DNA REPAIR 7 (2008) 663–669

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journal homepage: www.elsevier.com/locate/dnarepair

Brief report

Mutagenesis of uracil-DNA glycosylase deficient mutants of the extremely thermophilic eubacterium *Thermus thermophilus*

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ARTICLE INFO

Article history:
Received 20 September 2007
Received in revised form
7 January 2008
Accepted 14 January 2008

Keywords:
Uracil-DNA glycosylase
Thermus thermophilus
Mutator
Bgl assay

ABSTRACT

Thermus thermophilus is an extremely thermophilic, aerobic, and gram-negative eubacterium that grows optimally at 70–75 °C, pH 7.5. In extremely high temperature environment, DNA damages in cells occur at a much higher frequency in thermophiles than mesophiles such as *E. coli*. When temperature rises, the deamination of cytosine residues in double-strand DNA is expected to increase greatly. *T. thermophilus* HB27 has two putative uracil-DNA glycosylase genes (*udgA* and *udgB*). Expression level of *udgA* gene was 2–3 times higher than that of *udgB* at 70, 74, and 78 °C when it was monitored by β-glucosidase reporter assay. We developed $hisD_{3110}$, $hisD_{3113}$, $hisD_{3115}$, and $hisD_{174}$ marker allele that can specifically detect G:C → A:T, C:G → A:T, T:A → A:T, and A:T → G:C base-substitutions, respectively, by His^+ reverse mutations. We then disrupted *udgA* and *udgB* by thermostable kanamycin-resistant gene (*htk*) or *pyrE* gene insertion in each *hisD* background, and their spontaneous His^+ reversion frequencies were compared. A *udgA,B* double mutant showed a pronounced increase in G:C → A:T reversion frequency compared with each single *udg* mutant, *udgA* or *udgB*. Estimated mutation rates of the *udgA,B* mutant cultured at 60, 70, and 78 °C were about 2, 12, and 117 $His^+/10^8$/generation, respectively. At 70 °C culture, increased ratio of the mutation rate compared with the udg^+ strain was 12-fold in *udgA*, 3-fold in *udgB*, and 56-fold in *udgA,B* mutant. On the other hand, no difference was observed in other mutations of C:G → A:T, T:A → A:T, and A:T → G:C between *udgA,B* double mutant and the parent udg^+ strain. The present results indicated that gene products of *udgB* as well as *udgA* functioned in vivo to remove uracil in DNA and prevent G:C → A:T transition mutations.

1. Introduction

Thermus thermophilus HB27, isolated from a Japanese thermal spa, is an extremely thermophilic eubacterium that grows at 55–82 °C [1]. It is a nonsporulating, gram-negative, aerobic, obligate heterotroph that grows optimally at 70–75 °C and pH 7.5. Because of the rapid growth rate of *T. thermophilus* and the ease of purifying its proteins, its thermostable enzymes have been extensively studied in vitro, including in a structural genomics project [2]. The genome of the strain HB27

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doi:10.1016/j.dnarep.2008.01.006

has been sequenced [3] and consists of about 2200 putative genes divided between a 1.89 Mbp chromosome and a 0.23 Mbp megaplasmid (pTT27). *T. thermophilus* shows natural transformation competence throughout its growth phase with efficiency on the order of 10^4 transformants/μg DNA [4]. Although available antibiotic resistance genes at 70 °C are limited, a host–vector system was developed using the cryptic plasmid pTT8 (copy number: 8/cells) with a thermostable kanamycin-resistant *htk* gene [5]. On the other hand, a chemically defined medium for HB27 has been developed [6]. It provides a genetic method using auxotroph mutants.

Our major interest is DNA repair systems that function in extremely high temperature environments and the mechanisms of mutagenesis in *T. thermophilus*, because we would expect endogenous DNA damage such as deamination, depurination, methylation, oxidation, and single strand breaks to occur at a much higher frequency in thermophiles than in mesophiles. The spontaneous hydrolytic deamination of cytosine to uracil occurs frequently in the intracellular environment. A mismatched base pair G:U resulting from cytosine deamination generates an A:U base pair after replication. Subsequent replication of an A:U base pair should result in G:C → A:T transition mutation [7,8]. To prevent the occurrence of mutations by uracil in DNA, a base excision repair pathway has an important role [9]. Uracil-DNA glycosylase (UDG) catalyzes the first step in the repair pathway for uracil-containing DNA. The enzyme releases uracil from DNA by hydrolyzing the bond between the base and a deoxyribose [10]. Based on the amino acid sequences similarity and differences in substrate specificity, UDGs are classified into five families [9,11]. Family 1 UDGs (Ung family) excise uracil base from single-stranded DNA and double-stranded DNA. *E. coli* Ung is the representative of this family. Family 1 UDGs are found in many organisms including bacteria, yeast, mammalian cells, and plant cells, but not in archaea and insects. Family 2 UDGs (Mug/TDG family) consist of bacterial mismatch-specific uracil-DNA glycosylases (Mug) [12] and eukaryotic thymine-DNA glycosylases (TDG) [13,14]. They remove 3, N^4-ethenocytosine as well as uracil when mispaired with guanine. It has been reported that *E. coli* mug mutant showed no effect on G:C → A:T mutations [15]. Therefore, the principal role of family 2 UDGs may be the removal of 3, N^4-ethenocytosine. Family 3 UDGs (SMUG family) are single-strand-specific monofunctional uracil-DNA glycosylases (SMUG) and are identified in vertebrates and insects. They act on uracil and 5-hydroxymethyluracil in DNA [16]. Quite recently a bacterial SMUG ortholog has been reported in *Geobacter metallireducens* [17]. Family 4 UDGs (TmUDG) are thermostable UDG family found in thermophilic archaea and bacterial species. *Thermotoga maritima* UDG (TmUDG) is the representative of this family [18]. Family 4 enzymes from hyperthermophile archaea *Archaeoglobus fulgidus* [19] and *Pyrobaculum aerophilum* [20] are also characterized. They remove uracil from duplex DNA and single-stranded DNA containing uracil. Family 5 UDGs (UDG-b family) are found only in hyperthermophilic archaeon *P. aerophilum* [21] and eubacterium *T. thermophilus* [22]. They act on uracil in duplex DNA. PaUDG also catalyzes the removal of hypoxanthine, a product of adenine deamination. This enzyme, therefore, may play an important role against deamination of both cytosine and adenine. In addition to the above five UDG families, a novel UDG has been identified from hyperthermophilic archaeon *Methanococcus jannaschii* [23]. The MjUDG catalyzes the excision of 8-hydroxyguanine as well as uracil from DNA.

T. thermophilus HB27 has two putative UDG genes, *udgA* (TTC0366) and *udgB* (TTC0784) [22]. The former belongs to the family 4 UDGs and the latter is the family 5 UDGs. Since there is no report on isolation and characterization of *udg*-deficient mutants of *T. thermophilus*, little is known about the function and complementation of the two genes in vivo at growing temperature. We first investigated expression level of the *udgA* and *udgB* genes at different temperature by a newly developed β-glucosidase (Bgl) reporter assay. We demonstrate the constitutive expression of both the genes. Then, we developed a set of *hisD* reverse mutation assay system to determine the mutation spectrum and used it to investigate mutator phenotype of *udg* mutants. We report here the evidence that gene products of *udgB* as well as *udgA* functioned in vivo to prevent G:C → A:T transition mutations.

2. Materials and methods

2.1. Bacterial strains, culture media, and chemicals

Table 1 shows the strains of *T. thermophilus* used in this study. Bacteria were cultured in PY medium (0.8% polypeptone, 0.4% Difco yeast extract, 0.2% NaCl, 0.35 mM $CaCl_2$, and 0.4 mM $MgCl_2$) at 70 °C with shaking. MSG minimal medium (pH 7.5) consisted of 2% sucrose, 2% sodium glutamate, 0.2% NaCl, 0.05% $(NH_4)_2SO_4$, 0.05% K_2HPO_4, 0.025% KH_2PO_4, 88 μg/ml $CaCl_2{\cdot}2H_2O$, 35 μg/ml $MgCl_2{\cdot}6H_2O$, 1.2 μg/ml $Na_2MoO_4{\cdot}2H_2O$, 0.5 μg/ml $MnCl_2{\cdot}4H_2O$, 0.1 μg/ml $VOSO_4{\cdot}nH_2O$, 0.06 μg/ml $ZnSO_4{\cdot}7H_2O$, 0.08 μg/ml $CoCl_2{\cdot}6H_2O$, 0.015 μg/ml $CuSO_4{\cdot}5H_2O$, 0.002 μg/ml $NiCl_2{\cdot}6H_2O$, 6.7 μg/ml $FeSO_4{\cdot}7H_2O$, 0.1 μg/ml biotin, and 1 μg/ml thiamine. Medium was solidified with 1.5% agar for culture at 70 °C or 1.5% gellan gum for culture at 74 °C. Top agar contained 0.6% agar without NaCl. *Escherichia coli* strain XL1-Blue MRF' and vector plasmids pCR4-TOPO (Invitrogen Japan K.K., Tokyo) were used for plasmid construction. Gellan gum, 5-fluoroorotic acid (FOA), and 2-nitrophenyl-β-D-glucopyranoside (2NPGlc) were obtained from Wako Pure Chemical Industry, Tokyo. Kanamycin sulfate was purchased from Sigma–Aldrich Co., MO, USA.

2.2. Construction of $hisD_{3110}$, $hisD_{3113}$, and $hisD_{3115}$ mutation allele

The *hisD* gene encodes the enzyme L-histidinol dehydrogenase. The active site residue His-327 in PEHL motif of *E. coli* HisD protein participates in acid–base catalysis, whereas Glu-326 is responsible for the activation of a water molecule [24]. Since the corresponding Glu-311 in PEHL motif of *T. thermophilus* HisD protein would be expected to be indispensable for catalytic activity, we substituted the amino acid to obtain His auxotroph mutants. About 1800 bp fragment containing *hisD* was amplified by PCR from HB27 DNA and cloned onto pCR4-TOPO. GAG codon for Glu-311 in *hisD* gene was changed to GGG, GCG, or GTG codon for Gly, Ala, and Val, respectively, by site-directed mutagenesis. The HB27 cells were transformed