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Roles of the Y-family DNA Polymerase Dbh in Accurate Replication of the *Sulfolobus* Genome at Extremely High Temperature

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Abstract

The intrinsically thermostable Y-family DNA polymerases of *Sulfolobus* spp. have revealed detailed three-dimensional structure and catalytic mechanisms of trans-lesion DNA polymerases, yet their functions in maintaining their native genomes remain largely unexplored. To identify functions of the Y-family DNA polymerase Dbh in replicating the *Sulfolobus* genome under extreme conditions, we disrupted the *dbh* gene in *Sulfolobus acidocaldarius* and characterized the resulting mutant strains phenotypically. Disruption of *dbh* did not cause any obvious growth defect, sensitivity to any of several DNA-damaging agents, or change in overall rate of spontaneous mutation at a well-characterized target gene. Loss of *dbh* did, however, cause significant changes in the spectrum of spontaneous forward mutation in each of two orthologous target genes of different sequence. Relative to wild-type strains, *dbh*[−] constructs exhibited fewer frame-shift and other small insertion-deletion mutations, but exhibited more base-pair substitutions that converted G:C base pairs to T:A base pairs. These changes, which were confirmed to be statistically significant, indicate two distinct activities of the Dbh polymerase in *Sulfolobus* cells growing under nearly optimal culture conditions (78–80 °C and pH 3). The first activity promotes slipped-strand events within simple repetitive motifs, such as mononucleotide runs or triplet repeats, and the second promotes insertion of C opposite a potentially miscoding form of G, thereby avoiding G:C to T:A transversions.

Keywords

Trans-lesion DNA synthesis; Y-family DNA polymerase; *Sulfolobus* DNA polymerase Dbh; DNA-damage sensitivity; Spontaneous mutation spectra

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1 INTRODUCTION

1.1 Y-family DNA Polymerases

Trans-lesion DNA synthesis (TLS) enables DNA replication to proceed past an unrepaired template lesion, and involves recruitment of low-processivity DNA polymerases to the replication fork, where it synthesizes a short tract of DNA opposite the lesion [1]. One of the first TLS DNA polymerases to be characterized in functional terms was the UmuD₂C complex (DNA Pol V) of *E. coli*, which is required for UV-induced mutagenesis [2]. This and a number of other TLS DNA polymerases, including *E. coli* DNA Pol V and DNA Pol IV, yeast DNA Pol η , and human DNA Pol ι and DNA Pol κ , belong to a group of related DNA polymerases designated family Y. Consistent with the mutagenic role of *E. coli* DNA Pol V, all these enzymes yield high rates of base misincorporation *in vitro*, i.e., about 10^{-3} - 10^{-4} per nt [1,3]. These high error rates appear to reflect the intrinsic properties of Y-family DNA polymerases, which lack 3'-5' proofreading activity and have active sites that are relatively large, and thus structurally accommodating [4, 5]. Based on these properties, Y-family and other TLS DNA polymerases have been often viewed as providing a damage-coping strategy of last resort, in which the risk of generating a mutation represents the price paid for successful replication of a damaged genome.

1.2 Roles in Mutagenesis

Although error-prone *in vitro*, Y-family DNA polymerases are not considered major sources of replication errors on undamaged DNA *in vivo*, since few of the corresponding mutants exhibit obvious anti-mutator phenotypes [6-9]. The discrepancy between the high error rates observed *in vitro* and the minimal contribution to spontaneous mutation observed *in vivo* argues that TLS DNA polymerases are effectively excluded from replication forks during normal DNA synthesis. Conversely, several TLS DNA polymerases have been found to insert the correct base preferentially opposite particular template lesions, suggesting that these polymerases can suppress mutagenesis if recruited specifically to the appropriate DNA lesions [10-13]. The possibility that TLS polymerases provide lesion-specific accurate by-pass may account for the multiple TLS DNA polymerases with distinct functional properties found in some organisms [10]. At least four distinct Y-family DNA polymerases have been identified in humans, for example, and several of the remaining human DNA polymerases have also been implicated in TLS [14].

1.3. Structural biology of TLS

In contrast to humans, hyperthermophilic archaea have only a single Y-family DNA polymerase, despite the fact that the extremely high optimal growth temperatures of these prokaryotes should accelerate DNA damage by orders of magnitude relative to mesophilic organisms [15], which may, accordingly, create a relatively high concentration of DNA lesions able to interfere with genome replication. The Y-family DNA polymerases of two *Sulfolobus* spp., Dpo4 of *Sulfolobus solfataricus* and Dbh of *Sulfolobus acidocaldarius*, crystallize readily, which has facilitated extensive structural analysis of TLS, including crystallography of putative intermediates of the by-pass process [5]. Various catalytic properties of the *Sulfolobus* enzymes have also been analyzed in solution, including the specificity and kinetics of mis-incorporation and lesion bypass [16-19]. The *Sulfolobus* enzymes have thus become valuable models for the various microbial Y-family DNA polymerases that have been analyzed genetically, as well as for the human enzymes, several of which are implicated in disease [14].

Despite the detailed structural and catalytic data available for these two *Sulfolobus* DNA polymerases, basic questions about their native biological function have not been addressed. It remains unclear, for example, what DNA lesions are by-passed by Dpo4 and Dbh *in vivo*,

and whether certain mutations correlate with particular classes of lesions. To what extent are Dpo4 and Dbh excluded from undamaged template during normal DNA replication, and by what mechanisms does the *Sulfolobus* cell control this access? These questions concern how TLS DNA polymerases which are well understood in biochemical terms function to preserve their corresponding genomes against spontaneous and environmentally induced DNA damage. A Dpo4 mutant of *S. solfataricus* has been characterized with respect to cisplatin resistance [13], but, due to various genetic limitations, other functional analyses of the *Sulfolobus* DNA polymerases *in vivo* corresponding to those of bacterial and eukaryotic TLS enzymes have not been performed.

1.4 Goals of this study

In order to identify biological functions of a *Sulfolobus* Y-family DNA polymerase in its native context, we disrupted the gene encoding the Dbh DNA polymerase of *S. acidocaldarius*. We then compared disruptants to corresponding Dbh⁺ strains with respect to sensitivity to DNA damaging agents and rates of spontaneous mutation. To elucidate more completely the role that Dbh plays in spontaneous mutation, we also compared the types and positions of mutations in the Dbh⁻ and Dbh⁺ strains. The results demonstrate that the Dbh DNA polymerase has multiple functions affecting the stability of the *Sulfolobus* genome, suppressing certain mutations at particular sites and promoting other mutations elsewhere.

2 MATERIALS AND METHODS

2.1 Gene cloning and disruption

To clone the *S. acidocaldarius dbh* gene (Saci0554) onto a high-copy plasmid, a DNA fragment containing this gene plus fragments of the upstream and downstream genes (Saci_0553 and Saci_0555) was amplified by PCR using pOS21 [20] as a template, and oligonucleotide primers dbh.nhe.f and dbh.nhe.r (Table 1). After digesting with *NheI*, this fragment was ligated into *XbaI*-cut pUC19, creating pS12. To insert the *S. solfataricus pyrEF* (*SsopyrEF*) genes into the *dbh* gene, a DNA fragment containing *pyrE* (Sso1_1679), *pyrF* (Sso1_1680), and part of the upstream gene (Sso1_1678) was amplified using pMJ03 [21] as template and oligonucleotide primers pyr.bamH1.f and pyr.bamH1.r (Table 1). After digesting with *BamHI*, this fragment was ligated into *BglII*-cut pS12, creating pS17. The orientation of the *pyr* genes relative to *dbh* on pS17 was determined by diagnostic restriction digests.

Mutations in *dbh* were created using the QuikChange site-directed mutagenesis system (Stratagene) with oligonucleotides dbh.mut.forward and dbh.mut.reverse (Table 1). This procedure created a silent mutation at A₁₀₂, missense mutations D₁₀₅A, E₁₀₆A, which are in the conserved active site of Dbh, and a unique *NotI* site at nucleotides 313-320 of the *dbh* coding sequence. The construction of the resulting plasmid, pS40, was confirmed by restriction digests and sequencing using oligonucleotides dbh.forward and dbh.reverse (Table 1). A deletion of nucleotides 315 to 799 of the *dbh* coding sequence was then created by digesting pS40 with *EcoM* and *NotI*, blunting the ends using Klenow DNA polymerase, and ligating. The construction of the resulting plasmid, pS42 (Fig. 1A), was confirmed by sequencing using oligonucleotides dbh.forward2 (which is complementary to sequences 5' to the Dbh coding region) and dbh.reverse2 (Table 1).

2.2 Strain constructions

For electroporation into *S. acidocaldarius*, plasmid pS42 was protected from restriction by the *SuaI* endonuclease [22] by propagation in *E. coli* strain ER1821 harboring the compatible low-copy-number plasmid pM.EsaBC4I (New England Biolabs, Inc.). This *E. coli* strain modifies GGCC tetranucleotides with the same methylation pattern as the *SuaI*

methylase of *S. acidocaldarius* [23], thus preventing restriction in *S. acidocaldarius*. Modification was confirmed by resistance to *HaeIII*, and the plasmid DNA was electroporated into *S. acidocaldarius* strain MR31. Cells were plated on xylose-tryptone medium lacking uracil, and Pyr⁺ colonies were streaked for isolation on the same selective medium. Insertion of functional *SsopyrEF* into the mutated *dbh* gene was confirmed by PCR of genomic DNA. The corresponding strain was designated DG250.

Strain DG251 was constructed in the analogous manner by transformation of *S. acidocaldarius* strain MR31 to Pyr⁺ with purified DNA of plasmid pLK4a, which contains the *S. solfataricus pyrE* gene inserted into the *SpeI* site of the *S. acidocaldarius trpC* gene (Saci_1427) [24]. Disruption of the *trpC* gene was confirmed by phenotypic analysis (tryptophan auxotrophy) and PCR. Dbh⁻ strain DG259 is a derivative of DG250 with a large deletion (263 nt) of the *S. solfataricus pyrE* gene. The deletion was identified by screening 5-fluoroorotic acid (FOA)-resistant (Foa^R) mutants of DG250 for stability and confirmed by sequencing. Dbh⁻ strain CS2 was constructed from DG259 by transforming with plasmid pSAPE5, which contains the entire *pyrE* coding sequence plus flanking sequences [25], and selecting Pyr⁺ recombinants. Restoration of the *S. acidocaldarius pyrE* (*SacpyrE*) gene in CS2 was confirmed by PCR and sequencing.

2.3 Assays of sensitivity to DNA damage

MICs of chemical agents were determined by inoculating serial 1:2 dilutions in growth medium containing various concentrations of the agent, and scoring whether the culture grew, as described previously [24]. To assay survival of UV-C radiation, cultures were grown in liquid medium to exponential phase, harvested, and resuspended in UV-transparent dilution buffer to 1×10^8 cells/ml. Suspensions were irradiated as a thin (~2 mm) layer under a germicidal UV lamp and aliquots were withdrawn at regular intervals, diluted and plated under a dim red light before incubation (in the dark) to determine viable counts [24]. To assay the survival of cells as a function of cisplatin concentration, 10^5 cells were suspended in 0.5 ml of sterile dilution buffer 'Sdil' [26] containing varying concentrations of cisplatin (0-150 µg/ml). After overnight exposure at room temperature, the number of viable cells was determined by diluting and plating.

2.4 Assays of spontaneous mutation

Spontaneous mutation rates for *pyrE* were determined for each strain by fluctuation assays. Approximately 30 independent cultures (0.2 ml) were grown under non-selective conditions for 3-4 days to densities of about 10^8 cells/ml. The entire culture was then plated on selective medium containing FOA and uracil, and the resulting Foa^R colonies were counted. Total viable cells were estimated by averaging colony counts from 3-4 cultures per fluctuation test. Three replicates of fluctuation tests were completed for each strain.

The identities of the spontaneous Foa^R mutations were determined by PCR amplification and sequencing of the *pyrE* gene from one Foa^R colony isolated from each culture during fluctuation test assays. The -1 frameshifts mapping to the 7A tract at nt 545-551 have been shown to yield a leaky phenotype, causing the efficiency of their recovery to vary with slight changes in the stringency of selection [27]; these mutations therefore were excluded from the present analyses. Each mutation spectrum includes 50 to 110 independent mutants; about half of the mutants of DG185 were generated in this study, and the rest were recovered in a previous study using similar conditions [27].

2.5 Calculations

Spontaneous mutation rates were calculated using the Ma-Sandri-Sarkar maximum likelihood estimator (MSS-MLE), as implemented by the FALCOR interface [28]. Input

data were obtained from the fluctuation assays, and included the observed number of mutants per culture (**r**) and the average number of cells per culture (**Nt**). The samples of mutational spectra were compared for statistically significant differences by a Monte-Carlo approximation of Fisher's exact hypergeometric test, implemented by the Hyper-G program [29]. Calculation of the expected values and standard errors of the ratios given in Fig. 2B were as given in Rice [30].

3 RESULTS

3.1 Disruption of *S. acidocaldarius dbh* gene

To analyze the function of the thermostable Y-family DNA polymerase Dbh in its native context, we disrupted the *dbh* gene (Saci_0554) in the hyperthermophilic archaeon *S. acidocaldarius*. The *dbh* gene cloned in an *E. coli* plasmid was inactivated by point mutations removing active-site residues and by a large internal deletion that was replaced by two UMP biosynthetic genes (*pyrEF*) from the related species *S. solfataricus* (Fig. 1A). The multiply mutated *dbh* gene was transferred to the chromosome of *S. acidocaldarius pyrE* strain MR31 by homologous recombination between the sequences remaining on either side of the selectable cassette and the chromosomal sequences of the recipient cells. Putative integrants were purified clonally and integration was confirmed by PCR analysis and sequencing (Fig. 1B).

3.2 Assays of DNA repair capacity

To determine whether inactivating the Dbh DNA polymerase caused obvious deficiencies of DNA repair, *dbh*⁺ and *dbh*[−] strains were compared with respect to their sensitivity toward various DNA-damaging agents. The set of chemicals chosen produces a diversity of DNA lesions (notably cross-linked, covalently modified, or oxidized bases), and includes compounds that have been reported to distinguish *din*⁺ and *dinB* mutant strains of *E. coli* [31a, 31b]. MIC values of all compounds were very similar for both the *dbh*⁺ and *dbh*[−] strains (Table 2), thus indicating minimal impact of Dbh for tolerance of the corresponding DNA lesions. Analogous experiments measured the sensitivity to short-wavelength UV radiation, and revealed indistinguishable rates of killing as a function of UV dose (Fig. 2A). Also, in preparing various cell suspensions, we observed that ratios of viable cell concentration to turbidity were consistently identical for untreated cultures of *dbh*⁺ and *dbh*[−] strains, indicating that the *dbh*[−] strains do not accumulate a significant proportion of dead cells during normal growth.

Wong *et al.* [13] recently reported that a disruptant of the *dpo4* gene of *S. solfataricus* exhibited a detectable sensitivity to 10-25 ug/ml cisplatin. We therefore re-evaluated cisplatin sensitivity of *S. acidocaldarius dbh*[−] strains by using colony counts to quantify cell survival after overnight treatment of cells with various concentrations of cisplatin. The dose-response data for the wild-type strain DG185 versus the *dbh* mutant strains DG250 and CS2 indicated that the lethal effect of cisplatin was not statistically different between mutant and control strains across a wide range of concentrations, however (Fig 2B).

3.3 Assays of replication accuracy

To determine whether Dbh affects the accuracy of *S. acidocaldarius* genome replication, we analyzed forward (loss-of-function) mutation both quantitatively and qualitatively. In the first set of assays, the mutational target was *SsopyrE* inserted into the *dbh* gene (*dbh*[−] strain DG 250) vs. *SsopyrE* inserted into an unrelated gene (*trpC*) in the *dbh*⁺ control strain DG251; mutations inactivating *SsopyrE* were selected by the resulting resistant to FOA. The observed rates of spontaneous mutation for DG250 and DG251 were $3.2 (+0.4) \times 10^{-7}$ and $2.6 (+0.8) \times 10^{-7}$ events per cell division, respectively, indicating that there was no

significant difference in spontaneous mutation rates. The *SsopyrE* target was not in the same genomic location in these two strains, however, raising the possibility that differences in the gene context influenced the mutation rates.

To overcome this complication, we constructed strain CS2, in which the *SsopyrE* cassette inserted into *dbh* is inactivated whereas the function of the native *S. acidocaldarius pyrE* gene (*SacpyrE*) has been restored. This strain is isogenic to the wild-type strain DG185, with both strains providing the native *SacpyrE* as a mutational target. The spontaneous mutation rates to the *Foa^R* phenotype in CS2 and DG185 were $3.6 (+2.3) \times 10^{-7}$ and $2.8 (+1.5) \times 10^{-7}$ events per cell division, respectively. The results from both strain pairs (DG250 vs. DG251 and CS2 vs. DG185) therefore suggest that *Dbh* activity does not increase the total number of spontaneous mutations during normal growth under laboratory conditions. In fact, functional *Dbh* may decrease the overall rate of spontaneous mutation slightly, although the differences seen in our measurements were not statistically significant.

For each strain, the corresponding mutation spectrum was sampled by sequencing one randomly chosen *pyrE* mutant from each of a series of independent cultures (Fig. 3). In contrast to the similarity of overall mutation rates, this comparison revealed differences between the *dbh⁻* and *dbh⁺* strains with respect to the types of spontaneous mutations generated (Table 3). In particular, in the *dbh⁻* mutant strains the frequency of base-pair substitutions (BPSs) was higher, and contractions or expansions of triplet repeats were lower, than in the *dbh⁺* strains (Table 3). This pattern of offsetting effects is consistent with the minor impact of *dbh* disruption on the overall rate of spontaneous mutation, and suggests that during normal growth of *S. acidocaldarius*, *Dbh* suppresses BPSs but promotes the formation of frameshifts and small indels.

Differences among the spectra were confirmed by statistical methods. For example, χ^2 analyses confirmed that inactivating *dbh* increased the relative proportion of BPS versus frameshift mutations, and of transversion versus transitions among the BPSs (Table 4). This shift was evident when comparing the *pyrE* mutations of each strain pair individually, and when the mutations were pooled according to *dbh* genotype. An alternative Monte-Carlo analysis compared the complete spectra of each *dbh⁻/dbh⁺* strain pair, as well as the pooled results, according to the position of the *pyrE* mutations or the molecular nature of the sequence change. The results of this test (Table 5) indicated that both of these properties of spontaneous *pyrE* mutations responded to *Dbh* inactivation. Thus, both methods (χ^2 and Monte Carlo) indicated significant effects of *Dbh* inactivation for both the CS2/DG185 and DG250/DG251 strain pairs.

3.4 Effect of *Dbh* polymerase on specific mutational events

More detailed examination showed that *Dbh* inactivation increased the frequency of BPSs primarily by causing a higher frequency of transversions, nearly all of which were G:C to T:A events (Table 3). The impact was large in both strain pairs, increasing the percentage of G:C to T:A transversions from a level of 1-2% of all *pyrE* mutations in the two *Dbh⁺* strains to 19% and 33% in the *Dbh⁻* strains CS2 and DG251, respectively. These large increases in G:C to T:A transversions were seen in two target genes with rather different sequences (*SacpyrE* and *SsopyrE*), suggesting that this represents a general characteristic of inactivating *Dbh* *S. acidocaldarius*. Although G:C to T:A transversions occurred throughout *SacpyrE* in strain CS2, at least one hotspot was the center G in a TGGGA sequence at nt 248-252 (Fig. 3A). There was similar distribution of G:C to T:A transversions in *SsopyrE*, with GC to TA hotspots at the first G of the AGGA sequence at nt 198-201 and the G in the AGA sequence at nt 495-497 (Fig. 3C).

The G:C to T:A transversions in *Dbh*⁻ strains were further examined with respect to the sequence context. The probability of G:C to T:A transversions at a site generally increased with the number of G:C pairs at the site, but the probability per G:C base pair remained similar across these features in both target genes (at about 0.13 transversion events per G:C base pair). The single exception was a 5G hotspot in *SacpyrE* gene (where the transversion probability per bp was 0.6). Based on a total of 18 events at GG sites in the *Dbh*⁻ strains, transversions occurred preferentially at the 5' G (13 vs. 5 events). This pattern may reflect the mechanism by which certain bases are bypassed by *Dbh* or the relative susceptibility of the bases in short runs to damage. This latter possibility is supported by the fact that the TGGGGGC sequence at nt 198 to 204 in *SacpyrE* was a hotspot for a variety of mutational events in both the *dbh*⁻ and the *dbh*⁺ strains (Fig. 3A, 3B).

In the native *SacpyrE* target, *Dbh* inactivation revealed a different type of BPS hotspot at nt 353; the sequence motif (TGGTGGT) was similar to that of transversion hotspot at nt 250, but the BPSs at this site were G:C to A:T transitions (Fig. 3A). The *dbh*⁻ mutant strain (CS2) also exhibited a decrease in frameshift and small indel events. The affected sites are distributed throughout the target, but occur preferentially in two mononucleotide runs (Fig. 3A, 3B; Supplemental Data, Table S1). At the first site (six As at nt 28-33), loss of *Dbh* decreased the frequency of expansions of the run, whereas, at the second site (five Gs at nt 199-203), it decreased the frequency of contraction events. The results suggest that *Dbh* is required for about half of the +1 events at the 6 A run (nt 28-33) and half of the -1 events at the 5 G run (nt 199-203) that are normally observed in wild-type cells.

Further analysis of -1/+1 frameshift events as a function of the immediate sequence context (i.e., nature and length of mononucleotide run suggested that in both *dbh*⁻ and *dbh*⁺ strains, +1 frameshifts occur without obvious bias among the four bases, whereas -1 frameshifts at T's appear to be under-represented. Specifically, 21 single-A deletions are distributed over 427 A residues in the non-transcribed strand of the two target genes, compared to one single-T deletion distributed over 345 T residues in the same two genes (Supplementary Data, Table S1). This latter effect is thus strand-specific but independent of *Dbh* status.

Comparison of events that added or removed 3 or more bp (which we classified as indels) suggested that *Dbh* also promoted slipped-strand events at short triplet repeats. The site at nt 344-350 in *SacpyrE* (CTACTACT) was prone to expansion in strain DG185 but not in strain CS2 (Table 3, Fig. 3A, 3B). A similar, and more dramatic, effect was seen in *SsopyrE*, where spontaneous -3 events at a slightly longer triplet repeat (ATTATTATTA at nt 34-42) decreased nearly 10-fold in frequency in the *dbh*⁻ mutant strain relative to the *dbh*⁺ strain (Table 3, Fig. 3C, 3D).

4 DISCUSSION

4.1 Roles of the *Dbh* polymerase in *Sulfolobus* genome stability

The properties of *Dbh* and the related Dpo4 DNA polymerase have been well documented *in vitro*. High-resolution crystal structures of these DNA polymerases complexed with various substrates have allowed their properties to be interpreted in terms of conformations of template, incoming dNTPs, and active-site residues [5, 11, 12, 17, 18]. However, the extreme growth requirements of *Sulfolobus* species have prevented most of these analyses from being performed at physiological temperatures [11, 12, 16-19, 32-34]. Our analysis of *dbh*-disruption mutant strains of *S. acidocaldarius* provides the first systematic phenotypic analysis of a *Sulfolobus* TLS DNA polymerase in its native cellular context, and provides new evidence regarding the roles of these enzymes in enforcing the fidelity of genome replication.

Deleting *dbh* caused no measurable increase in sensitivity toward a diverse array of DNA-damaging treatments (Table 2). This result suggests either that the lethal DNA lesions induced by these various treatments are not usually bypassed by Dbh *in vivo*, or that another DNA polymerase or DNA repair pathway compensates very effectively for the absence of Dbh in the disruptants. Similarly, fluctuation assays using a well-characterized selection for forward mutation did not detect an obvious mutator function for Dbh under our normal laboratory growth conditions. Lack of a strong mutator role of the Dbh DNA polymerase *in vivo* is consistent with observed phenotypes of *S. cerevisiae* DNA Pol *eta* mutants [35] and *E. coli* *dinB* mutants [8, 9].

4.2. Effects on spontaneous mutagenesis

Despite the similarity of overall spontaneous mutation rates, comparison of spontaneous mutation spectra revealed differences in the molecular nature of spontaneous replication errors of *dbh*⁺ vs. *dbh*⁻ strains. In the *SacpyrE* target, Dbh was required for about half of the expansion events at the tract of six As at nt 28-33 and about half of the contraction events at the tract of five Gs at nt 199-203. In the alternative target (*SsopyrE*), Dbh was required for about 90% of the events that shortened a triplet repeat by one unit. Conversely, in *SacpyrE*, Dbh suppressed G:C to T:A transversions at nt 250 and G:C to A:T transitions at nt 353, and suppressed G:C to T:A transversions at multiple sites in *SsopyrE*. Perhaps significantly, both of the sites where Dbh suppressed BPS in *SacpyrE* were at short runs of G's (GGG and GG, respectively), suggesting that in the absence of Dbh some other DNA polymerase replicates these sites erroneously. Thus, *dbh*⁻ strains generally exhibited lower frequencies of small indels, but higher frequencies of BPSs. This indicates that the *Sulfolobus* TLS DNA polymerase Dbh plays at least two roles *in vivo*: one that generates small indels, and one that avoids BPSs.

4.3 Implications for DNA damage in the *Sulfolobus* genome

The existence of Dbh-suppressed mutations suggests that *Sulfolobus* genomes accumulate DNA lesions that are bypassed accurately by Dbh but erroneously by one or more other DNA polymerases. The alternative polymerase activities may be constitutive, or they may be suppressed when Dbh is present, or induced or activated by the loss of Dbh. G:C to T:A transversions accounted for nearly all of the Dbh-suppressed mutations observed in the two analyzed *pyrE* genes, and the molecular events leading to these mutations have important implications for the biological role of the Dbh DNA polymerase in its native context.

Prominent among the candidates for spontaneous DNA lesions are abasic sites, which impede replication and can pose a threat to genome stability. The temperature-dependence of depurination rates of pure DNA in buffered solutions [36] predicts that abasic sites should form at extremely high rates in hyperthermophiles. For example, the genomic DNA of *Pyrococcus abyssi*, a neutrophilic archaeon that grows at 95°C, is expected to depurinate at nearly 3,000 times the rate of the DNA of *E. coli* growing at 37°C [37]. However, the steady-state densities of abasic sites in growing and stationary-phase *P. abyssi* cells have been found to average only 10-fold higher than in *E. coli* cells [38]. This relatively modest difference in damage levels suggests that *P. abyssi* has a repair capacity that exceeds that of *E. coli*, but that this higher capacity does not compensate completely for the accelerated depurination at the higher growth temperature [38]. A similar situation may prevail in *Sulfolobus*, as the pH- and temperature-dependence of DNA depurination kinetics in solution [36] predicts a rate about 2000-fold higher in growing *Sulfolobus* cells than in *E. coli* cells.

Depurination at G could, in principle, generate the G:C to T:A transversions we observed in the absence of Dbh. Both *S. solfataricus* DNA Polymerase B1 [32] and the corresponding

Pyrococcus DNA polymerase [38] have been shown to bypass abasic sites using the ‘A-rule’, i.e., inserting dAMP opposite the abasic lesion. By this scheme, an abasic site generated by loss of guanine and replicated by DNA PolB1 would dictate insertion of dA, yielding a G:C to T:A transversion at that position after another round of replication. High temperature promotes loss of A at about 73% the rate of G loss [39a], but *Sulfolobus* genomes have 32-38 mol% G+C [39b], making A is nearly twice as abundant as G. As a result, non-enzymatic depurination is predicted to remove A about 25% more frequently than G, on average. The sites of A loss are predicted to be converted by DNA PolB1 (and any other A-rule DNA polymerase) into A:T to T:A transversions, yet such transversions are not observed in spontaneous mutation spectra of the *dbh*-mutant strains (Fig. 3). Furthermore, Dpo4 has itself been found to incorporate dAMP opposite abasic sites [33]. If Dbh behaves similarly, bypass of abasic sites by Dbh would not produce a different outcome than bypass by DNA PolB1 or another A-rule polymerase.

An alternative lesion that might account for the spontaneous mutational specificity we observed is 7,8-dihydro-8-oxo-guanine (8oxoG), which forms as a result of normal aerobic metabolism [40a]. Since *Sulfolobus* spp. are obligate aerobes, 8oxoG is expected to form relatively frequently in their genomes, although it may also be repaired with reasonable efficiency (*S. acidocaldarius* gene Saci_1367 encodes a putative OGG2-type DNA glycosylase; the related protein of *S. solfataricus* (encoded by SSO0904) is presumably the one crystallized and analyzed structurally by Faucher et al. [40b]). In other organisms, 8oxoG is bypassed by most DNA polymerases with the insertion of A, resulting in G:C to T:A transversions [41-44]. In contrast, Y-family DNA polymerases, including Dbh and closely related archaeal enzymes, have been shown to bypass 8oxoG accurately *in vitro* by inserting C [11, 12, 35]. Thus, the following hypothesis accounts for the available molecular data: Dbh can correctly insert C opposite 8oxoG, but in the absence of Dbh, more 8oxoG lesions are bypassed by one or more *Sulfolobus* DNA polymerases that follow the “A rule”;

Dbh also suppressed G:C to A:T transitions at the hotspot at nt 353 in *SacpyrE*. This result suggests that, at this site, either the specificity of by-pass is different or a different lesion occurs than occurs at the more common sites of G:C to T:A transversions. Thus, if the lesion at nt 353 were a damaged G, insertion of T opposite this lesion by a DNA polymerase other than Dbh would be required to generate the transitions observed. Alternatively, C may be the damaged residue, to which one of the most plausible routes would be deamination. The resulting dU could, in principle, be replicated accurately, although based on analyses *in vitro* this is predicted to be slow and preceded by stalling of the replicative DNA polymerase [45, 46]. Alternatively, the dU may be converted to an abasic site *via* one of the uracil DNA glycosylases that have been detected in *Sulfolobus* [47, 48]. In the absence of Dbh, the expected result at either an unrepaired dU or an abasic site is insertion of A by the replicative family-B DNA polymerase [38, 49], which also would result in the observed G:C to A:T mutation. The damaged-C scenario thus seems to provide a plausible mechanism for the observed transitions, but it implies that in the wild-type strain Dbh would insert G opposite either dU or an abasic site at this position. To our knowledge, such a property has not been observed for Dpo4 or Dbh *in vitro*.

In contrast to Dbh-suppressed mutations, molecular properties of the spontaneous Dbh-dependent mutations do not specifically implicate apurinic sites or 8oxoG lesions. For example, the most prominent hotspot in our analyses was the tract of five guanines at nt 199-203 in *SacpyrE*; at this site, Dbh enhanced –1 frameshifts (Fig. 3A vs. 3B). *In vitro*, both Dpo4 and Dbh bypass abasic sites with decreased efficiency and generate a variety of mutations, including not only GC to TA transversions but, also, –1 frameshifts [33, 34]. On an intact template *in vitro*, however, the most common frameshifts are –1 deletions in short mononucleotide runs in a template sequence of the form 5′ G (Y)_n (where Y=T or C). The

bias favoring these events is very strong, such that Dbh promotes such events at frequencies approaching 50% [16]). The sequence of the hotspot in *SacpyrE* fits this deletion-prone motif, suggesting that the mutations observed there result from Dbh replicating undamaged template. However, Dbh only contributed about half of these mutations, indicating that at least one other DNA polymerase makes similar errors at similar frequencies at this site. Furthermore, mononucleotide runs of four G:C base pairs occurring elsewhere in *SacpyrE* and *SsopyrE* are not obvious hotspots for Dbh-dependent –1 frameshifts (Fig. 3B, 3D).

4.4 Conclusions

S. acidocaldarius Dbh and the related DNA polymerase Dpo4 have provided some of the most detailed biochemical and structural data currently available regarding TLS by Y-family DNA polymerases. This reflects, in part, the relative ease of purifying and crystallizing these intrinsically thermostable enzymes, which are adapted to function in archaeal cells that grow optimally at about 80° C. This thermal adaptation has the technical consequence that catalytic and structural properties of Dpo4 and Dbh are typically measured at non-physiological temperatures, which may obscure biologically important functions. The availability of Dbh-disruption strains and quantitative genetic assays in *S. acidocaldarius* provide a basis for experimental analysis of the roles which Dbh and other Y-family DNA polymerases of *Sulfolobus* spp. play in maintaining genome stability at extremely high temperatures. Our phenotypic analysis of mutants indicates that Dbh avoids the majority of G:C to T:A transversions that would otherwise accumulate in *S. acidocaldarius* genomes. We hypothesize that many of these sites of potential transversions are spontaneously damaged G lesions, and that Dbh suppresses mutation by inserting dC opposite them, as demonstrated previously *in vitro* by incorporation assays and crystal structures. Our data also show, however, that Dbh also generates deleterious mutations, primarily 1- or 3-bp indels at simple repeats. Purified Dbh is known to promote frameshifts (especially –1 events) at extremely high frequency on undamaged templates *in vitro*, but to our knowledge –3 events have not been observed *in vitro*, and may reflect an activity evident only at physiological temperature or only *in vivo*. The site-specificity and relatively low frequency of the Dbh-dependent indels we observed *in vivo* suggest restricted access to the replication fork or additional forms of control, which, as in other TLS systems, should be crucial to the overall success of TLS as a damage-coping strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights (Sakofsky et al.)

Dbh is the Y-family trans-lesion DNA polymerase of *Sulfolobus acidocaldarius*

Crystal structures of this polymerase provide valuable information on TLS

To analyze its biological function, the *S. acidocaldarius dbh* gene was disrupted

The phenotype of disruptants indicates both mutagenic and anti-mutagenic roles

A:

B:

Figure 1.**Construction of *dbh*⁻ strain CS2**

Panel A. Cloning and disruption of *dbh*. Diagram shows major features of construct pS42, containing the disruption of *dbh* (Saci_0554) that was transferred to the *S. acidocaldarius* genome. For details of construction, see Materials and Methods.

Panel B. Confirmation of *dbh* disruption. PCR products amplified from genomic DNA of wild-type *S. acidocaldarius* (lanes 1 and 3) and *dbh* disruption mutants (lanes 2 and 4). Lanes 1 and 2 show amplification of the whole gene and lanes 3 and 4 show amplification of an internal portion of the gene. The primers used to amplify the whole gene were Saci0554f1 and Saci0554r1 and the primers used to amplify the internal portion were Saci0554f3 and Saci0554r3 (see Table 1). MW: molecular weight marker (bacteriophage λ DNA digested with BstEII); fragment lengths in bp are shown in the left-hand margin.

Figure 2.

Sensitivity to killing by UV or cisplatin

Panel A shows the fraction of cells surviving the indicated fluence of UV-C; error bars indicate standard deviations. Panel B shows the fraction of cells surviving overnight room-temperature treatment of cisplatin at the indicated concentration ($\mu\text{g/ml}$); error bars indicate standard errors. Symbols: circles, strain CS2; triangles, strain DG250; squares, strain DG185; diamonds, strain DG251.

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Figure 3.

Spontaneous *pyrE* mutation spectra in disruptant and control strains.

The sequence of the *S. acidocaldarius* (A and B) or *S. solfataricus* (C and D) *pyrE* coding region with markings indicating the spontaneous mutations recovered from (A) CS2 (B) DG185 (C) DG 250 (D) DG 251. BPSs are denoted with an A, T, C, or G above a base in the sequence. Deletion mutations (> 3bp) are represented by bold italicized text. Tandem duplications (> 6bp) are indicated by an underline or overline. A '+' or a 'Δ' sign above the sequence denotes a small insertion or deletion (< 6bp), respectively, of the base(s) below it. Placement of these symbols above mono-nucleotide runs is arbitrary, as all positions of a run are genetically equivalent. Single base pair insertions that differ from their adjacent bases is shown by a 'v' sign positioned in between two bases on the sequence with the insertion above it. Bracketed text with a '+' or 'Δ' indicates insertions or deletion mutations, respectively, occurring in a single mutant.

Table 1

Strains and DNAs used in this study.

DESIGNATION	GENETIC FEATURES	SOURCE OR DERIVATION
<i>S. acidocaldarius</i> strains		
DG185	wild-type	obtained as ATCC 33909
MR31	<i>pyrE131</i> (18-bp internal deletion)	[50]
DG 250	<i>pyrE131 dbh::SsopyrE⁺</i>	this work
DG 251	<i>pyrE131_{Sac} trpC::SsopyrE⁺</i>	this work
DG259	<i>pyrE131 dbh::SsopyrE⁻</i>	spontaneous Foa ^R of DG250
CS2	<i>dbh::SsopyrE⁻</i>	DG259 transformed to Pyr ⁺
<i>E. coli</i> Plasmids		
pS42	<i>dbh</i> disrupted by Sso <i>pyrE⁺</i>	this work
pSAPE5	<i>SacpyrE⁺</i>	[25]
pPCBE12	3' portion of <i>trpC</i> in pUC19	Phil Clark (unpublished)
pMJ03	<i>Sulfolobus</i> shuttle vector	[21]
pLK4a	<i>SsopyrE</i> in <i>trpC</i> insert of pPCBE12	this work
PCR primers		
SsoPEAvrKpnI	GCAGCCTAGGTACCGATATGAGAGAGGTTTATCCATTGC	
SsoPEFKpnAvrfI	GCACCTAGGACGTGTCTTAATCTCACAAAGCCCTTAT	
Saci0554f1	CGC ATT TAA TTA TGC GTA TGT GC	
Saci0554r1	GCT ATT AAA GGA AAG AAG GCA GT	
Saci0554f3	GCA ATA TAT GTG CCT ATG AGA A	
Saci0554r3	ACA TCT CTT GTG TTA TAG GGT A	
dbh.nhe.f	TAGTT GCTAGC TTT AGA TAC AAC AGG TGC T	
dbh.nhe.r	TAATT GCTAGC CCT GTC GTG AAC ATA CTA G	
pyr.bamH1.f	TCTC GGATCC GAA TAA TGC TGC C	
pyr.bamH1.r	TTAC GGATCC TTC CTC GTG TAG ATT TTC	
dbh.mut.forward	GAA GTA GCT AGT ATA GCG GCC GCT TAC TTA GAC GTA AC	
dbh.mut.reverse	GTT ACG TCT AAG TAA GCG GCC GCT ATA CTA GCT ACT TC	
dbh.forward	GTT TGC GTA TAT TCC GGT AGA ACC	
dbh.reverse	TGC CTA ATT CAT TTA GTC TCC TAG CC	
dbh.forward2	GGA TCT TCC ATA ATA GCT ATA ACA G	
dbh.reverse2	GGC ATT CTC AAA TAG GAT AAT GAA C	
XhoI pS42 1423	ATTAAGT CTCGAG CGA AAT TCA TAC GTA GAG ACT CTG	
XhoI pS42 2689	GAAACG CTCGAG TAA CAT TAA TAG GGG AAA ATC TAC	
pyrEF.F-Nhe	TCTCGCTAGCGAATAATGCTGCC	
pyrEF.R-NheI	TTACGCTAGCTTCCTCGTGTAGAT	

Table 2Sensitivity of *dbh*⁺ and *dbh*⁻ strains to DNA-damaging chemicals^a

Chemical	Type of Damage	DG185 <i>dbh</i> ⁺	DG 251 <i>dbh</i> ⁺	DG 250 <i>dbh</i> ⁻
Cisplatin (cis platinum(II) diammine dichloride)	intra- and inter- strand crosslinks	37.5	37.5	37.5
MNNG (N-methyl N'-nitro N-nitrosoguanidine)	methylation	37.5	37.5	37.5
Mechlorethamine (2-chloro-N-(2-chloroethyl) -N-methyl-ethanamine)	intra- and inter- strand crosslinks	300	300	300
Metronidazole (2-(2-methyl-5-nitro-1H -imidazol-1-yl) ethanol)	bulky adduct	2.5×10 ³	--- ^b	2.5×10 ³
Nitrofurazone	bulky adduct	50	50	50
4- nitroquinoline N-oxide	bulky adduct	0.3	0.6	0.6
Butadiene diepoxide	intra-strand crosslinks	75	75	75
Hydrogen peroxide	oxidation	62.5	62.5	62.5

^a MIC values (μg/ml) were determined from 3 replicates with each chemical except hydrogen peroxide, which was determined from 2 replicates. All MIC values among replicates agreed within one dilution of each other; the mode or median is shown.

^b Measurement not done.

Table 3

Summary of spontaneous *pyrE* mutations in *Dbh⁻* vs. *Dbh⁺* strains

Mutational target	CS2	DG 185	DG250	DG 251	Total <i>Dbh⁻</i>	Total <i>Dbh⁺</i>	Motif
	<i>SacpyrE</i>	<i>SacpyrE</i>	<i>SsopyrE</i>	<i>SsopyrE</i>			
<i>Dbh</i> genotype	<i>dbh⁻</i>	<i>dbh⁺</i>	<i>dbh⁻</i>	<i>dbh⁺</i>			
Total Mutations	111	109	84	52	195	161	
BPS	48	28	52	14	100	42	
Transitions	17	18	10	12	27	30	
Transversions	31	10	42	2	73	12	
Frameshift	53	68	28	20	81	88	(see Table 4)
+1 bp in runs	25	33	14	8	39	41	
-1 bp in runs	18	22	3	4	21	26	
+1 bp not in runs	8	11	7	6	15	17	
-1 bp not in runs	1	2	2	2	3	4	
+2 bp at dinucleotide repeats	1	0	2	0	3	0	(AT) _n
Indels	10	13	4	18	14	31	
Deletions >3 bp	1	2	0	1	1	3	
-3 bp at trinucleotide repeat	0	0	2	12	2	12	(ATT) _n
Duplications >6 bp	9	8	2	5	11	13	
+3 or +6	0	3	0	0	0	3	(CTA) _n

Table 4

χ^2 and p-values for mutation comparisons of *dbh*⁻ versus *dbh*⁺ strains

	CS2 vs. DG185 <i>SacpyrE</i>		DG250 vs. DG251 <i>SsopyrE</i>		<i>dbh</i> ⁻ vs. <i>dbh</i> ⁺	
Fraction of Total	χ^2	P	χ^2	P	χ^2	P
BPS vs. Frameshifts vs. Indels [*]	7.0	0.03	16	2.2×10 ⁻⁶	27	1.5×10 ⁻⁶
Fraction of BPS						
Ts vs. Tv [*]	5.9	0.02	19	1.3×10 ⁻⁵	24	8.3×10 ⁻⁷
Fraction of Frameshifts [†]						
+1 vs. -1	0.04	0.85	0.72	0.40	0.16	0.69
Ins vs Dels	0.06	0.81	0.07	0.79	1.9	0.17
Eliminating Indels						
BPS vs. Frameshifts [*]	6.4	0.01	5.6	0.018	15	1.0×10 ⁻⁴

Yate's correction was applied if the value of any cell was 5

^{*} The difference between *dbh*⁻ and *dbh*⁺ is significant at p 0.05

[†] The small number of +2 frameshifts were not included

Table 5
Hypergeometric Statistical Analyses of Mutation Spectra^a

	CS2 vs. DG185 <i>Sac pyrE</i>	DG250 vs. DG251 <i>Sso pyrE</i>	Dbh ⁻ vs. Dbh ⁺
+ 1 Frameshifts ^b	0.36	0.61	0.74
BPS ^c	0.0009 [*]	< 0.0001 [*]	< 0.0001 [*]
Indels ^d	0.55	0.53	0.10
Hotspots ^e	0.0041 [*]	0.0002 [*]	0.0001 [*]
Mutation Position ^f	0.0095 [*]	0.044 [*]	----- ^h
Mutation Type ^g	0.0015 [*]	< 0.0001 [*]	< 0.0001 [*]

^aEstimated p-values from hyper-geometric tests [29] comparing the occurrence of the indicated categories of mutation in strain CS2 or DG250 to those of the corresponding *dbh*⁺ strain.

^{*} Asterisks indicate significance at 0.05 level.

^b Single-frameshift categories were +A, -A, +C, -C, +G, -G, +T, -T

^c BPS categories were AT → TA; CG → GC; AT → GC; AT → CG; GC → AT; CG → AT

^d Indel categories included deletions of 4-10 or 21-30 bps and tandem duplications of 4-10, 11-20, 21-30, 31-40, 41-50, or 51- 60 bps

^e Hotspots were defined as 3 or more mutations occurring at a particular locus

^f Mutation position was considered to be the first nucleotide affected

^g Mutation type categories included ±1 and ±2 frameshifts, ±3 bps, BPS and indels as described above

^h not applicable