Thermostable archaeal O^6-alkylguanine-DNA alkyltransferases

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ABSTRACT Archaea represent some of the most ancient organisms on earth, and they have relatively uncharacterized DNA repair processes. We now show, using an *in vitro* assay, that extracts of two Crenarchaeota (*Sulfolobus acidocaldarius* and *Pyrobaculum islandicum)* and two Euryarchaeota (*Pyrococcus furiosus* and *Thermococcus litoralis*) contain the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (ATase). The ATase activities found in the archaea were extremely thermostable, with half-lives at 80°C ranging from 0.5 hr (*S. acidocaldarius*) to 13 hr (*T. litoralis*). The temperature optima of the four proteins ranged from ≈75 to ≈100°C, although activity was seen at 37°C, the temperature optimum of the *Escherichia coli* and human ATases. In all cases, preincubaton of extracts with a short oligonucleotide containing a single O^6-methylguanine residue caused essentially complete loss of ATase activity, suggesting that the alkylphosphotriester-DNA alkyltransferase activity seen in some prokaryotes is not present in Archaea. The ATase from *Pyrobaculum islandicum* had an apparent molecular mass of 15 kDa, making it the smallest of these proteins so far described. In higher organisms, ATase is responsible for the repair of toxic and mutagenic O^6-alkylguanine lesions in alkylated DNA. The presence of ATase in these primitive organisms therefore suggests that endogenous or exogenous exposure to agents that generate appropriate substrates in DNA may be an early event in evolution.

The DNA repair protein O^6-alkylguanine-DNA alkyltransferase (ATase) is considered to be the principal mechanism of cellular resistance to the toxic and mutagenic effects of DNA damage produced by certain monofunctional alkylating agents (1–3). ATase operates by the transfer of the offending alkyl groups from the O^6 position of guanine and the O^4 position of thymine in damaged DNA to a cysteine residue at the active site of the protein. This is an irreversible process that results in the stoichiometric inactivation of the protein, the repair of other lesions requiring additional active molecules (4, 5).

ATases have been characterized for a number of prokaryotes and eukaryotes, and their presence in such diverse organisms may imply that exposure of DNA to those agents that generate the corresponding substrate lesions in DNA is widespread. It has been suggested that endogenous generation of such agents is likely, and there is some indication that *S*-adenosylmethionine, the methyl donor molecule for a number of cellular biomethylation processes, may be the agent responsible (6, 7). While O^6-methylguanine is potentially a cytotoxic lesion, the precise mechanism of this cytotoxicity is not defined, although it is clear that the toxic effects are manifested only after DNA replication. It may be that the lesion results in DNA chain termination (8) or that the O^6-methylguanine·thymine mispairs that occur upon replica-

tion are recognized by the postreplication mismatch repair system (9) and that this results in cell death, possibly via a "futile" repair cycle (10) or by triggering a programmed (apoptotic) cell death pathway (11). Irrespective of the killing mechanism(s), the survival advantage that ATase confers as a consequence of repair of such lesions may be the basis of its evolution and conservation.

For the purposes of structure–activity studies and the possibility of obtaining useful reagents, we have screened a number of Archaea for ATase activity, and we describe here the presence of these proteins in extracts of four of these organisms. The phylogenetically lower Crenarchaeota were represented by the hyperthermophile *Pyrobaculum islandicum* and by the thermoacidophile *Sulfolobus acidocaldarius,* which have optimal growth temperatures of 100°C and 78°C, respectively (12, 13). The phylogenetically higher Archaea, Euryarchaeota, were represented by the hyperthermophilic *Pyrococcus furiosus* and *Thermococcus litoralis*, both belonging to the subgroup Thermococcales, which have optimal growth temperatures of 100°C and 88°C, respectively (14, 15).

MATERIALS AND METHODS

Organisms. The Archaea were grown at the Centre for Applied Microbiology and Research under conditions described elsewhere (16). *Escherichia coli* BS21, a strain constitutive for the synthesis of the Ada protein (17), was grown in LB medium (1% Bacto-tryptone/0.5% yeast extract/1% NaCl) with shaking at 37°C.

Human ATase Protein. Overexpression of the human ATase in *E. coli* was carried out as described (18). After cell disruption by sonication in buffer I (50 mM Tris·HCl, pH 8.3/1 mM EDTA/3 mM DTT) containing leupeptin (2 μg/ml) and the addition of phenylmethylsulfonyl fluoride (PMSF) to 87 μg/ml, buffered saturated ammonium sulfate was added to a final concentration of 30% (wt/vol) and stirred at 6°C for 1 hr. After centrifugation, the supernatant was removed and the ammonium sulfate concentration was increased to 60%. The mixture was again centrifuged and the resulting pellet was resuspended in buffer A [10 mM Tris·HCl, pH 7.5/1 mM DTT/2 mM EDTA/10% (vol/vol) glycerol], dialyzed overnight, and applied to a DEAE-cellulose column equilibrated in the same buffer. The human ATase was eluted with a linear NaCl gradient (0–0.1 M) and active fractions were pooled.

ATase Assay. Frozen cells (≈0.1 g) were suspended in 1 ml of buffer I containing leupeptin (5 μg/ml) and sonicated (two pulses of 25 sec at 18 μm and 20 μm peak-to-peak distance, respectively); PMSF was added to a final concentration of 50 μM immediately after the second sonication. Sonicates were clarified by centrifugation (18,000 × *g*, 15 min, 4°C) and supernatants were stored at 0°C and assayed for ATase activity. Increasing amounts of cell extract were incubated with

Abbreviation: ATase, O^6-alkylguanine-DNA alkyltransferase.
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substrate DNA (2 μg of [^{3}H]methylnitrosourea-methylated DNA; 17.3 Ci/mmol [1 Ci = 37 GBq], containing 90 fmol of O^6-[^{3}H]methylguanine) in a total volume of 300 μl of buffer I containing 1 mg/ml BSA (IBSA) in capped tubes at the temperatures and for the times indicated. The reaction mixture was then transferred to a 5-ml scintillation vial insert (LIP Ltd. Shipley, W. Yorkshire, U.K.) tube and 100 μl of BSA (10 mg/ml in buffer I), 100 μl of 4 M perchloric acid and 2 ml of 1 M perchloric acid were added and residual substrate was hydrolyzed to acid solubility at 75°C for 50 min. Protein was recovered by centrifugation at 2,300 × g for 10 min, washed with 4 ml of 1 M perchloric acid, and resuspended in 0.3 ml of 10 mM NaOH and 3 ml of scintillation cocktail. Radioactivity in the samples was determined by scintillation counting. The protein concentration in the crude extracts was determined as described (19).

Temperature Optima. The optimal temperature for methyl transfer was determined by using protein-limiting amounts of cell extracts by incubation with substrate DNA at the temperatures indicated for 20 min (*S. acidocaldarius, T. litoralis*) or 10 min (*P. islandicum, Pyrococcus furiosus*).

Effect of Oligonucleotides on ATase Activity. Crude cell extracts were preincubated under substrate-limiting conditions with 16-bp oligonucleotide (25 pmol) containing a single O^6-methylguanine [5′-d(GTGGGCGCTGXAGGCG)-3′ (X = O^6-methylguanine) kindly provided by R. C. Moschel, Frederick Cancer Research Facility, Frederick, Maryland] annealed to its complement by heating in 10 mM Tris·HCl/1 mM EDTA, pH 8.0, at 70°C for 10 min and allowed to cool slowly to room temperature. Control oligonucleotides contained guanine in place of O^6-methylguanine. Preincubation with archaeal extracts was performed for 1 hr at 53°C except in the case of *T. litoralis* extracts (which was for 8 hr) in 200 μl of IBSA. *E. coli* extracts and human ATases were preincubated at 37°C for 2 hr. After preincubation, excess ^{3}H-methylated substrate DNA was added and the samples were assayed for remaining ATase activity at 37°C or 53°C as appropriate. Results were expressed as percent of ATase activity remaining in comparison with the control oligonucleotide.

Fluorography. For the determination of the molecular mass of ATases, crude extracts that had been incubated with [^{3}H]methylnitrosourea-methylated substrate DNA in IBSA were precipitated with trichloroacetic acid and the precipitates were dissolved in SDS/PAGE loading buffer (60 mM Tris·HCl, pH 6.8/50 mM DTT/2% SDS/0.01% bromophenol blue/10% glycerol) by heating at 100°C for 10 min. Proteins were separated in a Bio-Rad Protean system through a 5% stacking and 16% resolving polyacrylamide gel in 25 mM Tris·HCl/192 mM glycine/0.035% SDS, pH 8.3 (20, 21). The proteins were then transferred to nitrocellulose membranes by electroblotting (20 V, 10 hr) in 25 mM Tris·HCl, pH 8.3/192 mM glycine/20% (vol/vol) methanol. The nitrocellulose was dried in air, wet with scintillation fluid, wrapped in Saran Wrap, and exposed to x-ray film at −80°C for up to 2 weeks.

RESULTS

Archaeal Extracts Contain ATase Activity. Extracts of the four thermophilic archaeal strains were able to transfer radio-
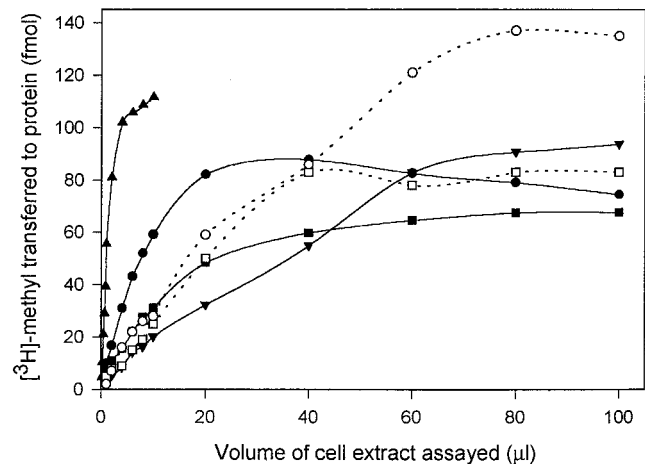

FIG. 1. Demonstration of ATase activity in archaeal cell extracts. The standard ATase assay was performed at the optimal growth temperatures of the individual strains; ●, *S. acidocaldarius* (78°C); ▲, *P. islandicum* (100°C); ▼, *Pyrococcus furiosus* (100°C); ■, *T. litoralis* (88°C); ○, *E. coli* BS21 (37°C); □, *E. coli* overexpressing human ATase (37°C). Cell extracts were incubated at these temperatures with ^{3}H-labeled substrate DNA for 20 min (*S. acidocaldarius, T. litoralis*), 10 min (*P. islandicum, Pyrococcus furiosus*) or 60 min (BS21, human). See text for details.

activity from ^{3}H-methylated substrate DNA to protein during incubation at the optimal growth temperatures for the respective Archaea (Fig. 1). Under protein-limiting conditions transfer was essentially linearly proportional to the amount of protein assayed, and this region of the protein-dependence curve was used to calculate ATase specific activities, which ranged from tens of fmol/mg of total protein in *T. litoralis* extracts up to several thousands of fmol/mg in *P. islandicum* (≈10^3 to ≈$7 × 10^4$ molecules per cell; Table 1).

Methyl Transfer Kinetics. The kinetics of [^{3}H]methyl transfer were generally dependent on the incubation temperature— i.e., the higher the temperature the more rapid the transfer (Table 1). However, for *Pyrococcus furiosus*, which was the most active at 60°C, there was only a slight (≈40%) increase in activity when the temperature was increased from 60°C to 80°C, whereas for *T. litoralis* and *P. islandicum* extracts, initial transfer rates at 80°C were almost twice those at 60°C and for *S. acidocaldarius* activity increased more than 3-fold. *S. acidocaldarius* cell extracts were detectably active at 37°C.

Thermostability. The thermostability of the archaeal ATase activities was determined by preincubation of cell extracts at various temperatures. Residual ATase activity was then measured by incubation with excess substrate at 75°C for 15 min. At 37°C all thermophilic ATases retaining 100% of activity for at least 8 hr, after which activity decreased slowly with half-lives ranging from 32 to 72 hr (Table 1). At 60°C, *Pyrococcus furiosus* ATase activity was extremely thermoresistant, retaining full activity after 8-hr preincubation, whereas the other three strains were less stable. At 80°C *S. acidocaldarius* ATase activity was the most thermosensitive, retaining

Table 1. Activity, kinetic, and stability characteristics of archaeal ATases

Strain	Molecules per cell	Specific activity, fmol/mg protein	Kinetics, fmol/min*				Stability† $t_{1/2}$, hr		
			37°C	60°C	72°C	80°C	37°C	60°C	80°C
S. acidocaldarius	6,700	720	1.2	3.3	ND	11.4	32	16	0.5
Pyrococcus furiosus	1,100	85	ND	8.2	9.3	11.2	60	40	5
P. islandicum	66,700	4,280	ND	1.2	1.8	2.3	38	12	5.5
T. litoralis	10,800	380	ND	1.5	2.2	2.9	72	18	13

*Initial rates of transfer of [^{3}H]methyl groups from substrate DNA to protein *in vitro*. ND, not determined.
†Protein-limiting amounts of cell extracts were preincubated in IBSA at the temperatures indicated. Residual ATase activity was determined after addition of ^{3}H-labeled substrate DNA and incubation at 75°C for 15 min. See text for details.
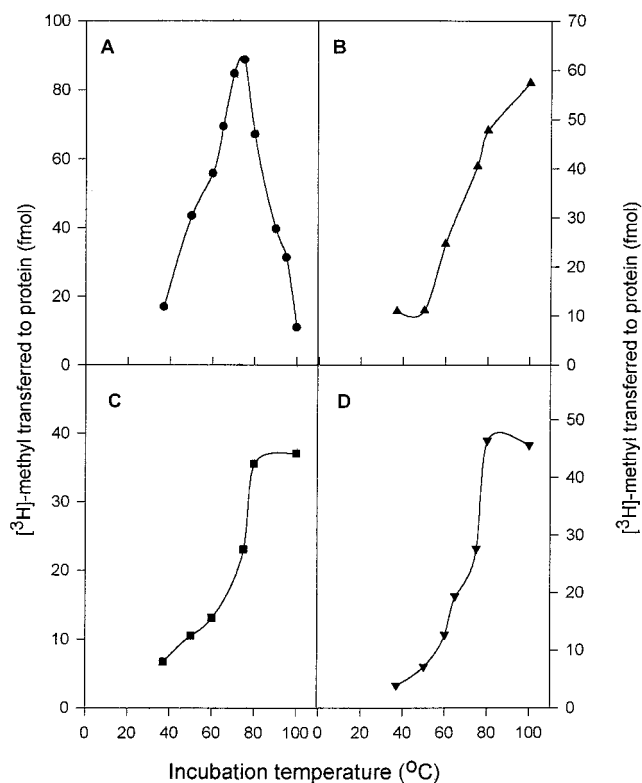
FIG. 2. Temperature dependence of archaeal ATase activity. Extracts of *S. acidocaldarius* (*A*), *P. islandicum* (*B*), *Pyrococcus furiosus* (*C*), and *T. litoralis* (*D*) were incubated with ³H-labeled substrate DNA under the conditions described in the text.

only 15% and <2% of activity after 1 and 4 hr preincubation, respectively, whereas that of *T. litoralis* was most thermoresistant, losing only ≈60% of activity by 24 h.

Temperature Optima. The optimal temperature for methyl transfer was determined by using a fixed amount of cell extract and time of incubation with substrate DNA. The optimal temperature for *S. acidocaldarius* ATase was around 75°C—i.e., very close to the optimal growth temperature for this organism (Fig. 2*A*, Table 2). However, at temperatures above 78°C, *S. acidocaldarius* ATase activity was much lower, with ≈45% residual activity obtained at 90°C and complete loss of activity at 100°C. This may be a consequence of the thermal instability of the protein either before or after methyl transfer. However, denaturation of the substrate DNA will occur as temperatures approach 94°C, and it may be that *S. acidocaldarius* ATase has an absolute requirement for a double-stranded DNA substrate. In contrast, the hyperthermophilic Archaea with optimal growth temperatures of 100°C, *P. islandicum* and *Pyrococcus furiosus*, have maximal ATase activity at approximately 100°C (Fig. 2 *B* and *C*), and *T. litoralis* ATase activity was maximal at around 80°C (Fig. 2*D*) with only a slight decrease in activity at 100°C. These latter ATases would thus
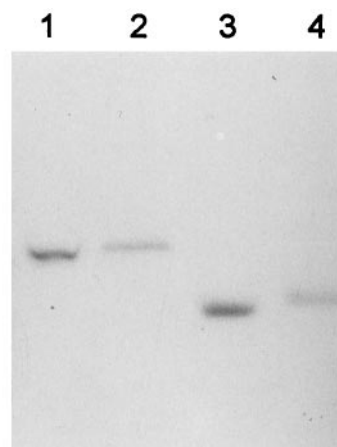

FIG. 3. SDS/PAGE fluorography of archaeal ATases. Extracts of the archaea were incubated with excess ³H-labeled substrate DNA and subjected to gel electrophoresis and fluorography as described in the text. Lane 1, *Pyrococcus furiosus*; lane 2, *T. litoralis*; lane 3, *P. islandicum;* and lane 4, *S. acidocaldarius*.

appear to be able to act very effectively on single-stranded DNA.

Archaeal Extracts Do Not Contain Alkylphosphotriester-DNA Alkyltransferase Activity. The presence of ATase activity and not alkylphosphotriester-DNA alkyltransferase activity in the archaeal extracts was indicated by the amounts of radioactivity transferred to protein under substrate-limiting conditions (Fig. 1). These amounts were closely similar to the amount found when extracts of *E. coli* overexpressing the human ATase were used and were 60% of the amount found when extracts of *E. coli* BS21, which constitutively expresses the *ada* gene and thus contains equimolar amounts of ATase and alkylphosphotriester-DNA alkyltransferase activities (22), were used (Fig. 1). To confirm the nature of the alkyltransferase activity in the Archaea, cell extracts were preincubated at 53°C with a double-stranded oligonucleotide containing a single *O*⁶-methylguanine residue, and residual ATase activity was then measured by incubation with ³H-labeled substrate DNA (53°C, 30 min). For comparison, purified recombinant human ATase and extracts of *E. coli* BS21 cells were used. In these cases, incubation was at 37°C because of the thermolability of the ATases. Essentially complete inactivation of the human ATase and the ATase in the archaeal extracts confirms the suggestion that, under the conditions used, only ATase activity is detectable in these organisms. In contrast, the *E. coli ada* ATase was only partially inactivated by preincubation with the oligonucleotide, consistent with the presence of the additional alkylphosphotriester-DNA alkyltransferase activity (Table 2).

Molecular Size of the Thermostable ATases. After incubation with ³H-methylated substrate DNA, extracts of the archaeal ATases contained only a single, well defined, radioactive band as shown by SDS/PAGE and fluorography (Fig. 3). The smallest ATase was seen in the *P. islandicum* extract and

Table 2. Temperature optima, size, and methylated oligonucleotide inactivation of ATases

Strain	Size,* kDa	Optimum temp, °C		Inactivation by *O*⁶-methylguanine oligonucleotides, % of control
		Growth	ATase	
S. acidocaldarius	16	78	≈75	91
Pyrococcus furiosus	22	100	≈100	94
P. islandicum	15	100	≈100	96
T. litoralis	23	88	≈90	94
E. coli BS21	39	37	37	34
Human	24	37	37	96

*As determined by polyacrylamide gel electrophoresis; see Fig. 3 and text.

had an apparent molecular mass of 15 kDa (Table 2). The extreme stability of the ATase activities shown in Table 1 suggests that the size estimates are not influenced by proteolysis.

DISCUSSION

ATases are widespread in nature, having been found and in some cases characterized in several prokaryota and eukaryota. The present report is, to our knowledge, the first functional demonstration of ATase activities present in representatives of the third domain of life, the Archaea.

Archaea represent some of the most ancient organisms on earth; apart from methanogens, most of them are extremophilic, adapted to harsh environmental conditions, including high temperature, extremely acidic or alkaline pH, high salt, or a combination of these, living mainly in hot springs and submarine geothermal vents. However, they have recently also been found to be widely distributed in cool parts of the ocean. While Archaea share a number of features with eubacteria (overall genome design, gene organization), many characteristics, particularly those involved with gene expression, are shared between the Archaea and eukaryotes. In DNA metabolism, RNA metabolism, and protein synthesis Archaea are closer relatives of the eukaryotes than they are of eubacteria. On the basis of 16S rRNA sequence information Archaea can be divided into two main groups: Crenarchaeota (of which *Sulfolobus acidocaldarius* and *Pyrobaculum islandicum* are examples) and Euryarchaeota (of which *Pyrococcus furiosus* and *Thermococcus litoralis* are examples). Several genes with significant homology to the DNA repair genes in Archaea have been described. The hyperthermophilic archaeon *Pyrococcus* contains an ORF that encodes a polypeptide with a high homology to the *E. coli* dinF (DNA damage inducible) gene product, suggesting that an SOS repair system might operate in Archaea (23). RecA-like genes from three archaeal species (*Sulfolobus solfataricus, Haloferax volcani*, and *Methanococcus jannaschii*) have been cloned, but the putative proteins encoded by these genes are more similar in sequence to Rad51 and Dmc1 proteins than they are to the bacterial RecA proteins (24). DNA photolyase found in the archaeon *Methanobacterium thermoautotrophicum* has no homology with the microbial photolyases, but it is similar to metazoan photolyases (25). Most recently, the sequencing of the entire *Methanococcus jannaschii* genome has been completed, and the genome has been found to contain an ORF with high amino acid homology to *Haemophilus influenzae* methylated-DNA-protein-cysteine methyltransferase. However, no biochemical characterization of the putative activity was reported (26).

In the present report, all four archaeal cell extracts exhibited ATase activity in an *in vitro* assay that was maximal at, or very close, to the optimal growth temperature of the respective strain. It is worth noting that we found very high ATase activities in *P. islandicum* and *Pyrococcus furiosus* at 100°C because, in the *in vitro* assay, the substrate DNA would be single-stranded at this temperature and all known ATases act much more rapidly on double-stranded substrates (27–31). The archaeal ATases thus appear to act on methylated single-stranded DNA more efficiently than mesophilic ATases. *In vivo*, protection of DNA against thermal denaturation could be achieved by various mechanisms—e.g., *S. acidocaldarius* contains a helix-stabilizing nucleoid protein (HSNP-C′) that binds strongly and cooperatively to double-stranded DNA and may condense and stabilize DNA at higher temperatures.

The kinetics of methyl transfer were temperature dependent: in general, the higher the temperature, the more rapid the reaction. Transfer was also measurable in *S. acidocaldarius* extracts at 37°C. All of the archaeal ATase activities examined were extremely thermostable at 60°C, but *S. acidocaldarius* ATase lost some activity at 80°C and had undetectable activity

at 100°C. This finding is not unexpected, given the optimal growth temperature of this strain (78°C). However, the intact hyperthermophiles may harbor mechanisms that also stabilize proteins: for example, the archaeon *Methanothermus fervidus* contains very high intracellular concentration of the anion 2,3-diphosphoglycerate, which affords protection against thermal denaturation of proteins. These thermophilic ATase activities represent an opposite extreme in nature to the yeast ATase protein, which is temperature sensitive, having a half-life of 4 min at 37°C (32). Similarly, human ATase (as a purified protein) had no detectable activity when assayed at 78°C (data not shown).

The amounts of [^{3}H]methyl groups transferred to protein under substrate-limiting conditions and the complete inactivation of the activity by preincubation with an oligonucleotide containing a single O^6-methylguanine residue suggest that the archaeal strains studied transfer alkyl groups from O^6-alkylguanine and not, or undetectably, alkylphosphotriesters in alkylated DNA. The principle activity thus resembles the *E. coli* Ogt (33) and the mammalian (e.g., ref. 34) ATases. This resemblance is supported by our observations that the archaeal ATase proteins were generally not greatly different in size from the *E. coli* Ogt protein (19 kDa) and the human ATase (24 kDa), and only single bands were seen on fluorography. It is worth noting that the *P. islandicum* protein has a molecular mass of ≈15 kDa, making this the smallest ATase so far found in nature. Whether there is an adaptive response pathway analogous to that controlled by the *ada* gene in *E. coli* might be technically difficult to establish, given the growth conditions for these organisms and the instability of agents that are known to induce the adaptive response in *E. coli* (35). However, the recent report of UV-induced error-prone repair and genetic recombination in *S. acidocaldarius* (36) is suggestive of an SOS-like function in this organism.

The conservation of ATase activity throughout evolution indicates a very strong selection pressure for this function, probably because of the toxic, but possibly also the mutagenic, effects of the substrate lesions in DNA. Given the diversity of the environments in which ATase-expressing organisms may be found, it seems unlikely that environmental agents provide the selective pressure to conserve ATase, but it is reasonable to suggest that some equally highly conserved endogenous process is the source of these lesions in DNA.

In summary, we think that this demonstration of functionally active thermostable alkyltransferases in extremophiles raises interesting questions about the basis of the evolution of such a function and what might generate the substrate in host DNA. In addition, the isolation, sequencing, and overexpression of the genes encoding the thermostable ATases will enable mechanistic and structure–activity studies that will be relevant to the clinical use of ATase-inactivating agents in cancer chemotherapy (2, 3).

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