

**Studies on the Oxidative Stress and Heat Stress Response Systems
in a Hyperthermophilic Archaeon**

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PREFACE

This is a thesis submitted by the author to Kyoto University for the degree of Doctor of Engineering. The study presented here has been performed under the supervision of Professor Tadayuki Imanaka in the Laboratory of Biochemical Engineering, Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, during 2005-2008.

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GENERAL INTRODUCTION

1. Hyperthermophilic archaea.

In previous times, taxonomical classification of living organisms was mainly based on comparisons of morphology and biochemical properties. Progress in genetic technology has brought about novel methods to classify living organisms, and has provided means to construct molecular phylogenetic trees. Sequence comparisons of 16S and 18S ribosomal RNA genes are one of the most powerful tools for classification that can evaluate the evolutionary relationships among both closely related and widely diverse organisms. From these comparisons, all living organisms on our planet can be divided into three domains, *Bacteria*, *Eucarya* and *Archaea* (Fig. 1) (34). The structure of the phylogenetic tree indicates that *Bacteria* and *Archaea* first separated from the last common ancestor, and that *Eucarya* evolved from pre-existing *Archaea*. The domain *Archaea* can further be classified into two major kingdoms; *Crenarchaeota* and *Euryarchaeota*.

Hyperthermophiles are defined as organisms that grow optimally at temperatures above 80°C (30). Most hyperthermophilic organisms are members of the domain *Archaea* with the exception of several bacterial genera, *Aquifex*, *Thermotoga* and *Geothermobacterium*. All hyperthermophiles, regardless of whether they are archaeal or bacterial, are positioned near the root of the phylogenetic tree (Fig. 1). In addition, in the early periods of earth when life originated approximately 3.5 billion years ago, it is presumed to have been a thermal environment. These indications have led many to suppose that life originated in the form of hyperthermophiles.

Along with the classical biochemistry and molecular cloning of structural genes, complete genome analyses in recent years have provided a new perspective on the means to

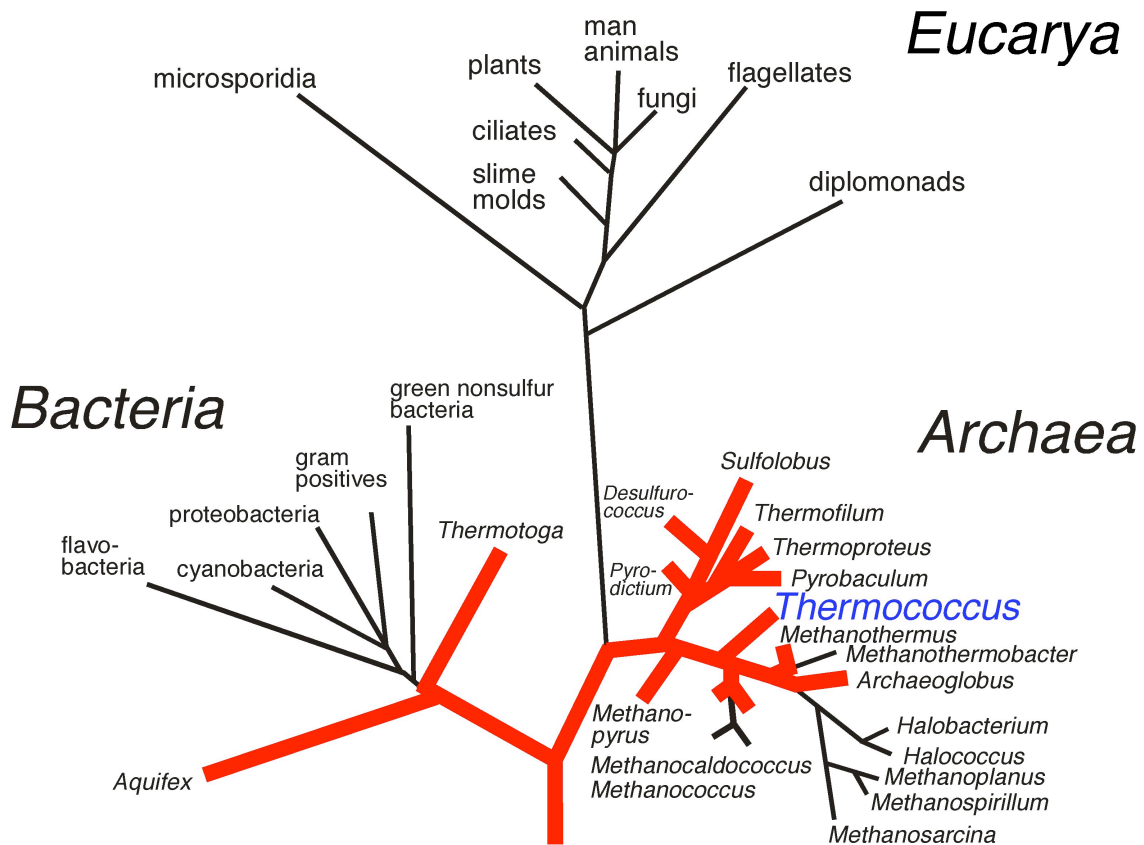


Fig. 1. The phylogenetic tree of life based on 16S/18S ribosomal RNA sequences. The red, bold lines denote hyperthermophiles.

understand the hyperthermophilic archaea. So far, entire genome sequences of 49 archaeal strains, including 20 hyperthermophiles, have been determined. This has revealed that genomes of the hyperthermophilic archaea are rather small in size. Therefore, regardless of their evolutionary position, it is a fact that hyperthermophiles are primitive organisms with a very small number of genes. Compared to higher eukaryotic organisms (over 20,000 genes) (7), the lower eukaryotic *Saccharomyces cerevisiae* (over 6,000 genes) (13) and even the bacterial *Escherichia coli* (over 4,000 genes) (4), hyperthermophilic archaea harbor only approximately 2,000 genes. The small number of genes suggests that their metabolism and biological machinery are simple, which should provide an advantage in examining the basic mechanisms of various biological phenomena. Studies on hyperthermophiles are also

attractive in terms of enzyme application. All proteins in a hyperthermophile must endure and properly function at high temperature ranges, making them much more (thermo)stable when compared to the conventional enzymes from mesophiles utilized at present (18, 29).

2. *Thermococcus kodakaraensis* KOD1.

Thermococcus kodakaraensis KOD1 is a sulfur-reducing hyperthermophilic archaeon, isolated from a solfatar on the shore of Kodakara Island, Kagoshima, Japan (Fig. 2) (3). The archaeon grows under strict anaerobic conditions and can utilize a number of organic compounds for growth such as amino acids, peptides, oligosaccharides and pyruvate. Compared to other hyperthermophiles, the temperature range at which *T. kodakaraensis* can grow is relatively broad, and growth has been confirmed at temperatures as low as 60°C. The optimum growth temperature of the strain is 85°C. The entire genome sequence of *T. kodakaraensis* has been determined (11), and moreover, a gene disruption system has been developed (27, 28). It is therefore possible to insert or disrupt particular genes of interest, and directly evaluate the effects brought about their presence or absence *in vivo*.

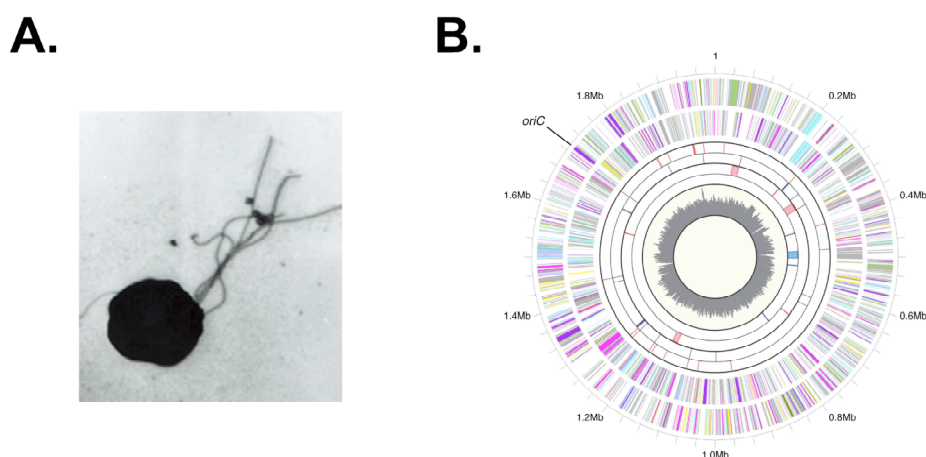


Fig. 2. *Thermococcus kodakaraensis* KOD1 and its genome. (A) An electron micrograph of *T. kodakaraensis*. (B) Map of the *T. kodakaraensis* genome. Colored lines indicate the individual predicted open reading frames (ORF) on the two DNA strands.

3. Oxidative stress.

The generation and accumulation of oxygen on the earth has been estimated to have initiated about 2.4 billion years ago (15). This change in the global environment can be considered to have brought about a drastic effect on the evolution of life thereafter. A group of organisms, which have now evolved to the aerobes, took advantage of the presence of oxygen and obtained the ability to respire using oxygen as a terminal electron acceptor. Other organisms evolved the means to detoxify oxygen or physically escape from aerobic environments. Oxygen potentially lays stress on all biological macromolecules (nucleic acids, proteins and lipids) in the cell as a source of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radical (19). To deal with the stress from ROS, living organisms have evolved defense systems composed of enzymes such as thioredoxin and glutaredoxin, peroxiredoxin, hydroperoxidase, NADH oxidase and superoxide dismutase (25).

Reduction of the superoxide anion is one of the most vital steps in protecting the cell from oxidative stress (Fig. 3). Life has evolved two enzymes that can carry out the reduction of superoxide to hydrogen peroxide; superoxide dismutase (SOD) and superoxide

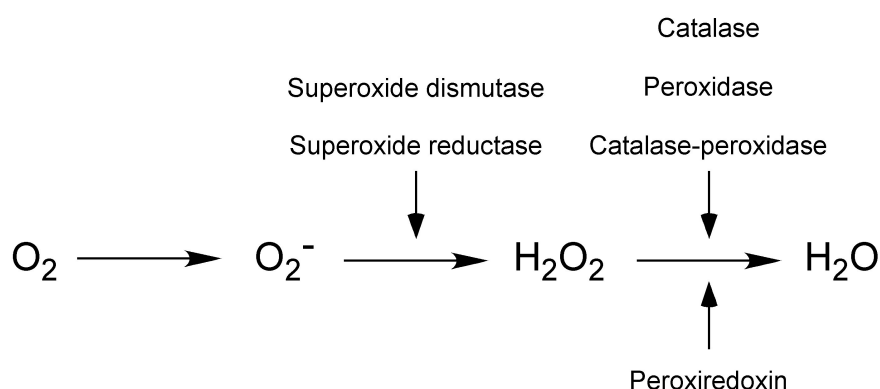


Fig. 3. Enzymes involved in the detoxification of superoxide.

Table 1. Distribution of genes involved in biological defense systems against oxidative stress.

| Organism | Kingdom | Oxygen Req. | Range | Trx | Prx | Cat | SOD | SOR |
|---|---------|--------------|-------|-----|-----|-----|-----|-----|
| <i>Metallosphaera sedula</i> | C | Aerobic | T | | | | | |
| <i>Sulfolobus acidocaldarius</i> | C | Aerobic | T | | | | | |
| <i>Picrophilus torridus</i> | E | Aerobic | T | | | | | |
| <i>Thermoplasma acidophilum</i> | E | Aerobic | T | | | | | |
| <i>Thermoplasma volcanium</i> | E | Aerobic | T | | | | | |
| <i>Methanosaeta thermophila</i> | E | Anaerobic | T | | | | | |
| <i>Methanothermobacter thermautotrophicus</i> | E | Anaerobic | T | | | | | |
| <i>Aeropyrum pernix</i> | C | Aerobic | H | | | | | |
| <i>Pyrobaculum aerophilum</i> | C | Aerobic | H | | | | | |
| <i>Pyrobaculum calidifontis</i> | C | Aerobic | H | | | | | |
| <i>Sulfolobus solfataricus</i> | C | Aerobic | H | | | | | |
| <i>Sulfolobus tokodaii</i> | C | Aerobic | H | | | | | |
| <i>Caldivirga maquilingensis</i> | C | Microaerobic | H | | | | | |
| <i>Hyperthermus butylicus</i> | C | Anaerobic | H | | | | | |
| <i>Ignicoccus hospitalis</i> | C | Anaerobic | H | | | | | |
| <i>Pyrobaculum arsenaticum</i> | C | Anaerobic | H | | | | | |
| <i>Pyrobaculum islandicum</i> | C | Anaerobic | H | | | | | |
| <i>Staphylothermus marinus</i> | C | Anaerobic | H | | | | | |
| <i>Thermofilum pendens</i> | C | Anaerobic | H | | | | | |
| <i>Archaeoglobus fulgidus</i> | E | Anaerobic | H | | | | | |
| <i>Methanocaldococcus jannaschii</i> | E | Anaerobic | H | | | | | |
| <i>Methanopyrus kandleri</i> | E | Anaerobic | H | | | | | |
| <i>Pyrococcus abyssi</i> | E | Anaerobic | H | | | | | |
| <i>Pyrococcus furiosus</i> | E | Anaerobic | H | | | | | |
| <i>Pyrococcus horikoshii</i> | E | Anaerobic | H | | | | | |
| <i>Thermococcus kodakaraensis</i> | E | Anaerobic | H | | | | | |
| <i>Nanoarchaeum equitans</i> | N | Anaerobic | H | | | | | |

The shaded boxes indicate the presence of homologs on the corresponding genomes.

Abbreviations: (*Proteins*) Trx: thioredoxin, Prx: peroxiredoxin, Cat: catalase, SOD: superoxide dismutase, SOR: superoxide reductase. (*Kingdom*) C: *Crenarchaeota*, E: *Euryarchaeota*, N: *Nanoarchaeota*. (*Growth temperature range*) T: thermophile, H: hyperthermophile.

reductase (SOR). SOD converts two molecules of superoxide to hydrogen peroxide and O₂, whereas SOR reduces superoxide to hydrogen peroxide with electrons provided by NADPH (1, 20). The latter reaction provides an advantage for anaerobes, as the reaction does not result in the generation of molecular oxygen. SOD or SOR homologs are found in a majority of the thermophilic and hyperthermophilic archaea (Table 1). SOD is found on the genomes of thermophilic archaea and some aerobic hyperthermophilic archaea. However, SOD homologs are absent in the anaerobic hyperthermophilic archaea, and are apparently replaced by SOR homologs. Although both SOD and SOR generate hydrogen peroxide as a result of superoxide reduction, only very few thermophilic and hyperthermophilic archaea such as *Pyrobaculum calidifontis*, *Pyrobaculum aerophilum*, *Pyrobaculum arsenaticum* and *Archaeoglobus fulgidus* harbor catalase homologs on their genomes (Table 1). This implies that peroxiredoxins may

play an active role in the further reduction of hydrogen peroxide in the thermophilic and hyperthermophilic archaea.

4. Methionine sulfoxide reductase.

Methionine (Met) is one of the amino acids most susceptible to oxidative stress. Methionine residues are oxidated to methionine sulfoxide (MetO), often resulting in a decrease or abolishment of protein function (33). Methionine sulfoxide reductase (Msr) is an enzyme that repairs the oxidized methionine, and catalyzes the thioredoxin-dependent reduction of MetO to Met (6). The oxidation of Met results in the formation of two asymmetric molecules, Met-*S*-O and Met-*R*-O. Each MetO is reduced by a specific and structurally distinct enzyme, Met-*S*-O by MsrA and Met-*R*-O by MsrB. Msr proteins are considered to play vital roles in maintaining the intracellular redox balance and in the repair of oxidized proteins (5, 10). Consistent with their important roles in dealing against oxygen or oxidative stress, Msr proteins are widely distributed in nature and can be found in all three domains of life, *Eucarya*, *Bacteria* and *Archaea* (8).

5. Heat shock response.

When the cell faces drastic changes in its environment, the cell tries to adapt to, or at least survive through, these changes by activating various defense systems. Oxidative stress, which has been described above, is one typical example of a stress environment which cells often encounter. Another well known example is temperature stress; high (or in some cases low) temperature stress triggers various intracellular changes that the cell must overcome, such as protein denaturation and membrane destabilization. The defense systems that cope with protein denaturation are by far the most extensively examined, as intracellular protein

Table 2. Distribution of genes encoding heat shock proteins in the archaea.

| Organism | Kingdom | Range | HSP100 | HSP90 | HSP70 | HSP40 | GrpE | HSP60 | PFD | sHSP |
|---|---------|-------|--------|-------|-------|-------|------|-------|-----|------|
| <i>Aeropyrum pernix</i> | C | H | | | | | | | | |
| <i>Pyrobaculum aerophilum</i> | C | H | | | | | | | | |
| <i>Pyrobaculum caldifontis</i> | C | H | | | | | | | | |
| <i>Sulfolobus solfataricus</i> | C | H | | | | | | | | |
| <i>Sulfolobus tokodaii</i> | C | H | | | | | | | | |
| <i>Archaeoglobus fulgidus</i> | E | H | | | | | | | | |
| <i>Hyperthermus butylicus</i> | C | H | | | | | | | | |
| <i>Ignicoccus hospitalis</i> | C | H | | | | | | | | |
| <i>Methanocaldococcus jannaschii</i> | E | H | | | | | | | | |
| <i>Methanopyrus kandleri</i> | E | H | | | | | | | | |
| <i>Nanoarchaeum equitans</i> | N | H | | | | | | | | |
| <i>Pyrobaculum arsenaticum</i> | C | H | | | | | | | | |
| <i>Pyrobaculum islandicum</i> | C | H | | | | | | | | |
| <i>Pyrococcus abyssi</i> | E | H | | | | | | | | |
| <i>Pyrococcus furiosus</i> | E | H | | | | | | | | |
| <i>Pyrococcus horikoshii</i> | E | H | | | | | | | | |
| <i>Staphylothermus marinus</i> | C | H | | | | | | | | |
| <i>Thermococcus kodakaraensis</i> | E | H | | | | | | | | |
| <i>Thermofilum pendens</i> | C | H | | | | | | | | |
| <i>Caldivirga maquilensis</i> | C | H | | | | | | | | |
| <i>Metallosphaera sedula</i> | C | T | | | | | | | | |
| <i>Picrophilus torridus</i> | E | T | | | | | | | | |
| <i>Sulfolobus acidocaldarius</i> | C | T | | | | | | | | |
| <i>Thermoplasma acidophilum</i> | E | T | | | | | | | | |
| <i>Thermoplasma volcanium</i> | E | T | | | | | | | | |
| <i>Methanosaeta thermophila</i> | E | T | | | | | | | | |
| <i>Methanothermobacter thermautotrophicus</i> | E | T | | | | | | | | |
| <i>Haloarcula marismortui</i> | E | M | | | | | | | | |
| <i>Halobacterium sp.</i> | E | M | | | | | | | | |
| <i>Natronomonas pharaonis</i> | E | M | | | | | | | | |
| <i>Candidatus Methanoregula boonei</i> | E | M | | | | | | | | |
| <i>Haloquadratum walsbyi</i> | E | M | | | | | | | | |
| <i>Methanobrevibacter smithii</i> | E | M | | | | | | | | |
| <i>Methanococcoides burtonii</i> | E | M | | | | | | | | |
| <i>Methanococcus aeolicus</i> | E | M | | | | | | | | |
| <i>Methanococcus maripaludis</i> C5 | E | M | | | | | | | | |
| <i>Methanococcus maripaludis</i> C6 | E | M | | | | | | | | |
| <i>Methanococcus maripaludis</i> C7 | E | M | | | | | | | | |
| <i>Methanococcus maripaludis</i> S2 | E | M | | | | | | | | |
| <i>Methanococcus vannieli</i> | E | M | | | | | | | | |
| <i>Methanocorpusculum labreanum</i> | E | M | | | | | | | | |
| <i>Methanoculleus marisnigri</i> | E | M | | | | | | | | |
| <i>Methanosarcina acetivorans</i> | E | M | | | | | | | | |
| <i>Methanosarcina barkeri</i> | E | M | | | | | | | | |
| <i>Methanosarcina mazei</i> | E | M | | | | | | | | |
| <i>Methanosphaera stadtmanae</i> | E | M | | | | | | | | |
| <i>Methanospirillum hungatei</i> | E | M | | | | | | | | |
| <i>Uncultured methanogenic archaeon RC-1</i> | E | M | | | | | | | | |
| <i>Cenarchaeum symbiosum</i> | C | P | | | | | | | | |

The shaded boxes indicate the presence of homologs on the corresponding genomes.

Abbreviations: (Proteins) PFD: prefoldin, sHSP: small HSP. (Kingdom) C: Crenarchaeota, E: Euryarchaeota, N: Nanoarchaeota. (Growth temperature range) H: hyperthermophile, T: thermophile, M: mesophile, P: psychrophile.

denaturation/aggregation is now known to be the cause of a number of diseases (9, 35).

Exposing cells to heat shock is a direct method to trigger intracellular protein denaturation,

and results in the so-called heat shock response. In the response, the amount of various heat shock proteins (HSPs), which include molecular chaperones, ATPases and proteases, dramatically increase. The HSPs are often classified according to their molecular masses: HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs (sHSP) (22). They are highly conserved between *Bacteria* and *Eucarya*, but not all proteins are conserved in the thermophilic and hyperthermophilic archaea (Table 2). For example, the HSP100 protein is only found in a single archaeon, *Methanothermobacter thermautotrophicus*, and HSP90 is not present on any of the genomes of thermophilic and hyperthermophilic archaea. The HSP70 (DnaK), HSP40 (DnaJ) and GrpE system, which is also highly conserved among *Bacteria* and *Eucarya*, can only be found in a limited number of thermophilic archaea. The HSP60 (chaperonins), prefoldins and sHSP are highly conserved between all thermophilic and hyperthermophilic archaea (Table 2).

Although a number of regulatory systems and regulation factors that govern the heat shock response have been identified in the *Bacteria* and *Eucarya*, very little is known about the mechanisms that function in the *Archaea*. In particular, the (hyper)thermophilic archaea thrive in environments that can be expected to constantly pose a challenge to maintaining proper protein structure. These organisms may harbor rapid and sensitive systems to deal with the harsh temperature shifts that occur in their native, hydrothermal surroundings.

Previous studies have clarified that the heat shock response indeed occurs in (hyper)thermophilic archaea. Regulator proteins that participate in the heat shock response have been identified in *Pyrococcus furiosus* (32) and *Archaeoglobus fulgidus* (26). In *P. furiosus*, a heat shock regulator Phr has been identified. Phr is a 24 kDa protein and forms a homodimer that inhibits the transcription *in vitro* by tightly binding to the 5'-upstream regions of heat shock genes such as AAA⁺ ATPase gene, a small heat shock protein gene (21) and the

Phr gene itself (32). It has been speculated that dissociation of Phr, and subsequent derepression of transcription, is brought about by the thermal denaturation of the protein itself at elevated temperatures. In *A. fulgidus*, HSR1, a protein distantly related to Phr in terms of primary structure, has been identified. As in the case of Phr, HSR1 also binds to the 5'-upstream regions of genes or gene clusters encoding the AAA⁺ ATPase gene, small heat shock proteins and HSR1 (26). The involvement of Phr or HSR1 in the regulation of other major HSPs, including the chaperonins (HSP60), is currently unknown.

6. Chaperonin.

Members of the HSP60 family, the chaperonins, assist the refolding of partially denatured proteins, and are considered one of the most important factors in the quality control of intracellular proteins. Chaperonins form a multi-subunit cylindrical complex that provides an environment in which the folding process of a protein is not disturbed by intermolecular interaction and enhanced by conformational changes of the chaperonins themselves coupled to ATP hydrolysis (16). Chaperonins are classified into two groups in terms of primary structure (17). Group I chaperonins are known as the GroEL proteins, which are found in bacteria, the eukaryotic mitochondria and chloroplasts, as well as in some mesophilic archaea. This group of chaperonins cooperates with a separate polypeptide GroES (Hsp10 family) in assisting the refolding of protein substrates. Group II chaperonins are found in the eukaryotic cytosol and in the *Archaea*. Group II chaperonins form a similar cylindrical complex to those of Group I proteins, but do not rely on any additional proteins for their function.

The systems that regulate the induction of these chaperonins in response to heat shock are well studied in the bacteria. In the gram-negative bacteria such as *E. coli*, transcription of the chaperonin subunit genes encoding Hsp60 and Hsp10 is under the control

of the σ^{32} factor (2, 14). The σ^{32} protein physically interacts with the DnaK/DnaJ/GrpE system under normal conditions and is directed to the degradation pathway mediated by the membrane-bound protease FtsH (31). Under stress conditions in which misfolded proteins accumulate in the cell, the DnaK/DnaJ/GrpE system is sequestered by these proteins, resulting in the release of free σ^{32} and induction of chaperonin gene transcription (12). In contrast, expression of the HSP60 chaperonins is regulated by a repression/derepression system in gram-positive bacteria (24). Under non-stress conditions, the repressor protein HrcA, through interactions with chaperonins, represses gene expression by binding to *cis*-acting elements known as CIRCE elements. The accumulation of denatured proteins leads to the dissociation of HrcA from the chaperonins in an inactive form, resulting in the release of transcriptional repression (23).

Based on genome sequence data, the HSP60 proteins are apparently present in all (hyper)thermophilic archaea. The intracellular levels of these proteins have also been shown to respond to heat shock in a number of (hyper)thermophilic archaea including *T. kodakaraensis*. However, mechanisms that are involved in this response are currently unknown.

7. Objectives of this study.

As in the case of the majority of hyperthermophilic archaea, *T. kodakaraensis* was isolated from a marine environment. It can therefore be expected that these organisms must constantly cope with dramatic changes in their environments. The two most prominent environmental parameters should be temperature, and to the many obligate anaerobic hyperthermophiles, oxygen concentration. The author took interest in how these hyperthermophiles, with only a relatively small number of genes, can deal with these ever-

changing surroundings. For example, deep-sea hydrothermal vent regions, where many hyperthermophiles thrive, include waters with temperatures ranging from 5°C to 300°C. The hyperthermophiles in shallow marine environments are exposed to cool and boiling waters, and in addition, the oxygen dissolved from the atmosphere.

By examining the genome sequence data, the author focused on the unusual presence of a putative methionine sulfoxide reductase gene on the *T. kodakaraensis* genome. As this gene could be involved in a defense system against oxidative stress, the author set out to examine the biochemical and physiological properties of the protein. In order to study the response mechanism against temperature change, the author did not apply conventional methods such as heat or cold-shock. Taking advantage of the gene disruption/insertion technology developed for this archaeon, a novel approach was pursued by examining the response of *T. kodakaraensis* when a thermolabile protein was produced in the cell.

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SYNOPSIS

In this study, the author has taken interest in how hyperthermophilic archaea respond towards conditions of stress. The organism studied in this thesis was an obligately anaerobic, hyperthermophilic archaeon, *Thermococcus kodakaraensis*. Taking into consideration the stress conditions that most likely occur in the native habitat of an anaerobic hyperthermophile, the author focused on oxidative stress and heat stress. In Chapters 1 and 2, a defense mechanism that functions against oxidative damage of proteins was characterized. In Chapter 3, the author examined the response of chaperonins when a thermolabile protein was present in the cell, which resembles a condition of heat stress.

In Chapter 1, the author performed a biochemical analysis on the methionine sulfoxide reductase (Msr) from *T. kodakaraensis*. Msr catalyzes the thioredoxin-dependent reduction and repair of methionine sulfoxide (MetO), which is generated through oxidative damage of methionine residues. Although Msr genes are ubiquitously found in a wide range of organisms, they are not present in most hyperthermophile genomes. As an exception, an Msr homolog encoding a putative MsrA-MsrB fusion protein (MsrAB_{TK}) was present on the genome of *T. kodakaraensis*. Recombinant proteins corresponding to MsrAB_{TK} and the individual domains (MsrA_{TK} and MsrB_{TK}) were produced, purified, and biochemically examined. MsrA_{TK} and MsrB_{TK} displayed strict substrate selectivity for Met-S-O and Met-R-O, respectively. MsrAB_{TK}, and in particular the MsrB domain of this protein, displayed an intriguing behavior for an enzyme from a hyperthermophile. While MsrAB_{TK} was relatively stable at temperatures up to 80°C, a 75% decrease in activity was observed after 2.5 min at 85°C, the optimal growth temperature of this archaeon. Moreover, maximal levels of MsrB activity of MsrAB_{TK} were observed at a strikingly low temperature of 30°C, which was also

observed for MsrB_{TK}. The optimal temperature of the MsrA_{TK} reaction was also low at 60°C. While measuring background rates of the Msr enzyme reactions, the author observed significant levels of MetO reduction at high temperatures without enzyme. The occurrence of non-enzymatic MetO reduction at high temperatures might provide an explanation for the specific absence of Msr homologs on the genomes of most hyperthermophiles. Together with the fact that the presence of Msr in *T. kodakaraensis* is exceptional among the hyperthermophiles, this raised the possibilities that the enzyme represents a novel strategy for this organism to deal with low-temperature environments in which the dissolved oxygen concentrations increase.

In order to evaluate this hypothesis and gain insight on the function of MsrAB_{TK} *in vivo*, in Chapter 2 the author first examined the presence of the MsrAB_{TK} protein in *T. kodakaraensis* cells. By performing a Western blot analysis using antisera raised against the purified recombinant protein, the author found that the MsrAB_{TK} protein in *T. kodakaraensis* was present only at sub-optimal growth temperatures. At these lower temperatures, the intracellular levels of MsrAB_{TK} responded against increases in dissolved oxygen, but the response was not observed at higher temperatures. The results supported the possibilities that MsrAB_{TK} is actually involved in the response against oxidative stress at sub-optimal growth temperatures *in vivo*. Furthermore, utilizing the gene disruption system developed in this archaeon, the author disrupted the MsrAB_{TK} gene, and examined whether the absence of the gene had any effect on the growth of *T. kodakaraensis* under various degrees of oxidative stress. The author observed that the mutant strains displayed less tolerance towards dissolved oxygen compared to the wild-type strain, specifically at lower growth temperatures. This change in phenotype indicates that MsrAB_{TK} participates in protecting the cell from oxidative stress and confirms the findings in Chapter 1 that Msr is a unique protein from a

hyperthermophile that is regulated and designed to function exclusively at sub-optimal growth temperatures.

In Chapter 3, the author aimed to gain insight on further defense mechanisms in *T. kodakaraensis* that function to maintain proper protein function in the cell. The author focused on the regulation mechanisms of chaperonins, proteins that assist the folding and/or assembly of newly synthesized polypeptide chains as well as mature proteins that have lost their optimal conformation due to various conditions of stress. The author introduced *pyrF* (encoding orotidine-5'-phosphate decarboxylase) genes from *Pyrococcus furiosus* (*pyrF_{Pf}*) and *Thermoplasma volcanium* (*pyrF_{Tv}*) into *T. kodakaraensis* KUW1 ($\Delta pyrF$, $\Delta trpE$), a strain that displays pyrimidine and tryptophan auxotrophy. While *T. kodakaraensis* exhibits an optimal growth temperature of 85°C, those of *P. furiosus* and *T. volcanium* are 100°C and 60°C, respectively. The growth characteristics of the wild-type *T. kodakaraensis* KOD1, the *pyrF_{Pf}*-integrated strain (KPF), and the *pyrF_{Tv}*-integrated strain (KTV) were examined. No difference was detected among the three strains in nutrient-rich medium in which *pyrF* function is not necessary. Under conditions in which pyrimidine biosynthesis was essential, strain KPF displayed uracil prototrophy at all temperatures examined, while growth of strain KTV was limited to temperatures at or below 75°C. By examining the thermostability of the three proteins, the author found that *PyrF_{Tv}* was by far the most thermolabile, with a half life of only 3 min at 80°C. Genome sequence data and previously reported results have indicated that *T. kodakaraensis* harbors two chaperonin subunits, CpkA and CpkB. At 75°C, an intracellular accumulation of the two chaperonin subunits CpkA and CpkB was observed in strain KTV, but not in strain KOD1 or KPF. CpkB is usually up-regulated at temperatures above the optimal growth temperature of *T. kodakaraensis*, and the protein levels decrease at lower temperatures. As 75°C is well below the optimal growth temperature, the accumulation

of CpkB observed in strain KTV cannot be due to high temperature *per se*. This raises the possibilities that CpkB expression is responding to the intracellular presence of partially denatured PyrF_{TV} protein, a mechanism similar to the unfolded protein response previously observed only in bacteria and eukaryotes.

CHAPTER 1

Biochemical properties of methionine sulfoxide reductase from *Thermococcus kodakaraensis*, an enzyme designed to function at sub-optimal growth temperatures

INTRODUCTION

Reactive oxygen species (ROS) are harmful to the cell as they oxidize various cell components such as lipids, nucleic acids and proteins. Among the amino acids, methionine residues are known to be particularly susceptible to oxidative stress, and are easily oxidized to methionine sulfoxides. Methionine sulfoxide reductase (Msr) is an enzyme that repairs the oxidized methionine, and catalyzes the thioredoxin-dependent reduction of methionine sulfoxide (MetO) to methionine (Met) (6). The oxidation of Met results in the formation of two asymmetric molecules, Met-*S*-O and Met-*R*-O. Each MetO is reduced by a specific and structurally distinct enzyme, Met-*S*-O by MsrA and Met-*R*-O by MsrB (Fig. 1). Msr proteins are considered to play vital roles in maintaining the intracellular redox balance and in the repair of oxidized proteins (5, 9). In mammals, defects in the function of Msr have been reported to result in neurological disorders and in some cases, a decrease in lifespan (30, 31).

Although structurally distinct, MsrA and MsrB catalyze the reduction of MetO with basically similar mechanisms (2, 4, 20, 33). A cysteine residue, designated as CysA, acts as a

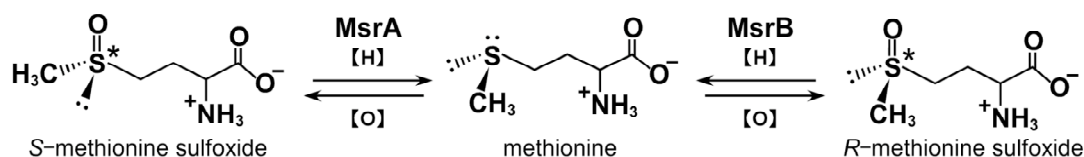


Fig. 1. Redox reaction of L-methionine. The asterisk indicates the chiral center generated on the side chain of *S*-methionine sulfoxide and *R*-methionine sulfoxide.

nucleophile that attacks the oxidized sulfur atom of MetO. A tetrahedral transition state is formed, followed by a rearrangement that releases the repaired Met and results in the formation of a sulfenic acid intermediate on the CysA side chain. A second cysteine residue, CysB, then attacks the oxidized CysA and, along with the release of a water molecule, forms a disulfide bond with CysA. Other cysteine residues may participate in the steps to follow, but the enzyme is eventually reduced in a thioredoxin-dependent manner, completing the reaction.

MsrAs can be classified into three main groups by the number and positions of cysteine residues that are proposed to be involved in the catalytic mechanism (20). The first group (MsrA_I) utilizes three Cys residues in catalysis, and the nucleophilic CysA residue is conserved in a GCFWG motif. The CysB residue is conserved in a GYCG sequence. A third Cys residue (CysC) is present in MsrA_I and resides downstream of a glycine-rich sequence. The second group of MsrA proteins (MsrA_{II}) utilizes only two Cys residues, basically corresponding to the CysA and CysB of MsrA_I enzymes. The third group of enzymes (MsrA_{III}) harbors both CysA and CysB residues in a single GCFWC motif. The MsrB proteins are classified into two groups by the presence (Form I) or absence (Form II) of two CxxC motifs that have been shown to participate in binding to a divalent zinc cation in the MsrB from *Drosophila* (26). Although mutations in any one of these four Cys residues leads to a non-active protein, this cluster seems to play a structural role in the enzyme. All MsrB proteins from eukaryotes and archaea are Form I enzymes, while bacteria harbor either Form I or II, depending on the species.

As described in the general introduction, Msr proteins are broadly distributed in nature and can be found in all three domains of life, *Eucarya*, *Bacteria* and *Archaea*. However, the recent accumulation of genome sequences indicate that almost all hyperthermophiles, from both the *Bacteria* and the *Archaea*, do not harbor Msr genes. The only exceptions are the

putative MsrAB fusion gene from *Thermococcus kodakaraensis* (3, 11) and the putative MsrA gene on the *Sulfolobus solfataricus* genome (43) (Table 1).

The genera *Pyrococcus* and *Thermococcus* both belong to the family Thermococcales, and consist of heterotrophic, sulfur-reducing anaerobes that share common metabolism and energy-generating mechanisms (1, 11, 48). The major distinction between *Thermococcus* and

Table 1. The presence of Msr homologs in various archaea along with their growth temperatures.

| Organism | MsrA | MsrB | Growth temperature (optimum) (°C) | Ref. |
|--|-----------------------|-----------------------|-----------------------------------|----------|
| <i>Methanococcus maripaludis</i> | CAF30404 | – | Mesophilic | |
| <i>Methanosarcina acetivorans</i> | AAM04846 | AAM03895 | Mesophilic | |
| <i>Methanosarcina mazei</i> | AAM32095 | AAM31330 | Mesophilic | |
| <i>Haloarcula marismortui</i> | AAV46883 AAV46414 | AAV45184 | (40-50) | (34) |
| <i>Halobacterium sp.</i> | AAG19555 | AAG19724 | 22-50 (49-50) | (15, 38) |
| <i>Thermoplasma acidophilum</i> | – | – | 45-63 (59) | (42) |
| <i>Thermoplasma volcanium</i> | – | – | 33-67 (59) | (42) |
| <i>Picrophilus torridus</i> | AAT42728 | – | 45-65 (60) | (41) |
| <i>Methanothermobacter thermotrophicus</i> | AAB85041 | AAB85216 | 40-70 (65) | (44) |
| <i>Sulfolobus solfataricus</i> | AAK41726 | – | 65-86 ^a (80) | (14) |
| <i>Sulfolobus tokodaii</i> | – | – | 70-85 (80) | (45) |
| <i>Archaeoglobus fulgidus</i> | – | – | 60-95 (83) | (24) |
| <i>Methanocaldococcus jannaschii</i> | – | – | 50-86 ^a (85) | (19) |
| <i>Thermococcus kodakaraensis</i> | BAD85008 [†] | BAD85008 [†] | 60-100 (85) | (3) |
| <i>Nanoarchaeum equitans</i> | – | – | 70-98 ^b | (17) |
| <i>Aeropyrum pernix</i> | – | – | 90-98 (95) | (21) |
| <i>Pyrococcus abyssi</i> | – | – | 67-102 ^c (96) | (8) |
| <i>Pyrococcus horikoshii</i> | – | – | 85-105 (98) | (12) |
| <i>Methanopyrus kandleri</i> | – | – | 84-110 (98) | (27) |
| <i>Pyrococcus furiosus</i> | – | – | 70-103 (100) | (10) |
| <i>Pyrobaculum aerophilum</i> | – | – | 75-104 (100) | (49) |

When a homolog is present, its protein accession number is given.

^aGrowth has been observed in this temperature range, and does not necessarily reflect the temperature limits.

^bSymbiotic growth with *Ignicoccus*.

^cGrowth under 200 kPa pressure.

[†]MsrA-MsrB fusion protein.

–, no homolog is present on the genome.

Pyrococcus is in their growth temperatures, the former with optimal growth temperatures between 75 and 93°C, while those of the latter ranging from 95 to 103°C (1, 18). The *Pyrococcus* have received relatively more attention in terms of biochemical and genome research; the genome sequences of *Pyrococcus furiosus* (37), *Pyrococcus abyssi* (7) and *Pyrococcus horikoshii* (22) have been determined. However, environmental studies have indicated that the *Thermococcus* seem to be by far the predominant genus distributed throughout the hydrothermal environments on our planet (16, 18, 35). The *Thermococcus* also seem to be much more diverse (18), including members that can grow at alkaline pH (*Thermococcus alcaliphilus*) (23), extremely low salinity (*Thermococcus waiotapuensis*) (13), and at temperatures as low as 40°C (*Thermococcus sibiricus*) (29). In the natural environment, a decrease in temperature usually brings about an increase in dissolved oxygen concentration. Thus the *Thermococcus*, which generally grow at lower temperature ranges, may harbor additional defense mechanisms against oxygen that are not present in the *Pyrococcus*. As an Msr gene is present on the *T. kodakaraensis* genome, but absent on the three *Pyrococcus* genomes, there is a possibility that Msr represents one of these additional mechanisms. In this chapter, the author has focused on MsrAB_{TK} in *T. kodakaraensis* and has examined the biochemical properties of MsrAB_{TK} from *T. kodakaraensis*.

MATERIALS AND METHODS

Phylogenetic analyses of Msr sequences.

MsrA and MsrB sequences were selected from various organisms and aligned, respectively, using the ClustalW program (47) provided by the DNA Data Bank of Japan. Core regions displaying homology among the MsrA sequences (residues 9 to 163 in MsrAB) and MsrB sequences (residues 219 to 322) were used for each phylogenetic analysis.

Phylogenetic trees were constructed with the neighbor-joining method (39) using the ClustalW program mentioned above.

Microorganisms, plasmids and media.

Escherichia coli DH5 α and plasmid pUC118 were used for general DNA manipulation and sequencing. *E. coli* BL21-Codon-Plus(DE3)-RIL (Stratagene, La Jolla, CA) and pET21a(+) (Novagen, Madison, WI) were used for gene expression. *E. coli* strains were cultivated in Luria-Bertani (LB) medium (10 g L⁻¹ of tryptone, 5 g L⁻¹ of yeast extract and 10 g L⁻¹ of NaCl (pH 7.0)) at 37°C with an addition of 100 μ g mL⁻¹ ampicillin (40).

DNA manipulation and sequencing.

Restriction and modification enzymes were purchased from Toyobo (Osaka, Japan) or Takara (Ohtsu, Japan). KOD Plus (Toyobo) was used for PCR. Plasmid DNA was isolated with a Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany). DNA fragments were recovered from agarose gels with a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK). DNA sequencing was performed with a BigDye Terminator Cycle Sequencing Kit v.3.0 and a Model 3100 capillary DNA sequencer (Applied Biosystems, Foster City, CA).

Expression and purification of MsrA_{TK}, MsrB_{TK}, and MsrAB_{TK}.

Expression plasmids for MsrA_{TK}, MsrB_{TK}, and MsrAB_{TK} were constructed as follows. The respective genes were amplified with *T. kodakaraensis* genomic DNA as a template and two oligonucleotide primers (MsrA_{TK}: sense, 5'-GTGCATATGGGTGGTATCAAATTGAACC-3', and antisense, 5'-

AGGAATTCACCTGAAGTGCCGGTTTTTCTC-3', MsrB_{Tk}: sense, 5'-
TCCATATGTTCCCTGAGAGAGAGGGCTAC-3', and antisense, 5'-
AGGAATTCTACTTAAAAATCCCCTCGTAAG-3', MsrAB_{Tk}: sense, 5'-
GTGCATATGGGTGGTATCAAATTGAACC-3', and antisense, 5'-

TTGAATTCGACTTAAAAATCCCCTCG-3'). NdeI and EcoRI sites were incorporated in the sense and antisense primers, respectively. An artificial initiation codon in the case of MsrB_{Tk} and an artificial stop codon for MsrA_{Tk} were introduced, respectively. The amplified fragments were inserted into pUC118 and sequenced. After confirming the absence of unintended mutations, the NdeI-EcoRI digested fragments were inserted into pET21a(+) and used to transform *E. coli* BL21-CodonPlus(DE3)-RIL. The recombinant cells were grown in LB medium at 37°C, and gene expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside at the mid-exponential growth phase with further incubation for 4 h. Cells were harvested by centrifugation (4,500 x g, 4°C, 20 min), resuspended in 10 mM sodium phosphate buffer (pH 7.0) and sonicated for 5 min (output:pause = 10:20 s; output power = ~80 W) with a Misonix Sonicator Ultrasonic Cell Processor XL2020 (Misonix, Farmingdale, NY). The lysates were centrifuged (20,000 x g, 4°C, 20 min) and the respective supernatants were subjected to heat treatment (MsrA: 5 min at 65°C, MsrB: 10 min at 80°C, MsrAB: 10 min at 75°C). After removing heat-labile proteins by ultracentrifugation (100,000 x g, 4°C, 30 min), the supernatants were applied to an anion exchange column Resource Q (6 mL; GE Healthcare) equilibrated with Buffer A (10 mM sodium phosphate buffer (pH 7.0), 1 mM DTT). Recombinant proteins were eluted with a linear gradient of 0 to 1 M NaCl in Buffer A. In the case of MsrA_{Tk}, samples were further applied to a CHT2-I hydroxyapatite column (2 mL; Bio-Rad, Hercules, CA) equilibrated with Buffer A and eluted with a linear gradient of 10 to 500 mM sodium phosphate buffer (pH 7.0) containing 1 mM DTT. Fractions

containing MsrB_{TK} or MsrAB_{TK} after Resource Q were applied to a hydrophobic column Resource ISO (6 mL; GE Healthcare) equilibrated with 1 M (NH₄)₂SO₄ in Buffer A and eluted with a linear gradient of 1 to 0 M (NH₄)₂SO₄. Finally, all three protein solutions were subjected to a gel filtration column Superdex 200 HR 10/30 (GE Healthcare) with a mobile phase of 50 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl and 1 mM DTT at a flow rate of 0.3 mL min⁻¹. Protein concentrations were determined with the Protein Assay Kit (Bio-Rad) with bovine serum albumin as a standard.

Activity measurements of the recombinant Msr proteins.

Activity measurements were performed using MetO or dabsyl-MetO as a substrate. Dabsyl-MetO was synthesized as follows: 100 μL of 100 mM Met-O solution was mixed with 100 μL of 200 mM bicarbonate buffer pH 9.0, and then with 800 μL of 5 mM dabsyl-Cl in acetonitrile. The solution was placed at 70 °C until the disappearance of precipitate. After 10 min additional incubation at 70 °C, the solution was applied to silica gel column for the separation of dabsyl-MetO from excess Met-O and dabsyl-Cl. Using methanol:acetonitrile (1:1) as separation solvent, fractions which included only the dabsyl-MetO were collected. After evaporation of solution under vacuum, the powder was dissolved in 50 mM sodium phosphate buffer (pH 7.0) (28). Unless otherwise stated, reaction mixtures (50 μL) contained 50 mM sodium phosphate buffer (pH 7.0), 20 mM dithiothreitol (DTT), 3-6 μg of purified enzyme, and substrate. MetO was used at various concentrations in the kinetic analysis, and dabsyl-MetO was used at 1 or 2 mM. Enzyme reactions were terminated by adding 5 μL of TFA (10%, v/v). With MetO as a substrate, this solution was further centrifuged (15,000 x g, 10 min), and 10 μL aliquots of the supernatant were applied to HPLC using a C18 column 5C18-AR-II (Nacalai tesque, Tokyo, Japan) equilibrated with 50 mM sodium phosphate

buffer (pH 4.5) at a flow rate of 1 mL min⁻¹. The amount of the reduced product, Met, was quantified by measuring the absorbance at 215 nm. In the case of dabsyl-MetO, 200 μ L ethanol was added to the terminated reaction mixture, followed by centrifugation (15,000 x g, 10 min), and 10 μ L aliquots of the supernatant were applied to HPLC using a chiral column AD-H (Daicel chemical industries, Osaka, Japan), which could separate dabsyl-Met-*S*-O, dabsyl-Met-*R*-O and dabsyl-Met. The column was equilibrated with 3:1 (v/v) *n*-hexane:ethanol containing 0.1% (v/v) acetic acid at a flow rate of 1 mL min⁻¹. The dabsyl derivatives were detected by measuring the absorbance at 436 nm. In all measurements, experiments were performed in the absence of enzyme, and the nonenzymatic reduction of MetO or dabsyl-MetO was subtracted in calculating enzyme activity.

The effects of pH on the MsrA and MsrB activities of MsrAB_{TK} were examined in standard reaction mixtures with the following buffer replacements: 200 mM Mes-NaOH (pH 5.5-6.5), 200 mM sodium phosphate (pH 6.0-7.5), or 200 mM Tris-HCl (pH 7.5-9.0). The reaction mixture was incubated at 50°C for 4 min, and the substrate reduced by the enzyme was analyzed as described above. The effect of temperature on the activity of each domain was examined in standard reaction mixtures. The reaction mixture was incubated at various temperatures for 1, 3, 5, and 7 min and the consumption of substrates was measured. When examining protein thermostability, purified proteins (3-6 μ g) were incubated in 50 mM sodium phosphate buffer (pH 7.0) at 70°C or 80°C for various periods of time. After incubation, residual enzyme activities were measured with standard procedures at 50°C.

Determination of nonenzymatic reduction of MetO.

The rates of free Met-*R,S*-O reduction were determined at various temperatures in the presence of DTT without enzyme. The reaction mixture (50 μ L) contained 1 mM Met-

R,S-O, 20 mM DTT, and 50 mM sodium phosphate buffer (pH 7.0). The reaction mixture was centrifuged, and 10 μ L aliquots of the supernatant were applied to a C18 column 5C18-AR-II (Nacalai tesque) equilibrated with 50 mM sodium phosphate buffer (pH 4.5) at a flow rate of 1 mL min⁻¹. The amount of the reduced product, Met, was quantified by measuring the absorbance at 215 nm.

Circular dichroism.

A J-820 spectropolarimeter (Jasco, Tokyo, Japan) was used to measure ellipticity as a function of wavelength from 250 nm to 195 nm in 0.2-nm increments using a 0.1-cm cylindrical quartz cuvette. MsrAB_{TK} was solved in 50 mM sodium phosphate buffer (pH 7.0) at a concentration of 0.8 mg mL⁻¹. The thermal denaturation curve at 222 nm was measured with a temperature elevation rate of 0.5°C min⁻¹, and data were collected every 0.2°C.

Fluorescence Spectra.

Intrinsic fluorescence spectra were measured with a fluorescence spectrophotometer capable of maintaining the cuvette at desired temperatures between 30 and 100°C (model F-2500, Hitachi, Tokyo, Japan). MsrAB_{TK} was solved in 50 mM sodium phosphate buffer (pH 7.0) at a concentration of 0.05 mg mL⁻¹. The fluorescence spectra of MsrAB_{TK} solution excited at 260 nm and 295 nm were measured in the range of 300 to 450 nm at various temperatures.

RESULTS

The MsrAB fusion protein from *T. kodakaraensis* displays unique structural features among the archaeal Msr proteins.

From the complete genome sequence of *T. kodakaraensis*, the author found an open reading frame that encoded a protein (TK0819) whose N-terminal and C-terminal regions displayed similarity with previously characterized MsrA and MsrB proteins, respectively. The author designated the gene as *msrAB_{TK}*, and its translation product as MsrAB_{TK}. MsrAB_{TK} was composed of 338 amino acids, and the molecular weight was calculated to be 39,119 Da. Although the N-terminal region of MsrAB_{TK} was shorter than the eukaryotic and bacterial enzymes, the possibilities that translation initiates further upstream are extremely low. A putative ribosomal binding site was present from positions -8 to -12 (relative to the initiation codon), a TATA sequence from -34 to -39, and an in-frame stop codon was found from -52 to -54. The MsrA-domain (designated as residues 1–184) was 49% identical to the MsrA from *E. coli*, and 33% to that from *Saccharomyces cerevisiae*. The MsrB-domain (residues 186–338) was 44% identical to the MsrB from *E. coli*, and 31% to that from *S. cerevisiae*. Sequence alignments of the respective domains with various Msr proteins/domains are shown in Fig. 2A (MsrA) and Fig. 2B (MsrB). The MsrA-domain of MsrAB_{TK} possesses a GCFWC sequence near its N-terminus (residues 17-21), indicating that this domain is a member of the third group of MsrA proteins (MsrA_{III}). As this domain does not harbor any further Cys residues, it is most likely that catalysis is carried out by these two residues. The MsrB-domain of MsrAB_{TK} does not contain the CxxC motifs, indicating that it is a Form II MsrB. This is noteworthy as Form II enzymes have previously been found only in bacteria. The strictly conserved catalytic CysA residue of MsrB proteins was present (Cys311), but the second CysB residue, conserved in a majority of MsrB proteins, was not found. Additional Cys

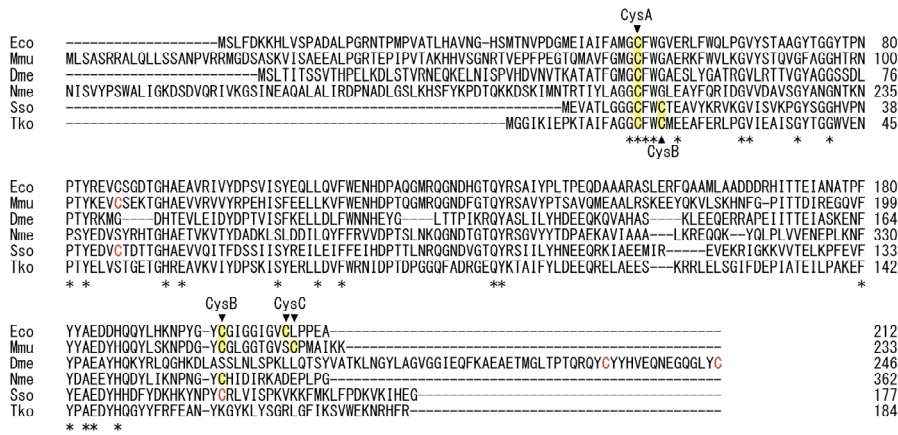
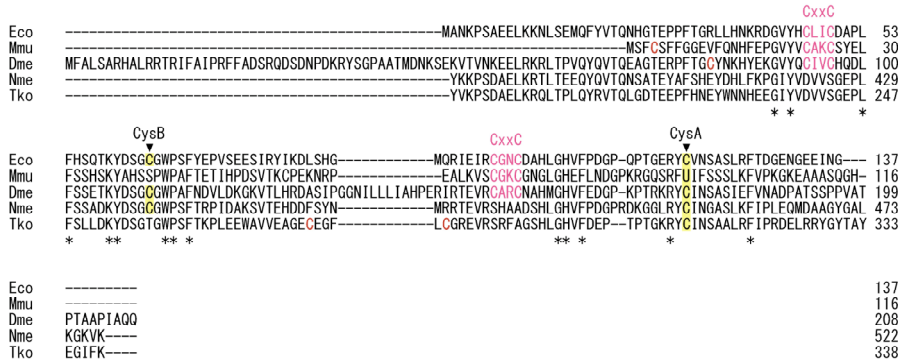
A.**B.**

Fig. 2. Sequence alignment of MsrA proteins/domains (A) and MsrB proteins/domains (B). Alignments were constructed with the following sequences; MsrA; *Azoarcus sp.*, CAI06587; *Bacillus cereus*, AAS44469; *Bacillus licheniformis*, AAU41183; *Bacillus subtilis*, CAB14087; *Bdellovibrio bacteriovorus*, CAE78986; *Drosophila melanogaster*, AAN28311; *Escherichia coli*, AAC77176; *Haloarcula marismortui* (1), AAV46883; *Haloarcula marismortui* (2), AAV46414; *Halobacterium sp.*, AAG19555; *Heliobacillus mobilis*, AAN87501; *Homo sapiens*, AAP97154; *Idiomarina loihiensis*, AAV82363; *Methanococcus maripaludis*, CAF30404; *Methanosarcina acetivorans*, AAM04846; *Methanosarcina mazei*, AAM32095; *Methanothermobacter thermotrophicus*, AAB85041; *Mus musculus*, AAH89311; *Mycobacterium bovis*, CAD93006; *Neisseria gonorrhoeae*, CAA32146; *Neisseria meningitides*, AAF40515; *Picrophilus torridus*, AAT42728; *Pseudomonas aeruginosa*, AAG08403; *Saccharomyces cerevisiae*, AAT92817; *Sulfolobus solfataricus*, AAK41726; *Symbiobacterium thermophilum*, BAD40746; *Thermococcus kodakaraensis*, BAD85008; *Vibrio cholerae*, AAF96516; *Vibrio vulnificus*, AAO08507. MsrB; *Azoarcus sp.*, CAI09433; *Bacillus cereus*, AAS44469; *Bacillus licheniformis*, AAU41182; *Bacillus subtilis*, CAB14086; *Bdellovibrio bacteriovorus*, CAE79257; *Drosophila melanogaster*, AAN13491; *Escherichia coli*, AAC74848; *Haloarcula marismortui*, AAV45184; *Halobacterium sp.*, AAG19724; *Heliobacillus mobilis*, AAN87501; *Homo sapiens*, AAQ88596; *Idiomarina loihiensis*, AAV82363; *Methanosarcina acetivorans*, AAM03895; *Methanosarcina mazei*, AAM31330; *Methanothermobacter thermotrophicus*, AAB85216; *Mus musculus*, AAH96447; *Mycobacterium bovis*, CAD94878; *Neisseria gonorrhoeae*, CAA32146; *Neisseria meningitides*, AAF40515; *Pseudomonas aeruginosa*, AAG06215; *Saccharomyces cerevisiae*, CAA42383; *Symbiobacterium thermophilum*, BAD40746; *Thermococcus kodakaraensis*, BAD85008; *Vibrio cholerae*, AAF96516; *Vibrio vulnificus*, AAO08507. Representative sequences were selected and are shown. Amino acid residues presumed to act as CysA, CysB and CysC are indicated by arrowheads and highlighted in yellow. Other Cys residues that may be involved in catalysis are indicated in red. Cys86 of MsrA from *E. coli* has been experimentally verified not to be involved in catalysis. The two CxxC motifs that bind to a divalent zinc cation in MsrB are indicated in pink. Conserved residues are indicated with asterisks. Abbreviations are Dme, *Drosophila melanogaster*; Eco, *Escherichia coli*; Mmu, *Mus musculus*; Nme, *Neisseria meningitides*; Sso, *Sulfolobus solfataricus*; Tko, *Thermococcus kodakaraensis*.

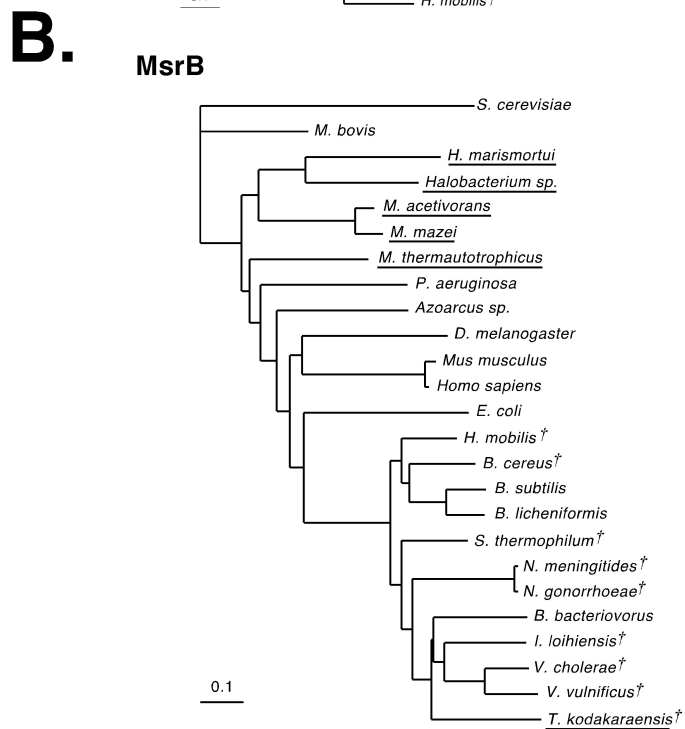
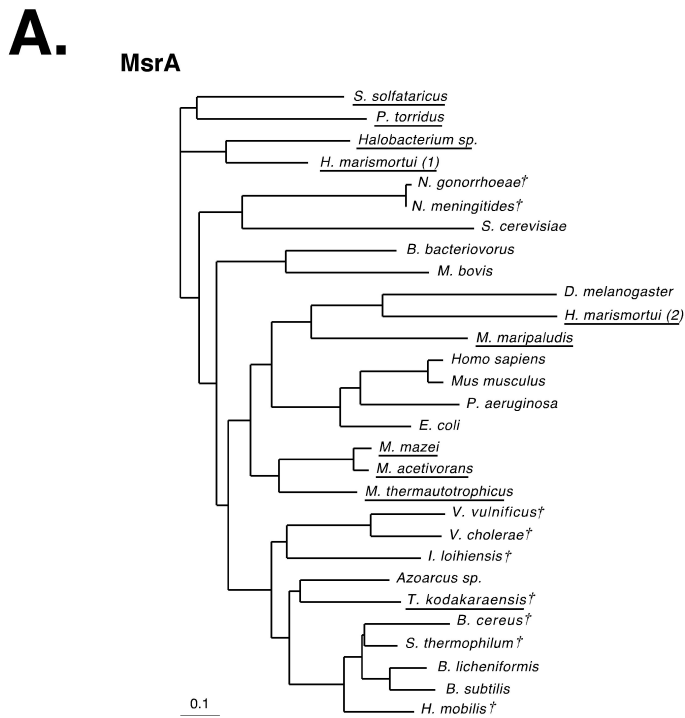


Fig. 3. Phylogenetic analyses of Msr proteins. Unrooted phylogenetic trees for MsrA (A) and MsrB (B) proteins/domains were constructed with the neighbor-joining method. Homologous core regions in MsrA (corresponding to residues 8 to 177 in MsrAB_{TK}) and MsrB (residues 201 to 338) from various sources were used. Proteins deriving from archaeal strains are underlined. MsrA-MsrB fusion proteins are indicated with daggers (†). The accession numbers of sequences use for constructing phylogenetic trees are described in the legend of Fig. 2.

residues at positions 278 and 283 may act as the CysB in the MsrB-domain (Fig. 2B).

Phylogenetic analyses of Msr proteins was performed and the resulting trees are illustrated in Figs. 3A (MsrA) and 3B (MsrB). In the case of fusion proteins, only sequences

of the corresponding domains were utilized. Nevertheless, the MsrA domains within fusion proteins were found to be phylogenetically related to one another, forming a large cluster in the tree. Two exceptions, the proteins from the pathogenic *Neisseria gonorrhoeae* and *N. meningitides*, are both rare examples in which the MsrA and MsrB domains are fused with the further addition of a thioredoxin domain. The MsrA domain of MsrAB_{Tk}, although an archaeal Msr, was more related with the MsrA domains found in bacterial fusion proteins. The same tendency was found in the phylogenetic analysis of the MsrB proteins/domains, with the MsrB domain of MsrAB_{Tk} clustering with the bacterial fusion proteins, and not the archaeal MsrB proteins. Together with the fact that the MsrB domain is a bacterial type Form II enzyme, this suggests that the MsrAB_{Tk} gene in *T. kodakaraensis* is a result of horizontal gene transfer from (mesophilic) bacteria harboring MsrAB fusion genes.

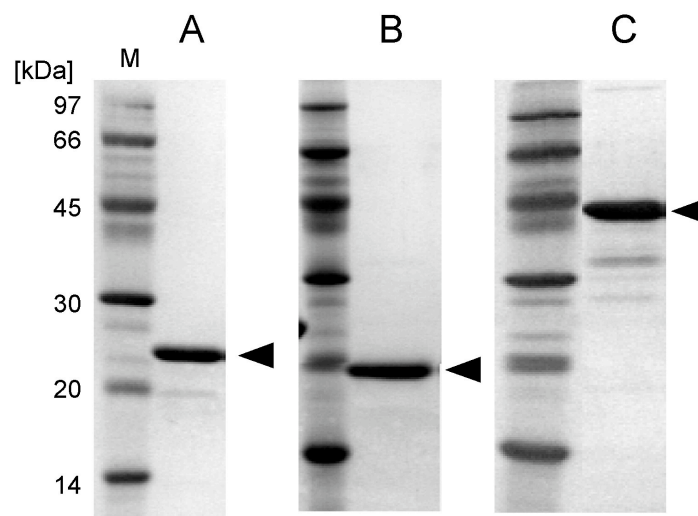


Fig. 4. Purification of MsrA_{Tk} (A), MsrB_{Tk} (B), and MsrAB_{Tk} (C). Fractions after the purification procedures were applied to SDS-PAGE, and the gels were stained with Coomassie Brilliant Blue.

Expression and purification of recombinant MsrA_{TK}, MsrB_{TK}, and MsrAB_{TK}.

The *msrAB_{TK}* gene was expressed in *E. coli*, and the recombinant protein was purified by heat treatment, anion exchange, hydrophobic, and gel-filtration chromatographies. In order to clarify the stereoselectivity of the respective domains, the individual domains were also produced separately in *E. coli*. The protein corresponding to the N-terminal domain was identical to the N-terminal 184 residues of MsrAB_{TK}, and designated MsrA_{TK}. An artificial Met residue was inserted at the N-terminal side of residue 186Phe to produce the recombinant C-terminal domain, designated as MsrB_{TK}. Both proteins were purified as described in Materials and Methods. SDS-PAGE analysis showed that the recombinant proteins were purified to apparent homogeneity, respectively, with the expected molecular weight (Fig. 4).

Stereoselectivity of MsrA_{TK} and MsrB_{TK}.

The author first examined the Msr activity of the recombinant proteins with several electron donors. By measuring the production of Met from Met-*R,S*-O with HPLC, the author found that the recombinant MsrA_{TK} and MsrB_{TK} proteins exhibited Msr activity in the presence of 20 mM DTT (MsrA_{TK}, 3.5 $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$; MsrB_{TK}, 1.9 $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$). In the case of MsrB_{TK}, the author also observed activity with 20 mM cysteine (0.6 $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$). No activity was observed in either protein with 20 mM reduced glutathione. The recombinant MsrAB_{TK} protein also exhibited activity in the presence of DTT. Further enzymatic examinations were thus performed with DTT as the electron donor.

The author examined the stereoselectivity of MsrA_{TK}. A partially enriched Met-*S*-O solution (*S*:*R*=73:27) and Met-*R*-O solution (*S*:*R*=5:95) were prepared from L-methionine sulfoxide as described elsewhere (32). The enrichment of either one of the epimers was confirmed by polarimetric measurements (data not shown). These solutions and a racemic

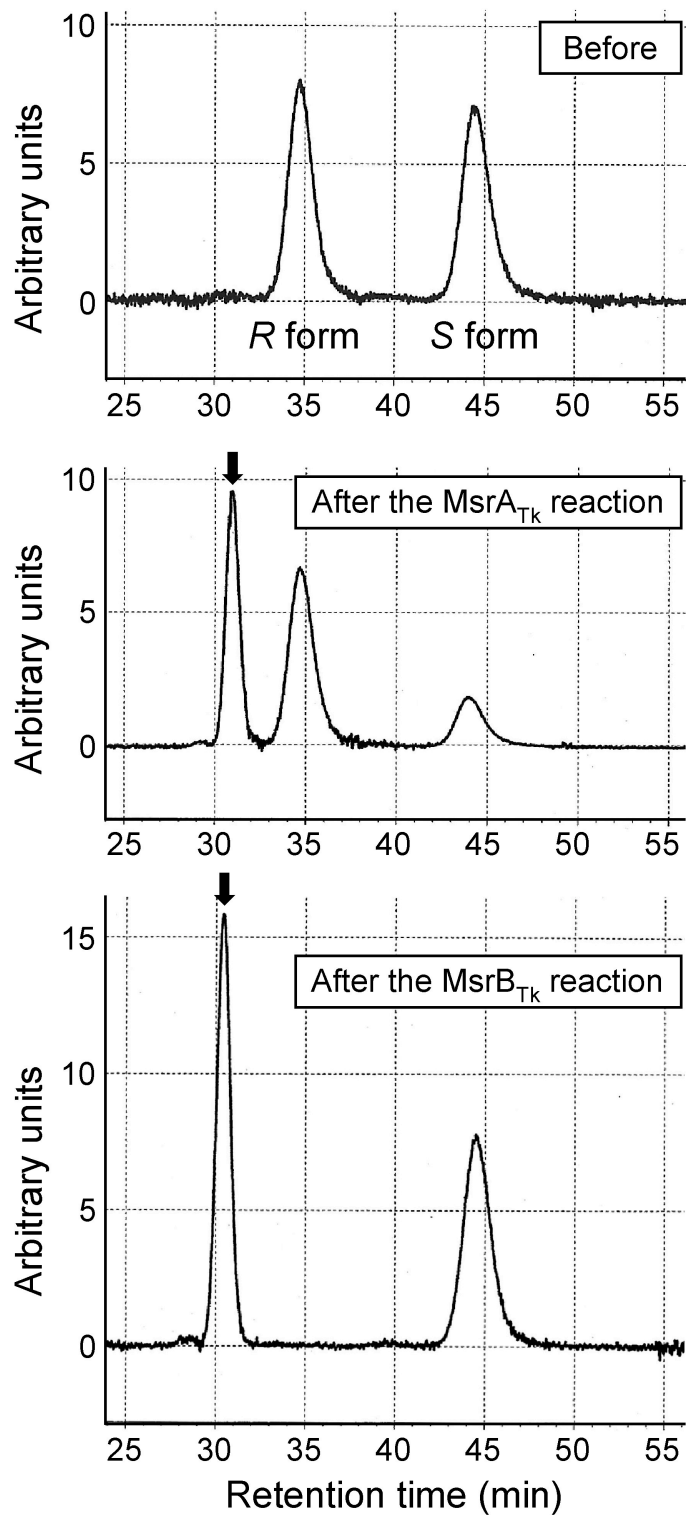


Fig. 5. Stereoselectivity of MsrA_{TK} and MsrB_{TK}. Reactions were carried out as described in Materials and Methods with 1 mM dabsyl-MetO and 6 μ g of purified protein. The MsrA_{TK} and MsrB_{TK} reactions were performed for 30 min at 60 and 30°C, respectively. The arrow indicates the peak of the reaction product, dabsyl-Met.

solution (*S*:*R*=50:50) were used as substrates for MsrA_{TK}. MsrA_{TK} displayed highest activity with the Met-*S*-O enriched solution, followed by the racemic solution, and displayed relatively low levels of activity against the Met-*R*-O enriched solution. In order to clarify whether the preference for Met-*S*-O was strict, further experiments were carried out using dabsyl-MetO. Dabsyl-MetO and acetyl-MetO are often used as model substrates that are presumed to better represent MetO residues within polypeptide chains (25). Dabsylated MetO also enables the separation of the two epimers, dabsyl-Met-*S*-O and dabsyl-Met-*R*-O, with a chiral column, as shown in Fig. 5. After the reaction with MsrA_{TK}, the author observed the reduction of only one of the two epimers with no detectable decrease of the other. Taking into account the preference of MsrA_{TK} for Met-*S*-O, the author could conclude that the domain was strictly specific for the reduction of Met-*S*-O. When MsrB_{TK} was examined in the same manner, the author found specific reduction of the opposite epimer. This clearly indicates that MsrB_{TK} is specific for Met-*R*-O.

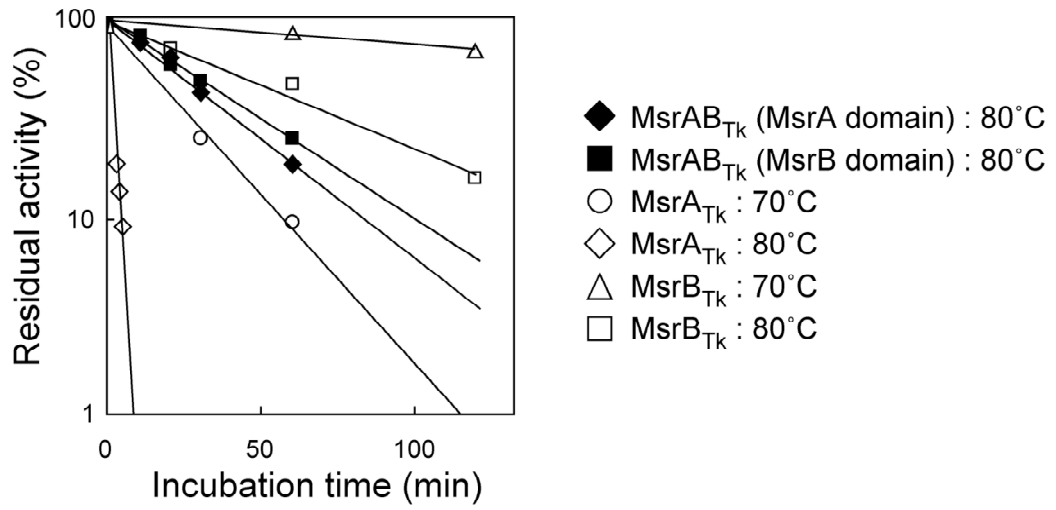
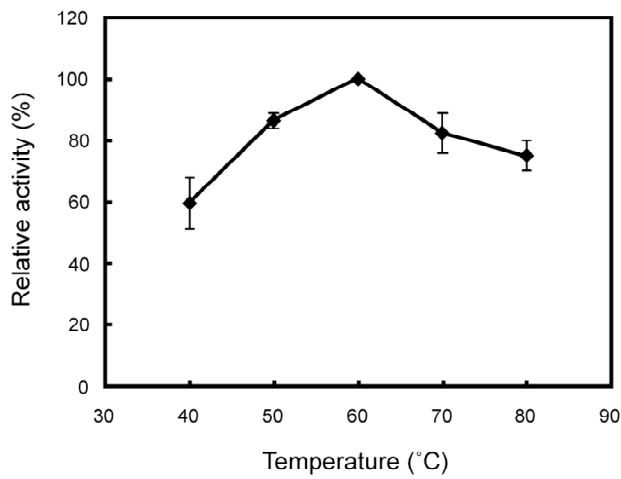
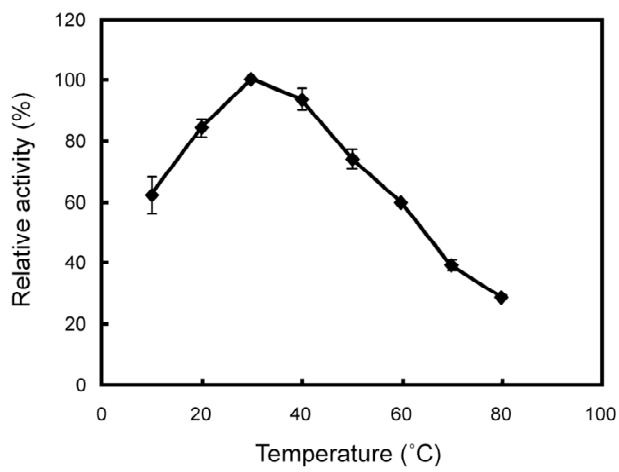
Thermostability of MsrA_{TK}, MsrB_{TK}, and MsrAB_{TK}.

The thermostabilities of MsrA_{TK}, MsrB_{TK}, and MsrAB_{TK} were examined at various temperatures (Fig. 6A). By using the dabsyl-MetO substrates, the author could examine the thermostabilities of the two domains of the MsrAB_{TK} fusion protein individually. While the recombinant MsrB_{TK} protein was relatively stable at 80°C ($T_{1/2} = 48$ min), the MsrA_{TK} protein was surprisingly thermolabile ($T_{1/2} \ll 1$ min). Interestingly, the MsrA domain within the MsrAB_{TK} protein exhibited higher thermostability ($T_{1/2} = 27$ min) than the individual MsrA_{TK} protein, suggesting that the domain is stabilized through its physical interaction with the more thermostable MsrB domain ($T_{1/2} = 31$ min). However, although MsrAB_{TK} is much more stable than a protein from a mesophile, the thermostabilities of both domains were relatively low

compared to other proteins from this hyperthermophile, which grows optimally at 85°C. The author observed a 75% decrease in total activity after 2.5 min at 85°C.

Optimum pH and temperature of MsrAB_{TK}.

The effect of pH on the reactions catalyzed by each domain of recombinant MsrAB_{TK} was examined with dabsyl-MetO. The author found that the MsrB domain exhibited highest activity between pH 6.0 and 7.0, while the optimum pH of the MsrA domain was slightly higher, in the range of 7.5-8.0 (data not shown). The author further examined the effects of temperature on the respective reactions. Using dabsyl-MetO, the MsrA domain of MsrAB_{TK} exhibited highest activity at a relatively low temperature of 60°C (Fig. 6B). Moreover, in the case of the MsrB domain, activity was measured from 10 to 80°C, and maximum activity levels were observed at a surprisingly low 30°C (Fig. 6C). In these experiments, the author could not apply saturating concentrations of substrate. As the reaction product is the same dabsyl-Met, it was necessary to quantify the decrease in substrate concentration (dabsyl-Met-*S*-O or dabsyl-Met-*R*-O) in order to distinguish the activities of the two domains in MsrAB_{TK}. In order to estimate the specific activities of each domain, the author applied non-dabsylated, free MetO substrates, and measured the reaction rates with MsrA_{TK}, MsrB_{TK} and MsrAB_{TK} at 30 and 60°C. In the presence of 20 mM DTT and 50 mM MetO, MsrA_{TK} displayed specific activity levels of 0.16 and 2.7 $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$ at 30 and 60°C, respectively. The results for MsrB_{TK} were 1.4 (30°C) and 0.34 (60°C) and those for MsrAB_{TK} were 0.54 (30°C) and 2.2 (60°C) $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$. Consistent with the results of the MsrB domain using dabsyl-MetO, the author found that MsrB_{TK} also exhibited higher levels of activity at 30°C than at 60°C using high concentrations of MetO. The author performed a kinetic analysis of the MsrA_{TK} and MsrB_{TK} reactions towards MetO and DTT. Examinations were performed at

A.**B.****C.**

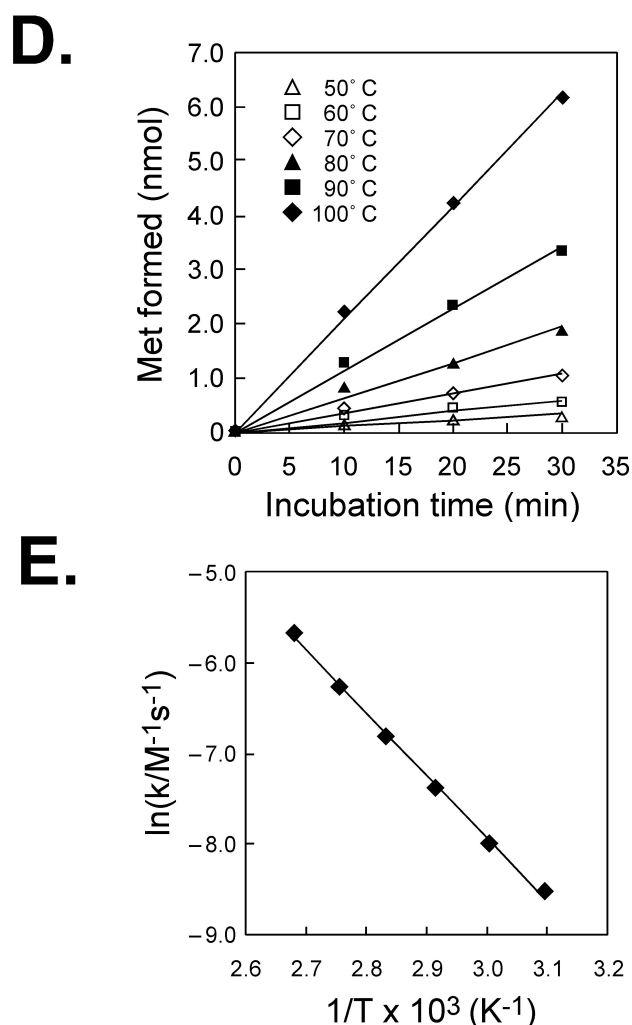


Fig. 6. Enzymatic characterization of MsrAB_{TK}. (A) Thermostability of MsrA_{TK}, MsrB_{TK}, and MsrAB_{TK}. Purified proteins (3-6 μ g) were incubated in 50 mM sodium phosphate buffer (pH 7.0) at 70°C or 80°C for various periods of time. After incubation, residual enzyme activities were measured at 50°C. (B, C) Effect of temperature on activity levels of the MsrA domain (B) and MsrB domain (C). Reactions were performed for 5 min at substrate concentrations of 2 mM (B) and 1 mM (C). (D) Non-enzymatic reduction of MetO. The reaction mixture (50 μ L) contained 1 mM Met-*R,S*-O, 20 mM DTT, and 50 mM sodium phosphate (pH 7.0). The produced Met was detected and quantified by HPLC. Aliquots (10 μ L) of the reaction mixture after centrifugation were applied to a C18 column 5C18-AR-II column (Nacalai tesque) equilibrated with 50 mM phosphate buffer (pH 4.5) at a flow rate of 1 mL min⁻¹. The amount of Met was monitored by absorbance at 215 nm. (E) An Arrhenius plot of the results of (D).

the optimal reaction temperatures for MsrA_{TK} (60°C) and MsrB_{TK} (30°C). The K_m and V_{max} values are shown in Table 2.

The higher levels of MsrB activity at lower temperatures were intriguing taking into

Table 2. Kinetic parameters of the reactions catalyzed by MsrA_{TK} and MsrB_{TK}.

| Enzyme | Substrate | Apparent K_m (mM) | V_{max} ($\mu\text{mol min}^{-1} \text{mg protein}^{-1}$) |
|--------------------|-----------------------------|---------------------|---|
| MsrA _{TK} | MetO ^a (Met-S-O) | 19 ± 3 (9.3 ± 1.3) | 7.8 ± 0.3 |
| | DTT ^b | 15 ± 2 | |
| MsrB _{TK} | MetO ^c (Met-R-O) | 67 ± 6 (33 ± 3) | 3.0 ± 0.1 |
| | DTT ^b | 2.0 ± 0.2 | |

^aIn the presence of 100 mM DTT.

^bIn the presence of 80 mM MetO.

^cIn the presence of 20 mM DTT.

account that the domain (and the MsrB_{TK} protein) is relatively thermostable in this temperature range ($T_{1/2}$ =31 min at 80°C). In order to confirm that these results accurately reflected the initial velocity of the reaction, the author measured the consumption of substrate (dabsyl-Met-R-O) after various intervals of time. The author found that substrate consumption was proportional with time, ruling out the possibility that enzyme inactivation at higher temperatures led to these results. Generation of dabsyl-Met was observed in these measurements, indicating that the substrate consumption was not due to an enzyme independent degradation of substrate. Furthermore, the author also measured the reaction in the absence of enzyme (see below), and found that chemical degradation of MetO is actually lower at lower temperatures. There is little possibility that an enzyme-independent chemical conversion of the substrate is a major factor, as this would lead to similar results with the MsrA and MsrB domains, which is clearly not the case. The results indicate that the MsrB domain of MsrAB_{TK} is a unique example of a thermostable protein that exhibits higher catalytic efficiency at lower temperatures.

Nonenzymatic reduction of MetO at various temperatures.

While measuring Msr activity at various temperatures, the author observed that the MetO reduction rates in the presence of DTT without enzyme became surprisingly high at

higher temperature ranges. The rates of nonenzymatic MetO reduction at various temperatures are shown in Fig. 6D. As in the case of the enzyme-dependent reaction, Met generation was observed with the consumption of MetO, and peaks other than those appearing on the chromatogram after the enzyme-dependent reaction were not observed. Furthermore, MetO degradation was not observed in the absence of DTT, ruling out the possibility that MetO is converted to compounds other than Met in the reaction mixture. As shown in Fig. 6D, a clear elevation in reaction rates was observed, reaching $>0.2 \text{ nmol min}^{-1}$ in the presence of 1 mM MetO and 20 mM DTT. An Arrhenius plot of the reaction gave a linear plot in the temperature range examined (Fig. 6E), and revealed that the activation energy of the reaction was 57.5 kJ mol^{-1} , comparable to values observed in enzyme-catalyzed reactions.

Circular dichroism analysis.

The intriguing effect of temperature on the MsrB activity of MsrAB_{TK} can be explained by a reversible change in conformation of the protein at elevated temperatures. In order to examine whether this change could be observed at the secondary structure level, the author carried out a CD analysis of MsrAB_{TK} at various temperatures. A gradual increase in ellipticity was observed when temperatures were elevated from 40 to 50, 60 and 70°C (Fig. 7A). This change was reversible, as the CD spectrum was indistinguishable to the original spectrum when the temperature was lowered back to 40°C (Fig. 7B). However, as the observed changes in the spectra were slight, the author could not strongly relate these changes to the effect of temperature on activity. The author next performed thermal denaturation experiments by following the change in ellipticity at 222 nm. The protein (0.8 mg mL^{-1}) was solved in 50 mM sodium phosphate buffer (pH 7.0) in the presence (not shown) or absence (Fig. 7C) of 20 mM DTT. In each case, MsrAB_{TK} displayed a two-state folding transition with

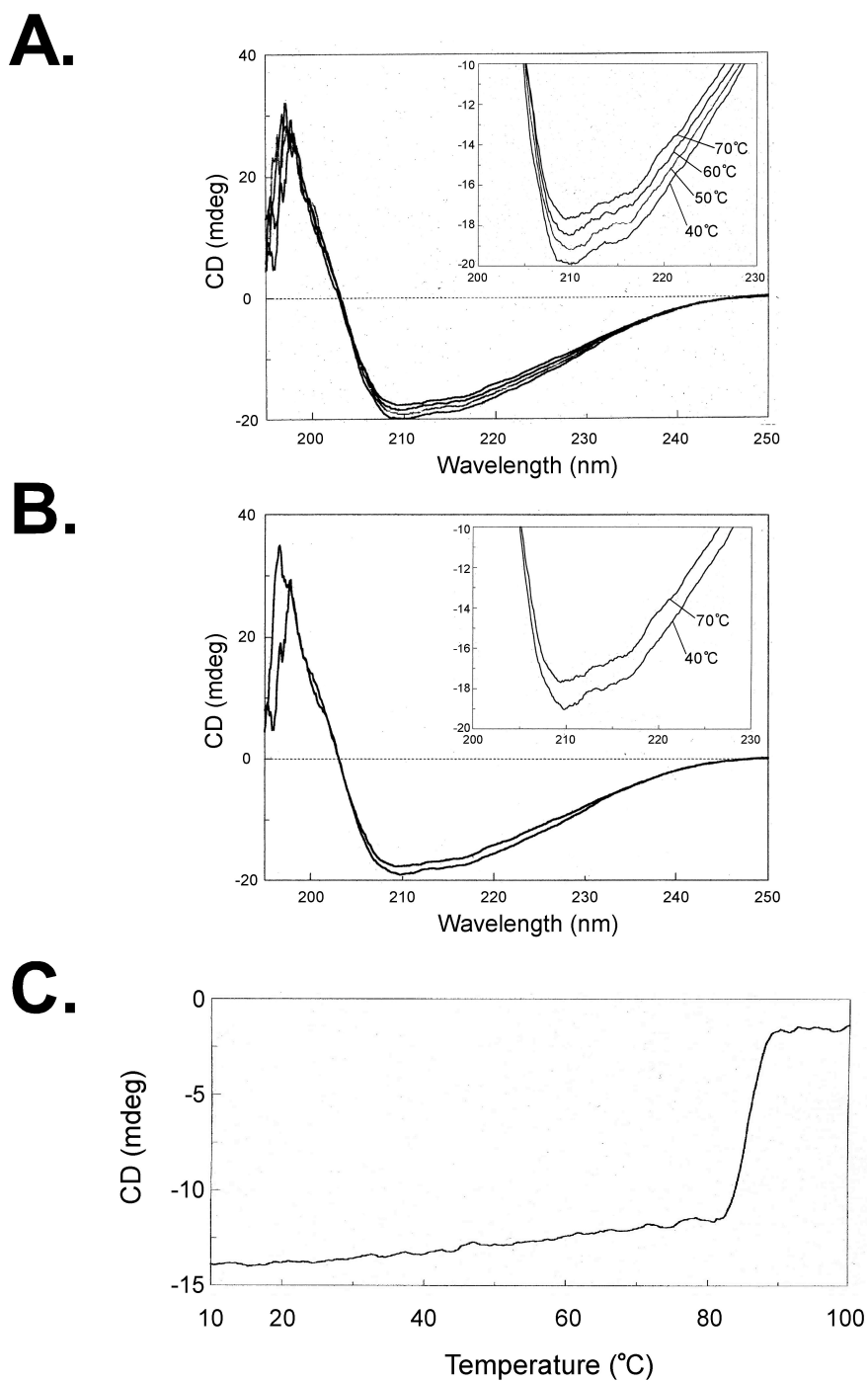


Fig. 7. Circular dichroism analysis of MsrAB_{TK}. (A) CD spectra of MsrAB_{TK} between 195 nm and 250 nm at 40, 50, 60, and 70°C. The ellipticity between 200 and 230 nm is enlarged. (B) CD spectrum of MsrAB_{TK} at 70°C, and that of the same protein sample after shifting the temperature to 40°C. The ellipticity between 200 and 230 nm is enlarged. (C) A thermal denaturation curve of MsrAB_{TK} at 222 nm measured with a temperature elevation rate of 0.5°C min⁻¹. In all cases, MsrAB_{TK} was present in 50 mM sodium phosphate buffer (pH 7.0) at a concentration of 0.8 mg mL⁻¹. The y axis represents the measured ellipticity, in millidegrees (mdeg).

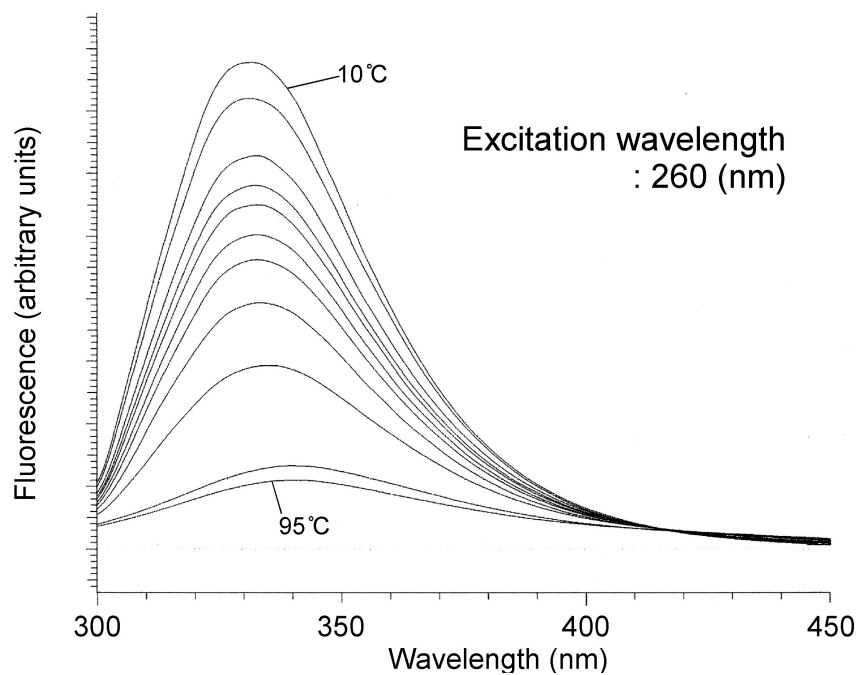
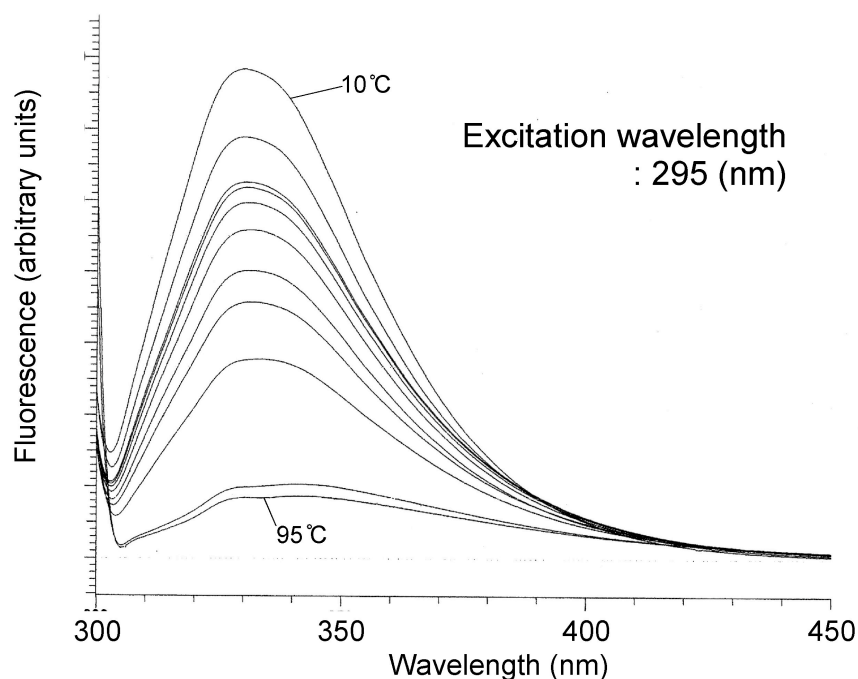
A.**B.**

Fig. 8. Intrinsic fluorescence spectra of MsrAB_{TK} at various temperatures. MsrAB_{TK} was solved in 50 mM sodium phosphate buffer (pH 7.0) at a concentration of 0.05 mg mL⁻¹. The fluorescence spectra of MsrAB_{TK} solution excited at 260 nm (A) and 295 nm (B) were measured in the range of 300 to 450 nm at 10, 20, 30, 35, 40, 50, 60, 70, 80, 90 and 95°C. In both cases, peak intensity decreased with the elevation in temperature.

a midpoint of denaturation at approximately 83°C. This further supports that the protein is relatively stable at temperatures up to 80°C, but readily denatured at 85°C, the optimal growth temperature of *T. kodakaraensis*. The results also indicate that the reversible change in conformation presumed to convert the enzyme to a less active form at higher temperatures does not involve a major change in the secondary structure of the protein.

Fluorescence emission spectra of tryptophan and tyrosine residues.

The author next examined the fluorescence emission spectra of tryptophan and tyrosine residues at various temperatures. Excitation wavelengths of 260 nm (Fig. 8A) and 295 nm (Fig. 8B) were applied for tyrosine and tryptophan, respectively, as well as 280 nm (data not shown). Emission spectra were examined between 300 nm and 450 nm at temperatures of 10, 20, 30, 35, 40, 50, 60, 70, 80, 90 and 95°C. At lower temperatures, the maximum emission intensity was observed between 330 and 333 nm, and a gradual decrease in intensity was observed with an elevation in temperature. At 90°C, a temperature at which the protein unfolds irreversibly, a drastic decrease in intensity, along with a characteristic red-shift of the emission peak, was observed. When comparing the spectra at various temperatures, the author could not detect any significant changes that seemed to correlate with the activity levels of the protein at low temperatures. The results agreed well with those obtained with CD spectrum analyses.

DISCUSSION

In this chapter, the author has examined the biochemical properties of an MsrA-MsrB fusion protein from *T. kodakaraensis* KOD1, whose presence is exceptional among the hyperthermophiles. The two domains of MsrAB_{Tk} exhibited strict substrate selectivity, MsrA

for Met-S-O and MsrB for Met-R-O. The most striking feature of the protein was that MsrB activity levels were found to be maximal at 30°C, well below the optimal growth temperature of this hyperthermophile (85°C). Consistent with this behavior, the protein readily denatures at 85°C *in vitro*. There is a possibility that the protein exhibits higher thermostability within the cell by the interaction with various intracellular compounds such as compatible solutes or potassium glutamate (46).

The MsrB domain of MsrAB_{Tk} exhibited maximum activity at a strikingly low temperature of 30°C. This was also observed in the recombinant MsrB_{Tk} protein. This was not due to (irreversible) thermal inactivation of the proteins at higher temperatures, as both MsrAB_{Tk} and MsrB_{Tk} displayed half-lives of over 30 min at 80°C. This indicates that the MsrB domain undergoes a reversible change in conformation at higher temperatures that brings about a decrease in activity. The decrease in activity is most likely due to a decrease in k_{cat} , as the specific activity of the MsrB_{Tk} protein under saturating concentrations of MetO also displayed higher activity at lower temperatures. As the CD and fluorescence emission spectra did not point to large or global changes in structure, the changes in activity levels of the enzyme at different temperatures are most likely due to structural alterations localized near the active site.

In the literature, a novel concept for enzymes has been proposed and experimentally verified, introducing a third intrinsic thermal parameter for enzymes, T_{eq} (36). The concept is based on the presumption that enzymes are at a reversible equilibrium between active (E_{act}) and inactive forms (E_{inact}), and that irreversible inactivation of the enzyme to the thermally denatured state (X) initiates only from the inactive form ($E_{act} \rightleftharpoons E_{inact} \rightarrow X$). The concentration of active enzyme can always be expressed as $[E_{act}] = ([E_0] - [X]) / (1 + K_{eq})$, where $[E_0]$ is the initial total concentration of enzyme, and K_{eq} is the equilibrium constant between

active and inactive forms of the enzyme ($K_{eq} = [E_{inact}]/[E_{act}]$). T_{eq} is defined as the temperature at which $K_{eq} = 1$, or $[E_{act}] = [E_{inact}]$. This concept has been experimentally verified for at least five enzymes, and consequently reveals that enzymes intrinsically harbor an optimal temperature for activity that is no longer dependent on thermal stability (36). This can easily be envisioned for an enzyme with a flexible catalytic core mounted on a rigid, global scaffold with high thermal stability. The MsrB domain of MsrAB_{TK} may very well be one extreme example of this kind of enzyme behavior, with a catalytic core structure that is optimal at 30°C, while the overall protein does not display significant thermal denaturation until temperatures above 80°C.

At high temperatures, the function of Msr may not be necessary, as high rates of MetO reduction in an enzyme-independent manner were observed. Although the rates of the enzyme-independent reaction greatly rely on the concentration and redox potential of the native electron donor (thioredoxin_{red}) *in vivo*, the high reaction rates observed in the reaction mixtures at elevated temperatures may be related to the absence of Msr homologs in most hyperthermophiles, particularly those that grow only at extremely high temperature ranges. *T. kodakaraensis* and *S. solfataricus*, the only hyperthermophiles harboring an Msr, exhibit relatively low optimal growth temperatures compared to other hyperthermophiles (3, 14). The function of the enzyme would therefore be more relevant in these organisms, which grow at temperatures lower than those that support the majority of other hyperthermophiles.

In addition to its function in growing cells, MsrAB_{TK} may also function in cells that are in the process of, or recovering from, exposure to low-temperature environments that can no longer sustain growth. A decrease in temperature brings about an increase in dissolved oxygen concentrations. Oxidative damage of biomolecules can therefore be expected to accumulate at these low temperatures, particularly when the cells are dormant. Production of MsrAB_{TK} at

sub-optimal growth temperatures would prepare the cell to tolerate further, more drastic temperature decreases and would also help the cell to recover from these conditions by initiating the repair of oxidized Met residues as soon as possible. MsrAB_{TK} exhibits activity at temperatures much lower than those expected to support growth of *T. kodakaraensis*. There would be a great advantage if the enzyme were to function in the cells throughout the temperature range observed in the *in vitro* experiments in this chapter. However, the lower temperature limit for the enzyme to function and turnover *in vivo* may well be restricted not by the enzyme itself, but by the activity levels of thioredoxin reductase and other enzymes that provide the reducing power to repair MetO. The effect of temperature on the activities and expression levels of these proteins *in vivo* are of high interest, and have been examined in the studies described in Chapter 2.

SUMMARY

Methionine sulfoxide reductase (Msr) catalyzes the thioredoxin-dependent reduction and repair of methionine sulfoxide (MetO). Although Msr genes are not present in most hyperthermophile genomes, an Msr homolog encoding an MsrA-MsrB fusion protein (MsrAB_{TK}) was present on the genome of the hyperthermophilic archaeon, *Thermococcus kodakaraensis*. Recombinant proteins corresponding to MsrAB_{TK} and the individual domains (MsrA_{TK} and MsrB_{TK}) were produced, purified, and biochemically examined. MsrA_{TK} and MsrB_{TK} displayed strict substrate selectivity for Met-S-O and Met-R-O, respectively. MsrAB_{TK}, and in particular the MsrB domain of this protein, displayed an intriguing behavior for an enzyme from a hyperthermophile. While MsrAB_{TK} was relatively stable at temperatures up to 80°C ($T_{1/2} = \sim 30$ min at 80°C), a 75% decrease in activity was observed after 2.5 min at 85°C, the optimal growth temperature of this archaeon. Moreover, maximal levels of MsrB activity

of MsrAB_{Tk} were observed at a strikingly low temperature of 30°C, which was also observed for MsrB_{Tk}. While measuring background rates of the Msr enzyme reactions, the author observed significant levels of MetO reduction at high temperatures without enzyme. The occurrence of non-enzymatic MetO reduction at high temperatures may explain the specific absence of Msr homologs in most hyperthermophiles. Together with the fact that the presence of Msr in *T. kodakaraensis* is exceptional among the hyperthermophiles, the enzyme may represent a novel strategy for this organism to deal with low-temperature environments in which the dissolved oxygen concentrations increase.

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CHAPTER 2

A genetic examination on methionine sulfoxide reductase of the hyperthermophilic archaeon, *Thermococcus kodakaraensis*

INTRODUCTION

As mentioned in the previous chapter, methionine residues are known to be particularly susceptible to oxidative stress, and are easily oxidized to methionine sulfoxides. This can have a detrimental effect towards enzyme activity, and in some cases complete inactivation of the enzyme is observed (6, 11). Methionine sulfoxide reductase (Msr) is an enzyme that repairs oxidized methionine residues, and catalyzes the thioredoxin-dependent reduction of methionine sulfoxide (MetO) to methionine (Met) (5). The oxidation of Met results in the formation of two enantiomers, Met-*S*-O and Met-*R*-O. The individual enantiomers are reduced to Met via the function of two structurally distinct enzymes, MsrA (for Met-*S*-O) and MsrB (for Met-*R*-O) (4, 8).

As Msr plays a major role in protecting proteins from oxidative damage, Msr proteins are widely distributed in nature and can be found in all three domains of life, *Eucarya*, *Bacteria* and *Archaea*. The author has previously observed that although Msr homologs are present in many of the archaeal genomes, they are absent in almost all of the genomes of the hyperthermophilic archaea. The only two exceptions are the MsrA homolog found in *Sulfolobus solfataricus* (16), and the MsrA-MsrB fusion homolog on the genome of *Thermococcus kodakaraensis* (9). In addition, Msr homologs are not found on the genomes of hyperthermophilic bacteria such as *Aquifex aeolicus* (7), *Carboxydothemus hydrogenoformans* (18), *Thermotoga maritima* (12) and *Thermoanaerobacter tengcongensis*

(3).

In the previous chapter, the author has examined the biochemical properties of the MsrA-MsrB fusion protein (MsrAB_{TK}, BAD85008) from the hyperthermophilic archaeon, *T. kodakaraensis*. *T. kodakaraensis* exhibits optimal growth at 85°C and is an obligate anaerobe and heterotroph (2). A phylogenetic analysis of the individual domains of MsrAB_{TK} with various MsrA and MsrB sequences suggested that the MsrA and MsrB domains of MsrAB_{TK} were more closely related with the domains of bacterial MsrAB fusion proteins, rather than the sequences from mesophilic archaea. This suggested that the *msrAB_{TK}* gene in *T. kodakaraensis* is most likely a result of horizontal gene transfer from mesophilic bacteria harboring *msrAB* fusion genes. Supporting this, the MsrAB_{TK} protein exhibited relatively low thermostability compared to other proteins from this hyperthermophile, with a half-life in activity of less than 2.5 min at 85°C. In addition, maximum activity was observed at strikingly low temperatures (MsrA activity: 60°C, MsrB activity: 30°C). The results suggest that MsrAB_{TK} is an enzyme from a hyperthermophile that has been designed to function only at sub-optimal temperatures.

Although the *in vitro* examinations of MsrAB_{TK} raise the possibility that the enzyme is involved in protecting the cell from oxidative stress specifically at sub-optimal growth temperatures, there was the possibility that the *msrAB_{TK}* gene is a pseudogene that is not expressed in *T. kodakaraensis* cells. There was also the possibility that even when the gene is expressed in the cells, the protein is too thermolabile to actually function in *T. kodakaraensis*. In order to gain insight on the function of MsrAB_{TK} *in vivo*, in this chapter the author first examined the presence of the MsrAB_{TK} protein in *T. kodakaraensis* cells. Furthermore, utilizing the gene disruption system developed in this archaeon (14, 15), the author has disrupted the MsrAB_{TK} gene, and examined whether the absence of the gene has any effect on

the growth of *T. kodakaraensis* under various degrees of oxidative stress.

MATERIALS AND METHODS

Strains and growth conditions.

Escherichia coli DH5 α was used for plasmid amplification and construction. *E. coli* cells were cultivated as described in Chapter 1. The wild-type *T. kodakaraensis* KOD1 and the *msrAB_{Tk}* gene disruption strain (Δ *msr*) were inoculated and grown in ASW-YT medium with 0.5% sodium pyruvate (ASW-YT-Pyr). ASW-YT medium is composed of a 1.25-fold dilution of artificial seawater (13) [20 g of NaCl, 3 g of MgCl₂·6H₂O, 6 g of MgSO₄·7H₂O, 1 g of (NH₄)₂SO₄, 0.2 g of NaHCO₃, 0.3 g of CaCl₂·2H₂O, 0.5 g of KCl, 0.42 g of KH₂PO₄, 0.05 g of NaBr, 0.02 g of SrCl₂·6H₂O, 0.01 g of Fe(NH₄) citrate per liter] (0.8 x ASW), 5.0 g L⁻¹ yeast extract and 5.0 g L⁻¹ tryptone. For standard anaerobic cultures, the ASW-YT medium after autoclaving was placed in an anaerobic atmosphere and sodium sulfide (0.02 or 0.1 g L⁻¹) and resazurin (0.25 mg L⁻¹) were added to the medium. To prepare media representing various degrees of oxidative stress, the ASW-YT medium after autoclaving was first incubated at 25°C in an aerobic environment for 24 h under sterile conditions. Afterwards, various amounts of sodium sulfide were added to the medium to remove the dissolved oxygen to different extents. After inoculation, the culture tubes were sealed with screw caps, butyl rubber stoppers or silicone plugs. Cultivation temperatures ranged from 60 to 90°C. In the case of plate culture, 10 g L⁻¹ Gelrite (Wako Pure Chemicals, Osaka, Japan) was added to solidify the media together with 2.0 mL L⁻¹ polysulfide solution (10 g Na₂S·9H₂O and 3.0 g sublimed sulfur per 15 mL). The cells inoculated on the plate medium were incubated at 85°C under an anaerobic atmosphere.

DNA manipulation and DNA sequencing.

Plasmid DNA was purified using Quantum Prep Plasmid Miniprep Kit (Bio-Rad, Hercules, CA). Restriction and modification enzymes were purchased from Toyobo (Osaka, Japan) or Takara (Ohtsu, Japan). KOD-Plus- (Toyobo) was used as a polymerase for PCR, and a Wizard SV Gel and PCR Clean-Up system (Promega, Madison, WI) was used to recover DNA fragments from agarose gels. DNA sequencing was performed using a BigDye Terminator Cycle Sequencing Kit ver. 3.1 and a Model 3130xl capillary DNA sequencer (Applied Biosystems, Foster City, CA).

Construction of the *msrAB_{Tk}* gene disruption plasmid.

The *msrAB_{Tk}* gene together with its flanking regions of about 1,000 bp was amplified from the genomic DNA of *T. kodakaraensis* KOD1 using the primer set msr-1/msr-2 (msr-1, 5'-CTCACCATCGCCCTCGCTCCAGCGTACCACGC-3'; msr-2, 5'-GGGAACGGCTACGTTGACCTCAGCAATGCC-3'). The fragment was inserted into the SmaI site of plasmid pUD2. pUD2 is a previously described plasmid in which the *pyrF* marker gene is inserted into the HincII site of pUC118 (14). Inverse PCR was performed using the primer set msr-3/msr-4 (msr-3, 5'-TATCACATCTCCAACCGAATGATGTCGGAA-3'; msr-4, 5'-GGTGAGGGTTCATGGGAGTGGTAATACGGGGG-3'). pDmsr was obtained by self-ligation of the obtained fragment.

Transformation of *T. kodakaraensis* strain KUW1.

All steps involved in the genetic manipulation of *T. kodakaraensis* were performed under anaerobic conditions with the exception of centrifugation for cell harvesting. *T. kodakaraensis* KUW1 ($\Delta pyrF$, $\Delta trpE$) (14) was cultivated in ASW-YT liquid medium with

elemental sulfur (S^0) for 14 h and cells were harvested ($5,800 \times g$, 5 min) from a 3 mL volume of culture broth and resuspended in 200 μ L of 0.8 x ASW, and kept on ice for 30 min. Plasmid DNA (3 μ g) was added into the suspension, and the cells were incubated on ice for 1 h. After incubation, cells were cultured in 20 mL of ASW-AA- S^0 medium in the absence of uracil. ASW-AA medium consisted of 0.8 x ASW supplemented with a 5.0 mL L^{-1} concentration of modified Wolfe's trace minerals [0.5 g $MnSO_4 \cdot 2H_2O$, 0.1 g $CoCl_2$, 0.1 g $ZnSO_4$, 0.01 g $CuSO_4 \cdot 5H_2O$, 0.01 g $AlK(SO_4)_2$, 0.01 g H_3BO_3 , and 0.01 g $NaMoO_4 \cdot 2H_2O$ per liter] (13), a vitamin mixture (5.0 mL L^{-1}) (13), 20 amino acids (250 mg cysteine·HCl·H₂O, 75 mg alanine, 125 mg arginine·HCl, 100 mg asparagine·H₂O, 50 mg aspartic acid, 50 mg glutamine, 200 mg glutamic acid, 200 mg glycine, 100 mg histidine·HCl·H₂O, 100 mg isoleucine, 100 mg lysine·HCl, 75 mg methionine, 75 mg phenylalanine, 125 mg proline, 75 mg serine, 100 mg threonine, 75 mg tryptophan, 100 mg tyrosine, and 50 mg valine per liter), 10 μ M $Na_2WO_4 \cdot 2H_2O$, and 2.5 g L^{-1} elemental sulfur. Cells were grown in the same medium three times to increase the population of the transformants, which should exhibit uracil prototrophy. The cells were then spread on ASW-YT plate medium supplemented with 7.5 g L^{-1} 5-fluoroorotic acid (5-FOA) in order to select transformants that performed a second, pop-out recombination (Fig. 2). After cultivation for 2 days at 85°C, colonies formed on the plate medium exhibiting 5-FOA resistance were isolated. Strains with a genotype identical to the host strain and strains with an *msrAB_{Tk}* gene disruption were obtained, and those exhibiting the latter genotype were selected for further analysis.

Measurements of dissolved oxygen concentration.

Dissolved oxygen concentrations were measured with a dissolved oxygen meter Sension6 (Hach, Loveland, CO) with methods recommended by the manufacturer. When

measuring medium at temperatures above 50°C, the medium was incubated at the desired temperature for a sufficient period of time, and then tightly sealed without headspace, and incubated at 30°C for 20 min. The vials were opened and dissolved oxygen concentrations were immediately and continuously monitored. The initial rates in the increase of dissolved oxygen concentration were linear and used to calculate the original dissolved oxygen concentration in the medium.

RESULTS

Presence of MsrAB_{TK} protein in *T. kodakaraensis*.

As described in Chapter 1, the optimal temperatures of MsrA_{TK} and MsrB_{TK} catalysis were unusually low for an enzyme from *T. kodakaraensis*. The author thus examined the presence of the MsrAB_{TK} protein in *T. kodakaraensis* at various temperatures. Polyclonal antibodies were raised against the purified recombinant MsrAB_{TK} and used to detect the protein in cells grown in ASW-YT-Pyr at temperatures between 60 and 90°C (Fig. 1A). An abundant amount of MsrAB_{TK} protein was observed in cells grown at 60 to 70°C, indicating that the gene is expressed in *T. kodakaraensis*. A steady decrease of the protein level was observed with temperature elevation and the protein was not detectable at the optimal growth temperature of *T. kodakaraensis* (85°C). The results clearly indicate that MsrAB_{TK} is present only in cells grown at sub-optimal temperatures, which is consistent with the biochemical properties of MsrAB_{TK} described in Chapter 1.

As the function of MsrAB_{TK} can be presumed to be involved in the response against oxygen or oxidative stress, the author next focused on whether protein levels of MsrAB_{TK} were affected by the presence of oxygen. The author examined the growth of *T. kodakaraensis* inoculated or grown under different culture conditions, taking into account the dissolved

Table 1. Growth of *T. kodakaraensis* under various conditions of oxygen stress.

| Atmosphere during inoculation | Anaerobic | | | | | | Aerobic | | | | | | | | | | | |
|---|--------------|-----------|-----------|----------------|----------------|----------------|--------------|-----------|-----------|------------|------------|------------|-------------------------|-------------------------|-------------------------|------------|------------|----------------|
| Plug | Butyl rubber | | | | | | Butyl rubber | | | | | | Silicone sponge | | | | | |
| Addition of 0.1 g L ⁻¹ Na ₂ S | Yes | | | No | | | Yes | | | No | | | Yes | | | No | | |
| Temperature (°C) | 65 | 75 | 85 | 65 | 75 | 85 | 65 | 75 | 85 | 65 | 75 | 85 | 65 | 75 | 85 | 65 | 75 | 85 |
| Color of resazurin during inoculation | - | - | - | + ^a | + ^a | + ^a | - | - | - | + | + | + | - | - | - | + | + | + ^a |
| Initial DO at 25°C (mg L ⁻¹) | 0.01 | 0.01 | 0.01 | 2.86 ±0.22 | 2.86 ±0.22 | 2.86 ±0.22 | 0.01 | 0.01 | 0.01 | 5.62 ±0.09 | 5.62 ±0.09 | 5.62 ±0.09 | 0.01 | 0.01 | 0.01 | 5.62 ±0.09 | 5.62 ±0.09 | 5.62 ±0.09 |
| DO at incubation temperature (mg L ⁻¹) | <0.01 | <0.01 | <0.01 | <2.86 | <2.86 | <2.86 | <0.01 | <0.01 | <0.01 | <5.62 | <5.62 | <5.62 | 0.61 ^b ±0.07 | 0.57 ^b ±0.05 | 0.23 ^b ±0.06 | 3.66 ±0.01 | 2.53 ±0.03 | 1.34 ±0.03 |
| Growth | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No | No | No | Yes | Yes | Yes | No | No | Yes |
| Condition number | <i>C1</i> | <i>C2</i> | <i>C3</i> | <i>C4</i> | <i>C5</i> | <i>C6</i> | <i>C7</i> | <i>C8</i> | <i>C9</i> | <i>C10</i> | <i>C11</i> | <i>C12</i> | <i>C13</i> | <i>C14</i> | <i>C15</i> | <i>C16</i> | <i>C17</i> | <i>C18</i> |

^aRapid loss of color is observed after initiation of growth.

^bMeasured after 12 h incubation at the respective temperatures.

oxygen (DO) concentrations and the color of resazurin in the medium. The color of resazurin in the medium at 0.25 mg L^{-1} starts to fade at DO concentrations of 0.1 mg L^{-1} , and becomes completely transparent at 0.05 mg L^{-1} . Under standard growth conditions (Table 1, condition no. *C1–C3*), the medium is reduced with 0.1 g L^{-1} sodium sulfide at room temperature under an anaerobic atmosphere, and cells are inoculated in this anaerobic environment. Culture flasks are sealed with butyl rubber stoppers. The resazurin was transparent and the DO concentration at 25°C was 0.01 mg L^{-1} . The alternative growth conditions considered were combinations of (i) omitting the addition of sodium sulfide (*C4–C6*, *C10–C12*, *C16–C18*), (ii) inoculating under an aerobic atmosphere (*C7–C18*), and (iii) using silicone sponge plugs instead of butyl rubber stoppers (*C13–C18*). Cultures were performed at 65 , 75 and 85°C without shaking and the occurrence of growth under these conditions are summarized in Table 1. When the addition of sodium sulfide was omitted, the color of the resazurin was red regardless of the atmosphere and plugs used. Without sodium sulfide, the author observed growth only when cells were inoculated under an anaerobic atmosphere and the flasks were sealed with butyl-rubber stoppers (*C4–C6*), or when cells were grown in an aerobic atmosphere with silicone sponge plugs at 85°C (*C18*). Under conditions *C4–C6*, the DO concentration at $65–85^\circ\text{C}$ can be expected to be considerably lower than $2.86 \pm 0.22 \text{ mg L}^{-1}$, as this is the value observed at 25°C prior to inoculation. Under condition *C18*, medium without cell inoculation displayed a DO concentration of $1.34 \pm 0.03 \text{ mg L}^{-1}$ at 85°C . As conditions *C16* and *C17* could not support growth, the threshold of DO that *T. kodakaraensis* can overcome to initiate growth is most likely between concentrations of 1.34 and 2.53 mg L^{-1} . Once growth was observed, resazurin rapidly became transparent ($<30 \text{ min}$), indicating that growing cells are actively removing oxygen from their environment. When sodium sulfide was added, all media displayed initial DO concentrations of 0.01 mg L^{-1} , and cell growth was

observed in all cases. The DO values under conditions *C13–C15* were 0.61 ± 0.07 , 0.57 ± 0.05 and 0.23 ± 0.06 mg L⁻¹, respectively, after 12 h, corresponding to the period of time between inoculation and initial cell growth.

Under all conditions in which cell growth was observed, the author examined the presence of MsrAB_{Tk} by Western blot analysis (Fig. 1B). As described above, MsrAB_{Tk} is

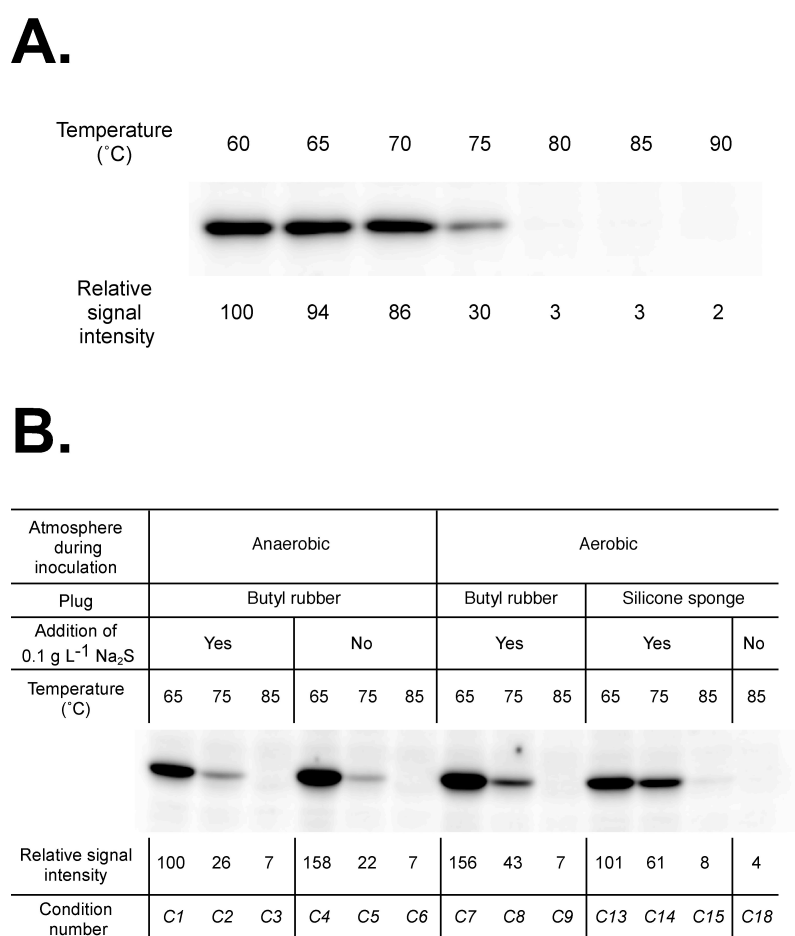


Fig. 1. Detection of MsrAB_{Tk} in *T. kodakaraensis* cells. (A) Western blot analysis was performed on cell-free extracts (10 µg) from cells grown under standard growth conditions in ASW-YT-Pyr at the temperatures indicated above the membrane. (B) Western blot analysis was performed on cell-free extracts (10 µg) obtained from *T. kodakaraensis* cells grown under various conditions that are described above the panel. Conditions numbers indicated below the panel correspond to those of Table 1. Relative signal intensities (A, 60°C=100; B, *C1*=100) are indicated below each lane.

only observed at suboptimal temperatures when cells are grown under standard growth conditions (C1–C3). When the addition of sodium sulfide was omitted, the author observed a slight increase of MsrAB_{Tk} in cells grown at 65°C (C4). When cells were inoculated under aerobic conditions, the author observed increases of the protein at 75°C (C8, C14). MsrAB_{Tk} could not be detected in cells inoculated and grown under aerobic conditions at 85°C (C15, C18). The results indicate two points. The medium under standard growth conditions contains <0.01 mg L⁻¹ oxygen. The author can thus conclude that the presence of MsrAB_{Tk} clearly responds to temperature even in the absence of oxygen (C1–C3). The second point is that the presence of MsrAB_{Tk} also responds to the presence of oxygen. This can be observed when comparing proteins levels in cells grown at 75°C under different growth conditions (C2, C5, C8, C14). However, as MsrAB_{Tk} was not present at detectable levels under aerobic conditions at 85°C, a condition with a higher DO than that of condition C14, MsrAB_{Tk} is regulated primarily by temperature, and the response towards DO occurs only at sub-optimal growth temperatures.

Construction of an *msrAB_{Tk}* gene disruption strain.

As the MsrAB_{Tk} protein was detected in *T. kodakaraensis* cells and its expression responded to temperature and oxygen concentrations, the possibilities increased that the protein participates in the response against oxidative stress *in vivo*. The author thus set out to disrupt the gene and examine the phenotypic changes brought about by gene disruption. The *msrAB_{Tk}* gene corresponds to TK0819, and although the gene does not seem to be included in an operon, it shares its 5'-flanking region with a gene cluster on the opposite strand that includes a hypothetical gene, pyruvate:ferredoxin oxidoreductase and peroxiredoxin. In the 3'-flanking region, a hypothetical open reading frame is located 167 bp downstream in the

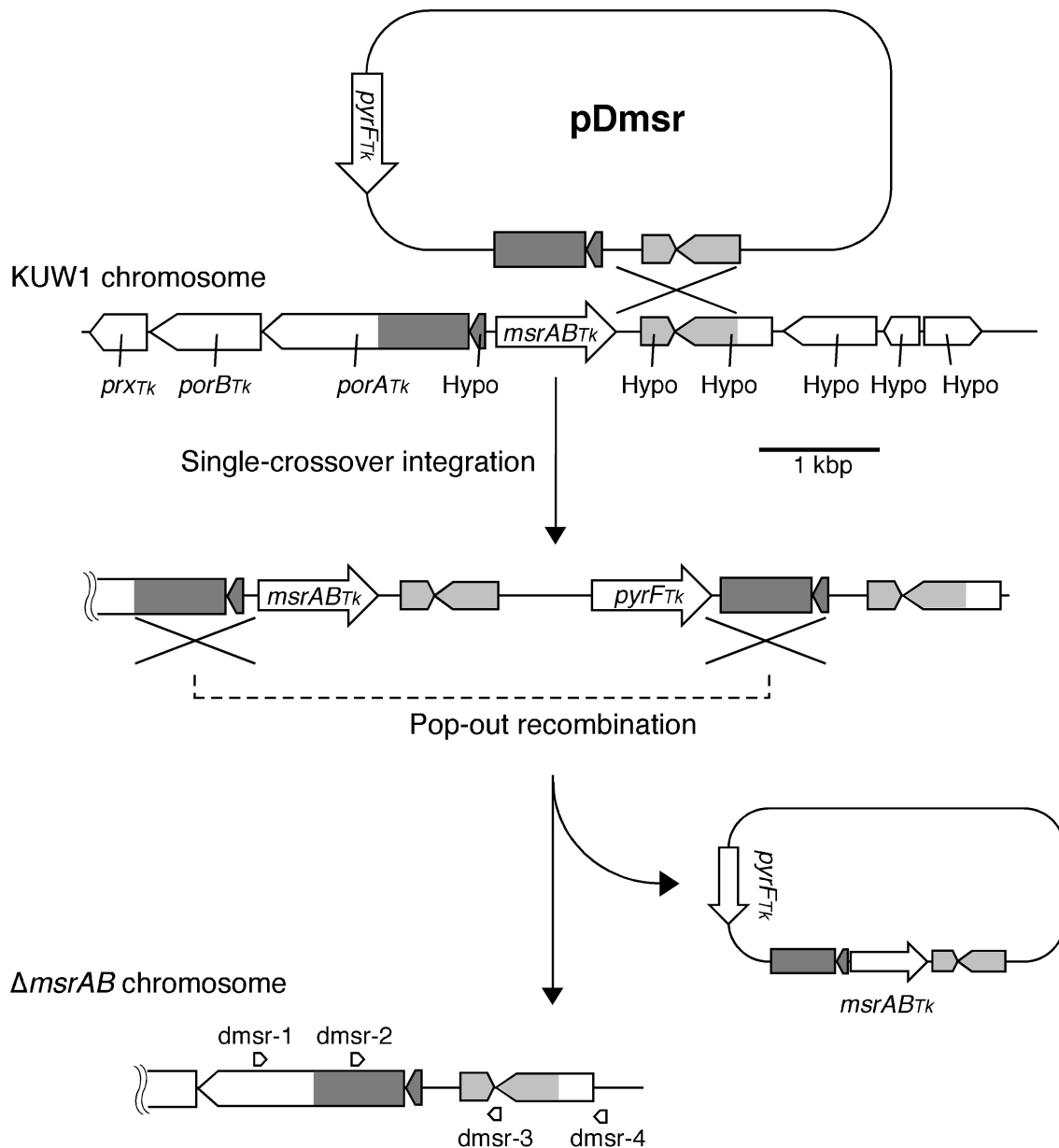


Fig. 2. A schematic diagram illustrating the strategy for gene disruption of *msrAB_{Tk}*. The homologous regions between the plasmid *pDmsr* and the chromosome of *T. kodakaraensis* KUV1 are shaded. The positions of the primers used for the PCR analyses of the *msrAB_{Tk}* locus (*dmsr-1*, *dmsr-2*, *dmsr-3*, and *dmsr-4*) are indicated by arrowheads. Abbreviations: *porA_{Tk}*, pyruvate:ferredoxin oxidoreductase a subunit; *porB_{Tk}*, pyruvate:ferredoxin oxidoreductase b subunit; *prx_{Tk}*, peroxiredoxin; Hypo, hypothetical protein.

same direction. A gene disruption plasmid, *pDmsr*, was designed to precisely remove the coding region of *msrAB_{Tk}* via single crossover integration, followed by pop-out recombination

(Fig. 2). The strain *T. kodakaraensis* KUW1 ($\Delta trpE$, $\Delta pyrF$) was utilized as the host strain. The *pyrF* gene encoding orotidine-5'-phosphate decarboxylase was used as the selection marker. As *pyrF* is necessary for *de novo* pyrimidine biosynthesis, cells that have undergone single crossover integration can be selectively grown in a liquid medium in the absence of uracil. The uracil prototrophs are then spread on a solid, nutrient-rich medium supplemented with 5-FOA. In the presence of an intact *pyrF* gene, 5-FOA is converted to the toxic 5-fluorouridylate, and thus only strains that have undergone a second (pop-out) recombination step that removes the *pyrF* gene can grow. In principle, among the 5-FOA resistant strains, half should harbor a genotype equivalent to the original host strain, whereas the other half should result in a genotype where the *msrAB_{Tk}* gene is deleted. After introducing pDmsr into the KUW1 strain, cells were grown twice in synthetic medium (in the absence of uracil), and spread on ASW-YT plate medium including 7.5 g L⁻¹ 5-FOA. Sixteen colonies were selected and PCR analyses indicated that 8 colonies corresponded to the *msrAB_{Tk}* gene disruption

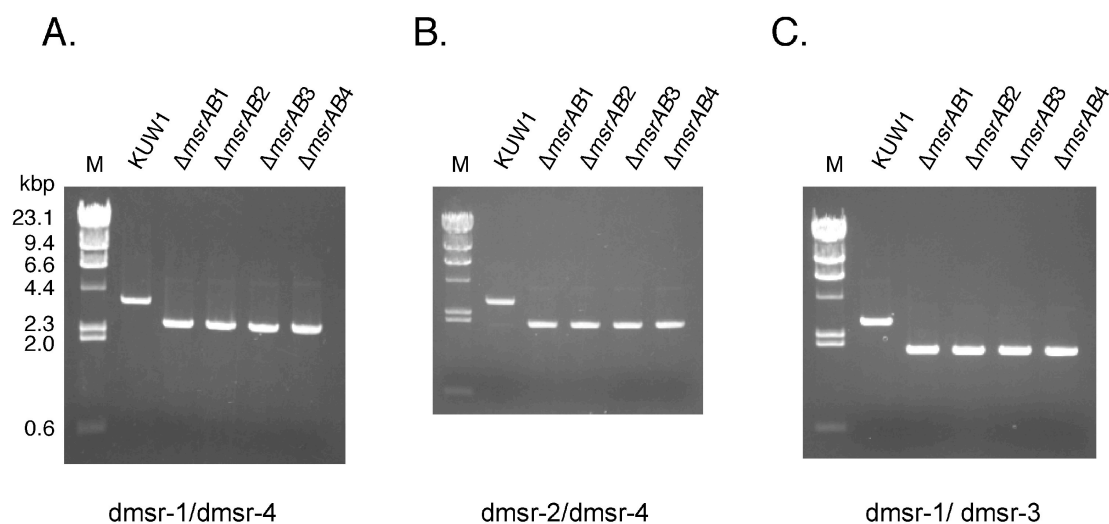


Fig. 3. PCR analyses of the *msrAB_{Tk}* locus of *T. kodakaraensis* KUW1 and four gene disruption strains. Amplification of the *msrAB_{Tk}* locus in strain KUW1 (host cell) and the $\Delta msrAB1\sim4$ strains using dmsr-1/dmsr-4 (left), dmsr-2/dmsr-4 (middle) and dmsr-1/dmsr-3 (right) are shown. M, Hind III-digested λ DNA.

strain. Four transformants were selected ($\Delta msrAB1-4$) for further examination. The results of the PCR analyses of these four strains, along with that of the host strain (KUD1), are shown in Fig. 3.

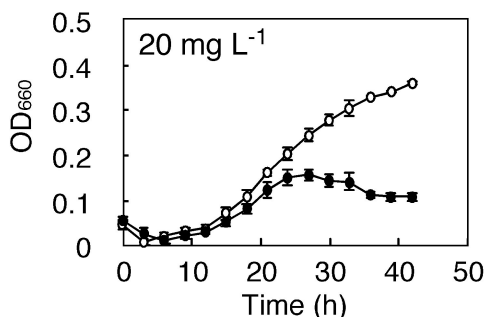
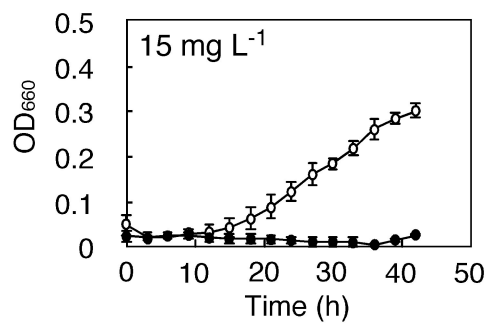
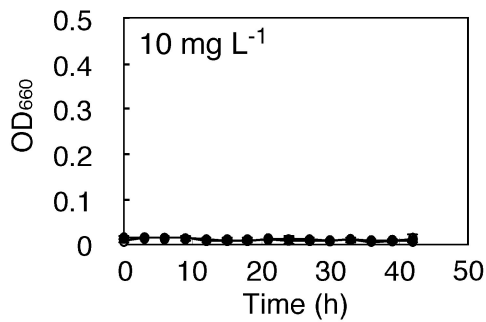
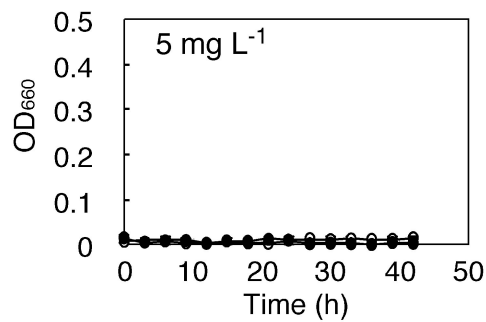
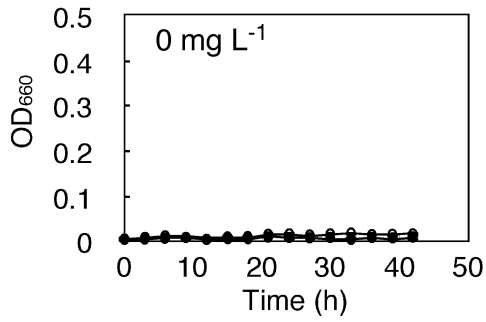
Growth comparisons between the $msrAB_{Tk}$ gene disruption strains and the wild-type KOD1.

As described in the previous chapter, the biochemical properties of MsrAB_{Tk} indicated that the protein prefers to function at low temperatures (30-60°C) compared to the optimal growth temperature of *T. kodakaraensis* (85°C). The results described above have revealed that the presence of the protein in the cell-free extracts could only be observed at sub-optimal growth temperatures (65-75°C), and could not be detected at 85°C. It can thus be expected that if MsrAB_{Tk} were to function against oxygen stress in *T. kodakaraensis* cells, effects brought about by the absence of the protein would be greater at lower growth temperatures.

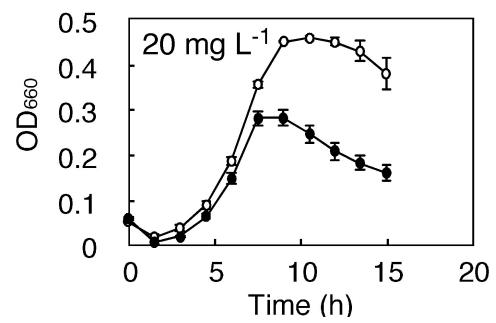
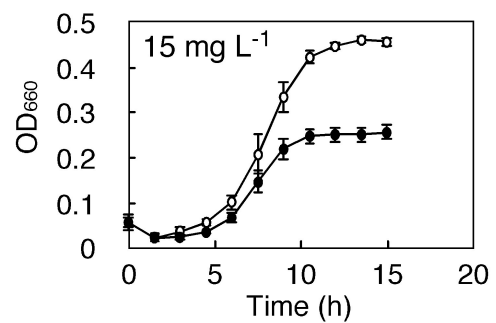
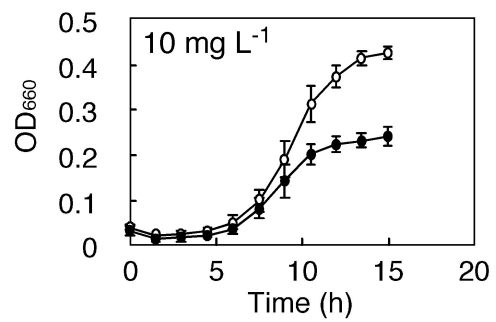
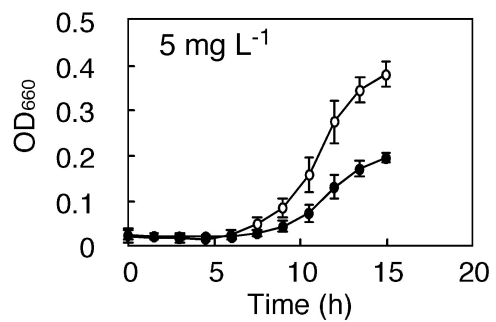
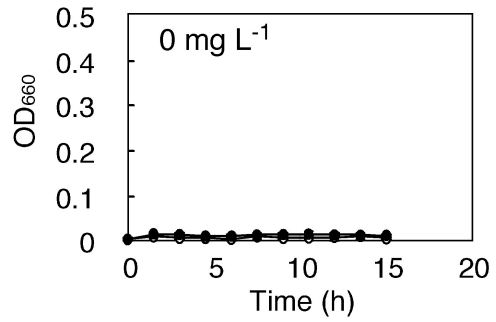
When *T. kodakaraensis* cells are grown under standard conditions, cells are inoculated in an anaerobic environment, and 20 or 100 mg L⁻¹ sodium sulfide is added to reduce the dissolved oxygen in the medium. The results shown in Table 1 indicated that wild-type cells could not grow in sealed culture bottles when inoculated under an aerobic environment without the addition of sodium sulfide. In order to prepare environments with

Fig. 4. Growth curves of the wild-type *T. kodakaraensis* KOD1 and the $\Delta msrAB$ strains at sub-optimal (65°C) and optimal (85°C) growth temperatures. The amounts of sodium sulfide added to medium are indicated in each panel. Inoculation methods and culture conditions are described in the text. KOD1, open circles; $\Delta msrAB$, closed circles. Error bars represent standard deviations of three independent growth experiments in the case of the wild-type KOD1, and those of the four individual cultures for each mutant strain in the case of $\Delta msrAB$ strains.

65° C



85° C



different degrees of oxidative stress, the author thus chose to inoculate cells under an aerobic environment with the addition of various amounts of sodium sulfide (0, 5, 10, 15, and 20 mg L⁻¹). Culture tubes were immediately sealed and cell growth was monitored by measuring the optical density of the culture at 660 nm. Cells were cultured in a nutrient-rich ASW-YT medium with 0.5% sodium pyruvate at the sub-optimal 65°C and optimal 85°C.

The results of the growth experiments are shown in Fig. 4. At 85°C, the wild-type KOD1 and the mutant strains displayed similar responses to oxidation stress. As in the case of condition *C12*, both could not grow in medium without the addition of sodium sulfide. However, both strains displayed growth in media supplemented with sodium sulfide at concentrations of 5 mg L⁻¹ or higher. The maximum specific growth rates of the wild-type strain and mutant strains were similar. Twenty and 100 mg L⁻¹ sodium sulfide (not shown) were sufficient to support cell growth equivalent to that observed under standard anaerobic conditions.

One unexpected observation was that the $\Delta msrAB$ cells had a tendency to aggregate and sediment in the culture tubes. Measuring the optical density after gently shaking the culture tubes indicated that the cell densities of the mutant cell cultures were equivalent to those of the wild-type strain, indicating that growth of the mutant cells continued even after the apparent plateau in OD₆₆₀ values. At 65°C, the author observed a clear difference in growth between the wild-type strain and the $\Delta msrAB$ strains. All strains could not grow with the addition of 0, 5, or 10 mg L⁻¹ sodium sulfide. However, while the wild-type strain displayed rapid growth in the presence of 15 mg L⁻¹ sodium sulfide, all four of the $\Delta msrAB$ strains displayed a lag phase of over 40 h. After 72 h, cell growth of the $\Delta msrAB$ strains could be observed. As in the case at 85°C, 20 and 100 mg L⁻¹ sodium sulfide (not shown) were sufficient to support cell growth equivalent to that observed under standard anaerobic

conditions.

DISCUSSION

In this chapter, the author has examined the presence of the MsrAB_{Tk} protein in the hyperthermophilic archaeon *T. kodakaraensis*, and its response towards temperature and oxidative stress. The results were consistent with the function and biochemical properties of the protein. The amount of MsrAB_{Tk} protein increased when cells were exposed to higher concentrations of oxygen, and moreover, the protein was abundant only at sub-optimal growth temperatures, correlating well with the fact that MsrAB_{Tk} exhibits relatively low thermostability. Gene disruption of the *msrAB_{Tk}* gene further revealed that the protein does participate in the response against oxygen stress, specifically at sub-optimal growth temperatures. As the primary structure of the protein strongly suggests that the *msrAB_{Tk}* gene was obtained via gene transfer from mesophilic bacteria, it can be presumed that the gene has evolved in *T. kodakaraensis* to obtain thermostability sufficient for it to function at the lower growth temperatures of this archaeon. It is not clear at present whether the different amounts of protein observed under different growth conditions are brought about by regulation at the transcription level. It does not seem likely that the absence of MsrAB_{Tk} at higher growth temperatures is merely due to protein denaturation. The author performed a Western blot analysis of MsrAB_{Tk} in wild-type *T. kodakaraensis* KOD1 cells grown at various temperatures in the presence of 20 mg L⁻¹ sodium sulfide (Fig. 5). While the protein was clearly detected at 65°C and to a lesser extent at 75°C, the presence of MsrAB_{Tk} protein was not observed in the whole cell extracts, nor in the soluble or insoluble fractions, of cells grown at 85°C (Fig. 5).

A tendency of cells to aggregate was observed in the $\Delta msrAB_{Tk}$ strains. This is most

likely due to a polar effect in the gene disruption strain resulting in changes in expression levels of nearby genes. This is supported by the fact that cell aggregation was observed at both 65 and 85°C, the latter being a temperature at which the MsrAB_{TK} protein cannot be observed in *T. kodakaraensis* cells. As noted above, the author confirmed the absence of MsrAB_{TK} in wild-type *T. kodakaraensis* KOD1 cells grown at 85°C in the presence of 20 mg L⁻¹ sodium sulfide (Fig. 5). It is difficult to imagine that the disruption of the *msrAB_{TK}* gene *per se* would lead to a dramatic change in phenotype under growth conditions in which the protein is originally not detectable.

Thermococcus and *Pyrococcus* are both major genera in the order Thermococcales, along with a third genus *Palaeococcus* (17). The Thermococcales represent sulfur-reducing, heterotrophic archaea and are obligate anaerobes. They share common metabolism and energy-generating mechanisms, with the only major distinction in their growth temperatures. The former with optimal growth temperatures between 75 and 93°C, while those of the latter ranging from 95 to 103°C (1, 10). As environmental studies have indicated a broad distribution of the *Thermococcus* throughout the global environment, it can be presumed that

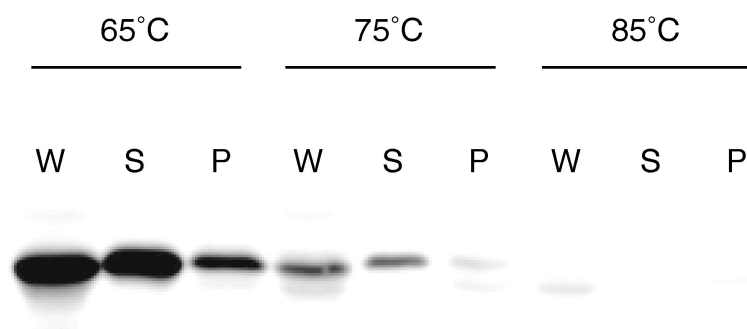


Fig. 5. Western blot analysis of whole cell extracts (W), and the soluble (S) and insoluble (P) fractions of *T. kodakaraensis* KOD1 cells using antisera raised against MsrAB_{TK}. Cells were grown at 65, 75, and 85°C in the presence of 20 mg L⁻¹ sodium sulfide. Each lane contains protein levels equivalent to those obtained from a 1 mL volume of culture broth with an OD₆₆₀ of 0.3.

the *Thermococcus* have the ability to cope with or adapt to a broader range of environmental conditions. The biochemical properties and regulated expression of MsrAB_{TK} clearly point to a function of the protein specific to low-temperature environments. In the natural environment, microorganisms must endure and survive drastic and continuous changes in their environment. The Msr from *T. kodakaraensis* reveals a novel example of a protein functionally designed and regulated to cope with redox changes in its surroundings accompanied by decreases in temperature. If the presence of Msr were a common trait of the *Thermococcus*, this might also be one of the factors contributing to the broad, global distribution of the *Thermococcus* compared to the *Pyrococcus*, which do not harbor Msr.

SUMMARY

Methionine sulfoxide reductase (Msr) catalyzes the thioredoxin-dependent reduction of methionine sulfoxide to methionine, and plays an important role in the repair of proteins damaged due to oxidation. Although hyperthermophiles in general do not harbor Msr, the author has characterized an Msr (MsrAB_{TK}) from the anaerobic hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1. MsrAB_{TK} exhibited relatively low thermostability and strikingly low optimal reaction temperatures *in vitro*. In this chapter, the author found that the MsrAB_{TK} protein in *T. kodakaraensis* was present only at sub-optimal growth temperatures, and that the intracellular levels of MsrAB_{TK} responded against increases in dissolved oxygen only at these lower temperatures. The results raised the possibilities that MsrAB_{TK} is involved in the response against oxidative stress *in vivo*. To confirm this, the author disrupted the gene of this archaeon and compared the growth properties between the wild-type strain and mutant strains under various degrees of oxidative stress. The author observed that the mutant strains displayed less tolerance toward dissolved oxygen,

specifically at lower growth temperatures. This change in phenotype suggests that MsrAB_{TK} participates in protecting the cell from oxidative stress and confirms his previous proposal that Msr is a protein regulated and designed to function exclusively at sub-optimal growth temperatures.

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CHAPTER 3

Chaperonin expression responds to the presence of unfolded proteins in the hyperthermophilic archaeon, *Thermococcus kodakaraensis*

INTRODUCTION

In the previous chapters, the author has identified and examined the biochemical and physiological properties of a methionine sulfoxide reductase (MsrAB_{TK}) from *Thermococcus kodakaraensis* KOD1. MsrAB_{TK} was found to represent a defense system against oxidative damage of proteins that had not been previously identified in the hyperthermophilic archaea. In this chapter, the author aimed to gain insight on further defense mechanisms in *T. kodakaraensis* that function to maintain proper protein function in the cell.

Molecular chaperones are a group of proteins that assist the folding and/or assembly of newly synthesized polypeptide chains as well as mature proteins that have lost their optimal conformation due to various conditions of stress (10). Many members of this group are also up-regulated by heat shock, and referred to as the heat shock proteins (HSPs). The HSP60 family of chaperones, or chaperonins, forms a multi-subunit cylindrical complex that provides an environment in which the folding process of a protein is not disturbed by intermolecular interaction and enhanced by conformational changes of the chaperonins themselves coupled to ATP hydrolysis (12). The mechanisms responsible for the induction of these chaperonins under conditions of stress have attracted much attention. In the gram-negative bacteria, expression of chaperonin subunits (Hsp60 and Hsp10) is under the control of a positive regulator protein, the σ^{32} factor, which is encoded by the *rpoH* gene (2, 9). Under normal conditions, σ^{32} interacts with the DnaK/DnaJ/GrpE system, which results in rapid

degradation of the protein by the membrane-bound protease FtsH (24). When cells are exposed to heat shock or other conditions which result in protein misfolding, the DnaK/DnaJ/GrpE system is sequestered by the misfolded proteins, thereby releasing σ^{32} and subsequently leading to the induction of chaperonin gene transcription (8). In the gram-positive bacteria, expression of the HSP60 chaperonins is under the control of the HrcA repressor (18). Under non-stress conditions, HrcA, through interactions with the chaperonins, represses gene expression by binding to *cis*-acting elements known as CIRCE elements. Similar to the case above, the accumulation of denatured proteins leads to the release of HrcA from the chaperonins in an inactive form, resulting in the derepression (or induction) of the chaperonin genes (17).

In the hyperthermophilic Archaea, although the group II chaperonins are known to be up-regulated in response to heat shock (5, 6, 13-15, 19, 23), the mechanisms governing this regulation are not known. Regulators involved in heat shock response have been identified in *Pyrococcus furiosus* (25) and *Archaeoglobus fulgidus* (19). In *P. furiosus*, a heat shock regulator, Phr, has been shown to regulate the AAA⁺ ATPase gene, a small heat shock protein gene (16) and the Phr gene itself in response to temperature (25). In *A. fulgidus*, HSR1, a protein distantly related to Phr, has been found to similarly bind to the 5'-upstream regions of genes or gene clusters encoding the AAA⁺ ATPase gene, small heat shock proteins and HSR1 (19). In both cases, it has been proposed that the regulators repress transcription under normal conditions by binding to an operator sequence. In the case of Phr, it has been further suggested that dissociation brought about by the thermal denaturation of the protein triggers increase in transcription at elevated temperatures. The involvement of Phr or HSR1 in the regulation of chaperonin (HSP60) gene expression in the respective host cells is currently unknown.

MATERIALS AND METHODS

Strains and growth conditions.

The strains used in this study are listed in Table 1. *T. kodakaraensis* and its derivatives were cultivated as described in Chapter 2. *Escherichia coli* DH5 α used for general DNA manipulation was cultivated as described in Chapter 1. *Thermoplasma volcanium* GSS1 and *Pyrococcus furiosus* Vc1 were purchased from the Japan Collection of Microorganisms (JCM) at Riken and grown with the recommended media.

DNA manipulation and DNA sequencing.

All enzymes, reagents, methods and apparatus applied for DNA purification, plasmid construction and sequence confirmation are described in the MATERIALS AND METHODS section of Chapter 1.

Construction of gene insertion plasmids.

The plasmids used for double-crossover homologous recombination in *T. kodakaraensis* were constructed as follows. DNA fragments corresponding to the 5'-flanking

Table 1. Archaeal and bacterial strains used in this study.

| Strains | Relevant characteristics | Source or reference |
|------------------------------------|---|---------------------|
| <i>Escherichia coli</i> | | |
| DH5 α | <i>supE44</i> Δ <i>lacU169</i> (Φ 80 <i>lacZ</i> Δ <i>M15</i>) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i> | Stratagene |
| <i>Thermococcus kodakaraensis</i> | | |
| KOD1 | Wild type | (3) |
| KUW1 | Δ <i>pyrF</i> Δ <i>trpE</i> | (20) |
| KPF | KUW1, Δ <i>chiA</i> :: <i>trpE</i> _{TK} -P _{pyrF} - <i>pyrF</i> _{Pf} | this study |
| KTV | KUW1, Δ <i>chiA</i> :: <i>trpE</i> _{TK} -P _{pyrF} - <i>pyrF</i> _{Tv} | this study |
| Csg-KPF | KUW1, Δ <i>chiA</i> :: <i>trpE</i> _{TK} -P _{csg} - <i>pyrF</i> _{Pf} | this study |
| Csg-KTV | KUW1, Δ <i>chiA</i> :: <i>trpE</i> _{TK} -P _{csg} - <i>pyrF</i> _{Tv} | this study |
| <i>Thermoplasma volcanium</i> GSS1 | Wild type | (22) |
| <i>Pyrococcus furiosus</i> Vc1 | Wild type | (7) |

P_{pyrF}: The 5'-upstream region of the operon which includes the *pyrF* gene of *T. kodakaraensis*.

P_{csg}: The 5'-upstream region of *csg* gene of *T. kodakaraensis*.

region (998 bp) of the *chiA* gene of *T. kodakaraensis* and the 3'-portion of the *chiA* gene itself (919 bp), were amplified from genomic DNA with the primer sets chiA-1/chiA-2 and chiA-3/chiA-4, respectively (chiA-1, 5'-ACGAACCTTATTCCTTCTGCATAC-3'; chiA-2, 5'-GTGCTCGGGAAACTAGTACAACACCCCTTGAGCTTTG-3'; chiA-3, 5'-GGGGTGTGTACTAGTTTCCCGAGCACTTCTTCGCCC-3'; chiA-4, 5'-GGTCAAACTGGAACTGCAACTGCC-3' [SpeI sites are underlined]). Sequences upstream of the SpeI sites in chiA-2 and chiA-3 were designed so that the amplified fragments would overlap with one another, allowing their fusion through a second PCR step. pUC118 (Takara) was digested with EcoRI and SalI followed by blunt ending, and ligated with the fused DNA fragment obtained above, resulting in the plasmid pchiA-NC. The *trpE* marker cassette (*pyrF* promoter-*trpE* gene fusion) was amplified from pUMT2 (21) with the primer set trpE1/trpE2 (5'-AGTCTCTAGACCGCAACGCGCATTTTTGCTCACCC-3'/ 5'-GACTACTAGTTCATTCCCTCACCCCCAGCG-3' [XbaI and SpeI sites are underlined]), digested with XbaI and SpeI, and inserted into pchiA-NC at the SpeI site, resulting in the plasmid pchiA-NCtrpE. The *pyrF* gene from *P. furiosus* (*pyrF_{Pf}*) was amplified from genomic DNA with the primer set PF1/PF2 (5'-GGTATGCATATGATTGTACTAGCGTTGGA-3'/ 5'-GCGACTAGTTTATCTTGAGCTCCATTCTT-3' [EcoT22I and SpeI sites are underlined]), and inserted into pUC118 at the HincII site. The *pyrF* promoter (from *T. kodakaraensis*) was amplified from the plasmid pchiA-NCtrpE with the primer set PpyrF1/PpyrF2 (5'-TAATCTAGACCGCAACGCGCATTTTTGCTC-3'/ 5'-GCTATGCATCACCTTTTAAACGGCCCTCA-3' [XbaI and EcoT22I sites are underlined]), and inserted into pUC118 at the HincII site. Both plasmids were digested with EcoT22I/SphI and the *pyrF_{Pf}* fragment was inserted downstream of the *pyrF* promoter. The *pyrF_{Pf}* expression cassette was excised with XbaI and SpeI, and inserted downstream of the *trpE*

cassette in pchiA-NCtrpE. The *pyrF* gene from *T. volcanium* (*pyrF_{Tv}*) was amplified from genomic DNA with the primer sets TV1/TV2 (5'-TTAAAAGGTGAGTACCGTGGATAAGAGAATTATAG-3'/5'-ATTACTAGTTTAACTGTTTTTAAGTCTCTCCC-3' [SpeI site is underlined]). The *pyrF* promoter was amplified from the plasmid pchiA-NCtrpE with the primer set PpyrF3/PpyrF4 (5'-AGTCACTAGTCCGCAACGCGCATTTTGCTCACCC-3'/5'-CTCTTATCCACGGTACTCACCTTTTTAACGGCCCTCT-3' [SpeI site is underlined]). The *pyrF* promoter was fused with the *pyrF_{Tv}* gene through a second PCR step. The *pyrF_{Tv}* expression cassette was digested with SpeI, and inserted downstream of the *trpE* cassette in pchiA-NCtrpE. The *csg* promoter-*pyrF_{Tv}* fusion and *csg* promoter-*pyrF_{Pf}* fusion were constructed as follows. The *csg* promoter was amplified from genomic DNA with the 5' terminal phosphorylated primer set Pcsg1/Pcsg2 (5'-TATCGGCAAAAGGCGAATTATGTGTAGGCA-3'/5'-TCCACAACACCTCCTTGGGTTGTTGGGGCT-3'). Inverse PCR was performed using the primer set TV3/TV4 (5'-TCATTCCCTCACCCCCAGCGCCTTCAGAAC-3'/5'-GTGGATAAGAGAATTATAGTAGCCTTGGATG-3') to the pchiA-NCtrpE with *pyrF_{Tv}* promoter-*pyrF_{Tv}* plasmid and primer set TV3/PF3 (5'-TCATTCCCTCACCCCCAGCGCCTTCAGAAC-3'/5'-ATGATTGTAAGTAGCGTTGGACGTGTATGAG-3') to the pchiA-NCtrpE with *pyrF_{Tv}* promoter-*pyrF_{Pf}* plasmid. *Csg* promoter fusion was obtained by ligation of *csg* promoter fragment and inverse PCR fragment. All plasmids were confirmed to harbor the expected sequences.

Transformation of *T. kodakaraensis* strain KUW1.

Transformation of *T. kodakaraensis* KUW1 was performed basically as described in Chapter 2. After the plasmid was added into the cell suspension and incubated on ice for 1 h, cells were directly spread on a selective synthetic ASW-AA plate medium supplemented with $10 \mu\text{g mL}^{-1}$ uracil but depleted of tryptophan. After cultivation for 6 days at 85°C , colonies formed on the plate medium exhibiting tryptophan prototrophy were isolated.

Enzyme activity measurements.

T. kodakaraensis KOD1 and its mutant strains were cultivated in ASW-YT-S⁰ liquid medium for 18, 16, 14 and 12 h, in the case 70, 75, 80 and 85°C respectively. Cells were harvested ($9,820 \times g$, 4°C , 15 min), and then washed with $0.8 \times$ ASW. The cell pellet was resuspended in assay buffer composed of 100 mM Tris-HCl (pH 8.6) and 1.5 mM MgCl_2 . Cells were sonicated for 8 min (output:pause=0.1:0.9s; output power=2) with an Ultrasonic Disruptor UD-201 (TOMY, Tokyo, Japan). The lysates were centrifuged ($20,000 \times g$, 4°C , 15 min), and the supernatants were centrifuged again ($100,000 \times g$, 4°C , 60 min). The supernatants were used as cell-free extracts. Protein concentration was determined using a Bio-Rad protein assay system with bovine serum albumin as a standard.

Orotidine-5'-monophosphate decarboxylase (OMPdecase) activity was measured by monitoring the decrease in absorbance at 285 nm derived from the conversion of orotidine-5'-monophosphate (OMP) to uridine-5'-monophosphate ($\Delta\epsilon_{285}=1,380 \text{ M}^{-1} \text{ cm}^{-1}$) (4). The assay mixture was composed of 100 mM Tris-HCl (pH 8.6), 1.5 mM MgCl_2 , 0.125 mM OMP and the enzyme solution in a total volume of 1 mL. After preincubation of the assay mixture in a capped cuvette at 60°C for 3 min, the reaction was started by addition of the enzyme solution and was monitored at the same temperature for 10 min.

Western blot analysis.

Samples were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5% acrylamide concentration) followed by blotting to a polyvinylidene fluoride membrane (HybondTM-P, GE life sciences, Buckinghamshire, UK). Anti-CpkA or anti-CpkB antiserum that can distinguish CpkA and CpkB was used along with HRP-rec-Protein G (Zymed Laboratories, San Francisco, CA). For detection, the ECL AdvanceTM Western Blotting Detection System (GE life sciences), HyperfilmTM (GE life sciences) and Lumi vision PRO 400EX (AISIN, Kariya, Japan) were used.

RESULTS

Design of the plasmids for introduction of heterologous *pyrF* genes into *T. kodakaraensis*.

The heterologous gene(s) to be introduced into *T. kodakaraensis* were the *pyrF* genes from *P. furiosus* (*pyrF_{Pf}*) and *T. volcanium* (*pyrF_{Tv}*). The host strain was *T. kodakaraensis* KUW1, a strain which displays strict auxotrophy towards both uracil and tryptophan ($\Delta pyrF$, $\Delta trpE$). By placing the endogenous *trpE* gene of *T. kodakaraensis* on the same plasmid along with the heterologous *pyrF* gene, gene insertion strains can be selected based on tryptophan prototrophy, in case the *pyrF* genes do not function. The plasmid structures are shown in Fig. 2A. The site for gene insertion was the *chiA* locus which encodes chitinase, a non-essential protein involved in chitin assimilation. As *P. furiosus* has a higher optimal growth temperature (100°C) than *T. kodakaraensis* (85°C), it can be expected that *pyrF_{Pf}* will be able to complement the uracil auxotrophy of *T. kodakaraensis* KUW1 at all temperatures examined. In contrast, *T. volcanium* exhibits an optimum growth temperature of 60°C and depending on the thermostability of the PyrF_{Tv} protein, the uracil prototrophy

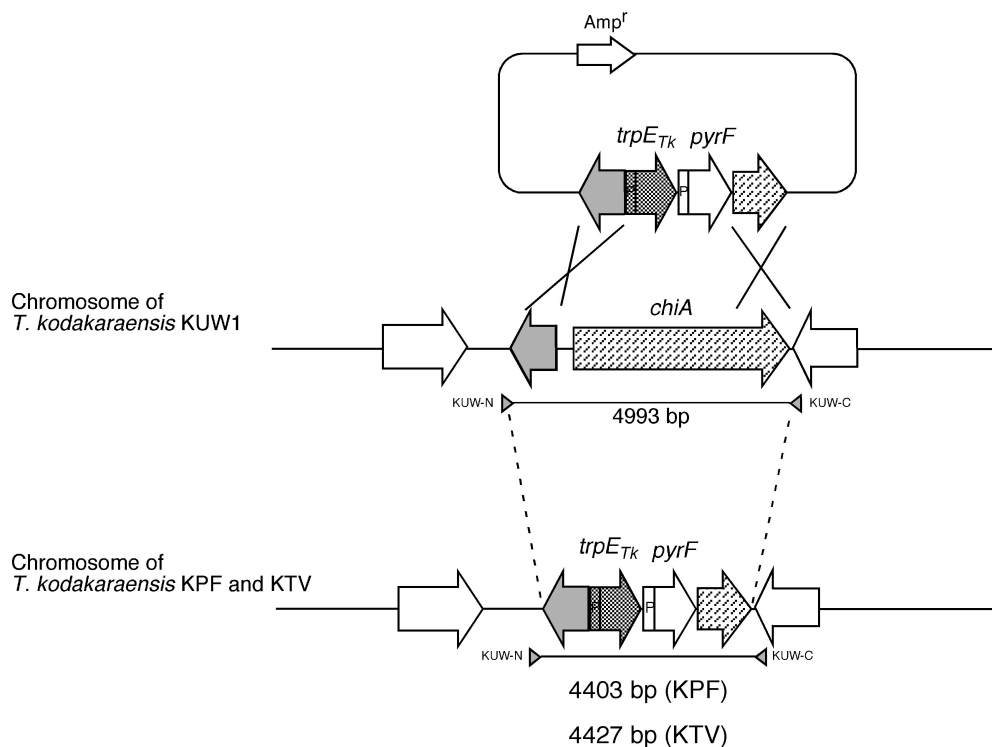
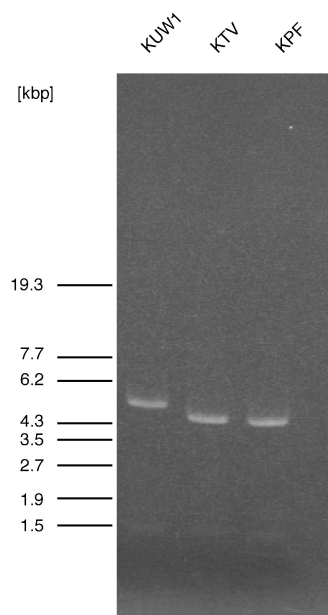
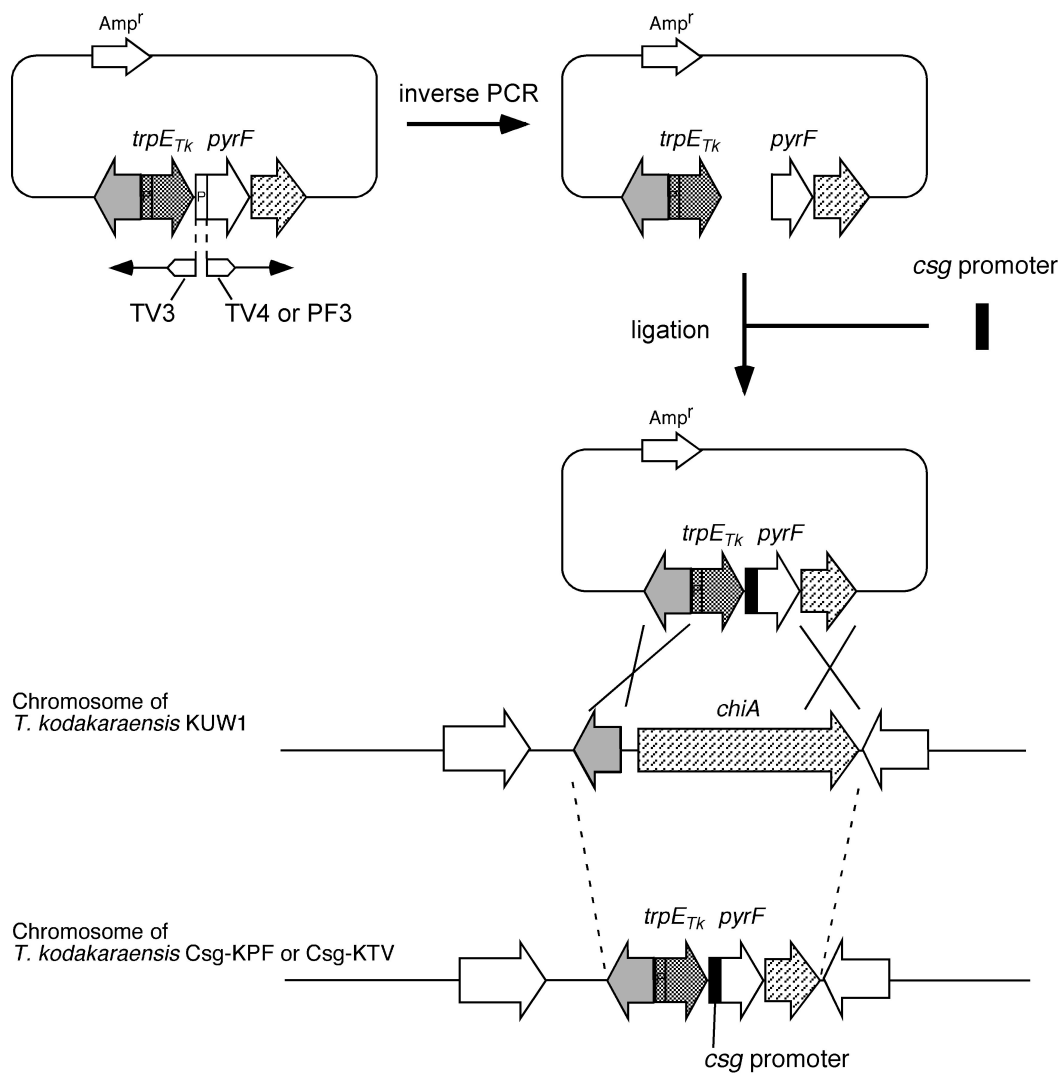
A.**B.**

Fig. 2. Introduction of heterologous *pyrF* genes into the KUW1 chromosome. (A) A diagram illustrating the gene insertion strategy. A *pyrF* gene was isolated from either *P. furiosus* or *T. volcanium*. The plasmids were designed so that the *trpE_{TK}-pyrF* construct replaced the non-essential chitinase gene of *T. kodakaraensis*. (B) PCR analysis confirming the recombination at the chitinase gene locus. Primers indicated in (A) were used. The calculated lengths of each fragment are indicated in (A). (C) A diagram illustrating the *csg* promoter exchange strategy. The *csg* promoter was amplified from *T. kodakaraensis* KOD1 genomic DNA.

brought about by introducing this gene may be limited to lower growth temperatures. The two plasmids were individually introduced into *T. kodakaraensis* KUW1, and recombinant strains were selected for their tryptophan prototrophy. Among 10 colonies that exhibited tryptophan prototrophy using the plasmid harboring *pyrF_{Tv}*, 8 were selected and their genotypes

C.

(Fig. 2. continued)

examined. PCR analysis was performed on the *chiA* locus, and indicated that the intended recombination had occurred in all cases (a representative result shown in Fig. 2B). Successful introduction of the $pyrF_{Pf}$ gene was also confirmed by PCR. One transformant was selected for each gene, and designated as *T. kodakaraensis* KPF ($pyrF_{Pf}$) and KTV ($pyrF_{TV}$).

The uracil prototrophy of *T. kodakaraensis* KPF and KTV.

The author first examined the ability of strains KPF and KTV to grow in a synthetic

medium (ASW-AA) in the absence of uracil and tryptophan. Taking into account the growth temperature of *T. volcanium*, strains were grown at 70°C. Both strain KPF and KTV displayed uracil prototrophy, indicating that the heterologous *pyrF* genes could function in *T. kodakaraensis* and that complementation experiments with heterologous genes are possible in this archaeon.

Effect of temperature on the growth of *T. kodakaraensis* KPF and KTV.

The author examined the uracil-independent growth of *T. kodakaraensis* KOD1 (wild type) and the two transformants at various temperatures. Cells were grown in synthetic medium without uracil and tryptophan at 70°C until the stationary phase and then inoculated into the same medium and incubated at various temperatures. The specific growth rates for each strain at 70, 75 and 80°C are shown in Table 2. The optimal growth temperature of *T. kodakaraensis* KOD1 is 85°C, and increases in growth rate were observed with increases in temperature. Similar tendencies were also observed for strain KPF harboring *pyrF_{Pf}*. In the case of strain KTV, growth was observed at 70 and 75°C, but not at 80°C. All strains, including KTV, exhibited similar growth characteristics at 85°C when grown in nutrient-rich medium, a condition in which the function of *pyrF* is not necessary (data not shown). The results indicate that the PyrF_{TV} protein brings about limitations on the growth temperature of strain KTV.

Table 2. Specific growth rates of KOD1, KPF and KTV at 70, 75 and 80°C.

| Growth temperature | Specific growth rates (h ⁻¹) | | |
|--------------------|--|-------------|-------------|
| | KOD1 | KPF | KTV |
| 70°C | 0.043±0.004 | 0.055±0.005 | 0.033±0.003 |
| 75°C | 0.057±0.002 | 0.074±0.003 | 0.065±0.012 |
| 80°C | 0.071±0.005 | 0.086±0.008 | no growth |

Thermostability of the heterologous PyrF proteins.

As the growth experiments implied that the thermostability of the PyrF_{TV} protein was insufficient to support growth at temperatures of 80°C or higher, the author examined the thermostability of the PyrF proteins by measuring OMPdecase activity present in the cell-free extracts. In order to obtain sufficient protein levels for the analyses, the author exchanged the promoter of *pyrF_{TV}* and *pyrF_{PF}* to the *csg* promoter, the 5'-flanking region of the cell surface glycoprotein gene in *T. kodakaraensis*. The *csg* promoter is a constitutive promoter that displays one of the strongest signal intensities on a transcriptome analysis of *T. kodakaraensis*. The plasmids and strategy applied for promoter exchange are shown in Fig. 2C. Incorporation of the *csg* promoter was confirmed by PCR amplification of the region and DNA sequencing. Transformants with the *pyrF* genes under the control of the *csg* promoter (Csg-KPF and Csg-KTV, see Table 1) were grown in ASW-YT-S⁰ medium at 70°C and the cell-free extracts were examined for OMPdecase activity. The levels of OMPdecase activity in the cell-free extracts were sufficient to examine the thermostability of PyrF_{TV} and PyrF_{PF}. Cell-free extracts were incubated at 70, 75, 80 and 85°C for various periods of time, and residual activities were measured. The results are shown in Fig. 3. OMPdecase activities in the wild-type KOD1 and Csg-KPF extracts displayed high thermostability, with no decrease in activity levels observed under the conditions examined (Fig. 3A, 3B). In contrast, PyrF_{TV} in the Csg-KTV strain was less stable at all temperatures, particularly at 80°C ($t_{1/2} = 3$ min), a temperature at which strain KTV no longer displayed growth (Fig. 3C).

The author also measured OMPdecase activity in the cell-free extracts of cells grown at various temperatures. Measurements were performed at 60°C in order to prevent denaturation of PyrF_{TV} during the assays. The results are shown in Fig. 4. OMPdecase activity in the wild-type KOD1 cells grown at 70, 75 and 80°C were observed at comparable levels

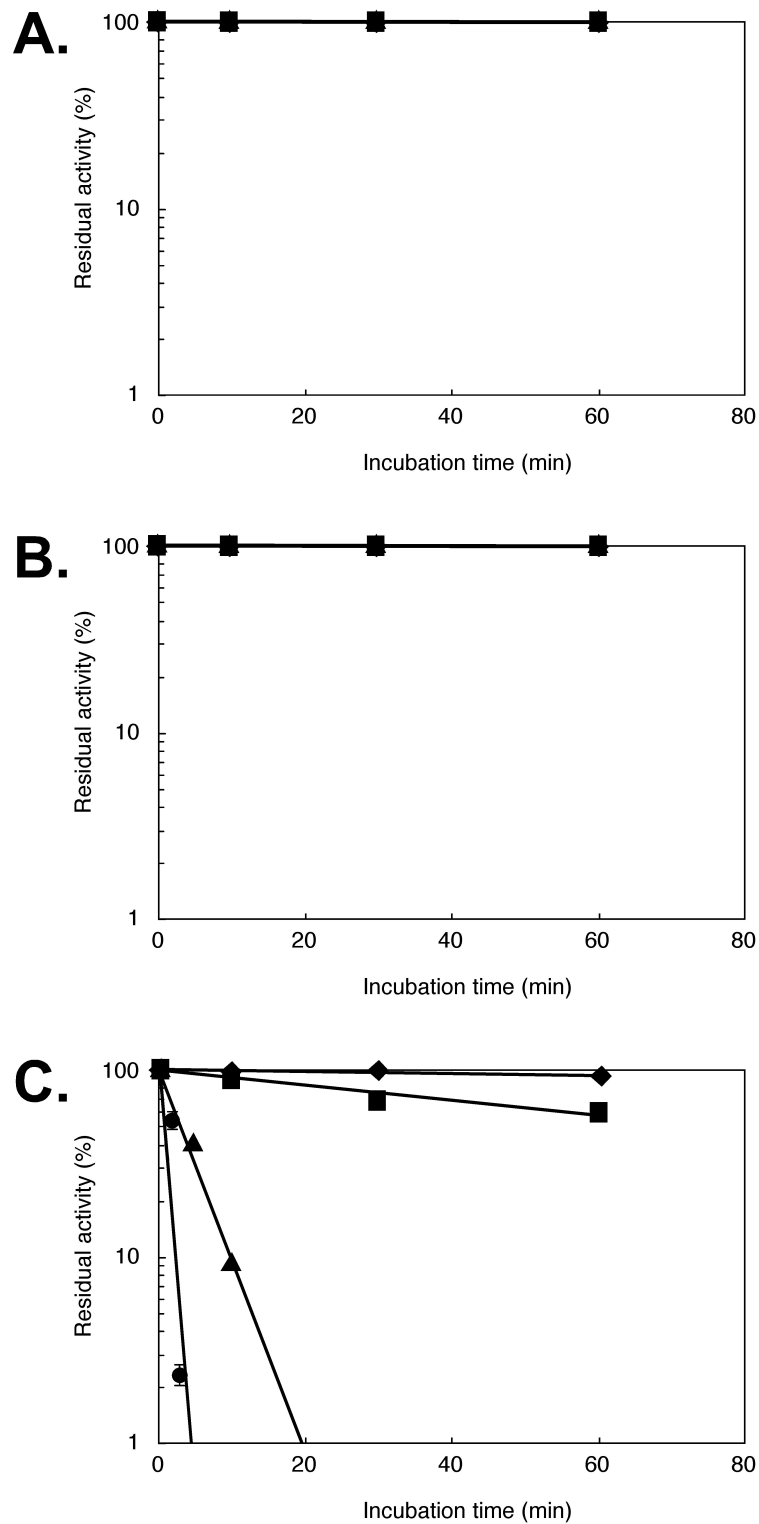


Fig. 3. Thermostability of PyrF activity in the cell-free extract of *T. kodakaraensis* (A) KOD1, (B) Csg-KPF, and (C) Csg-KTV. Cell-free extracts were incubated at 70 (diamonds), 75 (squares), 80°C (triangles) or 85°C (circles), various periods of time. After incubation, residual enzyme activities were measured at 60°C.

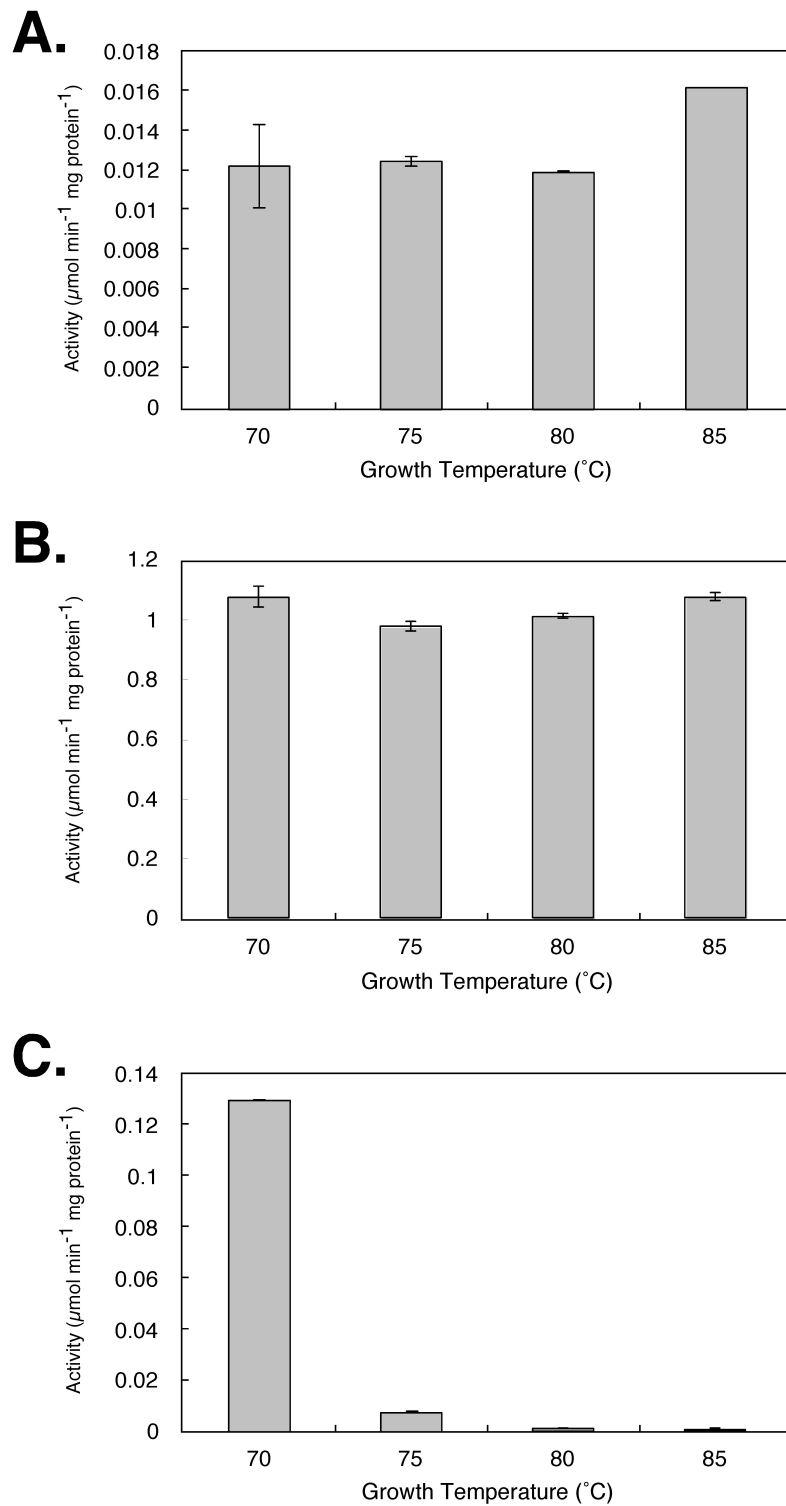


Fig. 4. OMPdecase activity in the cell-free extracts of cells grown at various temperatures. (A) KOD1, (B) Csg-KPF, (C) Csg-KTV.

and a slight increase in activity level was observed in cells grown at 85°C. Activity levels in the Csg-KPF strain were similar regardless of the growth temperature. On the other hand,

activities in the Csg-KTV strain displayed a dramatic decrease when cells were grown at temperatures at or above 75°C compared to that observed in cells grown at 70°C.

Induced expression of molecular chaperonins in *T. kodakaraensis* KTV.

The results described above clearly indicate that the thermostability of PyrF_{TV} is much lower than those of the PyrF proteins from *T. kodakaraensis* and *P. furiosus*, and is most likely the reason why strain KTV cannot grow at high temperatures in the absence of uracil. The author therefore took interest in the response of the host cell in dealing with the presence of a thermolabile protein that is necessary to support growth. The author focused on the chaperonins, a group of proteins involved in the refolding of partially denatured proteins. Two chaperonin subunits, CpkA and CpkB, both members of the HSP60 family, have been identified and studied in *T. kodakaraensis*. Although the functions of the two subunits cannot be distinguished, it has been shown that expression levels of CpkA and CpkB respond differently towards growth temperature. CpkA is the abundant chaperonin in *T. kodakaraensis* when cells are grown at temperatures lower than the optimal growth temperature of 85°C, while CpkB is mainly expressed at higher temperatures (Fig. 5A). The two subunits can interact with one another to form hetero-oligomeric complexes (13).

The author examined the protein levels of CpkA and CpkB in the strains KTV, KPF and KOD1 grown at different temperatures. Each strain was grown in the synthetic medium ASW-AA at 70°C until the stationary phase, and then inoculated into the same medium and incubated at 70°C or 75°C. Western blot analysis was carried out using antibodies specific to CpkA and CpkB. When compared to the wild type KOD1, the author found that both CpkA and CpkB were induced in strain KTV at 75°C (Fig. 5B). This was not observed in strain KPF, which harbors the thermostable PyrF protein from *P. furiosus*. As CpkB is usually only

expressed at temperatures near or above the optimal growth temperature, the abundant presence of CpkB in strain KTV at 75°C was unexpected and, suggests that the expression of the chaperonin responds to the presence of unfolded proteins in the cell.

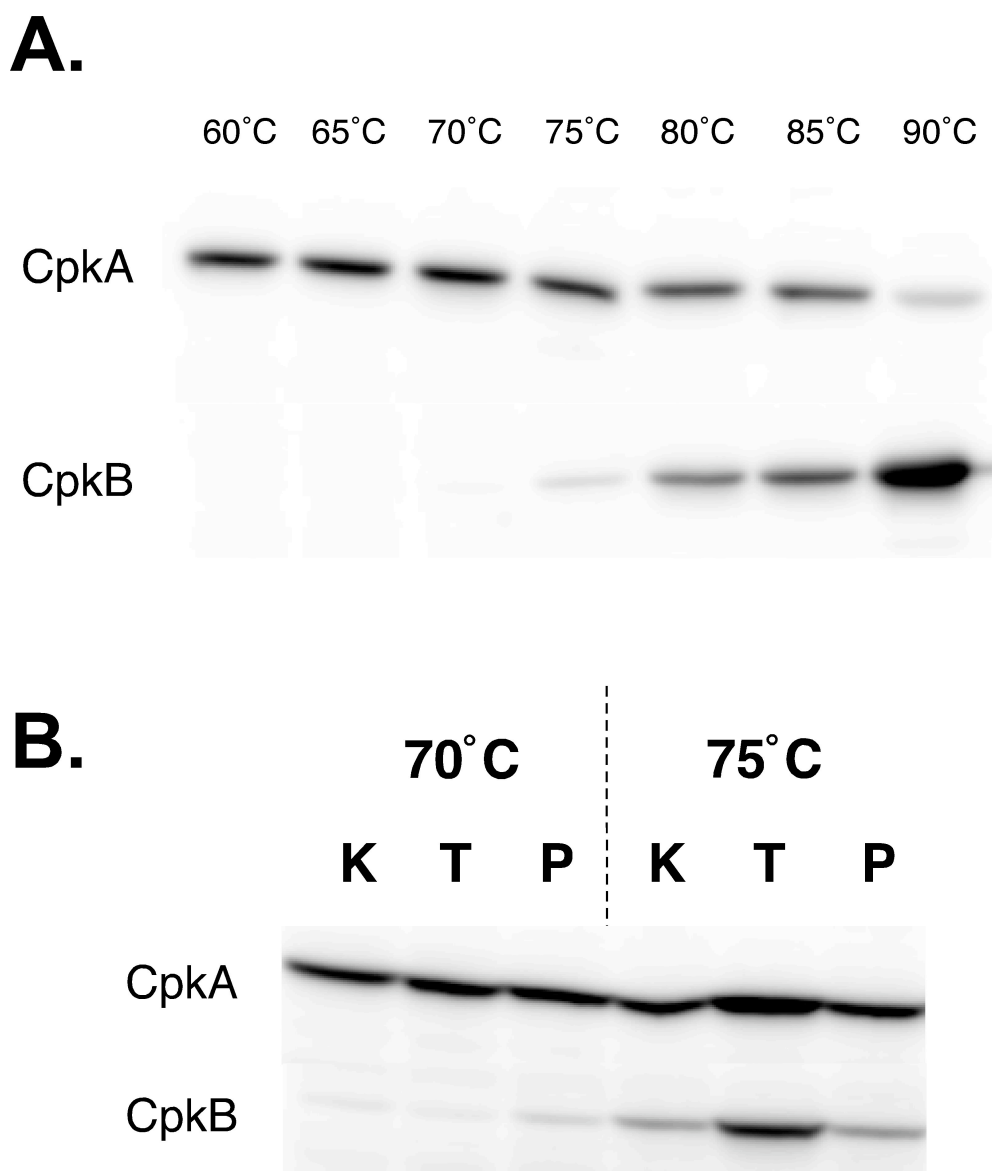


Fig. 5. Chaperonin production in response to temperature and thermolabile proteins. (A) Western blot analysis of CpkA and CpkB in the cell-free extracts (10 µg) of wild type *T. kodakaraensis* KOD1 grown at various temperatures. (B) Western blot analysis of CpkA and CpkB in the cell-free extracts (10 µg) of *T. kodakaraensis* KOD1 (K), KTV (T), KPF (P) grown at 70 and 75°C.

DISCUSSION

In this chapter, the author has examined the response of *T. kodakaraensis* towards the presence of a thermolabile protein in the cell. Taking advantage of the broad growth temperature range of *T. kodakaraensis*, the author was able to confirm the functional expression of *pyrF* genes from both *P. furiosus* ($T_{\text{opt}} = 100^{\circ}\text{C}$) and *T. volcanium* ($T_{\text{opt}} = 60^{\circ}\text{C}$). It is most likely that genes from a variety of (hyper)thermophilic archaea can be expressed and their functions examined in *T. kodakaraensis* cells. As a versatile gene disruption system has been developed in this archaeon, this strain has the potential to be utilized as a model host as in the cases of *E. coli* for the Gram-negative bacteria, *Bacillus subtilis* for the Gram-positives and *Saccharomyces cerevisiae* for the eukaryotes.

The author's results clearly indicate that the expression of the chaperonins CpkA and CpkB responds to the presence of unfolded proteins in the cell. The response mechanism seems to be sensitive, as only a single thermolabile protein (PyrF_{TV}) was expressed, and the promoter used was not a strong promoter. When cell-free extracts were compared among the wild-type strain KOD1 and KPF and KTV strains, the author could not detect bands corresponding to recombinant PyrF_{Pf} or PyrF_{TV} when SDS-PAGE gels were stained with Coomassie Brilliant Blue (data not shown).

The results also indicate that in the case of the chaperonins CpkA and CpkB, there is at least a regulation mechanism that is distinct to the mechanism postulated with Phr from *P. furiosus* or HSR1 from *A. fulgidus* as the regulator. First of all, the author could not find an HSR1 homolog on the *T. kodakaraensis* genome, indicating the absence of a regulatory system dependent on HSR1. In contrast, a closely related homolog of Phr is present on the *T. kodakaraensis* genome. However, as it is assumed that the denaturation of Phr itself triggers

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|-------------------------|------|------------------|-------------------------|---|
| | | | BRE TATA-box | |
| AAA ⁺ ATPase | -81 | TCACCTTAAGAAAAAT | <u>CGAAAAAGTTTATAAA</u> | GGGTTTGTCTTTAGTAACTTACGGTAACCAAAAATAAG -12 |
| | | | BRE TATA-box | |
| Phr | -72 | ACCACCAGCTCAAAC | <u>CGAAAAAGTTTAAATA</u> | CTTTTTACTAACTTTGGGTTAGGGAAAGGGGGTGAGAGCT -1 |
| | | | BRE TATA-box | |
| Hsp20 | -72 | TTGGTAACTTTCCGAG | <u>GAGAAAAATTTTTAAA</u> | GGGCTATTCTTTAATAACAATTGGTGAGAAAAAGCGGGGG -1 |
| | | | BRE TATA-box | |
| CpkA | -78 | TATGAACTTTTGAC | <u>CCAAAAGCTTTTATA</u> | GAGCACTAACGAAGCGTTTCTGCAAAAAGCAATTCGATAG -6 |
| | | | BRE TATA-box | |
| CpkB | -115 | GCTTTAATCATTAC | <u>CGAAAAGTTTATAAA</u> | TAGCACAAAAGAACAATAGCGGGAAAACACGAATTGTAA -50 |

Fig. 6. Promoter regions of the AAA⁺ ATPase, Phr, Hsp20, CpkA and CpkB genes from *T. kodakaraensis*. Sequences corresponding to putative B-recognition element (BRE) and TATA elements are boxed. Sequences similar to the binding sites reported for the Phr from *P. furiosus* (25) are underlined.

the release of gene repression, transcription levels should be directly dependent on the thermostability of Phr and temperature. The up-regulation of the chaperonins CpkA and CpkB was observed at temperatures below the optimal growth temperature of *T. kodakaraensis*, and in particular, CpkB expression was induced at temperatures well below the temperature at which CpkB is usually induced in the wild type strain (Fig. 5). This indicates that the up-regulation brought about by the expression of *pyrF_{Tv}* is not dependent on temperature *per se*, ruling out the possibilities of a scenario solely dependent on the thermal denaturation of Phr. There does remain the possibility that the induction of CpkB at high temperatures is dependent on a Phr dependent mechanism. The author searched the 5'-flanking regions of the AAA⁺ ATPase, small heat shock protein (Hsp20) and Phr homolog genes of *T. kodakaraensis*, and found the presence of the sequences recognized by the Phr of *P. furiosus* (25) (Fig. 6). As the Phr proteins from the two strains are 62% identical, it is most likely that the AAA⁺ ATPase, Hsp20 and Phr genes of *T. kodakaraensis* are also regulated by the Phr in this archaeon. However, no such sequence could be identified in the 5'-flanking regions of the CpkA and CpkB genes. In terms of CpkA, this is not surprising, as the enzyme is present in cells that are

grown at relatively lower temperatures (Fig. 5A). As for CpkB, together with the results of this study, gene expression may be regulated by a mechanism independent of Phr regulators that responds to the presence of unfolded proteins in the cell. Bacteria have been found to display multiple networks that respond to heat shock such as the σ^{32} and σ^{24} systems in *E. coli* (1, 2) and the HrcA, σ^B and the CtsR systems of *B. subtilis* (11). The author's results provide evidence suggesting the presence of a second regulatory system in the Thermococcales in addition to the Phr system first identified in *P. furiosus*.

SUMMARY

Thermococcus kodakaraensis is a hyperthermophilic archaeon for which a gene disruption/integration system has been developed. In this study, the author introduced *pyrF* genes from *Pyrococcus furiosus* (*pyrF_{Pf}*) and *Thermoplasma volcanium* (*pyrF_{Tv}*) into *T. kodakaraensis* KUW1 ($\Delta pyrF$, $\Delta trpE$), a strain that displays pyrimidine and tryptophan auxotrophy. While *T. kodakaraensis* exhibits an optimal growth temperature of 85°C, those of *P. furiosus* and *T. volcanium* are 100°C and 60°C, respectively. The author examined the growth characteristics of the wild-type *T. kodakaraensis* KOD1, the *pyrF_{Pf}*-integrated strain (KPF), and the *pyrF_{Tv}*-integrated strain (KTV). No difference was detected among the three strains in nutrient-rich medium in which *pyrF* function is not necessary. Under conditions in which pyrimidine biosynthesis is essential, strain KPF displayed uracil prototrophy at all temperatures examined, while growth of strain KTV was limited to temperatures at or below 75°C. By examining the thermostability of the three proteins, the author found that PyrF_{Tv} was the most thermolabile ($t_{1/2} = 3$ min, 80°C), while activity levels in strains KOD1 and KPF hardly decreased after 1 h at 85°C. At 75°C, an intracellular accumulation of the two chaperonin subunits CpkA and CpkB was observed in strain KTV, but not in strain KOD1 or

KPF. CpkB is usually up-regulated at temperatures above the optimal growth temperature of *T. kodakaraensis*, and the protein levels decrease at lower temperatures. As 75°C is well below the optimal growth temperature, the accumulation of CpkB observed in strain KTV cannot be due to high temperature *per se*. This raises the possibilities that CpkB expression is responding to the intracellular presence of partially denatured PyrF_{TV} protein, a mechanism similar to the unfolded protein response previously observed only in bacteria and eukaryotes.

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GENERAL CONCLUSIONS

In this study, the author has examined two stress response systems in the hyperthermophilic archaeon, *Thermococcus kodakaraensis*. The organism is an obligate anaerobe, and thus the first system studied was that against oxidative stress. Besides the thioredoxin/peroxiredoxin system and the superoxide reductase system reported in several anaerobic hyperthermophilic archaea, the author identified a methionine sulfoxide reductase homolog (*msrAB_{Tk}*) on the genome of *T. kodakaraensis*. The homolog is not found on the genomes of hyperthermophiles, and is the only exception along with that found on the *Sulfolobus solfataricus* genome. Phylogenetic analysis of MsrAB_{Tk} indicated that the presence of the gene was a result of horizontal gene transfer from mesophilic bacteria. Consistently, the protein product displayed relatively low levels of thermostability, implying that the protein would be rapidly denatured at the optimal growth temperatures of this archaeon. Activity levels at various temperatures also clearly indicated that the protein functions better at sub-optimal growth temperatures. In order to determine whether MsrAB_{Tk}, with its intriguing biochemical properties, actually functions *in vivo*, the author examined the native protein in *T. kodakaraensis* cells. The author found that the presence of MsrAB_{Tk} was observed only at sub-optimal growth temperatures, which correlated well with the results of *in vitro* analyses of the protein. At these sub-optimal growth temperatures, the author found that the protein levels increase with the elevation of oxidative stress, suggesting that MsrAB_{Tk} is involved in the repair system against oxidative damage. These hypotheses were confirmed by generating a *msrAB_{Tk}* knockout strain of *T. kodakaraensis*. The strain, although exhibiting similar behavior to the wild-type strain against oxidative stress at optimal growth temperatures, displayed higher degrees of sensitivity to oxygen at sub-optimal growth temperatures. The results

clearly reveal that in *T. kodakaraensis*, MsrAB_{Tk} represents a novel defense system against oxidative stress that functions specifically at lower temperatures. The presence of this system may be one of the reasons why the organism has evolved to grow at an extremely broad temperature range with its lower limits at 60°C or even lower. This also may be related to the fact that the *Thermococcus* are by far one of the most abundant hyperthermophile species distributed throughout hydrothermal environments on our planet.

In terms of heat stress, the only known mechanism for the induction of heat shock proteins in the hyperthermophilic archaea was the Phr-dependent repression/derepression system. Although this system had been found to govern the expression of several heat shock proteins, it is not involved in the regulation of the chaperonins (HSP60). By deliberately producing a thermolabile protein in *T. kodakaraensis* cells, the author found that the chaperonins of this organism (CpkA/CpkB) responded to the presumed intracellular accumulation of denatured proteins. As the response was observed at temperatures below the optimal growth temperature of *T. kodakaraensis*, the induction was not triggered by heat shock *per se*, but most likely the presence of unfolded proteins. This is a mechanism similar to the unfolded protein response previously observed only in bacteria and eukaryotes. The results of this study also provide the first clues as to how the chaperonins of the hyperthermophilic archaea respond to heat shock.

LIST OF PUBLICATIONS

1. Methionine sulfoxide reductase from the hyperthermophilic archaeon *Thermococcus kodakaraensis*, an enzyme designed to function at sub-optimal growth temperatures.
Eiji Fukushima, **Yasuhiro Shinka**, Toshiaki Fukui, Haruyuki Atomi, and Tadayuki Imanaka.
Journal of Bacteriology. 2007. **89** (19), 7134-7144.
2. A genetic examination on methionine sulfoxide reductase of the hyperthermophilic archaeon, *Thermococcus kodakaraensis*.
Yasuhiro Shinka, Eiji Fukushima, Haruyuki Atomi, and Tadayuki Imanaka.
Under revision for publication in the FEMS Microbiology Letters.
3. Chaperonin expression responds to the presence of unfolded proteins in the hyperthermophilic archaeon, *Thermococcus kodakaraensis*.
Yasuhiro Shinka, Keigo Sato, Toshiaki Fukui, Shinsuke Fujiwara, Haruyuki Atomi, and Tadayuki Imanaka.
Under revision for publication in the Journal of Bacteriology.