Nucleic Acids Research, 1996, Vol. 24, No. 10 **1799–1801**

Tsp49I (ACGT↓), a thermostable neoschizomer of the Type II restriction endonuclease *Mae*II (A↓CGT), discovered in isolates of the genus *Thermus* from the Azores, Iceland and New Zealand

Simon G. Welch* and Ralph A.D. Williams

Department of Biochemistry, Faculty of Basic Medical Sciences, Queen Mary and Westfield College, University of London, Mile End Road, London E1 4NS, UK

Received March 15, 1996; Accepted March 28, 1996

ABSTRACT

One hundred and forty eight isolates of the genus *Thermus*, from neutral and alkaline hot water springs on four continents, have been screened for the presence of restriction endonuclease activity. An isolate (SM49) from the island of Sao Miguel, in the Azores, showed a high level of restriction endonuclease activity when a cell-free extract was incubated with λ phage DNA at 65°C. A Type II restriction endonuclease (*Tsp*49I) has been partially purified from this isolate and the recognition and cleavage site determined. *Tsp*49I recognizes the four base sequence ACGT, which is the same as the recognition sequence of the mesophilic Type II restriction endonuclease *Mae*II. However, unlike *Mae*II, which cleaves DNA between the first and second base of the recognition sequence (A↓CGT), *Tsp*49I hydrolyses the phosphodiester bond in both strands of the substrate after the last base of the recognition sequence 5′-ACGT↓-3′, producing four base 3′-OH overhangs (sticky ends). The enzyme has a pH optimum of 9.0, requires 2 mM $MgCl_2$ for maximum activity and retains full enzyme activity following incubation for 10 min at temperatures up to 80°C. Two further examples of the same restriction endonuclease specificity as *Tsp*49I were detected in *Thermus* isolates from Iceland (*Tsp*IDSI) and New Zealand (*Tsp*WAM8AI). The three *Mae*II neoschizomers, *Tsp*49I, *Tsp*IDSI and *Tsp*WAM8AI, exhibit similar pH optima, heat stabilities and $MgCl_2$ requirements, but differ in their requirements for NaCl and KCl.

INTRODUCTION

In the 28 years since the discovery of restriction endonuclease activity in the bacterium *Escherichia coli* (1), thousands of bacterial strains have been screened for these site-specific endonucleolytic enzymes. Restriction endonucleases have provided the molecular biologist with a range of site-specific tools for analysis, rearrangement, cloning and sequencing of DNA. At least 2800 bacterial restriction endonucleases have been described and this number is continually being updated (2). Of these, only some 240 are enzymes with different and unique specificities. Most are enzymes either with the same recognition sequence and cleavage site as the prototype (isoschizomers), but from different bacterial genera, species or strains, or they are enzymes with the same recognition sequence, but an alternative cleavage site (neoschizomers).

The majority of the restriction endonucleases have been isolated from mesophilic bacteria and while stable at temperatures below 45°C, they are usually denatured at higher temperatures. Restriction endonucleases that will withstand higher temperatures represent a useful addition to the molecular biologist's toolkit. Thermally stable restriction endonucleases occur naturally in organisms that thrive at high temperatures. The thermophilic genus *Thermus* and its type species *Thermus aquaticus* (3) are aerobic, non-sporulating heterotrophic rods with optimum growth temperatures in the region of 70°C. Strains of *Thermus* have been isolated from neutral and alkaline hot water environments world wide (4). Valid species of the genus include *T.aquaticus* , *T.thermophilus*, *T.ruber*, *T.filiformis*, *T.brockianus* and *T.scotoductus*, but the variability of phenotype often renders the identification of species problematical (4). Twenty two restriction endonucleases, each with a different DNA recognition site, have been discovered within the genus *Thermus* (2) and of these, nine have no known mesophilic isoschizomers (*Taq*I, *Taq*II, *Tfi*I, *Tsp*EI, *Taq*52I, *Tsp*RI, *Tsp*45I, Tsp4CI and *Tth*111II; REBASE 603, February 1996).

In the present experiments we have screened 148 isolates of the genus *Thermus*, collected from hot springs on four continents. We report the discovery and characterization of three Type II restriction endonucleases, *Tsp*49I, *Tsp*IDSI and *Tsp*WAM8AI, with the same recognition and cleavage sites, ACGT↓, and suggest that because of its specificity and extreme thermal stability, these three neoschizomers of *Mae*II represent a useful addition to the small list of heat-stable restriction endonucleases.

MATERIALS AND METHODS

The DNA substrates λcI857Sam7 phage DNA (unmethylated), pBR322, pUC18, M13mp18 and ϕX174 were purchased from NBL Gene Sciences (Cramlington, UK). SV40 virus DNA and

* To whom correspondence should be addressed

the DNA markers *Hin*dIII-digested λ DNA and the 123 bp ladder were from Sigma (Dorset, UK). Adenovirus Type 2 DNA was purchased from BRL, USA. DNA grade agarose, buffers, urea and Kodak Biomax MR film were from IBI Ltd (Cambridge, UK). 'One-Phor-All' buffer was obtained from Pharmacia UK. Synthetic 26mer primer 5309 (5′-CTAACAACTAATAGATTAGAGCCGTC-3′) was synthesized by Oswel DNA Services, UK. The TAQuence version 2.0 DNA sequencing kit and [α-^{35}S]dCTP (1000 Ci/mmol) were purchased from Amersham Life Sciences, UK. The PC/GENE molecular biology programmes and databases were purchased from IntelliGenetics (Europe) (Geel, Belgium).

Thermus isolate SM49 was isolated from a hot water run-off from the volcanic region of Furnas in the south east of the island of Sao Miguel, in the Azores. At the time of sampling the water temperature was 81°C at a pH of 8.0. Cells were grown overnight at 65°C in 500 ml shake flask cultures containing 0.3% tryptone, 0.1% yeast extract (Difco) and Castenholtz mineral salts (5) and harvested by centrifugation at 6000 *g* for 30 min.

A 22.5 g cell pellet was suspended in 100 ml 20 mM Tris–HCl, 0.1 mM EDTA, 2 mM dithiothreitol, pH 7.6, and disrupted by sonication (3 × 30 s) in ice, followed by centrifugation at 16 000 *g* for 1 h. The cell-free extract (100 ml) was adjusted to 0.2 M NaCl and 3.3 ml 10% polyethyleneimine, pH 7.5, was added with stirring and the suspension centrifuged for 1 h at 16 000 *g*. The supernatant was adjusted to 70% saturation by the addition of solid ammonium sulphate and the precipitate collected by centrifugation at 16 000 *g* for 1 h. The pellet was suspended in 55 ml 20 mM K_2HPO_4, 1 mM EDTA, 5 mM 2-mercaptoethanol, 4.0% glycerol (v/v), pH 7.5, and dialysed against 3000 ml of the same buffer for 16 h. The sample was applied to a phosphocellulose (P11) column (13 × 3.2 cm) and eluted with a KCl gradient (0–1.0 M). Fractions were tested for restriction endonuclease activity against λ DNA as substrate. The fractions containing the peak of enzyme activity (0.6–0.8 M KCl) were concentrated to 25 ml by pressure filtration and dialysed for 16 h against 20 mM Tris–HCl, 5 mM 2-mercaptoethanol, 1 mM EDTA, 5.0% glycerol (v/v), pH 8.5. The sample was applied to a DEAE–Sephacel column (12 ×1.2 cm) and eluted step-wise with 20 ml aliquots of the same buffer containing 0.02, 0.05, 0.1, 0.2, 0.3, 0.5 and 0.7 M NaCl respectively. The fraction containing the peak of restriction endonuclease activity (0.2 M NaCl) was concentrated by pressure filtration and chromatographed on a Sephadex G-200 column (80 ×1.5 cm) eluted with 20 mM Tris–HCl, 50 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM EDTA, 5% glycerol, pH 7.5, at a flow rate of 6 ml/h. The fractions containing the peak of enzyme activity were pooled, concentrated by pressure filtration to 0.5 ml, adjusted to 50% (v/v) with glycerol and stored at –20°C. Enzyme activity was initially measured in 'One-Phor-All' buffer using λ phage DNA as substrate. One unit of enzyme activity is defined as the amount of restriction endonuclease required to totally digest 1 µg λ DNA in 1 h at 65°C, in a reaction volume of 50 µl.

From the gel filtration Sephadex G-200 column, previously calibrated with the molecular mass markers human serum transferrin (79.5 kDa), bovine serum albumin (65 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (29 kDa) and horse heart cytochrome c (12.4 kDa), the peak of *Tsp*49I activity was eluted at an elution volume corresponding to a molecular weight of 35 kDa. The final yield from a 22.5 g cell pellet was 14 000 U. The pH optimum of *Tsp*49I was estimated to be 9.0 in 50 mM Tris–HCl.

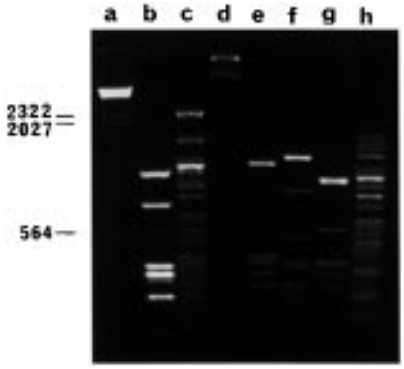

Figure 1. Digestion of seven DNA substrates by *Tsp*49I. Aliquots of 0.5 µg of each of the DNA substrates were incubated for 3 h at 65°C with 5 U partially purified *Tsp*49I restriction endonuclease: (**b**) ϕ X174; (**c**) adenovirus; (**d**) SV40 virus; (**e**) pUC18; (**f**) M13mp18; (**g**) pBR322; (**h**)λ phage. Size markers (**a**) *Hin*dIII-cleaved λ. Reaction products separated by electrophoresis in a 1.8% agarose gel.

In the absence of $MgCl_2$, no cleavage of λ phage DNA was detected. Maximum enzyme activity occurred at a $MgCl_2$ concentration of 2 mM, with considerable inhibition of activity at $MgCl_2$ concentrations above 16 mM. *Tsp*49I required neither the presence of NaCl nor KCl in order to exhibit full activity. However, at NaCl or KCl concentrations above 150 mM severe inhibition of enzyme activity was detected.

RESULTS AND DISCUSSION

The restriction fragment patterns produced by *Tsp*49I as a result of the digestion of λ phage DNA, pBR322, pUC18, ϕX174, M13mp18, SV40 virus DNA and adenovirus DNA are shown in Figure 1. The enzyme failed to cut SV40 DNA and for the substrates pBR322, ϕX174 and M13mp18 the number of restriction fragments >1000 bp were found to be 0, 1 and 2 respectively. Of the 224 possible four, five and six base palindromes (6), only 19 would result in one pBR322 fragment >1000 bp. Of these 19 potential recognition sites, only three are not present in SV40 and of these, only two (ACGT and CGCG) would produce only a single restriction fragment >1000 bp with ϕX174. Since in M13mp18 the sequence CGCG would generate no fragments >1000 bp, whereas ACGT would produce one fragment >1000 bp, only the sequence ACGT remained as a putative recognition sight for *Tsp*49I out of the original 224 possible four, five and six base palindromes. Further evidence to support the suggestion that the recognition sequence of *Tsp*49I could be ACGT, which incidentally is the same as the recognition sequence for the Type II restriction endonuclease *Mae*II (7), was provided by the following observations. Computer-generated fragment sizes (PC/GENE-RESTRI) from a range of DNA substrates for the four base sequence ACGT were in close agreement with the fragment sizes observed experimentally for *Tsp*49I. Secondly, the observed fragment patterns generated by *Tsp*49I were found to match with the computer-generated fragment patterns for *Mae*II (8). Thirdly, restriction fragment patterns for seven DNA substrates digested with *Tsp*49I and a commercial source of *Mae*II (Boehringer Mannheim, Germany) were indistinguishable.

Table 1.

Enzyme	Recognition sequence	Cleavage site	pH optima	$MgCl_2$ (mM)	NaCl (mM)	KCl (mM)	Heat stability[a] (°C)
*Tsp*49I	ACGT	ACGT↓	9.0	2.0	0	0	82
*Tsp*IDSI	ACGT	ACGT↓	9.5	2.0	50	100	78
*Tsp*WAM8AI	ACGT	ACGT↓	8.5	2.0	0	0	80
*Mae*II	ACGT	A↓CGT	10.0	12.0	150	150	55

[a]The maximum temperature that 10 U enzyme can withstand for 10 min in 50 µl OPA buffer without any loss of enzyme activity.

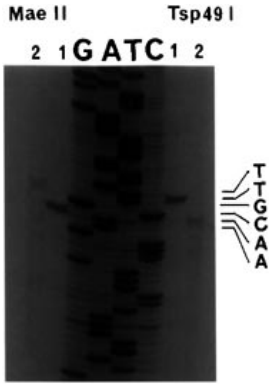

Figure 2. DNA sequencing gels to determine the cleavage site of *Taq*52I. Dideoxy sequencing ladder (lanes G, A, T and C). *Tsp*49I and *Mae*II digests of the labelled primer extension 5309 before (lane 1) and after (lane 2) incubation with T4 DNA polymerase.

The recognition site (ACGT) for *Tsp*49I was subsequently confirmed during the course of the experiments carried out to determine the precise location of the DNA cleavage site using the procedure of Brown and Smith (9). We designed a synthetic 26mer primer (5309) that would bind to a single-stranded M13mp18 template and initiate DNA synthesis 79 bases from position 4634, a putative recognition sequence (ACGT) of *Tsp*49I and the published recognition sequence (ACGT) of *Mae*II (7). Following the procedure of Brown and Smith (9), the results obtained (Fig. 2) confirmed the cleavage site for the enzyme *Mae*II as being between the first and second bases of the recognition sequence A↓CGT. For the enzyme *Tsp*49I, the ^{35}S-labelled product from the primer extension cut with *Tsp*49I migrates in the sequencing gel to a position level with the fourth base (T) of the recognition sequence ACGT. This should generate a four base 3′-OH overhang on the labelled strand of the double-stranded product. T4 DNA polymerase in the presence of the four dNTPs was found (Fig. 2) to have removed all four bases from the 3′-OH overhang, as expected.

From these results we deduce that *Tsp*49I is a neoschizomer of *Mae*II (A↓CGT) and that the enzyme cuts both strands of the DNA substrate immediately after the last base of the recognition sequence:

5′-ACGT↓-3′
3′-↑TGCA-5′

Subsequent to the discovery of the restriction endonuclease *Tsp*49I in a *Thermus* isolate (SM49) from the Azores, two further *Thermus* isolates examined in this laboratory, one from New Zealand (WAM8A) and the other from Iceland (IDS), have been found to contain a restriction endonuclease with the same recognition and cleavage site as *Tsp*49I. The three *Mae*II neoschizomers could be distinguished one from another on the basis of their pH optima and magnesium and salt requirements (Table 1).

*Tsp*49I represents a useful addition to the small list of extremely thermostable Type II restriction endonucleases. Although it is a neoschizomer of *Mae*II, the enzyme *Mae*II is one of three restriction endonucleases within the archaebacterium *Methanococcus aeolicus* (7), contributing only 3% of the total endonuclease activity. From 35 g of cells, Schmid *et al*. isolated 300 U *Mae*II, whereas the yield of *Tsp*49I reported in this present paper is 11 000 U from 22.5 g cells.

REFERENCES

1 Meselson,M. and Yuan,R. (1968) *Nature*, **217**, 1110–1114.
2 Roberts,R.J. and Macelis,D. (1996) *Nucleic Acids Res*., **24**, 223–235.
3 Brock,T.D. and Freeze,H. (1969) *J. Bacteriol*., **98**, 289–297.
4 Williams,R.A.D. and Sharp,R.J. (1995) In Sharp,R and Williams,R. (eds), *Thermus Species*. Plenum Press, New York, NY, pp. 1–42.
5 Ramaley,R.F. and Hixson,J. (1970) *J. Bacteriol*., **103**, 527–528.
6 Fuchs,C., Rosenvold,E.C., Honigman,A. and Szybalski,W. (1980) *Gene*, **10**, 357–370.
7 Schmid,K., Thomm,M., Lamminet,A., Lane,F.G., Kessler.C., Stetter,K.O. and Schmidtt,R. (1984) *Nucleic Acids Res*., **12**, 2619–2628.
8 Brown,T.A. (1991) In Rickwood, D and Hames, B.D. (eds), *Essential Molecular Biology: A Practical Approach*, Vol. 1. IRL Press, Oxford, UK.
9 Brown,N.L. and Smith,M. (1980) *Methods Enzymol*., **65**, 391–404.