Methanosarcina acetivorans C2A Topoisomerase Illα, an Archaeal Enzyme with Promiscuity in Divalent Cation Dependence

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Abstract

Topoisomerases play a fundamental role in genome stability, DNA replication and repair. As a result, topoisomerases have served as therapeutic targets of interest in Eukarya and Bacteria, two of the three domains of life. Since members of Archaea, the third domain of life, have not been implicated in any diseased state to-date, there is a paucity of data on archaeal topoisomerases. Here we report Methanosarcina acetivorans TopoIllα (MacTopoIllα) as the first biochemically characterized mesophilic archaeal topoisomerase. Maximal activity for MacTopoIllα was elicited at 30–35°C and 100 mM NaCl. As little as 10 fmol of the enzyme initiated DNA relaxation, and NaCl concentrations above 250 mM inhibited this activity. The present study also provides the first evidence that a type IA Topoisomerase has activity in the presence of all divalent cations tested (Mg2+, Ca2+, Sr2+, Ba2+, Mn2+, Fe2+, Co2+, Ni2+, Cu2+, Zn2+ and Cd2+). Activity profiles were, however, specific to each metal. Known type I (ssDNA and camptothecin) and type II (etoposide, novobiocin and nalidixic acid) inhibitors with different mechanisms of action were used to demonstrate that MacTopoIllα is a type IA topoisomerase. Alignment of MacTopoIllα with characterized topoisomerases identified Y317 as the putative catalytic residue, and a Y317F mutation ablated DNA relaxation activity, demonstrating that Y317 is essential for catalysis. As the role of Domain V (C-terminal domain) is unclear, MacTopoIllα was aligned with the canonical E. coli Topol 67 kDa fragment in order to construct an N-terminal (1–586) and a C-terminal (587–752) fragment for analysis. Activity could neither be elicited from the fragments individually nor reconstituted from a mixture of the fragments, suggesting that native folding is impaired when the two fragments are expressed separately. Evidence that each of the split domains plays a role in Zn2+ binding of the enzyme is also provided.

Introduction

Topoisomerases manage DNA supercoiling and are classified according to the mechanism of action employed [1–4]. In general, topoisomerases form a phosphotyrosine intermediate while cleaving one strand (type I or odd-numbered) or two strands (type II or even-numbered) of DNA. Before the initial transesterification is reversed and a ligated DNA backbone regenerated, type I and II enzymes change the DNA linking number [Lk] by 1 and 2, respectively. Type I topoisomerases are ascribed further distinction based on the polarity of the covalent intermediate formed. Type IA (like Type II) forms a 3′-phosphotyrosine adduct while type IB forms 3′-phosphotyrosine covalent adduct. Thus, the structure and mechanism of each class are distinct.

The characterization of the first topoisomerase E. coli Topol (EcoTopol), originally titled to protein, was an earnest exploration into a field that has grown to be of burgeoning biological importance [5]. Since then, topoisomerases have been demonstrated to be vital components of the cellular machinery in a wide array of processes that are essential for life, such as DNA replication, DNA repair and chromosome segregation [6–8]. While these molecular machines have been identified in every genome sequenced to date, irrespective of domain, eukaryotic and bacterial topoisomerases have received greater scrutiny given their role in diseased states such as Cancer and infections [9,10].

Interestingly, studies from bacteria and both lower and higher eukaryotes have demonstrated that ablation of TopoIII (a subfamily of type IA) activity in vivo without some compensatory mechanism leads to genomic instability and/or abnormal growth [11–16]. In spite of this dire evidence that TopoIII is vital for normal development, the majority of archival studies have focused on the non-essential Reverse Gyrase (a subfamily of type IA) given its unique phylogenetic distribution in thermophiles and hyperthermophiles [17–20]. Furthermore, the only data published on archaeal TopoIII are from hyperthermophilic organisms of the archaeal subdomain Crenarchaeota [21–23].

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The archaean subdomain Euryarchaeota contains a large group of economically important organisms, including both the mesophilic and hyperthermophilic methane-producing organisms. The methane-producing genus *Methanococcus* is known to harness all known substrates (including acetate, H₂ and CO₂, formate, methylimine and methanol) to produce methane, a greenhouse gas [24]. Recent reports also demonstrate that methanogens may be important in human health, especially from the nutritional standpoint [25,26].

With the largest sequenced archaeal genome, *Methanosarcina acetivorans* [NP_616517, NP_616516] [24]. Based on sequence homology, MacTopoIIIα (six genes) tentatively named MacTopoIIIα, MacTopoIIIβ [NP_616517, NP_616519, NP_616520], MacTopoIVα [NP_616515, NP_616516] [24]. Based on sequence homology, MacTopoIIIα is classified as a type IA topoisomerase III protein. In the present report, we characterize MacTopoIIIα, as the first mesophilic archaean TopoIII studied. The attributes shared with orthologs from the other domains and those potentially specific to Archaea are discussed.

**Results**

**Expression and purification of MacTopoIIIα**

In order to characterize MacTopoIIIα, the encoding gene was amplified, cloned, expressed, and purified from *E. coli* cells as a fusion protein with an N-terminal hexa-Histidine (6-His) tag. Purification of the recombinant protein was achieved through affinity chromatography, ion-exchange chromatography and size-exclusion chromatography. Based on the polypeptide sequence, the recombinant protein has an estimated molecular mass of ~86 kDa. As expected, the highly purified MacTopoIIIα migrated between the 66 and 116 kDa molecular mass markers via a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (Fig. 1A, lane 2) [27].

**Catalytic properties of MacTopoIIIα**

The putative topoisomerase was tested for the ability to relax negatively supercoiled pUC18 (300 ng). As little as 10 fmol of enzyme initiated relaxation upon addition to the reaction mixture (Fig. 2A). Whereas 200 fmol of enzyme showed modest activity, 5 pmol of enzyme elicited maximal activity under standard assay conditions. Analysis of aliquots of samples collected from the last step (gel filtration column) demonstrated similar activities in all fractions.

Maximal relaxation activity was observed around 30°C, and at temperatures of 50°C and above, no relaxation was observed (Fig. 2B). *M. acetivorans* C2A is an anaerobic mesophile that grows optimally at 35–40°C. Therefore, the optimal temperature for activity of MacTopoIIIα falls within the expected range of activity [26]. Both Na⁺ and Mg²⁺ are required for growth of this archaean, and the optimal concentration of NaCl required was 200 mM. In the present study, at NaCl concentrations of 400 mM or higher, the activity of MacTopoIIIα was almost not observable (Fig. 2C). A similar observation was made for the effect of KCl concentration on MacTopoIIIα activity. Initiation of activity by the enzyme was observable as early as 15 seconds after addition of MacTopoIIIα to the reaction mixture, and maximal activity was observed within 5 min (Fig. 2D).

The effects of different divalent cations on MacTopoIIIα activity

Topoisomerase activity was assessed as a function of divalent cation concentration in a semi-logarithmic range of concentrations from 50 μM to 10 mM (Fig. 3). We observed that each divalent cation elicited a unique activity profile. As in previous studies on topoisomerases, Mg²⁺ was able to elicit maximal activity at concentrations greater than or equal to 1 mM. Ca²⁺ was the only other cation that maintained a profile comparable to Mg²⁺, with concentrations greater than 1 mM showing near maximal relaxation. In fact, relaxation activity of MacTopoIIIα at concentrations above 1 mM appeared to be higher for Ca²⁺ compared with Mg²⁺, Ba²⁺ and Sr²⁺ profiles were similar, but were not able to relax the supercoiled substrate as effectively as Mg²⁺ and Ca²⁺. The ability of these Group II alkaline metals to elicit DNA relaxation activity at low cation concentration was, therefore, as follows: Mg²⁺ > Ca²⁺ >> Ba²⁺ > Sr²⁺.

In the presence of Zn²⁺, activity was observed in a narrow window of 100 μM. Zn²⁺ concentrations of 50 μM or less and 500 μM or more elicited no activity even when the reaction was performed in a 1:1 enzyme:DNA molar ratio (data not shown). To a lesser extent, Cd²⁺ was also able to elicit activity within a similar narrow range. Relaxation activity was observed at 0.1 mM and 0.5 mM concentrations of Co²⁺, and at a concentration of 1 mM or higher, either no activity was detectable or divalent cation-catalyzed DNA degradation was observed. Fe²⁺ required a concentration of 1 mM or higher for activity. However, DNA degradation was also significant at concentrations above 1 mM. These results are consistent with metal catalyzed DNA degradation seen with certain transition metals that are capable of promoting the Haber-Weiss reaction [29,30]. Relaxation activity in the presence of Mn²⁺ or Ni²⁺ or Cu²⁺ was also elicited under a narrow range (100 μM – 1 mM).

**Classification of MacTopoIIIα**

To determine the class of MacTopoIIIα in the topoisomerase family, its response to known topoisomerase inhibitors, with different mechanisms of action, was investigated. Table 1 shows a summary of the inhibitors tested. Ethidium bromide (EtBr) and M13 ssDNA inhibited activity. The type I prokaryotic inhibitor spermidine and the type I eukaryotic inhibitor camptothecin were unable to inhibit the activity of MacTopoIIIα. Furthermore, the type II inhibitors (etoposide, nalidixic acid and novobiocin) also had no effect on the relaxation activity of MacTopoIIIα. Relaxation activity was, however, inhibited by high concentrations of KCl and NaCl (>400 mM). Based also on preliminary analytical gel filtration experiments, MacTopoIIIα was estimated to exist as a monomer in solution, which is consistent with the oligomerization state of type IA topoisomerases [3].

**Catalytic Tyrosine of MacTopoIIIα**

A sequence alignment using *Sulfolobus solfataricus* topoisomerase III (SsoTopoIII) and the entire archaean TopoIII subfamily II (of which MacTopoIIIα is a member) was created to provide a hint at the potential catalytic tyrosine (Fig. 1C), because SsoTopoIII is the only archaean TopoIII with a characterized active site tyrosine [9,22]. The alignment suggested Tyr317 as the potential active site residue of interest. Site-directed mutagenesis was used to create a Y317F mutant. Size exclusion chromatography of MacTopoIIIα Y317F yielded similar elution volume to that of the wild-type protein, suggesting that the mutant also exists as a monomer in solution (data not shown). The purified wild-type and mutant proteins (Fig. 1A, lanes 2 and 3) were subjected to circular dichroism scan to determine whether the mutation grossly impacted the structure of MacTopoIIIα Y317F (Fig. 1C). No gross differences between the structures could be discerned, suggesting that the secondary structures of both enzymes are comparable, outside of the point mutation. By subjecting the
Figure 1. Purification and domain analysis of MacTopoIII. (A) SDS-PAGE of the purified recombinant proteins. Samples were loaded as follows: 1, Protein molecular mass markers (Fermentas); 2, MacTopoIII wild-type; 3, MacTopoIII Y317F; 4, MacTopoIII N1-586; 5, MacTopoIII C587–752. (B) Schematic representation of MacTopoIII wild-type, mutant (MacTopoIII Y317F) and truncation (MacTopoIII C587–752 and MacTopoIII N1-586) proteins, showing the five canonical domains with Domains I-IV distinguished from the C-terminal Domain V. The red asterisk indicates location of the Y317F mutation. The domains are not drawn to scale. (C) Alignment of the active site of Archaeal TopoIII SubFamily II Topoisomerases with Sulfolobus solfataricus P2 TopoIII. MacTopoII, Methanosarcina acetivorans C2A TopoII [NP_617416]; MmaTopoI, Methanosarcina mazei TopoI [AAM32772]; MbaTopo, Methanosarcina barkeri strain Fusaro TopoI [YP_305910]; MbuTopo, Methanococcoides burtonii DSM 6242 TopoI [ABE51114]; MthTopoII, Methanospirillum hungatei JF-1 TopoII [ABD40761]; RxyTopoI, Rubrobacter xylanophilus DSM 9941 TopoI [ABG04911]; PtoTopo, Picrophilus torridus DSM 9790 Topo [YP.023525]; TvoTopo, Thermoplasma volcanium G551 TopoI [NP_110538]; SsoTopoIII, Sulfolobus solfataricus P2 TopoIII [NP_342400]. The GenBank accession numbers are in brackets. The conserved and similar amino acids are shaded black and gray, respectively. The putative catalytic residue is shaded red. (D) Circular Dichroism (CD) spectra of purified MacTopoIII wild-type and the Y317F mutant. Triplicate data sets were collected from samples at a concentration of 0.5 mg/ml in a buffer containing 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 2 mM DTT. All data sets were normalized against baseline readings from buffer containing no protein.

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puriﬁed mutant protein to the relaxation reaction, it was revealed that MacTopoIII
Y317F was unable to relax the negatively supercoiled substrate under the same conditions where the wild-
type protein demonstrated activity (Fig. 4A). The products seen in lane 5 are likely to be due to iron catalyzed DNA degradation via
the Haber-Weiss reaction (refer to Fig. 3, last lane under Fe2+
).

Truncation mutants

Two truncations of MacTopoIII
were constructed based on
sequence alignment with the 67 kDa fragment of EcoTopoI [31]. Based on this sequence alignment, the MacTopoIII
sequence was spliced at a position between two small aliphatic residues (A586 and I587) where no secondary structure was apparent based on
secondary structure prediction (PredictProtein: http://www.
predictprotein.org/). We constructed two fragments, N1-586 and C587–752, containing the N-terminal 586 amino acid residues and the C-terminal 166 amino acid residues, respectively. Both truncations were cloned, overexpressed and highly puriﬁed. Neither the N1-586 nor the C587–752 fragment was able to relax the same substrate as the wild-type under standard assay conditions (Fig. 4B and 4C). To determine whether relaxation activity could be reconstituted by mixing the two fragments, they were incubated brieﬂy prior to addition to the reaction mixture at varying ratios (50:1, 5:1 and 1:1). However, no relaxation activity was observed (Fig. 4C). To determine whether higher concentrations of the two fragments will elicit topoisomerase activity, we examined concentrations up to ﬁve times the maximum concentration used for the wild-type protein; however, no

Figure 2. Characterization of activity based on protein concentration, salt and time. (A) Concentration dependent relaxation of negatively supercoiled pUC18 DNA by MacTopoIII
wild-type. Standard reaction mixtures with 90 mM NaCl were incubated with 0.3 μg of pUC18 for 30 min at 37°C with 10 mM MgCl2. From left to right, lanes contain increasing amounts of enzyme as labeled. fl, form I; flI, form II. (B) Temperature dependent relaxation of negatively supercoiled pUC18 DNA by MacTopoIII
wild-type. Standard reaction mixtures containing 5 pmol of enzyme were incubated with 0.3 μg of pUC18 for 30 min with 10 mM MgCl2. Lane C contains no divalent cation and was incubated at 37°C. The remaining lanes were incubated for 5 min at the indicated temperature prior to the addition of enzyme. (C) Salt dependent relaxation of negatively supercoiled pUC18 DNA by MacTopoIII
wild-type. Standard reaction mixtures containing 5 pmol of enzyme were incubated with 0.3 μg of pUC18 for 30 min at 37°C with 10 mM MgCl2. Lane C contains 90 mM NaCl. From left to right, remaining lanes contain increasing amounts of NaCl or KCl as labeled. (D) Time dependent relaxation of negatively supercoiled pUC18 DNA by MacTopoIII
wild-type. Standard reaction mixtures of enzyme were incubated with pUC18 at 37°C with 10 mM MgCl2. Lane C contains no divalent cation. Time points of the reaction were taken as indicated.
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Figure 3. Cation-dependent DNA relaxation activity profiles of MacTopoIIIα wild-type. Relaxation of negatively supercoiled pUC18 DNA by MacTopoIIIα wild-type. Standard reaction mixtures with 90 mM NaCl containing 5 pmol of enzyme were incubated with 0.3 μg of pUC18 for 30 min at 37°C with the corresponding divalent cation. Lanes 1–8 contain increasing amounts of the indicated divalent cation: Lane 1, 0 μM; Lane 2, 50 μM; Lane 3, 100 μM; Lane 4, 500 μM; Lane 5, 1 mM; Lane 6, 2.5 mM; Lane 7, 5 mM; Lane 8, 10 mM. fI, form I; fII, form II.

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relaxation of the substrate was observed either by the individual polypeptides or their mixture (Fig. S1). The polypeptides, especially the C587–752 polypeptide, demonstrated DNA binding activity at high concentrations. Further experiments will be designed to characterize this property of the split MacTopoIIIα.

**Zinc Content Measurements**

In an effort to assess the Zn$^{2+}$ binding ability of this enzyme, samples of wild-type and the two truncation mutants were subjected to ICP-MS analysis. The wild-type enzyme was found to contain $1.29 \pm 0.01$ mol of Zn$^{2+}$. The MacTopoIIIα N1-586 truncation was found to contain $0.62 \pm 0.02$ mol, and the C587–752 truncation mutant was found to contain $0.49 \pm 0.01$ mol of Zn$^{2+}$ (Fig. 5).

**Discussion**

MacTopoIIIα is promiscuous

A fundamental property of type IA topoisomerases is the absolute requirement of Mg$^{2+}$ for the large conformational change required during DNA relaxation, but other divalent cations can substitute [5,32]. Recent publications have proposed a two metal mechanism for type IA and type II topoisomerases wherein one may bind acidic residues within the TOPRIM motif causing the necessary large conformational changes while the other may play a transient role in stabilizing the transition state in preparation for

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**Table 1. Summary of inhibition assays conducted with MacTopoIIIα wild-type.**

| Chemical                | Inhibition |
|-------------------------|------------|
| Ethidium Bromide        | Yes        |
| M13 ssDNA               | Yes        |
| Spermidine              | No         |
| Camptothecin            | No         |
| Etoposide               | No         |
| Nalidixic acid          | No         |
| Novobiocin              | No         |
| High [KCl]              | Yes        |
| High [NaCl]             | Yes        |

Three independent trials were conducted to determine the ability of different agents to inhibit DNA relaxation activity at increasing concentrations. doi:10.1371/journal.pone.0026903.t001

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![Figure 4. Mutant and truncation DNA relaxation assays.](image-url)
And while the role of Mg$^{2+}$ is usually well examined, it is commonplace for other divalent cations to receive less scrutiny (Table 2). As one example, EcoTopoI DNA relaxation activity was investigated in the presence of 0–10 mM Mg$^{2+}$ while Ca$^{2+}$, Co$^{2+}$, Mn$^{2+}$ and Zn$^{2+}$ were investigated at 2 mM only [39]. In another study, while the activity of human topoisomerase IIIζ (hTopoIIIζ) was again tested in the presence of a range of MgCl$_2$ concentrations, hTopoIIIζ was deemed inactive in the presence of other divalent cations based on one concentration (5 mM) [40]. We demonstrate for the first time that MacTopoIIIζ is promiscuous with regard to divalent cation (Ba$^{2+}$, Ca$^{2+}$, Cd$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Fe$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, Zn$^{2+}$) and that each elicits a unique activity profile. Unlike results with other topoisomerases, we observe DNA relaxation activity in the presence of Zn$^{2+}$ albeit under a narrow range [22,32]. These results are not plasmid-specific as confirmed in the presence of 300 ng of either pUC18 or pGEM-T (data not shown). While further studies are needed to determine if all divalent cations may substitute in the mechanism for all topoisomerases, our data clearly demonstrates that there is a need to assess bivalent metal ion profiles and not simply examine activity at a singular concentration. As a result, it is possible that different divalent cations not previously reported to elicit activity for any given topoisomerase indeed do elicit activity, although it may be under a narrow range of concentrations. It also remains to be seen whether these divalent cation profiles can act as a tool to distinguish topoisomerases or whether this activity is specific to Archaea or to this particular archaeon in the present study.

**MacTopoIIIζ is a type IA topoisomerase**

Nalidixic acid, etoposide and novobiocin are specific inhibitors of type II topoisomerases with different mechanisms of action [10]. Camptothecin is a known eukaryotic type I inhibitor that stabilizes the binary protein-DNA complex intermediate thereby impeding DNA relaxation [41]. None of these inhibitors were able to inhibit the DNA relaxation activity of MacTopoIIIζ. Ethidium bromide induces positive supercoiling [42]. Type IA topoisomerases have a preference for ssDNA and preferentially relax negatively supercoiled DNA. Ethidium bromide and M13 ssDNA were each able to inhibit activity. Thus, the sequence homology in combination with the results from the inhibitor studies clearly demonstrates that MacTopoIIIζ is a type IA topoisomerase.

**Table 2. Summary of divalent cation studies.**

| Enzyme                  | Mg$^{2+}$ | Ca$^{2+}$ | Sr$^{2+}$ | Ba$^{2+}$ | Mn$^{2+}$ | Fe$^{2+}$ | Fe$^{3+}$ | Fe$^{4+}$ | Co$^{2+}$ | Ni$^{2+}$ | Cu$^{2+}$ | Zn$^{2+}$ | Cd$^{2+}$ | Ref     |
|-------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|---------|
| MacTopoIIIζ             | Y         | Y         | M         | M         | Y         | Y         | -         | -         | Y         | M         | Y         | M         | M       | This paper |
| DmeTopoIIβ              | Y         | Y         | Y         | -         | -         | N         | -         | -         | N         | N         | N         | -         | -       | [53]    |
| EcoTopoI                | Y         | Y$^{10}$  | -         | -         | N$^{10}$  | -         | -         | L$^{10}$  | -         | L$^{10}$  | -         | -       | [32]    |
| FisTopoI                | Y         | N$^{10}$  | -         | -         | N$^{10}$  | -         | -         | -         | -         | N$^{10}$  | N$^{10}$  | -       | [45]    |
| HsaTopoIIIz             | Y         | -         | -         | N         | -         | N         | N         | N         | N         | N         | N$^{5}$  | -       | [40]    |
| MetTopoI                | Y         | Y         | M         | -         | Y         | Y         | Y         | -         | Y         | N         | -         | -       | [49]    |
| NtaTopoI                | Y         | Y$^{10}$  | -         | -         | M$^{10}$  | -         | -         | -         | N$^{10}$  | N$^{10}$  | -       | -       | [54]    |
| RcaTopoI                | Y         | Y         | Y         | -         | Y         | -         | -         | -         | N         | N         | N         | -       | [55]    |
| SauTopoI                | Y         | Y         | Y         | -         | Y         | -         | -         | -         | Y         | -         | -       | -       | [46]    |
| SsoTopolloI             | Y         | M         | Y         | -         | N         | -         | N         | N         | N         | N         | N         | -       | [22]    |

A literature review was conducted on previously published studies where divalent cations other than Mg$^{2+}$ were utilized in type IA topoisomerase DNA relaxation assays. The findings are summarized herein. Methanococcus acetivorans C2A, Mac; Drosophila melanogaster, Dme; Echerichia coli; Eco; Fervidobacterium islandicum, Fis; Homo sapiens, Hsa; Methylophaga sp. strain 3, Met; Nicotiana tabacum, Nta; Rhodobacter capsulatus, Rca; Staphylococcus aureus, Sau; Sulfolobus solfataricus, Sso; $^{10}$, data not shown; $^{2}$, Mg$^{2+}$SO$_4$ used in place of Mg$^{2+}$; Y, high activity; M, moderate activity; L, low activity; N, no activity. 

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Spermidine had no effect on MacTopoIII\(\text{z}\) at concentrations up to 100 mM. Spermidine was initially postulated as a prokaryotic polyaniline topoisomerase inhibitor given its inhibitory effect on both EcoTopoI and EcoTopoIII at physiological concentrations [43,44]. However, the data actually suggests that it is a case by case scenario within the domain Bacteria and the only archaecal TopoIII tested to-date, SsoTopoIII, demonstrated no susceptibility [22,45–49]. The data reveal that there is no definite trend for polyaniline susceptibility amongst bacterial type IA topoisomerases and no demonstrated effect to-date in the domain Archaea.

**Catalytic tyrosine is located at Y317**

We assayed the Y317F mutant of MacTopoIII\(\text{z}\) for activity under conditions that were demonstrated to elicit relaxation activity for wild-type. After analyzing the CD spectra of MacTopoIII\(\text{z}\) Y317F to ensure that there were no gross structural differences compared to the wild-type, we demonstrated that MacTopoIII\(\text{z}\) Y317F was inactive under conditions where wild-type had activity. This result confirmed our prediction that Tyr317 is indeed essential in the mechanism of DNA relaxation by MacTopoIII\(\text{z}\).

**N-terminal Zn\(^{2+}\)-binding activity**

An open question about TopoI and TopoIII is the role that the C-terminal Domain V plays in the context of Zn\(^{2+}\)-binding. While lacking the C-terminal (Domain V), the EcoTopo fragment (Domains I–IV) retained the ability to cleave oligonucleotides, but lacking the C-terminal (Domain V), the EcoTopoI fragment affected DNA relaxation activity [4]. No TopoI crystal structures have been reported to-date examining the role of Zn\(^{2+}\) in TopoI (TmTopoI) only minimally affected DNA relaxation activity [4]. No TopoI crystal structures have been identified with Zn\(^{2+}\) positioned even when it was detected in the mother chloroform [50]. The story is further confounded by the fact that there are several bacterial type IA topoisomerases that contain no homologous Zn\(^{2+}\)-binding motif or Zn\(^{2+}\)-binding ability [37,48,51]. A reasonable conclusion from the current data available is that type IA topoisomerase Zn\(^{2+}\)-binding motifs, when present in a given bacterial organism, play a structural role that may or may not be essential for DNA relaxation activity.

Given that MacTopoIII\(\text{z}\) contains a C-terminal Cys-X\(_7\)-Cys-X\(_7\)-Cys-X\(_7\)-Cys motif and that there have not been any studies to date examining the role of Zn\(^{2+}\)-binding in archaeal TopoIII, we set out to determine whether MacTopoIII\(\text{z}\) contains Zn\(^{2+}\) in order to delineate any potential differences with the well-characterized bacterial topoisomerases. The results demonstrated that MacTopoIII\(\text{z}\) binds to approximately one mole of Zn\(^{2+}\) per mole of protein. We then constructed, expressed and purified N-terminal and C-terminal fragments to assess the Zn\(^{2+}\)-binding ability of each segment. To our surprise, both the C-terminal fragment and the N-terminal fragment demonstrated the ability to chelate approximately a half mole of Zn\(^{2+}\). To our knowledge, this is the first report of an N-terminal fragment of a TopoIII or TopoI having this ability to coordinate Zn\(^{2+}\). It is likely that residues in the two fragments coordinate Zn\(^{2+}\) in the wild-type. Mutational studies are currently underway to investigate the Zn\(^{2+}\)-binding role that any of the eight His residues in the N-terminal and eleven Cys/His residues in the C-terminal may play.

In this report, MacTopoIII\(\text{z}\), a mesophilic archaeal TopoIII, was biochemically characterized in the context of well-established properties of eukaryotic and bacterial type IA topoisomerases. We demonstrate that MacTopoIII\(\text{z}\) is a monomer in solution, binds Zn\(^{2+}\) and exhibits susceptibility to inhibitors in a manner similar to other type IA topoisomerases. MacTopoIII\(\text{z}\) is a distributive topoisomerase that has a high affinity for ssDNA and is inhibited by high salt concentrations. Properties that may be unique to this enzyme are the ability of all divalent cations tested to elicit DNA relaxation activity and the ability of this enzyme to bind Zn\(^{2+}\) via the N- and C-terminal. Further studies are needed in the domain Archaea and beyond to determine if these attributes are domain or organism specific.

**Materials and Methods**

Cloning and expression of *M. acetivorans* C2A TopoIII\(\text{z}\) (MacTopoIII\(\text{z}\)) gene

The gene for the putative topoisomerase, MacTopoIII\(\text{z}\) (NP_617416), was amplified from the *M. acetivorans* C2A genomic DNA using PCR primers containing restriction sites for Ndel and XhoI in the forward (`5'–CATATGGCACCTTATGTTAACG-GAAAAATATA-3') and reverse (`5'–CTGGAGCTATAGG TCCTCAACATGCGCGTTACA-3') primers, respectively. The size of the PCR product was verified through agarose gel electrophoresis and cloned into pGEM-T, a TA-cloning vector (Qiagen). The recombinant plasmid was purified using a Qiagen gel extraction kit, and after confirming the integrity of the sequence of the DNA insert, the MacTopoIII\(\text{z}\) gene was transferred into a modified pET28a expression vector. The pET28a vector has its kanamycin resistance gene replaced with an ampicillin resistance gene [52]. Therefore, the ligated products were transformed into *E. coli* JM109 cells and transformants were selected on lysogeny broth (LB) plates supplemented with ampicillin at a final concentration of 100 \(\mu\)g/mL. The plates were incubated overnight at 37 °C, and a single colony was picked and cultured in 10 mL LB containing the same antibiotic and grown at 37°C for 8 hours. The plasmid was extracted from the *E. coli* culture and examined for the potential of the MacTopoIII\(\text{z}\) gene through DNA sequencing. The recombinant plasmid was named pET28a/MacTopoIII\(\text{z}\). All DNA sequencing in the present report were carried out at the W. M. Keck Center for Comparative and Functional Genomics (University of Illinois at Urbana-Champaign).

The recombinant plasmid pET28/MacTopoIII\(\text{z}\) was used in transforming *E. coli* BL21 codon plus RIPL cells (Stratagene) and plated on LB plates supplemented with ampicillin (100 \(\mu\)g/mL) and chloramphenicol (50 \(\mu\)g/mL). A transformant was cultured at 37°C in 10 mL LB medium supplemented with both antibiotics at the same concentrations stated above until the optical density (O.D.) at 600 nm reached 0.3. The expression of the MacTopoIII\(\text{z}\) gene was then induced by adding isopropyl \(\beta\)-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. The temperature was decrease to 16°C and cell culturing was continued for 12–18 hours. The recombinant *E. coli* cells were then collected through centrifugation at 6500 rpm for 10 minutes.

**Site-directed mutagenesis**

Site-directed mutagenesis was carried out using the Quikchange site-directed mutagenesis kit (Stratagene) according to the instructions of the manufacturer. The PCR primer (`5’–AACGGGG-TATATATCTTTCGCCAGGAGCGAAT-3’) was utilized to convert MacTopoIII\(\text{z}\) Tyr317 into Phe317. Mutagenized plasmid was transformed into JM109 cells after digestion of the parental plasmid with DpnI. The *E. coli* transformants were selected on LB agar plates supplemented with ampicillin (100 \(\mu\)g/mL) overnight.
at 37°C. Plasmids were extracted from individual colonies after growth in liquid LB cultures supplemented with ampicillin [100 μg/mL]. Plasmids were sequenced as described above, and the desired plasmid containing the Y317F mutation was selected for gene expression as described above for the wild-type gene.

Truncation mutagenesis

A truncated gene containing the DNA sequence for the N-terminal 586 amino acids (N1-586) was generated using an iProof™ High-Fidelity PCR kit with a forward primer (5’-CATATGCACTTTATGTCGACGGGAACT-3’) and reverse primer (5’-CTCGAGCTATGCTGGAGGCT-3’) according to the manufacturer’s instructions. A truncated gene containing the DNA sequence for the C-terminal 166 amino acids (C587–752) was generated in the same manner with the forward primer (5’-CATATGGGTCTCAGG-GAAAGCAAGATCATAGGCAAC-3’) and the reverse primer (5’-CTCGAGCTATAGGCTCTCAACTATGCGCGGTAC-3’). The size of the PCR product was verified through agarose gel electrophoresis and cloned into the pGEM-T vector (Qiagen). Purification of MacTopoIIIα

For MacTopoIIIα wild-type protein and the Y317F mutant, the harvested recombinant E. coli cells were re-suspended in Buffer A (50 mM Sodium phosphate, pH 7.0, 300 mM NaCl), and the cell contents were released by a French pressure cell (American Instruments Co). The cell debris was removed through centrifugation at 9500 rpm for 15 min at 4°C. The supernatant was subsequently filtered using a 0.22 μm filter and applied to a His-Trap™ HP column (GE Healthcare) pre-equilibrated with 75% buffer A and 25% buffer B (50 mM Sodium phosphate, pH 7.0, 300 mM NaCl, 500 mM imidazole), and eluted using a linear gradient of buffer B.

The protein fractions from the HisTrap™ HP column were pooled and dialyzed against buffer A (50 mM Sodium phosphate, pH 7.0, 300 mM NaCl) overnight at 4°C. Samples were then applied to a HiTrap™ SP column (GE Healthcare) pre-equilibrated with 90% buffer A and 10% buffer B with a linear gradient of 100% buffer A until used. Aliquots of eluted fractions from all chromatographies were examined through SDS-PAGE.

Preparation of DNA substrates for topoisomerase assays

E. coli JM109 cells were heat shocked to uptake the pUC18 plasmid, and transformants were cultured in ampicillin supplemented (100 μg/mL) LB medium to amplify the plasmid. The pUC18 plasmid was purified from cell pellets with a commercial kit (Qiagen), and electrophoresed on a 1.5% agarose gel containing 1X TBE (89 mM Tris, 89 mM Boric acid, 2 mM EDTA). Subsequently, the gel was stained with ethidium bromide and imaged with a UV illuminator. The plasmid DNA migrating the fastest was excised and purified utilizing a gel extraction kit (Qiagen) according to the manufacturer’s specifications. The purified negatively supercoiled DNA was verified using a 2-Dimensional gel with EcoRI linearized pUC18 and ethidium bromide treated pUC18 as standards. The M13 ssDNA was from a commercial source (New England Biolabs).

DNA relaxation assay

Unless otherwise stated, the method is the same as previously described [22]. Briefly, reaction volumes contained 300 ng negatively supercoiled pUC18 DNA in the presence of 5 pmol of MacTopoIIIα wild-type (or its mutants) in 50 mM Tris-HCl, pH 8.8, 1 mM DTT, 0.1 mM EDTA, 90 mM NaCl, 30 μg of BSA/mL, and 12% (vol/vol) ethylene glycol with the indicated divalent cation. After 30 min incubation at 37°C (unless indicated otherwise), reactions were terminated by adding 4 μL of DNA loading dye (25% Glyceral, 0.2% Bromophenol Blue, 50 mM EDTA) and the products were resolved with 0.5X TPE (44.6 mM Tris, 0.13% Phosphoric acid, 1 mM EDTA) buffer. DNA bands were visualized through ethidium bromide staining and imaging with a UV illuminator.

The levels of negatively supercoiled DNA that remained or were relaxed (partially and completely) were determined by densitometry using AlphaEaseFC™ software. For each divalent ion, the values for a given lane were normalized to that obtained without any divalent.
cation present (Fig 3, Lane 1). Activity for each ion was assessed according to the maximal percentage of substrate that was relaxed (partially and completely) relative to the percentage of negatively scDNA when no ion was present. High activity (Y) was utilized to denote greater than or equal to 95% activity while medium activity (M) denotes activity falling below 95%.

Determination of zinc content by ICP-MS
The zinc content in purified wild-type MacTopoIIIα and variants was determined at the University of Illinois Microanalysis Laboratory using the SCIEX ELAN DRCe ICP-MS (PerkinElmer Life Sciences). Briefly, two aliquots of purified protein, 1 ml each, at a concentration of approximately 0.5 mg/ml, were digested in nitric acid. The resulting solutions were diluted to 25 ml and analyzed. Two isotopes of zinc, $^{64}$Zn and $^{66}$Zn, were analyzed with gallium as the internal standard.

Estimation of subunit organization by gel filtration
Since MacTopoIIIα was expressed with a N-terminal 6-His tag, the protein eluted from the Hitrap™ SP column was incubated with the protease thrombin (1 unit/ml) during dialysis in buffer GF (50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0) in order to remove the tag. The dialyzed was loaded onto a Superdex 200 HR 10/300 gel column pre-equilibrated with buffer GF. The chromatography was developed with the same buffer at a flow rate of 0.5 ml/min and fractions of 0.5 ml were collected and analyzed by SDS-PAGE. To generate a standard curve, the column was calibrated by analyzing a set of gel filtration standards (thyroglobulin, 669 kDa; bovine serum albumin, 66 kDa; chicken ovalbumin, 44 kDa; equine myoglobin, 17 kDa; and vitamin B12, 1.35 kDa) under the same conditions as the MacTopoIIIα.

Materials
Methanosarcina acetivorans C2A genomic DNA was used for amplification of the topoisomerase. E. coli JM109 competent cells were used as host cells for amplification of the plasmids pGEM-T and pUC18. The negatively supercoiled pGEM-T and pUC18 DNA were isolated with the Qiagen Mini Plasmid kit according to the specification of the manufacturer and used in the topoisomerase assay as substrate DNA. Reagents for SDS-PAGE, 1 protein molecular weight standards, 1 DNA molecular mass standards, etoposide, camptothecin, EDTA, nalidixic acid, novobiocin, spermidine, ethidium bromide and DTT were purchased from Sigma. HiTrap Q HP, HiTrap S HP, and Superdex 200 HR 10/30 columns were from GE Healthcare. BSA, single-stranded M13 ssDNA was from New England Biolabs. Gel filtration standards were obtained from Bio-Rad.

Supporting Information

Figure S1 Relaxation of negatively supercoiled pUC18 DNA by MacTopoIIIα N1-586 and C587-752 at higher concentrations. Standard reaction mixtures with 90 mM NaCl were incubated with 0.3 μg of pUC18 for 30 min at 37°C with 10 mM MgCl₂. The only exception is Lane 1 which contains no divalent cation. Lanes 1 and 2 contain 5 pmol of MacTopoIIIα wild-type only. Lane 3 contains 50 pmol of N1-586 only. Lane 4 contains 50 pmol of C587-752 only. Lanes 5–11 contain varying ratios of MacTopoIIIα C587-752 to MacTopoIIIα N1-586 in pmol ranging from 50:1, to 50:5 to 50:10 to 50:50 to 10:50 to 5:50 to 1:50, respectively. Lanes 12–18 contain varying ratios of MacTopoIIIα C587-752 to MacTopoIIIα N1-586 in pmol ranging from 10:5, to 1:10 to 10:10 to 5:10:1 to 1:10 to 0:5:10, respectively. (TIF)

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Author Contributions
Conceived and designed the experiments: RM IC. Performed the experiments: RM PS JZ. Analyzed the data: RM IC. Contributed reagents/materials/analysis tools: RM IC. Wrote the paper: RM IC.

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