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Cloning and Expression of the MutM Gene from Obligate Anaerobic Bacterium *Desulfovibrio vulgaris* (Miyazaki F)*

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The gene encoding a MutM from *Desulfovibrio vulgaris* (Miyazaki F) was cloned and expressed in *Escherichia coli*. A 5.9-kb DNA fragment, isolated from *D. vulgaris* (Miyazaki F) by *Xho*I and *Pvu*II, contained a MutM gene and other open reading frames. The nucleotide sequence of the MutM gene indicated that the protein was composed of 336 amino acids. The amino-acid sequence deduced from the MutM gene was highly homologous with the MutM of other bacteria; however an additional insert consisted of 64 amino acids. An expression system for the MutM gene under the control of the T7 promoter was constructed in *E. coli*. From the kinetic analysis results, the purified His-tagged MutM showed 8-oxoguanine-DNA glycosylase activity comparable with that of MutM from *E. coli*. In this study, the amounts of mRNA and protein for MutM were scant in the *D. vulgaris* (Miyazaki F). MutM activity may be induced by oxidative stress. However, its induction may not be frequently generated because sulfate-reducing bacteria generally grow in anaerobic conditions. MutM might play a role in the protection against the mutagenicity of oxygen when oxygen stress exceeded the capacity of the defense systems against oxygen toxicity.

Key words: MutM, obligate anaerobe, oxidative stress, recombinant, sulfate-reducing bacteria.

Abbreviations: *D., Desulfovibrio*; ICP-AE, inductively coupled plasma-atomic emission; ORF, open reading frame; 8-oxoG, 8-oxo-7,8-dihydro-2′-deoxyguanine; SRB, sulfate-reducing bacteria.

Oxidative DNA damage is generated by a variety of environmental and endogenous agents, including ionizing radiation, certain chemicals, and the products of aerobic metabolism (*1*). It seems likely that the oxidation of guanine residues to 8-oxo-7,8-dihydro-2′-deoxyguanine (8-oxoG) in DNA is one of the most abundant types of spontaneous, directly premutagenic events in living cells. Because 8-oxoG mispairs with adenine during replication, this oxidative lesion is a source of G·C to T·A transversion mutations (*2*, *3*). Nearly all organisms have the ability to recognize 8-oxoG·C pairs and to excise 8-oxoG bases. The MutM protein from *Escherichia coli* shows this ability and removes not only 8-oxoG but also 2,6-diamino-4-hydroxy-5-(*N*-methyl)-formamidopyrimidine and several structurally related lesions from damaged DNA (*4*). MutM is a component of the 8-oxoG resistance pathway that comprises MutY, a mismatch adenine-DNA glycosylase, and MutT, an 8-oxodGTPase; *E. coli* strains deficient in any of these genes are strong mutators (*5*–*7*).

Sulfate-reducing bacteria (SRB) are anaerobic prokaryotes found ubiquitously in nature. They typically use sulfate as a terminal electron acceptor for the respiration of hydrogen or various organic acids to produce sulfide, a highly reactive and toxic end product. SRB, which are generally classified as obligate anaerobes, were long believed to be incapable of surviving under aerobic conditions. Various enzymes involved in substrate oxidation, isolated from SRB, were oxygen sensitive (*8*, *9*). However, some SRB have been observed to live at the oxic-anoxic interface rather than under perfectly anoxic conditions (*10*–*12*). Some strains of *Desulfovibrio* (*D.*) and other sulfate reducers possess a degree aerotolerance. Although all aerobic organisms have evolved mechanisms of defense against oxidative damage, little is known about these mechanisms in anaerobic bacteria, including *D. vulgaris* (Miyazaki F). To understand the defence mechanisms against oxygen toxicity in anaerobes, we carried out a series of genetic studies on oxygen-related proteins in *D. vulgaris* (Miyazaki F) as well as previously cloned cytochrome *c* oxidase-like protein gene (*13*), rubredoxin and desulfoferrodoxin gene (*14*), catalase gene (*15*) and periplasmic superoxide dismutase gene (*16*). Genome analysis of *D. vulgaris* Hildenborough (*17*), *Desulfotalea psychrophila* (*18*) and *D. desulfuricans* G20 reveals the existence of these genes in all three organisms except for the catalase gene in *D. desulfuricans* G20. Genome analysis of these three SRB also revealed the existence of an oxidative damaged DNA repair enzyme gene, *mutM*. In this article, we report the molecular cloning of the MutM gene from *D. vulgaris* (Miyazaki F) and the overproduction and characterization of recombinant MutM.

*The nucleotide sequence data reported in this article will appear in the DDBJ, EMBL and Genbank nucleotide sequence databases with the accession number AB290343.

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EXPERIMENTAL PROCEDURES

Bacterial Strains and Materials—*E. coli* strain JM109 *recA* 1, Δ(*lac-proAB*), *endA* 1, *gyrA*96, *thi*-1, *hadR* 17, *relA* 1, *supE* 44[F′ *traD* 36, *proAB*+, *lacI*^{q} ZΔM15]) was used for cloning, and JM109(DE3), which carried the T7 RNA polymerase gene, was used to express the MutM gene. *D. vulgaris* (Miyazaki F) was grown anaerobically with lactate as a carbon source (*19*) and used for the genomic DNA and mRNA preparation. Restriction endonucleases and DNA modifying enzymes were purchased from New England Biolabs, Nippon Gene and Toyobo. [γ-^{32}P]ATP (185 TBq/mmol) was obtained from MP Biomedicals, Inc. A rabbit polyclonal antibody against recombinant MutM from *D. vulgaris* (Miyazaki F) was prepared by Hokkaido System Science Co. Ltd. The anti-rabbit immunoglobulin G alkaline phosphatase conjugate antibody was from Promega. All other reagents were of the highest available purity.

Oligonucleotide Synthesis—The common solvents and reagents for the DNA synthesizer (Applied Biosystems 310) were purchased from Applied Biosystems. The four normal phosphoramidite units for DNA synthesis were purchased from Glen Research. Substrate DNA oligonucleotides were synthesized using the established phosphoramidite approach and purified by reversed-phase HPLC (*20*). Oligonucleotide concentrations were determined spectrophotometrically at 260 nm with extinction coefficients calculated using pairwise base extinction data (*21*).

Cloning and Sequencing—The oligonucleotide sequences used in this study are summarized in Table 1. Genomic DNA isolated from *D. vulgaris* (Miyazaki F) was prepared by Saito and Miura's method (*22*). We searched for the published amino-acid sequences of other bacteria because the amino-acid sequence of MutM from *D. vulgaris* (Miyazaki F) was unknown. Since attempts to amplify a part of the MutM gene using PCR failed, we adopted another strategy using a highly conserved gene, which was assumed to be upstream or downstream of the MutM gene in *D. vulgaris* (Miyazaki F). We learned that the phenylacetatecoenzyme A ligase gene, whose amino-acid and nucleotide sequence were highly conserved across SRB species, exists in the sense strand of the upstream of the MutM gene (Fig. 1). Then two primers, mutM11 and mutM12, were designed based on the conserved regions of the amino-acid sequence of the phenylacetatecoenzyme A ligase gene from *D. vulgaris* Hildenborough, and a part of the phenylacetatecoenzyme A ligase gene was amplified using PCR with a KOD-Plus-(TOYOBO) DNA polymerase and a *D. vulgaris* (Miyazaki F) genomic DNA template. PCR was carried out for 35 cycles of 94°C for 15 s, 50°C for 30 s, and 68°C for 1 min after 94°C for 2 min. The PCR products carried out agarose gel electrophoresis, and a fragment of ~720 bp was extracted using a MinElut Gel Extraction Kit (QIAGEN). We determined the nucleotide sequence of this fragment by direct sequencing, and its nucleotide-derived amino-acid sequence resembled the amino-acid sequence of another phenylacetatecoenzyme A ligase from sulfate-reducing bacteria. DNA probe 1 (Table 1) was synthesized corresponding to the amino-acid sequence of D-L-S-Y-I-D-R-S-P-C-P (Fig. 1). We carried out Southern hybridization with this [γ-^{32}P] ATP labelled oligonucleotide at 60°C and detected a band hybridizing to an ~3.0 kbp *Xho*I-*Eco*RI fragment using a bioimage analyser, BAS1000 (FUJIX). We digested the genomic DNA with *Xho*I and *Eco*RI, and the digest was separated into several fractions on an agarose gel by size. The separated fragments were ligated into *Sal*I and *Eco*RI sites of pUC18, and *E. coli* JM109 was transformed with the resulting ligation mixture. One such transformant was found by a colony hybridization method to harbor a plasmid carrying the ~3.0 kbp *Xho*I-*Eco*RI fragment of *D. vulgaris* (Miyazaki F) DNA, which was named pMM-1000. The nucleotide sequence of the inserted fragment was determined by sequencing its restriction fragments that were cloned into the multicloning site of pUC18. We used the dideoxy chain termination method with a DNA sequencer (Applied Biosystems 310). The *Xho*I-*Eco*RI fragment did

Table 1. **Sequences of oligodeoxynucleotides used in this study.**

Name	Sequence
mutM9	5′-CCTGGATCCCATATGCCTGAATTACCCGAAG-3′
mutM10	5′-CGCATAAGCTTCTTCTGGCACTGCCGAC-3′
mutM11	5′-GGCCCCATATTCGACCCCGAAGA-3′
mutM12	5′-GGGTAGACGAACATGCCCTTGAT-3′
Probe1	5′-CCTGTCCTACATCGACCGTTCGCCCTGCCC-3′
Probe2	5′-TGGTGGTGGCGGCGATTCGGGATCTCTGCG-3′
mutM15	5′- ACATCTAGACATATGCCCGCCCGCGACAC -3′
mutM16	5′- GGGAAGCTTCCGGTACGGCGCGAAGCGCAG -3′
mutMOG	5′- GGTGGGCGCCGXCGGTGTGGGCA -3′
mutMC	5′- TGCCCACACCGCCGGCGCCCACC -3′
mutM-RT1	5′-ATGCCTGAGTTGCCAGAGGTGGAG-3′
mutM-RT2	5′-CGCGTCTGGCGGAACATTGGTTCC-3′
eno3	5′-GCCGAAGCCATCGTGGGGCTGGAT-3′
eno4	5′-GAAGGTGGCCGCGCCGATGGGCAT-3′
67-eno7	5′-GTTAGTTTTTTGCGGGGGAGGGAC-3′
67-eno8	5′-CGGGCGGCACCACGGGGGCAGCAT-3′

X; 8-oxodG.

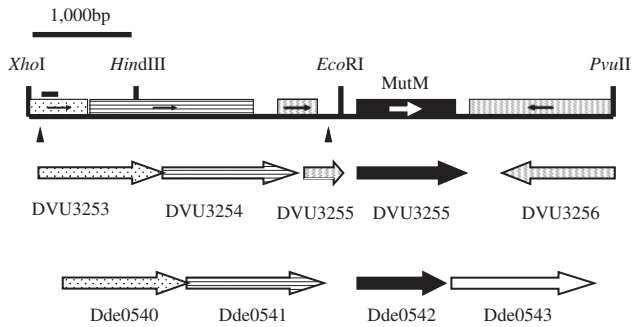

Fig. 1. **Genomic organization of *D. vulgaris* (Miyazaki F) and partial restriction map of a 5.9-kb *Xho*I-*Pvu*II DNA fragment.** Some restriction sites and the relative positions of MutM gene with other putative ORFs located upstream and downstream from the MutM gene are indicated. Black bars indicate the PCR fragment. Triangles indicate the positions of DNA probes for Southern hybridization. Left triangle is DNA probe 1. Right triangle is DNA probe 2. Homologs of other SRB are indicated under each gene. DVU and Dde are locus tags of *D. vulgaris* Hildenborough and *D. desulfuricans* G20, respectively.

not contain MutM gene, but the *Hin*dIII site was found. So DNA probe 2 (Table 1) was synthesized corresponding to a sense strand closer upstream of the MutM gene (Fig. 1). We carried out Southern hybridization again with this [γ-^{32}P] ATP-labelled oligonucleotide at 60°C and detected a band hybridizing to an ~4.8 kbp *Hin*dIII-*Pvu*II fragment. We digested the genomic DNA with *Hin*dIII and *Pvu*II, and the digest was separated into several fractions on an agarose gel by size. The separated fragments were ligated into *Hin*dIII and *Sma*I sites of pUC18, and *E. coli* JM109 was transformed with the resulting ligation mixture. One such transformant was found by a colony hybridization method to harbor a plasmid carrying an ~4.8 kbp *Hin*dIII-*Pvu*II fragment of *D. vulgaris* (Miyazaki F) DNA, which was named pMM-2000. The nucleotide sequence of the inserted fragment was determined by sequencing its restriction fragments that were cloned into a multicloning site of pUC18.

Construction of Expression Vectors—We constructed a high-level expression system in *E. coli* by connecting a T7 promoter to the upstream region of the MutM gene and added a His-tag at the COOH-terminus. The coding region of the MutM gene was amplified using PCR with a KOD-Plus-(TOYOBO) DNA polymerase. *D. vulgaris* (Miyazaki F) genomic DNA was used as a template in the PCR, and mutM15 and mutM16 (Table 1) were used as PCR primers. PCR was carried out for 35 cycles of 94°C for 15 s, 50°C for 30 s and 68°C for 90 s after 94°C for 2 min. The PCR product was digested with *Xba*I and *Bam*HI and ligated into the same sites of pUC18. The cloned fragment was sequenced, digested with *Nde*I and *Bam*HI, and then ligated into pET-21a to give the expression vector pET-cMutMF. The MutM gene from *E. coli* was prepared based on a similar procedure using primers mutM9 and mutM10 (Table 1). The resulting expression vector was designated as pET-cMutME.

Expression and Purification of Recombinant MutM—*E. coli* was transformed with pET-cMutMF, and the transformants were grown in 1.7 ml of LB medium containing 90 μg/ml ampicillin for 9 h at 37°C. Twelve flasks containing 167 ml of the same medium were inoculated with 1.7 ml of the culture and incubated overnight with agitation at 37°C. Cells were harvested by centrifugation at 6,000 rpm for 10 min. The cell pellet was suspended in a 10 mM Tris–HCl buffer (pH 8.0). The cell suspension was sonicated with a Model 201M sonicator (KUBOTA) at 9,000 Hz, 200 W for 10 min and then ultracentrifuged at 40,000 rpm for 2 h at 4°C. Affinity chromatography using a HisTrap HP column (0.7 × 2.5 cm, GE Healthcare) was then carried out. We used a wash buffer containing 20 mM imidazole and an elution buffer containing 500 mM imidazole in a 500 mM NaCl and 20 mM phosphate buffer (pH 7.4). The fraction containing MutM was loaded onto the gel filtration column (Superdex 75 HR10/30) and equilibrated with a 200 mM NaCl/10 mM Tris–HCl buffer (pH 8.0) at a flow rate of 0.5 ml/min. The purified MutM was collected and concentrated with a centriprep YM-30 (Millipore).

Analytical Methods—SDS–PAGE was carried out based on Laemmli's method with a gel concentration of 10% (*23*). The molecular weight of MutM in its native state was determined by gel filtration chromatography (Superdex 75 HR10/30). Bovine serum albumin (66,200), ovalbumin (43,000), carbonic anhydrase (29,000) and cytochrome *c* (12,400) were used as molecular weight markers. UV-visible absorption spectrum was recorded using a Hitachi U-3000 spectrophotometer. Metal contents for zinc, iron and chrome were determined by inductively coupled plasma-atomic emission analysis using a HORIBA JY38S. The wavelengths were set at 213.9, 259.9 and 283.6 nm, respectively.

MutM Activity Measurement—We determined protein concentrations using the Micro BCA Protein Assay Reagent Kit (Pierce). Bovine serum albumin was used as a standard, and we measured the absorbance at 280 nm. 5′-end-labelling of the 23mer oligodeoxynucleotide that contained 8-oxoG (20 pmol) was carried out using T4 polynucleotide kinase and [γ-^{32}P] ATP (MP Biomedicals, Inc.) for 1.5 h at 37°C, in a buffer containing 5 mM Tris–HCl (pH 7.6), 1 mM $MgCl_2$, and 1 mM 2-mercaptoethanol at final concentrations. After purification with a disposable reversed phase column (YMC Dispo SPE), the labelled oligodeoxynucleotide was mixed with non-labelled oligodeoxynucleotide (180 pmol) for the cleavage reaction. The oligodeoxynucleotide was annealed with unlabelled complementary oligodeoxynucleotide at 90°C for 2 min and cooled to room temperature for 60 min (*24*). The substrate oligonucleotides were mutMOG and mutMC (Table 1). A solution of the 23mer duplex substrate (0.1 mM final concentration) was mixed with a reaction buffer containing 50 mM Tris–HCl (pH7.5), 30 μg/ml BSA, and 100 mM KCl (final concentrations) and preincubated at 37°C for 10 min. After the addition of MutM (20 nM final concentration) to start the cleavage reaction, the mixture was incubated at 37°C. Three microliters of aliquots was taken at specific times and 3 μl of loading buffer, which contained 10 M Urea, 0.1% bromophenol blue, 0.1% xylene cyanole and 20 mM EDTA (final concentrations), was added to stop the reaction. The mixtures were fractionated by PAGE on a denaturing 20% gel containing 8 M urea (*4*). Quantification of the radioactivity was performed with an imaging analyser (FUJIX). To define the 8-oxoguanine-DNA glycosylase activity of MutM from the *D. vulgaris* for DNA duplexes containing 8-oxodG:dC, 20–90 nM of DNA, duplexes containing 8-oxodG:dC were incubated with 20 nM of MutM from *D. vulgaris*. Activity, which was measured at various time points (1–10 min), was detected by changes in the oligonucleotide length of the strand containing 8-oxoguanine.

Immunoblot Analysis—The total protein concentration of cell lysate was also determined using the Micro BCA Protein Assay Reagent Kit (Pierce). Various concentrations of cell lysates or recombinant MutM were loaded on a 10% polyacrylamide SDS minislab gel. Blotting was carried out electrophoretically with a nitrocellulose filter, which was then blocked by shaking in 3% (w/v) bovine serum albumin in Tween TBS containing 10 mM Tris–HCl (pH 7.5), 100 mM NaCl and 0.1% Tween 20 for 1 h (*15*). After a wash with Tween TBS, it was incubated in Tween TBS for 1 hour with a 1:1,000 dilution of a polyclonal antiserum raised against the recombinant MutM from *D. vulgaris* (Miyazaki F). The blot was then thoroughly washed with Tween TBS and incubated with

15 ml of alkaline phosphatase-conjugated goat anti-rabbit IgG in Tween TBS. Following two washes, the first with Tween TBS and the second with alkaline phosphatase buffer [100 mM Tris–HCl (pH 9.5), 100 mM NaCl and 5 mM $MgCl_2$], the nitrocellulose filter was incubated with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate in 10 ml of alkaline phosphatase buffer. The blot was finally washed with water and dried.

Reverse Transcription PCR—Total RNA was prepared with an RNeasy mini Kit (Qiagen). Reverse transcription PCR (RT–PCR) was performed with Ready-To-Go RT-PCR beads (GE Healthcare). The primer sequences used in RT–PCR are summarized in Table 1: mutM-RT1, corresponding to nt 3312 to nt 3335, and mutM-RT2, corresponding to nt 3589 to nt 3612. Primers eno3, eno4, 67-eno7 and 67-eno8 corresponded to the sequences in another part of the genome. Using mutM-RT1 and mutM-RT2, 300 bp of part of the MutM gene must be amplified. Primers eno3 and eno4 were used as a positive control (*25*). The absence of contaminating DNA was confirmed by PCR and RT–PCR using primers 67-eno7 and 67-eno8. If the DNA were contaminated, product containing two putative terminators must be amplified. For RT–PCR, 100 ng of total RNA as a template and 25 pmol of each primer were used in a procedure done as described by the manufacturer. The PCR products were analysed by agarose gel electrophoresis.

RESULTS

Nucleotide Sequence of MutM Gene—To identify the MutM gene, a ~5.9-kbp *Xho*I-*Pvu*II fragment of *D. vulgaris* (Miyazaki F) was cloned and sequenced. The results are shown in Supplementary Fig. 1. The open reading frame (ORF) encoding the MutM is composed of 336 amino-acid residues (fourth ORF in Supplementary Fig. 1). A potential ribosome-binding site (GGAG) from nucleotides 3302 to 3305 in the *Xho*I-*Pvu*II fragment was present upstream of the initiation codon (ATG) as well as possible promoter regions from nucleotides 3161 to 3166 (TTCCAG) and 3181 to 3186 (TTCTCC) in the *Xho*I-*Pvu*II fragment. Nucleotides 4333–4379 might comprise the putative transcriptional terminator, forming a stem-and-loop structure. The results of a BLAST homology search indicated that the product of the fourth ORF was highly homologous with MutM from other bacteria, especially that from *D. vulgaris* Hildenborough, whose identity was 71%. The deduced amino-acid sequence of the first, second, third, and fifth ORF products displayed 95%, 39%, 66%, and 45% homology with the COOH-terminal regions of phenylacetate-coenzyme A ligase, PDZ domain-containing protein, CopG family transcriptional regulator, and the DNA internalization-related competence protein ComEC/Rec2 from *D. vulgaris* Hildenborough, respectively. Based on the genome sequence data of *D. vulgaris* Hildenborough, *D. vulgaris* (Miyazaki F) has similar gene orders as shown here (Fig. 1) (*17*).

Amino-Acid Sequence Comparison—The deduced amino-acid sequences of the *D. vulgaris* (Miyazaki F) MutM gene and other MutM genes are compared in Fig. 2. The deduced amino-acid sequence of the *D. vulgaris* MutM gene resembles those of MutM genes from other bacteria. A zinc finger motif, C-$(X)_2$-C-$(X)_{16}$-C-$(X)_2$-C from residues

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D. vulgaris (MiyazakiF)                    [1] MPELPEVETIACGLRPQLTGRRIVSVLVRNEGTVQG---DAAAFARCVPG 50
D. vulgaris Hildenborough                  [2] MPELPEVETIACGLRPALSGRRIVGVTVHNPGTLEGPLCTPAAFTEAVQG 50
T. thermophilus HB8                        [3] MPELPEVETTRRRLRPLVLGQTLRQVVHRDPARYRN--------TALAEG 50
B. stearothermophilus                      [4] MPELPEVETIRRTLLPLIVGKTIEDVRIFWPNIIRHPR-DSEAFAARMIG 50
E. coli                                    [5] MPELPEVETSRRGIEPHLVGATILHAVVRNGRLRWP----VSEEIYRLSD 50
                                               *********      *   *

[1] RVIAGVGRRGKLLLMELA---PPGGPDAPDATGGGAG---------GPDAAGAAGTGTNVPPDAADLMACR-DAAGK---MAGSSATG----ATG----- 150
[2] QRIADVGRRGKLLLVAFASLPPVGHAGQPRPEGLSSSTVRDFLVTHGFHAAGCATSVHACAPLLADGQQTRGRLAGHGDGMDGTSRTGSTLPGTGGTENS 150
[3] RRILEVDRRGKFLLFALEG-------------------------------------------------------------------G---------- 150
[4] QTVRGLERRGKFLKFLLDR---------------------------------------------------------------------------- 150
[5] QPVLSVQRRAKYLLLELPEG-------------------------------------------------------------------------- 150
           ** * *

[1] ---AAGGNRVPHLLGVHLKMTGRLFVYGPEVAPNTHTRVVFGLDDGNRLFFDDARKFGYVRALSDADLATWDFWRSLGPEPL--EIAAPDFAALFRGRRG 250
[2] DAVAVADDDTVLGLAFHLKMTGRLFIHPPATPAGIHTRVVFDLEGGTRLFFDDARKFGYVRCITRRSLALWPFWRDLGPEPL--ETEARGFAARLARRRG 250
[3] -----------VELVAHLGMTG-----GFRLEPTPHTRAALVLEG-RTLYFHDPRRFGRLFGVRRGDYREIPLLLRLGPEPLSEAFAFPGFFRGLKESAR 250
[4] -----------DALISHLRMEGRYAVASALEPLEPHTHVVFCFTDGSELRYRDVRKFGTMHVYAKEEADRRPPLAELGPEPLSPAFSPAVLAERAVKTKR 250
[5] -----------WIIIHLGMSGSLRILPEELPPEKHDHVDLVMSNGKVLRYTDPRRFG--AWLWTKELEGHNVLTHLGPEPLSDDFNGEYLHQKCAKKKT 250
                  ** * *           *           *   * * **                  ******

[1] RIKALLLDQTVIAGIGNIYADESLFRASIRPDAQAGELSPERLCVLHGHLVDVLRESIAECGSSIRD--YRDAHGDAGAFQNRFRVYGRSGQPCVACGRA 350
[2] RIKALLLDQKVVAGVGNIYADESLFRAGIRPDTQAHTLTPERLFALHGHLQDVLRESIAECGSSIRD--YRDAHGDAGAFQNSFRVYGRGGQPCRHCGTT 350
[3] PLKALLLDQRLAAGVGNIYADEALFRARLSPFRPARSLTEEEARRLYRALREVLAEAVELGGSTLSDQSYRQPDGLPGGFQTRHAVYGREGLPCPACGRP 350
[4] SVKALLLDQTVVAGFGNIYVDESLFRAGILPGRPAASLSSKEIERLHEEMVATIGEAVMKGGSTVRT--YVNTQGEAGTFQHHLYVYGRQGNPCKRCGTP 350
[5] AIKPWLMDNKLVVGVGNIYASESLFAAGIHPDRLASSLSLAECELLARVIKAVLLRSIEQGGTTLKD--FLQSDGKPGYFAQELQVYGRKGEPCRVCGTP 350
     *  * *    * ****  * ** *   *   *  *       *              *           *  * *    **** * **  **

[1] LTTGKVAGRTTVFCERCQKAK- 372
[2] LATAQVAGRTTVFCPQCQR--- 372
[3] VERRVVAGRGTHFCPTCQGEGP 372
[4] IEKTVVAGRGTHYCPRCQR--- 372
[5] IVATKHAQRATFYCRQCQK--- 372
         * * *  *  **
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Fig. 2. **Alignment of amino-acid sequences deduced from the *D. vulgaris* (Miyazaki F) MutM gene and other MutM genes.** Amino-acid sequences of MutMs from *D. vulgaris* (Miyazaki F) (this work), *D. vulgaris* Hildenborough (*17*), *T. thermophilus* HB8 (*27*), *B. stearothermophilusr* (*28*) and *E. coli* (*26*) are shown. Sequence alignment was carried out using the CLUSTAL W (version 1.83) program. Asterisks show amino-acid residues conserved in all sequences. The italic letters in the amino-acid sequence deduced from *D. vulgaris* MutM genes indicate the inserted sequences.

309 to 332, which is conserved among all MutM homologues, was also observed in the *D. vulgaris* MutM protein. However, ~60 amino-acid fragments were inserted in the amino-acid sequence of *D. vulgaris* MutM.

Expression, Metal Analysis and Molecular Weight of Recombinant MutM—We constructed *E. coli* expression systems of the MutM gene from *D. vulgaris*. Recombinant MutM was detected in transformed *E. coli* crude cell extract by SDS–PAGE (Fig. 3A). Through chromatographic steps on HisTrap and Superdex 75, 2.5 mg of the recombinant MutM was purified to homogeneity on SDS–PAGE (Fig. 3A). The molecular weight of the expressed MutM was estimated to be ~40,000 by SDS–PAGE, which was close to the value (36,738) calculated from the amino-acid sequence. We also estimated the molecular weight in the native state to be ~38,000 using a Superdex 75 gel filtration column (Fig. 3B). The value was about the same calculated from the amino-acid sequence, indicating that MutM's native form is a monomer. The absorption maximum of MutM from *D. vulgaris* was observed at 280 nm, and the absorption coefficient at 280 nm was calculated as 14,100 M^{-1} cm^{-1} using the results of a BCA protein assay. To quantify the bound metals, ICP-AE analysis was performed. The results indicated that MutM from *D. vulgaris* contained 0.85 zinc ions per protein. Iron and chrome ions were not detected (Fig. 4).

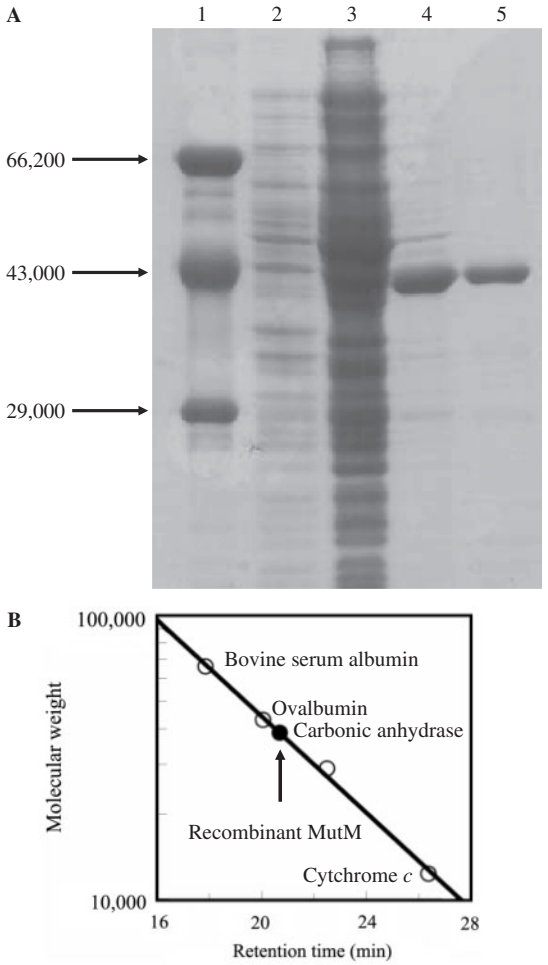

Fig. 3. **Estimation of MutM molecular weight.** (A) Purified MutM was analysed by SDS–PAGE using a gel concentration of 10.0%. Lane 1, standard proteins: bovine serum albumin (Mr 66,200), ovalbumin (Mr 43,000) and carbonic anhydrase (Mr 29,000); lane 2, pET21-a/JM109(DE3); lane 3, pET-cMutMF/JM109(DE3); lane 4, affinity chromatography fractions of pET-cMutMF/JM109(DE3); lane 5, purified recombinant MutM. Molecular weight of the monomer was estimated as 40,000. (B) Purified MutM and molecular weight markers were applied to Superdex 75 HR10/30 on an FPLC system (GE Healthcare Pharmacia Biotech UK), and elution was performed with 200 mM NaCl in 10 mM Tris-HCl (pH 8.0) at flow rate of 0.5 ml/min. Molecular weight markers were bovine serum albumin (66,200), ovalbumin (43,000), carbonic anhydrase (29,000) and cytchrome *c* (12,400).

MutM Activity—The 8-oxoguanine-DNA glycosylase activity of MutM was examined using a synthetic oligonucleotide containing 8-oxoguanine. The reaction rate for each substrate concentration was obtained, and the Michaelis constant (K_m) and the catalytic constant (k_{cat}) of the reaction catalysed by the MutM from *D. vulgaris* were estimated from the Lineweaver–Burk plots (Fig. 5). The K_m value of MutM from *D. vulgaris* for DNA duplexes containing 8-oxodG:dC was 165 nM, while their k_{cat} was 4 min^{-1}. MutM from *E. coli* showed slightly small K_m (72 nM) and k_{cat} (3 min^{-1}) values for DNA duplexes containing 8-oxodG:dC, resulting in similar catalytic efficiency (k_{cat}/K_m value) to that of MutM from *D. vulgaris*.

Immunoblot and RT–PCR—The recombinant MutM reacted with a polyclonal antibody raised against the recombinant MutM from *D. vulgaris* (Miyazaki F) and was detected by 1 ng per lane (Fig. 6A). However, the

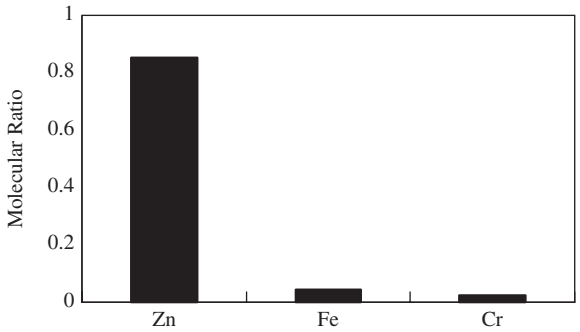

Fig. 4. **Metal analysis of MutM by ICP-AE.** Molecular ratios were normalized to that for MutM concentration (13.5 μM).

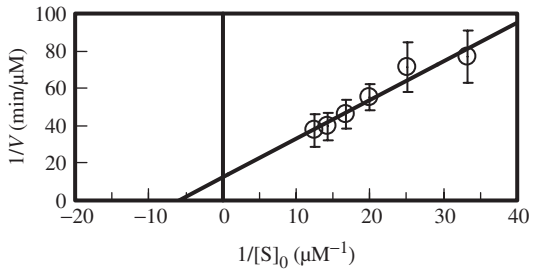

Fig. 5. **Lineweaver–Burk plot for kinetic analysis of recombinant MutM from *D. vulgaris* (Miyazaki F).** Assay conditions are described in EXPERIMENTAL PROCEDURES section.

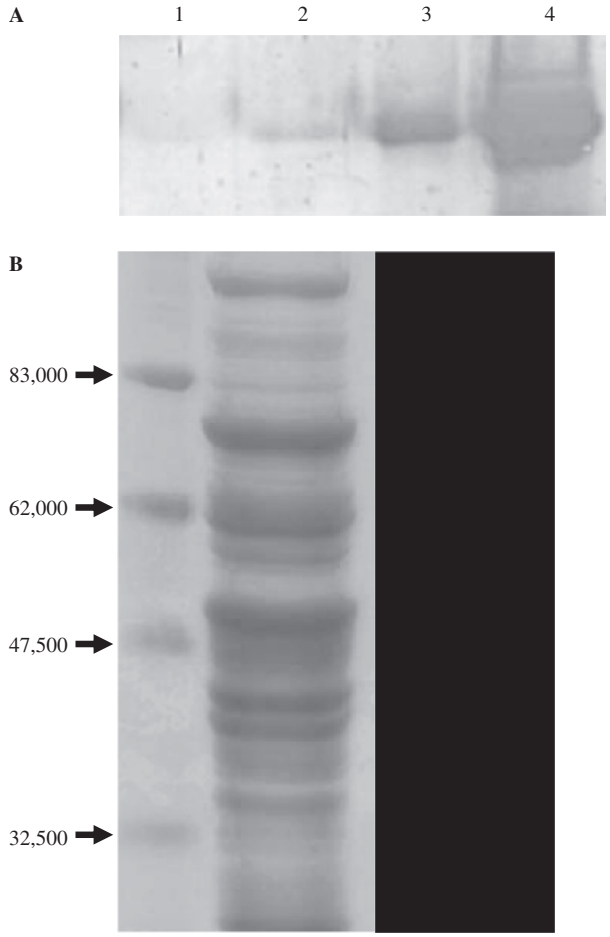

Fig. 6. **Immunoblot analysis of MutM content.** (A) Immnoblot of gel after SDS–PAGE. Lane 1, purified MutM 1 ng; lane 2, purified MutM 10 ng; lane 3, purified MutM 100 ng; lane 4, purified MutM 1 μg. (B) *Left:* CBB-stained gel after SDS–PAGE. lysate of *D. vulgaris* (Miyazaki F) 500 μg. *Right:* Immnoblot of gel as in left panel.

lysate of *D. vulgaris* (Miyazaki F), with total loading protein of 500 μg, was not detected (Fig. 6B). Then we assumed that the MutM was no more than 0.0002% by the amount of all proteins. We used RT–PCR methods with three sets of primers: mutM-RT1 and mutM-RT2, eno3 and eno4 as a positive control, and 67-eno7 and 67-eno8 as a negative control. The RT–PCR results are shown in Fig. 7. In every case, amplified 300-bp DNA was detected after PCR using 100 ng genomic DNA as a template. However, when using extracted RNA as a template, the amplified 300-bp DNA did not detect the cases of mutM-RT1/mutM-RT2 and 67-eno7/67-eno8, but eno3/eno4 was detected. These results indicate that mRNA of MutM was slight in *D. vulgaris* (Miyazaki F).

DISCUSSION

MutM is a bifunctional DNA glycosylase/lyase that catalyses complete excision of 8-oxoG lesion nucleosides when paired opposite C in DNA. The gene encoding MutM from a sulfate-reducing bacteria, *D. vulgaris*

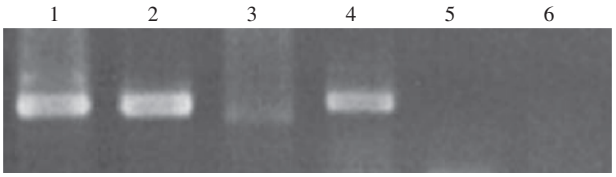

Fig. 7. **Analysis of amplified DNA from PCR and RT–PCR using genome DNA and total RNA of *D. vulgaris* (Miyazaki F), respectively.** Lane 1, amplified DNA using eno3 and eno4 by genomic DNA; lane 2, amplified DNA using mutM-RT1 and mutM-RT2 by using genomic DNA; lane 3, amplified DNA using 67-eno7 and 67-eno8 by using genomic DNA; lane 4, amplified DNA using eno3 and eno4 by RT–PCR using total RNA mixture; lane 5, amplified DNA using mutM-RT1 and mutM-RT2 by RT–PCR using genomic DNA; and lane 6, amplified DNA using 67-eno7 and 67-eno8 by RT–PCR using genomic DNA.

(Miyazaki F), was cloned and its nucleotide sequence was determined. The amino-acid sequence deduced from the *D. vulgaris* MutM gene was highly homologous with MutM from other bacteria; however an additional insert consisted of 64 amino acids (Fig. 2). The amino-acid sequences and the crystal structures of the MutM of *E. coli* (*26*), *Thermus thermophilus* (*27*) and *Bacillus stearothermophilus* (*28*) suggest that the insert was in the loop region of the N-terminal domain of MutM. The MutMs of *D. vulgaris* Hildenborough and *D. desulfuricans* G20 also contain inserts, composed from 92 amino acids and 21 amino acids, respectively, in the same loop region. An intein is defined as a protein sequence embedded in-frame within a precursor protein sequence that is spliced out at the protein level during maturation (*29*). All known inteins have sequences similar to homing endonucleases, while the MutM insertions lack such sequences. The MutM insertions from *Desulfovibrio* are not thought to be an intein, and they may form a new loop structure. The existence of the MutM gene suggests a horizontal transmission of the gene from some aerobic bacterium or that the sulfate-reducing bacterium might have been an aerobic bacterium at one time. Since aerobic bacteria are generally thought to have evolved from anaerobic bacteria, an essential enzyme of aerobic organisms may have evolved from that of an anaerobic bacterium. However, MutM from *D. vulgaris* (Miyazaki F) had an extra inserted sequence against the MutM of aerobic bacterium, which was unique to the *Desulfovibrio* species. It may be a piece of evidence to support the hypothesis that an anaerobic bacterium living today may have developed from an aerobic bacterium.

The calculated kinetic constants of purified MutM from *D. vulgaris* (Miyazaki F), overproduced in *E. coli*, were comparable with those of the recombinant MutM from *E. coli*. Although its K_m value was about twice as high as that of MutM from *E. coli*, its k_{cat} value was only somewhat higher than that of the MutM from *E. coli*. Its k_{cat}/K_m was only about 1.6 times lower than that of the MutM from *E. coli*. This k_{cat} value of the recombinant MutM from *E. coli* resembled the previous data ($4\,min^{-1}$), and its K_m value was higher than those of the previous data (12 nM) (*30*). This difference most likely results from the addition of a His-tag in the COOH terminal region to

the recombinant MutM of this study. The metal content detected by ICP-AE indicated that the recombinant MutM from *D. vulgaris* (Miyazaki F) contained only a single zinc, agreeing with the MutM data from *E. coli* (*26*). This suggests that the conserved Cys residues of the recombinant MutM formed a zinc-finger motif structure. DNA binding by MutM is abolished by the mutation of any of the four cysteines in the zinc finger (*31*, *32*), and this motif disruption would create conformational changes that affect the positioning of the conserved Arg-258 of the MutM from *E. coli* (*26*). Therefore, forming a zinc-finger motif structure may contribute to MutM activity or the stability of the overall structure. Metal-binding selectivity may be high, in contrast to that of the recombinant rubredoxin from *D. vulgaris*. Although native rubredoxin contains a non-haem iron, recombinant protein may contain an iron or a zinc (*14*). Anaerobic groups of SRB cultured in $ZnSO_4$/$FeSO_4$-containing media precipitated ZnS but not iron sulfides (*33*). It may be difficult that a protein forms the zinc-binding structure in SRB. Such difficulty may be one reason for the detection of little MutM in *D. vulgaris* (Miyazaki F).

It was reported that *Prevotella melaninogenica*, one of the strict anaerobes, is susceptible to oxygen and showed an increase in oxidative DNA damage upon exposure to oxygen. The induced 8-oxoG was not removed, even after anaerobic incubation for 24 h (*34*). Therefore, they concluded that 8-oxoG removal activity is probably absent or very low in *P. melaninogenica*, and the lack of a DNA repair system may also explain the oxygen sensitivity of *P. melaninogenica* (*35*). Accordingly, the high activity of MutM from *D. vulgaris* (Miyazaki F) may be one reason that SRB can actually tolerate oxygen. Superoxide dismutase and catalase activity were not detected in *P. melaninogenica* (*35*), which is highly sensitive to O_2 or H_2O_2. The increased 8-oxoG levels correlated well with the decreased survival (*34*). In the *Desulfovibrio* species, two superoxide scavengers, superoxide dismutase in the periplasm and superoxide reductase in the cytoplasm, have been characterized (*14*, *16*), and *D. vulgaris* is catalase positive (*15*). For *D. vulgaris* Hildenborough, the catalase gene is located on a *nif* gene-containing plasmid that is lost when the cells are cultured in an ammonium chloride-containing medium (*36*). O_2 bubbling of samples of *Bacteroides fragilis*, an aerotolerant anaerobe with catalase activity, did not result in higher levels of 8-oxoG (*34*). In this study, the detection of MutM gene expression might not have succeeded because the levels of 8-oxoG were very low in *D. vulgaris* (Miyazaki F), which possessed defense systems against oxygen toxicity. Actually, the cell lysate of *D. vulgaris* (Miyazaki F) did not show 8-oxoguanine-DNA glycosylase activity (data not shown). Superoxide dismutase genes are constitutively expressed in the *Desulfovibrio* species (*37*, *38*). In *D. gigas*, catalase activity increases as cells are exposed to increasing oxygen concentrations, and long exposure times to oxygen lead to higher catalase activity (*37*). In *E. coli*, MutM activity was induced by oxidative stress. Its regulation is under the negative control of the global regulatory genes (*39*). Further studies are needed to define the expression regulation of oxidative DNA lesion repair enzyme genes in the *Desulfovibrio* species.

We identified the MutM gene from obligate anaerobe sulfate-reducing bacterium for the first time. The activity of recombinant MutM from *D. vulgaris* (Miyazaki F) was comparable with that of MutM from aerobic bacteria. Even though *D. vulgaris* (Miyazaki F) was classified as an obligate anaerobe, it possessed defense systems against oxygen toxicity. So 8-oxoG lesions were not frequently generated in *D. vulgaris* (Miyazaki F). For this reason, the immunoblot and RT–PCR results are plausible. Intact MutM might be retained in *D. vulgaris* (Miyazaki F) to protect against the mutagenicity of oxygen, which occurred if oxygen stress exceeded the capacity of the defense systems against oxygen toxicity. These results suggest that the mutagenicity of 8-oxoG is a threat and that MutM is critical for living beings despite its obligate anaerobe.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

CONFLICT OF INTEREST

None declared.

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