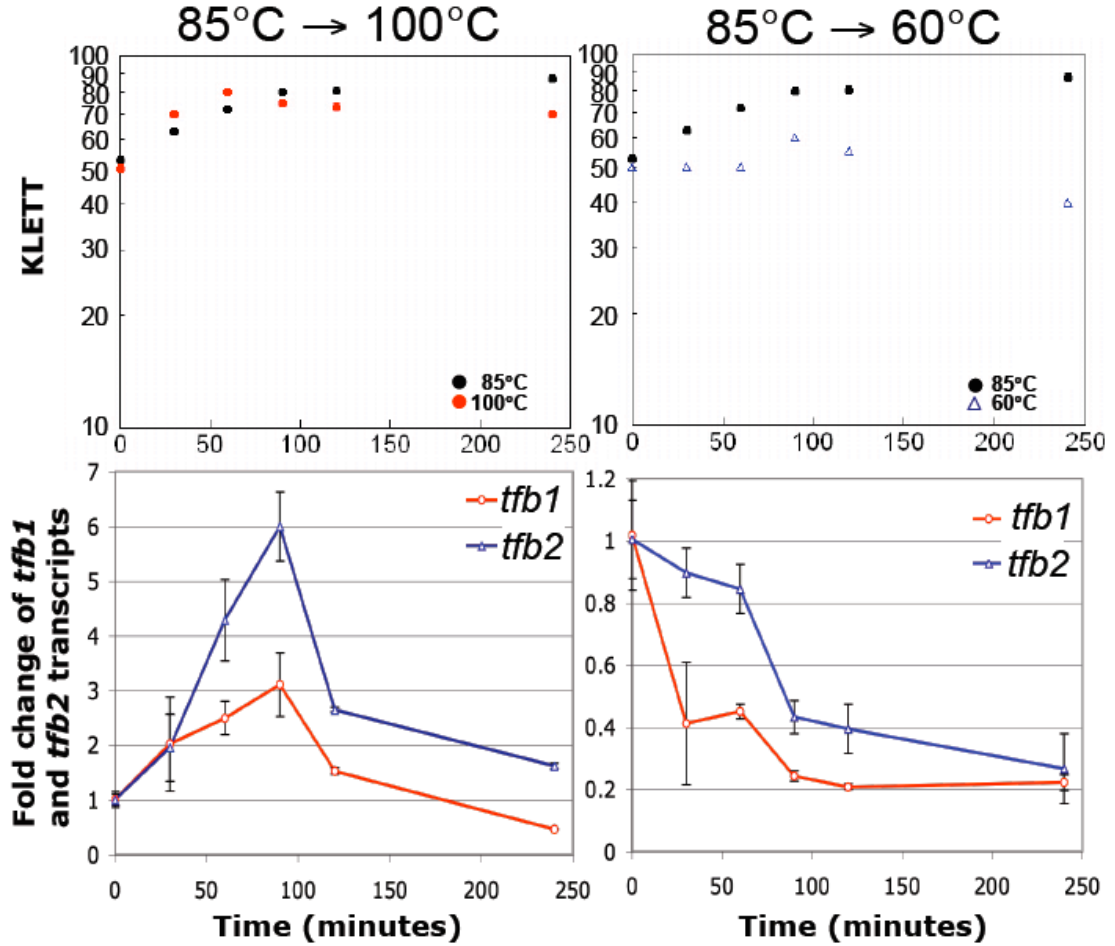
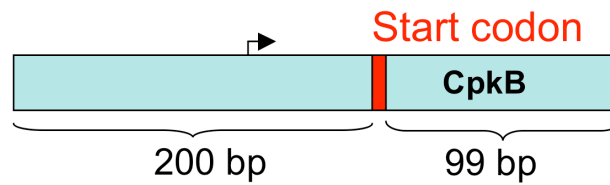


Fig. 2.5: Transcript abundance of *tfb1* and *tfb2* in wildtype *T. kodakarensis* KOD1 following temperature shift.

T. kodakarensis KOD1 cells were heat or cold shocked as described in materials and methods and harvested at various times during the shock for transcript quantification. Top panels show changes in turbidity during temperature shift. Black circles shown in both graphs are KLETT units reading of *T. kodakarensis* KOD1 grown at 85°C taken during separate experiments and shown as a reference. Bottom panels show fold changes of each transcripts, where mRNA abundance in the cells at time = 0 point was set to one. Cells were harvested at six time points (dots) and lines connecting dots are provided to follow changes in mRNA quantities over time. Three experimental replicates were prepared for each measurement and averages of the three readings were used for data analyses. Error bars correspond to one standard deviation of each point.

A



B

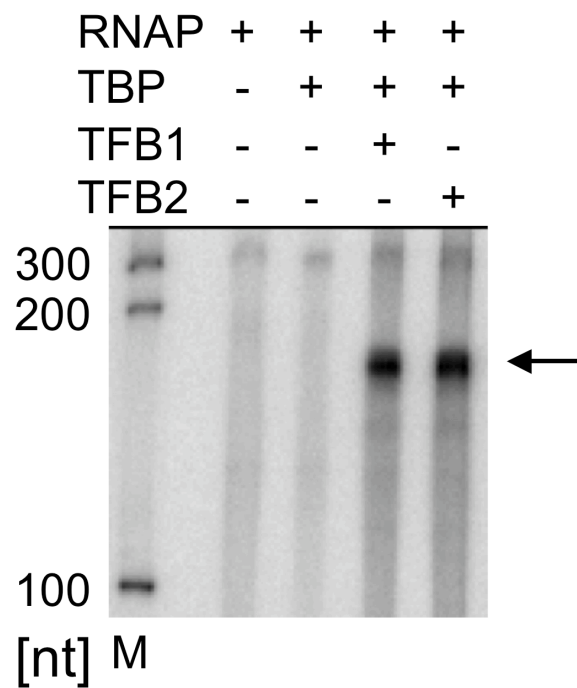


Fig. 2.6: Run-off transcription assay using *cpkB* promoter.

(A) Diagram illustrates a DNA template used in this assay. The templates contains 200 bp immediately upstream and 99 bp downstream of the *cpkB* start codon. (B) Run-off transcripts were synthesized as described in materials and methods and separated by gel electrophoresis. The position of run-off transcripts is indicated by a black arrow and approximately 300 nt bands shown in all lanes were likely the product from end-to-end transcription.

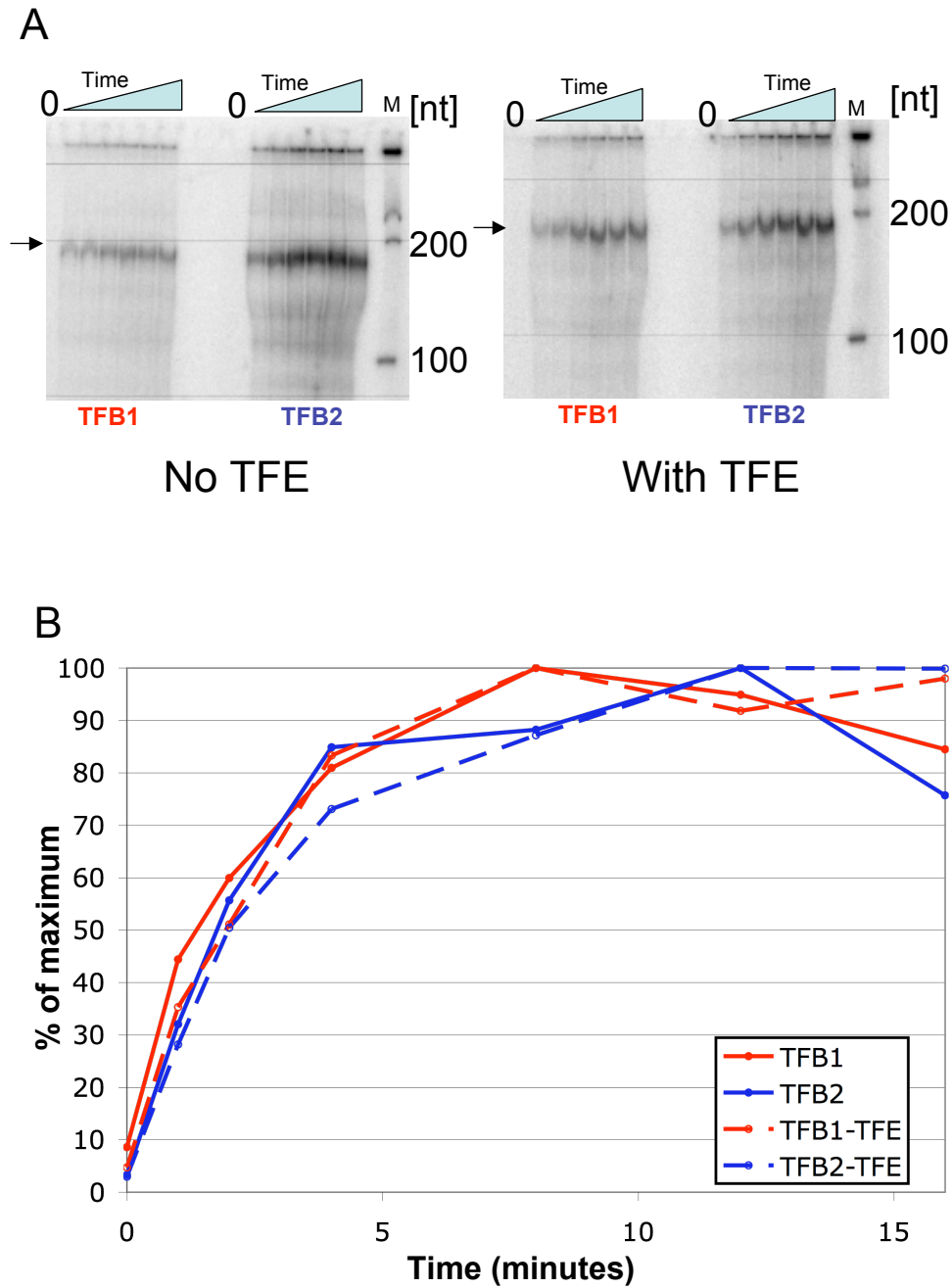


Fig. 2.7: Accumulation of transcripts from *cpkB* promoter.

(A) Time course experiments to monitor accumulation of transcripts. Transcription reaction was stopped at various time points (0, 1, 2, 4, 8, 12 and 16 minutes) as described in materials and methods and separated by gel electrophoresis. The position of run-off transcripts is indicated by black arrows. M indicates the location of a marker. Both images from the reactions with and without TFE are shown. (B) The graph shows the amount of transcripts synthesized determined from phosphorimaging, expressed as a percentage of the maximum synthesized.

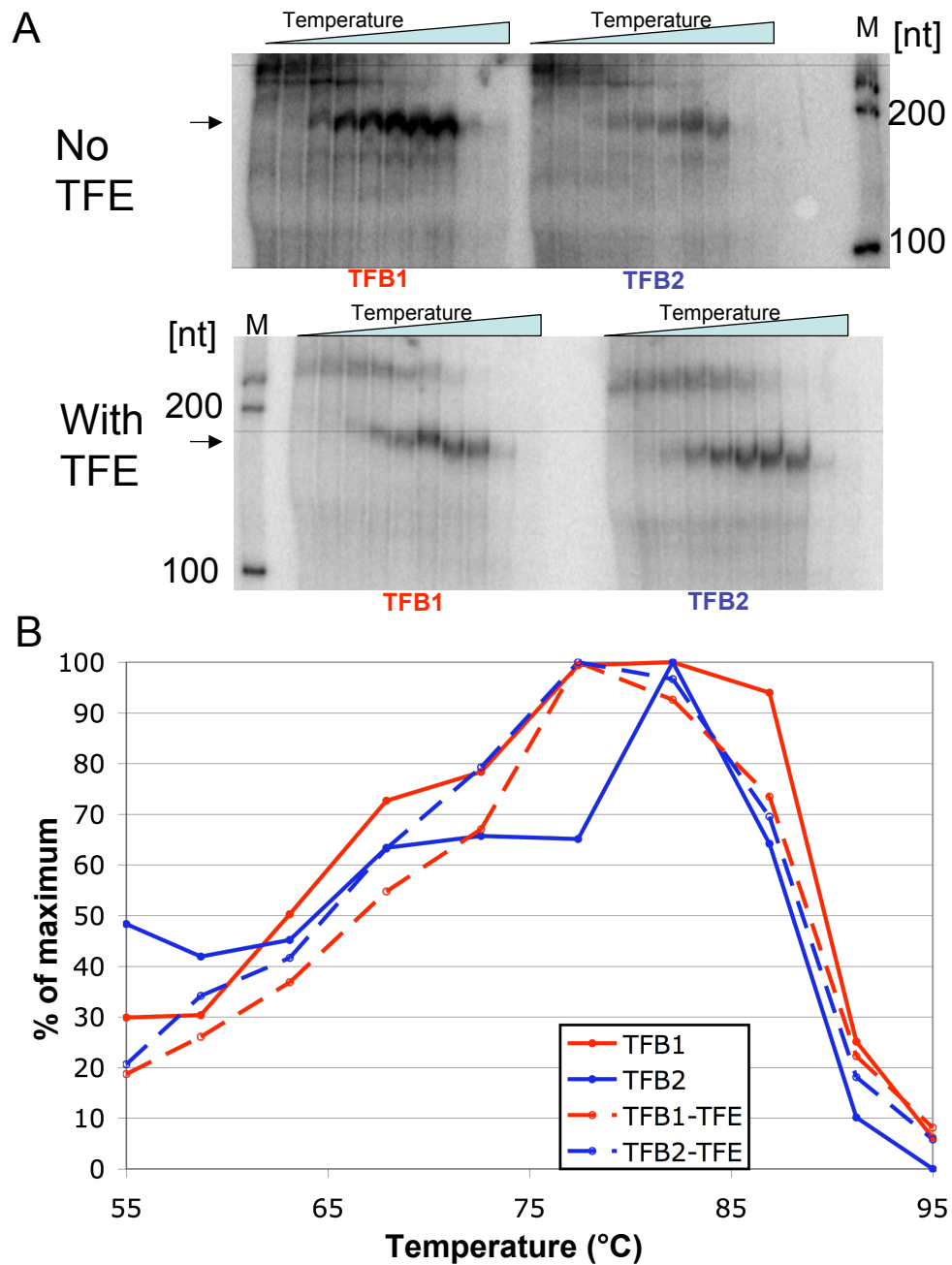


Fig. 2.8: Temperature effect of transcription from *cpkB* promoter.

(A) Transcription experiments were performed at various temperature (55, 58.7, 63.1, 67.9, 72.6, 77.4, 82.1, 86.9, 91.2 and 95 °C) as described in materials and methods and separated by gel electrophoresis. The position of run-off transcripts is indicated by black arrows. M indicates the location of a marker. Both images from the reaction with and without TFE are shown. (B) The graph shows the amount of transcripts synthesized determined from phosphorimaging, expressed as a percentage of the maximum synthesized.

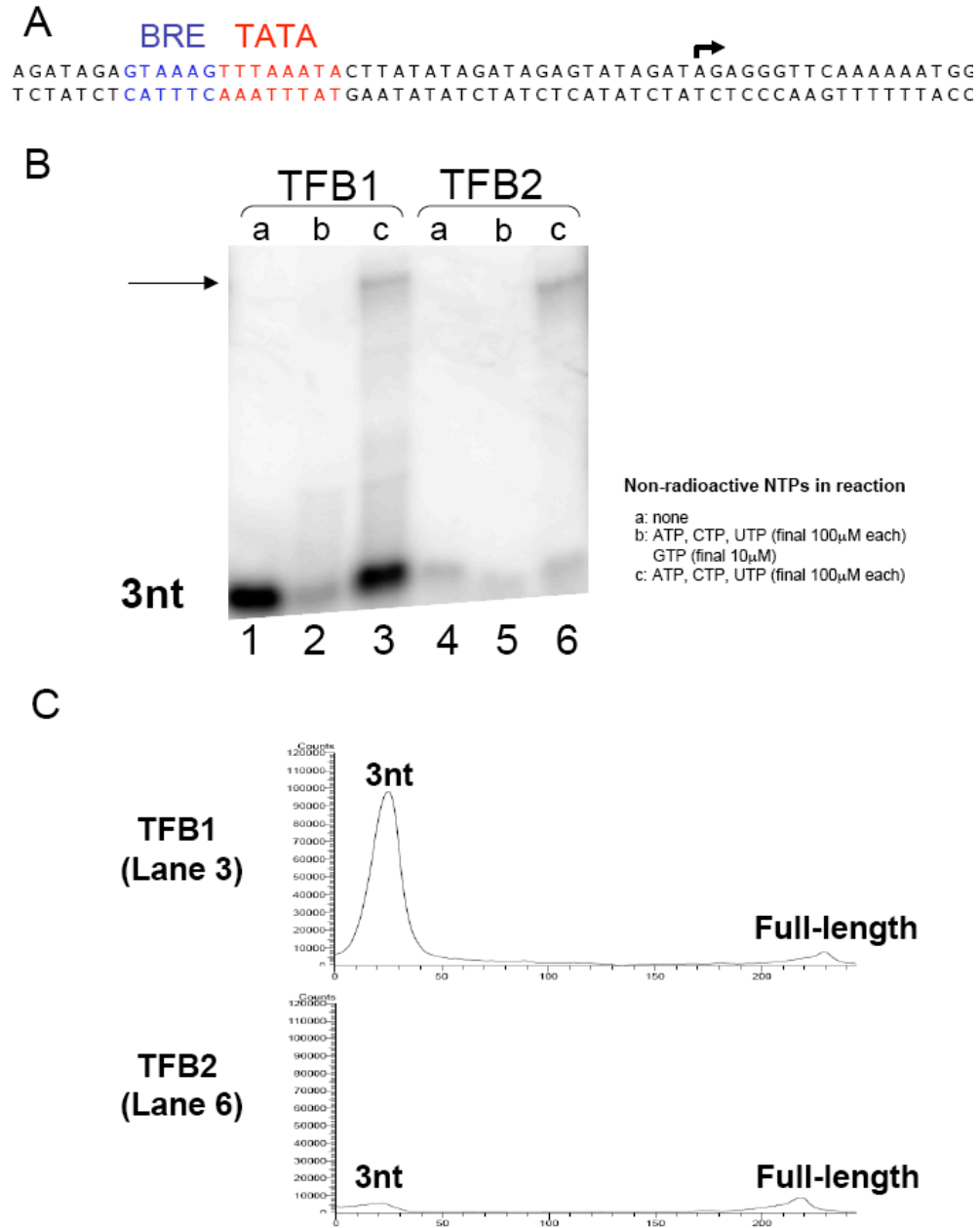
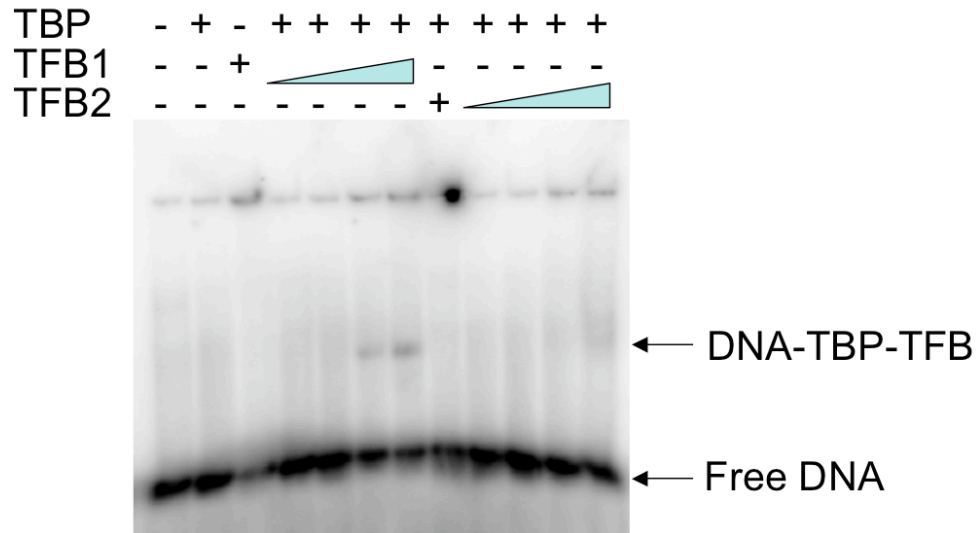


Fig. 2.9: Summary of *in vitro* transcription assays with strong archaeal promoter.

(A) A sequence of DNA template used in this assay. Strong archaeal promoter from *S. shibatae* virus was used [12]. Locations of DNA *cis*-acting elements are shown in blue (BRE) and red (TATA-box). The black arrow indicates transcription start site. (B) Comparison of the transcription activity of PIC containing TFB1 and TFB2. Transcripts were separated on a 24% acrylamide gel for TFB1-dependant transcription machinery (lane 1 through 3) and for TFB2-dependant machinery (lane 4 through 6). In addition to the dinucleotide (UpA) and radiolabeled GTP, nucleotide substrates indicated in the figure were included in each transcription reaction. Location of abortive product is marked as 3 nt, and putative full length product location is indicated by a black arrow. (C) and (D) Profiles of the two lanes of (B) (lane 3:(C) and lane 6:(D)), quantified by phosphorimaging. X-axis corresponds to the vertical distance from the bottom of lanes and y-axis shows the counts at each location.

A



B

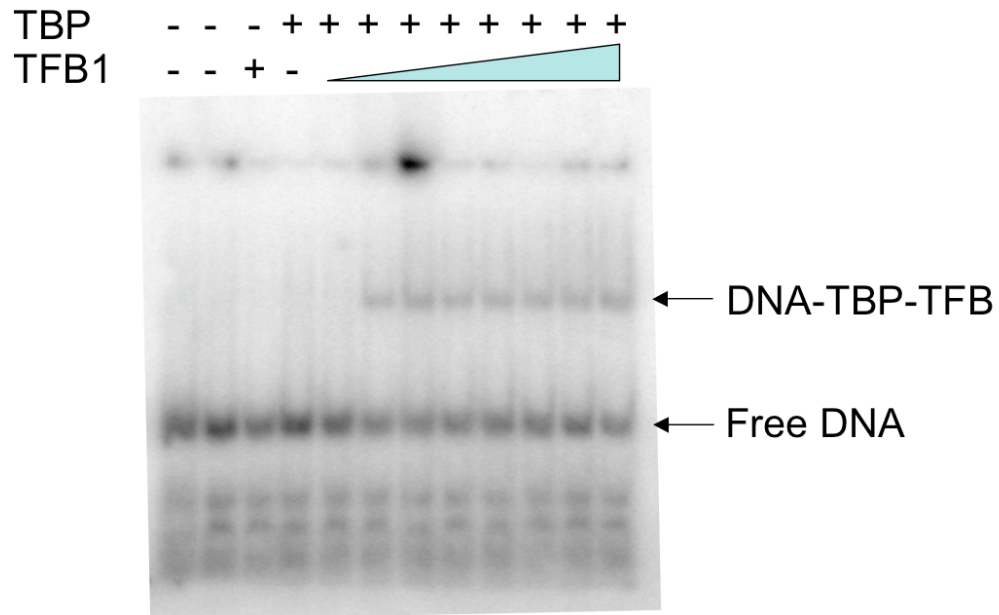
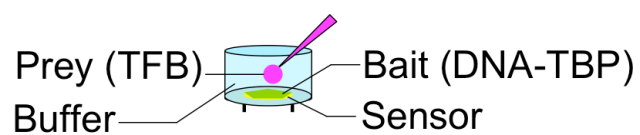


Fig. 2.10: EMSA analysis of DNA-TBP-TFB complex.

(A) TFB1 or TFB2 was titrated (1, 10, 125 and 250 nM) to determine the dissociation constant of TFB to DNA-TBP complexes. 250 nM TBP and 2.5 nM DNA were used. Location of the free DNA and DNA-TBP-TFB complex (putative) are indicated in the figure. (B) TFB1 concentration was titrated (1, 10, 20, 50, 100, 120, 150 and 200 nM) to observe changes in bound and unbound DNA concentration. 250 nM TBP and 2.5 nM DNA were used.

A



B

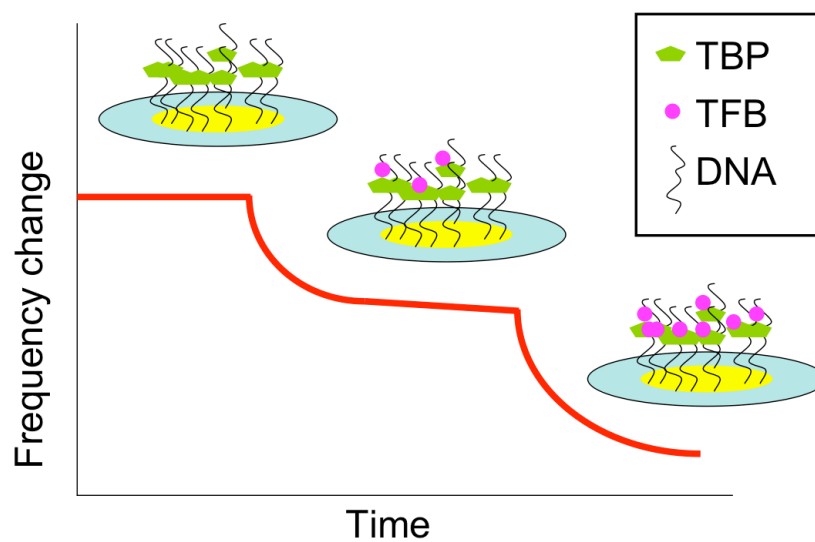


Fig. 2.11: Schematic representation of QCM assay.

(A) Illustration of sensor cell and an experimental set up to determine dissociation constant of TFB to DNA-TBP complex using QCM. (B) Illustration of an example of QCM response.

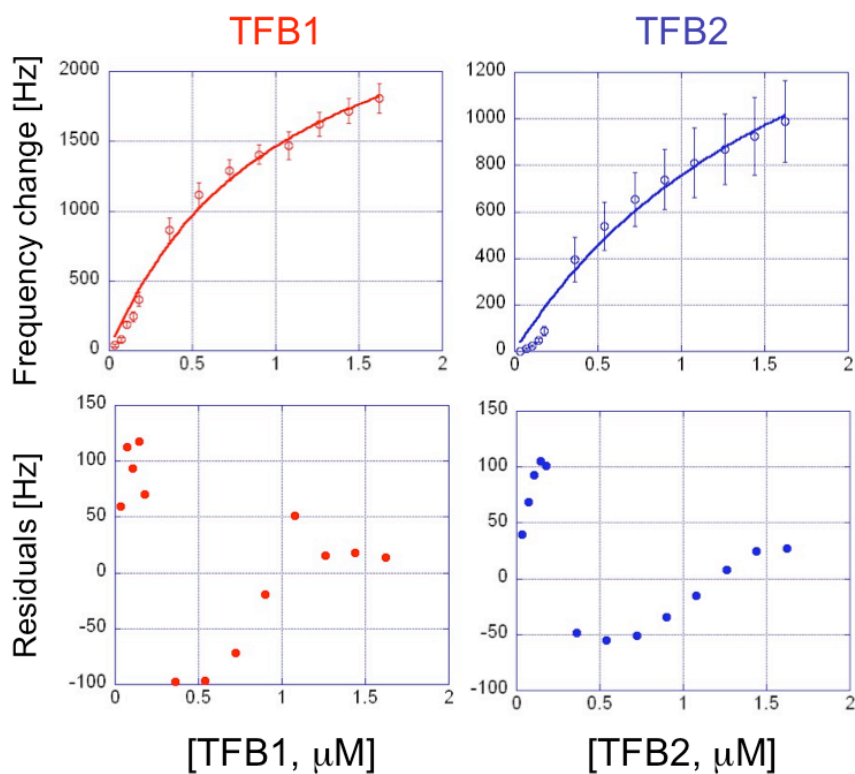


Fig. 2.12: QCM analyses of TFB dissociation constants.

Analyses of dissociation constant using QCM. Frequency profiles recorded by QCM was used to construct saturation curves. TFB concentration needed to reach half-maximum saturation was determined for TFB1 and TFB2. Top panels show change in frequency with TFB concentration increase and bottom panels shows residuals of observed value and fitted model.

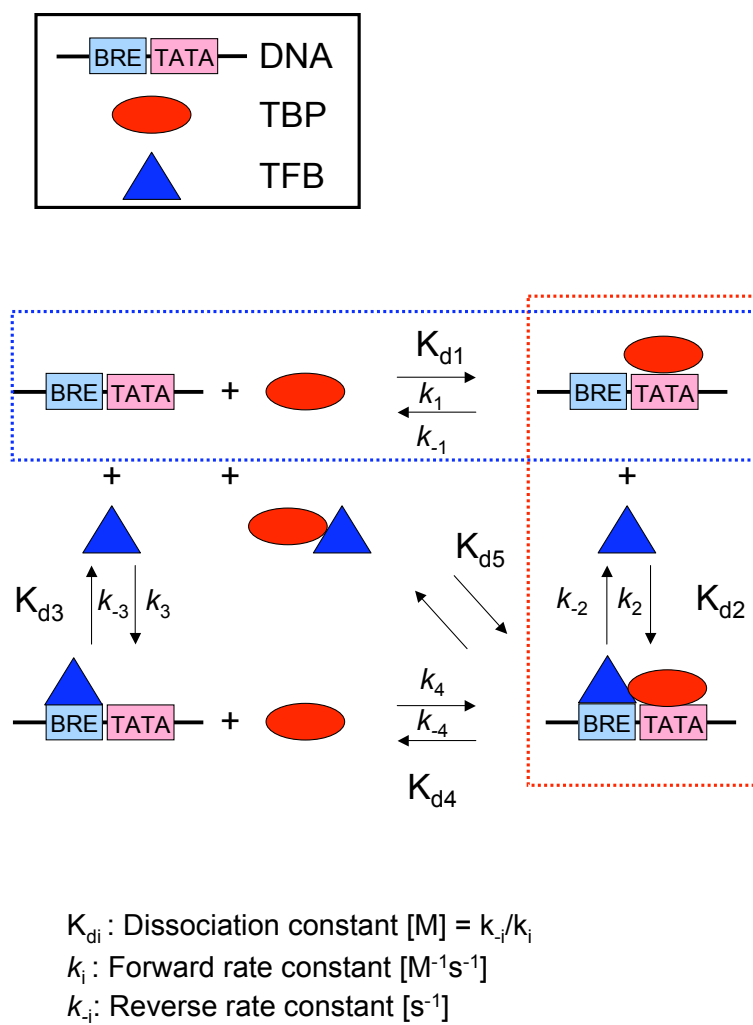


Fig. 2.13: Model of three molecule interactions.
Illustration of the interaction of three molecules (DNA, TBP and TFB) used in QCM analyses.

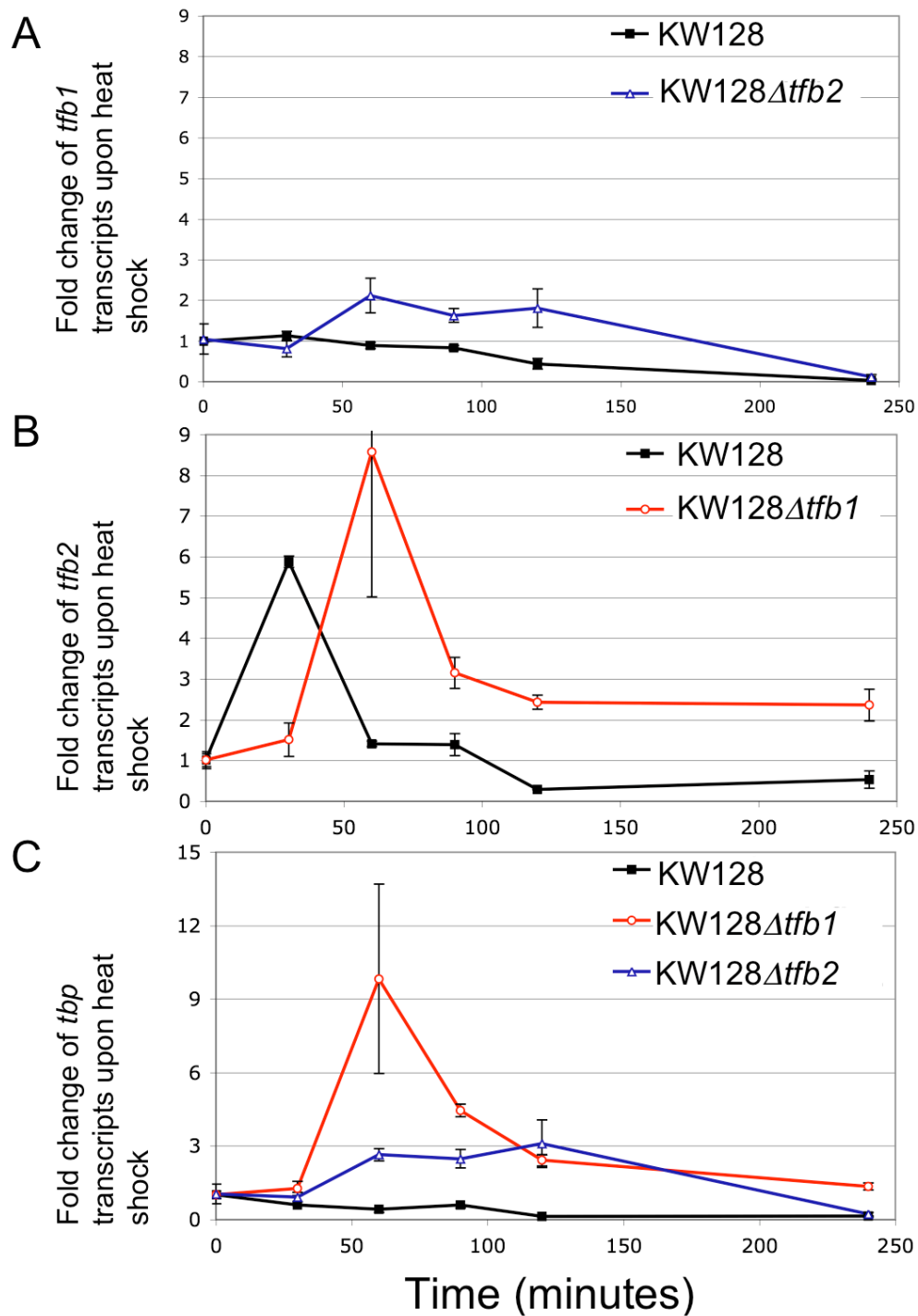


Fig. 2.14: Change in transcript relative abundances of GTFs in response to heat shock quantified by qRT-PCR.

Fold changes of transcripts for (A) *tfb1*, (B) *tfb2* and (C) *tbp* are shown. Three *T. kodakarensis* strains were heat shocked as described in materials and methods and harvested at various times during heat shock for transcript quantification. Quantifications were conducted at six time points (dots) and lines connecting dots are provided to follow changes in mRNA quantities over time. The y-axis corresponds to the fold changes of each transcripts from mRNA quantities in cells prior to the temperature shift. Three experimental replicates were prepared for each measurement and averages of the three readings were used for data analyses. Error bars correspond to one standard deviation of each point.

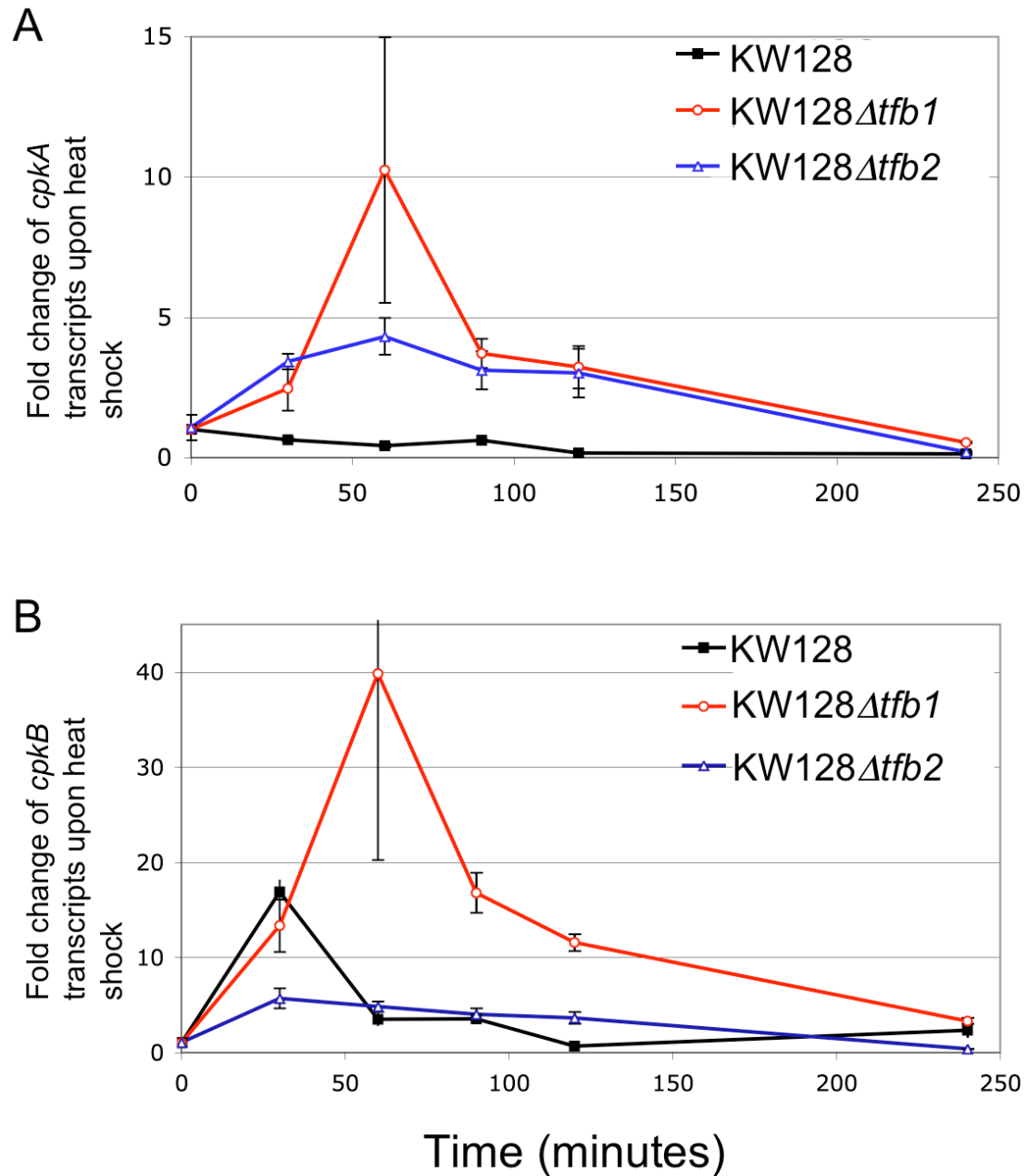


Fig. 2.15: Change in transcript relative abundances of thermosome subunits in response to heat shock quantified by qRT-PCR.

Fold changes of transcripts for (A) *cpkA* and (B) *cpkB* are shown. Three *T. kodakarensis* strains were heat shocked as described in materials and methods and harvested at various times during heat shock for transcript quantification. Quantifications were conducted at six time points (dots) and lines connecting dots are provided to follow changes in mRNA quantities over time. The y-axis corresponds to the fold changes of each transcripts from mRNA quantities in cells prior to the temperature shift. Three experimental replicates were prepared for each measurement and averages of the three readings were used for data analyses. Error bars correspond to one standard deviation of each point.

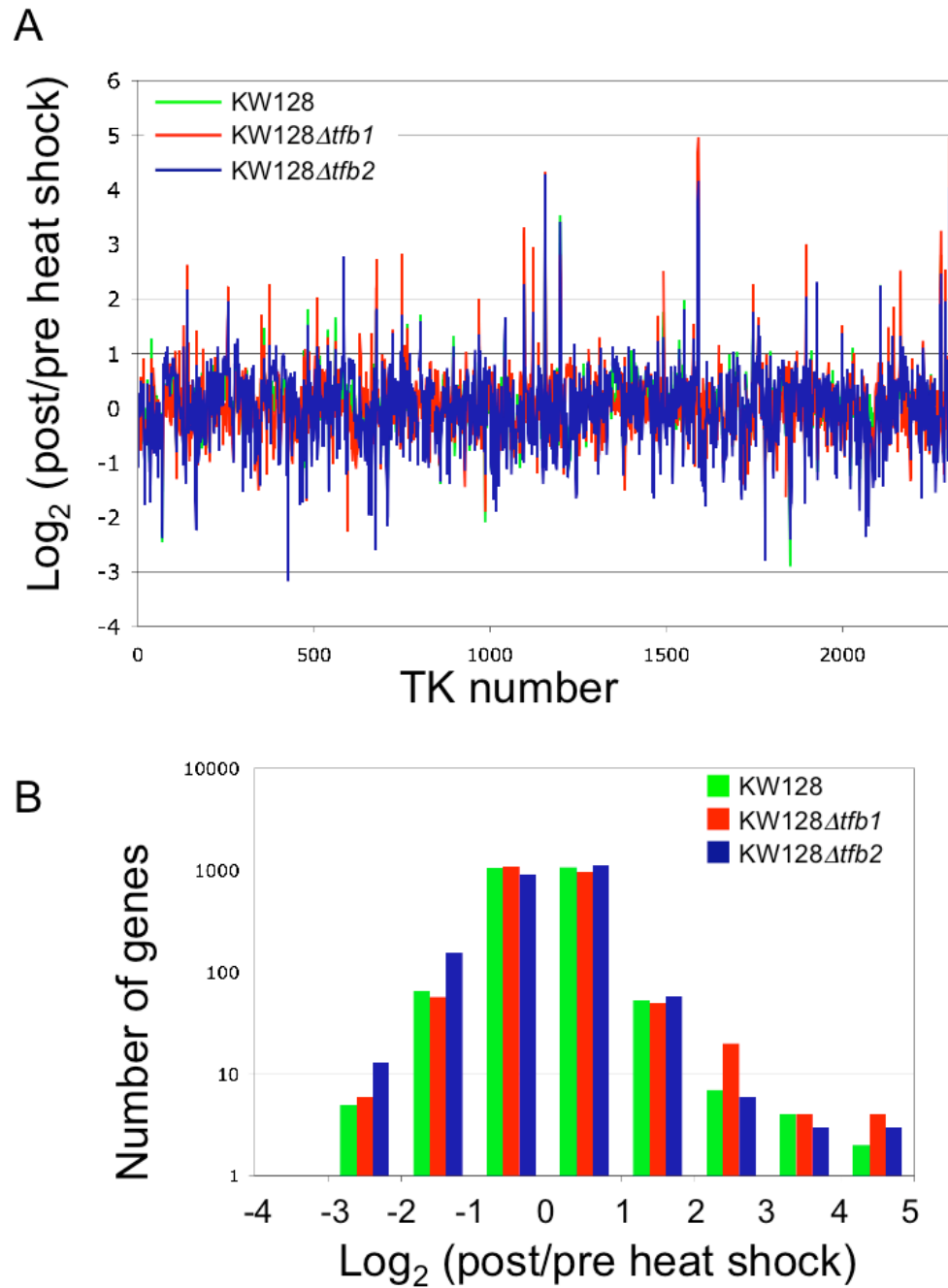


Fig. 2.16: DNA microarray analysis.

Three *T. kodakarensis* strains were heat shocked as described in materials and methods and changes in gene expression were analyzed by DNA microarray. (A) Fold changes following heat stress in KW128 (green), KW128 Δ tfb1 (red) and KW128 Δ tfb2 (blue) strains are summarized. X-axis corresponds to TK number (gene number) of *T. kodakarensis* and y-axis shows fold change. (B) The number of genes showing upregulation or downregulation in KW128 (green), KW128 Δ tfb1 (red), and KW128 Δ tfb2 (blue) are summarized.

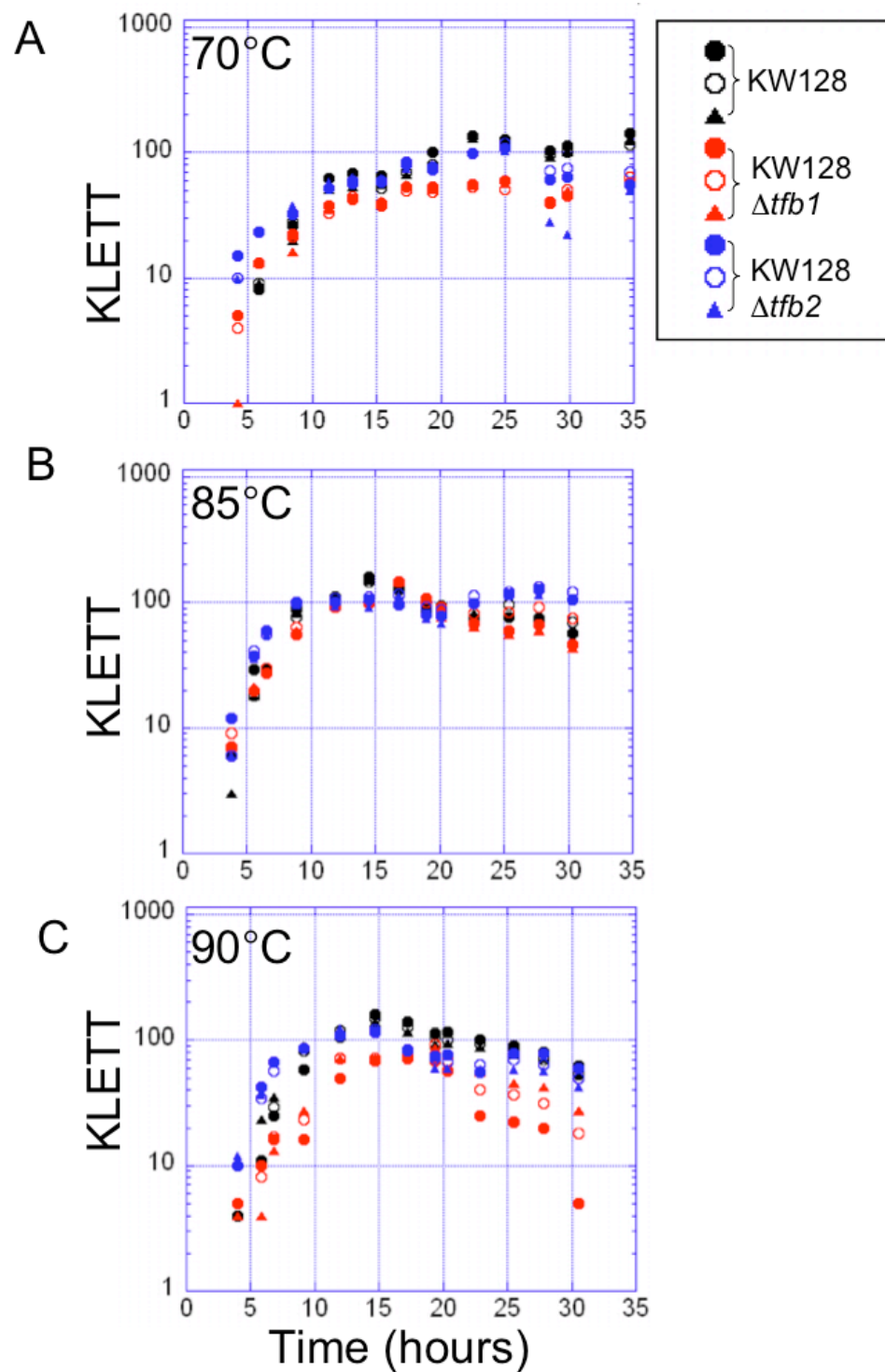


Fig. 2.17: Growth properties of *tfb1* or *tfb2* deletion strains. Cell growth of *T. kodakarensis* strains, KW128 (black), KW128 $\Delta tfb1$ (red) and KW128 $\Delta tfb2$ (blue) were monitored at various temperatures. The three strains were grown at (A) 70 °C, (B) 85 °C and (C) 90 °C in ASW-YT-S^o-pyruvate medium as described in materials and methods. Turbidity measurements were made using a KLETT spectrophotometer (100 KLETT units was equivalent to OD₆₀₀ of 0.65). Three experimental replicates were prepared for each condition and all data were shown in figures. Each experiment was repeated at least twice and representative measurements are shown.

Chapter 3

Development of a conditional gene expression system

3.1 Introduction

Since the discovery of *Archaea*, numerous efforts have been made to understand the life cycles and physiology of various archaeal species. Although there are various advantages to study archaea, such as a discovery of commercially useful enzymes (*e.g.* [78]), these organisms are less understood compared to bacteria and eukaryotes. One factor contributing is that development of genetic tools for the study of these organisms is far behind that of the bacteria and eukaryotes. Recently, an effective system for gene disruption was developed for the hyperthermophilic archaeon *Thermococcus kodakarensis* [67][69]. This system takes advantage of the organism's natural competency for foreign DNA uptake and its ability to incorporate foreign DNA into its genome by homologous recombination. These characteristics made various genetic approaches possible including the development of *in vivo* archaeal gene reporter system [65].

In order to understand how the expression of a given gene affect cell physiology, development of conditional gene expression system is crucial. In eukaryotes, gene rescue systems have been developed to test if over-expression of one gene can suppress phenotypes resulting from the loss of another gene (*e.g.* [73]). This chapter summarizes the design and construction of conditional gene expression system for *T. kodakarensis* [40] and its application to investigate consequences of TFB1 or TFB2 over-expression in the cell.

3.2 Results

3.2.1 Design and construction of conditional gene expression strains

Transcription of TK2164 that encodes fructose-1,6-bisphosphatase (FBPase) is known to be induced ~ 15 -fold under gluconeogenic versus glycolytic growth conditions [46]. On the plasmid vector designed for conditional gene expression, the gene targeted for expression was positioned immediately downstream from the TK2164 promoter (P_{fbp}) (Figure 3.1A). One of the two selection markers, *trpE* or *pyrF* was cloned adjacent to the promoter. This entire cassette was ligated between the 5' and 3' sequences from TK1765 (*chiA*, encodes for chitinase), a gene that is not required for *T. kodakarensis* growth in nutrient-rich media [K. Sato, T. Fukui, H. Atomi and T. Imanaka, unpublished result]. The TK1765 sequences were used for homologous recombination. Plasmids were introduced to parental strains lacking the gene used for positive selection and entire cassette was incorporated within TK1765 locus via homologous recombination. Transformants were isolated by growth without tryptophan or uracil depending on the selection marker. Protocols for transformant isolation for each selection marker and details of the experimental procedures are described in materials and methods.

3.2.2 Synthesis of RNA Polymerase subunits using conditional gene expression system

It has been found that *T. kodakarensis* strain lacking *rpoF*, gene encodes for RNA polymerase (RNAP) subunit F, has a temperature sensitive phenotype [40]. The conditional gene expression system was used to complement this deletion in order to verify that loss of *rpoF* was responsible for the phenotype, and to investigate whether expression of other RNAP subunits could suppress this phenotype.

The archaeal RNAP core consists of nine subunits and two additional subunits E and F form stalk protruding from the core. The subunit RPB4 and RPB7 of eukaryal RNA polymerase II (RNAPII) are orthologous to subunit E and F from archaea. These two subunits also form stalk structure protruding from RNAPII core. In yeast, a mutant lacking RPB4 exhibited a temperature sensitive phenotype [73] similar to the temperature sensitive phenotype in *T. kodakarensis* mutant lacking *rpoF* [40].

In yeast, it was discovered that the temperature sensitive phenotype in mutant lacking RPB4 could be suppressed by over-expressing RPB7 [73]. Therefore, it was of interest to determine if increased synthesis of subunit E suppress temperature sensitive growth of *rpoF* deletion strain, KUWF.

For conditional expression of RNAP subunits, gene encoding either subunit E (*rpoE*) or F (*rpoF*) were fused to FB Pase promoter in the expression vector described previously. Resulted strains were designated as KUWFE and KUWFF for conditional expression of *rpoE* and *rpoF*, respectively (See Table 3.1 for strains). Expression of subunit F in KUWFF strain under gluconeogenic conditions was confirmed by Western blotting [40]. Both KUW1 (parental strain) and KUWFF (*rpoF* expression strain) grew almost identically at 85 °C under gluconeogenic conditions, but under glycolytic growth conditions, KUWFF retained the temperature-sensitivity of KUWF [40]. Therefore, nutrient regulation of the P_{fbp} provide mechanism to regulate gene expression *in vivo* in *T. kodakarensis*.

Following isolation and genotype confirmation, all strains were grown at 85 °C in ASW-YT-S^o-pyruvate medium (gluconeogenic condition; P_{fbp} is active) to assess whether or not complementation of *rpoF* and/or over-expression of *rpoE* could rescue the temperature sensitive phenotype of KUWF (Figure 3.1B). Parental strain, KUW1, and *rpoF* expression strain, KUWFF

grew at the same rate indicating subunit F is necessary for effective growth at 85 °C. KUWFE (*rpoE* expression strain) grew slower than KUW1, but did reach ~74% of final cell density in this medium compared with KUW1. Culture of KUWF ($\Delta rpoF$) grew lower in comparison (~16% of KUW1). Therefore, the increase synthesis of subunit E partially rescue the temperature sensitivity resulted from subunit F deletion.

3.2.3 A study of transcription factor B conditional expression strains

The previous set of experiments demonstrated that the conditional gene expression system is capable of inducing expression of genes under gluconeogenic conditions. In order to observe the consequences of over-expression of the two transcription factor B (TFB) homologues (TFB1 and TFB2), two constructs were prepared for conditional gene expression of *tfb1* and *tfb2* as described previously [40] (Table 3.1, Figure 3.2).

3.2.3.1 Quantification of transcripts and proteins

To ensure the expression of *tfb1* and *tfb2* were achieved as expected, abundance of *tfb1* and *tfb2* transcripts were measured by quantitative real-time PCR (qRT-PCR) and proteins were quantified by Western blotting in cells grown at 85 °C under gluconeogenic conditions (Figure 3.3). Transcription of *tfb2* was induced ~20-fold and the TFB2 protein was induced ~11-fold. However, no induction of *tfb1* was observed at either the mRNA or protein level.

3.2.3.2 Growth properties of TFB conditional gene expression strain

Following isolation and genotype verification, *tfb1* expression strain, KUW11 and *tfb2* expression strain, KUW12 were grown in ASW-YT-S°-pyruvate medium (P_{fbp} is active) at 85 °C

and 90 °C. At 85 °C, KUW12 and the parental strain, KUW1 showed similar growth. However, a growth defect was observed in KUW11 after 6 hours incubation (Figure 3.4A). Growth defects were observed in both KUW11 and KUW12 compared to the parental strain at 90 °C (Figure 3.4B).

3.2.3.3 Verification of TFB1 conditional expression constructs

Because attempts to over-express *tfb1* were unsuccessful, DNA sequencing of KUW11 genome was conducted one more time to ensure that no mutation was introduced that would prevent transcription of *tfb1*. Both FB Pase promoter and *tfb1* sequences were found to be intact. Another concern was that random mutations or gene disruption could be introduced at some distant location during transformation, which potentially influence the transcription of P_{fbp} -*tfb1*. In order to eliminate the concern, three independent transformants were isolated from two separate transformations (designated as KUW11A through C). DNA sequencing of the inserted cassette and colony PCR showed incorporation of conditional gene expression cassette into their genomes. In three new strains, over-expressions of *tfb1* were observed, while induction of *tfb1* was not achieved in KUW11 (Figure 3.5A). This may suggest mutation may be introduced in KUW11, which lead to the suppression of over-expression activity from P_{fbp} . Interestingly, KUW11 and three new strains exhibited growth defect compared with the parental strain, KUW1 (Figure 3.5B). Because incorporation of P_{fbp} -*tfb2* did not affect cell growth at 85 °C, the growth defects in KUW11, KUW11A, KUW11B and KUW11C could result from the insertion of extra *tfb1* sequences into their genomes.

3.3 Discussion

A conditional gene expression system was developed and tested to investigate consequences of gene over-expression in the cell. This is the first system to control transcription *in vivo* in *T. kodakarensis* and one of the several archaeal systems for conditional gene expression. This system will provide various applications in genetic studies of *T. kodakarensis* and few examples of its application have been described in this chapter.

Using the conditional gene expression system, I observed over-expression of *tfb1* and *tfb2* influence cell growth. Contrary to the expectation that over-expression of *tfb1* will rescue the growth defect resulting from *tfb1* deletion, growth of the *tfb1* over-expression strain was not as efficient as its parental strain even at optimum growing temperature. The observation shows the over-expression of *tfb1* did not complement the loss of *tfb1*.

Both deletion and over-expression of *tfb1* showed deficient growth compared to parental strains. One explanation is that the concentration of intracellular TFB1 needs to be maintained at a strict range for optimal growth to occur. Conversely, phenotype effects of the deletion and over-expression of *tfb2* were less severe consistent with less significant control of the TFB concentration.

3.4 Materials and methods

3.4.1 Media used for *T. kodakarensis* transformation

For *T. kodakarensis* transformation, amino acid based medium ASW(Artificial Sea Water)-AA-S^o was used in order to select tryptophan or uracil prototrophs. ASW-AA-S^o medium (1 L) contains 0.8x artificial seawater, 1x amino acid solution, 5 ml of the vitamin mixture, and 5 ml

of Wolfe's trace mineral and 2 g of elemental sulfur (see Appendix A for media compositions) [2][67]. All media were autoclaved prior to use and transferred to an anaerobic environment ($\text{CO}_2:\text{H}_2:\text{N}_2$ (5:5:90)) for inoculation in Gloveless anaerobic chamber (Coy laboratory product Inc., Grass Lake, MI). As an indicator of oxygen presence, 0.8 mg of resazurin salt per liter was added to media. The media color was red when oxygen is present, so that 5% $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ was added to the medium until it restored the original color prior to the inoculation. In the case of plate culture, 1% (w/v) Gelrite (Sigma-Aldrich, St. Louis, MO) was added to solidify medium. In addition, 0.2% (v/v) of polysulfide solution (contains 10 g $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ and 3 g elemental sulfur per 15 ml) was added to the plate medium in place of elemental sulfur to reduce dissolved oxygen. Poured plates were placed in anaerobic chamber until their color changed from yellow to white. Cultures and plates were incubated in mechanical convection oven (DKN600, Yamato Scientific America, Inc., Santa Clara, CA) at the indicated temperature.

3.4.2 Construction of conditional gene expression strains

The required regions of the *T. kodakarensis* genome were PCR amplified from genomic DNA and cloned into pUC18 derivative by standard molecular biology technique.

Plasmids for conditional expression of RNAP subunits contain a 5' region of TK1765 (*chiA*), *trpE*, the intrinsic terminator from downstream of TK1431 (*gdh*), FBPase promoter, gene encode for RNAP subunit E or F, the 3' region of TK1765 and the down stream gene TK1766. These plasmids were amplified and purified from *Escherichia coli* Mach1-T1 and used to transform *T. kodakarensis* KUWF (Table 3.1) following a previously reported protocol [68]. Detailed procedures are as follows. KUWF was cultivated in ASW-YT-S° at 70 °C and 3 ml culture were harvested and the pellet was resuspended in 200 μl of 0.8x ASW. After placing suspended cells on ice for 30 minutes, 3 μg of plasmid was mixed and the cell-DNA mixture was kept

on ice for another 30 minutes. Then the tube was placed at 85 °C for 45 seconds and incubated on ice for 10 minutes. After the incubation, 1.3 ml of ASW-YT media and 2.6 μ l of polysulfide solution (See above for the composition) were added to the culture and the suspension was incubated at 70 °C for ~3 hours. Cells were then harvested (14,000 rpm, 5 minutes), resuspended in 200 μ l 0.8x ASW, and spreaded onto a selective ASW-AA palte lacking tryptophan. After incubating plates for 5 days at 70 °C, tryptophan prototroph, designated as KUWFE and KUWFF were selected (Table 3.1). The structure of the relevant region of the genomes of representative transformants were determined by PCR amplification [40]. To differentiate between single- and double-crossover events, primers were designed to anneal at outside of 5' and 3' franking regions (Figure 3.1A).

Plasmids for conditional expression of *tfb1* or *tfb2* contains a 5' region of TK1765 (*chiA*), *pyrF*, the intrinsic terminator from downstream of TK1431 (*gdh*), FBPase promoter, gene encode for TFB homologues, the 3' region of TK1765 and the down stream gene TK1766 (Figure 3.2A). To ensure *chiA* disruption does not affect cell growth, a control strain containing all cassette components without *tfb* genes was also constructed (Figure 3.2A, KUW1P). The growth of KUW1P strain did not exhibit any differences from the parental strain KUW1. *T. kodakarensis* strain KUW1 was transformed by a previously reported protocol [67] and uracil prototrophs were selected. Detailed procedures are as follows. KUW1 was cultivated in ASW-YT-S° at 85 °C until late exponential phase (12 - 16 hours). Approximately 4×10^8 cells (from 3 ml culture) were harvested and the pellet was resuspended in 200 μ l of 0.8x ASW. After placing suspended cells on ice for 30 minutes, 3 μ g of DNA (pUC18-ox_X vectors) was mixed and the cell-DNA mixture was kept on ice for another 30 minutes. Then the tube was placed at 85 °C for 45 seconds and incubated on ice for 10 minutes. After the incubation, all cells were inoculated to 20 ml ASW-AA-S° medium and incubated at 85 °C for about 40 hours. Cells were further inoculated to fresh ASW-AA-S° medium (1 to 100 ratio) and incubated at 85 °C for an additional 40 hours.

Inoculation and 40-hour incubation processes were repeated one more time. Following the final 40-hour incubation, 100 μ l broth was spread on ASW-AA plates for uracil prototroph isolation. After incubating plates for 3 - 4 days at 85 °C, several colonies were tested by PCR amplification (Figure 3.2B) and DNA sequencing of the PCR products for confirming the conditional gene expression cassette being incorporated.

3.4.3 Westernblotting

To quantify the TFB expression levels in conditional expression strains by Western blotting, three *T. kodakarensis* strains, KUW1, KUW11 and KUW12 were grown in ASW-YT-S⁻-pyruvate media until their growth reached late-log phase ($\sim$ 9 hours, OD₆₀₀ = 0.5 - 0.7). For each strain, cells were collected from approximately 2 ml culture by centrifugation (14,000 rpm for 5 minutes). Cell pellets from all strains were resuspended in 40 μ l buffer containing 20 mM phosphate buffer, pH 7.6, 7 mM β -mercaptoethanol, and 10% glycerol (v/v). Cells were, then disrupted by the addition of Triton X-100 to the final concentration of 0.1% (v/v). For each strain, 10 μ g of total proteins were separated on 12% SDS polyacrylamide gels (1/30th the concentration of acrylamide is N,Nmethylenebisacrylamide). Separated proteins were transferred to the PVDF membrane by electroblotting (Mini Trans-blot cell, Bio-rad laboratories Inc., Hercules, CA). Primary antibodies were prepared using two methods for protein quantification. The first method used purified recombinant TFB1 for antibody preparation (PA1024, Covance, Princeton, NJ). This antibody has a great sensitivity, although it cross-reacts with both TFB homologues. Also, several non-specific bands were observed with this antibody. Second primary antibody preparation used peptides unique to each protein (Covance, See Appendix C for peptides sequences. PA947 and PA948 for TFB1 and TFB2, respectively). The anti-TFB2 antibody, PA948, exhibited reasonable specificity. The anti-TFB1 antibody, PA947 did not have adequate specificity nor sensitivity, thus was not used for this study. The HRP linked secondary antibody

(ECL anti-rabbit IgG from Donkey, Amersham, GE Healthcare, Piscataway, NJ) and Supersignal West Pico substrate (Thermo scientific, Rockford, IL) were used for the chemiluminescent detection. Band intensities were determined by NIH image software (<http://rsbweb.nih.gov/niimage/>). Both PA1024 and PA948 antibodies showed similar results for assays of *tfb* conditional expression strains. Therefore, PA948 was used for TFB2 quantification because it produced less non-specific bands.

Cell growth experiments and qRT-PCR were conducted as described in Chapter 2.

3.5 Acknowledgements

I thank Dr. Tadayuki Imanaka and his group (Kyoto University) for some *T. kodakarensis* strains used in this study and also for guidance in manipulating the organism.

Table 3.1: *T. kodakarensis* strains and plasmids used for this study.

Strain	Relevant characteristics	References/construction
KUW1	$\Delta pyrF; \Delta trpE$	[68]
KUWF	$\Delta pyrF; \Delta trpE; \Delta rpoF::pyrF$	[40]
KUWFF	$\Delta pyrF; \Delta trpE; \Delta rpoF::pyrF;$ $\Delta chiA::trpE-P_{fbp}-rpoF$	[40]
KUWFE	$\Delta pyrF; \Delta trpE; \Delta rpoF::pyrF;$ $\Delta chiA::trpE-P_{fbp}-rpoE$	[40]
KUW1P	$\Delta pyrF; \Delta trpE; \Delta chiA::pyrF-P_{fbp}$	KUW1 with expression cassette without gene in <i>chiA</i>
KUW11	$\Delta pyrF; \Delta trpE; \Delta chiA::pyrF-P_{fbp}-tfb1$	KUW1 with <i>tfb1</i> (TK1280) expression cassette in <i>chiA</i>
KUW12	$\Delta pyrF; \Delta trpE; \Delta chiA::pyrF-P_{fbp}-tfb2$	KUW1 with <i>tfb2</i> (TK2287) expression cassette in <i>chiA</i>
Plasmid		
pUC18_ox_P	TK1765:: <i>pyrF</i> - <i>P_{fbp}</i> -TK1766 in pUC18	DNA fragment was inserted at <i>Hinc</i> II site
pUC18_ox_1	TK1765:: <i>pyrF</i> - <i>P_{fbp}</i> - <i>tfb1</i> -TK1766 in pUC18	DNA fragment was inserted at <i>Hinc</i> II site
pUC18_ox_2	TK1765:: <i>pyrF</i> - <i>P_{fbp}</i> - <i>tfb2</i> -TK1766 in pUC18	DNA fragment was inserted at <i>Hinc</i> II site

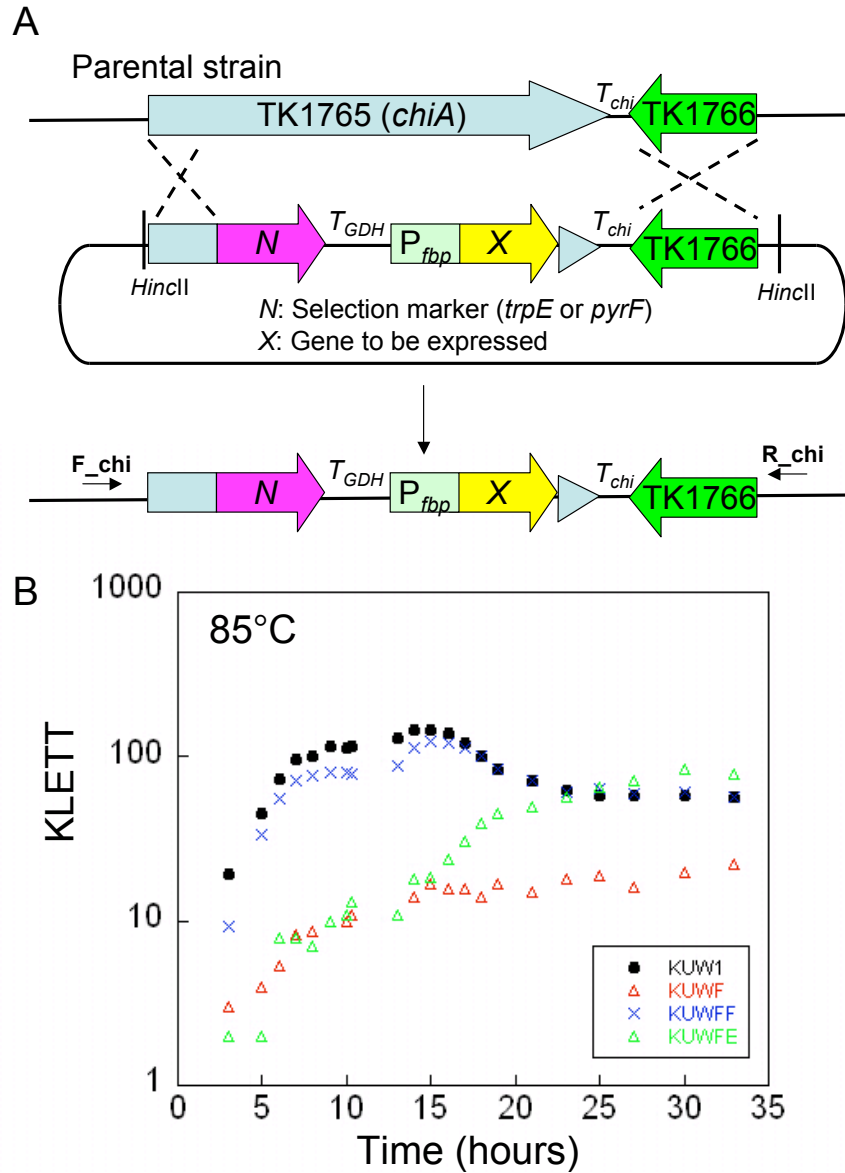


Fig. 3.1: Construction of conditional expression strains.

(A) Schematic diagram illustrating the expected homologous recombination. The plasmid for conditional gene expression includes: 1) 5' region of *chiA* gene (light blue); 2) selection marker *trpE* or *pyrF* (purple); 3) GDH terminator (T_{GDH}); 4) FBPase promoter (P_{fbp} , light green); 5) gene to be expressed (X , yellow); 6) 3' region of *chiA* gene (light blue); 7) *chiA* terminator (T_{chi}); and 8) TK1766 (downstream gene of chitinase, green). Location of primer set (F_{chi} and R_{chi}) used for the PCR assay were indicated as thin black arrows. (B) Partial suppression of the temperature-sensitive growth phenotype of KUWF($\Delta rpoF$) by *rpoE* over-expression. Cultures of *T. kodakarensis* KUW1 (black closed circles), KUWF (red open triangles), KUWFF (blue crosses) and KUWEE (green open triangles) were grown at 85 °C in ASW-YT-S-pyruvate (FB-Pase promoter is active). Turbidity measurement were made using a KLETT spectrophotometer (100 KLETT units was equivalent to and OD₆₀₀ of 0.65). Both figures are modified from [40].

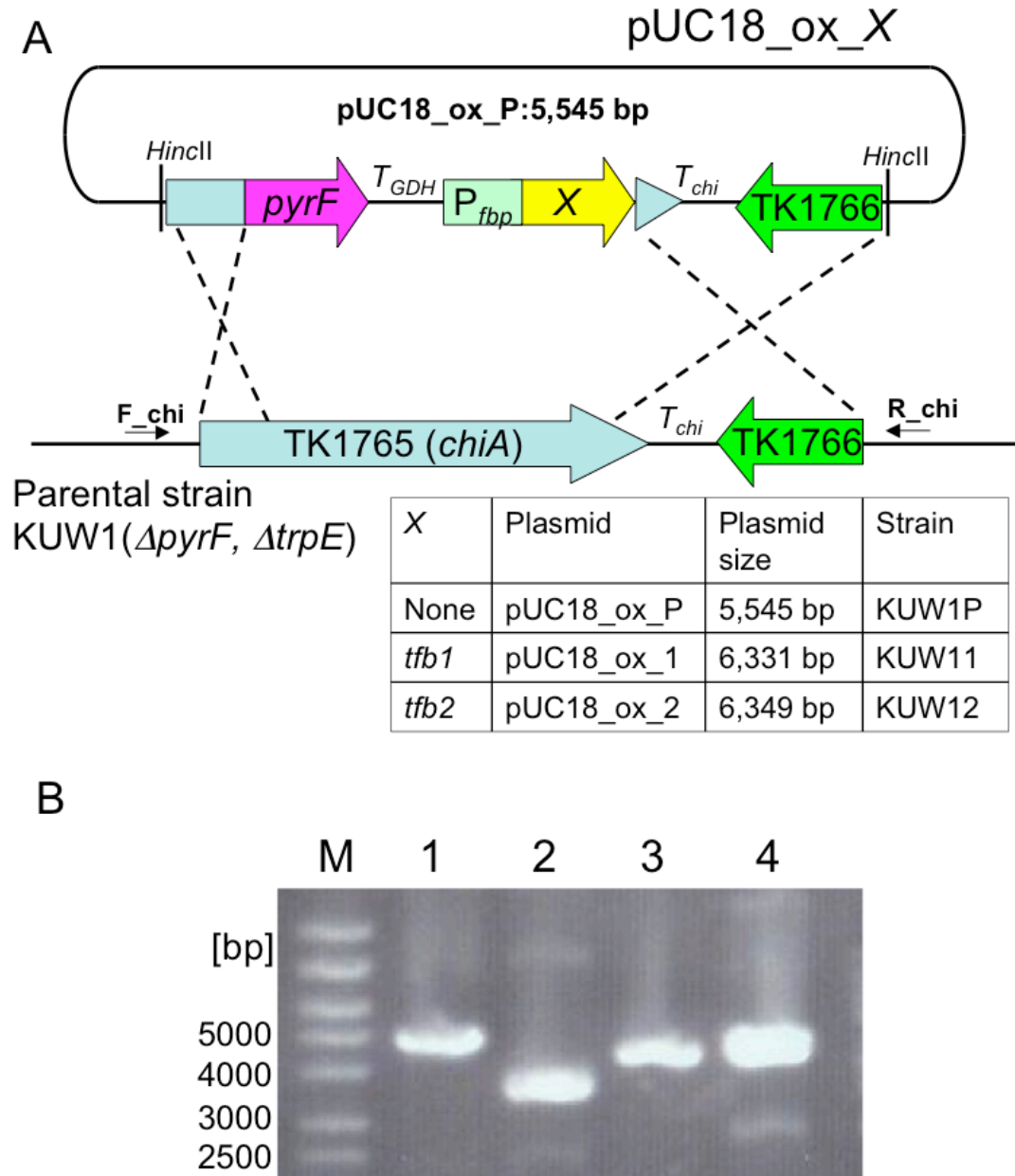
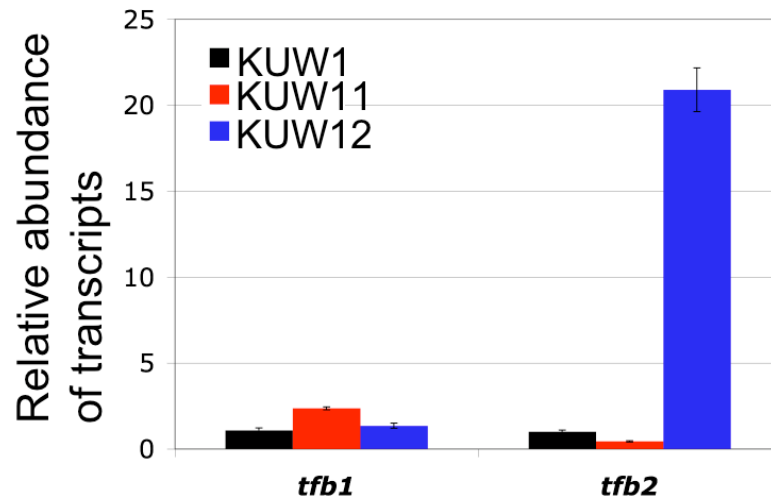


Fig. 3.2: Construction of *tfb* conditional expression strains.

(A) Schematic diagram illustrating the expected homologous recombination using pUC18_ox_X vectors. The pUC18_ox_X vector includes: 1) 5' region of *chiA* gene (light blue); 2) selection marker *pyrF* (purple); 3) GDH terminator (T_{GDH}); 4) FBPase promoter (P_{fbp} , light green); 5) gene to be expressed (*X*, yellow); 6) 3' region of *chiA* gene (light blue); 7) *chiA* terminator (T_{chi}); and 8) TK1766 (downstream gene of chitinase, green). (B) Genotype analyses of conditional expression strains by PCR. Location of primer set (F_chi and R_chi) used for the PCR assay were indicated as thin black arrows in (A). Expected sizes of the PCR products are 4,723 bp for KUW1 (lane 1), 3,446 bp for KUW1P (lane 2), 4,232 bp for KUW11 (lane 3) and 4,250 bp for KUW12 (lane 4).

A



B

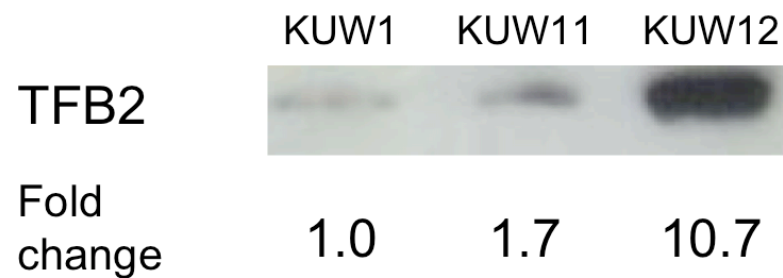


Fig. 3.3: Fold changes of transcripts and proteins in *tfb* conditional expression strains. (A) Relative quantification of transcript in conditional expression strains using qRT-PCR. The population of *tfb1* and *tfb2* transcript in parental strain (K UW1, black), *tfb1* conditional expression strain (K UW11, red) and *tfb2* conditional expression strain (K UW12, blue) are shown with respect to K UW1. (B) Western blotting analysis of TFB2 in conditional expression strains. Densities of each bands were shown as a fold changes from a parental strain K UW1.

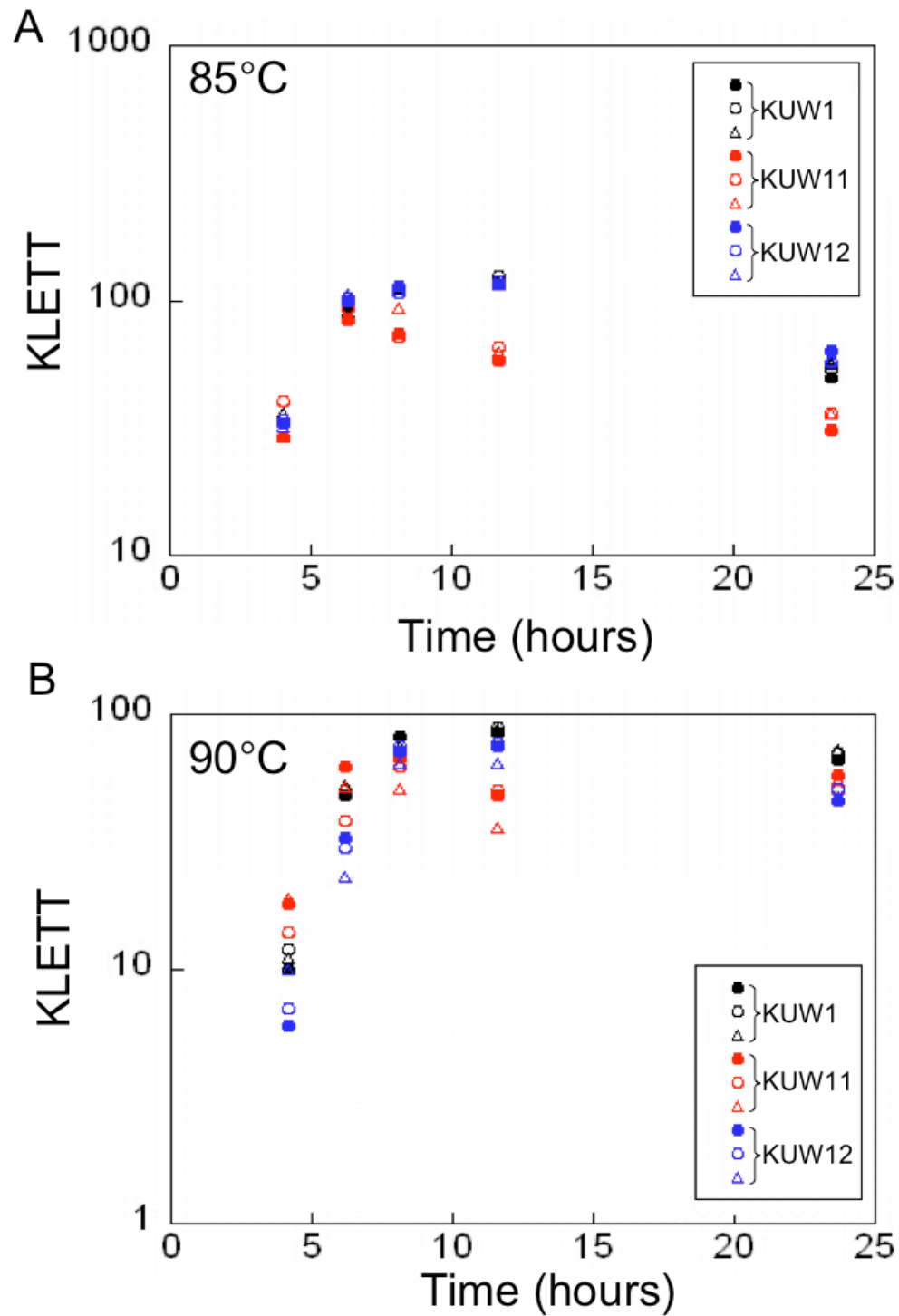


Fig. 3.4: Cell growth experiments with *tfb1* or *tfb2* conditional expression strains. Cell growth experiments with parental strain KUW1 (black), *tfb1* conditional expression strain KUW11 (red) and *tfb2* conditional expression strain KUW12 (blue) grown at (A) 85 °C and (B) 90 °C. All strains were grown in ASW-YT-S⁻-pyruvate medium as described in materials and methods. Turbidity measurement were made using a KLETT spectrophotometer (100 KLETT units was equivalent to OD₆₀₀ of 0.65). Three experimental replicates were prepared for each condition and measured turbidity of all samples are shown.

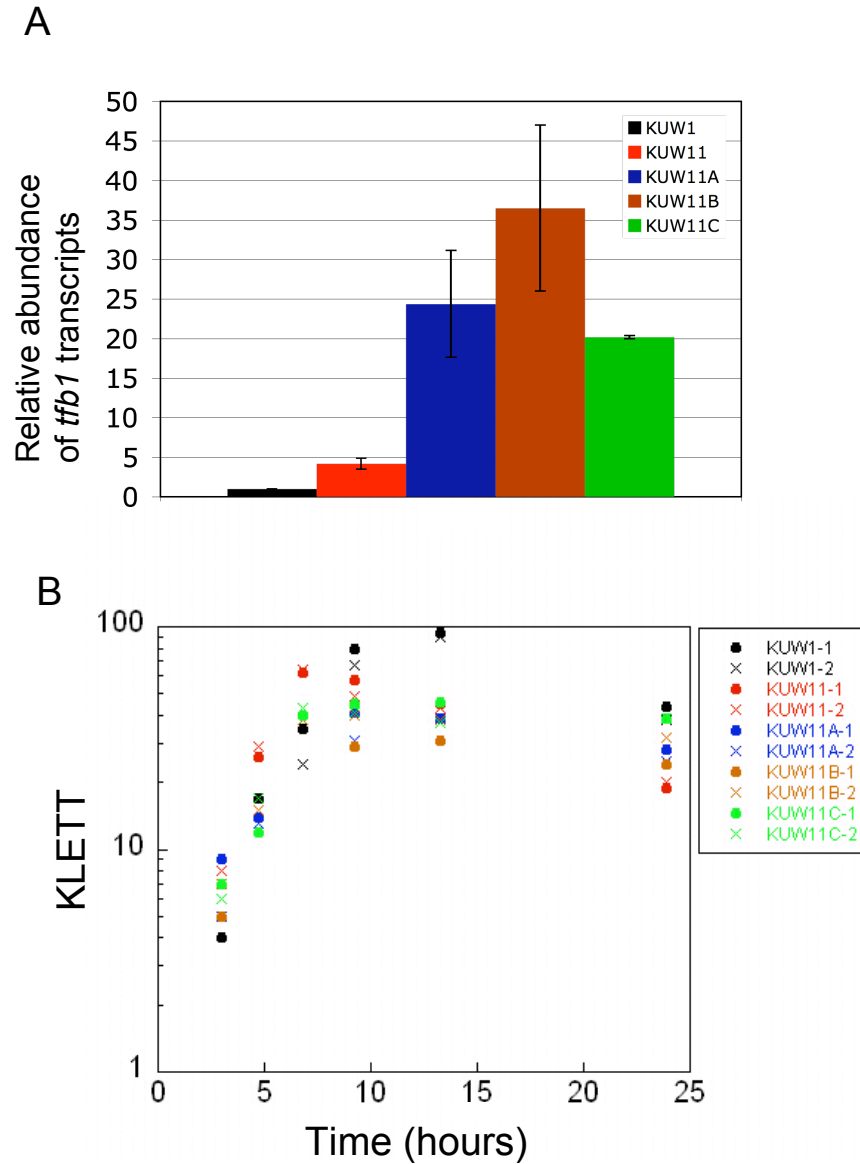


Fig. 3.5: Verification of *tfb1* conditional expression strains.

(A) Relative quantification of transcript in conditional expression strains using qRT-PCR. The population of *tfb1* transcript in parental strain (KUY1, black) and four *tfb1* conditional expression strains (KUY11 and KUY11A through C) are shown with respect to KUY1. (B) Growth properties of *tfb1* conditional expression strains. All strains were grown at 85 °C in ASW-YT-S°-pyruvate (FBPase promoter is active). Turbidity measurement were made using a KLETT spectrophotometer (100 KLETT units was equivalent to and OD₆₀₀ of 0.65). Two biological replicate measurement was taken for each strain and both KLETT readings are shown.

Chapter 4

Future directions

4.1 Introduction

In chapter 2, several lines of experimental evidence suggest the involvement of the two transcription factor B (TFB) homologues, TFB1 and TFB2, in transcription regulation of *Thermococcus kodakarensis*. Despite high sequence similarity, TFB1 and TFB2 appear to exert different effects on pre-initiation complex during transcription initiation. *In vitro* transcription assays suggest that these differences may be due to other factors. It is possible that differential interactions between each TFB and other putative regulatory proteins may play an important role in transcription regulation. To test this possibility, investigations of the interactions of TFB1 and TFB2 with other molecules in the cell will be required. Three ongoing experimental approaches to study interaction of each TFB homologue with other molecules in the cells are outlined in this chapter .

4.2 Identifying factors associating with TFB homologues

4.2.1 Introduction

In vitro transcription assays conducted thus far provide no evidence that the two *T. kodakarensis* TFB homologues have differences in promoter selectivity [66] (Chapter 2). Because only a limited number of promoters were assayed by *in vitro* transcription, and because these assays included only RNA polymerase (RNAP), TATA-binding protein (TBP) and TFB1 or TFB2, it was not possible to observe if TFB1 or TFB2 will differ in selectivity from promoters which

required additional transcription regulators.

A protein named TIP26 has been identified that was shown to interact with TBP in *Thermococcus kodakarensis* [52][53][86]. TIP26 binds to free or DNA-bound TBP, and when binds to TBP, it will inhibit transcription initiation [52]. Although not yet identified, it is possible that other proteins in *T. kodakarensis* are capable of forming similar interaction with TFB.

T. kodakarensis TFB1 and TFB2 have high amino acid sequence identity (67%) and residues which have been shown to interact with TBP and DNA based on the X-ray crystal structure [49] are well conserved (20 out of 25 residues are identical, Figure 4.1). To illustrate configurations of those conserved residues, a cartoon representation was prepared using previously determined *Pyrococcus woesei* X-ray crystal structure [49] (Figure 4.2). Identical residues between the two TFB homologues are colored in grey. In addition to the well-conserved TBP or DNA binding sites, several regions on the TFB surface are highly conserved suggesting they may potentially interact with common transcription factors in both TFB homologues.

To identify possible candidates for regulatory proteins, two experiments have been initiated. First experiment is a pull-down assay using tagged TFB1 or TFB2. Histidine tagged TFB1 or TFB2 encoding gene was introduced within TK1765 (chitinase) locus using conditional gene expression system described in Chapter 3. From cell lysates of conditional expression constructs, each TFB along with any interacting proteins can be purified using a Ni-affinity column. Proteins associating with each homologue will be visualized by SDS-PAGE and will be identified by mass spectroscopy.

The second approach is to identify factors interacting with promoter DNA other than TFB, TBP and RNAP. Cell lysates from KW128, KW128 Δ *tfb1* and KW128 Δ *tfb2* strains (See

Table 4.1 for construction of each strain) will be applied to the immobilized DNA containing a promoter from a heat shock gene, *cpkB*. Proteins that bind to the DNA will be separated on SDS-PAGE and will be identified by mass spectroscopy.

4.2.2 Materials and methods

4.2.2.1 Pull-down assay

In order to selectively pull down proteins associating with TFB1 or TFB2, *T. kodakarensis* strains which express either *tfb1* or *tfb2* gene with poly-histidine tag were constructed. The gene *tfb1* or *tfb2* with poly-histidine tag were fused to promoter from 1,6-bisphosphatase (FB-Pase), and introduced into the genome of the parental strain using conditional expression system described in Chapter 3. Isolated strains are designated as KUW11H and KUW12H for histidine-tagged TFB1 and TFB2 conditional expression, respectively (See Table 4.1 for construction of each strain). As described in Chapter 3, when both strains are grown in the media supplemented with pyruvate, expression of genes downstream of FBPase promoter is expected to be induced. Both conditional expression strains were grown in 300 ml ASW-YT-S° media supplemented with 1% sodium pyruvate for 16 hours at 85 °C. Cells were harvested by centrifugation and resuspended in 15 ml binding buffer (10 mM Tris-HCl, pH 8 at room temperature, 0.5 M NaCl, 10 mM imidazol, 5% glycerol, 5 mM β -mercaptoethanol and 1 mM PMSF) and sonicated. Sonication was conducted for 5 sets of 30-second program (on; 2 seconds, off; 1 second, power level 5.0) with microtip (Model S3000, Misonix Inc., Farmingdale, NY). The lyseate was centrifuged (16,000 rpm, 15 minutes) at 4 °C to remove cell debris and supernatant was used for following purifications. The supernatant was applied to His-trap column (5ml, GE Healthcare, Piscataway, NJ) or Hi-trap chealating column (5ml, GE Healthcare) charged with 0.1M NiSO₄. After wash with 20 ml each of binding buffer and wash buffer (10 mM Tris-HCl, pH 8 at room temperature, 50 mM NaCl, 10 mM imidazol, 5% glycerol), bound proteins were eluted with elution buffer

containing 250 mM imidazol. Eluted fractions from each strain were concentrated and separated by SDS-PAGE.

4.2.2.2 DNA-protein interaction assay

DNA promoter from *cpkB* was prepared by PCR using a primer set CpkB_F (5'-AGGTGCT TGAATTGGGAGAGAAGAAGAACTCG) and CpkB_R (5'-CCTGGCAGCAA GAATGTTGAGCC-biotin), which created 3'-biotynylated 299 bp-long fragments. The DNA fragments were then immobilized to Dynabeads M-280 (DynaL Biotech ASA, Oslo, Norway), which are the magnetic beads with covalently attached streptoavidin. Details of the protocol are as follows; For 2 reactions, 20 μ l of beads (contains 200 μ g beads) were transferred to 1.7-ml eppendorf tube from the stock. The tube was placed on magnet for 1 minute and supernatant was removed. Then, the beads were washed twice with binding and wash (BW) buffer containing 5 mM Tris-HCl, pH 8 at room temperature, 0.5 mM EDTA and 1 M NaCl. Following the wash, 16 μ l of BW buffer and 4 μ l of DNA (40 ng/ μ l) were added to the tube and incubated at the room temperature for one hour. Following the DNA binding reaction, unbound DNA fragments were removed by washing the beads for 3 times with BW buffer and 2 times with 10 mM Tris-HCl (pH 8, room temperature). The beads surface was blocked by addition of 34 μ l of buffer D (20 mM Hepes, pH 8 at room temperature, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT and 20% glycerol) and 6 μ l of BSA (10 mg/ml) and the tube was incubated at room temperature for 30 minutes. Unbound BSA was removed by the one time wash with buffer D. After removing the supernatant, beads were resuspended in 38 μ l buffer D and aliquoted to two tubes.

For the preliminary test, recombinant proteins were added to the beads. Both 50 ng recombinant TFB1 and 30 ng recombinant TBP were added to one tube, while TBP was omitted from another tube to compare TFB binding with or without TBP. Amount of protein solution

were adjusted, so that each tube had final volume of 20 μ l. Both tubes were incubated at 85 °C for 15 minutes and supernatant was removed (S). Then, the beads were washed with 20 μ l buffer D (W). Beads were washed additional 5 times with buffer D and final pellet was resuspended to 20 μ l buffer D (P). 5 μ l SDS (5x) solution were added to all samples (S, W and P), and samples were boiled for 5 minutes to denature proteins. The denatured samples were separated on 12% SDS-PAGE and analyzed by Western blotting using anti-TFB1 antibody (PA1024, See Chapter 3 for antibody preparation).

Streptoavidin conjugated to the beads may become unstable at high temperature. To exclude the concern, experiment was repeated using lower temperature (50 °C). For this experiment, negative control samples were prepared, for which water was added in place of DNA.

4.2.3 Preliminary results

4.2.3.1 Pull-down assay

Three strains, KUW1, KUW11H, KUW12H were grown on media supplemented with pyruvate (gluconeogenic condition; Inducible promoter, P_{fbp} is active). Histidine-tagged proteins were purified by Ni-affinity chromatography. Elution fraction were separated by SDS-PAGE (Figure 4.3A), and multiple bands were observed for all strains. A very strong band was found in all strains at around 40 kDa size. The contents of this bands has not been investigated. A band corresponding to histidine-tagged TFB2 was faint, suggesting over-expression of TFB2 may not occur. However, Western blotting analysis (Figure 4.3B) showed eluted fraction contains TFB1 or TFB2 in KUW11H and KUW12H indicating histidine-tagged TFB homologues were transcribed from P_{fbp} and purification of both tagged TFB homologues was achieved.

4.2.3.2 DNA-protein interaction assay

A preliminary assay with recombinant protein showed effective binding of TFB1 to the DNA containing *cpkB* promoter using magnetic beads (Figure 4.4A). However, there was no difference of TFB binding to the DNA with or without TBP. Experiments at 50 °C did not show any difference in binding protein stability compared with 85 °C, indicating high temperature was not a concern (Figure 4.4B). Concentrated KUW1 lysate was applied to immobilized DNA to identify proteins associating with the promoter. The experiment with the lysate did not work well as the test using recombinant proteins. I identified two reasons why this might be the case. The first problem was that TFB seems to bind non-specifically to the beads even when a BSA blocking step was employed (Figure 4.4C). The second concern was that the majority of TFB remains in supernatant indicating an adjustment of lysate to beads-DNA ratio may be necessary.

4.2.4 Future works

The pull-down assay with hisidine-tagged TFB needs further modifications to purification process in order to selectively purify histidine-tagged TFB. Previously, TIP26 was successfully isolated and from *T. kodakarensis* by loading cell lysate to an agarose column cross-linked to TBP [53]. Thus, a similar methodology could be used to find factors associating with TFB1 and TFB2.

DNA promoter binding assays require a larger amount of input proteins in order to visualize using SDS-PAGE stained with commassie brilliant blue or silver. However, increasing the input leads non-specific binding of proteins to the beads, which in turn make it difficult to interpret results from the DNA binding assay. Thus, blocking an uncoated surface with higher concentration of BSA or washing with higher salt concentrations to reduce non-specific binding

will be tested.

4.3 Structural study of TFB-RNAP complex

4.3.1 Introduction

X-ray crystallography is a powerful tool to investigate protein-protein interactions at the atomic level. Because the two TFB homologues in *T. kodakarensis* are quite similar, observations of each residue at a high resolution will be necessary in order to reveal residues or interactions, which lead to the functional variance between the two. Obtaining structural information of TFB1 or TFB2 in complex with other components within pre-initiation complex will provide key information for identifying residues, which need to be investigated more closely. Along with currently available X-ray crystal structures (*e.g.* archaeal RNAP [41], TFB-TBP-DNA [49] and eukaryal TFIIB-RNA polymerase II (RNAPII) [20]), a high resolution structure of archaeal TFB-RNAP will provide further insights into interactions among components of pre-initiation complex. Efforts to prepare crystals of TFB1-RNAP and TFB2-RNAP complexes are in progress.

4.3.2 Materials and methods

Recombinant TFB1 and TFB2 without histidine tag were prepared as described in Chapter 2 and RNAP was provided from Dr. Thomas Santangelo [66]. RNAP and TFB1 or TFB2 were mixed equimolar ratio and concentrated to approximately 3.5 mg/ml in exchange buffer containing 10 mM Tris-HCl (pH 8 at room temperature), 50 mM NaCl, 0.1 mM EDTA and 1 mM DTT. Two different screening conditions, Index HT and Salt RX HT (Hampton Research, Aliso Viejo, CA) were used and screening trays were prepared using crystallization robot (Penn

State X-ray core facility). Trays were placed either 4 °C and/or 21 °C incubator and crystallization status was observed routinely. No crystals were formed for structure analyses during three months from the tray preparations.

4.3.3 Future works

Two additional crystallization conditions will be tested in order to obtain crystals of RNAP-TFB complex. The first approach is to use core RNAP without the E and F subunits. X-ray crystal structure of archaeal RNAP showed protruding stalk consisting of subunit E and F [41]. Removal of these subunits may improve the crystal packing. The second method is to use only N-terminal domain of TFB for crystallization. Based on the structure of eukaryal RNAPII-TFIIB binary complex, it is expected that C-terminal domain of archaeal TFB will not associates with RNAP. Furthermore, it might be is flexible in the TFB-RNAP binary complex. The removal of flexible domain may improve crystallization.

4.4 Study of TFE functions

4.4.1 Introduction

TFE is an ortholog of eukaryal TFIIE α and its homologues have been identified in all archaeal genomes. However, TFE is not required to initiate promoter-specific transcription *in vitro* [38][60]. It has been shown that TFE co-purifies with RNAP from both *T. kodakarensis* [66] and *Pyrococcus furiosus* [54]. Previous *in vitro* transcription studies with *P. furiosus* showed TFE could improve transcription activity, when the TFB homologue lacking B-finger motif was used in the assay [54]. The observation is consistent with TFE interacting with TFB. It also has been

proposed that archaeal TFE is involved in promoter opening process during transcription initiation [55]. While the involvement of TFB and TBP in archaeal transcription initiation has been thoroughly investigated, limited studies have been reported for TFE. An investigation of TFE roles in *Archaea* would add to the understandings of the mechanisms of transcription initiation.

4.4.2 Experimental approaches

Attempts to delete TFE have not been successful using a standard homologous recombination procedure [T. Santangelo and J. N. Reeve, unpublished observation] indicating that it might be an essential gene in *T. kodakarensis*. I made attempts to disrupt *tfe* from original locus (TK2024) by inserting additional *tfe* gene with conditional gene expression cassette described in Chapter 3. The gene which encodes for TFE was fused to inducible promoter (P_{fbp}) and inserted within chitinase (*chiA*) locus using homologous recombination (Figure 4.5). The vector, pUC18_ox_E, used in the transformation contains: 1) 5' region of *chiA* gene; 2) selection marker *pyrF*; 3) GDH terminator; 4) FBPase promoter; 5) *tfe*; 6) 3' region of *chiA* gene; 7) *chiA* terminator; and 8) TK1766 (downstream gene of chitinase) and a strain KUW1E was obtained (See Table 4.1 for construction).

KUW1E was further transformed with the pUDE vector (Figure 4.6A). The pUDE was prepared by inserting a gene cassette, in which *tfe* (TK2024) was replaced by *trpE* (selection marker) in pUMT [69] (Table 4.1). The *trpE* is flanked by 1,321 bp upstream sequence (TK2022-2023) and 1,209 bp downstream sequence (TK2025-2026) of *tfe* (Figure 4.6A). Wildtype *tfe* was replaced by the selection marker *trpE* through homologous recombination and a strain KUW1E Δ *tfe* was isolated. The KUW1E Δ *tfe* strain contains only one *tfe* under the control

of the inducible promoter, P_{fbp} . The fact, being wildtype *tfe* could only be deleted when additional gene was expressed, is consistent with TFE being essential for cell viability.

Cell growth experiments were conducted using KUW1 (parental strain), KUW1E and KUW1E Δtfe strains in media supplemented with pyruvate (P_{fbp} is active) or maltodextrin (P_{fbp} is inactive). Under inducible conditions, all cells exhibited similar growth pattern at 85 °C (Figure 4.7A). Under non-inducible conditions, when sulfur was omitted from the media, all strains, including KUW1 did not show effective growth. Thus I was unable to make any conclusions with respect to growth phenotypes under the repressing condition. In order to investigate phenotypes of TFE deletion strains, further modification of the growth condition will be necessary.

The quantitative real-time PCR (qRT-PCR) analysis showed transcription of *tfe* took place from P_{fbp} in KUW1E Δtfe strains (Figure 4.7B). However, the *tfe* was not induced as much as expected from P_{fbp} activity [46].

4.4.3 Future work

Verification of KUW1E Δtfe strain will be carried out in order to ensure no suppressing mutation was introduced during transformation as in *tfb1* conditional expression strain (Chapter 3). If there are no mutation, there would be a post-transcription regulation mechanism to maintain the *tfe* level in the cells.

In addition to genetic approaches, *in vitro* transcription assays will be conducted using the two forms of *T. kodakarensis* RNAP. Wildtype RNAP contains 11 subunits and co-purifies with TFE. RNAP lacking two subunit E and F does not co-purify with TFE [40]. This suggests the E

and F subunits are required for RNAP to interact with TFE. Because *cpkB* was upregulated in the *T. kodakarensis* strain lacking subunit F [40], I speculated E and F subunits and TFE may have some roles in regulation of stress response gene transcription. To test this hypothesis, heat shock gene *cpkB* will be used as a template and transcription activity of wildtype RNAP and RNAP without subunit E and F will be compared.

4.5 Acknowledgements

I would like to thank Dr. Thomas Santangelo for providing us with the *T. kodakarensis* RNAP and pUDE plasmid.

Table 4.1: *T. kodakarensis* strains and plasmids used for this study.

Strain	Relevant characteristics	References/construction
KW128	$\Delta pyrF; \Delta trpE::pyrF$,	[68]
KW128 $\Delta tfb1$	$\Delta pyrF; \Delta trpE::\Delta tfb1::trpE$	[66]
KW128 $\Delta tfb2$	$\Delta pyrF; \Delta trpE::\Delta tfb2::trpE$	[66]
KUW1	$\Delta pyrF; \Delta trpE$	[68]
KUW11H	$\Delta pyrF; \Delta trpE; \Delta chiA::pyrF-P_{fbp}-tfb1-his_5$	KUW1 with histidine-tagged <i>tfb1</i> (TK1280) expression cassette in <i>chiA</i>
KUW12H	$\Delta pyrF; \Delta trpE; \Delta chiA::pyrF-P_{fbp}-tfb2-his_6$	KUW1 with histidine tagged <i>tfb2</i> (TK2287) expression cassette in <i>chiA</i>
KUW1E	$\Delta pyrF; \Delta trpE; \Delta chiA::pyrF-P_{fbp}-tfe$	KUW1 with <i>tfe</i> (TK2024) expression cassette in <i>chiA</i>
KUW1E Δtfe	$\Delta pyrF; \Delta trpE; \Delta chiA::pyrF-P_{fbp}-tfe; \Delta tfe::trpE$	KUW1E with <i>tfe</i> (TK2024) replaced by <i>trpE</i>
Plasmid		
pUC18_ox_1H	TK1765:: <i>pyrF-P_{fbp}-tfb1-his₅</i> -TK1766 in pUC18	DNA fragment was inserted at <i>HincII</i> site Used for the construction of KUW11H
pUC18_ox_2H	TK1765:: <i>pyrF-P_{fbp}-tfb2-his₆</i> -TK1766 in pUC18	DNA fragment was inserted at <i>HincII</i> site Used for the construction of KUW12H
pUC18_ox_E	TK1765:: <i>pyrF-P_{fbp}-tfe</i> -TK1766 in pUC18	DNA fragment was inserted at <i>HincII</i> site Used for the construction of KUW1E
pUMT2	pUC18 derivative (<i>P_{pyrF}::trpE</i>)	[69]
pUDE	TK2024::TK2026-TK2025- <i>trpE</i> -TK2023-TK2022 in pUMT2	DNA fragment was inserted between <i>SphI</i> and <i>KasI</i> sites (TK2026-TK2025) and <i>EcoRI</i> and <i>BamHI</i> sites (TK2023-TK2022)

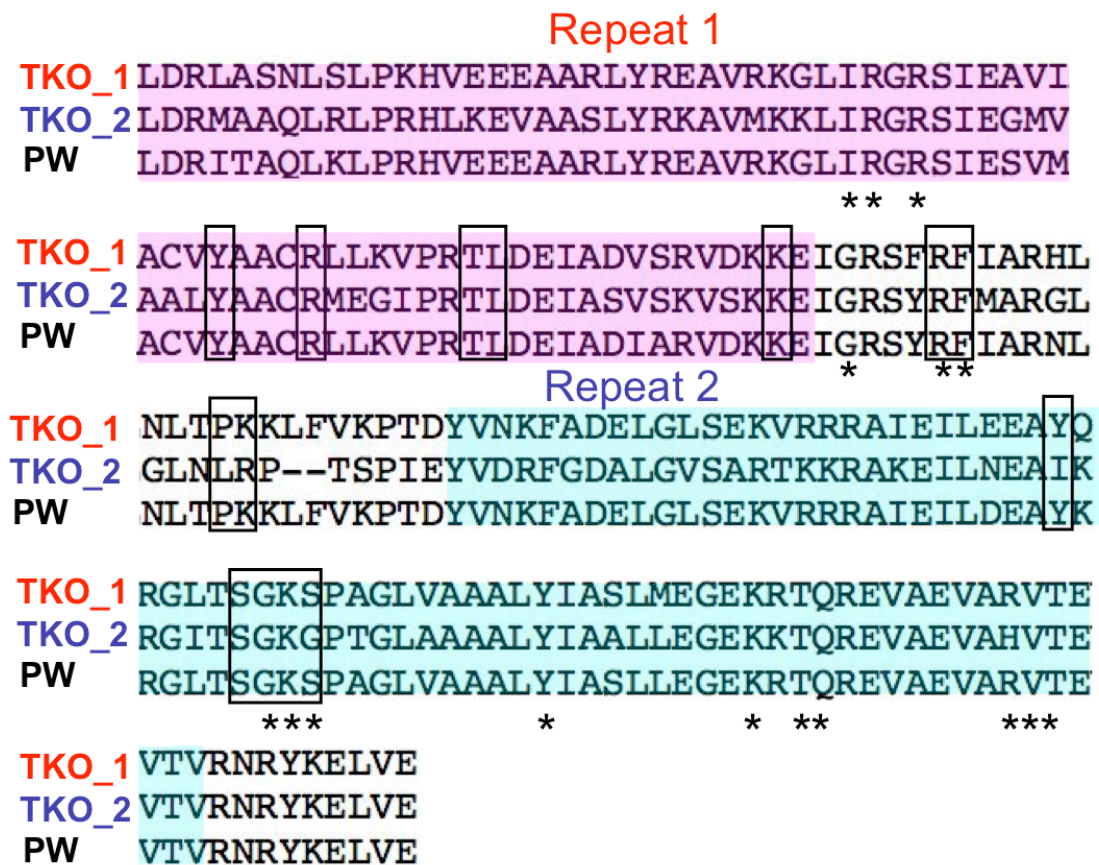


Fig. 4.1: Sequence alignment of TFB C-terminal helical repeats. C-terminal repeat sequences from *T. kodakarensis* (TKO_1:TFB1, TKO_2:TFB2) and *P. woesei* (PW) are aligned. Residues within the α -helices forming two motifs are colored in purple (repeat 1) and light blue (repeat 2). Boxed residues interact with TBP and asterisks indicate residues that interact with DNA identified by *P. woesei* X-ray crystal structure [49].

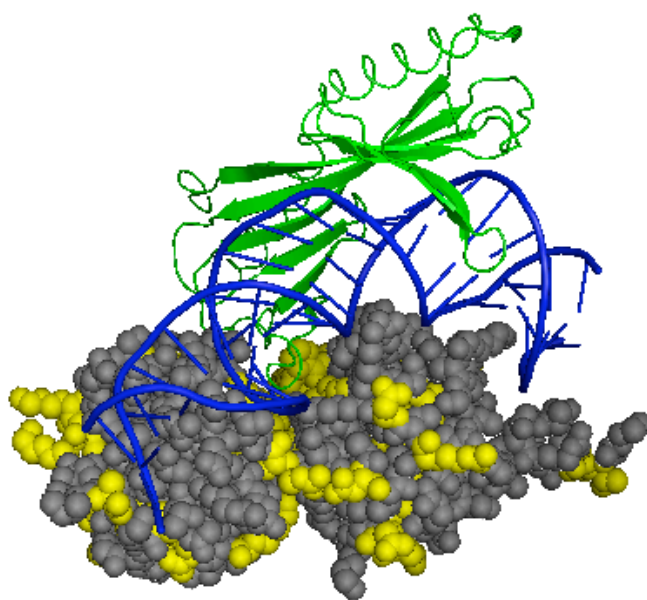
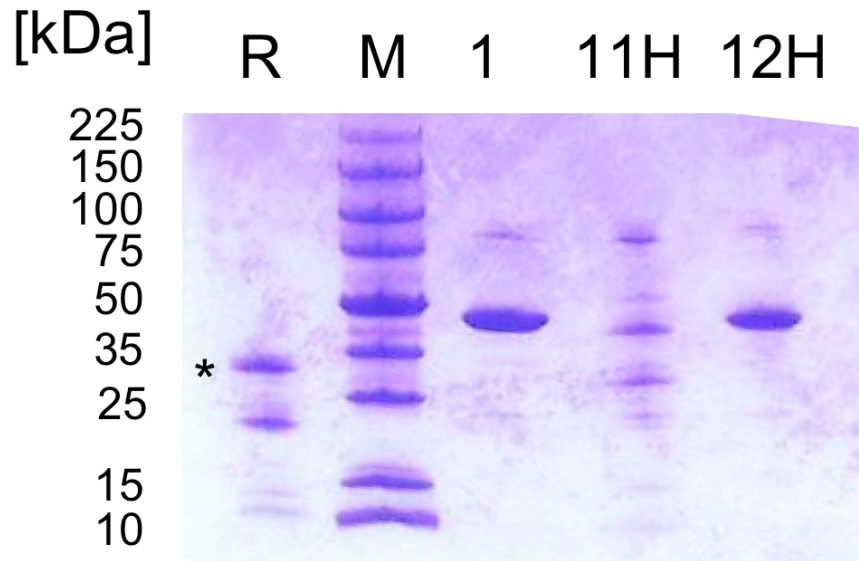


Fig. 4.2: Similarity of TFB homologues from *T. kodakarensis*. Structure of TFB C-terminal domain is shown as a surface model (from PDB:1D3U [49]). Identical residues between the two TFB homologues in *T. kodakarensis* are colored in grey and non-identical residues are shown in yellow. Cartoon representation of TBP (green) and DNA (blue) are also shown to illustrate location of each component.

A



B

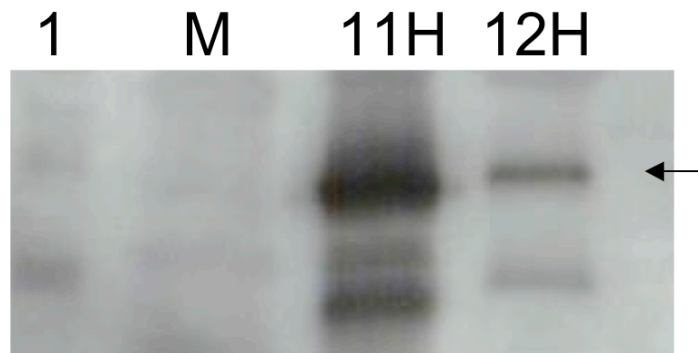


Fig. 4.3: Preliminary results from pull-down assay.

Histidine-tagged TFB1 and TFB2 were expressed in *T. kodakarensis* under gluconeogenetic condition. TFB homologues were purified by Ni-affinity chromatography as described in the text. (A) Commissie staining of the SDS-PAGE. An asterisk shows the location of histidine-tagged TFB1. (B) Western analysis of elution fraction using anti-TFB antibody (PA1024, see Chapter 3 for description). The location of band corresponds to histidine-tagged TFB is indicated by a black arrow. For both (A) and (B), M) location of the protein marker, R) recombinant histidine-tagged TFB1, 1) eluted fraction from KUW1, 11H) eluted fraction from KW11H (histidine-tagged TFB1 expression strain) and 12H) eluted fraction from KUW12H (histidine-tagged TFB2 expression strain).

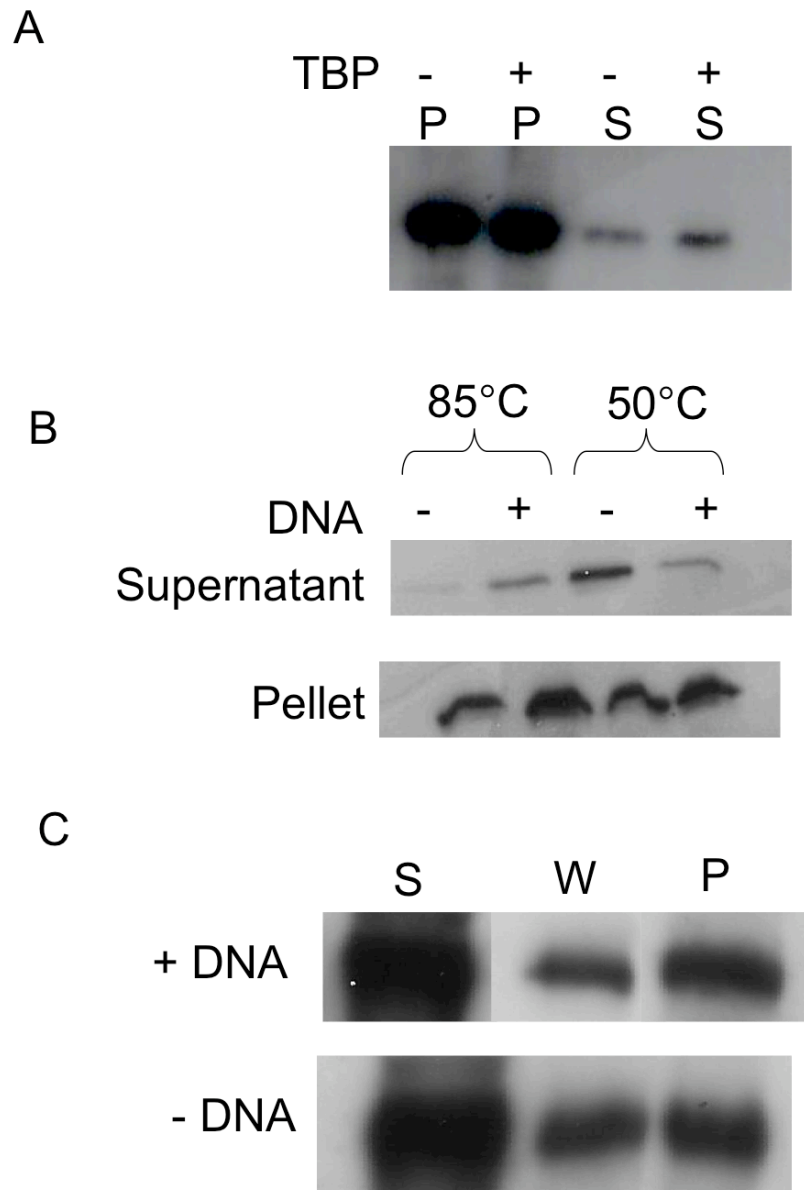


Fig. 4.4: Preliminary results from *cpkB* promoter binding assay.

(A) Western blotting result of preliminary experiment using recombinant TFB1 and TBP. Interaction of TFB1 and DNA was investigated with or without TBP. Pellet (P, proteins bound to DNA) and supernatant (S, unbound protein) fractions were separated on SDS-PAGE and detected by anti-TFB1 antibody. (B) Western blotting to examine temperature effect on protein stability. Binding experiments were conducted at two temperatures as shown in the figure. Reaction without DNA was prepared as a negative control. (C) Binding experiments with cell lysate. Concentrated cell lysate from KUW1 was mixed with DNA, which was conjugated to magnetic beads. Binding of TFB was monitored for supernatant (S), wash (W) and pellet (P) fractions by Western blotting. Reaction without DNA was prepared as a negative control.

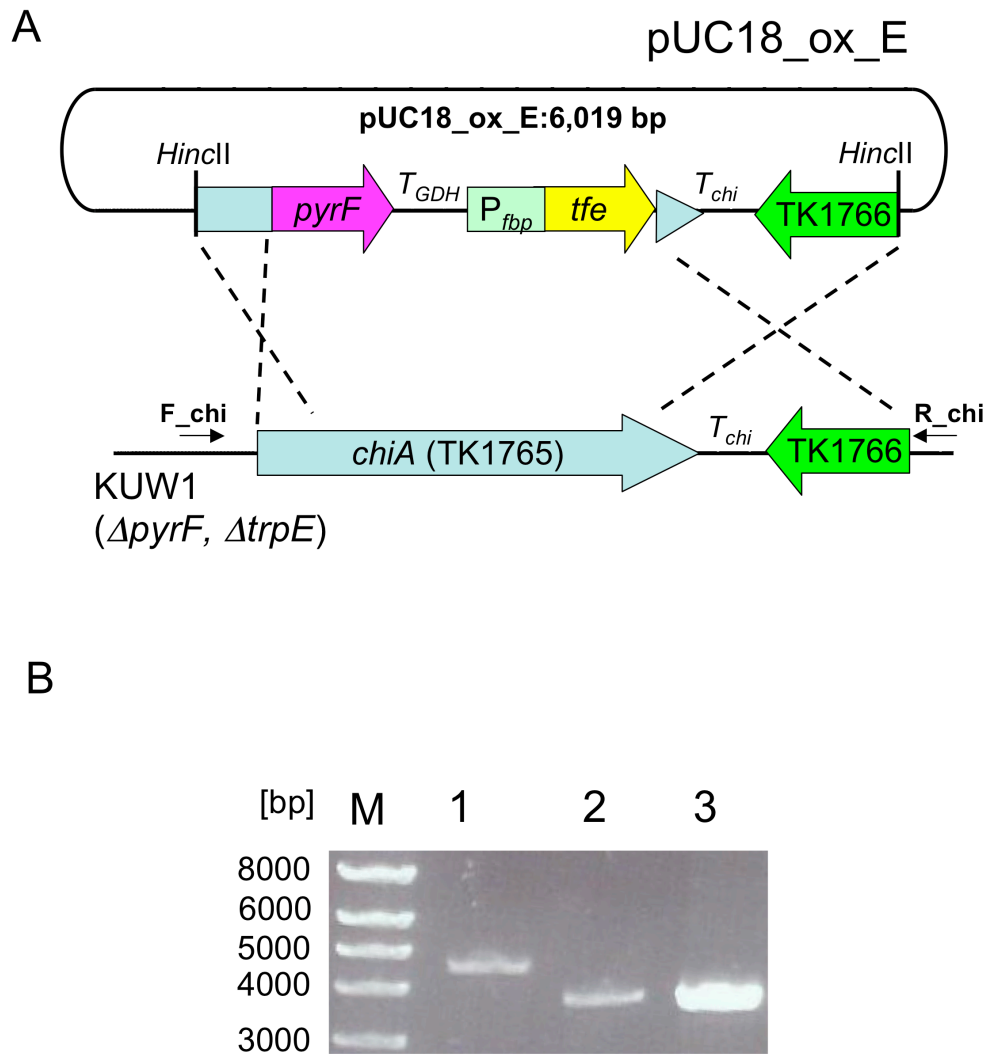


Fig. 4.5: Construction of a *tfe* conditional expression strain.

(A) Schematic diagram illustrating the expected homologous recombination using pUC18_ox_E vector. The pUC18_ox_E vector includes: 1) 5' region of *chiA* gene (light blue); 2) selection marker *pyrF* (purple); 3) GDH terminator (T_{GDH}); 4) FBPase promoter (P_{fbp} ; light green); 5) *tfe* (yellow); 6) 3' region of *chiA* gene (light blue); 7) *chiA* terminator (T_{chi}); and 8) TK1766 (downstream gene of chitinase, green). (B) Genotype analyses of conditional expression strains by PCR. Location of primer set (F_chi and R_chi) used for the PCR assay were indicated as thin black arrows in (A). Expected sizes of the PCR products are 4,723 bp for KUW1 (lane 1) and 3,890 bp for KUW1E (lane 2) and KUW1E Δtfe (lane 3).

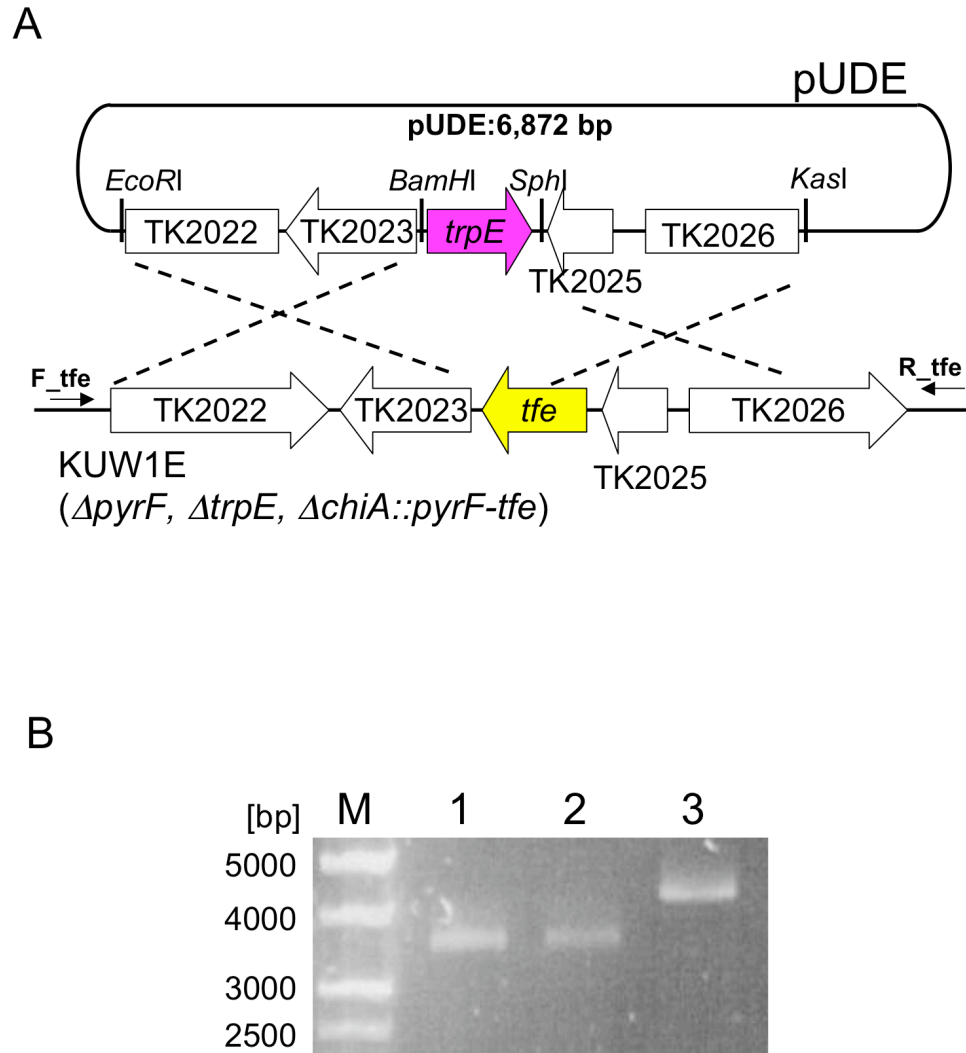


Fig. 4.6: Deletion of *tfe* from its original locus.

(A) Schematic diagram illustrating the expected homologous recombination using pUDE vector. pUDE was prepared by inserting gene cassette, in which *tfe* (TK2024) was replaced by *trpE* (selection marker) to pUMT plasmid [69]. The *trpE* is flanked by 1,321 bp upstream sequence (TK2022-2023) and 1,209 bp downstream sequence (TK2025-2026) of *tfe*. (B) Genotype analyses of conditional expression strains by PCR. Location for primer set (F_*tfe* and R_*tfe*) used for the PCR assay were indicated as thin black arrows in (B). Expected sizes of the PCR products are 3,595 bp for KUW1 (lane 1) and KUW1E (lane 2), 4,351 bp for KUW1EΔ*tfe* (lane 3).

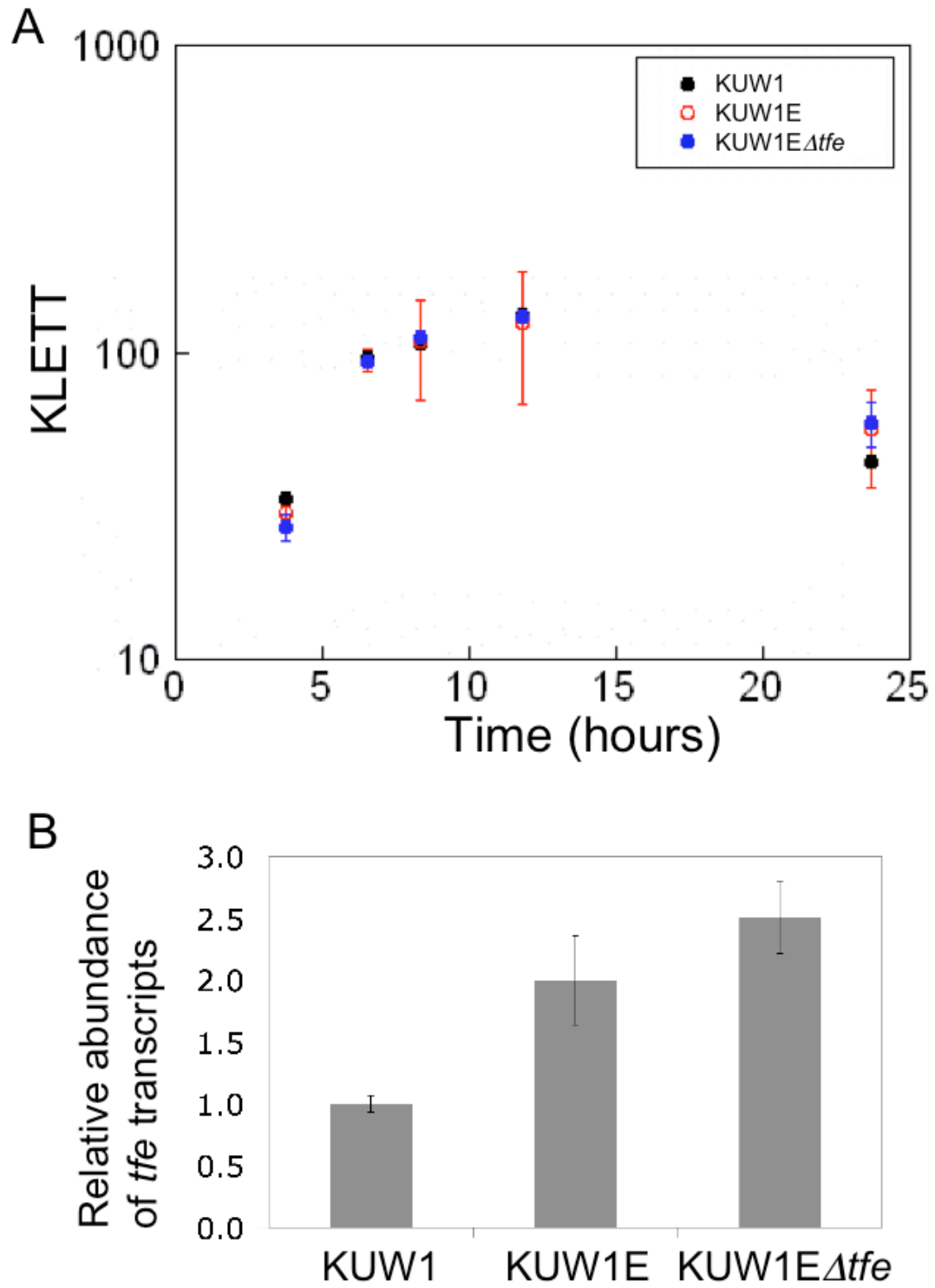


Fig. 4.7: Growth properties of *tfe* conditional expression strain.

(A) Cell growth of *T. kodakarensis* strains, KUW1 (black), KUW1E (red) and KUW1E Δtfe (blue) were monitored at 85 °C. (B) Relative quantification of *tfe* transcripts by qRT-PCR in KUW1, KUW1E and KUW1E Δtfe are summarized. Transcript abundances are shown as a fold changes relative to parental strain, KUW1.

Appendix A

Media compositions for *T. kodakarensis* studies

Table A.1: Artificial Sea Water (ASW)

	per 1 L
NaCl	20g
MgCl ₂ ·6H ₂ O	3 g
MgSO ₄ ·7H ₂ O	6 g
(NH ₄) ₂ SO ₄	1 g
NaHCO ₂	0.2g
CaCl ₂ ·2H ₂ O	0.3 g
KCl	0.5 g
KH ₂ PO ₄	0.42 g
NaBr	0.05 g
SrCl ₂ ·6H ₂ O	0.02 g
Fe(NH ₄)citrate	0.01 g

Table A.2: Mineral

	per 50 ml
MnSO ₄ ·2H ₂ O	0.5 g
CoCl ₂	0.1 g
ZnSO ₄	0.1 g
CuSO ₄ ·5H ₂ O	0.01 g
AlK(SO ₄) ₂	0.01 g
H ₂ BO ₃	0.01 g
NaMoO ₄ ·2H ₂ O	0.01 g

Table A.3: Vitamin

	per 50 ml
Niacin	10 mg
Biotin	4 mg
Pantothenate	10 mg
Lipoic acid	10 mg
Folic acid	4 mg
Aminobenzoic acid	10 mg
Thiamine(B ₁)	10 mg
Rivoflavin(B ₂)	10 mg
Pyridoxine(B ₆)	10 mg
Cobalamin(B ₁₂)	10 mg

Table A.4: Amino Acid (AA)

	per 1 L
Cystein	500 mg
Alanine	150 mg
Arginine	250 mg
Asparagine	200 mg
Asparatic Acid	100 mg
Glutamine	100 mg
Glutamic Acid	400 mg
Glycine	400 mg
Histidine	200 mg
Isoleucine	200 mg
Leucine	200 mg
Lysine	200 mg
Methionine	150 mg
Phenylalanine	150 mg
Proline	250 mg
Serine	150 mg
Threonine	200 mg
Tryptophan	150 mg
Tyrosine	200 mg
Valine	100 mg

*Adjust pH to 8 with potassium hydroxide.

Appendix B

Phylogenetic tree of TFBs from euryarchaeal species

An amino acid sequence alignment was conducted using TFB found in euryarchaeal species, whose genome sequences are available. Based on the sequence alignment, phylogenetic tree was constructed using geneious software (Biomatters Ltd., Auckland, New Zealand, <http://www.geneious.com/>) to illustrate evolutionary relationships of archaeal TFB.

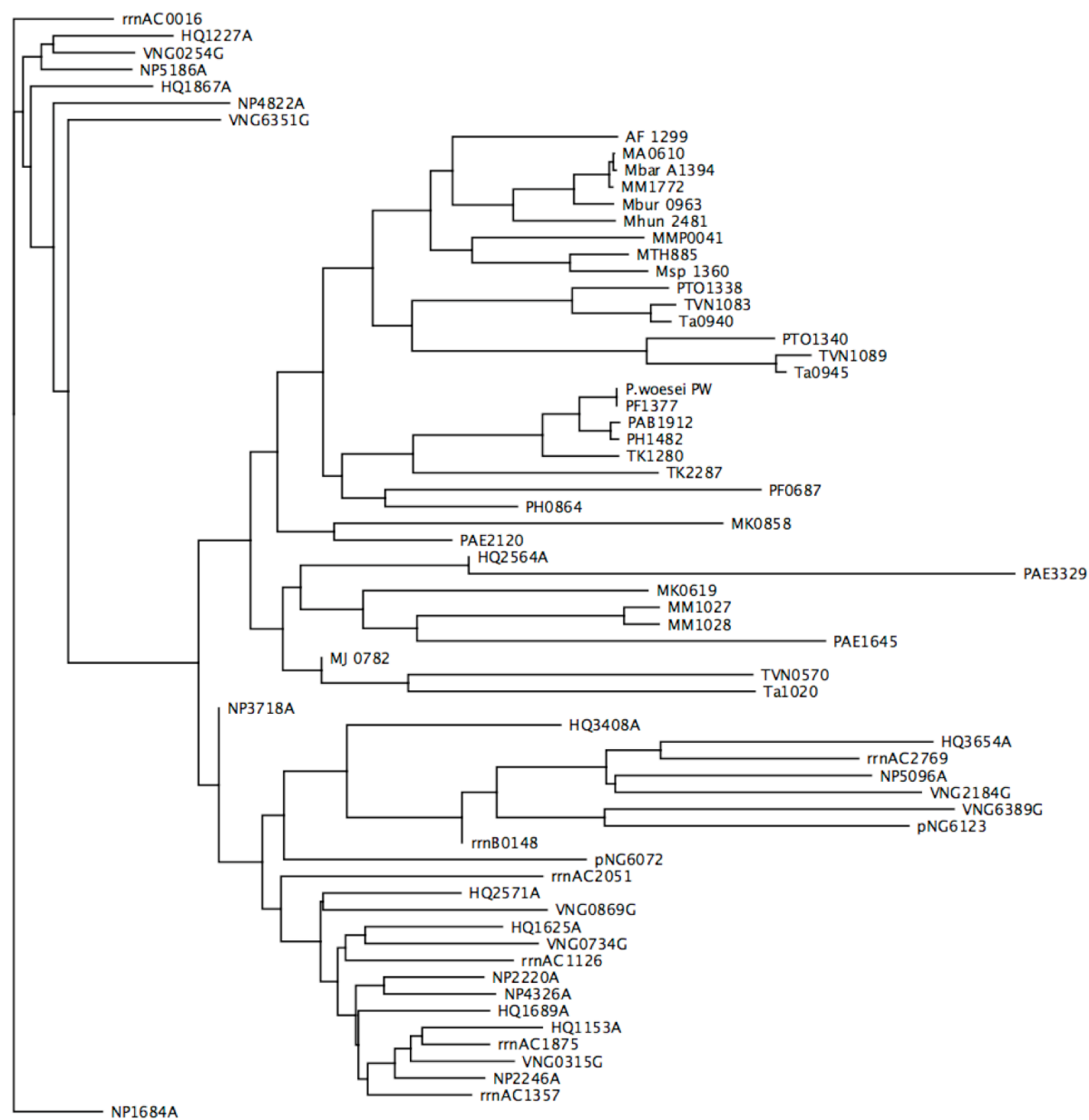


Fig. B.1: Phylogenetic tree of TFBs from euryarchaeal species.
See next page for species and abbreviations of TFB genes.

Organism name	Number of TFB isomers	Abbreviations
<i>Haloarcula marismortui</i> ATCC 43049	8	rrnA0016, rrnAC1126, rrnAC1357, rrnAC1875, rrAC205, rrcAC2769 rrcB0148, pNG6072, pNG6123
<i>Halobacterium</i> sp. NRC-1	7	VNG0254G, VNG0315G, VNG0734G, VNG0869G, VNG2184G, VNG6351G, VNG6389G
<i>Haloquadratum walsbyi</i> DSM 16790	9	HQ1153A, HQ1227A, HQ1625A, HQ1689A, HQ1867A, HQ2564A, HQ2571A, HQ3408A, HQ3654A
<i>Methanobacterium thermoautotrophicum</i> delta H	1	MTH885
<i>Methanococcoides burtonii</i> DSM 6242	1	Mbur_0963
<i>Methanococcus jannaschii</i> DSM2661	1	MJ_0782
<i>Methanococcus maripaludis</i> S2	1	MMP0041
<i>Methanopyrus kandleri</i> AV19	2	MK0619, MK0858
<i>Methanosarcina acetivorans</i> C2A	1	MA0610
<i>Methanosarcina barkeri</i> fusaro	1	Mbar_A1394
<i>Methanosarcina mazei</i> Go1	3	MM1027, MM1028, MM1072
<i>Methanosphaera stadtmanae</i> DSM 3091	1	Msp_1360
<i>Methanospirillum hungatei</i> JF-1	1	Mhun_2481
<i>Archaeoglobus fulgidus</i> DSM4304	1	AF_1299
<i>Natronomonas pharaonis</i> sp.	7	NP2220A, NP2246A, NP3718A, NP4326A, NP4822A, NP5096A, NP5186A
<i>Picrophilus torridus</i> DSM9790	2	PTO1338, PTO1340
<i>Pyrobaculum aerophilum</i> IM2	3	PAE1645, PAE2120, PAE3329
<i>Pyrococcus abyssi</i> GE5	1	PAB1912
<i>Pyrococcus furiosus</i> DSM 3638	2	PF0687, PF1377
<i>Pyrococcus horikoshii</i> shinkaj OT3	2	PH0864, PH1482
<i>Pyrococcus woesei</i>	1	P. woesei_PW
<i>Thermococcus kodakarensis</i> KOD1	2	TK1280, TK2287
<i>Thermoplasma acidophilum</i> DSM 1728	3	Ta0940, Ta0945, Ta1020
<i>Thermoplasma volcanium</i> GSS1	3	TVN0570, TVN1083, TVN1089

Appendix C

Amino acid sequences for antibody preparation

TFB1 KOD1	MSG---KRVCPVCGSTEFIYDPSRGEIVCKVCGYVIEENVVDEGPEWRA
TFB2 KOD1	MRGISPKRVCPICGSTEFIYDPRRGEIVCAKCGYVIEENVVDEGPEWRA
Similarity	
TFB1 KOD1	FDPGQREKRARVGAPESILLHDKGLSTDIGI-----DRSLTGLMREKM
TFB2 KOD1	FEPGQREKRARTGAPMTLMIHDKGLSTDI DWRDKDIHGNOITGM YRNKL
Similarity	
TFB1 KOD1	YRLRKWQSRLRVSDAAERNLAFALSELDRLASNLSLPKHVEEEAARLYR
TFB2 KOD1	RRLRMWQRRMRINDAAERNLAFALSELDRMAAQLRLPRHLKEVAASLYR
Similarity	
TFB1 KOD1	EAVRKGLIRGRSIEAVIAACVYAACRLLKVPRTLDEIADVSRVDKKEIG
TFB2 KOD1	KAVMKKLIRGRSIEGMVSAALYAACRMEGIPRTLDEIASVSKVSKKEIG
Similarity	
TFB1 KOD1	RSFRFIAR HLNLTPKKLFVKPTD YVNKFADDELGLSEKVRRAIEILEEA
TFB2 KOD1	RSYRFMARGLGLENLRP--TSPIEYVDRFGDALGVSAATKKRAKEILNEA
Similarity	
TFB1 KOD1	YQRGLTSGKSPAGLVAAALYIASLMEGEKRTQREVAEVARVTEVTVRNR
TFB2 KOD1	IKRGITSGKGPTGLAAAALYIAALLEGEKKTQREVAEVAHVTEVTVRNR
Similarity	
TFB1 KOD1	YKELVEKLNKLVPIA
TFB2 KOD1	YKELVEKLGINVPI-
Similarity	

Fig. C.1: Peptide sequences used for antibody preparation.

For antibody preparation of each TFB isomers in *T. kodakarensis*, 15-residue long peptides were used. Corresponding peptide sequences (TFB1: HLNLTPKKLFVKPTD, TFB2: DWRDKDIHGNOITGM) are boxed in red.

Appendix D

Protein purification profiles

Proteins from *T. kodakarensis* were purified following protocols described in materials and method section in Chapter 2. Elution profiles (reference OD₂₈₀, salt concentration, fraction number and elution buffer volume) and SDS-PAGE are summarized for each protein purification.

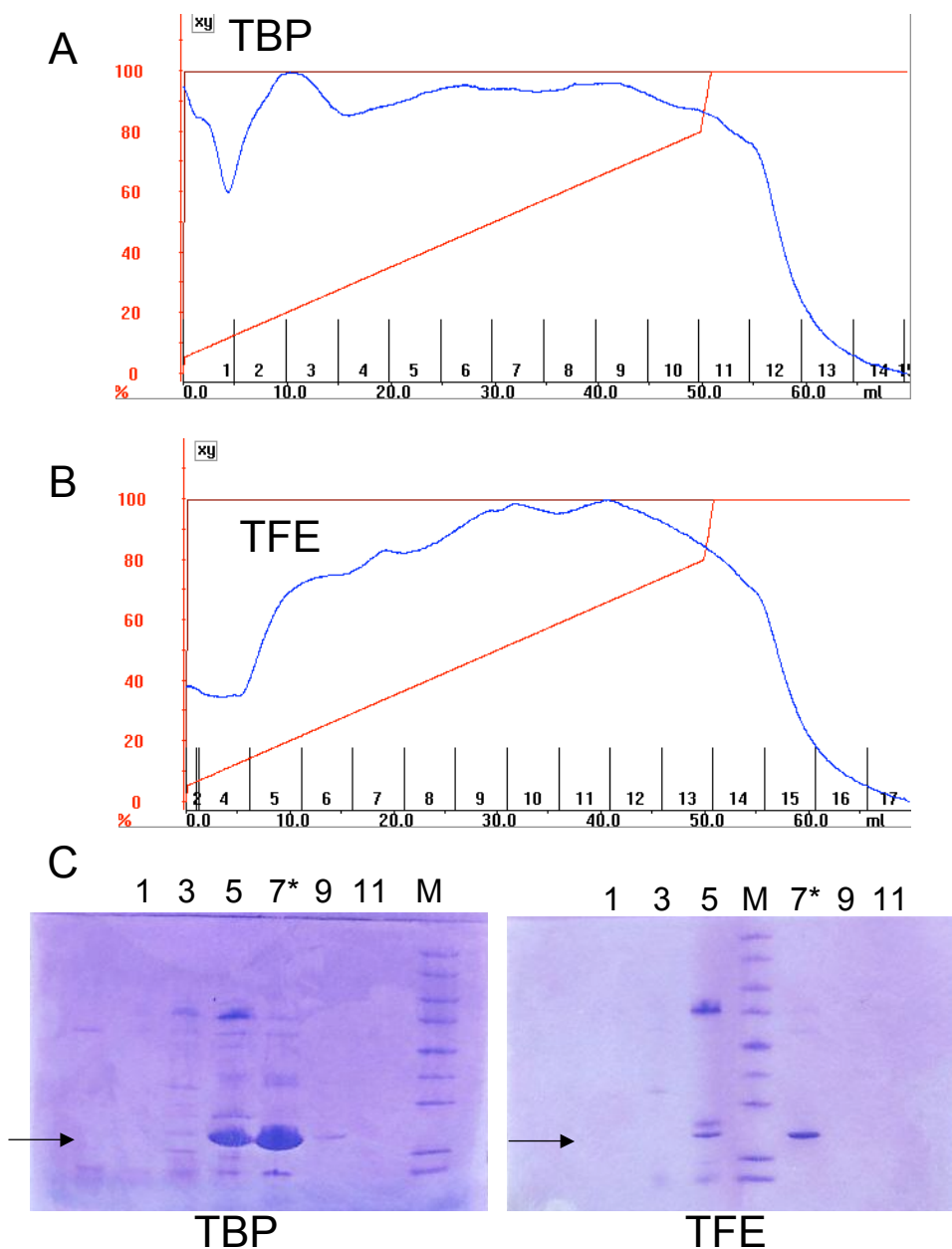


Fig. D.1: Elution profiles and SDS-PAGE of *T. kodakarensis* TBP and TFE. Elution profiles of *T. kodakarensis* (A) TBP and (B) TFE. In both figures, salt concentration in buffer (red lines, 100% equal to 1M NaCl), OD₂₈₀ reading (blue lines), fraction number and elution buffer volume are shown. (C) SDS-PAGE of TBP and TFE. Numbers on each lane corresponds to the fraction number, and M indicates the location of the marker. The location of target proteins are indicated by thin black arrows. Fractions with asterisk were used for biochemical assays.

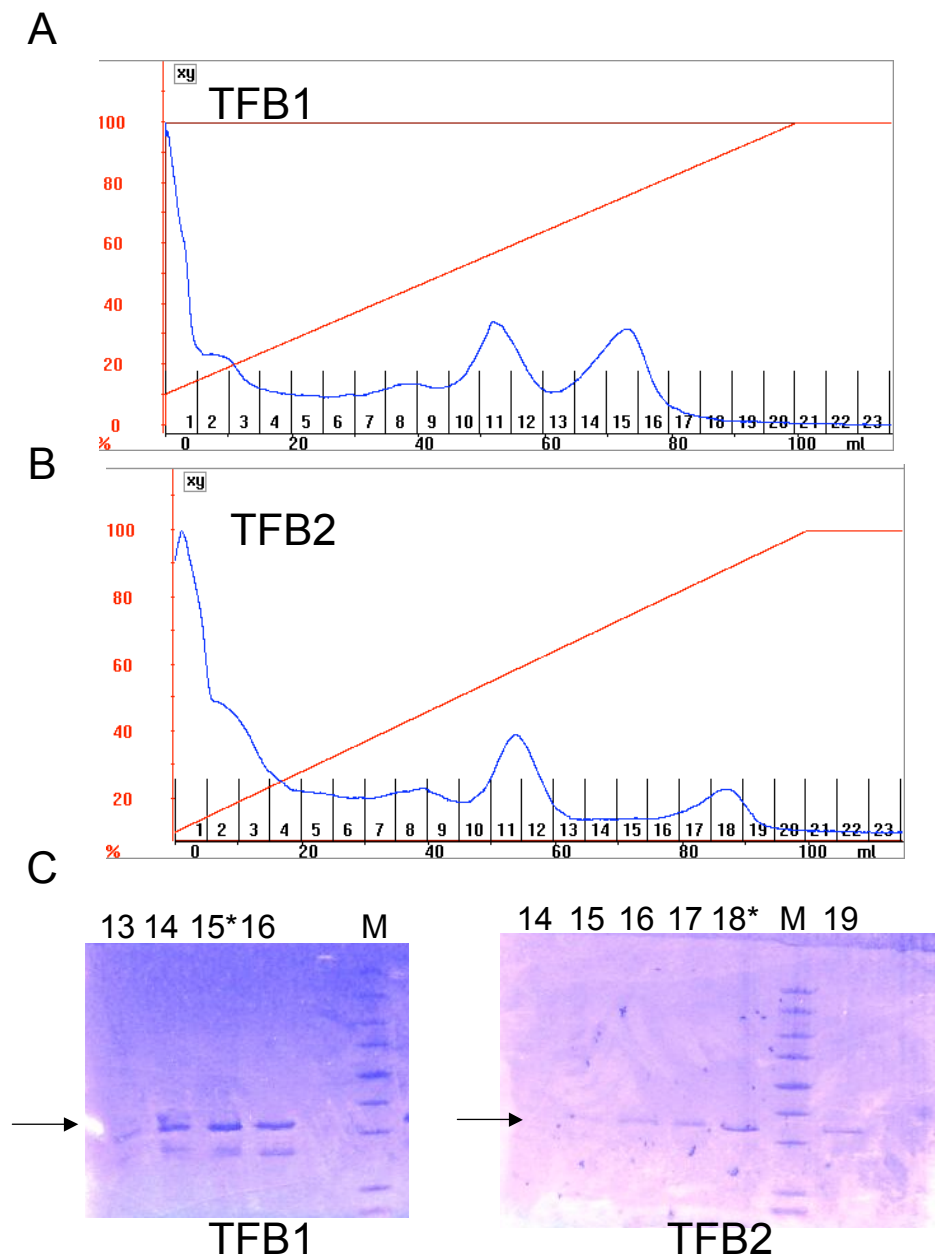


Fig. D.2: Elution profiles and SDS-PAGE of *T. kodakarensis* TFB1 and TFB2. Elution profiles of *T. kodakarensis* (A) TFB1 and (B) TFB2. In both figures, salt concentration in buffer (red lines, 100% equal to 1M NaCl), OD₂₈₀ reading (blue lines), fraction number and elution buffer volume are shown. (C) SDS-PAGE of TFB1 and TFB2. Numbers on each lane corresponds to the fraction number, and M indicates the location of the marker. The location of target proteins are indicated by thin black arrows. Fractions with asterisk were used for biochemical assays.

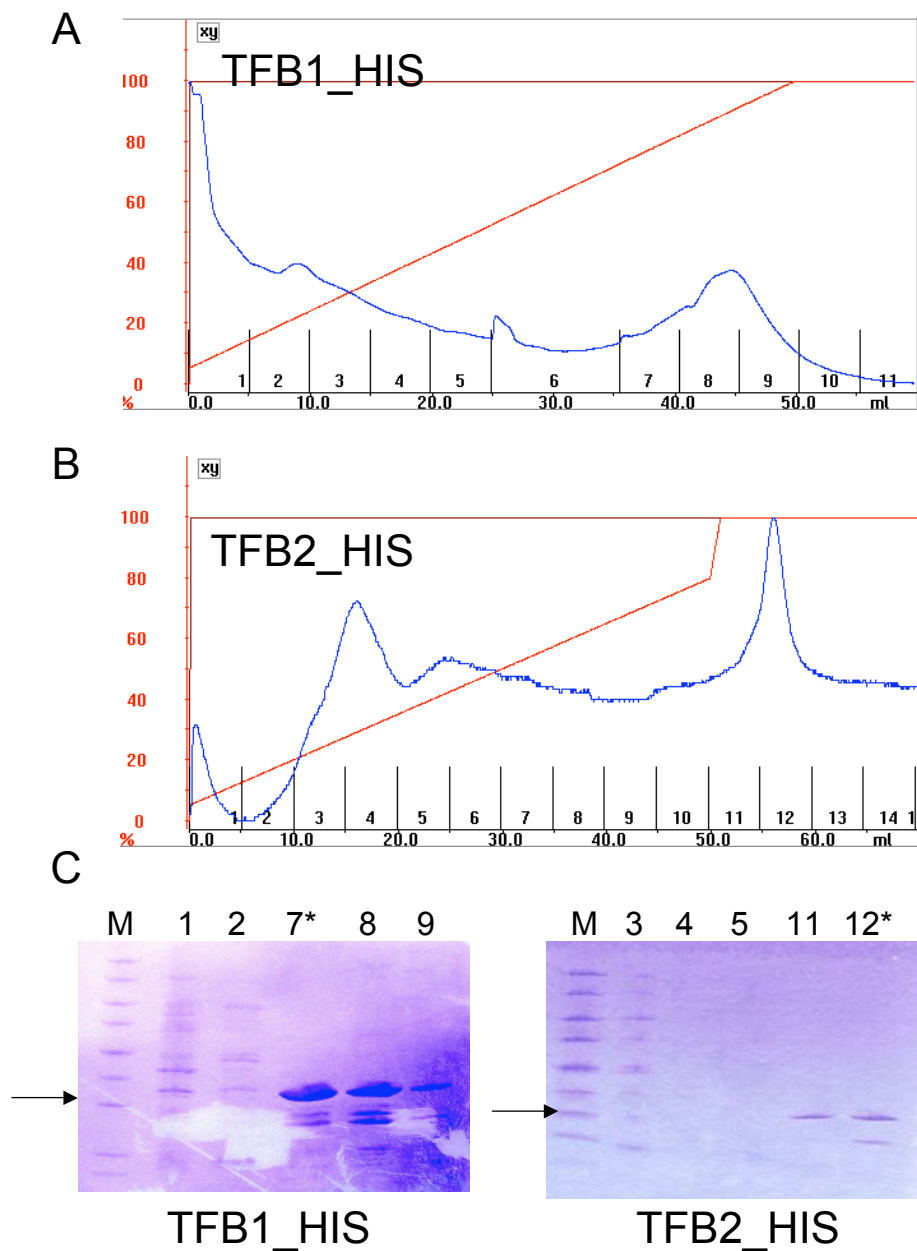


Fig. D.3: Elution profiles and SDS-PAGE of histidine-tagged *T. kodakarensis* TFB1 and TFB2. Elution profiles of *T. kodakarensis* (A) histidine-tagged TFB1 and (B) histidine-tagged TFB2. In both figures, salt concentration in buffer (red lines, 100% equal to 1M NaCl), OD₂₈₀ reading (blue lines), fraction number and elution buffer volume are shown. (C) SDS-PAGE of histidine-tagged TFB1 and TFB2. Numbers on each lane corresponds to the fraction number, and M indicates location of the marker. The location of target proteins are indicated by thin black arrows. The fractions with asterisk were used for biochemical assays.

Appendix E

QCM output

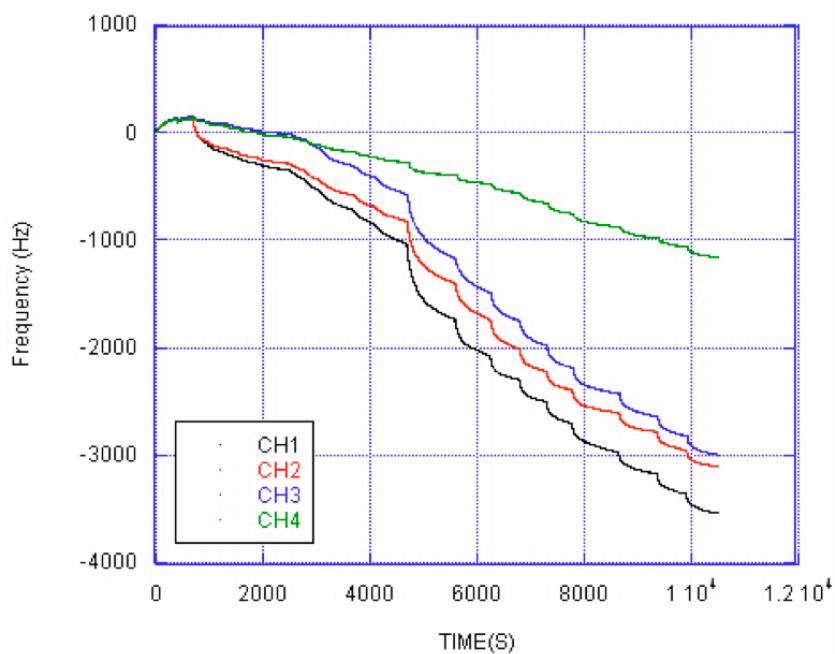


Fig. E.1: An example of QCM output.

QCM records frequency change over time as shown. The figure shows output data from an experiment using DNA-TBP (bait) and TFB1 (prey). TFB was started to titrated at approximately 2000-second time point. The green line showed response of sensor that contained no DNA (background). Black, red and blue lines are readings from other sensors containing all reaction components (DNA, TBP and TFB1).

Appendix F

Attempts to construct *tfb* deletion strain with other parental strains

Multiple attempts to construct *tfb1* or *tfb2* deletion strains were made using KUW1($\Delta pyrF$, $\Delta trpE$) strain. The deletion of *tfb1* was quite challenging and unsuccessful. Despite changing incubation temperatures and markers, single cross-over events dominate double cross-over recombination, which introduces difficulties for isolation of targeted deletion strains. On the other hand, deletion of *tfb2* from KUW1 with pUD2 plasmid was readily achieved using homologous recombination method described in Chapter 3 and deletion strain (KUW1 $\Delta tfb2$ _MT) was obtained (Figure F.1).

Several preliminary experiments were conducted using the KUW1 $\Delta tfb2$ _MT strain to search for conditions suitable for final observations. However, after several experiments, a critical error in the KUW1 $\Delta tfb2$ _MT strain was discovered; 84 bp from the gene preceding *tfb2* was also deleted due to a mistake in primer design. Because *tfb2* forms an operon with other two genes (Figure 2.3), transcription of all three genes in the *tfb2* operon had potentially been influenced by the extra base pair deletion. Thus, KUW1 $\Delta tfb2$ _MT strain was not used other than preliminary experiments described below.

Two noteworthy observations were made with the KUW1 $\Delta tfb2$ _MT strain, which contradict findings from KW128 $\Delta tfb2$ strain. Differences between the two strains appeared to be the lack of full transcription of the gene in the operon preceding *tfb2* (TK2286).

First, there was a defect in growth of KUW1 $\Delta tfb2$ -MT compared with parental strain at 90 °C (figure F.2A), whereas KW128 $\Delta tfb2$ and its parental strain did not exhibit any differences at the same condition (Figure 2.17C). I suspected one possible reason for the discrepancy was the differences in parental strains (KW128 versus KUW1). However, this was not the case as a similar observation was made for strains using another parental strain (KU216) (Figure F.2B). This observation also eliminates the possibility of a potential suppression mutations during *tfb2* deletion. Another possible reason is the polar effect from the *tfb2* deletion, although currently this has not been investigated.

Second, expression of the heat shock gene, *cpkB* showed a significant increase upon heat shock (Figure F.2C), while KW128 $\Delta tfb2$ showed only a moderate increase under the same condition (Figure 2.15B). Recently, it has been identified that the increase in *cpkB* was triggered by the presence of incomplete translation product [T. Kanai *et al.*, unpublished observations]. Thus, a possible cause of the *cpkB* increase would be the unfinished polypeptide of gene preceding *tfb2*. Because of the missing residues, the peptides could not fold to its final functional formation and somehow triggered a thermosome subunit (CpkB) production in *T. kodakarensis*.

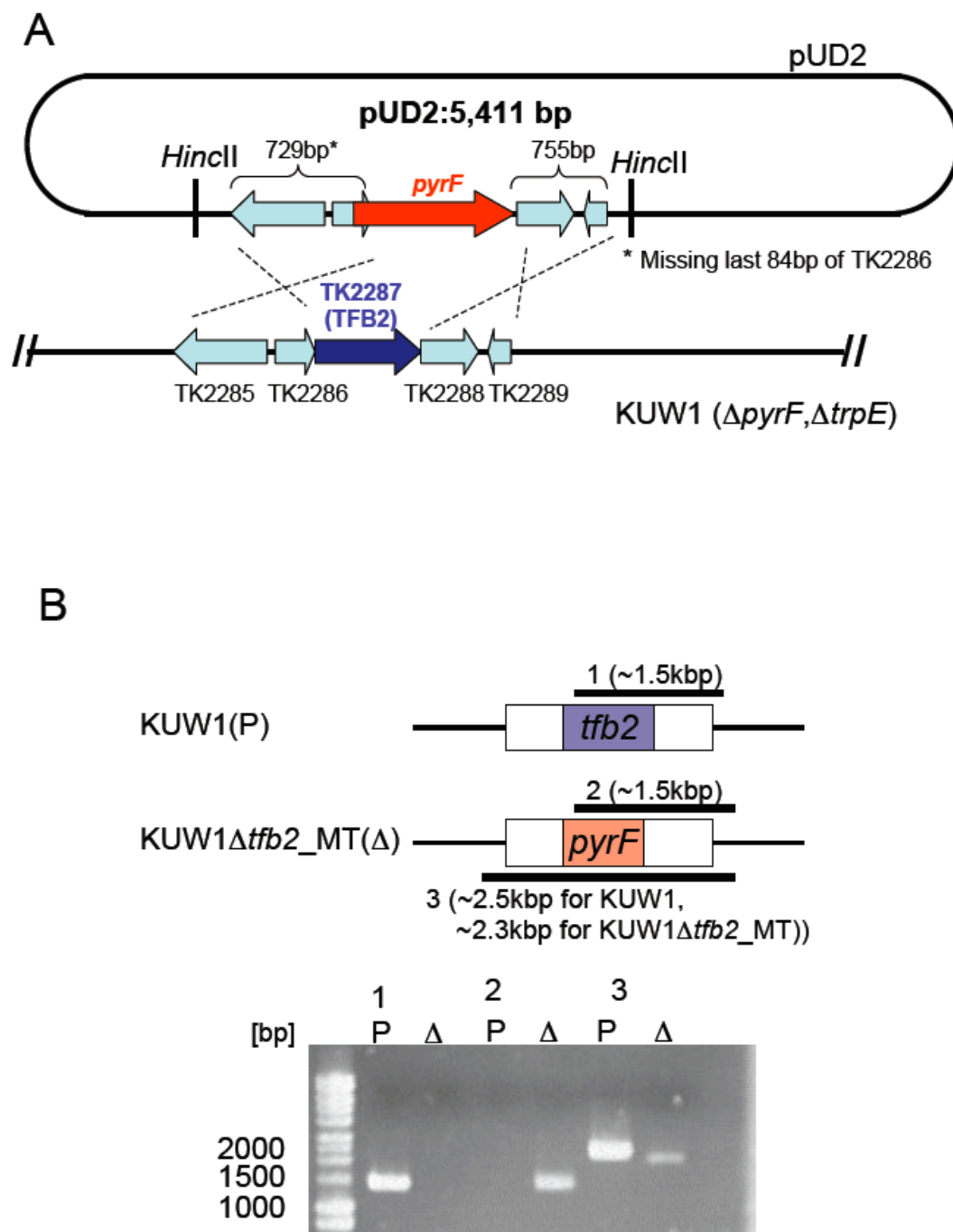


Fig. F.1: Construction of *tfb2* deletion strain.

(A) Schematic diagram illustrating the expected homologous recombination using pUD2 vector. The pUD2 vector contains 729 bp DNA sequence upstream of *tfb2* gene, selection marker *pyrF* and 755 bp downstream sequence. (B) Verification of *tfb2* gene disruption by PCR. Location and sizes for each amplicon is shown by black bars (primer sets 1 - 3). P or Δ on each lane indicates KUW1 and KUW1Δ*tfb2*_MT strain respectively.

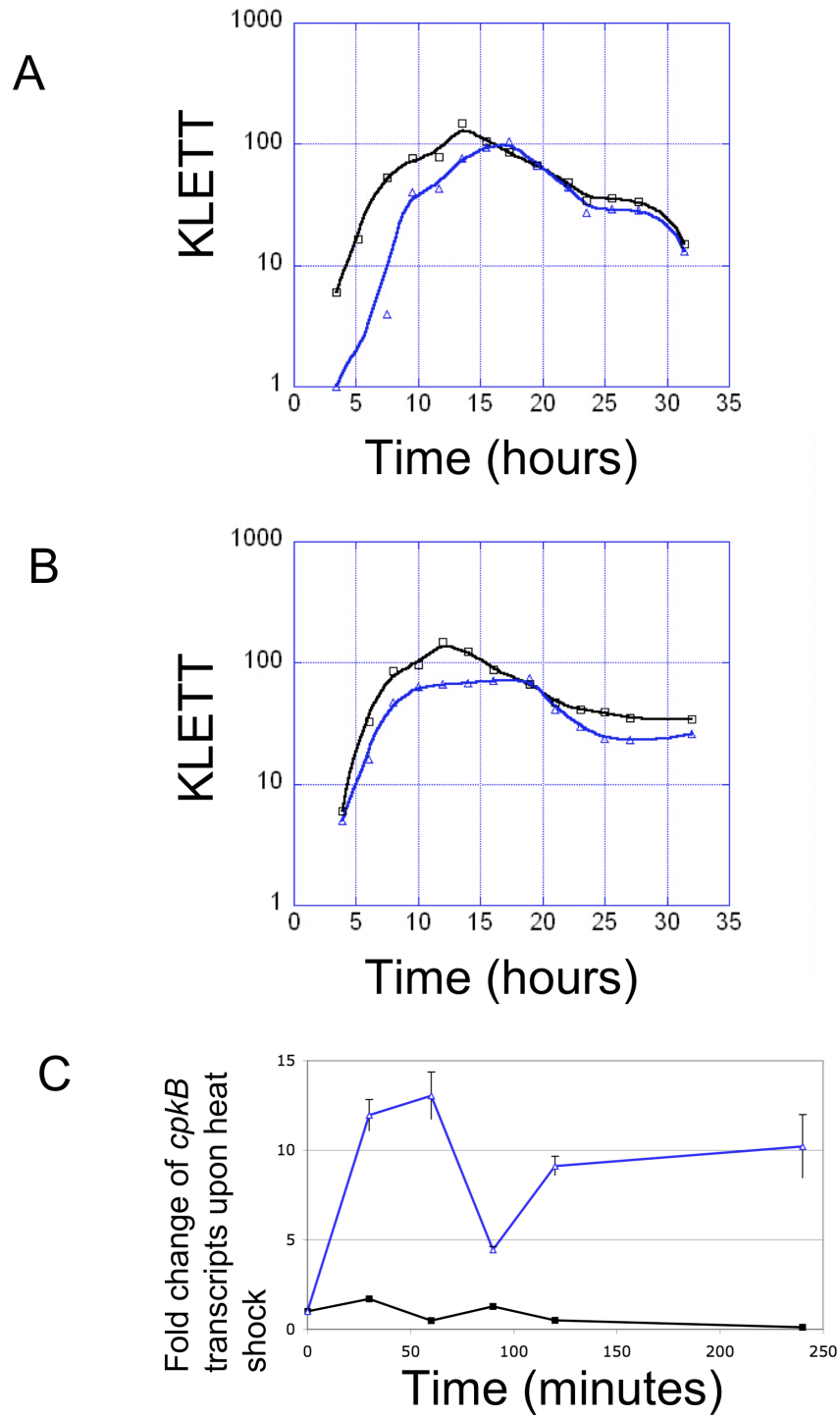


Fig. F.2: Phenotypes of *tfb2* deletion strains.

(A) Cell growth experiment for KUW1 (black) and KUW1Δ*tfb2*_MT (blue) strains at 90 °C. (B) Cell growth experiment for KU126 (black) and KU216Δ*tfb2*_MT (blue) strains at 90 °C. (C) Fold change of *cpkB* transcripts upon heat shock for KUW1 (black) and KUW1Δ*tfb2* (blue).

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Vita

Momoko Tajiri

Education

<i>The Pennsylvania State University</i>	State College, Pennsylvania	2004–2008
Ph.D. in Integrative Biosciences		
Chemical Biology Option		
<i>Georgia Institute of Technology</i>	Atlanta, GA	1999–2001
M.S. in Health Physics		
<i>University of Tsukuba</i>	Tsukuba, Japan	1992–1996
B.S. in Natural Sciences (major in Physics)		

Awards and Honors

Huck Institutes Fellowship	2004-2005
Health Physics Society Travel Grant	2000

Publications (The Pennsylvania State University)

Akira Hirata, Tamotsu Kanai, Thomas J. Santangelo, Momoko Tajiri, Kenji Manabe, John N. Reeve, Tadayuki Imanaka, Katsuhiko S. Murakami (2008) Archaeal RNA polymerase subunits E and F are not required for transcription *in vitro*, but a *Thermococcus kodakarensis* mutant lacking subunit F is temperature-sensitive. *Mol. Microbiol.* 70(3) 623-633

Momoko Tajiri, Akira Hirata, Tamotsu Kanai and Katsuhiko S. Murakami (2008) Archaeal general transcription factors, TFB1 and TFB2 in *Thermococcus kodakarensis* play distinct roles for heat shock response. (in preparation)

Publications (Georgia Institute of Technology)

Momoko Goto, Robert Rosson, J. Marion Wampler, W. Crawford Elliott, Steven Serkiz and Bernd Kahn (2008) Freundlich and Dual Langmuir Isotherm Models for Predicting ^{137}Cs Binding on Savannah River Site Soils. *J. Health Physics* 94(1) 18-32

Momoko Goto, Robert Rosson, J. Marion Wampler, W. Crawford Elliott, Steven Serkiz and Bernd Kahn (2008) Multiple Site Types for ^{137}Cs Sorption by Hydroxy-Interlayered vermiculite in Savannah River Site Soils. (in preparation)

Teaching Experience

<i>Teaching Assistant</i>	The Pennsylvania State University	2005-2006
BMB211 Elementary Biochemistry		