Trial of Efficient Protein Expression
Using Thermophilic Archaea as a Host

Thesis

Submitted to
Biology Master’s Program
To Obtain a Master of Science Degree (M.Si)

By:
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Biology Master’s Program
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ACKNOWLEDGEMENTS

There is no other expression other than a gratitude to the Lord Jesus Christ’s presence for His abundance of love to the author, so as to complete this final thesis. This thesis was written as a requirement to obtain Master degree from Kansei Gakuin University, Japan and at Satya Wacana Christian University, Indonesia.

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The end of the word, the author realizes that there is still a shortage of writing this thesis. Therefore, the author expects useful criticism and suggestions in this thesis. Hopefully with this thesis can be the foundation of further
knowledge and can be useful in its application to the community.

“And we know that all things work together for good to them that love God, to them who are called according to His purpose” Romans 8:28 (KJV)

Salatiga, November 22nd, 2017

Christina Nathalia Manuputty
ABSTRACT

Thermostable enzymes of (hyper)thermophiles which exhibit optimal growth at temperatures above 55°C have considerable potential for enzyme-based technologies as robust catalyst alternatives because most of them hardly unfold under various denaturing conditions. However, some thermostable proteins from hyperthermophiles often aggregate and form inclusion complex when they are expressed in mesophilic hosts such as Escherichia coli. A hyperthermophilic archaeon, Thermococcus kodakarensis grows fast and shows high cell density in nutrient medium and genetic technics including gene knockout and complementation by plasmid. T. kodakarensis contains a transcription repressor, SurR, which binds to the SurR-binding consensus sequence (SBS, GTTnAAC) by causing conformational change in the absence of element sulfur (S0). S0 was expected as an inducer for the expression of interest of gene in an expression system in T. kodakarensis. The aim of this research is to construct an efficient protein expression system in T. kodakarensis cells as a host.

To screen the suitable promoter in T. kodakarensis expression system, four promoters Tk0560, Tk1694, Tk2289 and Tk0895, have been chosen based on reported transcriptome analysis. Each DNA fragment containing the promoter region of these genes was cloned into Escherichia coli-T. kodakarensis shuttle plasmid pTKDR-His, yielding the plasmids pTKDR-pTk0560, pTKDR-pTk1694, pTKDR-pTk2289, and pTKDR-pTk0895 (designated as pCSG-His). Next, the T. kodakarensis DAD (ΔpyrF, ΔpdaD) cell harboring each plasmid was separately cultivated anaerobically at 85°C in nutrient-rich ASW-YT medium in the presence of S0. The cell extracts were applied to western blot analysis, performed by using antibody raised against Pc-Kat (Pyrobaculum calidifontis catalase gene as a reporter gene). The four candidate promoters contain a highly conserved 8-bp sequence element (TWTWTAWR, where W is A/T and R is A/C) and the purine-base-rich BRE (B recognition element), those of which are important for promoter strength. Among them, Tk0895 promoter led to the
highest expression of *Pc*-Kat.
To develop the switching system of expression in *T. kodakarensis*, SBS was tried to be inserted between TATA-Box and TSS in pCSG-His, but expected plasmid was not obtained. It is speculated that the high copy number SBS inserted in the plasmid has some toxic effect on *E. coli* cell growth. Each DNA fragment containing Tk0306 and Tk0460, which encodes RNA helicase, was cloned into the plasmid pCSG-His, yielding the plasmids pCSG-His-Tk0306 and pCSG-His-Tk0460, respectively. *T. kodakarensis* transformant harboring pCSG-His-Tk0306 or pCSG-His-Tk0460 were cultivated in ASW-YT media. Purification of the recombinant Tk0306 and Tk0460 proteins from cell extracts was performed using Ni-chelate affinity chromatography. The recombinant Tk0306 and Tk0460 were successfully obtained from *T. kodakarensis* extracts while Tk0460 showed smaller molecular mass than speculated size in SDS-PAGE, suggesting the recombinant Tk0460 was not fully denatured for SDS-PAGE in the present experimental conditions.
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Trial of Efficient Protein Expression Using Thermophilic Archaea as a Host

Christina Nathalia Manuputty¹,², Fujiwara Shinsuke¹

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Abstract

Thermostable enzymes of (hyper)thermophiles which exhibit optimal growth at temperatures above 55°C have considerable potential for enzyme-based technologies because most of them hardly unfold under various denaturing conditions. However, some thermostable proteins from hyperthermophiles often aggregate and form inclusion complex when they are expressed in Escherichia coli. T. kodakarensis contains a transcription repressor, SurR, which binds to the SurR-binding consensus sequence (SBS, GTTn₃AAC) by causing conformational change in the absence of element sulfur (S⁰). S⁰ was expected as an inducer for the expression of interest of gene in an expression system in T. kodakarensis. The aim of this research is to construct an efficient protein expression system in T. kodakarensis cells.

To screen the suitable promoter in T. kodakarensis expression system, four promoters Tk0560, Tk1694, Tk2289 and Tk0895, have been chosen. Each DNA fragment containing the promoter region of these genes was cloned into Escherichia coli - T. kodakarensis shuttle plasmid pTKDR-His, yielding pTKDR-Tk0560, pTKDR-Tk1694, pTKDR-Tk2289, and pTKDR-Tk0895. Next, the T. kodakarensis DAD (ΔpyrF, ΔpdaD) cell harboring each plasmid was separately cultivated anaerobically at 85°C in the presence of S⁰. The cell extracts were applied to western blot analysis, using antibody raised against Pc-Kat. Among them, Tk0895 promoter led to the highest expression of Pc-Kat.

To develop the switching system of expression in T. kodakarensis, SBS was tried to be inserted between TATA-Box and TSS in pCSG-His. Each DNA fragment containing Tk0306 and Tk0460, which encodes RNA helicase, was cloned into the plasmid pCSG-His, yielding pCSG-His-Tk0306 and pCSG-His-Tk0460. T. kodakarensis transformant harboring pCSG-His-Tk0306 or pCSG-His-Tk0460 were cultivated in ASW YT media and the purification...
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Keywords: Thermostable enzymes, *Escherichia coli*, *Thermococcus kodakarensis*, (hyper)thermophiles, plasmids, SurR-binding consensus sequence, protein.
Hal: Pemberitahuan artikel

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Tanggal: 28 November 2017
Lampiran:

Dengan hormat,

Bersama surat ini kami sampaikan bahwa abstrak dan makalah Ibu telah dipresentasikan dalam seminar dan pameran poster "The Law of Variation" pada tanggal 18 November 2017 dengan judul **Trial Of Efficient Protein Expression Using Thermophilic Archaea as A Host**

Demikian surat keterangan ini kami buat untuk digunakan sebagai mana mestinya...

Hormat kami,

Dhanang P., M.Si
Ketua Panitia
I. INTRODUCTION

1.1. Applications of thermostable enzyme in industry

Enzymes natural catalysts are useful in the chemical industry and other industrial applications. They exhibit high specificity and efficiency for their reactions and are applied in various industrial fields, including biofuel, detergent, brewing, food processing, paper manufacturing, and as tools for research and development. The representative enzymes for industrial applications are amylases, proteases, lipases, cellulases, xylanases and catalases, etc (Li et al, 2012). These are mostly derived from mesophilic organisms, which grow at normal temperature below 40°C. Most enzymes from mesophiles are relatively unstable and functional only in narrow ranges of temperature and pH, and may not be functional in the desired conditions. Enzymes are generally easy to denature in unnatural environment, and many industrial processes require enzyme to work at the high temperature, extreme pH, or at the chemical such as organic solvent. For example, in the food processing industries, amylase converts starch into more valuable products, such as dextrins, glucose, fructose, and maltose. High temperature is required to liquefy starch and make it accessible to enzymatic hydrolysis. To achieve its effective production, thermostable enzymes are needed (Robb et al, 2008). By the time, enzymes improve and generally modified to be more effective at the desirable temperature, pH or other manufacturing conditions, including the extreme conditions.

Thermostable enzymes from thermophilic organisms, which grow well above 55°C, have properties of high stability and optimal activity at temperature above 70°C (Vieille and Zeikus, 2001), and most of them hardly unfold at various denaturing conditions. One of the successful commercialized thermostable enzymes is DNA polymerase in PCR technique from several thermophiles, such as Thermus aquaticus and Thermococcus kodakarensis. There are many advantages of thermostable enzyme in industrial application like, decrease in viscosity, an increase in the diffusion coefficient of substrate, an increase in the solubility of substrate and products, improve catalytic reactions, and avoid the risk of contamination (Bouzas et al, 2006).
1.2. Thermostabilization mechanisms of enzymes from thermophiles

Extremophiles are organisms that thrive in extreme conditions. They thrive at extreme high or low temperature, in hydrothermal vent, acid and alkaline conditions, organic solvent, heavy metal, or in several extreme conditions. Thermophiles is one of extremophilic organisms that can grow at temperature above 55°C, and most of thermophiles are dominated by the archaebacteria (Rampelotto, 2013). Thermophilic organisms possess several adaptive mechanisms to maintain the stability of DNA at high temperature. All hyperthermophiles contain a DNA topoisomerase, and a reverse gyrase, which introduce positive supercoils in the molecules DNA concomitant with ATP hydrolysis, conferring a greater stability of the duplex structure of DNA and rendering it more resistant to thermal denaturation (Atomi et al, 2004b). Second, they involve some histones in DNA condensation to preserve the duplex structure of DNA, but it is not present in all hyperthermophiles (Topping and Gloss, 2004). Some hyperthermophilic methanogens, such as *Methanothermus fervidus*, accumulate large amounts of cyclic 2,3-potassium-biphospho-glycerate in their cytoplasm to protect DNA bases from depurinization, which is one of chemical damage (Bouzas et al, 2006).

Hyperthermophiles synthesize the unique thermostable enzymes, and they are optimally active at high temperature (Vieille and Zeikus, 2001). Thermostability and optimal activity at high temperatures are inherent properties of hyperthermophilic enzymes (Vieille and Zeikus, 2001). The thermostability includes thermodynamic and kinetic stabilities. Thermodynamic stability is defined by the enzyme’s free energy of stabilization (ΔG<sub>stab</sub>) and by its melting temperature (T<sub>m</sub>, the temperature at which 50% of the protein is unfolded). Thermostable enzymes show high activity at temperatures, usually 70 to 125°C. Hyperthermophilic enzymes are intrinsically stable, because their characteristics, such as surface ion pairs, decrease in solvent-exposed hydrophobic surface, and anchoring of “loose ends” (i.e., the N and C termini and loops) to the protein surface, contribute to the protein thermostability. While these characteristics, some external factors, such as salts, high protein concentrations, coenzymes, substrates, activators, or general stabilizers such as thermamine and polyamine, are key determinants to the protein thermostability (Vieille and Zeikus, 2001).
1.3. Heterologous gene expression system in *Escherichia coli* and thermophile

*Escherichia coli* is usually used for recombinant expression of protein, and also genes from hyperthermophiles have been cloned and expressed in *E. coli*. *E. coli* has many advantages as the host organism. High cell density cultures are easily achieved, transformation with exogenous DNA is fast and easy, and inexpensive to culture in simple media (Rosano and Ceccarelli, 2014). However, high level of expression of recombinant protein in *E. coli* often results in aggregation of the expressed protein molecules into inclusion bodies (Adrio and Demain, 2014; Singh et al, 2015). To avoid the formation of inclusion bodies, various approaches have been tried.

At present, heterologous gene expression system in *Sulfolobus* species is available because various *Sulfolobus*-*E. coli* shuttle vectors and genetic manipulation methods have been developed (Hwang et al, 2015). *Sulfolobus* species, an aerobic thermoacidophile, belong to the hyperthermophilic crenarchaeota in the archaeal kingdom. The system in thermophiles has a potential for obtaining functional proteins without forming aggregation unlike mesophile’s system because some thermophilic enzymes often require post-translational modifications, heat, or specific chaperones to be functional by stable folding (Vieille and Zeikus, 2001). However, *Sulfolobus* species grow slowly even in optimal condition and show low cell density in cultures. Therefore, more efficient system for heterologous gene expression in thermophile is required.

1.4. Phylogenetic location of a hyperthermophilic archaeon *Thermococcus kodakarensis*

Living organisms are divided into three domains, Bacteria, Archaea and Eucarya, based on 16s or 18s rRNA sequences. Domain of Archaea is divided into two groups with taxonomic rank of kingdoms: The Crenarchaeota (including all hyperthermophiles), and the Euryarchaeota, containing species with a variety of phenotypes (hyperthermophiles, mesophiles, methanogens, and halophiles) (Figure 1) (Petitjean et al, 2014). Archaea resembles Bacteria in that the genome size, genome organization and the absence of a nuclear membrane, but their genetic information storage and expression components are generally more closely
related that of eukaryotes than bacterial counterparts (Jäger et al, 2014). The small size genomes of hyperthermophiles define the lower limit for their genetic capacity. Based on the phylogenetic tree, many hyperthermophiles locate in the root of the tree, suggesting that hyperthermophiles locate closely to the most primitive life. In addition, hyperthermophilic archaea are very attractive microorganisms because they are sources of thermostable enzymes.

Figure 1. Phylogenetic tree of life based on 16S rRNA sequences. All microorganisms are divided into three domains of life, Archaea, Bacteria, and Eucarya.

A hyperthermophilic archaean **Thermococcus kodakarensis** was isolated from a solfatara on the shore of Kodakara Island, Japan (Atomi et al, 2004b). The stain, obligate anaerobe, has a wide growth temperature range between 60 to 100°C, with an optimal temperature at 85°C, and shows high competency (Hileman and Santangelo, 2012), when compared with other hyperthermophiles. A draft genome sequence of *T. kodakarensis* strain KOD1 has been determined (Fukui et al, 2005). Genetic technics including gene knockout and complementation by shuttle vector are available. Therefore, *T. kodakarensis* is recognized as a model organism of archaeal genetics. *T. kodakarensis*, when grows
in optimal condition, shows high cell density in cultures. In contrast to the *Sulfolobus* system, oxygen-labile thermostable protein will be applied to express in the *T. kodakarensis* system since *T. kodakarensis* is obligate anaerobe.

1.5. Sulfur dependency of cell growth of *Thermococcus kodakarensis*

*T. kodakarensis* grows in the presence or absence of S\(^0\). In the present of sulfur (S\(^0\)), *T. kodakarensis* utilizes amino acids as carbon and energy sources, that is, cyclo-octasulfur (chemical formula: S\(_8\)) as a terminal electron acceptor, while it can assimilate and grow on starch or pyruvate using H\(^+\) as a terminal electron acceptor, generating H\(_2\) in the absence of S\(^0\) (Atomi et al, 2004a). SurR (sulfur response regulator), which is an ArsR-type transcription regulator in the hyperthermophilic archaeon *P. furiosus*, was identified as a key regulator responsible for molecular hydrogen production through an S\(^0\)-dependent redox switch of the CXXC motif in its protein sequence (Lipscomb et al, 2009; Yang et al, 2011). *P. furiosus* can grow in the presence or absence of S\(^0\), depending on the available carbon source (Fiala and Stetter, 1986). SurR acts as a transcriptional repressor in the reduced form by binding to the SurR-binding consensus sequence (SBS, GTTn\(_3\)AAC) in the absence of S\(^0\), shown in Figure 2. (Lipscomb et al, 2009). By contrast, an intramolecular disulfide bond is thought to form in the SurR CXXC motif in the presence of S\(^0\), resulting in the loss of its ability to bind to the SBS. S\(^0\) is an oxidant of SurR CXXC motif to make the intramolecular disulfide bond as revealed by electrophoretic mobility shift assay (Yang et al, 2011). Thus, S\(^0\) can be utilized as an inducer for the expression of gene of interest in an expression system in *T. kodakarensis*. 
Figure 2. Molecular action of SurR. Reduced form of SurR acts as a transcriptional repressor by binding to the SurR-binding consensus sequence (SBS, GTTn$_3$AAC) in the absence of $S^0$.

1.6. Purposes of this study

In the present study, I have tried to express thermostable proteins in *T. kodakarensis*. Thermostable proteins are expected to be folded in a range of natural folding rate, resulting in decrease of inclusion complex when expressed in thermophiles. To develop a heterologous gene expression system in *T. kodakarensis*, I used an *E. coli*-*T. kodakarensis* shuttle vector, pTKDR, as a parental vector. First, I selected a promoter, which has high transcriptional activity. Next, I tried to introduce a switching operator SBS coupled with intrinsic transcriptional regulator, SurR. Finally, I evaluated gene expression system in *T. kodakarensis* to express RNA helicases, Tk0306 and Tk0460, from *T. kodakarensis*, which are known to form insoluble complex in *E. coli*. 
II. MATERIAlS AND METHODS

2.1. Strains and media

Strains were used in this study are shown in Table 1. The standard growth medium for cultivation of *Thermococcus kodakarensis* DAD (genotype, ∆pyrF ∆pdaD) (Fukuda et al, 2008) was used in nutrient-rich ASW-YT medium with supplemented with 2.0 g L⁻¹ S⁰ and 1 mM agmatine. ASW-YT medium was composed with 0.8 x artificial seawater, 0.5 g L⁻¹ yeast extract, and 0.5 g L⁻¹ tryptone, and additionally was added 0.5 g L⁻¹ pyruvate. The composition of 0.8 x artificial seawater (ASW) is 20 g NaCl, 3.0 g MgCl₂·6H₂O, 6.0 g MgSO₄·7H₂O, 1.0 g (NH₄)₂SO₄, 0.2 g NaHCO₃, 0.3 g CaCl₂·2H₂O, 0.5 g KCl, 0.42 g KH₂PO₄, 0.05 g NaBr, 0.02 g SrCl₂·6H₂O, and 0.01 g Fe(NH₄) citrate, per liter. *T. kodakarensis* cells were routinely grown under anaerobic conditions at 85°C.

*Escherichia coli* DH5α (Hanahan, 1983) (Takara Bio, Ohtsu, Shiga, Japan) was cultured in 5 ml LB medium in a test tube at 37°C for 12-16 hours and reciprocal shaking at 150 rpm. The composition of LB medium is 10 g of tryptone, 5.0 g of yeast extract, and 10 g of NaCl per liter. To make LB agar plate, 1% agar was added to LB medium. Ampicillin (50 μg mL⁻¹) was added to the LB medium to select transformants.

2.2. DNA manipulation and sequencing

General DNA manipulations were performed by following methods described by Green and Sambrook (Green and Sambrook, 2012). PCR was carried out using KOD-plus or KOD-plus Neo (Toyobo, Osaka, Japan) as DNA polymerase. The compositions of the 50 μl PCR mixture contained 10 μM each primer, 25 mM MgSO₄, 2 mM dNTPs, 10x buffer for KOD-plus, and KOD-plus polymerase (Toyobo, Osaka, Japan). Restriction and modifying enzymes were purchased from Takara Bio or Nippon Gene (Tokyo, Japan). PCR products were confirmed by agarose gel electrophoresis, and products were purified by using FastGene Gel/PCR Extraction Kit (Nippongene, Tokyo, Japan). Plasmid was extracted by using GenElute™ Plasmid Miniprep Kit by following manufacture’s instruction (Sigma-Aldrich, St. Louis, MO). Purity and quality of the plasmids were confirmed by measuring optical density NanoDrop ND-1000 Spectrophotometer (ThermoFisher, Waltham, MA) at 260 and 280 nm, and by agarose gel electrophoresis. DNA sequencing was performed using a BigDye
Terminator Cycle Sequencing kit, ver. 3.1, and a model 3130 capillary sequencer (Applied Biosystems, Foster City, CA, USA).

2.3. Construction of expression vectors containing candidate promoters

Plasmids pTKDR (Hidese et al, 2014a) and pCSG-PcKat (Nishikawa, Master Thesis, 2015) were used in this study as sources of plasmid variants. The nucleotide sequence of His-tag was inserted using the QuikChange protocol (Agilent Technologies, Santa Clara, CA) with the primers His insert-1-Fw and His insert-1-Rv into pTKDR, yielding pTKDR-His. The DNA fragments containing the 5'-flanking region of Tk0560, Tk1694, and Tk2289 were PCR-amplified with a set of primers Tk0560p-Fw and Tk0560p-Rv, Tk1694p-Fw and Tk1694p-Rv, and Tk2289p-Fw and Tk2289p-Rv, respectively, from T. kodakarensis genomic DNA. Each fragment was separately cloned into the EcoRV/SalI sites of pTKDR-His, yielding pTKDR-pTk0560, pTKDR-pTk1694, and pTKDR-pTk2289. The nucleotide sequence of His-tag was then inserted into pCSG-PcKat using the QuikChange with the primers His insert-2-Fw and His insert-2-Rv, yielding pCSG-His.
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<tbody>
<tr>
<td>E. coli</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, Φ80dlacZΔM15, Δ(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17[rK&lt;sup&gt;−&lt;/sup&gt;, mK&lt;sup&gt;+&lt;/sup&gt;], phoA, supE44, λ, thi&lt;sup&gt;−1&lt;/sup&gt;, gyrA96, relA1</td>
<td>Stratagene</td>
</tr>
<tr>
<td>DH5α</td>
<td>recA1, endA1, hsdR17[rK&lt;sup&gt;−&lt;/sup&gt;, mK&lt;sup&gt;+&lt;/sup&gt;], phoA, supE44, λ, thi&lt;sup&gt;−1&lt;/sup&gt;, gyrA96, relA1</td>
<td>Stratagene</td>
</tr>
<tr>
<td>BL21-Codon-Plus(DE3)-RIL</td>
<td>E. coli B F&lt;sup&gt;−&lt;/sup&gt; ompT hsdS&lt;sup&gt;rb&lt;/sup&gt;mg &lt;del&gt;dcm&lt;/del&gt; Tet&lt;sup&gt;+&lt;/sup&gt; gal λ(DE3) endA The [argU ileY leuW Cam&lt;sup&gt;+&lt;/sup&gt;]]</td>
<td>Stratagene</td>
</tr>
<tr>
<td>T. kodakarensis</td>
<td>ΔpyrF ΔpdaD</td>
<td>Fukuda et al, 2008</td>
</tr>
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</table>

<table>
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<th>Primers</th>
<th>Source or references</th>
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<tr>
<td>His insert-1-Fw</td>
<td>CGAATTCCTCTCCGATCATGTCGACCATCATCATCATCATCATAAGCGGCCATATGTACCTAAGAATAGACC</td>
</tr>
<tr>
<td>His insert-1-Rv</td>
<td>GATGATGATGGTCGACCATTCCACAACACTTGGTAAA</td>
</tr>
<tr>
<td>Tk0560p-Fw</td>
<td>TTTATCACCACGTTTAAGTC</td>
</tr>
<tr>
<td>Tk0560p-Rv</td>
<td>AAAAAAAACATATGTTCTGCGACCTCCGGAAGTT</td>
</tr>
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<td>Tk1694p-Fw</td>
<td>CCCATCTGTCTTTTTTTGT</td>
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<td>Tk1694p-Rv</td>
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</tr>
<tr>
<td>Tk0460-Rv</td>
<td>AAAAAACATATGATGAGTTTTAGAAGAATCTGACC</td>
</tr>
<tr>
<td>His insert-2-Fw</td>
<td>ACCCAAGGAGGTTGTGTAATGTGACACCACCATC TACATCATCATAGCGCCATATGACATTACCAAAACATATGTCGACCATCATAAGAAAT TAGACC</td>
</tr>
<tr>
<td>His insert-2-Rv</td>
<td>GATGATGATGGTCGACCATTCCACAACACTTGGTAAA</td>
</tr>
<tr>
<td>SBS-insert-Fw</td>
<td>TTTGCGCCGCTGAAAAGGTTAACAACCGGAAGAGTTATTAAACCCCAAAGTTGTGTAACGCTATAGA</td>
</tr>
<tr>
<td>SBS-insert-Rv</td>
<td>AAGTGTGTTCATACCGTTTCCAACACTTGGGTTTAAATACTTTTCGCGTGTGTAACGCTATAGA</td>
</tr>
</tbody>
</table>

This study
2.4. Transformation of *T. kodakarensis* DAD cells

The transformation of *T. kodakarensis* cells was performed using DAD strain *T. kodakarensis* (ΔpyrF ΔpdaD), which shows agmatine auxotroph, cultivated anaerobically in ASW-YT medium with S\(^0\) and 1 mM agmatine at 85°C (Fukuda et al, 2008). After cultivation, cells were harvested by centrifugation (5,000 x g, 4°C, 10 min) and washed twice by 0.8 x ASW. The cells were suspended in 200 μl of 0.8 xASW, and kept on ice for 30 min. After the addition of 5.0 μg plasmid into solution, further incubated on ice for 1 h, transformants were inoculated into 20 ml of ASW-YT medium with S\(^0\) and then grown at 85°C for 12 h. Transformants were selected in the absence of agmatine. Cells were maintained in ASW-YT-S\(^0\) medium.

2.5. Reporter assay

Transformants containing each plasmid, pTKDR-pTk0560, pTKDR-pTk1694, pTKDR-pTk2289 and pCSG-His, were separately grown in 40 ml ASW-YT-Pyr-S\(^0\) medium at 85°C. Cells grown at mid-log phase were collected by centrifugation (13,200 rpm, 4°C, 10 min). The cells were suspended by 20 mM Tris-HCl (pH 8.0) and disrupted by sonication to obtain crude extracts. The crude extracts (20 μg each) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF) (ATTO, Tokyo, Japan). Immunodetection was performed with both antibody raised against *Pc*-Kat and Alexa Fluor 700 goat anti-rabbit IgG (Invitrogen, Carlsberg, CA) by using Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

2.6. Trials of construction of switching system of expression in *T. kodakarensis*

The nucleotide sequence of SurR-binding sites (SBS, GAA\(\_\_\_\_\)TTC) was tried to insert using the QuikChange protocol with the primers SBS-insert-Fw and SBS-insert-Rv into pTKDR-His, yielding pTKDR-SBS-His.

2.7. Cloning of RNA helicases in *T. kodakarensis*

The DNA fragments containing the Tk0306 and Tk0460 were PCR-amplified with a set of primers Tk0306-Fw and Tk0306-Rv and Tk0460-Fw and Tk0460-Rv, respectively, from *T.*
kodakarensis genomic DNA. Each PCR-amplified DNA fragments encoding two kinds of RNA helicases (Tk0306 and Tk0460) were digested by NdeI/NotI and cloned into the same digestion sites of pCSG-His, yielding the pCSG-His-Tk0306 and pCSG-His-Tk0460.

2.8. Expression of helicases in T. kodakarensis

Each plasmid, pCSG-His-Tk0306 and pCSG-His-Tk0460, was introduced to T. kodakarensis DAD cells. The transformants were grown in 500 ml ASW-YT- S0 medium at 85°C. Cells were harvested by centrifugation (13,200 rpm, 4°C, 10 min) at mid-log phase. The cells were suspended by 20 mM Tris-HCl (pH 8.0) and disrupted by sonication. The extracts (20 µg each) were separated by SDS-PAGE.

2.9. Protein purification by Ni-affinity chromatography

The transformants containing each plasmid, pCSG-His-Tk0306 and pCSG-His-Tk0460, were separately grown in 500 ml ASW-YT-Pyr-S0 medium at 85°C for 12-16 hours. Cells grown at mid-log arithmic phase were harvested by centrifugation (8,000 rpm, 4°C, 10 min). The cells were suspended in sonication buffer (20 mM Tris-HCl pH 7.6, 1 mM mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 300 mM NaCl) and disrupted by sonication. The crude extracts were separately obtained by centrifugation (8,000 rpm, 4°C, 10 min) and then applied to Nickel-NTA affinity column (3 ml) (Qiagen, Hilden, Germany). After washing the protein-bound column with five column volume of sonication buffer, the recombinant Tk0306 or Tk0460 was eluted in stepwise with five column volume of each sonication buffer containing 5 mM imidazole, 10 mM imidazole, 50 mM imidazole, 100 mM imidazole, and 500 mM imidazole.
III. RESULTS

3.1. Screening of a promoter with high constitutive activity from transcriptome analysis

Archaeal promoters typically have a BRE (B Recognition Element) followed by a TATA-box and direct transcription initiation site at ~23 bp downstream from the TATA-box sequence (Jäger et al, 2014). In the present study, the mRNA level of all the genes were evaluated by transcriptome analyses by RNA-seq (Jäger et al, 2014). To screen the suitable promoter, which possesses efficient transcriptional activity, four promoters have been focused from ten candidate promoters, Tk0560, Tk1694, Tk2289 and Tk0895 as shown in Table 2, because TSS (Transcription Start Site) position is elucidated in these genes as shown in Figure 3. Nucleotide sequences of the four candidate promoters are highly conserved and they have BRE and TATA-box sequences (Figure 3). The four candidate promoters of Tk0560, Tk1694, Tk2289, and Tk0895 genes, contain a highly conserved 8-bp sequence element (TWTWTAWR, where W is A/T and R is A/C) that is recognized for TBP (TATA-binding protein) binding site. The purine-base-rich BRE, which is located immediately upstream of the TATA-box, is also important for promoter strength (Ao et al, 2013). Tk1311 and Tk1685 (FNOR2) genes, whose transcripts are low abundance in T. kodakarensis cells, do not have purine-base-rich BRE and conserved 8-bp sequence element in TATA-box sequences.

Table 2. List of promoter candidates

<table>
<thead>
<tr>
<th>TK#</th>
<th>P exp library</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>108 Tko-sR04 + hypothetical protein</td>
</tr>
<tr>
<td>2</td>
<td>1311 50S ribosomal protein L7 Ae</td>
</tr>
<tr>
<td>3</td>
<td>560 DNA/RNA-binding protein A1bA</td>
</tr>
<tr>
<td>4</td>
<td>1694 ferredoxin 1</td>
</tr>
<tr>
<td>5</td>
<td>1067 hypothetical protein</td>
</tr>
<tr>
<td>6</td>
<td>2289 histone B</td>
</tr>
<tr>
<td>7</td>
<td>1431 glutamate dehydrogenase</td>
</tr>
<tr>
<td>8</td>
<td>1331 Lrp/AsnC family transcriptional regulator</td>
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<tr>
<td>9</td>
<td>1484 hypothetical protein</td>
</tr>
<tr>
<td>10</td>
<td>895 S-layer protein</td>
</tr>
</tbody>
</table>

Protein-encoding genes in the pyruvate (P exp). T. kodakarensis cells were obtained after growing exponentially in ASW-YT medium with pyruvate (P exp). The TKxxxx are the numerical gene (c).
3.2. Evaluation of transcription activity

Cell extracts were obtained from *T. kodakarensis* harboring each plasmid pTKDR-pTk0560, pTKDR-pTk1694, pTKDR-pTk2289, and pTKDR-pTk0895 grown at 85°C. Catalase (*Pc*-Kat) encoded by *Pc*-kat from *Pyrobaculum calidifontis* (Amo et al, 2002) requires manganese for its activity. *Pc*-Kat was expressed as inactive-form, probably due to an inability of manganese cofactor-assembly into the protein in *T. kodakarensis*. However, *Pc*-Kat was applicable as a reporter to monitor gene expression by western blot analysis (Nagaoka et al, 2013). The expression level of *Pc*-Kat from each plasmid was shown in Figure 4. *T. kodakarensis* cells harboring pTKR was used as a negative control. The cell harboring plasmid pTKDR-pTk0895 showed the highest expression of *Pc*-Kat when compared to that with each plasmid, pTKDR-pTk0560,
pTKDR-pTk1694 and pTKDR-pTk2289 tested. Based on the obtained data, pTKDR-pTk0895 was used to develop an expression system in *T. kodakarensis*. I designed the plasmid pTKDR-pTk0895 as pCSG-His.

**Figure 4.** The heterologous expression of *Pc*-Kat protein of *T. kodakarensis*. **a)** Gel was stained with Coomassie Brilliant Blue (CBB). M: low molecular mass markers. **b)** Western Blot analysis using anti-*Pc*-Kat antibody. pTKR (promoter less plasmid) is a
negative control. M: low molecular mass markers. The arrow
indicates the band position of Pc-Kat protein (33 kDa). c). Relative
band intensity calculated by Pc-Kat expression level. The
expression level of pCSG-His was set to 1.

3.3. Trial of construction of switching system of expression in
*T. kodakarensis*

To achieve sulfur dependent expression control, I tried to
insert the SBS region between core promoter and transcriptional
start site. In the absence of S\(^0\), a reduced form of SurR binds to
SBS located in just upstream and downstream regions of TATA-
Box, resulting in the repression of transcription. By adding S\(^0\), a
conformational change of SurR is occurred to be an oxidized form,
a DNA-unbound form. Transcription is initiated by release of SurR
from promoter region as shown in Figure 5. The nucleotide
sequence of SurR-binding sites (SBS, GAAn\(_3\) TTC) was tried to
insert using the QuikChange protocol with the primers SBS-insert-
Fw and SBS-insert-Rv into pTKDR-His, yielding pTKDR-SBS-His.
The amplified DNA was self-ligated and introduced into *E. coli* cells.
The positive candidates were selected by ampicillin resistance.
However, no transformants appeared in selection plate. I
speculated that the high copy number of SBS have some toxic
effects on *E. coli* cell growth. Next I tried protein expression using
pCSG-His.
3.4. **Trial of expression of thermostable helicases in *T. kodakarensis***

Tk0306 is known to be a cold stress inducible RNA helicase (Shimada et al, 2009). When expressed in *E. coli* cells, Tk0306 often forms insoluble inclusion complex. Tk0460 another helicase, also forms inclusion complex in *E. coli* (Kawato, Bachelor Thesis,
These two RNA helicases presumably hold incorrectly in *E. coli* cells, probably due to the difference in protein folding machinery between archaea and bacteria. Thermostable protein generally folds rapidly at extremely high temperature above 80°C. However, in *E. coli* cells which grow at 37°C, thermostable recombinant protein might not fold properly. In the present study, I focused on these two helicases as a model protein. The primers Tk0306-Fw and Tk0306-Rv were used for PCR and Tk0306 gene was amplified using *T. kodakarensis* genomic DNA as a template. As well, Tk0460 was also amplified with primer Tk0460-Fw and Tk0460-Rv. The PCR products were digested with a set of restriction enzymes, NdeI and NotI, and introduced into the corresponding sites of plasmid pCSG-His. The constructed plasmids were named as pCSG-His-Tk0306 and pCSG-His-Tk0460, respectively. Details of experimental procedures were described in material and methods. Positive *E. coli* clones were examined by colony PCR using primers Tk0306-Fw and Tk0306-Rv for pCSG-His-Tk0306 selection, and primers Tk0460-Fw and Tk0460-Rv for pCSG-His-Tk0460. Two colonies were picked and the insertion of Tk0306 or Tk0460 in each plasmid was confirmed by PCR (Figure 6), and nucleotide sequences of Tk0306 and Tk0460 were confirmed.

**Figure 6.** Agarose gel electrophoresis to confirm construction of pCSG-His-Tk0306 and pCSG-His-Tk0460. The bands (1221 bp), indicated by
arrows, show the amplified DNA bands by PCR with a set of primers Tk0306-Fw and Tk0306-Rv. The bands (2223 bp) show the band by primers Tk0460-Fw and Tk0460-Rv. No. 1 and 2 indicate the number of candidate colonies.

3.5. Expression of helicases in *T. kodakarensis*

Plasmids, pCSG-His-Tk0306 and pCSG-His-Tk0460 were separately introduced into *T. kodakarensis* DAD strain. Cells were cultivated at 85°C in 1 liter of ASW-YT-Pyr-S0 medium for 12-16 hours. Plasmid pTKDR was used as a negative control (Hidese et al, 2014a). The grown cells were harvested by centrifugation. The collected cells were disrupted by sonication and crude extracts were obtained by centrifugation. To examine protein expression, cell extracts (20 µg each) was applied to SDS-PAGE. The bands correspond to Tk0306 and Tk0460 were not detected by CBB staining, as shown in Figure 7, suggesting that the expression level of the proteins is low. Since the expressed Tk0306 and Tk0460 contain Histidine-tag in N-terminus, the proteins were expected to be trapped by Ni-chelate affinity chromatography even though their expression levels were low.

![Figure 7](image)

**Figure 7.** The expression of N-terminal Histidine-taged recombinant Tk0306 and Tk0460 in *T. kodakarensis* strain DAD. The bands were
visualized by CBB staining. M: low molecular mass markers. The arrow indicates the band position of Tk0306 (46 kDa) and Tk0460 (85 kDa), respectively. pTKDR and pTKDR-His was used as a control.

3.6. Purification of helicases expressed in *T. kodakarensis*

Next, I carried out purification of the recombinant Tk0306 and Tk0460 proteins from *T. kodakarensis* cell extracts. The crude extracts were applied to 3 ml of Ni-chelate affinity chromatography. After washing the protein-bound column by sonication buffer, the candidate proteins were eluted by stepwise increase of imidazole concentration. Each eluate was applied to SDS-PAGE, and gel was visualized by CBB staining. As shown in Figure 8, a protein band was appeared in the position of 46 kDa at 10 mM imidazole eluate. The size shows a reasonable agreement with the molecular mass of the recombinant Tk0306. Thus, Tk0306 appears to be partially purified. As for Tk0460, protein band at position of 66 kDa in 10 mM imidazole eluate is smaller than that of Tk0460 (85 kDa), suggesting the recombinant Tk0460 was not fully denatured by SDS-PAGE in the present experimental condition.

**Figure 8.** Purification of the recombinant Tk0306 and Tk0460 by Ni-affinity chromatography of *T. kodakarensis* strain DAD. Gel was stained with CBB. Crude extract of the *T. kodakarensis* cells harboring pCSG-His-Tk0306 a) or pCSG-His-Tk0460 b) was applied to Ni-chelate affinity chromatography. The number in top of SDS-PAGE gel indicates each fraction (1 ml) eluted by sonication buffer containing 10 mM imidazole in order of eluate. The arrows indicate the position at 46 kDa a) and 85 kDa.
b), respectively. M: low molecular mass markers.
IV. DISCUSSION

In the present study, I have tried to construct gene expression system in *T. kodakarensis*. First, I selected a promoter, which has high transcriptional activity. Then, the system was applied to express RNA helicases, Tk0306 and Tk0460, from *T. kodakarensis*, which are known to form insoluble complex expressed in *E. coli*. Four candidate promoters of Tk0560, Tk1694, Tk2289 and Tk0895 genes have been selected because transcripts under the regulation of these promoters are abundantly detected by RNA-seq analysis (Jäger et al., 2014). Tk0895 gene encodes S-layer (surface layer) protein, which is a part of the cell envelope and presents in bacteria and almost all archaea (Sára and Sleytr, 2000). Tk0560 gene encodes DNA/RNA binding protein Alba (acetylation lowers binding affinity), which are the second most abundant chromatin protein in archaea (Crnigoj et al., 2013). Tk1694 gene encodes ferredoxin-1 protein that is very abundant in sulfur metabolizing archaea (Aono et al., 1989). Tk2289 gene encodes a histone B that facilitates DNA wrapping into nucleosome by its binding (Reeve et al., 1997).

The initiation of transcription in archaea starts with the multisubunit RNA polymerase (RNAP) and three basal transcription factors, TBP (TATA binding protein), TFB (transcription factor B), and TFE (transcription factor E) (Hidese et al., 2014b; Lipscomb et al., 2009). TBP and TFB recognize archaeal promoters by binding to the TATA-Box and the BRE, respectively, and then RNAP interacts with the TFB and TBP complex bound to the promoter to initiate transcription. TFE facilitates open complex formation during pre-initiation by interacting with the RNAP stalk and clamp and single-stranded DNA in the transcription bubble (Blombach et al., 2016; Schulz et al., 2016). The known transcriptional regulators are bacterial type (Leyn and Rodionov, 2015; Gindner et al., 2014).

Among promoter candidates, Tk0895 promoter showed the highest transcription activity and the promoter was hence selected for the construction of expression vector in *T. kodakarensis*. Interestingly, 8-bp TATA-box element (TWTWTAWR, where W is A/T and R is A/C) and the purine-base-rich BRE were highly conserved in the four promoters (Figure 3). However, the sequences found in these promoters are not found in promoters of Tk1311 and Tk1685 (FNOR2) gene, which shows lower transcript abundances in RNA-Seq analysis, indicating 8-bp TATA-box element and the purine-base-rich BRE are important for an
To develop the switching system of expression in *T. kodakarensis*, I tried to insert SBS into the position between TATA-Box and transcription start site (TSS) in plasmid pCSG-His. But I could not obtain any transformants of *E. coli* harboring the corresponding SBS-inserted plasmid. Because pCSG-His is one of pUC derivatives, which shows high-copy number, I speculate that the high copy number of SBS-inserted plasmid has some toxic effect on *E. coli* cell growth. A high plasmid number may impose a metabolic burden that inhibits *E. coli* growth and may introduce plasmid instability (Rosano and Ceccarelli, 2014).

The recombinant Tk0306 and Tk0460, RNA helicases, which are known to form insoluble inclusion complex when expressed in *E. coli* cells, were expected to express as soluble fraction in *T. kodakarensis* cells. I tried to purify the proteins by Ni-affinity chromatography. The recombinant Tk0306 seems to be successfully obtained. But, as for Tk0460, the protein was not detected by SDS-PAGE at an appropriate position for the suitable molecular mass (85kDa). Some of thermostable proteins are not denatured at boiling temperature in the presence of SDS. Partially denatured proteins sometimes show unique migration patterns which do not correspond to exact molecular mass. In the present experimental condition, Tk0460 might not be fully denatured and partially unfolded Tk0460 might showed a band whose size was not appropriate for the calculated molecular mass. To confirm that Tk0306 and Tk0460 are expressed in active form, enzyme characteristics of Tk0306 and Tk0460 need to be examined. Analyses of enzymatic activity and structural stability are required.
V. CONCLUSION

- Promoter of \textit{csg} (Tk0895) gene exhibited the highest transcriptional activity among promoters tested and was expected to apply for efficient expression system.
- SBS-inserted plasmid was not obtained in \textit{E. coli}, suggesting that SBS in high copy number plasmid has some toxic effect on \textit{E. coli} cell growth.
- The recombinant Tk0306 was expressed as a soluble form in \textit{T. kodakarensis} cells and successfully purified by means of Ni-affinity chromatography.
VI. REFERENCES


