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An alkyltransferase-like protein from *Thermus thermophilus* HB8 affects the regulation of gene expression in alkylation response

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Alkylation is a type of stress that is fatal to cells. However, cells have various responses to alkylation. Alkyltransferase-like (ATL) protein is a novel protein involved in the repair of alkylated DNA; however, its repair mechanism at the molecular level is unclear. DNA microarray analysis revealed that the upregulation of 71 genes because of treatment with an alkylating agent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine was related to the presence of TTHA1564, the ATL protein from *Thermus thermophilus* HB8. Affinity chromatography showed a direct interaction of purified TTHA1564 with purified RNA polymerase holoenzyme. The amino acid sequence of TTHA1564 is homologous to that of the C-terminal domain of Ada protein, which acts as a transcriptional activator. These results suggest that TTHA1564 might act as a transcriptional regulator. The results of DNA microarray analysis also implied that the alkylating agent induced oxidation stress in addition to alkylation stress.

Keywords: ATL protein/DNA microarray/nucleotide excision repair/O^6-methylguanine/transcriptional regulation.

Abbreviations: ATL protein, alkyltransferase-like protein; MBP, maltose-binding protein; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; NER, nucleotide excision repair; O^6-meG, O^6-methylguanine; Ogt, O^6-methylguanine-DNA methyltransferase; ORF, open reading frame; PCR, polymerase chain reaction; WT, wild type.

Cells are usually exposed to various stresses and possess a lot of mechanisms to adapt to these stressors. Alkylation is a type of stress that is fatal to cells. Under alkylation stress, many cellular components, including DNA and proteins, are alkylated. Presence of alkylated bases in DNA is harmful to cells because they can induce mutations and apoptosis. To protect the integrity of its genome, the ability of a cell to repair its DNA is essential (*1*).

O^6-methylguanine (O^6-meG) is one of the most harmful alkylated lesions. O^6-meG is repaired by O^6-methylguanine-DNA methyltransferase (Ogt), which accepts an alkyl group on a cysteine residue at the active site (PCHR) in a stoichiometric fashion (*2–4*). Although a wide range of organisms possess Ogt homologues, several species do not. Instead, these species possess Ogt-like proteins that lack a cysteine residue in their putative active site. These Ogt-like homologues are called alkyltransferase-like (ATL) proteins which are found in all three domains of life (*5*). It is known that ATL proteins recognize and bind to O^6-meG-including DNA, but they have no alkyltransferase activity (*6–8*).

ATL protein has been studied in several organisms ever since they were first reported in 2005 (*6–13*). The first report was that *Escherichia coli* ATL protein inhibited the activity of Ogt (*7*) and then the possibility was raised that ATL protein is involved in the repair of alkylation damage. Importantly, the relationship between ATL proteins and nucleotide excision repair (NER) proteins was reported in detail (*6*, *10–13*). We found that ATL protein homolog of *Thermus thermophilus* HB8, TTHA1564, directly interacts with a bacterial NER protein, UvrA (*6*). *Escherichia coli* ATL protein enhanced the repair by NER of the O^6-alkylguanine adducts *in vivo* (*10*). Recently, TTHA1564 was also suggested to function in the repair of alkylated lesions via an NER system (*12*). Together with other studies, it is becoming clear that ATL proteins act via the same pathway as NER proteins do *in vivo* (*11*, *13*). However, there is no experimental evidence to show that ATL directly recruits NER proteins to O^6-meG lesion. It is still unclear how ATL protein acts in repairing the lesion at the molecular level.

Fig. 1 Sequence alignment of TTHA1564 and C-Ada. The amino acids sequence of TTHA1564 displayed 28% identity and 40% similarity to C-Ada. The identical and similar residues are represented by white letters on black background.

We then considered the alternative possibility that ATL proteins are also involved in the indirect pathway of O^6-meG repair. A possible indirect pathway involves ATL proteins through transcriptional regulation. Ada protein, one of two O^6-meG-DNA methyltransferases of *E. coli*, also acts as transcription activator when its cysteine residue is methylated (*14*). The amino acid sequence of the C-terminal domain of Ada (C-Ada) is similar to those of ATL proteins (Fig. 1). Therefore, we used DNA microarray analysis to obtain a potential clue regarding the mechanism underlying ATL involvement in the repair process. Since *T. thermophilus* HB8 has only about 2,200 genes, its small genome is useful in the interpretation of DNA microarray data. Recently, there have been several studies using DNA microarrays with *T. thermophilus* HB8, and the microarray data were verified by quantitative polymerase chain reaction (PCR) (*15–17*). *Thermus thermophilus* HB8 is a Gram-negative bacterium that grows at temperatures above 75°C (*18*). It is the most thermophilic bacterium for which a gene manipulation system has been established (*19–21*). Proteins from this bacterium are stable against thermal denaturation and are suitable for physicochemical studies.

In this study, we used DNA microarray analysis to identify genes whose transcription levels changed in response to alkylation stress induced by *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG). Comparison between wild-type (WT) and *ttha1564* gene disruptant strains showed that the upregulation of several genes under alkylating stress condition is possibly related to TTHA1564. We further show that purified TTHA1564 interacts with purified RNA polymerase holoenzyme. Our results suggest that TTHA1564 affects the transcription of many genes upon alkylation treatment.

Materials and Methods

Materials

All strains were derivatives of *T. thermophilus* HB8 (ATCC27634). The *ttha1564* gene deletion mutant (*Δttha1564*) was reported previously (*6*). Yeast extract and polypeptone were from Nihon Pharmaceutical Co., Ltd (Tokyo, Japan) and Difco (Detroit, MI), respectively. The DNA oligomers were synthesized by BEX Co. (Tokyo, Japan). All other reagents used in this study were of the highest available commercial grade.

DNA microarray analysis

Thermus thermophilus HB8 WT and *Δttha1564* strains were cultured in TT medium [0.4%(w/v) polypeptone, 0.2%(w/v) yeast extract, 0.1%(w/v) NaCl, 0.4 mM $CaCl_2$ and 0.4 mM $MgCl_2$, (pH 7.5)] at 70°C until the OD_{600} reached 0.8. The cultures were then divided into two flasks and an equal volume of TT medium prewarmed at 70°C was added; 100 μg/ml MNNG was added to one of the media. We confirmed that the cells could continue to grow in TT medium containing 100 μg/ml MNNG, even though DNA is sufficiently alkylated (*12*). After further cultivation at 70°C for 10 min, the diluted cultures were collected into an equal of 100% ethanol and stored at −80°C. The procedures used for crude RNA extraction, cDNA synthesis, cDNA fragment labelling, hybridization to a TTHB8401a520105F GeneChip (Affymetrix) and data analysis were the same as those described previously (*17*, *22*). The *t*-test *P*-values of the observed differences in the normalized intensities between the WT and *Δttha1564* strains were calculated using the Subio Platform (Subio), and then from these values, their false discovery rates (*q* value) were calculated. To determine the mRNA expression level of each sample, image data for the three independent samples were processed. The DNA microarray data discussed in this study were deposited in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession no. GSE22962.

Quantitative real-time polymerase chain reaction (PCR)

Total RNAs extracted from the *T. thermophilus* HB8 cells were reverse transcribed with gene-specific primers (Table I) by using the same methods as the DNA microarray analysis. The cDNAs obtained were used as template for quantitative real-time PCR (qRT–PCR). The solutions for qRT–PCR contained SYBR Green PCR Master Mix (Applied Biosystems) and 400 nM of each primer. After incubation at 50°C for 2 min, the solutions were heated at 90°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All qRT–PCRs were carried out using an Applied Biosystems 7300 Real-Time PCR system. The abundance of mRNA was determined by comparing the values obtained from qRT–PCR to those in the standard curve. The TTHA1197 gene (*argC*) was used as a control because it is not affected by alkylation or deletion of *ttha1564*. Relative mRNA abundance was normalized with respect to that of each gene in the WT strain without alkylation and set as 1.

Protein purification

TTHA1564 fused with maltose-binding protein (MBP-TTHA1564) was purified as described previously (*6*). In this study, we used amylose-resin and TOYOPEARL-SuperQ columns for purification of MBP-TTHA1564 and MBP. RNA polymerase holoenzyme was purified as described previously (*23*).

Interaction assay

Approximately 4.38 nmol of MBP-TTHA1564 or MBP in interaction buffer [20 mM Tris–HCl (pH 8.0) and 50 mM NaCl] was applied to the amylose resin column (bed volume, 0.6 ml) that had been equilibrated with interaction buffer. Then, 17.7 μg of RNA polymerase holoenzyme was applied to the MBP-TTHA1564- or MBP-immobilized columns. After thoroughly washing the column with interaction buffer, bound proteins were eluted with interaction buffer containing 10 mM maltose.

Results

ttha1564 affects gene expression regulation

First, we performed DNA microarray analysis to identify proteins that are rapidly induced by alkylation in WT *T. thermophilus* HB8. Cells were treated with 100 μg/ml MNNG, a well-known O^6-alkylating agent, for 10 min. The overall mRNA expression of MNNG-treated *T. thermophilus* HB8 was analyzed at the open reading frame (ORF) level and compared to that of untreated cells. Comparison of the data resulted in the identification of 89 and 96 genes that

Table I. The sequences of primers used in this study.

Target	Sequence (5′–3′)
UvrA_forward	ACG GGA CGG TGA AGA TTG AG
UvrA_reverse	TCC ACC GTC ATG TCC AGA AC
UvrB_ forward	CCG GGT TTG TCC TCT TTC C
UvrB_reverse	TCT CCC AGA GCT CCT TCT CAA T
UvrC_forward	CCA CCG CCC CCT CTA CA
UvrC_reverse	GGC TCG TTG GTG AGC TTG A
TTHA0706_forward	AAC CCT GGT TAC GCT GGC TA
TTHA0706_ reverse	GCC ACG CTA TAA GCC TCC AG
TTHA1422_forward	CGA CCT TCG TCT TCC TCG TC
TTHA1422_reverse	ATC CAC ACG CAC CTC CTT TG
TTHA1718_forward	GTG ACC AAG GCC CTG AAG AA
TTHA1718_reverse	CAG AAC CTC GGC CTT GTA CC
TTHB174_forward	CTA CGC CTC CAT AGG GGA GA
TTHB174_reverse	TCG TCC AGA ACC TCC ACC TTA
TTHB186_forward	TCG AGG CCC TTC TTG AAC TC
TTHB186_reverse	CTG GTG GTA GAG GAG CCT TC
Cas3_forward	CTG GGA GGA GGG AAA GGA AC
Cas3_reverse	GCA GGA AAA GCT CCG TGA AG
Cas1_forward	TGG CGG AAG ACC TTC TGA AC
Cas1_reverse	CTC CAG GAT CAT CAC CAC CA
TTHB208_forward	CCT CGC CCT TTT CCT CTT CTC
TTHB208_reverse	GGA CGT TCT CCT CCT TGC TG

were up- and downregulated, respectively (Tables II and III). Among the upregulated genes (Table II), three genes encode proteins that are involved in DNA replication and repair. UvrB (TTHA1892) is an NER protein that acts in damage recognition (*24*). RadA (TTHA0541) is involved in homologous recombination (*25*). DNA gyrase subunit B (TTHA1586) is a topoisomerase II protein that introduces negative supercoils in the DNA (*26*). Among the genes involved in nucleotide metabolism, two subunits of ribonucleoside-diphosphate (NDP) reductase (TTHB208 and TTHB209) were also upregulated by more than 5-fold. Most of the other upregulated genes were classified into the categories other than DNA replication and repair. This point will be discussed later.

Next, to examine the effect of TTHA1564 on alkylation response, we performed DNA microarray analysis for the *Δttha1564* strain with and without MNNG treatment. Comparison of the microarray data resulted in the identification of thirty-five and nine genes that were up- and downregulated, respectively (Tables IV and V). The number of altered genes in *Δttha1564* was less than that of WT, suggesting that the transcriptional regulation of several genes is associated with TTHA1564. The numbers of commonly up- and downregulated genes shared between the WT and *Δttha1564* strains were twelve and two, respectively. These genes might be considered to be regulated independently of TTHA1564.

To further examine whether the presence of TTHA1564 affects the upregulation of genes under alkylating stress condition, we searched for upregulated genes whose fold-change values in the WT were significantly greater than those in *Δttha1564* strain. In this experiment, 71 genes met this criterion (Table VI). More than 79% of genes upregulated in WT strain could be related to TTHA1564.

Gene expression quantification by qRT–PCR

To validate the microarray data, we carried out qRT–PCR analysis for nine genes (Table VII). We selected seven genes as representatives of distinct categories from upregulated genes listed in Table II; these are *ttha0706*, *ttha1718*, *ttha1892*, *tthb174*, *tthb186*, *tthb187* and *tthb208*. If multiple upregulated genes were supposed to form an operon, the most-upstream gene was selected from them. Since ATL was suggested to be associated with NER pathway, two genes [*ttha1440* (*uvrA*) and *ttha1548* (*uvrC*)] involved in NER were also selected in addition to *ttha1892* (*uvrB*). The results of qRT–PCR are corroborated by those of the DNA microarray, although there were some differences in degree of fold change. Therefore, these nine genes were clearly upregulated by alkylating stress.

To further confirm the effect of TTHA1564 on gene expression regulation, we analyzed the above seven genes in *Δttha1564* by qRT–PCR. Among them, *ttha0706*, *ttha1892*, *tthb174* and *tthb208* did not show statistically significant difference between the WT and *Δttha1564* strains. However, *ttha1718, tthb186* and *tthb187* were significantly more upregulated in the WT than in the *Δttha1564* strain under alkylating condition (Fig. 2). These results support the notion that the expression of these genes is related to the presence of TTHA1564 under alkylating conditions.

TTHA1564 interacts with RNA polymerase

We presumed that TTHA1564 interacts with RNA polymerase to regulate gene expression. Based on the pull-down assay against *T. thermophilus* HB8 cell lysates, we previously suggested that TTHA1564 can interact with RNA polymerase (*6*). To confirm this interaction, we immobilized purified MBP-TTHA1564 in an amylose-resin column and subsequently applied purified RNA polymerase holoenzyme to the column. After resin was sufficiently washed, we eluted proteins with maltose-containing buffer. SDS–PAGE analysis revealed that RNA polymerase subunits RpoB and RpoC were eluted together with MBP-TTHA1564 along with maltose (Fig. 3A), but not with MBP (Fig. 3B). These results show that RNA polymerase binds to TTHA1564.

Discussion

Comparison of the DNA microarray data in the WT and *Δttha1564* strains resulted in the identification of 71 genes upregulated under alkylating condition were related to the presence of TTHA1564. These results lead to the hypothesis that TTHA1564 affects the gene expression regulation. The results of qRT–PCR and DNA microarray corroborate each other, supporting our hypothesis. It is unclear how TTHA1546 affected the gene expression regulation and also unknown whether this effect was direct or indirect. The direct interaction between TTHA1564 and RNA polymerase raises a possibility that TTHA1564 acts as a transcriptional regulatory protein directly. However, the alternative possibility cannot be ruled out at present that DNA damage remained unrepaired in the

Table II. Subset of genes upregulated in *T. thermophilus* HB8 WT strain with MNNG treatment.

Locus Tag	Fold	Annotation	*q*-values
TTHA0072	2.02	hypothetical protein	0.0073
TTHA0152	2.72	tungsten-containing aldehyde:ferredoxin oxidoreductase	0.0031
TTHA0164	3.12	thiol:disulfide interchange protein	0.0031
TTHA0166	2.00	hypothetical protein	0.0154
TTHA0212	2.79	hypothetical protein	0.0031
TTHA0359	3.94	cold shock protein 2	0.0058
TTHA0425	3.06	NADH oxidase (H_2O_2-forming)	0.0031
TTHA0479	2.25	trehalose-6-phosphate phosphatase	0.0336
TTHA0495	2.11	2-phosphoglycerate kinase	0.0034
TTHA0541	2.04	DNA repair protein RadA	0.0133
TTHA0570	2.05	putative oxidoreductase (glucose dehydrogenase)	0.0031
TTHA0607	6.45	biotin synthase (biotin synthetase)	0.0031
TTHA0655	2.11	hypothetical protein	0.0031
TTHA0665	2.45	N5,N10-methylenetetrahydromethanopterin reductase	0.0066
TTHA0677	2.71	putative oxidoreductase	0.0187
TTHA0688	2.44	sugar ABC transporter, substrate-binding protein	0.0031
TTHA0689	2.45	putative xylose repressor [C-terminal]	0.0347
TTHA0706	6.92	cation-transporting ATPase	0.0031
TTHA0707	2.21	glutamine amidotransferase (pyridoxine biosynthesis)	0.0031
TTHA0729	2.43	probable efflux transporter, AcrB/AcrD/AcrF family	0.0067
TTHA0731	2.73	hypothetical protein	0.0033
TTHA0742	17.09	hypothetical protein	0.0031
TTHA0743	35.87	glycosyltransferase related protein	0.0031
TTHA0744	10.84	hypothetical protein	0.0031
TTHA0757	2.92	hypothetical protein	0.0031
TTHA0770	2.00	ATP-dependent protease La (Lon protease)	0.0031
TTHA0771	3.02	hypothetical protein	0.0031
TTHA0798	2.83	ggdef domain protein	0.0031
TTHA0807	2.33	transcriptional regulator	0.0031
TTHA0837	2.26	metallo-beta-lactamase family protein	0.0032
TTHA0841	3.68	stage V sporulation protein R (SpoVR) related protein	0.0105
TTHA0842	3.71	hypothetical protein	0.0128
TTHA0843	5.24	serine protein kinase	0.0047
TTHA0945	2.43	hypothetical protein	0.0031
TTHA0986	3.16	hypothetical protein	0.0031
TTHA1044	2.07	diacylglycerol kinase	0.0073
TTHA1045	2.49	hypothetical protein	0.0034
TTHA1128	2.03	peptidase	0.0031
TTHA1133	2.56	ba_3-type cytochrome c oxidase polypeptide IIA	0.0034
TTHA1141	2.06	cation-transporting ATPase	0.0031
TTHA1147	2.07	hypothetical protein	0.0031
TTHA1216	2.58	prepilin-like protein	0.0063
TTHA1410	2.14	sulfite dehydrogenase cytochrome subunit SoxD	0.0315
TTHA1411	2.05	sulphite dehydrogenase SoxC precursor	0.0201
TTHA1412	3.66	sulphide dehydrogenase flavocytochrome C	0.0084
TTHA1413	2.06	hypothetical protein	0.0131
TTHA1414	3.45	putative sulfurtransferase	0.0202
TTHA1415	3.87	sulphur oxidation protein SoxA	0.0044
TTHA1416	3.65	cytochrome c (SoxX)	0.0101
TTHA1417	2.68	sulphur oxidation protein SoxB	0.0034
TTHA1418	3.82	putative cytochrome c	0.0044
TTHA1419	3.16	putative cytochrome c precursor	0.0034
TTHA1420	4.67	sulphur oxidation protein SoxZ	0.0056
TTHA1421	6.60	sulphur oxidation protein SoxY	0.0043
TTHA1422	5.75	disulphide isomerase (DsbC)	0.0034
TTHA1492	2.21	cell division protein FtsH	0.0031
TTHA1496	2.04	arginase	0.0031
TTHA1498	2.28	elongation factor G (EF-G-2)	0.0057
TTHA1499	2.05	MoxR-related protein	0.0031
TTHA1500	2.00	phosphoenolpyruvate synthase	0.0031
TTHA1506	2.20	glutamyl-tRNA reductase	0.0031
TTHA1527	2.10	NADPH-quinone reductase	0.0031
TTHA1586	2.07	DNA gyrase, subunit B	0.0031
TTHA1625	2.01	osmotically inducible protein OsmC	0.0031
TTHA1626	2.09	hypothetical protein	0.0130
TTHA1635	2.19	iron–sulphur cluster biosynthesis protein IscA	0.0031
TTHA1718	4.34	heavy metal binding protein	0.0045
TTHA1719	3.13	copper homeostasis operon regulatory protein	0.0058
TTHA1720	3.58	cation-transporting ATPase	0.0057
TTHA1776	2.12	hypothetical protein	0.0044
TTHA1803	2.14	pterin-4-alpha-carbinolamine dehydratase	0.0031
TTHA1871	2.13	hypothetical protein	0.0031
TTHA1892	2.47	excinuclease ABC subunit B (UvrB)	0.0031

Table II. Continued

Locus Tag	Fold	Annotation	*q*-values
TTHA1941	3.02	ZIP zinc transporter family protein	0.0031
TTHB089	2.17	hypothetical protein	0.0031
TTHB174	4.21	sensor histidine kinase-like protein	0.0039
TTHB175	2.59	ABC transporter, ATP-binding protein	0.0032
TTHB176	2.83	putative iron ABC transporter, permease protein	0.0034
TTHB177	2.69	iron ABC transporter, periplasmic iron-binding protein	0.0049
TTHB186	2.05	putative transcriptional regulator	0.0031
TTHB187	2.49	CRISPR-associated protein, Cas3	0.0031
TTHB207	6.02	hypothetical protein	0.0031
TTHB208	5.36	ribonucleoside-diphosphate reductase, beta subunit	0.0035
TTHB209	5.50	ribonucleoside-diphosphate reductase, alpha subunit	0.0031
TTHB243	2.31	hypothetical protein	0.0031
TTHC001	2.24	putative RepA protein	0.0323
TTHC010	2.32	hypothetical protein	0.0069
TTHC013	2.40	toxin-like protein	0.0057
TTHC014	2.12	hypothetical protein	0.0060

ORFs with a q value ≤ 0.05 and a fold change of ≥ 2-fold relative to without MNNG are listed.

Table III. Subset of genes downregulated in *T. thermophilus* HB8 WT strain with MNNG treatment.

Locus Tag	Fold	Annotation	*q*-values
TTHA0003	0.49	pyruvate kinase	0.0031
TTHA0056	0.34	ammonium transporter	0.0031
TTHA0057	0.40	nitrogen regulatory protein P-II	0.0031
TTHA0080	0.33	hypothetical protein	0.0041
TTHA0091	0.36	NADH-quinone oxidoreductase chain 8	0.0031
TTHA0092	0.47	NADH-quinone oxidoreductase chain 9	0.0031
TTHA0093	0.43	NADH-quinone oxidoreductase chain 10	0.0031
TTHA0094	0.33	NADH-quinone oxidoreductase chain 11	0.0031
TTHA0095	0.30	NADH-quinone oxidoreductase chain 12	0.0031
TTHA0096	0.30	NADH-quinone oxidoreductase chain 13	0.0031
TTHA0097	0.34	NADH-quinone oxidoreductase chain 14	0.0031
TTHA0098	0.48	arginyl-tRNA synthetase	0.0031
TTHA0144	0.43	sensor histidine kinase	0.0066
TTHA0145	0.43	phosphate regulon transcriptional regulatory protein PhoB	0.0031
TTHA0154	0.37	hypothetical protein	0.0031
TTHA0155	0.43	hypothetical protein	0.0051
TTHA0156	0.35	hypothetical protein	0.0040
TTHA0197	0.47	S-layer-like protein	0.0052
TTHA0431	0.41	sugar kinase	0.0032
TTHA0439	0.48	ABC transporter, ATP-binding protein	0.0036
TTHA0440	0.44	hypothetical protein	0.0031
TTHA0453	0.10	Mn^{2+}/Zn^{2+} ABC transporter, ATP-binding protein	0.0047
TTHA0454	0.27	Mn^{2+}/Zn^{2+} ABC transporter, permease protein	0.0064
TTHA0471	0.46	peptide ABC transporter, permease protein	0.0088
TTHA0515	0.48	hypothetical protein	0.0055
TTHA0560	0.48	hypothetical protein	0.0034
TTHA0561	0.41	outer membrane protein	0.0031
TTHA0630	0.45	heat shock protein HslU	0.0035
TTHA0631	0.41	heat shock protein HslV	0.0031
TTHA0684	0.48	probable TolQ-type transport protein	0.0057
TTHA0724	0.40	serine protease	0.0051
TTHA0796	0.39	hypothetical protein	0.0058
TTHA0828	0.42	hypothetical protein	0.0052
TTHA0859	0.42	uridylate kinase	0.0031
TTHA0957	0.23	dihydrodipicolinate synthase	0.0086
TTHA0958	0.24	2-hydroxyhepta-2,4-diene-1,7-dioate isomerase/5-carboxymethyl-2-oxo-hex-3-ene-1,7-dioate decarboxylase	0.0077
TTHA0959	0.22	5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase	0.0063
TTHA0960	0.24	4-hydroxyphenylacetate-3-hydroxylase	0.0055
TTHA0961	0.28	4-hydroxyphenylacetate 3-monooxygenase	0.0051
TTHA0962	0.17	homoprotocatechuate 2,3-dioxygenase	0.0042
TTHA0963	0.36	ABC tranpsorter ATP binding protein related protein	0.0125
TTHA0964	0.26	ABC tranpsorter ATP binding protein related protein	0.0035
TTHA0965	0.28	phenylacetic acid degradation protein PaaI	0.0075
TTHA0966	0.35	phenylacetyl-CoA ligase	0.0065
TTHA0967	0.36	hypothetical protein	0.0188
TTHA0968	0.36	phenylacetic acid degradation protein PaaZ	0.0143

(continued)

Table III. Continued

Locus Tag	Fold	Annotation	*q*-values
TTHA0969	0.30	phenylacetic acid degradation protein PaaD	0.0254
TTHA0970	0.35	phenylacetic acid degradation protein PaaC	0.0187
TTHA0971	0.36	phenylacetic acid degradation protein PaaB	0.0201
TTHA0972	0.40	phenylacetic acid degradation protein PaaA	0.0202
TTHA0973	0.31	transcriptional regulator, TetR family	0.0176
TTHA1004	0.37	conserved hypothetical membrane protein	0.0031
TTHA1005	0.37	hypothetical protein	0.0031
TTHA1006	0.43	hypothetical protein	0.0031
TTHA1007	0.40	ABC transporter, ATP-binding protein	0.0066
TTHA1008	0.34	ABC transporter, permease protein	0.0031
TTHA1012	0.41	hypothetical protein	0.0065
TTHA1080	0.49	hypothetical protein	0.0051
TTHA1085	0.46	UDP-N-acetylmuramate–alanine ligase (MurC)	0.0047
TTHA1101	0.44	hypothetical protein	0.0031
TTHA1102	0.45	hypothetical protein	0.0038
TTHA1170	0.50	amino acid ABC transporter, permease protein	0.0049
TTHA1172	0.43	alpha-glucan phosphorylase	0.0031
TTHA1332	0.49	branched-chain amino acid ABC transporter, permease protein	0.0031
TTHA1334	0.44	branched-chain amino acid ABC transporter, ATP-binding protein	0.0045
TTHA1337	0.43	peptide ABC transporter, permease protein	0.0045
TTHA1339	0.42	hypothetical protein	0.0058
TTHA1436	0.37	ABC-type transporter, ATP-binding protein	0.0031
TTHA1473	0.47	hypothetical protein	0.0061
TTHA1511	0.49	putative catechol 1,2-dioxygenase	0.0038
TTHA1578	0.42	1-Pyrroline-5-carboxylate dehydrogenase	0.0074
TTHA1579	0.28	putative proline dehydrogenase (monofunctional)	0.0043
TTHA1580	0.31	transcriptional regulator, GntR family	0.0035
TTHA1581	0.48	hypothetical protein	0.0041
TTHA1610	0.50	hypothetical protein	0.0102
TTHA1623	0.49	metallo-beta-lactamase superfamily protein	0.0310
TTHA1629	0.19	iron ABC transporter, permease protein	0.0310
TTHA1766	0.49	S-layer-like protein	0.0086
TTHA1837	0.45	hypothetical protein	0.0031
TTHA1840	0.42	SufD protein	0.0118
TTHA1841	0.46	putative dioxygenase ferredoxin subunit	0.0268
TTHA1877	0.40	sugar ABC transporter, periplasmic sugar-binding protein	0.0040
TTHA1942	0.29	putative cytochrome c oxidase assembly protein	0.0031
TTHA1943	0.38	hypothetical protein	0.0033
TTHA1963	0.49	tetrapyrrole methylase family protein	0.0038
TTHB014	0.14	probable phosphotransferase	0.0059
TTHB019	0.35	MaoC-related acyl dehydratase	0.0180
TTHB021	0.44	hypothetical protein	0.0037
TTHB022	0.50	putative acyl-CoA dehydrogenase	0.0158
TTHB023	0.47	transcriptional regulator, TetR family	0.0040
TTHB070	0.49	survival protein SurE	0.0115
TTHB168	0.46	hypothetical protein	0.0031
TTHB218	0.32	ABC transporter, ATP-binding protein	0.0487
TTHB219	0.31	hemin ABC transporter, permease protein	0.0459
TTHB221	0.23	hypothetical protein	0.0340
TTHB222	0.25	hypothetical protein	0.0369

ORFs with a q value $\leq$0.05 and a fold change of $\leq$0.5-fold relative to without MNNG are listed.

Δttha1564 strain indirectly affected the regulation of gene expression.

The direct interaction of ATL proteins and RNA polymerase was demonstrated for the first time in this study. This interaction seems similar to that of Ada protein in *E. coli*. The methylated N-terminal domain of Ada (N-Ada) specifically binds to the promoter regions of the *ada-alkB* operon, the *alkA* and *aidB* genes (*14*, *27*), whereas the C-terminal domain of Ada (C-Ada) binds to RNA polymerase; methylated Ada acts as transcriptional activator in this scheme (*28*, *29*). The amino acid sequence of C-Ada is similar to those of ATL proteins (*7*, *9*, *13*, *30*). The amino acids sequence of TTHA1564 displays 28% identity and 40% similarity to that of C-Ada. C-Ada has the methyl group-accepting cysteine residue (Cys321), whereas the corresponding residue in TTHA1564 is alanine (Fig. 1). However, tertiary structures of ATL and C-Ada are homologous (*13*, *31*). Therefore, we hypothesized that TTHA1564 acts as a direct transcriptional regulator similar to Ada protein. It should be noted here, however, that Ada has the N-terminal domain (N-Ada), which binds specifically to the promoter regions of the genes involved in the response to alkylation damage (*14*). At present, it is unknown whether TTHA1564 binds to specific region of DNA. Since TTHA1564 can specifically bind to DNA containing O^6-meG (*6*), TTHA1564 might bind to a

Table IV. Subset of genes upregulated in *T. thermophilus* HB8 *Δtth1564* strain with MNNG treatment.

Locus Tag	Fold	Annotation	*q*-values
TTHA0028	2.02	putative macrolide-efflux protein	0.0202
TTHA0105	2.18	hypothetical protein	0.0165
TTHA0109	2.08	ATP-dependent RNA helicase	0.0314
TTHA0359	5.59	cold shock protein 2	0.0262
TTHA0531	2.09	hypothetical protein	0.0150
TTHA0589	2.43	branched-chain amino acid ABC transporter permease protein	0.0232
TTHA0688	2.03	sugar ABC transporter, substrate-binding protein	0.0114
TTHA0799	2.77	prephenate dehydrogenase	0.0114
TTHA0800	3.11	phospho-2-dehydro-3-deoxyheptonate aldolase	0.0114
TTHA0843	2.00	serine protein kinase	0.0349
TTHA0948	2.48	fatty acid desaturase	0.0292
TTHA0979	2.51	hypothetical protein	0.0114
TTHA1211	2.32	probable ketol-acid reductoisomerase (IlvC)	0.0114
TTHA1212	2.91	acetolactate synthase, small subunit (IlvN)	0.0114
TTHA1213	2.88	acetolactate synthase, large subunit	0.0114
TTHA1359	2.38	transcriptional regulator, FNR/CRP family	0.0172
TTHA1420	2.25	sulphur oxidation protein SoxZ	0.0114
TTHA1421	2.43	sulphur oxidation protein SoxY	0.0121
TTHA1540	3.16	hypothetical protein	0.0440
TTHA1541	2.54	hypothetical protein	0.0199
TTHA1718	2.02	heavy metal binding protein	0.0118
TTHA1724	2.02	hypothetical protein	0.0114
TTHA1725	2.05	hypothetical protein	0.0262
TTHA1743	2.63	orotidine 5'-phosphate decarboxylase (PyrF)	0.0262
TTHA1803	2.12	pterin-4-alpha-carbinolamine dehydratase	0.0377
TTHA1868	2.32	extracellular serine protease	0.0114
TTHB074	2.20	putative C4-dicarboxylate transporter, periplasmic C4-dicarboxylate-binding protein	0.0128
TTHB088	2.02	Zn-dependent hydrolase	0.0114
TTHB097	2.10	hypothetical protein	0.0114
TTHB176	2.28	putative iron ABC transporter, permease protein	0.0252
TTHB177	2.58	iron ABC transporter, periplasmic iron-binding protein	0.0202
TTHB207	3.04	hypothetical protein	0.0236
TTHB208	3.48	ribonucleoside-diphosphate reductase, beta subunit	0.0260
TTHB209	4.16	ribonucleoside-diphosphate reductase, alpha subunit	0.0202
TTHB250	2.86	metapyrocatechase (catechol 2,3-dioxygenase)	0.0185

ORFs with a q value $\leq$0.05 and a fold change of $\geq$2-fold relative to without MNNG are listed.

Table V. Subset of genes downregulated in *T. thermophilus* HB8 *Δtth1564* strain with MNNG treatment.

Locus Tag	Fold	Annotation	*q*-values
TTHA0666	0.13	ATP sulphurylase (sulfate adenylyltransferase)	0.0114
TTHA0667	0.20	uroporphyrin-III C-methyl transferase	0.0114
TTHA0668	0.21	hypothetical protein	0.0114
TTHA0669	0.15	phosphoadenosine phosphosulphate reductase (CysH)	0.0114
TTHA0670	0.19	siroheme synthase (precorrin-2 oxidase/ferrochelatase)	0.0114
TTHA0671	0.20	hemD Uroporphyrinogen-III synthase	0.0114
TTHA0672	0.21	probable sulfite reductase	0.0114
TTHA0959	0.47	5-Carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase	0.0385
TTHA0962	0.46	homoprotocatechuate 2,3-dioxygenase	0.0392

ORFs with a q value $\leq$0.05 and a fold change of $\leq$0.5-fold relative to without MNNG are listed.

specific region containing DNA modification. Other factors, such as DNA-binding proteins, may recruit TTHA1564 to specific promoter regions. It is reported that most of the proteins that interact with TTHA1564 are DNA-binding proteins (*6*). A comparison of the findings of our previous interactome study and those of the present transcriptome study reveals one protein common to both results. DNA gyrase was upregulated by TTHA1564 under alkylating condition (Table VI) and is included in the list of proteins that interact with TTHA1564 (*6*). Recently, a fusion protein of Ogt and endonuclease V was reported in an archaeon (*32*). This bifunctional protein has an N-terminal domain that is homologous to C-Ada and a C-terminal domain that is homologous to endonuclease V as a DNA-binding domain. Considering the combination of these two domains, endonuclease V might affect the regulation system of TTHA1564 in *T. thermophilus* HB8.

As described in introduction, it is becoming clear that ATL proteins act via the same pathway as NER proteins *in vivo* to repair O^6-meG (*6*, *9–13*). We have proposed the hypothesis that ATL recruits NER protein(s) to a methylated site in DNA (*6*). To test this

Table VI. Subset of genes upregulated only in *T. thermophilus* HB8 WT with MNNG treatment.

Locus tag	Fold	Annotation	*q*-values	COG code
TTHA0152	2.72	tungsten-containing aldehyde:ferredoxin oxidoreductase	0.0031	C
TTHA0164	3.12	thiol:disulfide interchange protein	0.0031	O,C
TTHA0166	2.00	hypothetical protein	0.0154	Q,P
TTHA0212	2.79	hypothetical protein	0.0031	S
TTHA0425	3.06	NADH oxidase (H_2O_2-forming)	0.0031	C
TTHA0495	2.11	2-Phosphoglycerate kinase	0.0034	G
TTHA0541	2.04	DNA repair protein RadA	0.0133	O
TTHA0570	2.05	putative oxidoreductase (glucose dehydrogenase)	0.0031	G
TTHA0607	6.45	biotin synthase (biotin synthetase)	0.0031	H
TTHA0655	2.11	hypothetical protein	0.0031	K
TTHA0665	2.45	N5,N10-methylenetetrahydromethanopterin reductase	0.0066	C
TTHA0706	6.92	cation-transporting ATPase	0.0031	P
TTHA0707	2.21	glutamine amidotransferase (pyridoxine biosynthesis)	0.0031	H
TTHA0729	2.43	probable efflux transporter, AcrB/AcrD/AcrF family	0.0067	V
TTHA0731	2.73	hypothetical protein	0.0033	M,U
TTHA0742	17.09	hypothetical protein	0.0031	–
TTHA0743	35.87	glycosyltransferase related protein	0.0031	M,N,U
TTHA0744	10.84	hypothetical protein	0.0031	O
TTHA0757	2.92	hypothetical protein	0.0031	–
TTHA0770	2.00	ATP-dependent protease La (Lon protease)	0.0031	O
TTHA0771	3.02	hypothetical protein	0.0031	E,O,T
TTHA0798	2.83	GGDEF domain protein	0.0031	T
TTHA0807	2.33	transcriptional regulator	0.0031	K
TTHA0841	3.68	stage V sporulation protein R (SpoVR) related protein	0.0105	S
TTHA0842	3.71	hypothetical protein	0.0128	S
TTHA0843	5.24	serine protein kinase	0.0047	T
TTHA0945	2.43	hypothetical protein	0.0031	–
TTHA0986	3.16	hypothetical protein	0.0031	O
TTHA1044	2.07	diacylglycerol kinase	0.0073	M
TTHA1045	2.49	hypothetical protein	0.0034	R
TTHA1128	2.03	peptidase	0.0031	E
TTHA1133	2.56	ba_3-type cytochrome c oxidase polypeptide IIA	0.0034	–
TTHA1141	2.06	cation-transporting ATPase	0.0031	P
TTHA1147	2.07	hypothetical protein	0.0031	–
TTHA1410	2.14	sulphite dehydrogenase cytochrome subunit SoxD	0.0315	–
TTHA1412	3.66	sulphide dehydrogenase flavocytochrome C	0.0084	C
TTHA1415	3.87	sulphur oxidation protein SoxA	0.0044	–
TTHA1416	3.65	cytochrome c (SoxX)	0.0101	–
TTHA1418	3.82	putative cytochrome c	0.0044	C
TTHA1419	3.16	putative cytochrome c precursor	0.0034	C
TTHA1420	4.67	sulphur oxidation protein SoxZ	0.0056	–
TTHA1421	6.60	sulphur oxidation protein SoxY	0.0043	S
TTHA1422	5.75	disulphide isomerase (DsbC)	0.0034	O,C
TTHA1492	2.21	cell division protein FtsH	0.0031	O
TTHA1496	2.04	arginase	0.0031	E
TTHA1498	2.28	elongation factor G (EF-G-2)	0.0057	J
TTHA1500	2.00	phosphoenolpyruvate synthase	0.0031	G
TTHA1506	2.20	glutamyl-tRNA reductase	0.0031	H
TTHA1527	2.10	NADPH-quinone reductase	0.0031	C,R
TTHA1586	2.07	DNA gyrase, subunit B	0.0031	L
TTHA1625	2.01	osmotically inducible protein OsmC	0.0031	O
TTHA1626	2.09	hypothetical protein	0.0130	S
TTHA1635	2.19	iron–sulphur cluster biosynthesis protein IscA	0.0031	S
TTHA1718	4.34	heavy metal binding protein	0.0045	P
TTHA1719	3.13	copper homeostasis operon regulatory protein	0.0058	S
TTHA1720	3.58	cation-transporting ATPase	0.0057	P
TTHA1776	2.12	hypothetical protein	0.0044	–
TTHA1871	2.13	hypothetical protein	0.0031	–
TTHA1892	2.47	excinuclease ABC subunit B (UvrB)	0.0031	L
TTHA1941	3.02	ZIP zinc transporter family protein	0.0031	P
TTHB089	2.17	hypothetical protein	0.0031	–
TTHB175	2.59	ABC transporter, ATP-binding protein	0.0032	E
TTHB186	2.05	putative transcriptional regulator	0.0031	K
TTHB187	2.49	CRISPR-associated protein, Cas3	0.0031	R
TTHB207	6.02	hypothetical protein	0.0031	I
TTHB208	5.36	ribonucleoside-diphosphate reductase, beta subunit	0.0035	F
TTHB243	2.31	hypothetical protein	0.0031	S
TTHC001	2.24	putative RepA protein	0.0323	–
TTHC010	2.32	hypothetical protein	0.0069	–
TTHC013	2.40	toxin-like protein	0.0057	J,D
TTHC014	2.12	hypothetical protein	0.0060	–

The fold-change values of upregulation of these genes were higher in WT strain than in *Δttha1564* strain statistically. These genes showed ≥1.5-fold change in WT compared with in *Δttha1564* strain. ORFs with a *q*-value ≤0.05 and a fold change of ≥2-fold in WT strain are listed. Each gene function was categorized by using alphabets in COG database (http://www.ncbi.nlm.nih.gov/COG/old/palox.cgi?fun=all).

Table VII. The relative ratio of genes treated with and without MNNG in WT strain by qRT-PCR.

Gene	Product	Amplification efficiency	Fold	*P*-value	Fold (array)
TTHA0706	cation-transporting ATPase	2.00	2.1	0.008	6.92
TTHA1440	excinuclease ABC subunit A (UvrA)	1.98	5.1	0.0004	1.47
TTHA1548	excinuclease ABC subunit C (UvrC)	2.03	1.3	0.038	1.70
TTHA1718	heavy metal binding protein	2.05	4.7	0.005	4.34
TTHA1892	excinuclease ABC subunit B (UvrB)	2.12	2.5	0.0006	2.47
TTHB174	sensor histidine kinase-like protein	2.03	1.9	0.01	4.21
TTHB186	putative transcriptional regulator	2.00	2.0	0.009	2.05
TTHB187	CRISPR-associated protein, Cas3	2.10	3.1	0.028	2.49
TTHB208	ribonucleoside-diphosphate reductase, beta subunit	2.06	11.0	0.03	5.36

Numbers in 'Fold (array)' column show the fold-change values in DNA microarray analysis.

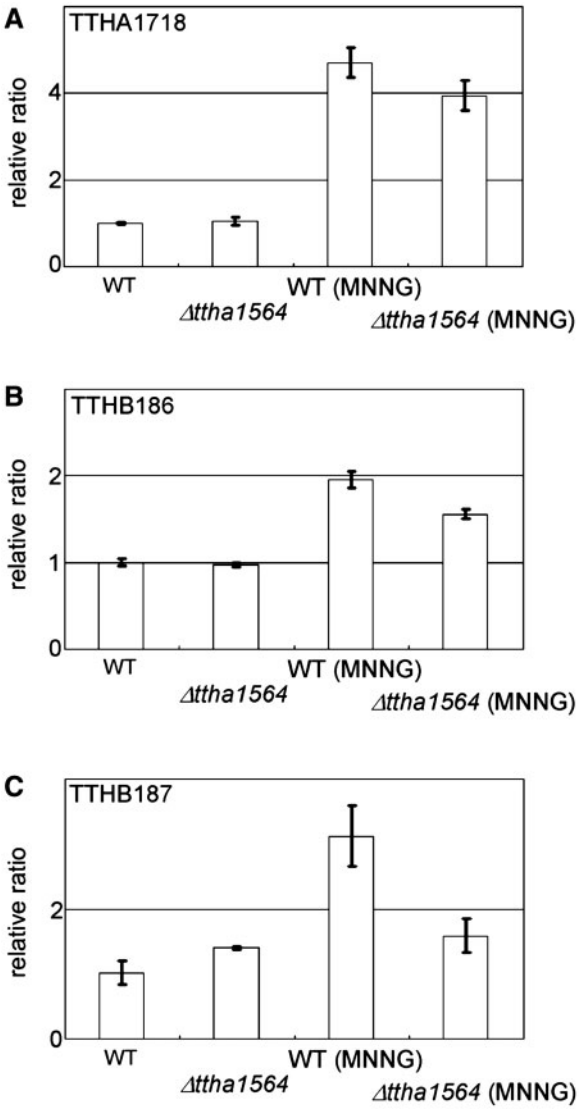

Fig. 2 qRT-PCR shows that alkylation upregulates *ttha1718*, *ttha186* and *ttha187* to a greater extent in the WT than in *Δttha1564*. The values of the WT strain were set at 1. WT (MNNG), WT strain under alkylation; *Δttha1564* (MNNG), *Δttha1564* strain under alkylation; TTHA1718, heavy metal-binding protein (copper homeostasis operon regulatory protein); TTHB186, putative transcriptional regulator; and TTHB187, CRISPR-associated protein (Cas3).

hypothesis, we established an *in vitro* reconstitution system with purified UvrA, UvrB, UvrC and TTHA1564 under the condition reported by Hori (*33*). However, we have detected no cleaved DNA fragment for the O^6-meG-containing DNA substrate (data not shown). UvrABC cleaved the DNA substrate containing the fluorescein-modified thymine as a positive control, but TTHA1564 inhibited this cleavage (data not shown). Such inhibition is likely due to the strong affinity of TTHA1564 for O^6-meG as well as the results of previous reports wherein ATL proteins were shown to inhibit the activity of Ogt and mismatch repair system (*7*, *8*, *11*). Direct pathway via which TTHA1564 anticipates in the repair, probably NER, of alkylated legions needs to be investigated further.

Comparison of the microarray data in WT strain resulted in the identification of 89 upregulated genes upon MNNG treatment. It is well-known that MNNG causes DNA alkylation and such damage is repaired by DNA repair systems (*34*). Among these upregulated genes, it is reasonable to consider that transcriptional regulation of UvrB, RadA and DNA gyrase genes is an alkylating stress response. As shown in Table VII, *uvrA* and *uvrC* were also upregulated. Upregulation of these NER genes is a notable change because ATL proteins are supposed to act via the same pathway as NER proteins. UvrABC was reported to be sufficient to cleave DNA including O^6-meG in *E. coli* (*35*, *36*). RadA is thought to be involved in homologous recombination process, but its molecular and cellular function is still unclear (*25*). Homologous recombination may act to prevent mutagenicity caused by recognition of O^6-meG:T by mismatch repair (*37*). It was suggested that DNA gyrase directly interacts with TTHA1564 (*6*). The expression level of *ttha1564* was not significantly changed.

As a response to alkylating stress, it is noteworthy that two genes of NDP reductase subunits (TTHB208 and TTHB209) were upregulated by >4-fold. NDP reductase is one of the most important enzyme in the biosynthesis of dNTP (*38*). In *E. coli*, it is reported that *nrdA* (an NDP reductase gene) is upregulated by DNA damage such as alkylation by MNNG (*39*). Furthermore, multiple factors in *E. coli*, such as FIS, IciA, DnaA, regulate the *nrdAB* operon and stalling of

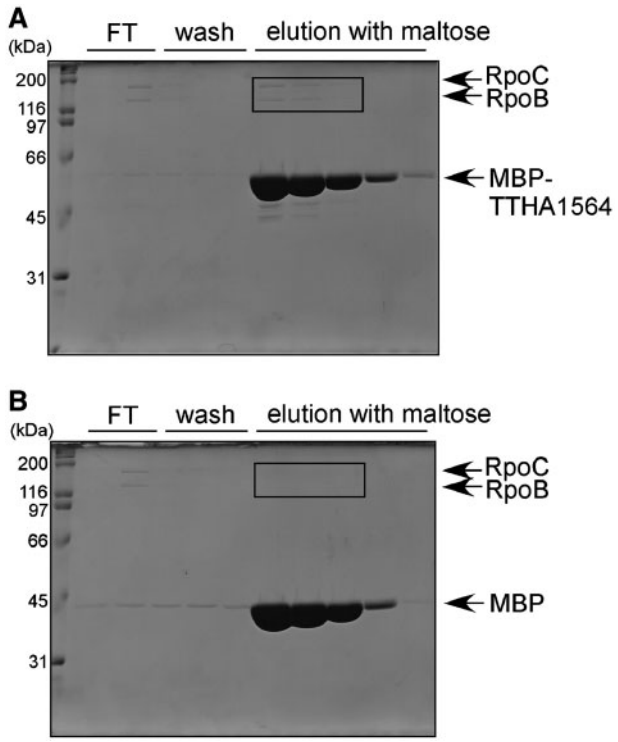

Fig. 3 Interaction between TTHA1564 and RNA polymerase. RNA polymerase was applied to MBP-TTHA1564- or MBP-immobilized columns. After sufficient washing, the immobilized protein was eluted with maltose. We collected the fractions of the flow through (FT), the wash (wash) and the eluted solution (elution with maltose). (A) MBP-TTHA1564 was immobilized in this column. RpoC and RpoB bands were detected in the square. RNA polymerase holoenzyme is comprised of RpoA, RpoB, RpoC and a sigma factor. (B) The bands of RNA polymerase were detected only in the FT lanes, and no band was detected in the square in the elution lanes.

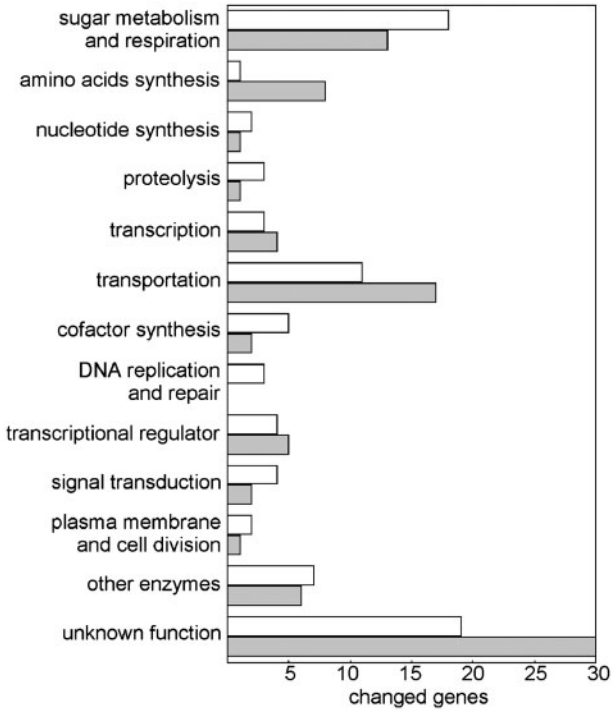

Fig. 4 Categorized changed genes in WT strain. Up- and downregulated genes in the WT strain are categorized by their function. White and gray bars show up- and downregulated genes, respectively.

the replication fork induces the upregulation of *nrdAB* (*40*, *41*). In *T. thermophilus*, the observed upregulation of *ttha208* and *ttha0209* can be considered to be a response to alkylation.

The *tthb187* gene is present in the CRISPR loci in the *T. thermophilus* HB8 genome (*15*). CRISPR systems are composed of CRISPRs (clustered regularly interspaced short palindromic repeats) and CRISPR-associated (Cas) genes. This system degrades exogenous DNA such as phage DNA. A recent report suggests that Cas proteins are involved in DNA repair (*42*). TTHB187 (Cas3) contains both DEAH and HD motifs, suggesting it is a helicase. Therefore, the observed upregulation of TTHB187 might be associated with the DNA repair of alkylated lesions.

The most drastic change (11-to 36-fold) in transcription level was observed in *ttha0742-ttha0744*, which encode hypothetical proteins. TTHA0743 includes a glycosyltransferase motif and other motif involved in a type II secretion system at the N-terminal region. Therefore, TTHA0743 (as well as the other two) may act in the extracellular compartment. It is known that DNA glucosylation functions in transcriptional regulation of phage-specific genes (*43*). However, it is unclear how TTH0743 (and the other two neighbours) is related to MNNG treatment or DNA repair.

The other genes that were upregulated upon MNNG treatment can be classified into the categories other than DNA-related one. Figure 4 shows the functional categories of the distribution of up- and downregulated genes in the WT. Most of these genes could be related to TTHA1564. It seems difficult to interpret these observed changes in conjunction with DNA alkylation and repair events. Thus, we consider the possibility that MNNG causes other effects beside DNA alkylation. In order to reveal a clue regarding the function of TTHA1564, we selected and considered several interesting genes. The results mentioned below suggest that MNNG treatment may induce other stresses such as oxidative stress in addition to alkylation.

Sox genes

Most of the genes encoding sulphur oxidation ('Sox') proteins (*ttha1410-to ttha1422*) were markedly upregulated by alkylation. Sox proteins encoded by these genes are involved in (thio)sulphur oxidation (*44*). The transcription of these Sox genes is regulated by a transcriptional factor, SoxR; it was recently reported that redox-cycling drugs could activate SoxR (Gu, 2011). Furthermore, it is known that MNNG reacts with sulphur compounds such as glutathione (*45*). Therefore, sulphur oxidation proteins may respond to the oxidation of sulphur compounds by MNNG, which can be considered as a response to oxidation stress.

Cation-transporting ATPases

The genes encoding cation-transporting proteins (*ttha0706*, *ttha1141* and *ttha1720*) were upregulated by 2- to 7-fold, although their loci are not close together. TTHA1718, TTHA1719 and TTHA1720 are proteins involved in the transport of copper ions as a copper ion-binding protein, a copper ion-specific regulator and a copper ion-transporting ATPase, respectively (*46*). TTHA0706 and TTHA1141 are also predicted to be cation-transporting ATPases. It is known that free copper ions produce hydroxyl radicals from hydrogen peroxide and singlet oxygen, which mediate oxidative damages to proteins and DNA (*47*). The upregulation of these genes also could be considered as a response to oxidation stress. *ttha1941* and *tthb176*, encoding zinc and iron transporter, respectively, may also be related to such a response.

Signal transduction

The genes encoding sensor histidine kinase-like protein (*tthb174*) and serine protein kinase (*ttha0843*) were upregulated by more than 4-fold. In prokaryotes, in order to adapt to environmental changes and regulate intracellular signal networks, a 'two-component regulatory system' acts in the cells (*48*); this is one of the major signal transduction systems in prokaryotes. Autophosphorylation of sensor kinases activates this system, which regulates some genes involved in adaptation to environmental conditions via a phosphorelay cascade. TTHA0843 is a bacteria-specific serine protein kinase, PrkA. Bacteria have protein phosphorylation–dephosphorylation networks that target serine, threonine, and/or tyrosine residues on proteins as well as eukaryotes. It is known that in *E. coli* the expression *prkA* homologue, *yeaG*, is stimulated under stress conditions (*49*). There is no report regarding a two-component system and Ser/Thr/Tyr protein kinase systems in response to alkylating stress. TTHB174 and TTHA0843 may be a candidate for it.

Transcriptional regulators

The genes encoding transcriptional regulators (*ttha0807* and *tthb186*) were upregulated. Although it is unclear which genes are regulated by these transcriptional regulators, it is possible to consider these regulate some of the genes discussed above. The relationship between these regulators and TTHA1564 may be important for the regulation of several genes.

In this study we suggested that TTHA1564 affects the regulation of gene expression. However, the mechanism behind this novel gene regulatory system remains unknown—specifically, the link between biochemical properties of TTHA1564 and the repair of O^6-meG lesion is missing. At present, *in vitro* studies alone have produced insufficient to propose any working hypothesis. To elucidate the mechanism of the O^6-meG repair system with ATL proteins *in vivo*, it is important to assess the effects of alkylating agent *in vivo* in greater details. However, this study also suggests that in addition to alkylation, MNNG has other stressful effects, which would make it difficult to assess its alkylating effect. Therefore, both *in vivo* and *in vitro* assays are essential for elucidating functional mechanisms of ATL proteins.

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Conflict of interest

None declared.

References

1. Morita, R., Nakane, S., Shimada, A., Inoue, M., Iino, H., Wakamatsu, T., Fukui, K., Nakagawa, N., Masui, R., and Kuramitsu, S. (2010) Molecular mechanisms of the whole DNA repair system: a comparison of bacterial and eukaryotic systems. *J. Nucleic Acids*, **2010**, ID 179594
2. Olsson, M. and Lidahl, T. (1980) Repair of alkylated DNA in *Escherichia coli*. Methyl group transfer from O^6-methylguanine to a protein cysteine residue. *J. Biol. Chem.* **255**, 10569–10571
3. Lindahl, T., Demple, B., and Robins, P. (1982) Suicide inactivation of the *E. coli* O^6-methylguanine-DNA methyltransferase. *EMBO J.* **1**, 1359–1363
4. Wiestler, O., Kleihues, P., and Pegg, A.E. (1984) O^6-alkylguanine-DNA alkyltransferase activity in human brain and brain tumors. *Carcinogenesis* **5**, 121–124
5. Tubbs, J.L. and Tainer, J.A. (2010) Alkyltransferase-like proteins: molecular switches between DNA repair pathways. *Cell. Mol. Life Sci.* **67**, 3749–3762
6. Morita, R., Nakagawa, N., Kuramitsu, S., and Masui, R. (2008) An O^6-methylguanine-DNA methyltransferase-like protein from *Thermus thermophilus* interacts with a nucleotide excision repair protein. *J. Biochem.* **144**, 267–277
7. Pearson, S.J., Ferguson, J., Santibanez-Koref, M.F., and Margison, G.P. (2005) Inhibition of O^6-methylguanine-DNA methyltransferase by an alkyltransferase-like protein from *Escherichia coli*. *Nucleic Acids Res.* **33**, 3837–3844
8. Pearson, S.J., Wharton, S., Watson, A.J., Begum, G., Butt, A., Glynn, N., Williams, D.M., Shibata, T., Santibáñez-Koref, M.F., and Margison, G.P. (2006) A novel DNA damage recognition protein in *Schizosaccharomyces pombe*. *Nucleic Acids Res.* **34**, 2347–2354
9. Aramini, J.M., Tubbs, J.L., Kanugula, S., Rossi, P., Ertekin, A., Maglaqui, M., Hamilton, K., Ciccosanti, C.T., Jiang, M., Xiao, R., Soong, T.T., Rost, B., Acton, T.B., Everett, J.K., Pegg, A.E., Tainer, J.A., and Montelione, G.T. (2010) Structural basis of O^6-alkylguanine recognition by a bacterial alkyltransferase-like DNA repair protein. *J. Biol. Chem.* **285**, 13736–13741
10. Mazon, G., Philippin, G., Cadet, J., Gasparutto, D., and Fuchs, R.P. (2009) The alkyltransferase-like *ybaZ* gene product enhances nucleotide excision repair of O^6-alkylguanine adducts in *E. coli*. *DNA Repair* **8**, 697–703
11. Mazon, G., Philippin, G., Cadet, J., Gasparutto, D., Modesti, M., and Fuchs, R.P. (2010) Alkyltransferase-like protein (eATL) prevents mismatch repair-mediated toxicity induced by O^6-alkylguanine

adducts in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **107**, 18050–18055
12. Onodera, T., Morino, K., Tokishita, S., Morita, R., Masui, R., Kuramitsu, S., and Ohta, T. (2011) Role of alkyltransferase-like (ATL) protein in repair of methylated DNA lesions in *Thermus thermophilus*. *Mutagenesis* **26**, 303–308
13. Tubbs, J.L., Latypov, V., Kanugula, S., Butt, A., Melikishvili, M., Kraehenbuehl, R., Fleck, O., Marriott, A., Watson, A.J., Verbeek, B., McGown, G., Thorncroft, M., Santibanez-Koref, M.F., Millington, C., Arvai, A.S., Kroeger, M.D., Peterson, L.A., Williams, D.M., Fried, M.G., Margison, G.P., Pegg, A.E., and Tainer, J.A. (2009) Flipping of alkylated DNA damage bridges base and nucleotide excision repair. *Nature* **459**, 808–813
14. Sedgwick, B. and Lindahl, T. (2002) Recent progress on the Ada response for inducible repair of DNA alkylation damage. *Oncogene* **21**, 8886–8894
15. Agari, Y., Kuramitsu, S., and Shinkai, A. (2010) Identification of novel genes regulated by the oxidative stress-responsive transcriptional activator SdrP in *Thermus thermophilus* HB8. *FEMS Microbiol. Lett.* **313**, 127–134
16. Mega, R., Manzoku, M., Shinkai, A., Nakagawa, N., Kuramitsu, S., and Masui, R. (2010) Very rapid induction of a cold shock protein by temperature downshift in *Thermus thermophilus*. *Biochem. Biophys. Res. Commun.* **399**, 336–340
17. Shinkai, A., Kira, S., Nakagawa, N., Kashihara, A., Kuramitsu, S., and Yokoyama, S. (2007) Transcription activation mediated by a cyclic AMP receptor protein from *Thermus thermophilus* HB8. *J. Bacteriol.* **189**, 3891–3901
18. Oshima, T. and Imahori, K. (1974) Description of *Thermus thermophilus* (Yoshida and Oshima) comb. nov. A non-sporulating thermophilic bacterium from a japanese thermal spa. *Int. J. Syst. Bacteriol.* **24**, 102–112
19. Hashimoto, Y., Yano, T., Kuramitsu, S., and Kagamiyama, H. (2001) Disruption of *Thermus thermophilus* genes by homologous recombination using a thermostable kanamycin-resistant marker. *FEBS Lett.* **506**, 231–234
20. Hoseki, J., Yano, T., Koyama, Y., Kuramitsu, S., and Kagamiyama, H. (1999) Directed evolution of thermostable kanamycin-resistance gene: a convenient selection marker for *Thermus thermophilus*. *J. Biochem.* **126**, 951–956
21. Yamagishi, A., Tanimoto, T., Suzuki, T., and Oshima, T. (1996) Pyrimidine biosynthesis genes (pyrE and pyrF) of an extreme thermophile, *Thermus thermophilus*. *Appl. Environ. Microbiol.* **62**, 2191–2194
22. Agari, Y., Kashihara, A., Yokoyama, S., Kuramitsu, S., and Shinkai, A. (2008) Global gene expression mediated by *Thermus thermophilus* SdrP, a CRP/FNR family transcription regulator. *Mol. Microbiol.* **70**, 60–75
23. Xue, Y., Hogan, B.P., and Erie, D.A. (2000) Purification and initial characterization of RNA polymerase from *Thermus thermophilus* strain HB8. *Biochemistry* **39**, 14356–14362
24. Theis, K., Skorvaga, M., Machius, M., Nakagawa, N., Van Houten, B., and Kisker, C. (2000) The nucleotide excision repair protein UvrB, a helicase-like enzyme with a catch. *Mutat. Res.* **460**, 277–300
25. Beam, C., Saveson, C., and Lovett, S. (2002) Role for *radA*/*sms* in recombination intermediate processing in *Escherichia coli*. *J. Bacteriol.* **184**, 6836–6844
26. Nöllmann, M., Crisona, N., and Arimondo, P. (2007) Thirty years of *Escherichia coli* DNA gyrase: from *in vivo* function to single-molecule mechanism. *Biochimie* **89**, 490–499
27. Landini, P. and Volkert, M.R. (2000) Regulatory responses of the adaptive response to alkylation damage: a simple regulon with complex regulatory features. *J. Bacteriol.* **182**, 6543–6549
28. Landini, P. and Volkert, M.R. (1995) RNA polymerase α subunit binding site in positively controlled promoters: a new model for RNA polymerase-promoter interaction and transcriptional activation in the *Escherichia coli ada* and *aidB* genes. *EMBO J.* **14**, 4329–4335
29. Sakumi, K., Igarashi, K., Sekiguchi, M., and Ishihama, A. (1993) The Ada protein is a class I transcription factor of *Escherichia coli*. *J. Bacteriol.* **175**, 2455–2457
30. Margison, G.P., Butt, A., Pearson, S.J., Wharton, S., Watson, A.J., Marriott, A., Caetano, C.M., Hollins, J.J., Rukazenkova, N., Begum, G., and Santibáñez-Koref, M.F. (2007) Alkyltransferase-like proteins. *DNA Repair* **6**, 1222–1228
31. Moore, M.H., Gulbis, J.M., Dodson, E.J., Demple, B., and Moody, P.C. (1994) Crystal structure of a suicidal DNA repair protein: the Ada O^6-methylguanine-DNA methyltransferase from *E. coli*. *EMBO J.* **13**, 1495–1501
32. Kanugula, S., Pauly, G.T., Moschel, R.C., and Pegg, A.E. (2005) A bifunctional DNA repair protein from *Ferroplasma acidarmanus* exhibits O^6-alkylguanine-DNA alkyltransferase and endonuclease V activities. *Proc. Natl Acad. Sci. USA* **102**, 3617–3622
33. Hori, M., Ishiguro, C., Suzuki, T., Nakagawa, N., Nunoshiba, T., Kuramitsu, S., Yamamoto, K., Kasai, H., Harashima, H., and Kamiya, H. (2007) UvrA and UvrB enhance mutations induced by oxidized deoxyribonucleotides. *DNA Repair* **6**, 1786–1793
34. Friedburg, E.C., Walker, G.C., Siede, W., and Schultz, R.A. (2006) *DNA Repair and Mutagenesis*., ASM Press, Washington DC
35. Samson, L. (1992) The suicidal DNA repair methyltransferases of microbes. *Mol. Microbiol.* **6**, 825–831
36. Voigt, J., Van Houten, B., Sancar, A., and Topal, M. (1989) Repair of O^6-methylguanine by ABC excinuclease of *Escherichia coli in vitro*. *J. Biol. Chem.* **264**, 5172–5176
37. Rajesh, P., Rajesh, C., Wyatt, M.D., and Pittman, D.L. (2010) RAD51D protects against MLH1-dependent cytotoxic responses to O^6-methylguanine. *DNA Repair* **4**, 458–467
38. Nordlund, P. and Reichard, P. (2006) Ribonucleotide reductases. *Annu. Rev. Biochem.* **75**, 681–706
39. Sitjes, J., Ysern, P., Barbe, J., and Llagostera, M. (1992) Induction of ribonucleoside diphosphate reductase gene transcription by chemicals in *Escherichia coli*. *Mutagenesis* **7**, 47–49
40. Olliver, A., Saggioro, C., Herrick, J., and Sclavi, B. (2010) DnaA-ATP acts as a molecular switch to control levels of ribonucleotide reductase expression in *Escherichia coli*. *Mol. Microbiol.* **76**, 1555–1571
41. Sun, L. and Fuchs, J.A. (1992) *Escherichia coli* ribonucleotide reductase expression is cell cycle regulated. *Mol. Biol. Cell* **3**, 1095–1105
42. Babu, M., Beloglazova, N., Flick, R., Graham, C., Skarina, T., Nocek, B., Gagarinova, A., Pogoutse, O., Brown, G., Binkowski, A., Phanse, S., Joachimiak, A., Koonin, E.V., Savchenko, A., Emili, A., Greenblatt, J., Edwards, A.M., and Yakunin, A.F. (2011) A dual function of the CRISPR–Cas system in bacterial antivirus immunity and DNA repair. *Mol. Microbiol.* **79**, 484–502
43. Moréra, S., Imberty, A., Aschke-Sonnenborn, U., Rüger, W., and Freemont, P.S. (1999) T4 phage β-glucosyltransferase: substrate binding and proposed catalytic mechanism. *J. Mol. Biol.* **292**, 717–730

44. Friedrich, C., Bardischewsky, F., Rother, D., Quentmeier, A., and Fischer, J. (2005) Prokaryotic sulfur oxidation. *Curr. Opin. Microbiol.* **8**, 253–259
45. Niknahad, H. and O'Brien, P.J. (1995) Cytotoxicity induced by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine may involve S-nitrosyl glutathione and nitric oxide. *Xenobiotica* **25**, 91–101
46. Sakamoto, K., Agari, Y., Agari, K., Kuramitsu, S., and Shinkai, A. (2010) Structural and functional characterization of the transcriptional repressor CsoR from *Thermus thermophilus* HB8. *Microbiology* **156**, 1993–2005
47. Hiniker, A., Collet, J.F., and Bardwell, J.C. (2005) Copper stress causes an *in vivo* requirement for the *Escherichia coli* disulfide isomerase DsbC. *J. Biol. Chem.* **280**, 33785–33791
48. Igo, M.M., Slauch, J.M., and Silhavy, T.J. (1990) Signal transduction in bacteria: kinases that control gene expression. *New Biol.* **2**, 5–9
49. Weber, H., Polen, T., Heuvelling, J., Wendisch, V.F., and Hengge, R. (2005) Genome-wide analysis of the general stress response network in *Escherichia coli*: σ^S-dependent genes, promoters, and sigma factor selectivity. *J. Bacteriol.* **187**, 1591–1603