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Rates of Spontaneous Mutation in an Archaeon from Geothermal Environments

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To estimate the efficacy of mechanisms which may prevent or repair thermal damage to DNA in thermophilic archaea, a quantitative assay of forward mutation at extremely high temperature was developed for *Sulfolobus acidocaldarius*, based on the selection of pyrimidine-requiring mutants resistant to 5-fluoro-orotic acid. Maximum-likelihood analysis of spontaneous mutant distributions in wild-type cultures yielded maximal estimates of $(2.8 \pm 0.7) \times 10^{-7}$ and $(1.5 \pm 0.6) \times 10^{-7}$ mutational events per cell per division cycle for the *pyrE* and *pyrF* loci, respectively. To our knowledge, these results provide the first accurate measurement of the genetic fidelity maintained by archaea that populate geothermal environments. The measured rates of forward mutation at the *pyrE* and *pyrF* loci in *S. acidocaldarius* are close to corresponding rates reported for protein-encoding genes of *Escherichia coli*. The normal rate of spontaneous mutation in *E. coli* at 37°C is known to require the functioning of several enzyme systems that repair spontaneous damage in DNA. Our results provide indirect evidence that *S. acidocaldarius* has cellular mechanisms, as yet unidentified, which effectively compensate for the higher chemical instability of DNA at the temperatures and pHs that prevail within growing *Sulfolobus* cells.

In the past 25 years, diverse microorganisms have been isolated from geothermal environments and have been shown to require temperatures of 75 to 105°C (depending on the species) for optimal growth; these organisms have been variously termed extreme thermophiles or hyperthermophiles (30). The fact that the preferred growth temperatures of these organisms can effectively denature the RNA, DNA, and protein of mesophilic microorganisms raises basic questions regarding the potential stability of biological macromolecules and the means by which extreme thermophiles and hyperthermophiles realize this potential stability in vivo. Considerable effort has been invested in understanding, in molecular terms, how the tertiary and secondary structures of enzymes and nucleic acids are preserved in these microorganisms. The fact that growth temperatures of 75 to 105°C should also disrupt the primary structure of biological macromolecules has received much less attention, despite indications that spontaneous processes of this type affect the growth and survival of bacteria growing at 37°C (16, 29).

The chemical instability of DNA is a major source of spontaneous mutation in mesophiles and has been studied extensively. Hydrolytic depurination represents the most frequent spontaneous decomposition of duplex DNA, followed by hydrolytic deamination of cytosine and adenine residues (19–22, 29). In aerobes, additional oxidative reactions form a variety of damaged bases (21). Biochemical and genetic studies using bacteria have shown that these various spontaneous reactions (i) occur in vivo (indeed, some metabolic processes actually contribute to them [21, 29]); (ii) are potentially mutagenic, i.e., they cause mutation if the lesions escape repair or are misrepaired (22, 29); and (iii) are accelerated several orders of magnitude by a temperature increase of 40 to 50°C (19–21). A variety of cellular mechanisms which minimize the resulting lesions in DNA, either by slowing their formation or by repairing them once formed, have been identified in bacteria. Abasic sites formed by depurination are repaired via excision initiated by apurinic (AP) endonucleases (21, 22). Also, an alternative to repair has been observed for bacterial spores, which contain small basic proteins that retard DNA depurination and other damage processes (28). Deamination products (i.e., uracil and hypoxanthine residues) are excised from cellular DNA by specific *N*-glycosylases. If not excised, these bases give rise to mutations, as demonstrated by the mutator phenotypes of bacterial strains lacking the *N*-glycosylases (21). Similarly, a DNA base damaged by oxidation is removed by a cognate *N*-glycosylase and loss of the enzyme causes an increase in the rate of spontaneous mutation (21, 29).

The biological importance of retarding and repairing spontaneous DNA damage has thus been well documented for mesophilic bacteria; it has not, however, been similarly documented for prokaryotes adapted to extremely high temperatures. In particular, most prokaryotes isolated from geothermal environments (the most thermophilic organisms known) belong to the domain *Archaea* (31). This group has many molecular features unknown in the domain *Bacteria*, including features previously believed to be exclusively eukaryotic (12). The ability of archaea from geothermal environments to accurately replicate and maintain their DNA is therefore a question of fundamental interest from a phylogenetic, as well as a physiological, perspective. Direct experimental study of this question has been hindered by the fact that most thermophilic archaea do not lend themselves to routine microbiological manipulation, much less to genetic analysis. To our knowledge, much of the available data regarding spontaneous mutation in thermophilic archaea concern an insertion sequence which inactivates the β-glycosidase gene of *Sulfolobus solfataricus* at high frequency (26). Whether archaea growing at extremely high temperatures can generally maintain a level of genetic stability similar to that of the mesophilic bacteria upon which much of our understanding of mutation and DNA repair is based has remained an unresolved question.

In the present study, we took advantage of the favorable properties of *S. acidocaldarius*, an archaeon isolated from acidic terrestrial hot springs (2), to develop a quantitative genetic assay of spontaneous mutation at extremely high tem-

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peratures. *S. acidocaldarius* grows optimally at about 80°C and pH 3 and forms isolated colonies on suitable solid media (6). The pyrimidine analog 5-fluoro-orotic acid (FOA) selects two distinct classes of *S. acidocaldarius* auxotrophs, and loss of particular enzyme activities of UMP biosynthesis has been shown to be responsible for the phenotypes of these mutants (7). We used this property to assay the rates of spontaneous forward mutation at the corresponding genetic loci of *S. acidocaldarius*. To our knowledge, the results provide the first experimental estimates of the genetic fidelity maintained by an archaeon growing at extremely high temperature. The values we obtained imply extremely efficient avoidance and repair of spontaneous damage to DNA in this organism.

MATERIALS AND METHODS

Strains and growth conditions. The strains used in this study have been previously described (9). Growth of auxotrophic strain DG40 is supported by acid-hydrolyzed casein but not by individual amino acids. The fact that this phenotype occurs and reverts at reasonable frequencies indicates that it results from a single mutation, which has been designated *caa-2* (9).

Unless otherwise noted, the growth medium used consisted of mineral base (8) supplemented with 2.0 g of D-xylose, 1.0 g of NZAmine AS (Sigma Chemical Co.), and 20 mg of uracil per liter; this medium is designated XTura. Cultures (2.5 ml each) were incubated under conditions of high aeration, except where noted otherwise. This was achieved by means of a rack that rotated 16-mm-diameter screw-cap tubes at 56 rpm, at radii of 7 to 17 cm, about an axis inclined at 12° from horizontal. The growth temperature was 75°C unless otherwise noted. Under these conditions, cultures grew with a doubling time of 5 h in the exponential phase.

Mutation rate assays. Maximum-likelihood calculations were applied to multiple independent cultures in Luria-Delbrück fluctuation tests (15) to estimate the rates of *pyrE* and *pyrF* mutant formation. The inoculum for each set of independent cultures was prepared by resuspending one isolated *S. acidocaldarius* colony in growth medium and incubating it until the cell density reached about 5×10^7/ml. An aliquot corresponding to 10^5 or fewer cells was then diluted into 100 ml of freshly prepared medium, and the resulting suspension was distributed among many identical culture tubes. The resulting cultures, containing less than about 2,500 cells each, were incubated under test conditions, except that aeration was delayed 8 to 10 h to decrease the possibility of killing the small inocula by initial oxygen stress (6).

Cultures were removed from incubation when the cell density reached or exceeded about 5×10^7/ml, and any minor volume losses due to evaporation were replaced with distilled water. The density of viable cells in each tube was calculated from the apparent A_{600} in 16-mm culture tubes (Milton-Roy Spectronic 21D) by the following empirically determined relationships: CFU per milliliter equals $(1{,}710A_{600} - 33) \times 10^6$ for strain DG6 and $(1{,}820A_{600} - 26) \times 10^6$ for strain DG185. One-fifth (0.50 ml) of each culture was plated on solid XTura medium containing 60 μg of FOA per ml and incubated at 75°C.

After 1 week of incubation, the numbers of small and large colonies (*pyrE* and *pyrF* mutants, respectively; see Results) were counted. The numbers of mutant cells per culture corresponding to the 25th, 50th, and 75th percentiles (Q_1, Q_2, and Q_3, i.e., the first, second and third quartiles) were calculated by interpolation. Each of these three quartile values was used to estimate the most probable number of mutational events per culture, m, by the method of Lea and Coulson (15) as calculated by Koch (13) for large values of Q. In a few cases, Koch's calculations did not include sufficiently large Q values and the following linear extrapolations of the curves of Fig. 2 in reference 13 were used: $m_1 = 0.1724(Q_1 + 150)$, $m_2 = 0.1429(Q_2 + 110)$, and $m_3 = 0.1190(Q_3 - 60)$. These equations produced results that were within 2.5% of Koch's computations for m values ranging from 70 to 120 and thus were taken to be valid for m values somewhat above 120. The three estimates of m were then used to calculate the corresponding estimates of the mutation rate as follows: $\mu = (m/N_{av})\ln 2$, where μ is the number of mutational events per cell per division cycle (i.e., per cell division) and N_{av} is the average population size (per culture) for the set of cultures.

Controls. Internal controls for efficiency of plating were included in each fluctuation test; from one culture of each set, aliquots ranging from 100 to 500 μl were plated on selective medium to confirm that the number of Foa^r colonies was proportional to the volume of culture plated.

Lag in the expression of the Foa^r phenotype was tested directly by chemical mutagenesis of auxotrophic strains (suggested by Koch [13]) as follows. Growing cultures of two amino acid auxotrophs, DG40 and DG55, were harvested at low cell density (approximately 10^8/ml), washed free of medium, resuspended in sterile, nonnutrient buffer, and divided into two equal portions. One portion was treated with 200 μg of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) per ml, and both were incubated at 45°C for 1 h. Following mutagenesis, both portions of cells were harvested from the treatment medium by centrifugation; the cells then were diluted into fresh XTura medium and aerated at 75°C. Subsequent growth of the cells was monitored by sampling the cultures and plating them on solid XTura medium. (It should be noted that the MNNG treatment caused a subsequent growth lag of several hours; see the legend to Fig. 2.) Foa^r mutants were similarly enumerated on XTura containing 60 μg of FOA per ml, whereas phenotypic revertants (i.e., prototrophs) were enumerated by plating on defined medium lacking all amino acids except L-glutamine.

The relative growth rates of *pyrE* mutant, *pyrF* mutant, and parental *S. acidocaldarius* strains were determined by (i) determination of turbidimetric growth curves of separate cultures and (ii) viable-count assays of competition among *pyrE*, *pyrF*, and parental strains in artificial mixtures. In both methods, a given genotype (*pyrE*, e.g.) was represented in a culture by combining three independent colonies from an actual fluctuation test in the inoculum. Use of these pools rather than individual clones was intended to ensure that the reconstructions resembled actual mutation rate assay cultures.

Growth curves were determined for five pools of wild-type *S. acidocaldarius* colonies, six pools of *pyrE* colonies, and five pools of *pyrF* colonies under the standard conditions used for fluctuation tests (see Materials and Methods). Growth rate constants were calculated by least-squares fitting of the exponential portion of each curve, and the mean value for the wild-type cultures was set equal to 1.0. Under these conditions, the mean values ± standard deviations were 1.2 ± 0.23 for *pyrE* pools and 1.2 ± 0.25 for *pyrF* pools.

Competition was determined for 12 cultures, each inoculated with different pools of *pyrE*, *pyrF*, and wild-type cells in ratios of approximately 1:1:100. Viable counts of *pyrE*, *pyrF*, and wild-type strains were determined before and after three to four generations of growth by plating appropriate dilutions on selective and nonselective plates. The proportion of *pyrE* mutants increased in six trials and decreased in six, whereas the proportion of *pyrF* mutants increased in five trials and decreased in seven. We interpreted these results as signifying no measurable deficiency in overall growth and recovery of Foa^r mutants relative to the parent strain under the conditions of the present study.

Estimates of uncertainty. The mutation rates determined in the present study incorporate the inherent uncertainty of estimating m by the maximum-likelihood method and the uncertainty of estimating N_{av} by measurement of culture turbidity. The former was calculated by Lea and Coulson as a function of m and the number of cultures in a fluctuation test (15). For 25 independent cultures and an m value of 40 (criteria exceeded by most of the fluctuation tests conducted in the present study), the uncertainty of m estimation is about 8% (15). We estimated the uncertainty of N_{av} estimation by comparing several standard curves relating A_{600} and viable counts; each of these curves was taken from an independent fluctuation test that covered a range of growth parameters (temperature, e.g.). The means and standard deviations of slopes and intercepts were then used to calculate the extreme values of N_{av} for a hypothetical culture of average cell density (i.e., $A_{600} = 0.15$ in a 16-mm culture tube). The resulting N_{av} deviations were ±13% of the mean for a hypothetical DG40 culture ($n = 3$) and ±23% of the mean for a hypothetical DG55 culture ($n = 5$). These results suggest an inherent uncertainty based on the limitations of measurement of about 30% for the μ values calculated in the present study.

RESULTS

FOA selection as a quantitative assay of forward mutation in *S. acidocaldarius*. Kondo et al. (14) were the first to report the selection of *Sulfolobus* mutants resistant to FOA. When this selection is applied to *S. acidocaldarius*, three phenotypically distinct classes of Foa^r mutants can be identified: (i) *fpy* mutants, which are prototrophs with general resistance to 5-fluoropyrimidine compounds; (ii) *pyrE* mutants, which are pyrimidine auxotrophs specifically resistant to FOA (MIC, 200 μg/ml); and (iii) *pyrF* mutants, which resemble *pyrE* mutants but have higher FOA resistance (MIC, >1,000 μg/ml) (7). The latter two phenotypes have been shown to be due to loss of the UMP-biosynthetic enzymes orotate phosphoribosyltransferase (OPRT) and orotidylate decarboxylase (ODC) in *S. acidocaldarius* (7). We therefore evaluated the formation of Foa^r clones during growth in nonselective liquid medium as a quantitative assay of forward (i.e., loss-of-function) mutations in this thermophilic archaeon.

Figure 1 shows typical results of plating of uracil-supplemented *S. acidocaldarius* cultures on solid medium containing uracil and 60 μg of FOA per ml. Two distinct colony sizes resulted, consistent with the distinct sensitivities to FOA exhibited by *pyrE* and *pyrF* mutants of *S. acidocaldarius*. To confirm a reliable correlation of colony size with the *pyr* genotype, 100 large Foa^r colonies and 95 small Foa^r colonies (4 to 6 colonies each from 20 independent liquid cultures) were

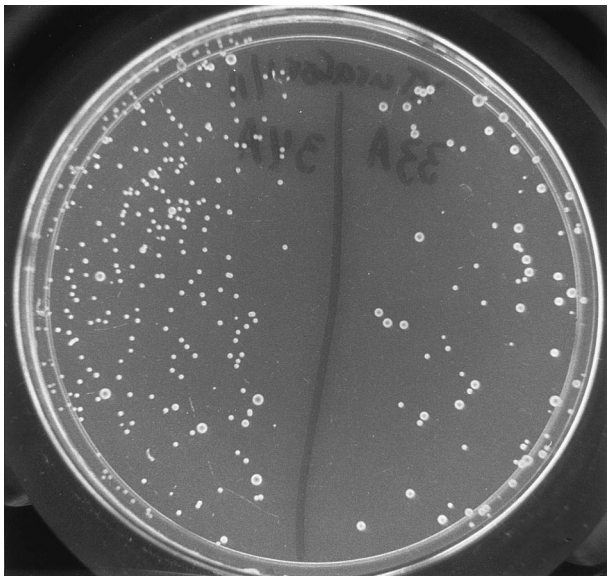

FIG. 1. Selection of spontaneous Foa^r mutants. Typical results of plating aliquots of liquid cultures on solid XTura medium containing 60 μg of FOA per ml. Two independent cultures were plated on opposite halves of a selective plate. The small colonies are *pyrE* mutants, and the large colonies (with dark centers) are *pyrF* mutants, according to phenotypic characterization (see text).

scored by colony transfer for pyrimidine auxotrophy and for resistance to 500 μg of FOA per ml. Four of 95 small colonies and 96 of 100 large colonies grew on medium containing 500 μg of FOA per ml, which is a defining characteristic of *S. acidocaldarius pyrF* mutants (7). Of the 195 small and large Foa^r colonies scored, 3 were prototrophic. The results thus demonstrated a negligible (<2%) contribution of *fpy* mutant formation to the total colony count and an acceptable (i.e., about 95%) accuracy of *pyrE* and *pyrF* mutant identification based upon colony size under these conditions. Similar results were obtained in smaller-scale experiments using serial dilutions to score the level of FOA resistance (32).

Physiological factors that can lead to underestimation of mutation rates by plating methods (5) were experimentally tested. The efficiency of colony formation can be sensitive to the number of cells plated, for example (5). Accordingly, the proportionality of the number of Foa^r colonies to the volume of culture plated was confirmed in all of the fluctuation tests scored (see Materials and Methods). Secondly, the rate of mutation will be underestimated if mutants grow more slowly than the parent strain. Therefore, the growth rates of *pyrE* and *pyrF* mutants relative to that of the parent strain were measured by two different methods (see Materials and Methods). Photometrically determined growth curves indicated approximately 20% faster growth of *pyrE* and *pyrF* mutants than the parent strain, whereas competition experiments did not show consistent differences in fitness between mutant and parent strains. We therefore found no evidence that the *pyrE* and *pyrF* mutants were at any growth disadvantage under the conditions used for fluctuation tests in this study.

Thirdly, a phenotypic lag significantly longer than one generation causes underestimation of mutation rates by preventing newly formed mutants from being detected. The maximum-likelihood method avoids the high sensitivity to phenotypic lag of other methods of mutation rate determination (24). In addition, Koch has suggested that the overall pattern of estimates based upon the three quartiles of the fluctuation tests provides an indicator of phenotypic lag, based on the assumption that a higher quartile is influenced less (i.e., is decreased less) by phenotypic lag than is a lower quartile (13, 24). Accordingly, we compared the three μ values from 13 independent experiments, including those of Table 2 (see below), and found that the higher quartile yielded a higher μ value in 87% of the *pyrE* cases and 77% of the *pyrF* cases. This bias was often numerically small and could, based upon our understanding of theoretical studies, perhaps be explained by a growth advantage of the mutant over the parent, which was consistent with some of our control experiments described above. However, to further test the validity of our measurements, we also tested for phenotypic lag of *pyrE* and *pyrF* mutants directly. Three independent trials used chemical mutagenesis of auxotrophic *S. acidocaldarius* mutants to compare the kinetics of appearance of Foa^r clones versus prototrophic clones arising by phenotypic reversion of the auxotrophic marker (Fig. 2). In all three experiments, chemically induced *pyrE* and *pyrF* mutations first appeared concurrently with, or slightly before, induced phenotypic reversions and well before the completion of one generation of growth (Fig. 2). The direct measurements therefore gave no evidence of a genetically significant phenotypic lag for either the *pyrE* or the *pyrF* mutation under the conditions of the present study.

Rates of forward mutation under normal growth conditions. The above results indicated that enumeration of the large and small Foa^r colonies obtained under our conditions provided a sensitive and accurate assay of spontaneous mutational events which inactivate the biosynthetic enzymes OPRT and ODC of *S. acidocaldarius* (7). We therefore used formation of Foa^r mutants in fluctuation tests to measure the rates of forward mutation in two wild-type strains under growth conditions typically used for *S. acidocaldarius* in the laboratory, i.e., XTura medium continuously aerated at 75°C (this temperature is near the midpoint of the organism's growth range [6]). Results from two sets of multiple independent cultures are shown in Table 1. As expected for a fluctuation test, the variance in mutant number per culture greatly exceeded the mean value. Furthermore, the numbers of *pyrE* and *pyrF* mutants varied independently of each other among the cultures (Table 1). These results are fully consistent with random spontaneous mutation at two or more genetic loci of *S. acidocaldarius.*

Multiple experiments of this type were analyzed by the maximum-likelihood method of Lea and Coulson (13, 15); the results are summarized in Table 2. We took the third quartiles of the distributions (Table 2) to represent maximal estimates of spontaneous mutation rates. These yielded means ± standard deviations of $(2.8 \pm 0.7) \times 10^{-7}$ and $(1.5 \pm 0.6) \times 10^{-7}$ mutational events per cell per division cycle for the *pyrE* and *pyrF* loci, respectively, in wild-type strains under these conditions. It should be noted that these upper estimates (i.e., μ_3 values) were not much higher than the averages of all three μ values: $(2.1 \pm 0.6) \times 10^{-7}$ for *pyrE* and $(1.2 \pm 0.6) \times 10^{-7}$ for *pyrF* (data of Table 2).

DISCUSSION

Our results demonstrate that FOA-plus-uracil selection provides a quantitative assay of forward (i.e., loss-of-function) mutation in the thermophilic archaeon *S. acidocaldarius*. Direct selections of this type are not common and have historically played important roles in the genetic manipulation and analysis of microorganisms. FOA selection should provide a more sensitive and general assay of mutational events in *S. acidocaldarius* than does phenotypic reversion of auxotrophic markers. Mutation at any one of many different nucleotide

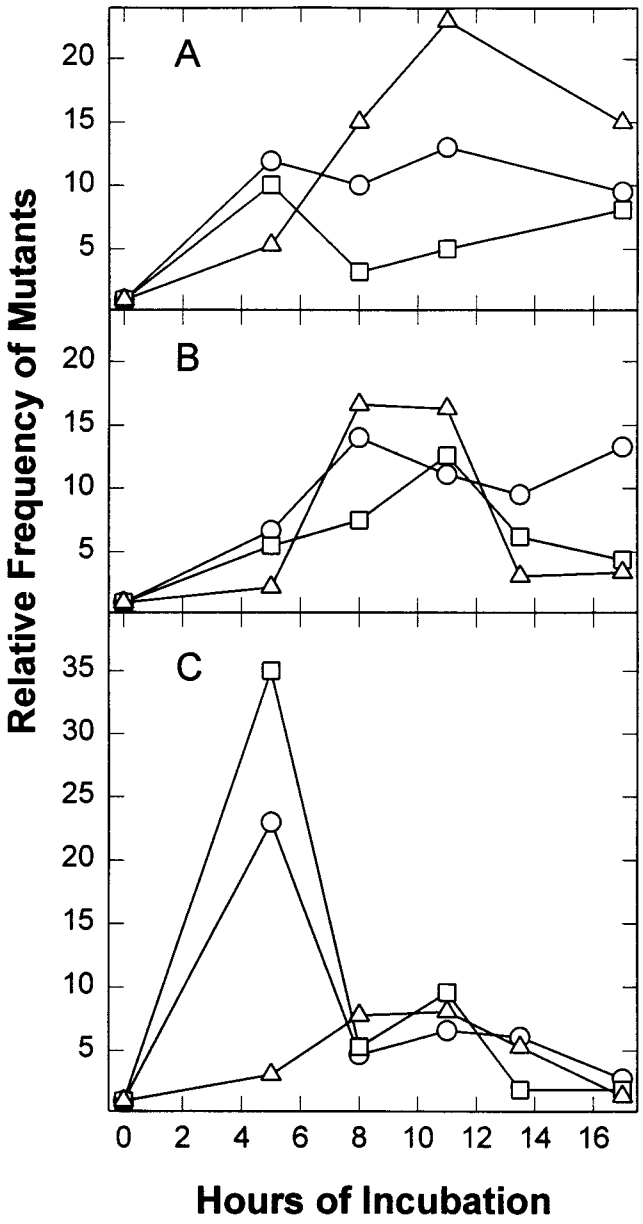

FIG. 2. Kinetics of appearance of the Foa^r phenotype. Results of three independent trials in which exponential-phase cultures of DG40 (A and B) or DG55 (C) were mutagenized with MNNG and diluted into fresh medium as described in Materials and Methods. The numbers of *pyrE*, *pyrF*, prototrophic, and total viable cells per milliliter were determined for each aliquot withdrawn from the culture at the indicated time. The frequencies of *pyrE*, *pyrF*, and phenotypic revertant cells in the mutagenized cultures were normalized to those of otherwise identical nonmutagenized controls. The ratios plotted depict the kinetics with which a chemically induced mutation became apparent. Under these conditions, the time required for mutagenized cultures to complete one generation of growth (measured as a doubling of the viable titer) was 14 h (A and B) or 12 h (C). Symbols: ○, *pyrF* mutants; □, *pyrE* mutants; △, prototrophs.

positions can inactivate a functional biosynthetic gene, whereas only a few particular mutations can revert or suppress a given mutant allele of such a gene. Furthermore, the observed rate of phenotypic reversion tends to vary tremendously as a function of the specific site of mutation within a gene due to "DNA context effects" (5). In the present study, use of direct selection facilitated large-scale fluctuation tests and their analysis by the maximum-likelihood method, which represents the most accurate and statistically efficient strategy widely used to measure mutation rates in microorganisms (15, 17). The growth capabilities of *S. acidocaldarius* also facilitated efforts to directly estimate the possible impact of differential growth rates and phenotypic lag on mutation rate determinations (5).

To our knowledge, these data provide the first accurate experimental measurements of the genetic fidelity maintained by an archaeon from geothermal habitats. Our maximal estimates of the number of forward mutational events per cell division in wild-type cells under typical growth conditions are $(2.8 \pm 0.7) \times 10^{-7}$ for the *pyrE* locus and $(1.5 \pm 0.6) \times 10^{-7}$ for the *pyrF* locus. These calculations do not take into account evidence that *pyrE* and *pyrF* mutants may grow faster that the parent strain, whereas they do attempt to correct for phenotypic lag, even though none was independently confirmed. We have thus tried to ensure that our measured rates are not underestimates.

DNA sequences encoding OPRTs (GenBank entries available in mid-1996, e.g.) for phylogenetically diverse eukaryotes and bacteria fall within a length range of 600 to 720 nucleotides, whereas ODC coding sequences comprise 700 to 1,200 nucleotides, depending on the species. Loss of either activity is

TABLE 1. Results of two typical fluctuation tests with wild-type *S. acidocaldarius*

Culture no.	No. of mutant cells			
	Strain DG6		Strain DG185	
	pyrE	*pyrF*	*pyrE*	*pyrF*
1	213	879	1,036	655
2	901	795	90	168
3	650	62	1,652	510
4	62	34	112	1,954
5	504	314	84	235
6	67	73	286	403
7	263	123	3,640	151
8	246	78	375	314
9	571	190	207	347
10	420	185	246	230
11	1,820	146	1,417	1,512
12	269	11	1,842	862
13	342	1,136	1,786	358
14	2,940	168	280	1,036
15	258	258	454	812
16	3,029	656	644	2,582
17	1,249	403	353	106
18	2,554	73	258	207
19	1,327	241	1,434	347
20	392	268	560	151
21	1,014	50	347	823
22	1,187	207	230	941
23	476	202	554	291
24	476	280	627	986
25	185	157	151	45
26	459	745	2,800	50
27	291	1,226	241	330
28	1,215	118	1,512	487
29	1,618	465	202	980
30	286	1,708	1,456	347
31	582	286	140	364
32	1,204	330	90	230
Mean	846	368	785	588
Variance	640,203	157,530	257,598	321,771
Q_1	281	122	224	230
Q_2	490	224	548	353
Q_3	1,207	419	1,421	833

TABLE 2. Summary of mutation rate determinations[a]

Strain	n^b	$N_{av}{}^c$	*pyrE*[d]				*pyrF*[d]			
			μ_1	μ_2	μ_3	μ_{av}	μ_1	μ_2	μ_3	μ_{av}
DG6	17	5.6	2.6	2.5	2.4	2.5	1.4	2.6	2.2	2.1
DG6	32	5.7	0.93	1.1	1.8	1.3	0.47	0.57	0.67	0.57
DG6	31	5.2	0.79	1.2	3.5	1.8	0.50	0.90	1.3	0.90
DG185	15	5.6	2.5	2.6	2.8	2.6	1.4	1.4	2.0	1.6
DG185	32	6.2	0.97	1.1	2.8	1.6	1.0	1.1	1.6	1.2
DG185	25	4.0	1.9	2.3	3.7	2.6	0.5	0.9	1.3	0.9

[a] Multiple independent cultures at 75°C in XTura medium with high aeration.
[b] Number of independent cultures assayed.
[c] Average number of cells per culture (10^8).
[d] Three mutation rate estimates (μ_1, μ_2, and μ_3; see Materials and Methods) and their average (μ_{av}) are shown. Units, 10^{-7} mutational events per cell per division cycle.

necessary and sufficient to confer the Foa^r phenotype for approximately 98% of the mutant colonies counted in the present study (7; see Materials and Methods). Therefore, without correcting for context effects and the proportion of silent mutations (which vary among genes and organisms [5]), the mutational target relevant to our study is probably at least 1.3 kbp of chromosomal DNA, i.e., the minimum used by other organisms to encode both ODC and OPRT. Our results for wild-type *S. acidocaldarius* thus provide a maximal estimate of $[(2.8 \pm 1.5)/1.3] \times 10^{-7} = 3.3 \times 10^{-7}$ observable mutational events per kbp per cell division at 75°C, on average, for these biosynthetic genes. For comparison, rates of spontaneous forward mutation in protein-encoding genes of *Escherichia coli* reported in the literature are 1.6×10^{-7} (average value for the histidine operon), 2.5×10^{-7}, 4.3×10^{-7} (independent estimates for the lactose operon repressor gene), and 2.0×10^{-7} (average for five arabinose utilization genes) mutational events per kbp per cell division (4, 5, 18). The results thus depict *S. acidocaldarius* as about as genetically stable as *E. coli*, despite its extremely harsh growth conditions. This is consistent with the fact that a variety of stable auxotrophic mutants of *S. acidocaldarius* have been isolated (7, 8). In particular, neither the *pyrE* nor the *pyrF* locus of *S. acidocaldarius* shows phenotypic evidence of high-frequency inactivation by insertion sequences, in contrast to what has been observed for a readily scored genetic marker of *S. solfataricus* P2 (26).

Recent studies argue that preservation of DNA primary structure in extreme thermophiles and hyperthermophiles is intrinsically more difficult than preservation of DNA secondary structure (23). Mechanisms by which thermophilic archaea compensate for the chemical instability of DNA at their optimal growth temperatures remain to be identified and should be of interest from both the biochemical and evolutionary points of view. To our knowledge, the only prior experimental evidence of DNA repair in vivo by hyperthermophilic or extremely thermophilic archaea comprises observations on genomic DNA of γ-irradiated *Pyrococcus furiosus* (3) and demonstration of photoreactivation of UV-treated *S. acidocaldarius* cells (10). Neither of these phenomena necessarily relates to the repair of thermally induced DNA damage, however, since γ radiation and UV radiation each induce a specific type of lesion different from many of those induced by high temperature (10, 23, 28). It will thus be of interest to develop and utilize genetic assays of DNA repair in these archaea.

In the latter context, we note that *Sulfolobus* spp. have demonstrated the greatest promise for routine genetic manipulation among thermophilic archaea. *S. solfataricus* P2 and *S. shibatae*, for example, serve as hosts for genetic elements such as viruses, plasmids, and insertion sequences (25, 27, 33). To our knowledge, however, no auxotrophic mutants of these two *Sulfolobus* isolates have been described; furthermore, FOA does not appear to effectively select pyrimidine auxotrophs of either isolate, in contrast to its effect on *S. acidocaldarius* (11). The present study may therefore have not been feasible with a thermophilic archaeon other than *S. acidocaldarius. S. acidocaldarius* also has the advantage of a natural mechanism of genetic exchange, which appears useful for performing simple strain constructions (9). Reports of transformation and stable maintenance in *S. acidocaldarius* of shuttle vectors (1) raise the prospect that more precise genetic manipulation of *S. acidocaldarius* may enable DNA repair mechanisms of archaea to be studied in molecular detail in vivo at extremely high temperatures.

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