

Cloning and characterization of the *TneDI* restriction–modification system of *Thermotoga neapolitana*

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Abstract A putative Type II restriction–modification system of *Thermotoga neapolitana*, *TneDI*, was cloned into *Escherichia coli* XL1-Blue MRF' and characterized. Gene CTN_0339 specifies the endonuclease R.*TneDI*, while CTN_0340 encodes the cognate DNA methyltransferase M.*TneDI*. Both enzymes were purified simply by heating the cell lysates of *E. coli* followed by centrifugation. The enzymes were active over a broad range of temperatures, from 42°C to at least 77°C, with the highest activities observed at 77°C. R.*TneDI* cleaved at the center of the recognition sequence (CG↓CG) and generated blunt-end cuts. Overexpression of R.*TneDI* in BL21(DE3) was confirmed by both SDS-PAGE and Western blotting.

Keywords *Thermotoga* · *TneDI* ·
Restriction–modification systems ·
Type II endonucleases

Background

Thermotoga spp. are a group of hyperthermophilic bacteria with optimal growth around 80°C. These strict anaerobes

hydrolyze a number of polysaccharides through fermentative catabolism and produce hydrogen gas as one of the final products (Schroder et al. 1994), which may be directly used as a clean fuel. In addition, many *Thermotoga* enzymes have been expressed in *E. coli* and display extraordinary stability and extended shelf life. There is an increasing demand for tractable tools to enable genetic analyses and manipulations of *Thermotoga*. A technical obstacle to any genetic engineering effort is the restriction–modification (R–M) systems of the host. If not properly modified, foreign DNA molecules will likely be restricted by host endonucleases as soon as they enter the new cell. Understanding the R–M systems of *Thermotoga* is a necessary step toward genetically modifying these organisms.

R–M systems represent a common mechanism that bacteria use to protect the integrity of their genetic material, DNA. Most R–M systems comprise pairs of intracellular enzymes with opposing catalytic activities: a restriction endonuclease (REase) and a modification methyltransferase (MTase) (Wilson and Murray 1991; Pingoud et al. 2005). The modification function adds a methyl group to an adenine or cytosine in a specific sequence context, i.e., a recognition site. In most cases, REases break DNA chains containing the recognition sites, but not if the site has been modified by the cognate MTases. Genes specifying REases are generally linked to the gene encoding the cognate MTases. R–M systems are often strain specific, allowing bacteria to differentially destroy invading DNA.

Thousands of R–M systems have been identified through massive screening of a large number of strains (Whitehead and Brown 1985; Hjorleifsdottir et al. 1996) or more recently by bioinformatic analysis of genome sequences (Matveyev et al. 2001; Ishikawa et al. 2005). These systems have been grouped into 4 classes (Types I, II, III, and IV)

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Table 1 Strains and vectors used in this study

Strain or plasmid	Description	Reference
<i>Thermotoga</i>		
<i>T. neapolitana</i> ATCC 49049	Isolated from a shallow submarine hot spring near Bay of Naples, Italy	(Belkin et al. 1986)
<i>E. coli</i>		
XL1-Blue MRF ^r	$\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac$ [F ^r proAB lacI ^q Z Δ M15 Tn10 (Tet ^r)]	Stratagene
BL21(DE3)	F ⁻ <i>ompT gal [dcm] [lon] hsdS_B (τ_B⁻ m_B⁻)</i> with DE3, a λ prophage carrying the T7 RNA polymerase gene	(Studier et al. 1990)
Plasmids		
pUC19	High-copy number <i>E. coli</i> cloning vector containing portions of pBR322 and M13mp19; Ap ^r	GenBank Accession #: L09137
pET-24a(+)	<i>E. coli</i> expression vector possessing an N-terminal T7-Tag [®] sequence and a C-terminal His-Tag [®] sequence; Kn ^r	Novagen
pACYC184	Low-copy number <i>E. coli</i> cloning vector containing the p15A origin of replication; Tc ^r ; Cm ^r	GenBank Accession #: X06403
pJC184	A pACYC184-derived plasmid with a <i>NdeI</i> – <i>Bam</i> HI linker inserted downstream the promoter of <i>tet</i> ; Cm ^r	This study
pJC339	Coding region of CTN_0339 (excluding the stop codon) inserted into the <i>NdeI</i> – <i>Hind</i> III sites of pET-24a(+); Kn ^r	This study
pJC340	Coding region of CTN_0340 inserted into the <i>NdeI</i> – <i>Bam</i> HI sites of pJC184; Cm ^r	This study
pDH22	Coding region of CTN_0339 was digested by <i>NdeI</i> and <i>R.Tne</i> DI and was inserted into the <i>NdeI</i> – <i>Sma</i> I sites of pUC19; Ap ^r	This study

(Wilson and Murray 1991; Roberts et al. 2003). In a Type I system, three subunits, R (restriction), M (modification), and S (specificity) form a holoenzyme acting as both a REase and an MTase. Their recognition sequences are asymmetric and bipartite. Cleavage happens at random positions far away from the recognition site (~100 nt), and ATP is required for this process. Type II systems are composed of just two proteins, R and M, and they usually act independently. They break DNA at or near the recognition sites at specific positions. The restriction activity requires Mg²⁺ but not ATP. Type III systems also have just two polypeptides: R and M. The M subunit can function independently as a modification MTase, but the restriction activity requires a complex containing both R and M subunits. DNA cleavage occurs at a site ~25 bp away from the asymmetrical recognition sequence in the presence of Mg²⁺ and ATP (Sears et al. 2005; Adamczyk-Poplawska et al. 2009). Type IV systems cleave only modified DNA. DNA fragments cut by the same REase or its isoschizomers can be rejoined by DNA ligase, allowing rearrangement of DNA sequences. R–M systems serve as important biotechnology tools in modern molecular biological research and genetic engineering practices, particularly Type II R–M systems, hundreds of which have been commercialized.

Based on sequence comparison to related genes, the Restriction Enzyme Database (REBASE) (Roberts et al.

2010) predicts that there are three methyltransferase genes in the genome of *T. neapolitana*: CTN_0340, CTN_1203, and CTN_1590. It further suggests that CTN_0339 and CTN_0340 constitute a Type II R–M system recognizing CGCG sequences with an unclear cleavage site. In the NCBI database, CTN_0339 is annotated as a hypothetical gene, and CTN_0340 as an m⁴C-MTase gene. These two genes are clustered on the chromosome with a convergent orientation. The purpose of this study is to validate the functional assignments of the two genes made by REBASE and to facilitate the construction of genetic tools for *Thermotoga*.

Materials and methods

Strains and cultivation conditions

Bacterial strains and vectors involved in this study are listed in Table 1. *T. neapolitana* ATCC 49049 (same as DSM 4359) was obtained from the American Type Culture Collection (<http://www.atcc.org/>) and was cultivated at 77°C in liquid SVO medium (Van Ooteghem et al. 2002). All DNA manipulations were carried out in *E. coli* XL1-Blue MRF^r, and the expression of CTN_0339 and CTN_0340 was studied in both XL1-Blue MRF^r and *E. coli*

BL21(DE3). *E. coli* strains were cultivated at 30, 37, or 42°C in Luria–Bertani (LB) medium (1% tryptone, 0.5% NaCl, 0.5% yeast extract) with 1.5% (w/v) agar for plates. Kanamycin and chloramphenicol were added when needed at 50 and 30 µg ml⁻¹, respectively. Growth of *E. coli* strains was measured as optical density at 600 nm (OD₆₀₀). For BL21(DE3) recombinant strains, 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to induce the expression of CTN_0339 when culture density reached about 0.4–0.6. Induced cultures were incubated for another 4 h prior to further analyses.

Cloning of CTN_0339 into pET-24a(+)

The complete genome sequence of *T. neapolitana* is available at <http://www.ncbi.nlm.nih.gov/genomeprj/21023>. A DNA fragment corresponding to the coding region of CTN_0339 (672 bp, excluding the stop codon) was amplified from the genomic DNA of *T. neapolitana* with primers 5'-GGAATTCATATGAGAAAACGGATCCTCTCAT-3', which allows the start codon to be embedded in an *NdeI* recognition site, and 5'-CACAAGCTTCTGTTGATATTTTTCTATCA-3', which introduces a *HindIII* site at the end of sequence. The PCR product (691 bp, including *NdeI* and *HindIII* linkers) was purified by ethanol precipitation, digested with the two restriction enzymes, and inserted to the same sites of the *E. coli* expression vector pET-24a(+) to generate pJC339. The expressed protein has an appended His tag for immunodetection.

Cloning of CTN_0340 into pACYC184

CTN_0340 was cloned to pACYC184 under the control of the promoter for the tetracycline resistance gene (*tet*). First, the coding region of the *tet* gene as well as its downstream region (1581–3603) was removed from plasmid pACYC184 by inverse PCR using primers 5'-GAATTCCATATGCGGTGCCTGACTGCGTTAGC-3' and 5'-GAATTCCATATGGAATTCCCGCGGATCCTGAGAAGCACACGGTCACTGCTTCC-3'. The PCR product carries an *NdeI* site at its 5' end and a *BamHI*–*NdeI* linker at its 3' end. After digestion with *NdeI*, the fragment was allowed to recircularize to form pJC184. Meanwhile, primers 5'-GAATTCCATATGAAGAGAAGAAAATCAACAAG-3' and 5'-CGCGGATCCTTATCAACAGTGATCTTCAA-3' were used to amplify the 927-bp coding region of CTN_0340 from the chromosome of *T. neapolitana*. The primers were designed to have the start codon embedded in an *NdeI* site and the stop codon followed by a *BamHI* site. PCR fragment of CTN_0340 was then digested with *NdeI* and *BamHI* and was inserted to the corresponding sites of pJC184 to generate pJC340.

Purification and analyses of CTN_0339 and CTN_0340 gene products

One gram of *E. coli* wet cells grown at 37°C, containing pJC339 or pJC340 or both, were resuspended in 3 ml of lysis buffer (10 mM Tris–HCl, 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT, pH 8.0) and were lysed with two passages through a French press at 14,000 pounds per square inch. After centrifugation at 27 k×g for 25 min, the supernatant was transferred to a new tube and heated at 77°C for 1 h to denature most of the host proteins, followed by another round of centrifugation at 17 k×g for 15 min. The supernatant containing purified *Thermotoga* proteins was stored at 4°C. Such extracts retained 100% of the restriction activity for up to 5 weeks, and ≥50% of the activity for at least 12 weeks. For expression analysis, heat-purified cell extracts were mixed with loading buffer and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in 15% (w/v) gels. HisDetector Western Blot Kit (Kirkegaard & Perry Laboratories, Inc., Maryland, USA) was used for colorimetric immunodetection of histidine-tagged CTN_0339 protein. For restriction assays, heat-purified cell extracts containing REase were serially diluted in 2-fold steps with the lysis buffer and incubated with 5 µg of substrate DNA in a reaction volume of 50 µl. After 1 h of incubation at 50, 65, or 77°C, the reaction was terminated with 5 µl of stopping buffer (50 mM EDTA, 50% (w/v) glycerol, 0.02% (w/v) bromophenol blue, pH 8.0). Digested DNA fragments were resolved on 1% (w/v) agarose gels. One unit of REase activity is defined as the quantity required to completely digest 1 µg of pUC19 DNA in 1 h at 77°C. For modification assays, heat-purified cell extracts containing the MTase were serially diluted in 2-fold steps with the lysis buffer and incubated with 5 µg of substrate DNA in a total volume of 50 µl. After 1 h of incubation at 77°C, the reaction mix was then incubated with 0.5 U of REase at 77°C overnight. One unit of the MTase activity is defined as the quantity required to completely protect 1 µg of pUC19 DNA from the cleavage of REase for 1 h at 77°C.

Determination of the cleavage site of REase

The PCR product of CTN_0339 was completely digested by heat-purified REase. Two fragments were generated and retrieved from agarose gels. The smaller piece was directly analyzed by Sanger sequencing; the larger piece was further digested by *NdeI* and inserted into pUC19 at the *NdeI*–*SmaI* sites to form pDH22. The insertion region of the constructed vector was sequenced.

Results

Growth of *E. coli* recombinant strains

Plasmids pJC339 and pJC340 were separately constructed in *E. coli* strain XL1-Blue MRF['] by screening the transformants at 30°C. The cloned sequence of CTN_0339 (putative REase) and CTN_0340 (putative MTase) were confirmed by both restriction digestions and DNA sequencing. Plasmid pJC339 was introduced into XL1-Blue MRF[']/pJC340 to obtain strain XL1-Blue MRF[']/pJC339+pJC340. On these plasmids, CTN_0339 and CTN_0340 are under the control of T7 and *tet* promoters, respectively. Unlike BL21(DE3), XL1-Blue MRF['] has no chromosomal T7 RNA polymerase gene, thus CTN_0339 is transcribed merely at a basal level in an XL1-Blue MRF['] background. The *tet* promoter is constitutively active in most *E. coli* laboratory strains, including XL1-Blue MRF['] and BL21(DE3).

XL1-Blue MRF['] harboring pJC339 or along with pJC340 was cultivated at 30, 37, or 42°C, and the growth was monitored hourly. All strains grew equally well at 30 and 37°C (Fig. 1) and displayed no signs of stress from expressing the *Thermotoga* proteins, which are not expected to be much active at these temperatures. At 42°C, cells carrying pJC339 and pJC340 propagated as well as the control strain. With pJC339, however, the growth ceased at a low culture density. Therefore, at elevated temperatures, CTN_0339 inhibits the physiological well-being of host cells, but this inhibition is countered by CTN_0340. These observations are in agreement with the speculated restriction and modification activities of the two proteins.

Restriction and modification assays of the *Thermotoga* proteins

To provide further evidence to the functions of the two *Thermotoga* proteins, we first tested the restriction activity of CTN_0339 at 50, 65, and 77°C using pUC19 plasmid DNA prepared from strain XL1-Blue MRF[']. Apparent REase activity was observed in cells expressing pJC339 at all tested temperatures, and the highest cleavage efficiency occurred at 77°C (Fig. 2). For example, 2.5 µl of CTN_0339 extract was required to completely digest 1 µg of pUC19 DNA within 1 h at 65 or 50°C, whereas only half of the amount was needed at 77°C (Fig. 2). The sizes of fully digested pUC19 fragments agree with the occurrence and locations of CGCG sites in this plasmid (Fig. 3).

We next tested whether CTN_0340 was able to protect pUC19 DNA from the digestion of REase. The substrate DNA was incubated with CTN_0340 extract at 77°C for

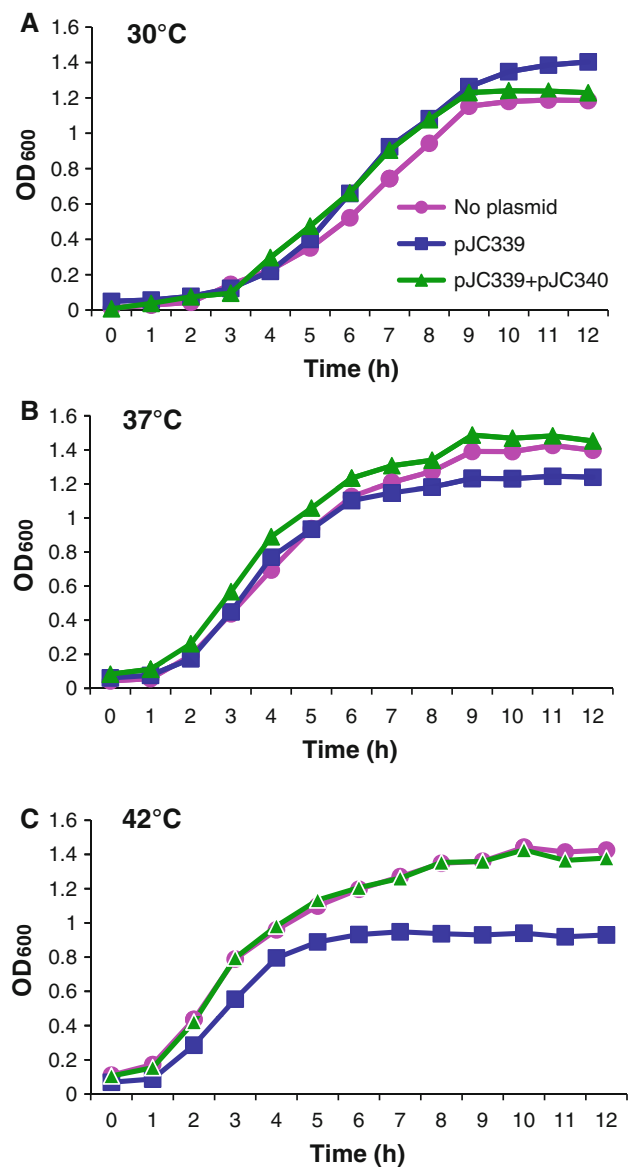


Fig. 1 XL1-Blue MRF['] recombinant strains grown at 30°C (a), 37°C (b), or 42°C (c)

1 h and then digested by REase. DNA treated with 10 µl of CTN_0340 extract was only partially digested by REase, even after overnight incubation at 77°C (Fig. 4a). If the CTN_0340 extract was diluted in 2-fold steps, the substrate DNA became more and more susceptible to the same amount of R.*Tne*DI, and full digestion eventually occurred (Fig. 4a). In another experiment, cell extract containing both CTN_0339 and CTN_0340 was used to treat pUC19 DNA (Fig. 4b). After 1 h of incubation at 55°C, the majority of the DNA was still intact (Fig. 4b), whereas under the same conditions, cell extract containing just CTN_0339 would have substantially degraded the DNA (Fig. 2a). Low levels of partial digestion were noticed in some samples in Fig. 4b, reflecting a competition between

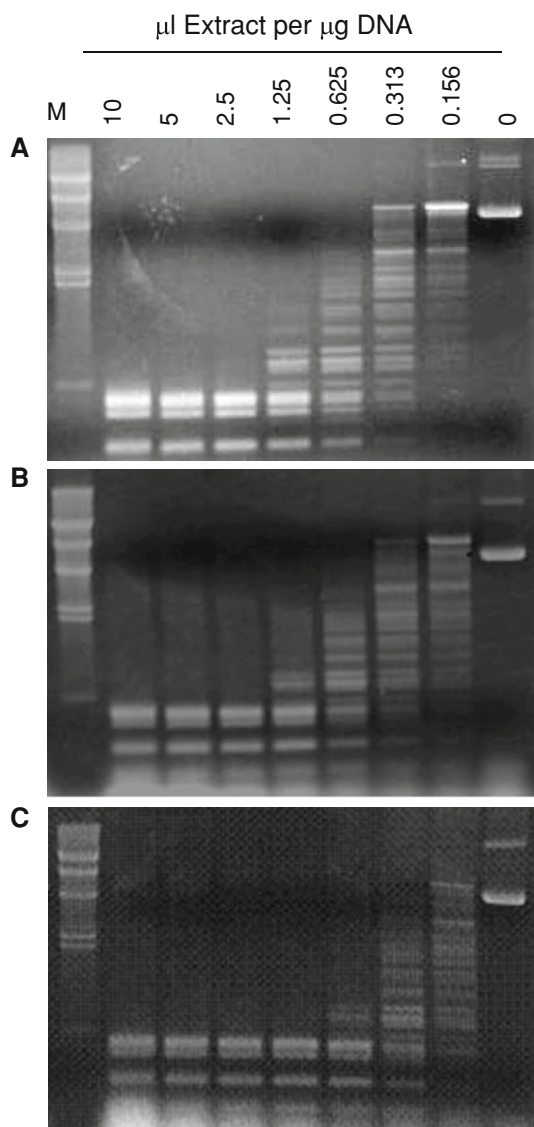


Fig. 2 Digestion of pUC19 DNA with extracts of XL1-Blue MRF' carrying pJC339 at 50°C (a), 65°C (b), or 77°C (c). The amount of cell extract per µg DNA is labeled on top of each lane (in µl). M λ HindIII. Analyzed with 1% (w/v) agarose gel

the antagonistic activities of CTN_0339 and CTN_0340. Taking together the above findings (Figs. 1, 2, 3, 4), it is conclusive that CTN_0339 encodes REase R.*TneDI*, and CTN_0340 the MTase M.*TneDI*.

Overexpression of R.*TneDI*

Although REase activity was detected in XL1-Blue MRF'/pJC339, we were unable to observe the R.*TneDI* protein with either SDS-PAGE or immunoblotting, probably due to its extremely low expression level. To prepare a large quantity of R.*TneDI* for further applications, pJC340 and pJC339 were co-transformed to BL21(DE3), and the cell extract of the recombinant strain

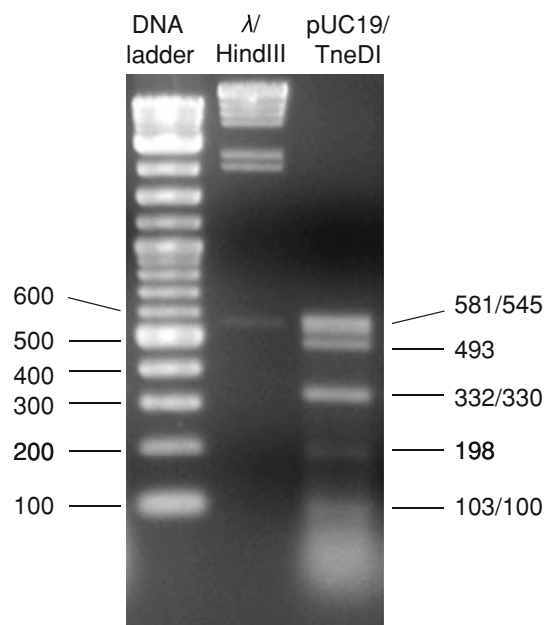


Fig. 3 R.*TneDI*-digested pUC19 DNA analyzed with 2% (w/v) agarose gel. Sizes of the fully digested fragments match the occurrence and locations of CGCG sites in the plasmid

was prepared and analyzed by SDS-PAGE. A distinctive band with an apparent size of ~29 kDa was revealed (Fig. 5, top). Western blotting analysis confirmed that the specific band was the histidine-tagged R.*TneDI* (Fig. 5, bottom), which has a theoretical molecular weight of 28.3 kDa. Thus, it is feasible to overproduce R.*TneDI* in an *E. coli* host and subsequently purify it with an affinity column. The synthesis of M.*TneDI*, which has a theoretical molecular weight of 35.3 kDa, was below the detection limit of SDS-PAGE in both hosts. This is not surprising though, since pACYC184 is a low copy number vector and the *tet* promoter is not typically used for overexpression. Immunoblotting for this MTase was not attempted.

Determination of the cleavage site of R.*TneDI*

Sequence analysis uncovered a recognition site (CGCG) in the coding region of R.*TneDI*. The PCR product of this region was digested by R.*TneDI*, generating an upstream fragment of 502 bp and a downstream fragment of 189 bp. Sequencing of the smaller piece revealed that R.*TneDI* cut immediately after the first G in its recognition sequence (CG↓CG) (Fig. 6a). To further confirm that R.*TneDI* is a blunt-end cutter, the larger fragment was digested with *NdeI* and inserted into the *NdeI*–*SmaI* sites of pUC19. Sequencing of the new vector in the insertion region showed that the two DNA pieces, cut by R.*TneDI* and *SmaI*, respectively, were joined together seamlessly (Fig. 6b).

Fig. 4 Protection of pUC19 DNA by *M.TneDI*. **a** Plasmid DNA was treated with various amounts of cell extract of XL1-Blue MRF⁺ expressing CTN_0340, as labeled on top of each lane. The DNA was then subjected to the digestion of *R.TneDI* (0.1 U per μg DNA). **b** Plasmid DNA was treated with various amounts of cell extract of XL1-Blue MRF⁺ expressing both CTN_0340 and CTN_0339. The amount of cell extract per μg DNA is labeled on top of each lane (in μl)

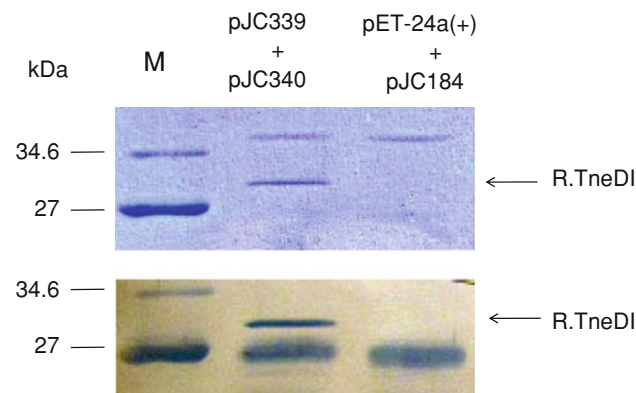
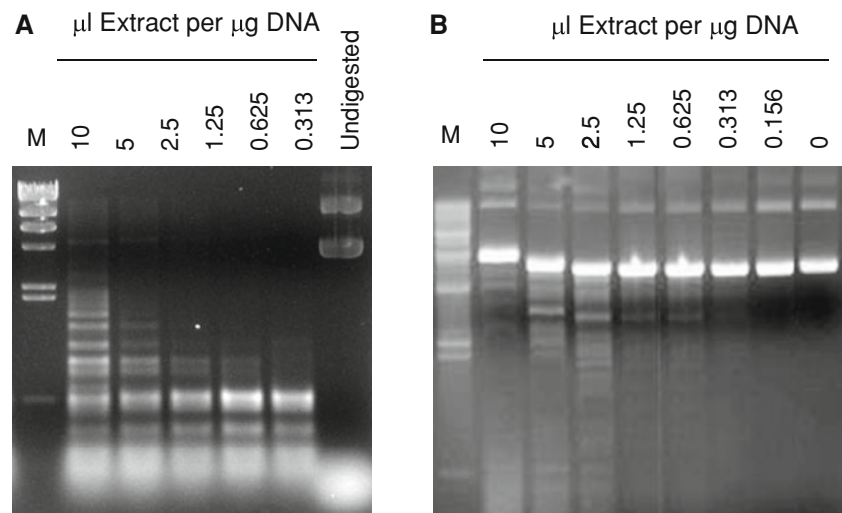


Fig. 5 SDS-PAGE (top) and Western blotting (bottom) analyses of extracts of BL21(DE3) carrying both pJC339 and pJC340. Arrows point to the position of *R.TneDI*. Cells carrying the parent plasmids pET-24a(+) and pJC184 were used as the control

Discussion

In this study, we demonstrated that genes CTN_0339 and CTN_0340 of *T. neapolitana* encode the *TneDI* Type II R–M system. *R.TneDI* may serve as a handy tool for specific cleavage of DNA molecules, and an *E. coli* strain containing *M.TneDI* should be a good host for preparing the DNA to be introduced to a *Thermotoga* host. Type II REases recognizing CGCG sequence have been discovered before, such as *AccII*, *FnuDII*, *ThaI*, *BsuE*, *Bsh1236I*, *BepI*, and *MvnI* (Lui et al. 1979; Strobl and Thompson 1984; Gaido and Strobl 1987; Gaido et al. 1988; Thomm et al. 1988; Venetianer and Orosz 1988), all of which cleave at the center of the recognition site and create blunt ends for their DNA substrates. A notable exception is *SelI*, which cleaves at the ends of the recognition site (\downarrow CGCG) (Miyake et al. 1992).

To protect host chromosomal DNA, REases usually have to be cloned in the presence of the cognate MTases

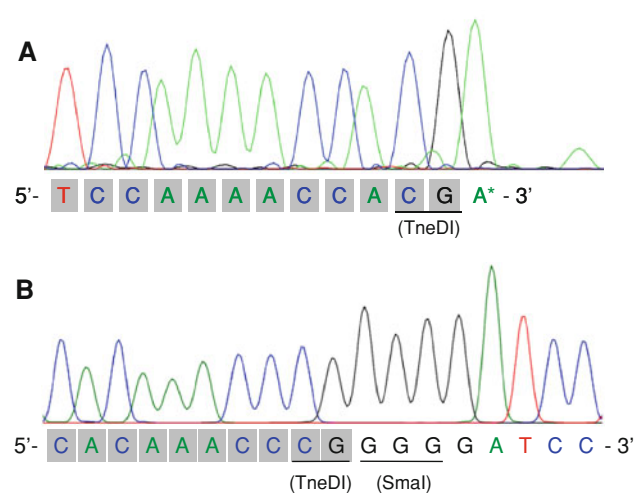


Fig. 6 *R.TneDI* cleaved at the center of its recognition sequence (CG \downarrow CG). **a** The sequence of the smaller fragment of CTN_0339/*R.TneDI* ended at CG. The extra A at the 3'-end, donated by an asterisk, was a template-independent addition by Taq polymerase (Clark 1988; Stier and Kiss 2010). **b** The larger fragment of CTN_0339/*R.TneDI* was ligated with pUC19/*SmaI* with their blunt ends. The half recognition sites of the enzymes are underlined. The shaded nucleotides represent CTN_0339 DNA sequences

(Howard et al. 1986). Alternatively, in vitro transcription and translation approaches could be used (Ishikawa et al. 2005). Here, we were able to directly clone *R.TneDI* to XL1-Blue MRF⁺ by imposing its expression under the control of the T7 promoter. The fact that *R.TneDI* is less active at mesophilic temperatures probably also contributed to the survival of the host cells. Similar success has been achieved before with other thermophilic R–M systems, such as *TaqI* (Slatko et al. 1987).

In a modification reaction, AdoMet (*S*-adenosyl-L-methionine) serves as the methyl donor and is thus an essential cofactor for methylases. In our modification assays (Fig. 4), supplementation of AdoMet was not attempted, because the

E. coli cell extracts were expected to carry enough of the cofactor, even after the heat treatment. Contrary to our common belief, AdoMet is reasonably stable in solutions. For instance, if crude yeast extract is incubated at 37°C, ~70% of the original AdoMet can be found after 6 days and ~25% for 15 days (Morana et al. 2002). Therefore, a fair amount of AdoMet is expected to survive after incubation at 77°C for 1 h in the cell extract of *E. coli*. Moreover, our cell extracts were prepared from 1 g of wet cells (equivalent to 1 L at OD₆₀₀ ~1.0) in 3 ml of lysis buffer. The cell density was extremely high. In our assays, the cell extracts were diluted for no more than 64 times. Consequently, even the most diluted sample should have enough AdoMet.

We have not determined which cytosine at the recognition site is modified by *M.TneDI*. Also unknown is the exact modification position of the cytosine, though motif analysis almost certainly identifies *M.TneDI* as an m⁴C MTase (Malone et al. 1995). Comparison of *M.TneDI* and *M.PvuII*, a m⁴C MTase with a known structure (Gong et al. 1997), revealed the conserved catalytic center (TSPPY/F)

(in motif IV) and the Rossman fold serving as the AdoMet binding site (FxGxG/N) (in motif I) (Fig. 7). The alignment is more gapped in the target recognition domains, which is not surprising as *PvuII* system recognizes CAGCTG instead of CGCG. It has been suggested that m⁴C is more common in thermophiles than m⁵C, because an m⁵C residue has a higher risk of deamination at elevated temperatures (Ehrlich et al. 1985).

Orthologs of the *TneDI* system have been found in other members of the *Thermotogaceae* family. The locus IDs and identities of the orthologs pertaining to the *TneDI* system at the protein level are summarized in Table 2. The neighborhood genes in the six *Thermotogaceae* genomes share nearly perfect synteny. Of even greater interest, given the low level of sequence conservation among REases in general, is the presence of an orthologous R–M system in the archaeon *Ferroglobus placidus*. While *F. placidus* is phylogenetically distant from *Thermotogaceae*, they live in the same high-temperature anaerobic environment, suggesting a recent horizontal gene transfer event between the two groups.

Fig. 7 Sequence alignment of *M.TneDI* and *M.PvuII* generated by CLUSTAL W (1.81) (Thompson et al. 1994) and shaded by Boxshade 3.3.1. Conserved residues are highlighted in black and similar residues in gray. Nine possible structural motifs (IV–III), which are well conserved among group β MTase (Malone et al. 1995), are identified in *M.TneDI* and underlined

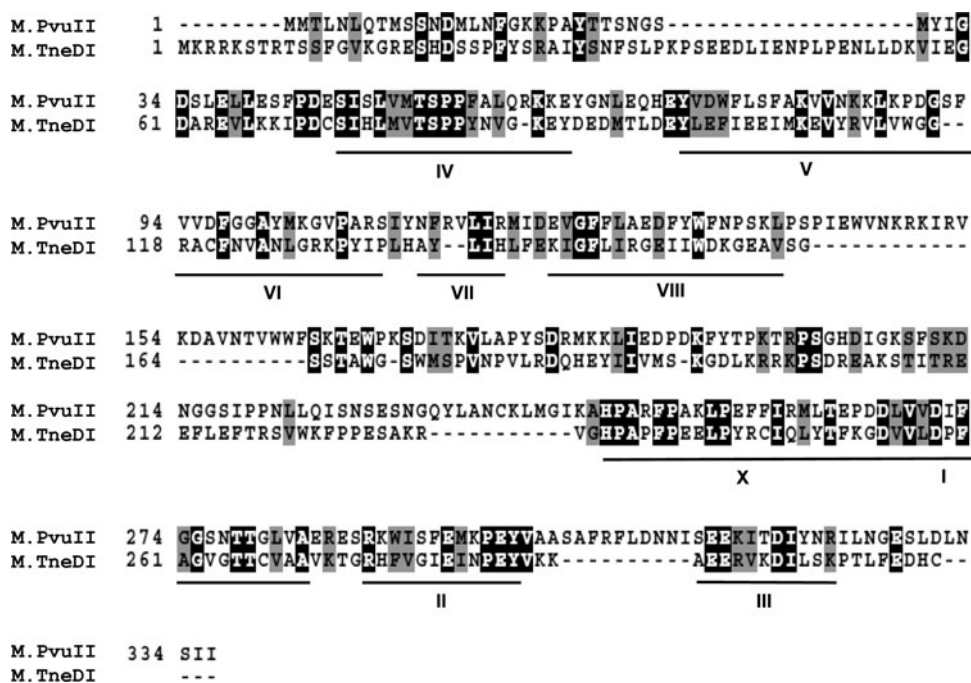


Table 2 Orthologs of the *TneDI* system

	<i>R.TneDI</i>	<i>M.TneDI</i>
<i>T. maritima</i>	TM0329 (100)	TM0328 (100)
<i>T. petrophila</i> RKU-1	Tpet_0590 (100)	Tpet_0591 (100)
<i>T. sp.</i> RQ2	TRQ2_0604 (100)	TRQ2_0605 (100)
<i>Marinitoga piezophila</i> KA3	MPKA3_1285 (100)	MPKA3_1161 (100)
<i>T. naphthophila</i> RKU-10	Tnap_0965 (93)	Tnap_0964 (98)
<i>F. placidus</i>	Ferp_1585 (38)	Ferp_1586 (76)

Identities of the protein products pertaining to *TneDI* system are listed as percentages in parentheses as aligned by BLASTP (Altschul et al. 1997)

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