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Effects of Overexpression and Antisense RNA Expression of Orf17, a MutT-Type Enzyme

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The *Escherichia coli* Orf17 (NtpA, NudB) protein, a MutT-type enzyme, hydrolyzes oxidized deoxyribonucleotides, including 8-hydroxy-2'-deoxyadenosine 5'-triphosphate and 8-hydroxy-2'-deoxyguanosine 5'-triphosphate, *in vitro*. To examine its *in vivo* role(s) in bacteria, plasmid DNAs containing the *orf17* gene in the sense and antisense orientations were introduced. When the Orf17 protein was overexpressed in *mutT* cells, the *rpoB* mutant frequency was decreased. On the other hand, similar effects were not observed when Orf17 was overexpressed in wild type and *orf135* cells. Expression of the antisense RNA of the *orf17* gene did not produce an obvious phenotype, such as increased mutant frequency and resistance to ionizing radiation. These results suggest that the role of the Orf17 protein is to back up the MutT function, and to assist in the elimination of 8-hydroxy-2'-deoxyguanosine nucleotides.

Key words Orf17; MutT; 8-hydroxy-dGTP; nucleotide pool sanitization; antisense RNA

Reactive oxygen species (ROS) seem to be involved in mutagenesis, carcinogenesis, aging, and neurodegeneration.^{1,2)} Many oxidative DNA lesions and oxidized DNA precursors are formed by ROS, and their mutagenic potentials have been studied.³⁾ Nucleotide pool sanitization enzymes hydrolyze oxidized deoxyribonucleotides and function as a defense system against them in prokaryotic and eukaryotic cells. The *Escherichia coli* MutT protein and its mammalian functional homologue (MTH1) hydrolyze 8-hydroxy-2'-deoxyguanosine 5'-triphosphate (8-OH-dGTP).^{4–6)} The mutation frequency in *mutT*-deficient strains is higher than that in wild type (wt) strains.^{7,8)} Increased tumor formation was found in MTH1 knock-out mice.⁹⁾ In addition, the MTH1 (-like) protein suppresses the mutagenesis induced by oxidized dATP, 2-hydroxy-2'-deoxyadenosine 5'-triphosphate (2-OH-dATP), in an *in vitro* HeLa extract replication system.¹⁰⁾ Thus, nucleotide pool sanitization is crucial for the prevention of mutagenesis by damaged DNA precursors.

Previously, activities as nucleotide pool sanitization enzymes have been identified for the two *E. coli* proteins, Orf135 (NudG) and Orf17 (NudB, NtpA), in our laboratory. The Orf135 protein hydrolyzes 2-OH-dATP and 8-OH-dGTP *in vitro*.¹¹⁾ In addition, the lack of the Orf135 protein and its overexpression in *E. coli* cells result in increased and decreased mutation frequencies, respectively.¹²⁾ These *in vivo* experiments indicate that the Orf135 protein also contributes to the suppression of mutagenesis by oxidized deoxyribonucleotide(s). The other protein, Orf17, degrades various oxidized deoxyribonucleotides, including 8-hydroxy-2'-deoxyadenosine 5'-triphosphate (8-OH-dATP) and 8-OH-dGTP, *in vitro*.¹³⁾ Thus, like MutT and Orf135, this nucleotide pool sanitization enzyme might act as a defense against damaged DNA precursors. Interestingly, Orf17 and MutT degrade both damaged nucleoside triphosphates and diphosphates, suggesting their important roles in nucleotide pool sanitization by their dual functions.^{13,14)}

In this study, we examined the *in vivo* roles of the Orf17 protein in *E. coli* cells. Since the destruction of the *orf17*

gene resulted in severe growth inhibition (Miki *et al.*, personal communication), we introduced plasmid DNA containing the gene in the antisense orientation, under an inducible promoter. In addition, plasmid DNA that overexpresses an Orf17 protein fusion with glutathione-S-transferase (GST) was introduced into various *E. coli* strains. A phenotype was observed when the Orf17 protein was overexpressed in *mutT*-deficient *E. coli* cells.

MATERIALS AND METHODS

Materials The *E. coli* strains KP7600 (*lacI*^q, *lacZ*ΔM15-*gal*⁻, *F*⁻), JD22899 (KP7600 but *orf135*::mini Tn10 (*kan*)) (Miki *et al.*, unpublished results), and KAM0003 (AB1157 but *mutT*::*Cm*) were used. The KAM0003 strain was constructed by P1 transduction, and will be reported elsewhere. pBluescript II SK(+) was from Stratagene (La Jolla, California, U.S.A.). The pGEX-6P-3 DNA was obtained from Amersham Biosciences (Piscataway, New Jersey, U.S.A.). The pGST-Orf17 plasmid, containing the gene for the GST-Orf17 fusion protein, was previously constructed.¹³⁾ Oligodeoxyribonucleotides were purchased from Sigma Genosys Japan (Ishikari, Japan) in purified forms.

Construction of the Plasmid Carrying the Antisense *orf17* Gene The suspension of *E. coli* KP7600 was boiled for 5 min and chilled. The supernatant after centrifugation was used as the template in a polymerase chain reaction (PCR) performed with *Taq* DNA polymerase (Toyobo, Osaka, Japan). The primers for *orf17* were 5'-dGGCAGT-CAATTAATAGGCAGCGTG (*orf17* *AseI*) and 5'-dCATAAAAATGTCGACAGATAGCCCTGC, in which the underlined sequences correspond to *AseI* and *SalI* sites, respectively. The amplified DNA was then treated with *AseI* and *SalI*.

The *lac* promoter was amplified by PCR with pBluescript II SK(+) as the template. The primers used were 5'-dTAAATCAGTCGACTAGCTGTTTCCTGTGTGAAATT and

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5'-dGGAATTCGCGCAACGCAATTAATGTGA, in which the underlined sequences correspond to *SalI* and *EcoRI* sites, respectively. The amplified DNA was then digested with *SalI* and *EcoRI*.

The amplified *orf17* gene and *lac* promoter were ligated at the *SalI* sites and then inserted into pBR322, which had been digested with *AseI* and *EcoRI*. These procedures produced the pBR-lacP-*orf17* plasmid containing the *lac* promoter, the antisense *orf17* gene, the β -lactamase (*bla*) gene terminator from pBR322, and the tetracycline resistance gene (Fig. 1A). The ligated DNA was transfected into *E. coli* strain DH5 α . The nucleotide sequence of the gene was confirmed by sequencing, using a BigDye Terminator Cycle Sequencing Kit and an ABI model 377 DNA sequencer (Applied Biosystems, Norwalk, Connecticut, U.S.A.). The plasmid containing the correct sequence was transfected into the KP7600 strain.

Detection of Antisense RNA by RT-PCR KP7600/pBR-lacP-*orf17* was inoculated into LB medium with tetracycline (50 μ g/ml) and isopropyl- β -D-thiogalactopyranoside (IPTG, 1 mM) and incubated at 37 °C until the turbidity at 570 nm reached 0.3. Total RNA was isolated with the TRIzol reagent (Invitrogen, Carlsbad, California, U.S.A.) and was precipitated with isopropanol. A reverse-transcription (RT) reaction was carried out with the Superscript II enzyme (Invitrogen) and a primer (*orf17* *AseI*, described above). The *atpB* mRNA was also reverse-transcribed with the *atpB* RT primer (5'-dCAGACGCCATCGACAGATAG). Each of the RNA transcripts was quantitated by a real-time quantitative PCR method, using an ABI 7500 Real Time PCR System and SYBR-Green chemistry. The primers were as follows: *orf17* upper: 5'-dCGGGTGCTGATGTTGCAGC, *orf17* lower: 5'-dAGCTTGCGGCGCGGTTTCA, *atpB* upper: 5'-dTCAGCTGGACCTGCGTACAT, *atpB* lower: 5'-dCAGCACCACCGAGAAGAACA.

Mutagenesis Assays A single colony, taken from an LB agar plate with an appropriate antibiotic, was inoculated into LB medium and incubated at 37 °C until the turbidity at 570 nm reached 0.2. *E. coli* cells harboring a plasmid encoding the antisense *orf17* gene or the control pBR322 plasmid were grown in LB medium with tetracycline (50 μ g/ml) and IPTG (1 mM). In the case of *E. coli* harboring the plasmid encoding GST (pGEX-6P-3), GST-Orf17, or GST-Orf135, ampicillin (50 μ g/ml) and IPTG (10 μ M) were added to the LB medium. For spontaneous mutations, the culture was centrifuged, and the pellet thus obtained was resuspended in a smaller volume of ice-cold LB medium. A portion of the suspension was diluted with ice-cold LB medium, transferred onto an LB agar plate (a titer plate), and incubated at 37 °C for 12 h. Another portion of the suspension was transferred onto an LB agar plate containing rifampicin (100 μ g/ml) (a selection plate), which was incubated at 37 °C for 20 h. The *rpoB* mutant frequency was calculated according to the numbers of colonies on the titer and selection plates. The statistical significance of the values was examined by the Student's *t*-test or a one-way ANOVA (analysis of variance) and SNK (Student–Newman–Keuls) test.

In the H₂O₂-treatment experiments, the *E. coli* culture was incubated at 37 °C until the turbidity at 570 nm reached 0.2, and then 1 ml of the culture was transferred into a 2-ml microtube. After H₂O₂ was added to a final concentration of 2 mM, the culture was incubated at 37 °C for 30 min. The *E.*

coli pellet obtained by centrifugation was resuspended in 1 ml of pre-warmed LB medium, cultured at 37 °C for 2 h, and then placed on ice. The *rpoB* mutant frequency was calculated as described above.

In the X-ray irradiation experiments, the *E. coli* culture was incubated at 37 °C until the turbidity at 570 nm reached 0.2, and then the *E. coli* pellet, obtained from 13 ml of the culture, was resuspended in 2 ml of Sorensen's phosphate buffer (KH₂PO₄ 3.5 g, Na₂HPO₄ 5.8 g per liter). The suspension was transferred to a 6-cm dish and exposed to 300 Gy of X-rays. Irradiation was performed with an X-ray generator (1.0 mm Al filter, 200 kVp, 20 mA, Shimadzu HF-350, Kyoto, Japan) at a dose rate of 16.6 Gy/min, which was determined by Fricke's chemical dosimeter. The *E. coli* pellet obtained by centrifugation was resuspended in 1 ml of pre-warmed LB medium, cultured at 37 °C for 3 h, and then placed on ice. The *rpoB* mutant frequency was calculated as described above.

RESULTS

Expression of the *orf17* Antisense RNA First, we examined the effects of suppressing *orf17* expression. However, the destruction of the *orf17* gene resulted in severe growth inhibition (Miki *et al.*, personal communication). This prompted us to use the antisense strategy to reduce the amount of the Orf17 protein. A plasmid containing the *orf17* gene in the antisense orientation was constructed (Fig. 1A) and introduced into the KP7600 *E. coli* strain. This gene is regulated by the *lac* promoter, and thus the antisense RNA can be inducibly expressed with IPTG. When the antisense

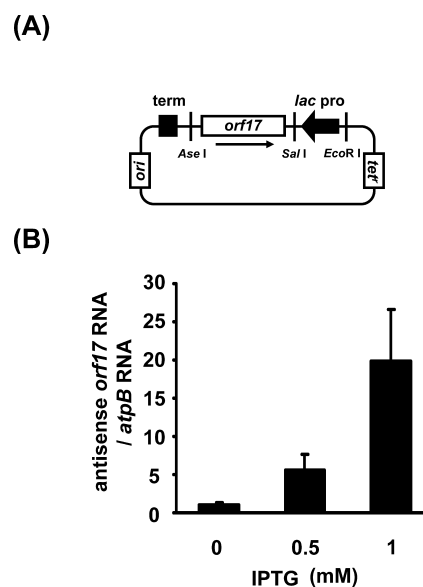


Fig. 1. Expression of the Antisense *orf17* RNA

(A) The constructed pBR-lacP-*orf17* vector, containing the antisense *orf17* gene. The *orf17* gene is located in the inverted orientation, downstream from the *lac* promoter. The restriction enzyme sites used for the construction are also indicated. *lac pro*, *lac* promoter; *term*, *bla* terminator; *tet^r*, *E. coli* tetracycline resistance gene; *ori*, *E. coli* replication origin. (B) Expression of *orf17* antisense RNA induced by IPTG, examined by real-time RT-PCR. The *E. coli* culture was incubated at 37 °C until the turbidity at 570 nm reached 0.3. Total RNA isolation and real-time RT-PCR were conducted as described in the Materials and Methods. The amount of antisense *orf17* RNA was normalized relative to that of the *atpB* RNA contained in each sample. Relative values to that of the sample without IPTG are shown. Experiments were repeated three times, and the data are expressed as the mean \pm S.D.

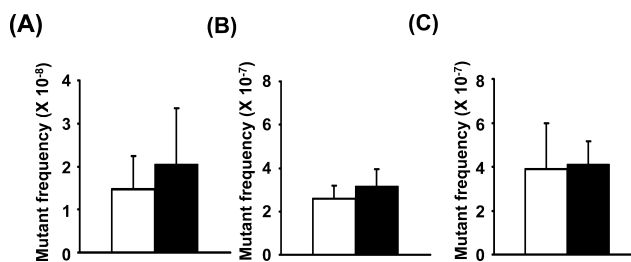


Fig. 2. (A) Spontaneous, (B) H₂O₂-, and (C) X-Ray-Induced Mutant Frequencies in the wt and *orf17*-Knock-Down *E. coli* Strains

A single colony taken from an LB agar plate was inoculated into LB medium and incubated at 37 °C until the turbidity at 570 nm reached 0.2. (*E. coli* cells were then plated onto titer and selection plates. The mutant frequency was calculated according to the numbers of colonies on the titer and selection plates, as described in the Materials and Methods. The *E. coli* culture was (B) treated with 2 mM H₂O₂, and (C) irradiated with 300 Gy of X-rays. The mutant frequency was calculated as described above. Open column, *E. coli* harboring the pBR322 control plasmid; closed column, *E. coli* harboring the plasmid carrying the *orf17* antisense gene. Experiments were repeated at least three times, and the data are expressed as the mean ± S.D.

RNA was quantitatively detected by PCR after cDNA synthesis, its amount was increased in an IPTG concentration-dependent manner (Fig. 1B). The addition of 1 mM IPTG increased the antisense RNA *ca.* 20-fold, as compared to the control (without IPTG). When 5–50 mM IPTG was added to the medium, the amount of the antisense RNA was similar to that obtained with 1 mM IPTG (data not shown). We estimated that the antisense RNA was *ca.* 1000-fold more abundant than the sense (endogenous) *orf17* mRNA (data not shown). Thus, amount of the Orf17 protein was expected to be lowered due to degradation of double-stranded RNA by RNase III and translation suppression. No antisense RNA was detected in the control KP7600/pBR322 strain.

Effects of *orf17* Antisense RNA Expression The *E. coli* Orf17 protein hydrolyzes oxidized purine deoxyribonucleoside 5'-triphosphates, 8-OH-dATP, 2-OH-dATP, and 8-OH-dGTP.¹³ Since ROS are endogenously formed in cells, these oxidized nucleotides may be present. Higher mutation frequencies are detected in strains lacking the MutT or Orf135 protein.^{7,8,12} Likewise, a reduction in the amount of the Orf17 protein might cause a mutator phenotype. The *orf17* antisense RNA was expressed in *E. coli* KP7600 by the addition of 1 mM IPTG, and its effects on the spontaneous mutation frequency were examined. The same strain with the pBR322 plasmid was used as a control. The *E. coli* culture was transferred onto rifampicin-containing agar plates to select cells with a mutation in the *rpoB* (RNA polymerase β -subunit) gene, and the *rpoB* mutant colonies on these plates were counted. As shown in Fig. 2A, the spontaneous mutant frequencies were 2.1×10^{-8} for the Orf17-knock-down strain and 1.5×10^{-8} for the control strain. Thus, the antisense RNA expression seems to increase the number of spontaneous mutations slightly. However, this difference was not statistically significant.

In addition, both strains of bacteria were treated with 2 mM H₂O₂, and the *rpoB* mutant frequencies were calculated. The treatment of the Orf17-knock-down strain and the control strain with H₂O₂ caused mutant colonies to appear at similar frequencies (Fig. 2B). The mutant frequencies were 3.2×10^{-7} for the knock-down strain and 2.6×10^{-7} for the control strain. Therefore, the expression of the antisense *orf17* RNA did not cause an increase in H₂O₂-induced mutations.

8-Hydroxyadenine is formed by ionizing radiation.^{15–17} Since deoxyribonucleotides with the 8-hydroxyadenine base are the best substrates,¹³ more obvious phenotypes might be observed upon subjecting the cells to ionizing radiation. Both

strains of bacteria were irradiated by 300 Gy of X-rays. However, no difference in the *rpoB* mutant frequency was observed (4.1×10^{-7} for the knock-down strain and 3.9×10^{-7} for the control strain) (Fig. 2C). Again, the expression of the antisense RNA for the *orf17* gene did not induce a clear mutator phenotype upon irradiation.

There was no difference in the number of viable cells between the wt and *orf17*-knock-down strains after the H₂O₂ and irradiation treatments (data not shown). Thus, the expression of the antisense *orf17* RNA has little, if any, influence on the sensitivity to these mutagens.

Recombinant Orf17 Protein Expression Suppresses Mutations in a *mutT* Strain We then examined whether the overexpression of the Orf17 protein reduces the mutant frequency. Plasmid DNAs bearing the gene for either the GST or GST-Orf17 fusion protein were transfected into the wt, *orf135*, and *mutT* strains. The expression of these genes was induced by a treatment with 10 μ M IPTG, and was confirmed by SDS-PAGE (data not shown). When >10 μ M IPTG was added into the medium, growth inhibition was observed (data not shown).

The expression of the recombinant Orf17 protein in the wt and *orf135* strains did not reduce the spontaneous mutant frequency, as compared with that of the GST protein (data not shown).

On the other hand, the expression of the recombinant Orf17 protein in the *mutT* strain reduced the mutant frequency, as compared with that of the GST protein (Fig. 3). The expression of the recombinant Orf17 protein decreased the mutant frequency two-fold (3.4×10^{-7} to 1.7×10^{-7}). Thus, the results of these experiments indicate that the Orf17 protein could eliminate the mutagenic oxidized deoxyribonucleotides, 8-OH-dGTP and its diphosphate derivative, which are substrates of the MutT protein. A similar reduction in the mutant frequency was observed when another MutT-type enzyme, the Orf135 protein, was expressed in the *mutT* strain (3.4×10^{-7} to 1.1×10^{-7} , Fig. 3). In this case, only the 8-OH-dGTP that accumulated due to the lack of MutT would be hydrolyzed by Orf135, since the diphosphates act as inhibitors of this protein.¹¹

DISCUSSION

Sanitization of the nucleotide pool is an important defense system against mutations induced by damaged DNA precursors. Previously, it was shown that the Orf17 protein hy-

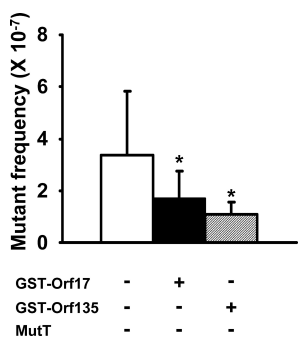


Fig. 3. Effects of Recombinant Orf17 Protein Overexpression on Mutant Frequencies in the *mutT*-Deficient Strain

Mutant frequencies of *rpoB* in the *mutT* *E. coli* strains harboring the plasmid for GST and GST-Orf17. The mutant frequency was calculated as described in the legend of Fig. 2A. Open column, *E. coli* harboring the plasmid for GST; closed column, *E. coli* harboring the plasmid for GST-Orf17; hatched column, *E. coli* harboring the plasmid for GST-Orf135. Experiments were repeated at least five times, and the data are expressed as the mean \pm S.D. * $p < 0.05$ (significant difference vs. the GST plasmid, ANOVA, SNK).

hydrolyzes 8-OH-dATP, 2-OH-dATP, and 8-OH-dGTP *in vitro*.¹³ In addition to these oxidized deoxyribonucleoside triphosphates, their diphosphate derivatives are also substrates of this protein. The objective of this study was to reveal the *in vivo* role(s) of the Orf17 protein. Bacteria deficient in the *mutT* and *orf135* genes show mutator phenotypes.^{7,8,12} A decrease in the amount of the Orf17 protein might also promote mutagenesis. To examine this possibility, we introduced a plasmid containing the antisense *orf17* gene. Although a slightly increased mutation frequency was observed for spontaneous mutations, no clear phenotype was observed for H₂O₂- and X-ray-induced mutations (Fig. 2). These results could be explained by the weak mutagenicity of 8-OH-dATP in *E. coli*,¹⁸ and by the fact that 2-OH-dATP and 8-OH-dGTP are degraded mainly by Orf135 and MutT, respectively.^{8,12,19,20} Taken together, the expression of the antisense *orf17* RNA did not lead to an evident mutator phenotype.

We also introduced the *orf17* gene into the wt, *orf135*, and *mutT* strains to examine whether the overexpressed Orf17 protein could degrade endogenous mutagenic deoxyribonucleotides. The overexpression of the Orf17 protein did not reduce the *rpoB* mutant frequencies in the wt and *orf135* strains (data not shown). On the other hand, the overexpressed Orf17 decreased the mutant frequency in the *mutT* strain (Fig. 3). These results suggest that the Orf17 protein could act as a backup protein for the MutT protein. Interestingly, Orf17 and MutT hydrolyze 8-OH-dGTP and its diphosphate derivative.^{13,14} The suppression of the mutant frequency by Orf17 would be due to its dual functions.

Likewise, the overexpression of the Orf135 protein suppressed mutations in the *mutT* strain (Fig. 3). Orf135 also degrades 8-OH-dGTP, and this activity could explain this suppression.¹¹ It has been reported that *E. coli* GTP cyclohydrolyase II may be a backup enzyme for MutT.²¹ Thus, three other enzymes, besides MutT, could eliminate the highly mutagenic 8-OH-dGTP^{22,23} from the nucleotide pool.

The overexpression of the Orf17 protein in the *orf135* strain did not reduce the *rpoB* mutant frequency (data not shown). Although both Orf17 and Orf135 have abilities to hydrolyze 8-OH-dGTP and 2-OH-dATP *in vitro*,^{11,13} Orf17

did not complement the *orf135*-deficiency. It might be explained by large contribution of the MutT protein to the 8-OH-dGTP-degradation and by the mild mutator phenotype of the *orf135* strain.¹²

The Orf17 protein belongs to the Nudix hydrolase superfamily.²⁴ Among the Nudix superfamily members, Orf17 is the most abundant of any *E. coli* Nudix hydrolase at the mRNA level, and its mRNA level is *ca.* 15-fold of that of *mutT*.²⁵ In a recent genome-wide study, Orf17 was found to be the only *E. coli* Nudix enzyme essential for aerobic growth in rich media (or at least to impart a substantial fitness advantage), suggesting that Orf17 has more than just an antimutator function and may be involved in regulating nucleotide pools for growth.²⁶ The Orf17 protein has dATPase and dADPase activities,^{13,27} and these functions might be related to its potential roles in nucleotide metabolism. However, the direct introduction of dATP into the *orf17*-knock-down strain did not affect its survival (data not shown). Thus, further studies will be required to elucidate the roles of Orf17 in nucleotide metabolism.

In this study, we found that overexpression of the Orf17 protein in the *mutT* strain decreased the mutation frequency. However, the expression of the *orf17* antisense RNA did not induce a clear mutator phenotype. The role of the Orf17 protein as an 8-OH-dGTPase is exerted when 8-OH-dGTP accumulates in the cells, as in the *mutT* strain.

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