Short Communication

Examining influence of DNA methylation and deamination on genomic evolution: Spotlight *Thermus thermophilus*

Nirupama Narayanan[1*], Matthew Levy[2] and John M. Greally[3]

[1]Department of Biology, New York School of Career and Applied Studies, Touro College, 27-33 W 23rd Street, New York, NY 10010
[2]Department of Biochemistry, Albert Einstein College of Medicine, Michael F. Price Center, 1301 Morris Park Avenue, Room 519, Bronx, NY 10461
[3]Department of Genetics (Computational Genetics), Albert Einstein College of Medicine, Michael F. Price Center, 1301 Morris Park Avenue, Room 322, Bronx, NY 10461
*Corresponding author

A B S T R A C T

Our goal was to examine the influence of DNA methylation and deamination on genomic evolution. Genomic DNA of thermophiles mostly exists in a single stranded form, thereby making them excellent models. We wanted to know if spontaneous deamination occurs in such DNA mediated by its own cytidine deaminases. Spontaneous deamination causes mutations, which would significantly impact genomic evolution of these organisms. *Cdd* - a cytidine deaminase from a thermophilic bacterium *Thermus thermophilus* was cloned and characterized. We found that *cdd* deaminates only single nucleotides and not single stranded DNA. Therefore, it appears unlikely to influence genomic evolution of these organisms.

Introduction

Cytidine deaminases play an important role in the pyrimidine salvage pathway of many organisms by catalyzing hydrolytic deamination of cytidine and cytidine derivatives into their respective uridine counterparts (Ashley and Bartlett, 1984; Bransteitter *et al.,* 2003; Cohen and Wolfenden, 1971). However, while much focus has centered on the activity and function of a number of mesophilic cytosine deaminases (Bransteitter *et al.,* 2003; Cohen and Wolfenden, 1971; Vincenzetti *et al.,*

1999; Ireton *et al.,* 2002; Conticello *et al.,* 2007; Vincenzetti *et al.,* 1996; Petersen-Mahrt and Neuberger, 2003; Li *et al.,* 2003; Pham *et al.,* 2003; Besmer *et al.,* 2006; Cascalho, 2004; Dickerson *et al.,* 2003; Ramiro *et al.,* 2003), very few characterizations have been reported for those produced by thermophilic organisms (Cambi *et al.,* 2001; Elshafei *et al.,* 2005; Li *et al.,* 2011; Cava *et al.,* 2009). To this end, we cloned the cytidine deaminase (*cdd*) from the thermophilic bacterium *Thermus*

thermophilus and characterized it in several biochemical assays.

Results and Discussion

A histidine tagged *cdd* T7 double stranded DNA (dsDNA) expression cassette template was generated by two successive PCRs (Roche Applied Science, IN). To facilitate rapid protein production, we used an *in vitro* transcription and translation system (TNT) derived from *E. coli* lysates (Roche Applied Science, IN). Translation reactions (50µL) were performed for 2 hours at 30°C using 200 ng of template after which *cdd* enzyme was purified with Nickel superflow resin (QIAGEN, CA) equilibrated with 1X TBST, pH-7.2 in a rotator for 4 hours at 4°C. 1X TBST supplemented with 10 and 20 mM imidazole served as wash buffers I and II, respectively.

Two washes were done with each buffer to remove any unbound protein. *Cdd* was then eluted with 1X TBST supplemented with 500 mM imidazole in a single step and finally dialyzed against 1X TBST overnight at 4°C. Enzyme purity was ascertained by a 4–20% SDS-PAGE gel (Bio Rad, CA). Molecular mass of *cdd* is roughly 15 kDa. There was no detectable protein loss in washes. After purification a 50 uL reaction typically produced 2 µg of protein (Fig. 2c).

We performed UV spectrophotometry to assess ability of *cdd* to deaminate cytidine. Deamination assays were carried out where 25 ng of *cdd* was incubated with varying cytidine concentrations (150 µM–1 mM in 1X PBS, pH-7.2) at 75°C for 5 min. Absorbance was measured at selected time points namely: 0, 0.5, 1, 2, 3, 4 and 5 min for each cytidine concentration. The reaction was then quenched by addition of 0.5M HCl and read under acidic conditions at 290 nm where absorbance of cytidine (λ_{max} = 280)

can be readily distinguished from that of the deamination product uridine (λ_{max} = 260). Selected experimental trials were repeated and absorbance values were averaged. Slope of absorbance versus time was used to calculate rate constant (Fig. 1a). Data obtained suggested that *cdd* obeys first order kinetics. Hence data was fit to Michaelis Menten equation and kinetic parameters like V_{max} and K_m were obtained (Table 1). Reciprocal of individual reaction velocities (1/v) was then plotted against reciprocal of respective cytidine concentrations (1/[S]). This was used to generate Lineweaver Burke plot (Fig. 1b) for final data representation (Cohen and Wolfenden 1971).

UDG (Uracil DNA Glycosylase) (New England Biolabs, MA) cleavage assays (Fig. 2a) were carried out to determine if *cdd* could deaminate cytidine from single stranded DNA (ssDNA) (Vincenzetti *et al.*, 1999). Thirty picomoles of FAM (fluorescein-6-carboxamidohexyl) labelled DNA was mixed with UDG buffer (20 mM Tris-HCl, 1 mM Dithiothreitol, 1 mM EDTA, pH 8.0) in a 10 µL reaction and incubated for 10 min at 37ºC. 0.5 µL of UDG was added to start the cleavage reaction.

After 30 min, the reaction was treated with 50 mM NaOH. *Cdd* was then heat inactivated at 95°C for 15 min. Samples were incubated with 2X formamide dye [90% formamide, 10 mM EDTA (pH 8.0), 0.1% xylene cyanol, 0.1% bromophenol blue] for 3 min at 90ºC and resolved on 15% denaturing polyacrylamide gels (Fig. 2b). Gels were imaged using a Typhoon variable mode molecular imager (Amersham Biosciences Corp., NJ). Lanes 4, 5 and 7 represent control samples. Sample in lane 3 was subjected to UDG treatment only and no deamination.
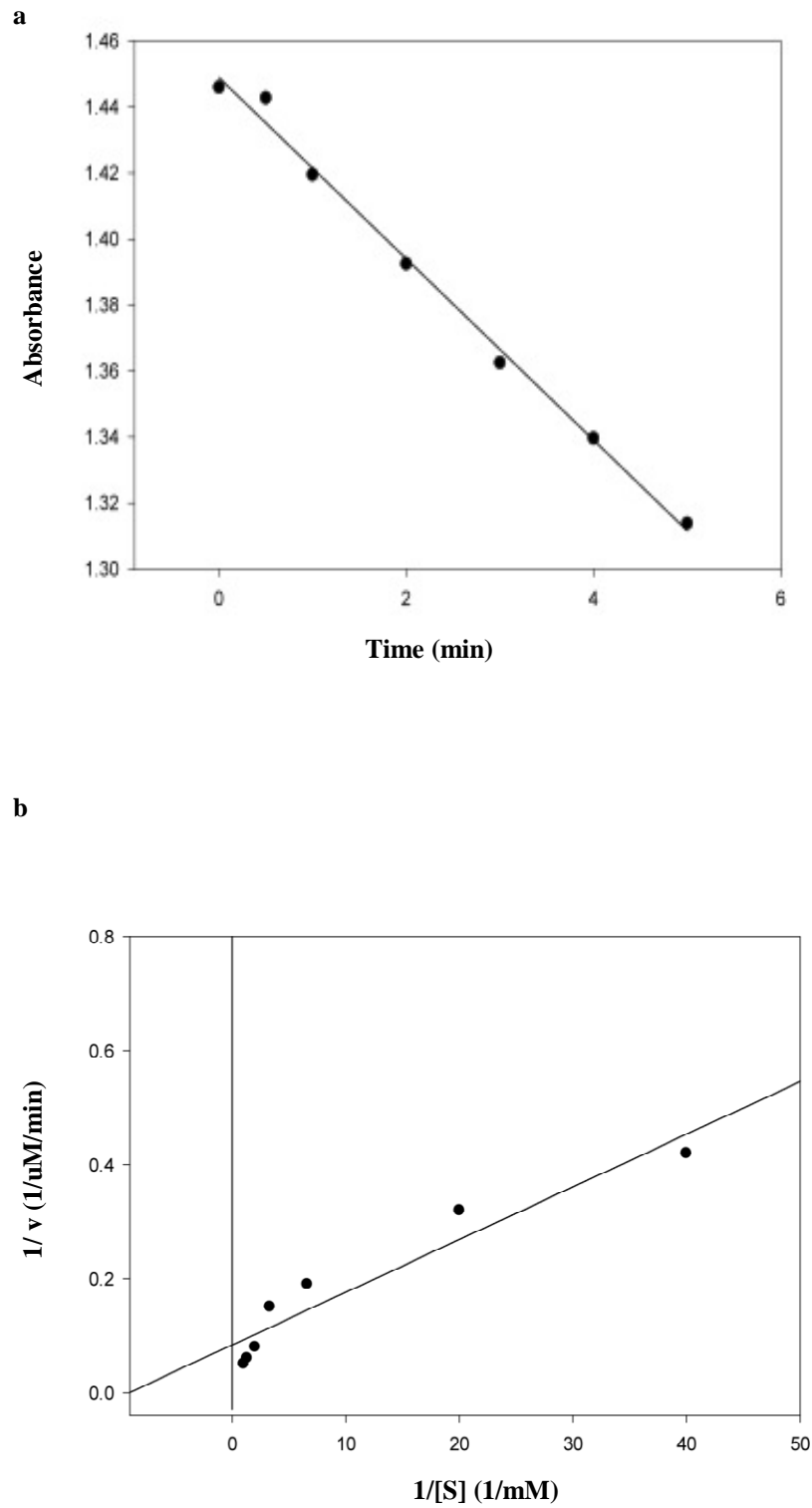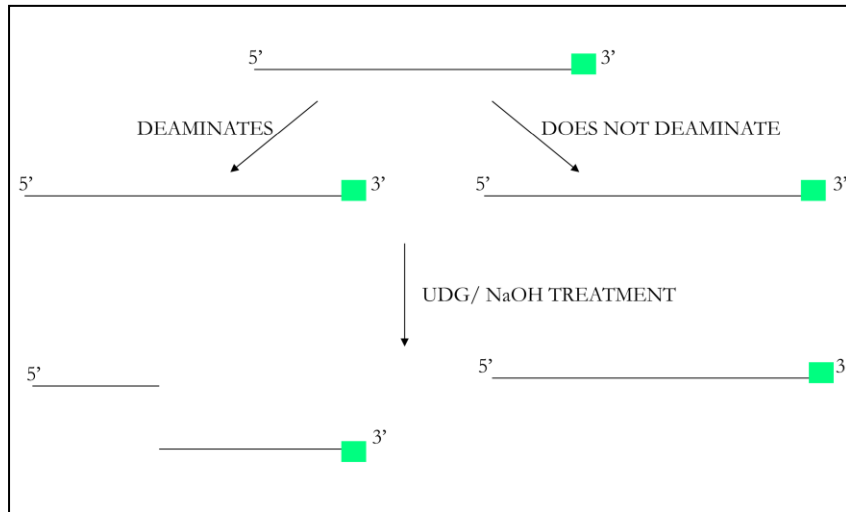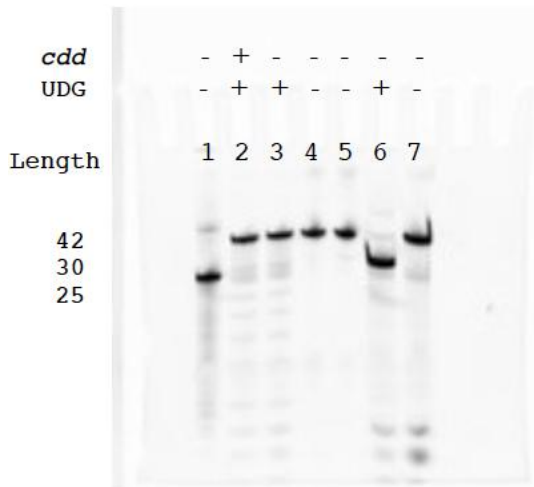
a

b

Fig.1   Determination of kinetic parameters of *cdd* enzyme **(a)** Sample plot showing rate constant calculation **(b)** Lineweaver-Burk plot between 1/v versus 1/[S] is used to determine kinetic parameters like Vmax and Km of *cdd*
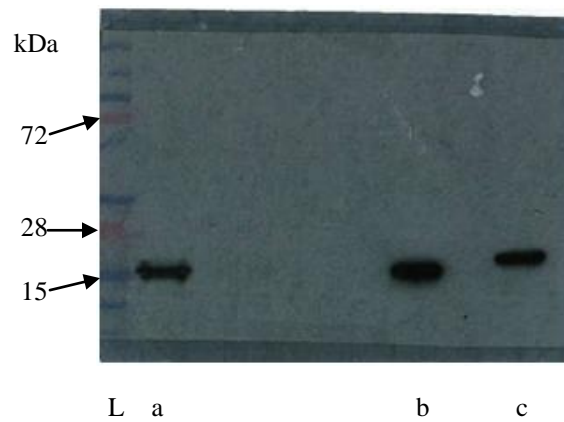
Fig. 2 UDG cleavage assay on single stranded DNA with *cdd* **(a)** Schematic showing UDG cleavage assay design **(b)** Resolution of UDG cleavage assay products on a 15% SDS-PAGE denaturing gel is shown: Lane 1–25 nucleotide DNA size standard, Lane 2 - Reaction sample, Lane 3 - Negative control with no *cdd* added, Lanes 4, 5 and 7 - FAM labelled oligonucleotides with no *cdd* or UDG added, Lane 6 - Positive control for UDG treatment being cleaved into shorter products **(c)** Western blot of fractions obtained during *cdd* enzyme purification is shown where only selected lanes are labeled: L – Protein ladder, a – *cdd* TNT reaction sample, b – *cdd* elution sample, c – protein run as histidine standard. Lanes between a and b – washes

Table.1 Comparison of *cdd* Km values for cytidine from various organisms.

	T. thermophilus cdd	*B. caldolyticus cdd*	*E. coli cdd*	*Arabidopsis CDA*	*Human cdd*
Cytidine	**1.3 mM**	2.1 mM	110 μM	150 μM	39 μM

Letters in bold indicate Km data from *T. thermophilus*

This negative control was done to rule out any spontaneous deamination. Positive control in lane 6 had a deoxyuridine at position 13; which upon UDG treatment cleaved into 2 products; each of length 29 and 13 nucleotides. A 25 nucleotide length DNA sample was run in lane 1 for size comparison. Sample in lane 2 was subjected to deamination by *cdd* and then UDG treatment, but it does not cleave into two fragments. Data from these results suggest that *cdd* does not exhibit any deamination activity on cytidine in the context of ssDNA.

In this study, we have attempted to understand how DNA methylation and deamination influences genomic evolution. Spontaneous deamination of DNA causes mutations (Glass *et al.,* 2007). Thermophiles are excellent models for investigating this, since most of their DNA exists in a single stranded form owing to high ambient temperatures (Cava *et al.,* 2009). We wanted to find out if spontaneous deamination occurs at these DNA locations catalyzed by their own deaminases. If so, why does this not lead to mutations? What is the DNA methylation status at these locations? Furthermore, we wanted to find out if these enzymes deaminated both single nucleotides and nucleotides in the context of single stranded DNA (ssDNA).

To our knowledge, our approach of using an *in vitro* TNT system for expressing and purifying a thermophilic enzyme is a novel and unique one. Classic protein expression relies on mesophilic bacterial hosts for expression and column chromatographic techniques for purification. Although these methods are relatively inexpensive and yield more protein; yield time, enzyme solubility, purity, stability and shelf life pose major challenges. Several standardization trials are needed to achieve optimum results (Vincenzetti *et al.,* 1999; Vincenzetti *et al.,* 1996; Gräslund *et al.,* 2008; Vita *et al.,* 1985; Woo *et al.,* 2002; Yang *et al.,* 1980; Zubay, 1973). Further, protein scale-up and purification can often be problematic. To circumvent these potential limitations, we employed an *in vitro* TNT system which poses several advantages including ease of use and rapid protein production. Indeed, dsDNA can be used directly as input into these reactions obviating the need for cloning, a factor which should facilitate screens in which multiple proteins are assayed (Sepp and Choo, 2005).

Cdd deaminates cytidine within the context of single nucleotides but not from ssDNA. Hence, it appears unlikely to influence genomic evolution of these organisms. Further biochemical characterization of *cdd* would explain several aspects of enzymatic activity. Obtaining a 3D crystal structure of *cdd* would be a great initial step. Exploring other relevant enzymes and proteins from this organism also hold much promise from an evolutionary standpoint.

References

Ashley, G.W., Bartlett, P.A. 1984. Purification and properties of cytidin e deaminase from *Escherichia coli*. *J. Biol. Chem.,* 259: 13615–13620.

Besmer, E., Market, E., Papavasiliou, F.N. 2006. The transcription elongation complex directs activation-induced cytidine deaminase-mediated DNA deamination. *Mol. Cell. Biol.,* 26: 4378–4385.

Bransteitter, R., Pham, P., Scharff, M.D., Goodman, M.F. 2003. Activation-induced cytidine deaminase deaminates deoxycytidine on single-stranded DNA but requires the action of RNase. *Proc. Natl. Acad. Sci.,* 100: 4102–4107.

Cambi, A., Vincenzetti, S., De Sanctis, G., Neuhard, J., Natalini, P., Vita, A. 2001. Cytidine deaminase from two extremophilic bacteria: Cloning, expression and comparison of their structural stability. *Protein Eng.,* 14: 807–813.

Cascalho, M. 2004. Advantages and disadvantages of cytidine deamination. *J. Immunol.,* 172: 6513–6518.

Cava, F., Hidalgo, A., Berenguer, J. 2009. *Thermus thermophilus* as a biological model. *Extremophiles,* 13: 213–231.

Cohen, R.M., Wolfenden, R. 1971. Cytidine deaminase from *E.coli*. *J. Biol. Chem.,* 246: 7561–7565.

Conticello, S.G., Langlois, M.A., Neuberger, M.S. 2007. Insights into DNA deaminases. *Nat. Struct. Mol. Biol.,* 14: 7–9.

Dickerson, S.K., Market, E., Besmer, E., Papavasiliou, F.N. 2003. AID mediates hypermutation by deaminating single stranded DNA. *J. Exp. Med.,* 197: 1291–1296.

Elshafei, A.M., Ali, N.H., Mohamed, L.A. 2005. Cytidine deaminase activity in *Penicillium politans* NRC-510. *J. Basic Microbiol.,* 45: 335–343.

Glass, J.L., Thompson, R.F., Khulan, B., *et al.* 2007. CG dinucleotide clustering is a species-specific property of the genome. *Nucleic Acids Res.,* 35: 6798–6807.

Gräslund, S., Nordlund, P., Weigelt, J., *et al.* 2008. Protein production and purification. *Nat. Methods,* 5: 135–146.

Ireton, G.C., McDermott, G., Black, M.E., Stoddard, B.L. 2002. The structure of *Escherichia coli* cytosine deaminase. *J. Mol. Biol.,* 315: 687–697.

Li, B., Chen, Y., Liu, Q., Hu, S., Chen, X. 2011. Complete genome analysis of *Sulfobacillus acidophilus* strain TPY, isolated from a hydrothermal vent in the Pacific Ocean. *J. Bacteriol.,* 193: 5555–5556.

Li, Z., Woo, C.J., Scharff, M.D. 2003. Mutations in AID and UNG extend the function of AID. *Nat. Immunol.,* 4: 945–946.

Petersen-Mahrt, S.K., Neuberger, M.S. 2003. In vitro deamination of cytosine to uracil in single-stranded DNA by apolipoprotein B editing complex catalytic subunit 1 (APOBEC1). *J. Biol. Chem.,* 278: 19583–19586.

Pham, P., Bransteitter, R., Petruska, J., Goodman, M.F. 2003. Processive AID-catalysed cytosine deamination on single-stranded DNA simulates somatic hypermutation. Nature, 424: 103-107.

Ramiro, A.R., Stavropoulos, P., Jankovic, M., Nussenzweig, M.C. 2003. Transcription enhances AID-mediated cytidine deamination by exposing single-stranded DNA on

the nontemplate strand. *Nat. Immunol.,* 4: 452–456.

Sepp, A., Choo, Y. 2005. Cell-free selection of zinc finger DNA-binding proteins using in vitro compartmentalization. *J. Mol. Biol.,* 354: 212–219.

Vincenzetti, S., Cambi, A., Neuhard, J., Garattini, E., Vita, A. 1996. Recombinant human cytidine deaminase: Expression, purification, and characterization. *Prot. Expr. Purif.,* 8: 247–253.

Vincenzetti, S., Cambi, A., Neuhard, J., Schnorr, K., Grelloni, M., Vita, A. 1999. Cloning, expression, and purification of cytidine deaminase from *Arabidopsis thaliana. Prot. Expr. Purif.*, 15: 8–15.

Vita, A., Amici, A., Cacciamani, T., Lanciotti, M., Magni, G. 1985. Cytidine deaminase from *Escherichia coli B.* purification and enzymatic and molecular properties. *Biochemistry,* 24: 6020–6024.

Woo, J.H., Heo, N.J., Ghim, S.Y., Kim, J.G., Song, B.H. 2002. Purification and characterization of thermostable cytidine deaminase encoded by the *Bacillus caldolyticus* cdd gene. *Enzym Microb. Technol.,* 30: 153-160.

Yang, H.L., Ivashkiv, L., Chen, H.Z., Zubay, G., Cashel, M. 1980. Cell-free coupled transcription-translation system for investigation of linear DNA segments. *Proc. Natl. Acad. Sci.,* 77: 7029–7033.

Zubay, G. 1973. In vitro synthesis of protein in microbial systems. *Annu. Rev. Genet.,* 7: 267–287.