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Extreme Resistance to Thermally Induced DNA Backbone Breaks in the Hyperthermophilic Archaeon *Pyrococcus furiosus*

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Pyrococcus furiosus **is a hyperthermophilic archaeon that grows optimally at 100°C. It is not conceivable that these organisms could survive with genomic DNA that was subject to thermal destruction, yet the mechanisms protecting the genomes of this and other hyperthermophiles against such destruction are obscure. We have determined the effect of elevated temperatures up to 110°C on the molecular weight of DNA in intact *P. furiosus* cells, compared with the effect of elevated temperatures on DNA in the mesothermophilic bacterium *Escherichia coli*. At 100°C, DNA in *P. furiosus* cells is about 20 times more resistant to thermal breakage than that in *E. coli* cells, and six times fewer breaks were found in *P. furiosus* DNA after exposure to 110°C for 30 min than in *E. coli* DNA at 95°C. Our hypothesis for this remarkable stability of DNA in a hyperthermophile is that this hyperthermophile possesses DNA-binding proteins that protect against hydrolytic damage, as well as other endogenous protective mechanisms and DNA repair enzyme systems.**

The chemical effects of elevated temperatures on DNA are believed to be hydrolytic: (i) phosphodiester bond scission (backbone breakage); (ii) cleavage of *N*-glycosyl bonds (rendered labile by the lack of the sugar 2′-OH bond), resulting in base elimination, producing an apurinic or apyrimidinic site that weakens the DNA chain, which also then enhances cleavage by β-elimination; and (iii) hydrolytic base deamination, particularly at the 4 position of cytosine (5). In a series of papers, Lindahl and coworkers have measured the rates of these hydrolytic changes in DNA caused by high temperature (5–9). It was calculated by extrapolation that DNA at 100°C would experience approximately 3,000 times more hydrolytic events than DNA at 37°C (5). For backbone breaks in depurinated DNA, no measurements above 70°C were described (9).

The problem of topological DNA stability in thermophiles and hyperthermophiles was the subject of a recent chapter (4); however, DNA backbone stability in hyperthermophiles has not previously been addressed. The purpose of our work was to determine the effects of temperatures higher than 90°C on DNA in vivo by comparing the integrity of DNA in a mesophilic cell to that in a hyperthermophile. Here we describe our comparison of DNA backbone breakage resulting from phosphodiester bond cleavage (including the production of AP sites leading to backbone cleavage by β-elimination).

Alkaline sedimentation of *P. furiosus* DNA. *P. furiosus* (DSM 3638) was grown to about 4×10^8 cells per ml in sealed culture tubes in an artificial seawater medium supplemented with yeast extract (1%) and tryptone (0.5%) modified from that described by Brown and Kelly (2). Yeast extract and tryptone were from Difco Laboratories (Detroit, Mich.), and maltose, deoxyadenosine, thymidine, proteinase K, and Sarkosyl were from Sigma Chemical Co. (St. Louis, Mo.). Radiolabelled thymidine was obtained from ICN Radiochemicals (Irvine, Calif.). Tungsten (3.75 μM $NaWO_4 \cdot 2H_2O$) and maltose (0.5%) were used in place of elemental sulfur. Cultures were incubated at 95°C for 16 h. For radioactive labelling the medium was supplemented with [^{3}H]thymidine (1 μCi/ml), deoxyadenosine (50 μg/ml), and carrier thymidine (5 μM) at the concentrations that were determined experimentally to produce more cell growth, better yield, and the highest incorporation of radioactivity. Labelling efficiency was low compared with that of *E. coli*, but it was sufficient to locate the position of the DNA in the gradients.

Labelled cells were pelleted by centrifugation, washed free of medium, and resuspended in phosphate-buffered saline. Aliquots (50 μl) of cell suspension were sealed in microcentrifuge tubes and held in an oil or water bath at the appropriate temperature. At the end of the treatment time, the cell suspension was cooled in ice and transferred to centrifuge tubes for lysis. The procedures for number-average DNA molecular mass (M_n) determination from sedimentation coefficients of DNA and calculation of the numbers of backbone breaks were exactly as described previously (11, 16). For *E. coli*, treated cells were lysed on top of 5 to 20% alkaline sucrose gradients, and the DNA was centrifuged in an SW55Ti swing-out rotor as described previously (11).

With *P. furiosus*, initial problems were experienced with the protocol because of unidentified proteins tightly complexed with the DNA that caused anomalous sedimentation behavior. To eliminate the problem, a more rigorous lysis procedure was employed in which the cells were lysed in the ultracentrifuge tubes in Tris-EDTA (10 mM Tris, 0.5 M EDTA) buffer (pH 8) containing 1 mg of proteinase K per ml and 1% *N*-lauroyl sarcosine (Sarkosyl) at 35°C for 24 h (12). To minimize shearing of the DNA by avoiding the need to pipette the lysate, the alkaline sucrose gradients were gently layered beneath the lysates, which were thus elevated to the top of the tube over the gradients. Centrifugation was done for 120 min at 38,000 and 25,000 rpm in an SS55Ti swing-out rotor for *P. furiosus* and *E. coli*, respectively, to position the differently sized genomes (see below) similarly in the gradients.

The sedimentation profiles for *P. furiosus* DNA were similar to those described previously for *E. coli* and *Bacillus subtilis* (11, 16), having a single DNA peak as illustrated in Fig. 1 for control (untreated) *P. furiosus* DNA (the low point of fraction 17 is an anomaly of this particular gradient). This sedimentation behavior translates into an M_n value of 2×10^4 kDa (62

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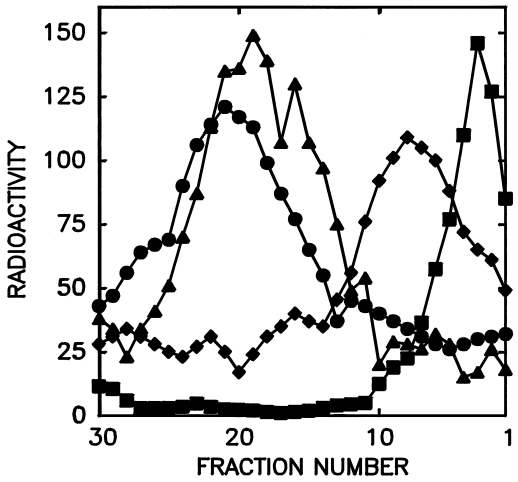

FIG. 1. Profiles of *P. furiosus* and *E. coli* DNA sedimented through alkaline sucrose gradients. The data have been reproduced, and Fig. 1 to 3 show the results of typical experiments. Unless otherwise stated, datum points are the means of two or three measurements. When more replicates were tested, the standard error of the mean was about 30% of the mean: for instance, DNA breaks in *E. coli* at 80°C were 4.6 ± 1.4 per 10^8 Da ($n = 7$). *E. coli* was grown, and its DNA was labelled with [^{3}H]thymidine as described previously (11). Circles, control *E. coli* DNA; triangles, control *P. furiosus* DNA; squares, *E. coli* DNA after 15 min of boiling; diamonds, *P. furiosus* after 180 min of boiling. Radioactivity, tritium counts; sedimentation, from right to left.

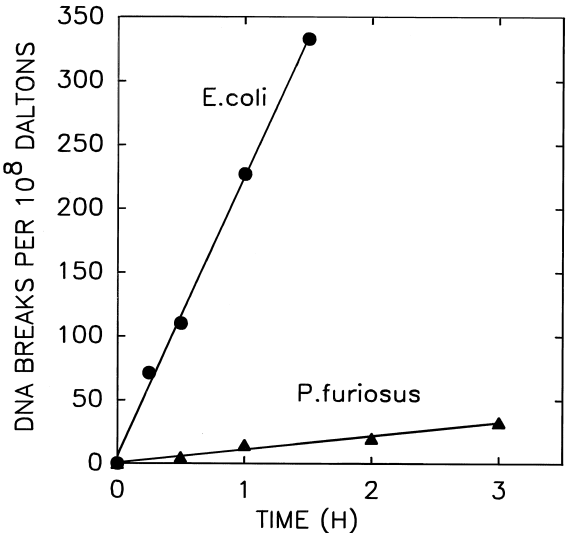

FIG. 2. Comparison of rates of DNA backbone breaks induced in *E. coli* DNA and in *P. furiosus* DNA by exposure of the cells to 100°C. Aliquots of cells prepared as described in the legend to Fig. 1 were removed from a boiling water bath at 30-min intervals for determination of the molecular masses of their DNAs. Sedimentation times were increased appropriately for the highly sheared DNA of boiled *E. coli.*

kb), compared with 400 kb determined for *E. coli* in these experiments. The size of the genome in this organism is 1.9 Mb (14a), compared with 4.69 Mb for *E. coli* (16), so the procedures of lysis, holding, and pouring the gradient under the lysate must have introduced about 30 nicks per genome into the backbone, about three times as many as are produced in *E. coli* DNA during its lysis procedure. This degree of nicking in control cells was reproducible from experiment to experiment. The figure clearly shows the extreme stability at 100°C of *P. furiosus* DNA compared with that of *E. coli*: 15 min of boiling of the cells relocates *E. coli* DNA almost to the top of the gradient, whereas 3 h of boiling of *P. furiosus* cells relocated the DNA peak from fraction 12 to fraction 22.

Stability of cellular DNA to thermal stress. Figure 2 shows a comparison of the rates of breakage of DNA when cells were held at 100°C for periods up to 3 h. The DNA in *E. coli* was broken more than 20 times faster than that in *P. furiosus*, which sustained about 10 breaks per 10^8 Da (15.5×10^5 breaks per Mb) after 1 h at 100°C. This corresponds to about 100 breaks per cell genome, compared with more than 4,000 produced in the DNA of an *E. coli* cell by the same treatment. Figure 3 demonstrates the yield of breaks in the two organisms held at a range of temperatures for 30 min. No breaks were detected in *P. furiosus* DNA at temperatures up to 95°C, whereas in the DNA of *E. coli* breaks were first detected at 65°C. Above 85°C in *E. coli*, the break yield increased dramatically to more than 60 per 10^8 Da per 30 min at 100°C, the highest exposure temperature used for this organism. By contrast, even at 110°C the *P. furiosus* DNA had sustained only about eight breaks per 10^8 Da per 30 min.

These experiments demonstrate for the first time the very low rates of accumulation of thermally induced hydrolytic events such as backbone breaks in DNA from hyperthermophiles. Not surprisingly, *P. furiosus*, which grows optimally at 100°C (3) and survives for at least 2 h at temperatures up to 105°C, has genomic DNA that is remarkably stable in vivo at high temperatures.

Two general mechanisms are feasible for this stability in *P. furiosus*: either the DNA is totally covered by a protective layer of DNA-binding molecules such as proteins and is inaccessible to water, or else there are exceptionally efficient and thermally stable repair systems in the cells. Although we have evidence that some proteins may be exceptionally tightly bound to DNA in *P. furiosus* (see above), the first alternative seems unlikely to be the sole mechanism. It is difficult to imagine maintaining the genome in a totally occluded condition in *P. furiosus* cells that are growing with generation times of less than 40 min (3),

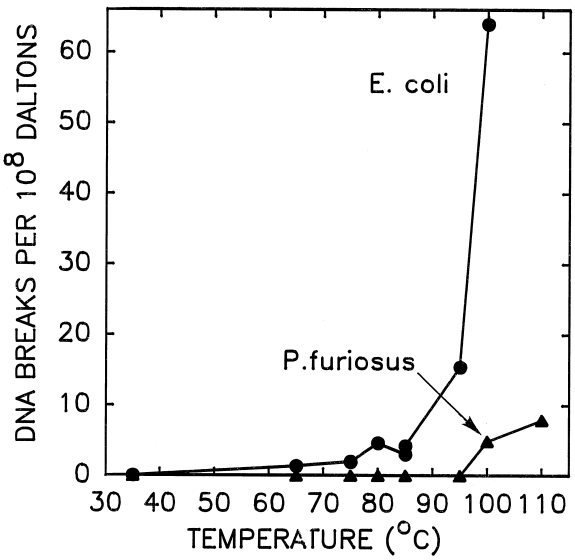

FIG. 3. Comparison of rates of DNA backbone breaks induced in *E. coli* DNA and in *P. furiosus* DNA by exposure of the cells to a range of temperatures for 30 min. Cells were held at the different temperatures for 30-min intervals before determination of the molecular masses of their DNAs. Symbols, as described in the legend to Fig. 1.

although one possibility might be that the DNA is transiently freed from the putative protective proteins (and is therefore vulnerable to breakage at that time) to allow functions such as replication and transcription to occur. On the other hand, the histone-like DNA-binding proteins found in the *Archaea* (10, 13) and the nucleosome-like structures reported in some hyperthermophiles (14) are not present in the large amounts that would be required to include the entire chromosome. One possibility is that the hyperthermophiles also elaborate rapid repair systems that allow them to maintain their genetic material at very high temperatures. However, in this case in our experiments the cells would require an endogenous energy source since they were kept in phosphate-buffered saline during holding at 100°C.

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