Specificity of Fur Binding to the Oxidative Stress Response Gene Promoter in the Facultative Anaerobic Archaeon *Thermoplasma volcanium*

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The genome of the facultative anaerobic thermoacidophilic archaeon *Thermoplasma volcanium* contains the open-reading frames (ORFs) *tvso*d and *tvog*g, which are predicted to encode a putative superoxide dismutase and an 8-oxoguanine DNA glycosylase, respectively. *Tvso*d is immediately upstream of *tvog*g, and these two ORFs are aligned in a head-to-tail manner in a single operon. A previous study showed that *T. volcanium* contains an ORF (TVN0292) encoding the ferric uptake regulator (Fur) and that the *T. volcanium* Fur protein (TvFur) binds to its own promoter in a metal-dependent manner *in vitro*. Here, we demonstrated that TvFur also binds to the *tvso*d-*tvog*g promoter and determined the TvFur-binding sequences in the *tvso*d-*tvog*g promoter by *DNase*I footprinting analysis. These results suggest that Fur is required for resistance against reactive oxygen species in this facultative anaerobic archaeon.

Key words archaea; ferric uptake regulator (Fur); superoxide dismutase gene promoter

Archaea¹⁾ is a taxon that constitutes a kingdom of life. Almost two-thirds of the archaea are thermophiles or hyperthermophiles,²⁾ and most of them are strict anaerobes.³⁾ The proteins encoded by their genomes are heat-stable, and some of them are utilized in bioscience research, for example, polymerase chain reaction (PCR) employs thermophilic DNA polymerases. Therefore, research on additional proteins encoded by thermophilic and hyperthermophilic archaea could likely be beneficial.

In general, eukarya cannot survive in the absence of oxygen because they require molecular oxygen to transfer the hydrogen ions (electrons) to that are produced by oxidation of nutrients through the electron transport pathway to generate energy. Nevertheless, reactive oxygen species (ROS) generated by these electron transport pathways are toxic because macromolecules such as proteins and DNA are readily oxidized by ROS. This explains why internal injuries from oxidative-stress are inevitable for organisms that use oxygen as a terminal electron acceptor. Therefore, establishing defense systems against this kind of oxidation is necessary for any organisms that live in an aerobic environment.

Among the vast number of macromolecules that are oxidized by ROS, DNA oxidation may result in the most serious consequences. In particular, the generation of 8-oxo-7,8-dehydro-2'-deoxyguanosine (8-oxoG) by oxidation of guanine is the most frequently observed lesion in DNA.^{4,5)} 8-oxoG has been shown to form a stable base pair with both cytosine and adenine; therefore, the 8-oxoG:A pair can undergo transversion from G:C to T:A after replication.^{6,7)} To prevent this transversion mutation, organisms have evolved base excision repair pathways that utilize a specific DNA glycosylase.

In anaerobic archaea, an enzyme called superoxide reductase (SOR) has evolved as a defense system that removes ROS without generating molecular oxygen.⁸⁾ Most organisms

that live in aerobic environments also possess another enzyme to detoxify ROS, superoxide dismutase (SOD). Under aerobic conditions, some archaea adopt oxygen as terminal electron acceptor.³⁾ *Thermoplasma volcanium* is one of these aero-anaero facultative archaea⁹⁾ that has a putative SOD gene (TVN0061, designated *tvso*d). The *T. volcanium* genome also encodes a putative 8-oxoG DNA glycosylase (TVN0062, designated *tvog*g) that removes the oxidized guanines as well as a uracil DNA glycosylase (TVN0827) that removes the uracils generated by deamination of cytosine,¹⁰⁾ and an AP endonuclease (TVN0046 and TVN0971) that restores an apurinic/aprimidinic site.¹¹⁾ In the genome of *T. volcanium*, *tvog*g is located in an operon with *tvso*d, and these two genes constitute a single transcription unit (Toda *et al.*, unpublished). This gene configuration makes it possible to rapidly respond to oxidative stress.

In numerous bacteria, the ferric uptake regulator (Fur) is a transcriptional regulator of genes encoding proteins that are involved in iron homeostasis and protection from the effects of iron toxicity, including oxidative stress under aerobic conditions.¹²⁾ *T. volcanium* contains a gene encoding Fur (TVN0292, designated *tvfur*), and TvFur, the *T. volcanium* Fur protein, binds to its own promoter region *in vitro*.¹³⁾ In this study, we investigated the binding specificity of TvFur to the *tvso*d-*og*g operon promoter by electrophoretic mobility shift assay (EMSA), and determined the TvFur binding sequence in this promoter by *DNase*I footprinting analysis. TvFur recognized a TATA-box like TA repeat sequences.

MATERIALS AND METHODS

Strains *Thermoplasma volcanium* GSS1 (NBRC 15438) was used as a source of whole genomic DNA. Cloning of the gene encoding TvFur, as well as the overexpression and purification of the TvFur protein have been described previously.¹³⁾

Electrophoretic Mobility Shift Assays (EMSA) A 204-

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bp DNA fragment including the *tvod-ogg* promoter and a portion of its coding region (from -132 to $+72$; designated as 5' *tvod-ogg*), corresponding to bases 62122–62325 of *T. volcanium* (GenBank accession no. NC_002689.2) was amplified from genomic DNA by PCR with the primers psod 5' (5'-AAC AGA ATT CAT CGC CTC CAG AG-3') and psod 3' (5'-TAT CTG TAC ATC TGA ATT CCT TC-3') carrying *EcoRI* restriction sites. The 5' *tvod-ogg* fragment was cloned into the pGEM-T Easy vector (Promega), and then digested with *EcoRI*. The digested 5' *tvod-ogg* fragment was gel purified and subcloned into pUC119 (TaKaRa) to generate pUC119-5' *tvod-ogg*. For EMSA, a DNA fragment containing 5' *tvod-ogg* was prepared by PCR amplification of pUC119-5' *tvod-ogg* with the primer pair M13-20-FAM (5'-6-FAM-GTT TTC CCA GTC ACG AC-3') and M13rev (5'-CAG GAA ACA GCT ATG AC-3'). As a negative control, 50S ribosomal protein L3P gene (TVN0324 rpl3p, first gene of ribosomal operon corresponded with 332101 to 332360 of NC_002689.2) was prepared. To determine the minimal recognition region for TvFur binding, a series of oligonucleotides containing TvFur binding sequences or those with several bases substitutions were prepared. TvFur binding sequences were determined by the *DNaseI* footprinting analysis described below. The 5' FAM labeled or non-labeled double-stranded DNA for EMSA was generated by annealing complementary oligonucleotides by incubation at 95°C for 5 min and then cooling to room temperature. EMSA was performed with $0.2\mu\text{M}$ or $0.4\mu\text{M}$ of DNA under essentially the same conditions as previously described¹³⁾ except electrophoresis buffer containing 1 mM dithiothreitol (DTT). The DNA bands were detected and analyzed with a Pharos FX molecular imager and Quantity One imaging software (Bio-Rad), and the dissociation constant (K_d) was calculated.¹⁴⁾

***DNaseI* Footprinting Analysis** *DNaseI* footprinting analysis was performed according to the method described by Riboulet-Bisson *et al.*¹⁵⁾ with some modification. The 5' *tvod-ogg* fragment was generated by PCR with pUC119-5' *tvod-ogg* as a template and a D4 dye end-labeled primer set of M13-20 (5'-D4-GTA AAA CGA CGG CCA GT-3') and M13rev (5'-D4-GGA AAC AGC TAT GAC CAT G-3'). The binding reaction contained 1.0 pmol of end-labeled DNA fragment and various amounts of TvFur protein (0.75–1.25 pmol) and was incubated at room temperature for 10 min in 35 μL of 40 mM KCl, 1 mM DTT, 100 μM MnCl_2 , 1 $\mu\text{g}/\text{mL}$ bovine serum albumin (BSA), 1 $\mu\text{g}/\text{mL}$ poly(dI-dC), 5% glycerol, and 10 mM Tris-HCl, pH 8.0. The product was then digested with 0.01 U of freshly diluted RNase free *DNaseI* (Roche) in the same buffer for 5 min at room temperature. The reaction was terminated by the addition of 5 μL of 250 mM ethylenediamine-tetraacetic acid (EDTA). The samples were analyzed with the CEQ8800 sequencing apparatus (Beckman Coulter). Determination of the DNA sequence of the protected region was performed according to a previously described method.¹³⁾

RESULTS AND DISCUSSION

TvFur Specifically Binds to the *tvod-ogg* Promoter Region In the TvFur 5' *tvod-ogg* DNA binding experiments, the bound fraction of the FAM-labeled 5' *tvod-ogg* DNA fragment increased as the amount of TvFur protein was raised from $1.0\mu\text{M}$ to $4.0\mu\text{M}$ (Fig. 1A, lanes 2–5). Bound TvFur

Fig. 1. Electrophoretic Mobility Shift Assay (EMSA) with $0.2\mu\text{M}$ of FAM-Labeled *tvod-ogg* Promoter DNA (Lanes 1–6) or the Promoter DNA of TVN0324 rpl3p, First Gene of Ribosomal Operon (Lanes 7 and 8) as a Negative Control Incubated with TvFur in the Presence of $100\mu\text{M}$ MnCl_2

(A) Lanes 1 and 7, no TvFur protein; lanes 2–5 contained 1.0 , 2.0 , 3.0 and $4.0\mu\text{M}$ of TvFur protein, respectively. Lane 6 contained $0.2\mu\text{M}$ of FAM-labeled *tvod-ogg* promoter DNA, $1.0\mu\text{M}$ of non-labeled *tvod-ogg* promoter DNA and $4.0\mu\text{M}$ of TvFur protein. C and F indicate TvFur bound and free (unbound) promoter DNA, respectively. (B) Binding profile of TvFur to $0.2\mu\text{M}$ of *tvod-ogg* promoter DNA. The TvFur concentration varied from 0 to $4.0\mu\text{M}$ as indicated above. Error bars represent the S.D. of three independent experiments. $\circ$, TvFur binding to the *tvod-ogg* promoter DNA; $\blacktriangle$, TvFur binding to the rpl3p gene promoter.

competed with fivefold excess of non-labeled promoter DNA (Fig. 1A, lane 6). The binding of TvFur to the 5' *tvod-ogg* DNA fragment was only observed in the presence of $100\mu\text{M}$ Mn^{2+} , while no binding was observed in metal ion-free binding buffer (data not shown). No unbound DNA was observed when the EMSA included $4.0\mu\text{M}$ of TvFur (Fig. 1A, lane 5). TvFur scarcely bind to the ribosomal protein L3 encoding gene promoter (Fig. 1A, lane 8), demonstrating that TvFur specifically binds to the *tvod-ogg* promoter region. From the EMSA results, the dissociation constant (K_d) was calculated as $1.3 \times 10^{-6}\text{M}$ (Fig. 1B). The dissociation constant for TvFur binding to its own promoter was approximately same value,¹³⁾ however that the constant for binding to the *tvhemB* promoter was 37% of this value (data not shown). These results indicate that TvFur binds to the former two promoters with higher affinities than to the *tvhemB* promoter region. Therefore, TvFur could robustly regulate the expression of *tvod-ogg* genes.

Estimation of the Essential Recognition Region for

Fig. 2. Identification of the TvFur Binding Region in the *tvsod-ogg* Promoter by *DNaseI* Footprinting Analysis

Fluorescence intensity (fragment abundance) is shown on the y axis and elution position (size) is shown on the x axis. (A) Full length electropherograms of 1.0 pmol of 5' *tvsod-ogg* DNA after *DNaseI* footprinting analysis without TvFur (1) or with 0.75 pmol (2), 1.0 pmol (3), and 1.25 pmol (4) of TvFur. The elution position between two dotted lines represents the region protected from *DNaseI* digestion by TvFur binding. (B) Full length electropherograms after *DNaseI* footprinting analysis performed without (lower panel) or with 1.25 pmol of TvFur (upper panel). The DNA region corresponding to 25–28 min after start (between the left and right dotted lines) was the region protected from *DNaseI* digestion by TvFur binding. The TvFur binding sequence identified by footprinting analysis is indicated at the bottom. The putative BRE and TATA-box are boxed and underlined, respectively.

TvFur Binding To determine the exact location where TvFur binds to the *tvsod-ogg* promoter, *DNaseI* footprinting was performed (Fig. 2). The protected regions in a *DNaseI* footprinting reaction with 1.0 pmol of 5' *tvsod-ogg* DNA and 0.75 pmol of TvFur were not changed by increasing the amount of TvFur to 1.25 pmol (Fig. 2A). The DNA sequence of the protected region were corresponded to from 25.0 min to 28.0 min on the electropherogram (Fig. 2B). This region corresponded to the 41-bp sequence of 5'-TCA ATT TAA CGA GTA TTA ATT AAT ATT AAT TCT TAC TTA AA-3' (Fig. 2B), which extended to positions –92 to –51 relative to the translational start site of *tvsod*. These results indicated that the TvFur binding region included the TATA-box or was in the vicinity of the TATA-box in the *tvsod-ogg* promoter. The TvFur binding sequence in the *tvfur* promoter region includes the 30-bp sequence 5'-GTT ATT ATG TTT ATA T AT TAA TTA GTA CAA-3' when 0.8 pmol of TvFur and 1 pmol of DNA was used in a *DNaseI* footprinting assay. In contrast to the results obtained for the *tvsod-ogg* promoter, the protected region of the *tvfur* promoter extended downstream towards the start codon depending on the amount of TvFur.¹³ The *T. volcanium* consensus BRE and TATA-box sequences are 5'-AAAA-3' and 5'-TTTATATA-3', respectively,¹⁶ and both of them are consistent with the archaeal consensus sequences 5'-WAAW-3' (BRE) and 5'-YTTWWAWW-3' (TATA-box).¹⁷ The putative BRE of the *tvsod-ogg* promoter, 5'-TAAT-3', was consistent with the consensus archaeal BRE sequence, whereas the putative TATA-box of the *tvsod-ogg* promoter, 5'-TAATATTA-3', differed from the archaeal consensus TATA-box sequence. This divergence between the putative TATA-box of the *tvsod-ogg* promoter and the archaeal consensus TATA-box might enable TvFur to regulate the transcriptional level of *tvsod-ogg*. To determine the precise region of where TvFur interacts with the *tvsod-ogg* promoter *in vitro*, three FAM labeled DNA con-

taining regions upstream or downstream of the BRE-TATA region (Fig. 3A, P1–P3), and three non-labeled DNA harboring base substitutions (Fig. 3A, S4–S6) were prepared and EMSAs were performed. TvFur obviously bound to 82.4% of the 20-bp DNA fragment P2 (5'-G TATTAAT TAATA TTAATT C-3'; Figs. 3B, C, P2). TvFur recognized a DNA fragment of the *tvsod-ogg* promoter without the BRE, P3, and bound to 65.6% of the DNA fragment without BRE (Figs. 3B, C, P3). The binding of TvFur to a *tvsod-ogg* promoter DNA fragment without the TATA-box, P1, was decreased and TvFur bound to 50.9% of P1 DNA (Figs. 3B, C, P1). Bound TvFur competed with fivefold excess of non-labeled DNA substituted 5'-GTT to 5'-GGG of P2 DNA (Figs. 3D, E, +S4), however, it could not be affected by the DNA of BRE and/or TATA box mutations (Figs. 3D, E, +S5, +S6). These results indicate that the BRE and TA repeats in the TATA-box are essential for *tvsod-ogg* promoter recognition by TvFur. Together with the results of the previous study,¹³ this suggests that TvFur recognizes these TA repeats.

Bacterial Fur proteins bind to a 19-bp inverted repeat sequence known as the classical Fur box.¹² The binding of TvFur to the 5'-G T ATTAAT TA-AT ATTAAT T C-3' sequence of the *tvsod-ogg* promoter DNA implies that it binds to an inverted repeat array. This repeat unit 5'-ATT AAT-3' is also found in the TvFur binding region of the *tvfur* promoter 5'-GTT ATT ATG TTT ATA T-AT TAA T-TA G-3'. The two inverted repeat units located in the *tvsod-ogg* promoter may allow TvFur to bind to this region.

A *fur* homolog is conserved in the genome of all known *Pyrobaculum* species, and an antisense RNA, designated *asR2*, co-exists with the *fur* transcript, which probably decreases Fur levels.¹⁸ In *Escherichia coli*, Fur down-regulates the *sodA* gene, which encodes Mn-superoxide dismutase, but up-regulates *sodB*, which encodes Fe-superoxide dismutase, *via* re-

Fig. 3. EMSA Analysis of TvFur Binding to *tvso*d-*ogg* Promoter DNA

(A) The sequence of the TvFur binding region in the *tvso*d-*ogg* promoter revealed by *DNase*I footprinting analysis (uppermost sequence, putative BRE and TATA-box are underlined and bolded, respectively), the sequences of the TvFur binding DNA fragments (P1, P2, and P3), and the sequences of DNA fragments containing base substitutions as competitors (S4, S5, and S6, substituted bases were underlined) employed for EMSAs. (B) EMSAs of TvFur binding to 2.0 pmol of the DNA fragments shown in Fig. 3A, P1 to P3 in the presence of 100 μ M MnCl₂. EMSA was performed with (right lanes) 40 pmol of TvFur. (C) TvFur binding in B is represented as the percentage of bound DNA (indicated in the columns) calculated by subtracting the band intensity of the unbound DNA from that of DNA incubated without TvFur. Error bars represent the S.D. of two independent experiments. (D) EMSA of 4.0 pmol of P2 was performed with 30 pmol of TvFur. Lanes +S4, +S5, and +S6 contained 30 pmol TvFur and fivefold excess of DNA of S4, S5, and S6 shown in A, respectively. (E) TvFur binding in D is represented as the percentage of bound DNA (indicated above the columns) calculated same as B.

pression of the small RNA RyhB, leading to rapid degradation of the *sodB* transcript.¹⁹ Several bacterial Fur deletion mutant strains are known to be sensitive to ROS.^{20–22} These reports indicate that Fur is required for resistance to ROS. This study suggests the possibility that TvFur functions in the same way in *Thermoplasma*.

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