Short Communication

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

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**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; Caschalho, 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 superflo 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 µL 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 ($\lambda_{\text{max}} = 280$) can be readily distinguished from that of the deamination product uridine ($\lambda_{\text{max}} = 260$). 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 $V_{\text{max}}$ and $K_m$ were obtained (Table 1). Reciprocal of individual reaction velocities (1/ν) 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 37°C. 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 90°C 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.
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.

<table>
<thead>
<tr>
<th></th>
<th>T. thermophilus cdd</th>
<th>B. caldolyticus cdd</th>
<th>E. coli cdd</th>
<th>Arabidopsis CDA</th>
<th>Human cdd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytidine</td>
<td>1.3 mM</td>
<td>2.1 mM</td>
<td>110 µM</td>
<td>150 µM</td>
<td>39 µM</td>
</tr>
</tbody>
</table>

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


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


