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Note

An Unusual Pattern of Spontaneous Mutations Recovered in the Halophilic Archaeon *Haloferax volcanii*

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ABSTRACT

Spontaneous mutations in the orotate:phosphoribosyl transferase (*pyrE2*) gene of the halophilic archaeon *Haloferax volcanii* were selected by 5-fluoroorotic acid plus uracil at a rate of $\sim 2 \times 10^{-8}$/cell division in fluctuation and null-fraction tests but $\sim 6 \times 10^{-8}$/cell division in mutation-accumulation tests. The corresponding genomic mutation rates were substantially lower than those observed for other mesophilic microbial DNA genomes on the basis of similar target genes. The mutational spectrum was dominated by indels adding or deleting multiples of 3 bp. Properties of the organism contributing to this unusual mutational pattern may include phenotypic lag caused by a high chromosomal copy number and efficient promotion of strand misalignments by short direct repeats.

ANALYSES of spontaneous mutation in diverse micro-organisms have provided important insights into the fundamental forces and molecular mechanisms determining genetic fidelity. In most of these studies, a selection is used to quantify the rate of forward (*i.e.*, inactivating) mutations in one or more chromosomal genes. Sequencing of representative mutants then reveals a spectrum of mutation, which enables the efficiency of mutation detection and other parameters to be estimated. Such analyses have shown that all mesophilic micro-organisms examined (including DNA viruses) share two mutational characteristics: (i) the rates of mutation per genome fall near 0.003/replication, despite large differences in mutation rates per base pair (Drake 1991; Drake *et al.* 1998; Drake and Hwang 2005), and (ii) ~70% of the observed mutations are base-pair substitutions (BPSs) (Grogan *et al.* 2001). However, extending this analysis to an archaeon from a geothermal environment revealed an apparently lower genomic rate ($\leq$0.0018) and a lower proportion of BPSs (33%) (Grogan *et al.* 2001). These results suggest that basic mutational properties of micro-organisms may adapt to unusual environmental conditions.

Halophilic archaea grow optimally at moderate temperatures in 1–4 M salt (Rodriquez-Valera 1995). They cope with these environments by maintaining high intracellular concentrations of potassium and chloride (Lanyi 1974), which can be expected to exert molecular stresses on genetic processes. Certain genetic methods have been developed for *Halobacterium salinarum* and *Haloferax volcanii* (Allers and Mevarech 2005; Soppa 2006), and various systems for repairing DNA damage have been reported in these two species (McCready 1996; Baliga *et al.* 2004; Kottemann *et al.* 2005), but neither halophile has been evaluated for the accuracy of genome replication. In this study, we demonstrated the ability of 5-fluoroorotic acid (FOA) to select spontaneous orotate:phosphoribosyl transferase (OPRTase) mutants of *H. volcanii*. We measured the rate of spontaneous mutation in the corresponding *pyrE2* gene, analyzed a number of mutants by DNA sequencing, and evaluated the implications for genomic mutation in this organism.

The *H. volcanii* wild-type strain DS70 (Wendoloski *et al.* 2001) was cultured on HYTU medium as described (Rodriquez-Valera 1995). Larger liquid cultures (25 ml) were swirled at ≈120 rpm in 50-ml flasks in a gyrorotary water bath, small cultures (3 ml) were rotated continuously on a New Brunswick Model TC-7 rotator in 16-mm screw-cap tubes, and very small cultures (0.2 ml) were incubated without shaking in microdilution plates. Following Rodriquez-Valera (1995), liquid cultures were incubated at 37° to minimize oxygen limitation, whereas agar plates were incubated at 41° to accelerate colony growth. The minimum inhibitory

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TABLE 1

Rates of spontaneous mutation in *H. volcanii pyrE2*

	Fluctuation test (quartile method)[a]			
Basis	Equal growth	Slower growth	P_0 method[b]	MA method[c]
Quartile 1	1.43 ± 0.84	1.32 ± 0.72		
Median	1.72 ± 0.62	2.01 ± 0.53		
Quartile 3	2.29 ± 0.87	2.63 ± 0.95		
Mean[d]	1.81 ± 0.84	1.99 ± 86	1.97 ± 0.57	5.87

[a] Values are for eight sets of 20 cultures, incorporating two types of replicates to smooth experimental variability: (i) four different culture sets were inoculated on different days, with different inocula and batches of medium, and (ii) within each, two culture sets differed with respect to the number of cells in the inocula (10^3 *vs.* 10^4). Calculations shown for slower growth are based on $k^m/k^w = 0.9$ (Koch 1982).

[b] Values are from six independent samples drawn at various cell densities from four cultures for a total of 24 measurements of N and f.

[c] Median of the six component rates 4.30, 4.97, 5.02, 6.73, 23.9, and 26.5 (all $\times 10^{-8}$).

[d] Global mean of all 24 determinations (three estimates from each of eight fluctuation tests) are listed in the two quartile columns.

concentration of FOA for strain DS70 was determined to be 156 μg/ml by twofold serial dilutions in liquid HYTU. On the basis of this result, selective plates contained HYTU supplemented with 200 μg/ml FOA. An isolated colony was suspended in HYTU medium, diluted such that 200 μl contained 10^3 cells, and dispensed into a microdilution plate to yield 20 replicate cultures; a corresponding set of 20 cultures was prepared with 10^4 cells as inoculum. Cultures were incubated until each contained $\sim 4 \times 10^7$ cells, at which point the average number of cells per culture, N_{av}, was determined by serial dilution and plating from at least three wells from different locations in the array of cultures. Each of the remaining cultures was plated *in toto* on HYTU+FOA plates to select FOA-resistant mutants. Mutant colony counts varied greatly from one culture to another (Luria and Delbrück 1943). Three estimates of the most probable number of mutational events per culture, m, were calculated on the basis of three quartiles of the mutant frequency distribution and the numerical solutions of Koch (1982).

To score uracil auxotrophy, each clonally purified FOA-resistant mutant was used to inoculate two 3-ml liquid cultures. For this purpose, the medium contained 1% Difco casamino acids in place of yeast extract and tryptone, and only one of the tubes was supplemented with uracil. On average, 80% of the colonies picked at random from independent cultures proved to be uracil auxotrophs. From each set of fluctuation cultures, the numbers of FOA-resistant colonies (sums of the two aliquots) were ranked and adjusted for the proportion (80%) of auxotrophs. In addition, sequencing revealed that only 17 of 23 uracil auxotrophs contained a mutation in the *pyrE2* mutation-reporter gene. All mutation rates (including that below) were therefore adjusted by factors of 0.8 and $17/23 = 0.739$ and are expressed as mutations/10^8 cell divisions ± sample standard deviation. The MA method used six 25-ml cultures grown from single colonies to produce exponential-phase populations of $\sim 10^{10}$ cells. Each culture was subsequently determined to have sustained ≥600 mutational events (*e.g.*, 10^{10} cells times $\geq 6 \times 10^{-8}$ mutations/cell division). Aliquots were plated on selective medium without dilution and on nonselective medium with dilution, and the resulting viable titers were used to calculate N, the number of cells per culture, and f, the ratio of mutants to total cells. Mutation rates were calculated using the expression $\mu = f/\ln(N\mu)$ and the median value of μ was reported (Drake 1991; Rosche and Foster 2000).

Genic mutation rates are shown in Table 1. In the fluctuation tests, we calculated three estimates of the most probable number of mutational events per culture, m, on the basis of the three quartiles of the mutant frequency distributions and the numerical solutions of Koch (1982). The mean and sample standard deviation across three quartiles and eight sets of independent cultures were $(1.81 \pm 0.84) \times 10^{-8}$ mutations/cell division (Table 1, column 2).

To determine whether the growth rates of mutants affected this estimate, we drew 11 uracil auxotrophs arbitrarily from the mutant collection, mixed a late-exponential liquid culture of each with a 10-fold excess of wild-type cells, and inoculated halophile–yeast extract–tryptone–uracil (HYTU) medium with the resulting binary mixtures. Before and after 24 hr of growth, samples of each binary mixture were diluted and plated on selective and nonselective media, and the ratio of specific growth-rate constants of the mutant and wild-type strain (k^m/k^w) was calculated for each mixture as $(\ln N_f^m - \ln N_i^m)/(\ln N_f^w - \ln N_i^w)$, where subscripts i and f represent initial and final times and superscripts m and w represent mutant and wild-type strains, respectively. The

measured ratios were 0.84, 0.86, 0.87, 0.87, 0.92, 0.93, 0.96, 1.00, 1.00, 1.03, and 1.16. The resulting mean, 0.95, has an ~5% probability of representing a true underlying value of 1.00. To estimate the effect of the slightly slower growth of mutants, we used a variant of Koch's method to account for the case when the mutant cells reproduce at 0.90 of the wild-type rate (Koch 1982). The resulting rate, $(1.99 \pm 0.86) \times 10^{-8}$ (Table 1, column 3), was then averaged with the uncorrected value to estimate the case in which mutants reproduce with an average growth rate 0.95 that of the parental strain, yielding 1.90×10^{-8} mutational events/cell division.

In another method, the null-class or P_0 method, the proportion of cultures yielding no mutants (P_0) is used to calculate $m = -\ln P_0$ (Luria and Delbrück 1943). This method could be applied to the same fluctuation cultures because a large fraction of them contained no mutants. The resulting rate, $(1.97 \pm 0.57) \times 10^{-8}$ (Table 1, column 4), is statistically indistinguishable from the quartile results, and the average of these two related methods is 1.93×10^{-8}.

To provide an experimentally independent assessment of mutation rate, we used the mutation-accumulation (MA) method. The resulting rate, 5.87×10^{-8} (Table, column 5), did not lend itself to variance analysis, but can be considered higher than the results from the other two methods because its lowest component value was twice the other calculated rates. The difference between the first two methods and the MA method suggests an effect of phenotypic lag, which is more severe for smaller populations.

To characterize the spectrum of mutations, we sequenced 23 mutants of independent origin drawn at random from the fluctuation-test cultures. Uracil auxotrophs, each from a different fluctuation-test culture (drawn, in turn, from multiple trials), were chosen for sequence analysis. To avoid bias on the basis of colony size, the back of each plate was marked with a single spot and the colony arising closest to that spot was picked. After clonal purification and confirmation of uracil auxotrophy, the strains were cryo-preserved at −70° (Rodriquez-Valera 1995). Genomic DNA purified from small liquid cultures by a modification of the method of Boom *et al.* (1990) served as template in separate standard PCR reactions to amplify the two genes previously designated "*pyrE1*" and "*pyrE2*" (Bitan-Banin *et al.* 2003). The upstream *pyrE1* amplification primer was 5′-GACGACGACACCACT-3′ and the downstream primer was 5′-CGGGTCTGCACTTCTT-3′. The *pyrE1* PCR reaction included a 9-min denaturing step at 95° and a 1-min preheat step at 95° with Taq DNA polymerase (Invitrogen, Carlsbad, CA). The reaction was then run for 30 cycles of 1 min at 94°, 1 min at 54°, and 1 min at 72°. This was followed by a final extension at 72° for 10 min. The upstream *pyrE2* amplification primer was 5′-ACCGCTCACCCGAACC-3′ and the downstream primer was 5′-TCCGGCTTCGTACTT-3′. The *pyrE2* PCR reaction included a 2-min denaturing step at 98° and a 30-sec preheat step at 94° with Taq DNA polymerase. The reaction was then run for 39 cycles of 30 sec at 94°, 30 sec at 53°, and 30 sec at 72°. This was followed by a final extension step at 72° for 10 min. The resulting PCR products were purified using QIAquick purification kit (QIAGEN, Valencia, CA). The BigDye-Terminator cycle sequencing kit was used to sequence the purified PCR products using the same primers. The sequencing cycle consisted of 10 min at 95° followed by 25 cycles of 30 sec at 94°, 30 sec at 53°, and 4 min at 60°.

TABLE 2

Spontaneous *pyrE2* mutations

Mutation	No.[a]	Sequence context[b]
$T^{185} \rightarrow G$	1	^{179}ACAAAC<u>T</u>GGCCGG^{191} (Leu to Arg)
$C^{206} \rightarrow A$	1	^{200}TCGGCG<u>C</u>GGTCCC^{212} (Ala to Glu)
−15 bp	2	^{300}<u>CGAGGGCG</u>[7 bp]<u>CGAGGGCG</u>^{322}
+51 bp	5	^{378}<u>CGTGGA</u>[45 bp]<u>CGTGGA</u>^{434}
$-C^{417}$	1	^{411}CGACCG<u>C</u>GTCGTC^{423}
−3 bp[c]	3	^{417}CGTCGTCGTCGTCGT^{431}
+3 bp[c]	1	^{417}CGTCGTCGTCGTCGT^{431}
−51 bp	3	^{457}<u>CTGCTGGCCGAC</u>[39 bp]<u>CTGCTGGCCGAC</u>^{519}

[a] Number of independent isolates containing this mutation.
[b] Superscripts indicate the position of the first and last posted nucleotide, where A in the initiating ATG is 1 (Bitan-Banin *et al.* 2003). For the multiple-base indels, the direct repeats defining the ends are underlined. The numbers in brackets indicate the numbers of bases deleted or duplicated, to which is added the number in one copy of the flanking sequence. For the three single-base mutations, six nucleotides are shown before and after the underlined wild-type base.
[c] Due to the repetitive nature of this region, the original event could have added or removed CGT, GTC, or TCG.

None of the spontaneous FOA-resistant uracil auxotrophs had mutations in the gene designated *pyrE1*, corroborating other genetic evidence that this ORF is not relevant to FOA metabolism (Bitan-Banin *et al.* 2003). Of the 23 mutants, 6 had no mutations in *pyrE2* and were not further analyzed, in part because PCR products amplified by *pyrF* primers were not the expected sequence (Bitan-Banin *et al.* 2003). The *pyrE2* mutations in the remaining 17 mutants are summarized in Table 2. Two contained BPSs generating amino acid substitutions. The remaining 15 contained insertions or deletions (indels), representing one −1 frameshift mutation and 14 in-frame mutations (8 deletions and six tandem duplications); all of the in-frame mutations were flanked by repeated sequences. None of the mutations were caused by transposable elements.

The observed rate and character of spontaneous mutations recovered in *H. volcanii* differ markedly from those in most unicellular microbes and DNA viruses examined so far. For example, all mesophilic DNA microbes with well-characterized spontaneous mutation

rates display a genomic mutation rate of $\mu_g \approx 0.0034$ (range 0.0025–0.0046)/chromosome replication (Drake 1991; Drake *et al.* 1998; Drake and Hwang 2005). On the basis of an *H. volcanii pyrE2* mutation rate of (1.93–5.87) $\times 10^{-8}$ and the coding sequence of 531 bp (including the termination codon), the midrange mutation rate per base pair is 7.34×10^{-11}. Before converting to a genomic rate, however, an observed rate should be corrected for the fraction of mutations that produce no phenotypic effect. This is usually done by assuming that all indels are detected and then by estimating the total number of BPSs either from the number of observed chain-termination mutations or by adjusting the number of observed BPSs by a fraction determined by BPS detection efficiency in several other microbes (~0.2) (Grogan *et al.* 2001). Because chain-terminating mutations were not represented in our mutant set, we used the latter approach of dividing observed BPSs by 0.2. The resulting rate estimate per base pair thus becomes $(7.34 \times 10^{-11})(15 + 2/0.2)/17 = 1.08 \times 10^{-10}$. On the basis of the current sequence assembly (http://www.tigr.org/tdb/mdb/mdbinprogress.html), the *H. volcanii* genome is ~4.2 Mbp, which yields an apparent genomic mutation rate of 0.00045. This value is ~7.5-fold lower than the consensus value of 0.0034 observed in other mesophilic microbes with DNA genomes.

With respect to the molecular character of mutations in other organisms, BPSs make up roughly two-thirds of the spontaneous mutations recovered, and the next most common class (excluding occasional extreme hotspots of indels and high proportions of transposon insertions) are ±1 frameshifts (Garcia-Diaz and Kunkel 2006). In *H. volcanii*, however, base substitutions were only 12% of detected mutations, and single-nucleotide frameshifts were only 7% of the indels. The remaining indels were flanked by repeated sequences and consisted of multiples of three bases. Flanking repeats are common among indels (Albertini *et al.* 1982; Halliday and Glickman 1991; Tran *et al.* 1995; Glaab *et al.* 2005; Garcia-Diaz and Kunkel 2006). In-phase indels are not prominent in published spectra based on laboratory studies, but have been observed to be enriched in human pseudogenes; in that context, a much greater number of indels were analyzed and the bias was much lower than observed here (Zhang and Gerstein 2003).

We are unaware of any other micro-organism that has exhibited a similar pattern of low genomic mutation rate, low proportion of BPSs, and predominance of in-frame indels. According to control experiments, the low rate should not be an artifact of slow growth or erratic efficiency of plating of the mutants. Alternatively, the possibility that *H. volcanii pyrE2* is an intrinsically insensitive mutational reporter for BPSs conflicts with the fact that several other mutation-detection systems use similar target genes and selections (*i.e.*, purine or pyrimidine phosphoribosyl transferases) and detect high frequencies of BPSs (Nalbantoglu *et al.* 1986; Glaab *et al.* 2005). However, attenuation of mutant recovery due to phenotypic lag may be a factor in the mutation-rate measurements on the basis of the mechanism of the selection—*pyrE2* mutations must substantially decrease the cellular OPRTase level to be selected by FOA—and on two observations. First, the MA method (based on large populations) yielded a higher mutation rate than did fluctuation tests (based on small populations), which is one consequence of phenotypic lag. Furthermore, quantitative Southern-blot and real-time PCR assays have indicated that *H. volcanii* maintains nearly 20 copies of its chromosome/cell in exponential growth and ~12 copies/cell in stationary phase (Breuert *et al.* 2006). These levels of polyploidy imply that, if FOA resistance normally occurs only after the generation of homozygous mutant cells, *pyrE2* mutations could require many generations to be detected with full efficiency. Regardless of the underlying mechanism, the low rate of mutant recovery that we documented has functional implications for natural *H. volcanii* populations. Establishing the mechanistic basis of these low rates may become feasible as genetic methods continue to be developed for *H. volcanii* (Allers and Mevarech 2005; Soppa 2006)

In contrast to the low rate, the extremely high ratio of indels to BPSs represented among the recovered mutants cannot be readily attributed to the effects of polyploidy or phenotypic lag. In theory, the possibility that the indels that we recovered represent dominant-negative alleles could explain both their preferential recovery and their preservation of the *pyrE2* reading frame. However, dominant-negative alleles of biosynthetic genes are rarely reported, presumably because they must incapacitate the wild-type polypeptide via subunit–subunit interactions (Gibbons *et al.* 1975); in addition, we know of no evidence that the *H. volcanii* OPRTase is multimeric.

An alternative explanation is suggested by the fact that all but one of the indels fit the pattern expected for templating by short direct repeats (DRs) (Albertini *et al.* 1982). According to this explanation, DRs promote strand misalignments during DNA replication, and their distribution thus determines the length, position, and relative frequency of spontaneous indels recovered in this gene. Our first test of this possibility involved comparing the DRs at the endpoints of the indels (underlined in Table 2) to all the DRs of equal length within *pyrE2*. Thus, the endpoints of the 51-nt tandem duplication (Table 2) were found to correspond to 1 of 35 DRs of 6 nt in *pyrE2*. Similarly, the 15-nt deletion corresponds to 1 of 12 DRs of 8 nt, the 51-nt deletion corresponds to the only 12-nt DR, and the site of recurring triplet indels represents the largest triplet repeat (15 nt) in *pyrE2* (Table 2). This pattern of DR utilization resembles those seen in other mutational targets (Albertini *et al.* 1982): as DR length increases, the abundance (frequency) of DRs within a gene decreases, but the probability that a given DR will be associated with a mutation increases,

TABLE 3

Short DRs in *H. volcanii* genes

Gene[a]	Putative function[a]	No. of DRs of indicated length[b] 8	9	10	11	12	>12	Total	% in frame
trpC	Indole-3-glycerol-phosphate synthase	16	5	3	2	0	0	26	65
smc1	Chromosome segregation protein	7	5	0	0	0	0	12	67
dnaK	Heat-shock protein	4	5	1	0	0	0	10	70
aspB1	Aspartate aminotransferase	12	4	2	0	0	0	18	78
eif2a	Translation initiation factor eIF-2 subunit	4	4	1	0	0	0	9	100
udp2	Uridine phosphorylase	2	0	1	1	0	0	4	100
flaB1	Flagellin B1 precursor	8	2	1	0	0	4	13	92
argB	Ornithine carbamoyltransferase	15	3	2	3	0	0	23	78
glyA	Glycine hydroxymethyltransferase	20	4	1	2	1	0	28	71
hisD	Histidinol dehydrogenase	24	6	4	2	0	0	36	78
thrS	Threonyl-tRNA synthetase	21	3	2	1	0	1	28	89
ftsZ3	Cell division protein	5	2	0	0	0	0	7	100
pyrD	Dihydroorotate dehydrogenase	10	3	2	2	1	0	18	56
rpoB	DNA-directed RNA pol subunit B	9	3	2	0	0	0	14	86
csg	Cell surface glycoprotein	9	3	0	0	0	1	13	100
(5′)	Region before *pyE2*	16	3	6	4	0	0	29	83
(3′)	Region after *pyrE2*	10	2	0	1	0	0	13	77
Column statistics:	Mean	11.3	3.4	1.7	1.06	0.12	0.24	17.7	81.8
	Sample SD	6.5	1.5	1.6	1.3	0.33	0.56	9.1	13.7
	Median	10	3	1	1	0	0	14	78.3
pyrE2	Orotate phosphoribosyl transferase	14	4	1	2	0	0	21	95.2

[a] The indicated gene of *Halobacterium* sp. NRC-1 (Ng *et al.* 2000) was used to query unpublished *H. volcanii* sequence via the TIGR BLAST server (http://www.tigr.org) and the first 540 nt of *H. volcanii* sequence matching the Halobacterium gene were analyzed.

[b] All DRs ≥8 nt were located in the 540-nt window using the RePuter program via the Bielefeld University Bioinformatics server (http://bibiserv.techfak.uni-bielefeld.de/reputer/).

consistent with the increased stability of the corresponding misaligned intermediates. What has not been reported for other systems, however, is the strongly phased spacing that we observed between the DRs in *pyrE*2: 87 and 93%, respectively, of the hexanucleotide and octanucleotide repeats in *pyrE2* are offset by multiples of three nucleotides. Finally, it should be noted that postreplicational mismatch repair may contribute to the unusual mutational pattern of the *H. volcanii pyrE2* gene by removing mispaired bases and single-nucleotide loops efficiently, but leaving the larger indel precursors unrepaired.

The apparent importance of DRs in the mutational spectrum of *pyrE2* nevertheless suggests that, in *H. volcanii*, the sequence of a gene should exert a strong influence on its mutational properties. This influence (specifically, the promotion of indels by DRs) accordingly provides a criterion for investigating whether mutational properties of the *H. volcanii* genome could be expected to resemble those of the *pyrE2* gene. Such an analysis is significant, as, to our knowledge, no other quantitative assay of forward mutation has been developed for *H. volcanii*. Although the complete *H. volcanii* genome sequence had not been released at the time of writing, individual gene sequences were available from The Institute for Genomic Research via BLAST server (http://www.tigr.org). We therefore used the *Halobacterium* sp. NRC-1 annotation (Ng *et al.* 2000) to recover sequences of 19 other *H. volcanii* loci (including the regions flanking *pyrE2*) and analyzed these with respect to intragenic DRs of 8 nt or longer. The results, shown in Table 3, argue that *pyrE2* is not unusual with respect to DRs in the *H. volcanii* genome. Specifically, the number of DRs of each length-class in *pyrE2*, and the proportion defining in-frame indels, were all within 1 standard deviation of the corresponding mean of the other *H. volcanii* loci. Thus, the spectrum of spontaneous mutations that we recovered from the *pyrE2* gene is expected to approximate those of these other loci and, by extension, the genome as a whole.

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