Lesion-induced Mutation in the Hyperthermophilic Archaeon Sulfolobus acidocaldarius and its Avoidance by the Y-family DNA Polymerase Dbh

Cynthia J. Sakofsky*1 and Dennis W. Grogan§

Department of Biological Sciences
University of Cincinnati

1Current address:
Department of Biology
University of Iowa
143 Biology Building
Iowa City, IA 52242-1324
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Corresponding author:
Dennis Grogan
Department of Biological Sciences
614 Rieveschl Hall, Clifton Court
Cincinnati Ohio 45221-0006
513-556-9748
grogandw@ucmail.uc.edu
ABSTRACT

Hyperthermophilic archaea offer certain advantages as models of genome replication, and Sulfolobus Y-family polymerases Dpo4 (S. solfataricus) and Dbh (S. acidocaldarius) have been studied intensively in vitro as biochemical and structural models of trans-lesion DNA synthesis (TLS). However, the genetic functions of these enzymes have not been determined in the native context of living cells. We developed the first quantitative genetic assays of replication past defined DNA lesions and error-prone motifs in Sulfolobus chromosomes, and used them to measure the efficiency and accuracy of bypass in normal and dbh-minus strains of Sulfolobus acidocaldarius. Oligonucleotide-mediated transformation allowed low levels of abasic-site bypass to be observed in S. acidocaldarius, and demonstrated that the local sequence context affected bypass specificity; in addition, most erroneous TLS did not require Dbh function. Applying the technique to another common lesion, 8-oxo-dG, revealed an anti-mutagenic role of Dbh. The efficiency and accuracy of replication past 8-oxo-dG was higher in the presence of Dbh, and up to 90% of the Dbh-dependent events inserted dC. A third set of assays, based on phenotypic reversion, showed no effect of Dbh function on spontaneous -1 frameshifts in mononucleotide tracts in vivo, despite the extremely frequent slippage at these motifs documented in vitro. Taken together, the results indicate that a primary genetic role of Dbh is to avoid mutations at 8-oxo-dG that occur when other Sulfolobus enzymes replicate past this lesion. The genetic evidence that Dbh is recruited to 8-oxo-dG raises questions regarding the mechanism of recruitment, since Sulfolobus spp. have eukaryotic-like replisomes but no ubiquitin.
INTRODUCTION

The fact that DNA damage occurs in all living cells poses a threat to the accurate replication and partitioning of their genomes. Cells counter this threat with an array of diverse damage-coping systems, some of which repair the DNA, whereas others enable replication to continue past persistent lesions. Lesion bypass, also termed damage tolerance, can follow various alternatives, which are broadly distinguished as error-free vs. error-prone (Lehman et al. 2007). The error-free pathways generally use recombination-associated functions to pair a strand, newly synthesized on intact template, to the damaged DNA strand; the mechanisms for this include transient reversal of the replication fork and strand exchange at gaps left behind the fork (Sale 2012). These mechanisms bypass lesions accurately, although they can promote other forms of genetic instability, such as ectopic recombination between dispersed repeats (Izhar et al. 2013).

Error-prone mechanisms of lesion bypass, in contrast, use specialized, trans-lesion synthesis (TLS) polymerases to continue strand elongation using the damaged template; most of these enzymes belong to the Y family of DNA polymerases (Sale et al. 2012). Large catalytic sites, combined with the absence of proofreading activity, allow these polymerases to insert nucleotides opposite diverse DNA lesions, but also limit the accuracy with which they replicate intact template (Yang & Woodgate 2007; Pata 2010). This latter property creates a risk of mutagenesis, which is mitigated somewhat by other features of these enzymes, including low processivity and a bias toward insertion of the correct nucleotide opposite certain DNA lesions (Jarosz et al. 2007). TLS polymerases are nevertheless subject to mechanisms which limit their access to template and co-ordinate TLS with other lesion-bypass strategies. The potential
complexity of this co-ordination, and its importance for achieving genetic stability, is illustrated in human cells, which have an extensive repertoire of TLS polymerases (Waters et al. 2009).

Y-family DNA polymerases of hyperthermophilic archaea have become valuable models for elucidating mechanisms of TLS, due in part to their favorable crystallization properties. As the third, deeply diverging, lineage of cellular organisms distinct from bacteria and eukaryotes, archaea provide a unique perspective on the diversity and evolution of DNA replication and damage-coping mechanisms. The scheme of DNA replication in archaea incorporates many eukaryotic features but is simpler than that of eukaryotes (Ishino et al. 2013). Archaeal replication origins are recognized by Cdc6/Orc1 homologues, and the replicative helicase is an MCM homologue that moves 3’ to 5’ on the leading-strand template (Barry & Bell 2006; Li et al. 2013). Two molecules of a B-family polymerase, one for each arm of the fork, are complexed to corresponding rings of PCNA, which attach to RFC and four GINS-like proteins to form a replisome (Li et al. 2013). Archaeal Okazaki fragments are short, and are processed for ligation by a Fen1 homologue (Barry & Bell 2006; Li et al. 2013; O’Donnell et al. 2013; Raymann et al. 2014).

The systems by which archaea cope with DNA damage and replication errors are less clear, however. Although several hyperthermophilic archaea encode the components necessary for complete systems of base excision repair (BER), homologous recombination, and TLS, all hyperthermophilic archaea lack certain nucleotide excision repair (NER) and mismatch repair (MMR) proteins that are broadly conserved in other organisms, including mesophilic archaea (White & Grogan 2008). For a few hyperthermophilic archaea, including Sulfolobus spp. (which
grow optimally at about 80° C and pH 3), the functional properties of genome-maintenance systems have been investigated by genetic analyses. Homologous recombination in *S. acidocaldarius* appears to rely heavily on a short-patch, gene-conversion process which is not affected by mismatches in the substrates, and thus seems capable of promoting ectopic events (Grogan & Stengel 2008; Grogan & Rockwood 2010; Rockwood *et al.* 2013).

Diverse DNA-damaging treatments induce mutation in *Sulfolobus* cells, providing functional evidence of error-prone TLS (Reilly & Grogan 2002). *Sulfolobus* species each have a single Y-family polymerase; the enzyme from *S. solfataricus* (Dpo4) and its counterpart from *S. acidocaldarius* (Dbh), exhibit the low fidelity typical of these enzymes, making them potential sources of mutations. The *S. acidocaldarius* polymerase Dbh, appears to be particularly mutagenic; when Dbh replicated short homopyrimidine tracts followed by G (i.e., 5'G(Y)_n template) *in vitro*, up to 50% of the extension products had single-nt deletions within the tract (Potapova *et al.* 2002).

Despite the multiple threats to genome stability posed by growth temperatures that accelerate DNA damage, the presence of an error-prone DNA polymerase, the lack of MutS and MutL proteins, and promiscuous recombination, *Sulfolobus* cells achieve remarkably accurate genome replication. *S. acidocaldarius*, for example, exhibits an overall error rate per genome replication below that of most MMR-proficient mesophilic micro-organisms (Grogan *et al.* 2001), and an intra-specific sequence divergence far below that of *Sulfolobus islandicus* (Mao & Grogan 2012b). The mechanistic basis of the observed replication fidelity remains unclear. In addition to the inability to identify certain repair genes in *Sulfolobus* genomes, the genetic consequences
of unrepaired lesions and the specific roles played by TLS polymerases in determining these consequences have not been defined experimentally.

Two widely occurring DNA lesions, abasic, or ‘AP’, sites and 7,8-dihydro-8-oxo-deoxyguanosine (8-oxo-dG), are expected to arise frequently in *Sulfolobus* chromosomes. The extremely high temperatures and mildly acidic conditions within actively dividing *Sulfolobus* cells (Meyer & Schäfer 1992) create abasic sites at high rates both directly, *via* spontaneous depurination (Lindahl & Nyberg 1972), and indirectly *via* enzymatic removal of deaminated bases, which also form spontaneously at high temperatures (Lindahl & Nyberg 1974). *In vitro*, chemically stabilized abasic sites impede Dbh progression (Gruz *et al.* 2001), and the residual bypass that does occur tends to skip over the lesion, generating a -1 bp deletion (Potapova *et al.* 2002). The catalytic properties of Dbh thus do not seem to implicate it specifically in coping with unrepaired abasic sites in the *S. acidocaldarius* genome. Similarly, 8-oxo-dG forms in the DNA of all aerobes by spontaneous oxidation of guanine, which is predicted to be accelerated under aerobic conditions by high temperature (Cadet *et al.* 1997). Unlike an abasic site, 8-oxo-dG does not significantly alter the helical structure of DNA, and does not block most polymerases (Freisinger *et al.* 2002), but its structure encourages insertion of dA, generating G:C to T:A transversions (Hsu *et al.* 2004). *In vitro*, Dbh and Dpo4 preferentially insert dC opposite 8-oxo-dG (Gruz *et al.* 2001; Rechkoblit *et al.* 2006; Zang *et al.* 2006; Maxwell & Suo 2012); this predicts that these enzymes would suppress transversion *in vivo* if recruited to 8-oxo-dG during replication of *Sulfolobus* chromosomes. Consistent with this prediction, disrupting the *dbh* gene (Saci_0554) resulted in increased rates of spontaneous G:C to T:A transversions in a chromosomal gene (*pyrE*) where forward mutations can be selected (Sakofsky *et al.* 2012).
The present study developed new genetic assays, based on existing selections for loss or restoration of pyrE gene function in *S. acidocaldarius* (Grogan and Stengel 2008), and used them to address two related questions: i) what genetic consequences result from placing abasic sites and 8-oxo-dG in the chromosome of an hyperthermophilic archaeon, and ii) what roles do the Y-family polymerase and other DNA polymerases of the organism play in determining these outcomes? The results provide evidence for lesion-specific recruitment of a Y-family polymerase in archaea, mutagenesis by B-family *Sulfolobus* polymerases, an influence of sequence context on the accuracy of bypass, and avoidance of 8-oxo-dG-promoted mutation mediated by Dbh.

**MATERIALS AND METHODS**

**Strains and growth conditions:** The strains and oligonucleotides used in this study are described in Supporting Information (Tables S1 - S3). *S. acidocaldarius* pyrE mutations were selected in wild-type and *dbh*− backgrounds via spontaneous 5-fluoro-orotic acid (FOA) resistance (Grogan & Gunsalus 1993), which is similar to the selection of *Saccharomyces cerevisiae ura3* mutants (Boeke *et al.* 1984). Mutants were confirmed phenotypically by pyrimidine auxotrophy, and the identity of the mutation was confirmed by dye-terminator sequencing of PCR products of the pyrE gene. Pyr− strains were grown on xylose-tryptone (XT) medium supplemented with uracil (20 mg/L) (Grogan and Gunsalus 1993). Pyr+ revertants or transformants were selected by plating on solid XT medium lacking uracil. For genotyping, Pyr+ transformants were clonally purified on selective plates, followed by growth in liquid culture and extraction of genomic DNA.
Genetic assays: Rates of reversion were measured by fluctuation assays of 16-20 independent cultures (0.2 mL each), grown from inocula of about $10^4$ cells to a final size of about $10^8$ cells. The average number of viable cells per culture was determined by dilution and plating on non-selective medium, and the number of Pyr$^+$ revertants was determined individually for each culture by harvesting the entire culture and plating on selective medium (xylose-tryptone without uracil). The rate of mutation was calculated using the Ma-Sandri-Sarkar maximum-likelihood estimator via the FALCOR web interface (Hall et al. 2009).

To analyze TLS, isogenic pairs of $dbh^+$ and $dbh^-$ strains were prepared in which each strain contained a non-synonymous BPS inactivating the pyrE gene next to a synonymous site in the coding sequence. Three alleles in the *S. acidocaldarius* pyrE gene satisfied these criteria and were used in the study (Fig. 1 (e-g)); a fourth site in a heterologous pyrE gene (*Sulfolobus solfataricus*) was used to evaluate 8-oxo-dG bypass only (Fig. 1(h, i)). Corresponding sets of commercially synthesized, single stranded (ss) oligonucleotides (Table S2) were prepared as 10μM or 20μM stock solutions and were electroporated into recipient cells as previously described (Grogan & Stengel 2008). All four bases at the query sites were confirmed to yield transformants; a strand bias favoring transformation by anti-sense oligonucleotides, corresponding to that observed for other pyrE alleles (Grogan & Stengel 2008), was also confirmed (Table S4).

Except for the assays depicted in Fig. 1 (h, i), lesion-bearing oligonucleotides electroporated into pyrE recipients carried a centrally located abasic site or 8-oxo-dG residue directly adjacent to the
nucleotide used for selection (Table S2). The abasic site was a tetrahydrofuran (THF) spacer incorporated by synthesis (Integrated DNA Technologies, Coralville, Iowa USA). Oligonucleotides containing 8-oxo-dG lesions were synthesized and purified by HPLC at the University of Utah DNA/Peptide Core Facility.

To identify the outcome of bypass events, genomic DNA was extracted from clonally purified transformants, and the S. acidocaldarius pyrE region was amplified by PCR (Table S4). The appropriate synonymous site within pyrE was then scored by ligase chain reaction (LCR; Wiedmann et al. 1994) using the pyrE PCR product as template, as follows. A set of four upstream oligonucleotides probed for each of the four nucleotides at the site of the lesion, and four corresponding LCR reactions were completed for each recombinant (Table S3). The ligation product was detected by analyzing a melting curve from 65°C to 95°C of the DNA upon completion of the LCR cycles. All transformants were confirmed to yield product in one, but only one, of the four nucleotide-specific LCR reactions.

**AP Endonuclease assays:** S. acidocaldarius cells were suspended in 50 mM MES buffer, potassium salt (pH 6.3), and disrupted by sonication; the resulting extracts were clarified by centrifugation (10 min, 13000 xg), and stored frozen until use. Each assay contained 5 μL extract (27 μg protein), 40 pmol fluorescein-labelled oligonucleotide (5'-FAM), 50 mM MES buffer (as above), and 2 mM MgSO₄. Sequences of the substrate oligonucleotides are given in Table S3. Duplex substrates were formed by annealing a fluorescently labeled ssDNA with a 20% excess of unlabeled complement.
Reactions were started by adding cell extract to the chilled mixture, followed by mixing and immediate incubation at 70°C. Reactions were stopped by rapid cooling to 25°C and adding an equal volume of 90% formamide-50 mM EDTA, followed by heating 5 min at 100°C. To detect strand scission products, the entire assay mixture was electrophoresed in a 15% polyacrylamide gel containing 7M urea in TE buffer. Electrophoresis standards consisted of unmodified fluorescent oligonucleotide, combined with the cleavage product generated by treatment of the corresponding dU-containing oligonucleotide with *E. coli* Endonuclease III (New England Biolabs) at 37°C for 30 min, followed by chemical cleavage with piperidine (90°C, 30 min).

**Quantitative Analyses:** Transformation efficiencies of oligonucleotides carrying abasic or 8-oxo-dG lesions are reported as medians in Dbh⁺ and Dbh⁻ backgrounds, and were calculated based on a minimum of six independent experiments. Statistical comparisons between transformation efficiencies of damaged DNA into Dbh⁺ and Dbh⁻ at each nt position were performed using the Mann-Whitney U test. Chi-square analyses were used to determine statistical differences between the distributions of nucleotides placed; Yate’s correction was used where appropriate. Frequencies of nucleotides at the query positions of the *S. acidocaldarius* pyrE gene were corrected by subtracting the background reversion frequencies for the corresponding recipient strain.

**RESULTS**

**Molecular assays of TLS in the Sulfolobus chromosome:** Oligonucleotide-mediated transformation (OMT) methods have been used to analyze the fate of DNA lesions at specific
sites in yeast and bacterial chromosomes. In this approach, incorporation of a lesion-containing DNA is selected by restoration or alteration of a chromosomal gene, and individual bypass events are documented by sequencing the region (Otsuka et al. 2002; Kroeger et al. 2004; Weiss 2008; Rodriguez et al. 2013). The properties of OMT in S. acidocaldarius (Kurosawa & Grogan 2005; Grogan & Stengel 2008; Mao & Grogan 2012a) suggested the feasibility of corresponding genetic assays that would enable both accurate and erroneous bypass events to be analyzed in vivo. In the method we developed for Sulfolobus (Fig. 1), a synthetic ssDNA restores the wild-type sequence at the site of a chromosomal pyrE mutation which inactivates the encoded UMP-biosynthetic enzyme (orotate:phosphoribosyl transferase). If the oligonucleotide is incorporated into the chromosome and replicated, the recipient cell gains the ability to grow in medium lacking uracil. A synonymous site adjacent to the selected nucleotide in the transforming DNA contains the DNA lesion to be evaluated (Fig. 1a, b). Any base-pair substitution at this “query position” yields the same amino acid as in the wild-type, so that pyrE gene function is restored, regardless of base inserted opposite the lesion at the first replication (Fig. 1c, d).

Eight S. acidocaldarius recipients, representing four alleles in a chromosomal pyrE gene (Fig. 1 (e – i)), provided query sites in different positions and sequence contexts, thus allowing the generality of bypass patterns to be evaluated. Each query and selection site was also provided in both a Dbh+ and Dbh− background, allowing the function of Dbh to be evaluated independently at each site (Table S1). Control oligonucleotides confirmed that any base placed at the query sites yielded transformants with high efficiency (Table S4). In addition, phenotypic reversion (whether spontaneous or induced by the electroporation procedure) (Berkner & Lipps 2008) was
confirmed to be rare (Table 1), and the distribution of nucleotides provided corrections for corresponding analyses of transformants (Table 2).

To enforce stringency in analyzing lesion bypass, the OMT assay (Fig. 1) was designed to make transformation sensitive both to repair activities and polymerase blockage. Even precise removal of the lesion from the annealed oligonucleotide by an endonuclease was expected to negate transformation, because a 1-nt, 5’ flap on the 3’ side of the repair gap provides a favorable substrate for removal by the *Sulfolobus* Fen1/XPG homolog (Horie et al. 2007; Hutton et al. 2008) but not for the highly selective *Sulfolobus* ligase (Lai et al. 2002). We evaluated the feasibility of OMT as a probe of lesion bypass in *Sulfolobus* experimentally by placing a chemically stabilized abasic site (THF spacer) in three transforming oligonucleotides. Prior *in vitro* studies have shown this lesion to impede progression of the Y-family polymerase Dbh of *S. acidocaldarius* and its *S. solfataricus* counterpart Dpo4 (Boudsocq et al. 2001, Gruz et al. 2001; Potapova et al. 2002; Kokoska et al. 2003; Boudsocq et al. 2004; Ling et al. 2004). In *vivo*, BER enzymes that act on ssDNA can also block OMT before the recombination step. Although THF spacers are not repaired by AP lyases (Kroeger et al. 2004), they are substrates for AP endonucleases, which have been detected in archaea (Grasso and Tell 2014). To determine the relevance of these enzymes to OMT in *S. acidocaldarius*, we incubated fluorescently labelled oligonucleotides with cell extracts and monitored backbone cleavage. The results confirmed that *S. acidocaldarius* has significant endonuclease activity on oligonucleotides containing a THF spacer (Fig. S1). Under the assay conditions, the DNA backbone was cleaved more rapidly in duplex than in single-stranded form, and the cleavage of dsDNA was not strongly influenced by the identity of the opposing base (Fig. S1). *In vivo*, therefore, the observed repair activity could
be expected to preclude OMT either before or after a transforming oligonucleotide has annealed to its complement in the recipient chromosome.

**Genetic consequences of abasic sites.** Consistent with all these properties, a THF spacer decreased the average transformant yield of the three oligonucleotides by a factor of 200 to 700, yet the lowest observed frequencies of transformation still remained nearly 10-fold higher than phenotypic reversion (Table 1), suggesting that most of the selected clones were generated by some form of lesion bypass. Identifying the nucleotide placed in the query site in transformants via ligase chain reaction (LCR) assays (see Methods) showed that the distributions of nucleotides placed opposite the DNA lesion were non-random (P< 0.05 for expected values of equal distribution of bases), and differed dramatically among the three sites tested (Table 2). The dominant outcome at nt 189 and 219 (A in the sense strand) maintained the bp found at the query site in the recipient (Table 2). The pattern at these two sites thus remained formally consistent with three mechanistically distinct alternatives, singly or in combination: i) single-nt repair of the lesion that preserved the selected nucleotide, ii) error-free damage tolerance (typically due to strand-exchange processes), or iii) TLS by one or more non-Dbh polymerases, with a strong intrinsic bias toward insertion of dA. At the third site (bp 348), neither (i) nor (ii) applied to the most common outcome, however, making the results more informative (Table 2). At this position, fewer transformants retained the original bp (reflecting either BER or error-free tolerance), and the primary effect of Dbh was to decrease two erroneous insertions (dA and dC; Table 3). The consistent recovery of a non-templated dA at position 348 is consistent with erroneous TLS but not error-free tolerance (Table 2, Table 3). Furthermore, because this result
was seen in Δdbh recipients, it could be attributed to one or more of the B-family enzymes of *S. acidocaldarius*.

**Genetic impact of 8-oxo-dG:** Although replicative DNA polymerases can proceed past 8-oxo-dG, they are prone to insert dA, which yields G:C to T:A transversions. In contrast, certain Y-family polymerases, including *E. coli* DinB and human Pol κ, insert dC opposite 8-oxo-dG (Waters *et al.* 2009), and thus avoid this mutagenesis. To investigate whether Dbh plays the latter role in *S. acidocaldarius*, we repeated the OMT assay with oligonucleotides that placed 8-oxo-dG at the three *pyrE* locations used previously. This lesion decreased genetic transformation relative to undamaged DNAs, but the decrease (15 to 100-fold) was smaller than that observed for abasic sites. Transformation by DNAs containing 8-oxo-dG at *pyrE* positions 219 and 348 was significantly higher for Dbh+ recipients than for Dbh−, indicating that Dbh increased the overall success of strand incorporation and replication (Table 1). In addition, the pattern of nucleotide insertion opposite 8-oxo-dG was distinct from that observed with abasic oligonucleotides, indicating that these transformants did not simply result from TLS past intermediates formed during BER of 8-oxo-dG. In particular, insertion of dC was the most frequent outcome under all but one condition in Dbh+ cells (Table 2). Since none of the recipients have C in the top strand at these sites, this result is consistent neither with repair nor error-free damage tolerance. The specificity of nucleotide insertion opposite 8-oxo-dG was strongly influenced by the functional status of Dbh at all target positions within the *pyrE* gene (nt189, *P* <0.0001; nt219, *P* <0.0001; nt348, *P* =0.0032). The primary impact of functional Dbh was to increase the frequency of dC insertion opposite 8-oxo-dG. This was accompanied by a decrease in insertion of dA (the native nucleotide) at positions 189 and 219 (Table 3).
The strong bias toward insertion of dC opposite 8-oxo-dG correlated with Dbh function in three different contexts and conformed to the behavior predicted for accurate TLS. To test the generality of this result, we evaluated a fourth set of isogenic (Δdbh vs. dbh⁺) S. acidocaldarius recipients, which placed the selected nt (defined by the pyrE mutation) on the 5’ side of the lesion (Fig 1h), so that polymerases encountered 8-oxo-dG before the selected nucleotide. In further contrast to the previous assays, one bp separated the selected and damaged sites, and the transforming oligonucleotide differed from the recipient at this intermediate position. This latter feature was designed to limit the efficiency of single-nt repair of the lesion via BER and strand-exchange processes, while providing a marker for any such events that did occur (Fig. 1h).

Normalized efficiencies of transformation were similar to those of the previous TLS assays, and were significantly elevated in the Dbh⁺ recipient (Table 1, entries for position 443). Correct insertion (dC opposite 8-oxo-dG) was about 9-fold more frequent in Dbh⁺ transformants than in Dbh⁻ transformants (Table 3).

**No detectable contribution of Dbh to spontaneous frameshift mutation:** *In vitro*, Dbh has a characteristic signature on undamaged template, dominated by single-nt deletions which are especially frequent opposite 5’G(Y)ₙ motifs (Potapova *et al.* 2002). Crystallography of putative intermediates, combined with single-nucleotide addition kinetics, indicate that these deletions occur when the nascent-strand terminus has extended into the homopyrimidine tract of a G(Y)ₙ template (Wilson & Pata 2008). The nascent strand re-aligns to exclude one of the template pyrimidines from the helix, and Dbh extends these intermediates efficiently, particularly the one in which the extra-helical template pyrimidine is three bp back from the nascent-strand terminus.
Wilson & Pata 2008). The catalytic and structural data therefore predict that about half of the Dbh molecules which gain access to G(Y)ₙ in vivo would relinquish back to the replicative polymerase a substrate with a bulge several bp back from the 3’ end. Such sub-terminal mismatches evade correction by polymerase proofreading, which accounts for the pronounced length-dependence of mononucleotide-repeat instability seen in eukaryotic and bacterial cells, and its further enhancement by inactivation of post-replicational MMR (Tran et al. 1997; Harfe & Jinks-Robertson 1999).

*S. acidocaldarius* appears to lack post-replicative MMR naturally (White & Grogan 2008; Mao & Grogan 2012), and G(Y)ₙ motifs are abundant in its chromosome. These two properties combine to argue that the *S. acidocaldarius* chromosome should be a particularly sensitive detector of Dbh-mediated mutagenesis. At least two hot-spots of spontaneous frameshifts occur within the *S. acidocaldarius pyrE* gene (Grogan et al. 2001; Sakofsky et al. 2012). One hotspot matches the Dbh deletogenic motif closely (5’GCCCCC on the minus strand), whereas the other lacks the characteristic G (minus-strand 5’ATTTTT). Both sites generate approximately equal proportions of +1 and -1 frameshifts in vivo (Grogan et al. 2001; Sakofsky et al. 2012), whereas purified Dbh makes -1 frameshifts almost exclusively (Potapova et al. 2002). Thus the deletogenic activity exhibited by Dbh on undamaged DNA in vitro is consistent with some, but not all, of the observed properties of spontaneous mutation at these sites.

In order to resolve the contribution of Dbh to this prominent form of spontaneous mutation in *S. acidocaldarius*, we assembled a set of *S. acidocaldarius pyrE* mutants that allowed four frameshifts on homopyrimidine templates to be detected individually in both Dbh⁻ and Dbh⁺
backgrounds: i) C added to GCCCC, ii) T added to ATTTTT, iii) C deleted from GCCCCCC, or iv) T deleted from ATTTTTTT (Table S1). Each of these events restored pyrE gene function, and could therefore be measured by selecting revertants. This was done in side-by-side fluctuation assays which included multiple strains with the same mutations, to ensure that the only difference between groups of cultures was the dbh genotype of the strains. Differences in the pyrE reversion rates for the corresponding Dbh− and Dbh+ strains could thus reveal the contribution, if any, of the Dbh polymerase to the corresponding frameshift event.

Similar to the pattern observed in other systems (Tran et al. 1997; Harfe & Jinks-Robertson 1999), the frameshift rates generally increased with tract length (Table 4), but in every case the rate measured in the dbh+ strain could not be distinguished from that measured in the isogenic Δdbh strain. These assays therefore detected no contribution of Dbh to spontaneous frameshifts at motifs similar to those which it replicates with extremely high frameshift rates in vitro. This result provided evidence that Dbh either does not have deletogenic properties in vivo, or normally does not gain access to this template during replication of the S. acidocaldarius chromosome.

A second test of Dbh-dependent -1 events at G(Y)n motifs was designed to exploit the apparent specificity of Dbh for 8-oxo-dG in vivo (Tables 1 and 2). We modified one of the OMT assays by designing two ssDNAs that contained an extra nucleotide and thus required a -1bp bypass event near the selected and query sites in order to generate transformants. One DNA contained undamaged G at the query site, the other contained 8-oxo-dG (Fig. 1(i)), and each was electroporated into corresponding isogenic Dbh+ and Dbh− recipients. Consistent with the assay
design, the undamaged oligonucleotide yielded Pyr+ clones at low efficiencies (Table 1), and all the transformants examined contained intragenic suppressor mutations in the form of 1-bp deletions uniformly distributed within about 5 bp of the selected nt. In all cases, these deletions occurred outside of the short homopyrimidine tract, and thus did not represent the type of slipped-strand events mediated by Dbh \textit{in vitro} (Potapova \textit{et al.} 2002). Replacing the G at the query site with 8-oxo-dG increased the yield of transformants about 10-fold, yielding a relative efficiency similar to that of in-frame OMT in both Dbh⁻ and Dbh⁺ recipients (Table 1). In addition, 15 of 16 transformants scored showed precise deletion of the query position, not of a subsequent G:C bp in the homopyrimidine tract. Thus, this assay detected 8-oxo-dG-promoted 1-bp deletions in \textit{S. acidocaldarius} that were both frequent and localized at a particular site, but not mediated by Dbh.

\textbf{DISCUSSION}

In Archaea, DNA replication and responses to DNA damage occur in the context of a relatively simple prokaryotic cell, yet these processes involve proteins and other molecular features homologous to those of eukaryotic cells, rather than bacteria (Ishino \textit{et al.} 2013). This situation makes archaea strategic for understanding the diversity and evolution of mechanisms that preserve genome integrity, but the archaeal mechanisms remain largely unexplored in their cellular context. In particular, despite extensive studies of \textit{Sulfolobus} DNA polymerases \textit{in vitro}, the contributions of \textit{Sulfolobus} Y-family vs. B-family DNA polymerases to mutagenesis by particular DNA lesions have not been defined in molecular terms. The present study is the first to investigate this question experimentally \textit{in vivo}.
The genetic consequences of DNA lesions transferred to the *S. acidocaldarius* chromosome differed as a function of DNA lesion, location within the gene sequence, and Dbh status (Table 2). Differences between the abasic-site and 8-oxo-dG patterns include a significant level of accurate bypass with little input from Dbh in the case of abasic sites vs. a strong dependence on Dbh for accurate bypass in the case of 8-oxo-dG. Both for abasic sites and 8-oxo-dG, the pattern of outcomes varied with the location of the damage within the target gene. Although the accuracy of oligonucleotide synthesis may have contributed to the site-specific differences, it does not account for the similarity seen for abasic and 8-oxo-dG oligonucleotides at different sites, and the differential impact of Dbh status across the sites (Table 2). The results thus suggest that *S. acidocaldarius*, and presumably other hyperthermophilic archaea, can use multiple mechanisms for lesion bypass, and that the identity of the lesion and its local sequence context determines which mechanism predominates.

As depicted in the scheme of Figure 2, the most consistent and biologically significant function of the Y-family DNA polymerase Dbh evident in this study was the avoidance of base-pair substitutions during replication past 8-oxo-dG. Dbh seems to be specialized in this respect; it did not contribute to TLS past abasic sites nor to spontaneous -1 events in frameshift hotspots. Both mutation avoidance at 8-oxo-dG and poor bypass of abasic sites are consistent with known enzymological properties of Dbh (Gruz *et al.* 2001). In contrast, the failure to promote -1 events at homo-pyrimidine tracts, which we documented in two distinct experimental contexts (normal growth and OMT), is not consistent with the strong deletogenic propensity of Dbh demonstrated repeatedly *in vitro* (Potapova *et al.* 2002; Wilson and Pata 2008). The formal possibility remains that deletogenesis on intact template reflects some artifact of *in vitro* assays, but this seems
unlikely, as this property has been linked to specific structural features of the polymerase (Boudocq et al. 2004; Pata 2010) and conformational dynamics of the template in the enzyme active site (Wilson & Pata 2008; Manjari et al. 2014). Conversely, deletogenesis seems unlikely to be masked in vivo by non-specific factors, such as cytoplasmic solutes or elevated temperature. Accordingly, we interpret the irrelevance of Dbh to spontaneous frameshifts in the pyrE gene as genetic evidence that Dbh is normally prevented, with some specificity, from replicating undamaged DNA in vivo. The extent to which Dbh promotes 1-nt deletions at persistent lesions in vivo remains unresolved, but the fact that Dbh function increases the rate of transformation by 8-oxo-dG-containing oligonucleotides argues that such lesion-triggered deletions would be relatively rare. Since a frameshift nullifies detection of the TLS event in most of the assays used, any such -1 events appear to be significantly outnumbered by in-frame TLS, for example.

Ironically, Dbh has been characterized biochemically as an extremely error-prone DNA polymerase, yet most of the erroneous bypass we observed in S. acidocaldarius did not respond to Dbh function. Sulfolobus spp. generally encode four DNA polymerases: a replicative enzyme responsible for the bulk of DNA synthesis (B-family), a Y-family polymerase implicated in TLS, and two additional B-family enzymes which remain relatively obscure. Biochemical studies of the latter enzymes of S. solfataricus indicate limited affinity for DNA substrate, limited processivity, and relatively low rates of polymerization (Choi et al. 2011). More recently, the DNA primase of a distantly related hyperthermophilic archaeon was reported to catalyze TLS in vitro (Jozwiakowski et al. 2015). In vivo, we observed several distinct types of mutagenesis by non-Dbh enzymes, including OMT-associated base substitution and single-bp deletion, and
spontaneous frameshift mutation. The mechanism of the second mutagenic process has yet to be defined, but its efficiency and specificity seems to pose an additional mutagenic threat to *Sulfolobus* genomes from 8-oxo-dG which, to our knowledge, has not been predicted by other studies.

At the four sites we evaluated, Dbh mediated 75% to 90% of the observed TLS past 8-oxo-dG, despite the ability of other enzymes to perform bypass in its absence. This observation complements the evidence of Dbh exclusion from undamaged template, indicating that some property favors Dbh over other alternative pathways in replicating 8-oxo-dG. Recruitment of TLS polymerases to unrepaired lesions is commonly associated with polymerase switching (Lehman *et al.* 2007). Although this process occurs both in bacteria and in eukaryotic cells, and involves corresponding replisome components (i.e., the sliding clamp), the bacterial and eukaryotic mechanisms differ dramatically. Bacterial polymerases appear to compete for preferred binding sites on the dimeric β clamp, where binding appears to be largely reversible, i.e., determined by the relative concentrations of these enzymes (Friedberg *et al.* 2006; Lovett 2007; Andersson *et al.* 2010; Kath *et al.* 2014). In eukaryotes, however, different patterns of ubiquitination of the trimeric PCNA dictate which damage-tolerance pathways will be activated. A diversity of ubiquitination patterns allows a range of distinct responses to be specified; the possible choices encompass several TLS polymerases and multiple error-free processes, including replication-fork regression or Rad51-dependent strand exchange (Nick McElhinny *et al.* 2008; Yang *et al.* 2013).
The eukaryotic features of archaeal replisomes include trimeric PCNAs instead of the dimeric β clamps found in bacteria. Since archaea encode no ubiquitin, however, it remains unclear whether archaea switch DNA polymerases in response to covalent modification of their PCNA. Ubiquitin alternatives, with corresponding ligation systems, do occur in archaea, but they have been implicated so far only in other processes, such as cofactor synthesis, tRNA modification, and proteolysis (Makarova & Koonin 2010). Conversely, properties observed in vitro raise the possibility that biologically relevant switching could occur via passive exchange among archaeal DNA polymerases, as has been proposed for bacteria. For example, Dpo1, the replicative polymerase of *S. solfataricus*, disengages from PCNA frequently (Bauer et al. 2013), and 8-oxo-dG in the template blocks its progress, but increases the catalytic efficiency of the TLS polymerase Dpo4 (Maxwell & Suo 2012). In this context, the present study demonstrates that genetic analysis of TLS in *S. acidocaldarius* can provide important functional insight into mutation avoidance, polymerase recruitment and other aspects of DNA damage tolerance modeled by the enzymes of hyperthermophilic archaea.

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Figure 1. Transformation assays of TLS in the *S. acidocaldarius* chromosome. Diagrams (a) through (d) depict the transfer of the lesion to the *Sulfolobus* chromosome by OMT, and its subsequent bypass. The transforming ssDNA (a) represents the anti-sense (minus) strand of the *S. acidocaldarius pyrE* gene. Near its midpoint, one nucleotide (filled symbol) corrects a point mutation in the recipient cell, while a DNA lesion replaces the adjacent nucleotide (x). This ssDNA is electroporated into recipient cells (b), where it anneals to a transient gap (Li et al.)
The resulting heteroduplex (b) is then replicated (c), which requires some form of bypass of the DNA lesion. Replication produces two completed chromosomes (i.e., sister chromatids) (d), but only daughter cells that retain the marker provided by the transforming ssDNA generate a colony.

The remaining panels show the central section of each transforming ssDNA (bottom strand) opposite the recipient chromosome (top strand); numbers indicate positions within the pyrE coding sequence. The mutant codon of the recipient is underlined, with the corresponding amino acid shown to the right in bracketed italic type; the corresponding WT codon provided by the transforming DNA is given for the bottom strand. The selected nucleotide and query site (x) are shown in boldface type. Panels (e) – (g) show sites in the pyrE gene of S. acidocaldarius; (h) and (i) represent the pyrE gene of S. solfataricus inserted into the S. acidocaldarius chromosome (see Table S1).
Figure 2. Dbh and bypass of 8-oxo-dG

The diagram depicts the most common responses to 8-oxo-dG ("oG" above) represented by bypass events in normal and Dbh-deficient mutants of *S. acidocaldarius*. Individual processes contributing to the bypass seen in *dbh* mutants were not resolved in this study, and may include repair, error-free (recombinational) damage tolerance, or polymerase switching among B-family enzymes.
Table 1. Effects of DNA lesions and Dbh function on overall efficiency of OMT (oligonucleotide-mediated transformation)

<table>
<thead>
<tr>
<th>DNA lesion</th>
<th>Position in pyrE</th>
<th>Transformation Efficiency</th>
<th>[median (range)]¹</th>
<th>Rel. Efficiency²</th>
<th>Effect of Dbh³</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>189</td>
<td>Δdbh</td>
<td>1720 (196 - 2436)</td>
<td>1012 (227 - 1722)</td>
<td>100 100</td>
</tr>
<tr>
<td>none</td>
<td>219</td>
<td>Δdbh</td>
<td>2280 (2120 - 2808)</td>
<td>1927 (1600 - 2790)</td>
<td>100 100</td>
</tr>
<tr>
<td>none</td>
<td>348</td>
<td>Δdbh</td>
<td>547 (451 - 817)</td>
<td>692 (510 - 1918)</td>
<td>100 100</td>
</tr>
<tr>
<td>none</td>
<td>443⁴</td>
<td>Δdbh</td>
<td>750 (317 - 1390)</td>
<td>484.5 (191 - 1200)</td>
<td>100 100</td>
</tr>
<tr>
<td>abasic site</td>
<td>189</td>
<td>Δdbh</td>
<td>9 (6 - 12)</td>
<td>5 (7 - 17)</td>
<td>0.52 0.49 0.94</td>
</tr>
<tr>
<td>abasic site</td>
<td>219</td>
<td>Δdbh</td>
<td>3 (1 - 5)</td>
<td>2 (1 - 5)</td>
<td>0.13 0.10 0.77</td>
</tr>
<tr>
<td>abasic site</td>
<td>348</td>
<td>Δdbh</td>
<td>3 (1 - 12)</td>
<td>1 (1 - 5)</td>
<td>0.55 0.14 0.25</td>
</tr>
<tr>
<td>C duplication⁵</td>
<td>443⁴</td>
<td>Δdbh</td>
<td>20 (7 - 41)</td>
<td>9 (6 - 34)</td>
<td>2.67 1.86 0.70 0.71</td>
</tr>
<tr>
<td>8-oxo-dG</td>
<td>189</td>
<td>Δdbh</td>
<td>38.5 (6 - 58)</td>
<td>45 (11 - 64)</td>
<td>2.24 4.45 1.99 0.093</td>
</tr>
<tr>
<td>8-oxo-dG</td>
<td>219</td>
<td>Δdbh</td>
<td>23.5 (18 - 49)</td>
<td>133.5 (95 - 171)</td>
<td>1.03 6.93 6.73 0.0022*</td>
</tr>
<tr>
<td>8-oxo-dG</td>
<td>348</td>
<td>Δdbh</td>
<td>14.5 (9 - 21)</td>
<td>29.5 (17 - 63)</td>
<td>2.65 4.26 1.61 0.045*</td>
</tr>
<tr>
<td>8-oxo-dG +C⁵</td>
<td>443⁴</td>
<td>Δdbh</td>
<td>6 (2 - 46)</td>
<td>18 (2 - 53)</td>
<td>0.80 3.72 4.64 0.0008*</td>
</tr>
<tr>
<td>8-oxo-dG +C⁵</td>
<td>443⁴</td>
<td>Δdbh</td>
<td>159 (62 - 376)</td>
<td>144 (41 - 389)</td>
<td>21.2 29.7 1.40 0.25</td>
</tr>
</tbody>
</table>
1 Transformants per electroporation, based on at least 6 independent experiments. Because the number of transformants did not correlate with amount of DNA per electroporation over the ranges tested (20 to 100 pmol for normal ssDNAs and 50 to 200 pmol for lesion-containing ssDNAs), results were pooled for each combination of DNA and recipient strain.

2 Calculated as 100 times the median transformation efficiency divided by the corresponding median for undamaged DNA in the same strain.

3 P values for variation with Dbh status were from Mann-Whitney U tests comparing transformant yields in Dbh⁻ and Dbh⁺ recipients, as corrected for differences in transformation efficiencies (by normalizing to the same cells transformed with undamaged DNA). Asterisks (*) denote statistical difference at the P< 0.05 level.

4 This query position was in the pyrE sequence of *S. solfataricus*, used as a selectable cassette to construct *S. acidocaldarius* strains DG250 and DG251 (see Table S1).

5 The transforming DNAs contained an extra C between the query site and the selected nucleotide (see Results), thus requiring a -1 replication error for successful transformation.
Table 2. Nucleotide specificity of lesion bypass<sup>a</sup>

<table>
<thead>
<tr>
<th>Pos.&lt;sup&gt;b&lt;/sup&gt;</th>
<th>(n)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>A</th>
<th>T</th>
<th>C</th>
<th>G</th>
<th>(n)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>A</th>
<th>T</th>
<th>C</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abasic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>189</td>
<td>54</td>
<td>98%</td>
<td>2%</td>
<td>0%</td>
<td>0%</td>
<td>60</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>219</td>
<td>60</td>
<td>98%</td>
<td>2%</td>
<td>0%</td>
<td>0%</td>
<td>54</td>
<td>96%</td>
<td>2%</td>
<td>2%</td>
<td>0%</td>
</tr>
<tr>
<td>348</td>
<td>49</td>
<td>32%</td>
<td>38%</td>
<td>5%</td>
<td>25%</td>
<td>49</td>
<td>22%</td>
<td>30%</td>
<td>2%</td>
<td>46%</td>
</tr>
<tr>
<td>oxo-dG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>189</td>
<td>66</td>
<td>48%</td>
<td>0%</td>
<td>52%</td>
<td>0%</td>
<td>61</td>
<td>3%</td>
<td>0%</td>
<td>97%</td>
<td>0%</td>
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<tr>
<td>219</td>
<td>69</td>
<td>36%</td>
<td>0%</td>
<td>63%</td>
<td>1%</td>
<td>63</td>
<td>2%</td>
<td>0%</td>
<td>98%</td>
<td>0%</td>
</tr>
<tr>
<td>348</td>
<td>59</td>
<td>48%</td>
<td>26%</td>
<td>24%</td>
<td>2%</td>
<td>62</td>
<td>29%</td>
<td>13%</td>
<td>56%</td>
<td>2%</td>
</tr>
<tr>
<td>443&lt;sup&gt;e&lt;/sup&gt;</td>
<td>15</td>
<td>0%</td>
<td>27%</td>
<td>27%</td>
<td>27%</td>
<td>13</td>
<td>0%</td>
<td>0%</td>
<td>69%</td>
<td>15%</td>
</tr>
</tbody>
</table>

<sup>a</sup> The nucleotide found and the indicated position was identified by LCR (see Methods), except as noted below. Values for each base are % occurrence among the transformants. Boldface numerals indicate the native nucleotide at that position, i.e., the one which could be templated.

<sup>b</sup> Nucleotide in the pyrE coding sequence
Values were corrected for the predicted contribution from spontaneous revertants in the indicated strain, as determined from DNA-less electroporation controls. This correction changed most entries about 1% or less.

The site and transforming oligonucleotides correspond to the pyrE gene of S. solfataricus (see Fig. 1h). For these transformations, insertion of dA was precluded by the selection, whereas additional outcomes not listed in the table were possible. As a result, the transformants were scored by dideoxy sequencing of PCR products. The most frequent of the additional events not depicted in the table accounted for about 13% of Δdbh and 8% of dbh' transformants; this was a complex event yielding CTTCACAAA in the coding strand, where the query site is underlined (see Fig. 1h).
Table 3. Normalized frequencies of bypass events$^a$

<table>
<thead>
<tr>
<th>pyrE position</th>
<th>$\Delta dbh$</th>
<th>$dbh^+$</th>
<th>ratio$^b$</th>
<th>$\Delta dbh$</th>
<th>$dbh^+$</th>
<th>ratio$^b$</th>
<th>$\Delta dbh$</th>
<th>$dbh^+$</th>
<th>ratio$^b$</th>
<th>$\Delta dbh$</th>
<th>$dbh^+$</th>
<th>ratio$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abasic site</td>
<td>189</td>
<td>0.533</td>
<td>0.592</td>
<td>1.11</td>
<td>0.01</td>
<td>und.$^c$</td>
<td>-</td>
<td>und.$^c$</td>
<td>und.$^c$</td>
<td>-</td>
<td>und.$^c$</td>
<td>und.$^c$</td>
</tr>
<tr>
<td></td>
<td>219</td>
<td>0.180</td>
<td>0.070</td>
<td>0.39</td>
<td>0.006</td>
<td>0.001</td>
<td>0.3</td>
<td>und.$^c$</td>
<td>0.001</td>
<td>-</td>
<td>und.$^c$</td>
<td>und.$^c$</td>
</tr>
<tr>
<td></td>
<td>348</td>
<td>0.20</td>
<td>0.060</td>
<td>0.28</td>
<td>0.074</td>
<td>0.057</td>
<td>0.8</td>
<td>0.032</td>
<td>0.005</td>
<td>0.15</td>
<td>0.15</td>
<td>0.12</td>
</tr>
<tr>
<td>8-oxo-dG</td>
<td>189</td>
<td>1.0</td>
<td>0.1</td>
<td>0.12</td>
<td>und.$^c$</td>
<td>und.$^c$</td>
<td>-</td>
<td>1.11</td>
<td>3.91</td>
<td>3.52</td>
<td>und.$^c$</td>
<td>und.$^c$</td>
</tr>
<tr>
<td></td>
<td>219</td>
<td>0.29</td>
<td>0.070</td>
<td>0.22</td>
<td>und.$^c$</td>
<td>und.$^c$</td>
<td>-</td>
<td>0.90</td>
<td>6.47</td>
<td>7.19</td>
<td>und.$^c$</td>
<td>und.$^c$</td>
</tr>
<tr>
<td></td>
<td>348</td>
<td>1.4</td>
<td>1.3</td>
<td>0.97</td>
<td>0.18</td>
<td>0.34</td>
<td>1.8</td>
<td>0.71</td>
<td>2.5</td>
<td>3.5</td>
<td>0.050</td>
<td>0.08</td>
</tr>
<tr>
<td>443$^d$</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.351</td>
<td>und.$^c$</td>
<td>-</td>
<td>0.35</td>
<td>3.05</td>
<td>8.68</td>
<td>0.35</td>
<td>0.0068</td>
</tr>
</tbody>
</table>

$^a$ Unless noted, numerical values are frequencies of the corresponding outcome, expressed as a percentage of the overall transformation efficiency of the corresponding undamaged oligonucleotide.
b Transformant yield in $dbh^+/yield$ in $Δdbh$, calculated to indicate the effect of Dbh function.

c Events that were allowed by the selection but not detected (see Table 2).

d Position in the $pyrE$ gene of $S. solfataricus$ (see Fig. 1(h)).
Table 4. Rates of mononucleotide-repeat expansion and contraction at *pyrE* frameshift hotspots<sup>a</sup>

<table>
<thead>
<tr>
<th></th>
<th>(G:C)&lt;sub&gt;4&lt;/sub&gt; expansion&lt;sup&gt;b&lt;/sup&gt;</th>
<th>(A:T)&lt;sub&gt;5&lt;/sub&gt; expansion&lt;sup&gt;b&lt;/sup&gt;</th>
<th>(G:C)&lt;sub&gt;6&lt;/sub&gt; contraction&lt;sup&gt;b&lt;/sup&gt;</th>
<th>(A:T)&lt;sub&gt;7&lt;/sub&gt; contraction&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rate</strong></td>
<td>(95% ci)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(95% ci)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(95% ci)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(95% ci)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Dbh&lt;sup&gt;−&lt;/sup&gt;</strong></td>
<td>2.6 (3.7 - 1.7)</td>
<td>1.5 (2.2 - 0.95)</td>
<td>33 (39 - 27)</td>
<td>43 (50 - 36)</td>
</tr>
<tr>
<td><strong>Dbh&lt;sup&gt;+&lt;/sup&gt;</strong></td>
<td>2.3 (3.2 - 1.5)</td>
<td>1.8 (2.6 - 1.1)</td>
<td>32 (38 - 27)</td>
<td>38 (45 - 32)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values represent the number of the indicated mutational (i.e., *pyrE* reversion) events per 10<sup>8</sup> cell divisions. Each rate was measured using four sets of 15-20 independent cultures each (see Methods). Some of the *pyrE* mutations were represented by multiple strains (CS2.3 and CS2.5, for example; see Methods), as a means to control for any genetic differences that might have arisen during initial isolation. No differences in reversion rates between corresponding mutants were observed, however, so the data were pooled to yield the analyses shown above.

<sup>b</sup> The base in the top (sense) strand is indicated first in each basepair.

<sup>c</sup> “ci” indicates confidence interval.