Two thermostable Type II restriction endonucleases from Icelandic strains of the genus *Thermus*: *Tsp*4C I (ACN/GT), a novel Type II restriction endonuclease, and *Tsp*8E I, an isoschizomer of the mesophilic enzyme *Bgl* I (GCCNNNN/NGGC)

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Sixteen isolates of thermophilic bacteria from the genus *Thermus*, isolated from neutral and alkaline hot water springs in the southwest region of Iceland, were tested for the presence of restriction endonucleases. Extracts from five of the isolates showed evidence of the presence of restriction endonuclease activity by producing discrete nucleotide fragments when incubated at 65 °C with λ phage DNA. Two of the isolates (*Tsp*4C and *Tsp*8E) were found to have particularly high levels of restriction endonuclease activity, and the respective enzymes from these two *Thermus* isolates were partially purified and characterized and their recognition and cleavage sites were determined. Enzyme *Tsp*4C I is a novel Type II restriction endonuclease recognizing the interrupted palindromic tetranucleotide sequence ACNGT, where N can be any one of the four bases in DNA. *Tsp*4C I, which retains full enzyme activity when incubated for 10 min at temperatures up to 76 °C, hydrolyses the phosphodiester bond in both strands of a double-stranded DNA substrate between the third and fourth bases of the recognition sequence (ACN/GT), generating fragments with a single base 3′-OH overhang. Enzyme *Tsp*8E I is a thermostable isoschizomer of the mesophilic Type II restriction endonuclease *Bgl* I (GCCNNNN/NGGC) [Lee, Clanton and Chirikjiam (1979) Fed. Proc. **28**, 294], generating fragments with a three base 3′-OH overhang. However, unlike *Bgl* I, *Tsp*8E I exhibits considerable thermal stability, retaining full enzyme activity when incubated for 10 min at temperatures up to 78 °C. Both *Tsp*4C I and *Tsp*8E I represent significant additions to the small but expanding list of the extremely thermostable restriction endonucleases.

INTRODUCTION

In the 26 years that have elapsed since the first discovery of restriction endonuclease activity in the bacterium *Escherichia coli* [1], many thousands of bacterial strains have been screened for the presence of these site-specific endonucleolytic enzymes. Restriction endonucleases have subsequently provided the molecular biologist with an extensive range of site-specific tools to assist in the analysis, rearrangement, cloning and sequencing of DNA. It has been estimated that at least 2800 bacterial restriction endonucleases have been described, and this number is continually being updated [2]. Of this vast number however, only some 230 are examples of enzymes with different and unique specificities, the remainder being enzymes with the same substrate specificity as the prototype (isoschizomers) but from different bacterial genera, species or strains.

The majority of the restriction endonucleases have been isolated from mesophilic bacteria, so whilst the enzymes are stable at temperatures below 45 °C, they usually become denatured rapidly at higher temperatures. Restriction endonucleases that will withstand higher temperatures represent an extremely useful addition to the molecular biologist's tool-kit. In recent years the search for novel and thermally stable restriction endonucleases has been extended to include organisms that normally thrive at high temperatures. The thermophilic genus *Thermus* and its type species *Thermus aquaticus* were first described in 1969 [3] as 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 in the U.S.A [4], Japan [5], Iceland [6], Belgium [7], Britain [8], New Zealand [9], Portugal [10], the Czech Republic [11] and Thailand [12]. Twenty-two restriction endonucleases, each with a different DNA recognition site, have been discovered within the species and strains of the genus *Thermus* [2] and four of these enzymes have no known mesophilic isoschizomers.

In the present experiments we have screened 16 isolates of the genus *Thermus* collected from hot springs in Iceland. We report the discovery and characterization of two novel Type II restriction endonucleases and suggest that because of their specificities and extreme thermal stability, *Tsp*4C I and *Tsp*8E I represent a useful addition to the small list of heat-stable restriction endonucleases.

EXPERIMENTAL

Materials

DNA substrates, λcI857Sam7 phage DNA (unmethylated), pBR322, pUC18, M13mp18 and φX174, were purchased from NBL Gene Sciences, Cramlington, U.K. DNA markers, *Hin*dIII λDNA and a 123 bp ladder were purchased from Sigma, Dorset, U.K. DNA-grade agarose, buffers, urea and Kodak Biomax MR film were obtained from IBI Ltd., Cambridge, U.K. 'One-Phor-All' universal buffer and the two specially designed synthetic 24-mer primers, 8E (5′-TTGAGGGGACGACGACCGTATCGG-

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3′) and 4C (5′-GTCACCGACTTGAGCCATTTGGGA-3′), were synthesized by Pharmacia, U.K. TAQuence version 2.0 DNA sequencing kit (M13) produced by USB Corporation, Cleveland, OH, U.S.A. and [α^{35}S]dCTP (1000 Ci/mmol) were purchased from Amersham Life Sciences, U.K. The PC/GENE molecular biology programs and databases were purchased from IntelliGenetics (Europe), Geel, Belgium.

Bacterial cultures

Thermus sp. 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, as described by Ramaley and Hixson [13]. Cells were harvested by centrifugation at 6000 *g* for 30 min and stored at −20 °C.

Screening bacterial isolates for restriction endonuclease activity

Cell pellets (3 g) were thawed, suspended in 10 ml of 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 16000 *g* for 1 h. Two 10 μl aliquots of the supernatant were removed and diluted 1 in 5 and 1 in 30 respectively. Each dilution (2 μl) was incubated in 12 μl of a 1 in 10 dilution of 'One-Phor-All' buffer containing 0.25 μg of λDNA at 65 °C for 1 h. Reactions were terminated by the addition of 3 μl of 'stop buffer' (4 g of sucrose/10 mg of Bromophenol Blue/740 mg of EDTA/10 ml of 0.25 M Tris-HCl, pH 8.0). Samples were electrophoresed in 1.4% agarose gels for 2 h; the agarose gel and electrode buffer (Tris/Borate/EDTA, pH 8.3) contained ethidium bromide (0.5 μg/ml). DNA fragments were detected by UV transillumination, and photographed.

Partial purification of restriction endonucleases *Tsp*4C and *Tsp*8E

Cell-free extract (10 ml) from 3 g of cell pellet was adjusted to 0.2 M NaCl, 300 μl of 10% polyethyleneimine (pH 7.5) was added with stirring and the suspension was centrifuged for 1 h at 16000 *g*. The supernatant was adjusted to 70% saturation by the addition of solid ammonium sulphate and the precipitate was collected by centrifugation at 16000 *g* for 1 h. The pellet was suspended in 5 ml of 20 mM K_2HPO_4/1 mM EDTA/5 mM 2-mercaptoethanol, pH 7.5, and dialysed against 800 ml of the same buffer for 16 h. The sample was applied to a phosphocellulose (P11) column (6 × 0.5 cm) and eluted in a step-wise fashion with 4 ml aliquots of the same buffer containing increasing concentrations of KCl: 0.12, 0.24, 0.48, 0.85 and 1.2 M. Fractions were tested for restriction endonuclease activity using λDNA as substrate. Those fractions containing the peak of enzyme activity were pooled and dialysed for 16 h against 10 mM Tris-HCl, pH 7.5. The sample was applied to a DEAE-Sephacel column (6 × 0.5 cm) and eluted in a step-wise fashion with 4 ml aliquots of the 10 mM Tris-HCl, pH 7.5, containing increasing concentrations of NaCl: 0.1, 0.2, 0.3, 0.5 and 0.8 M respectively. Fractions containing the peak of restriction endonuclease activity were pooled, concentrated by pressure filtration, applied to a gel-filtration column (Sephadex G150, 50 × 1.5 cm) and 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 and adjusted to 50% (v/v) with glycerol before storage at −20 °C.

Enzyme activity was measured in 'One-Phor-All' buffer using λphage DNA as substrate. One unit of enzyme activity is defined as the amount of restriction endonuclease required to totally digest 1 μg of λDNA in 1 h at 65 °C in a reaction volume of 50 μl.

Determination of optimum conditions for restriction endonuclease activity

Assays were carried out using λDNA as substrate in 50 mM Tris-HCl at a range of pHs (7.0–10.5) and concentrations of $MgCl_2$ (0–32 mM), NaCl (0–160 mM) and KCl (0–160 mM).

Thermal stability studies

1 unit of restriction endonuclease in 20 μl of the optimum reaction buffer was incubated for 10 min at temperatures from 45 to 100 °C. Following rapid cooling in ice, 1 μg of λDNA was added and the mixture was incubated for 1 h at 65 °C. Residual enzyme activity was detected by assessing the extent of substrate digestion, as revealed by agarose gel electrophoresis of the reaction products.

Determination of the recognition site

The partially purified restriction endonucleases *Tsp*4C I and *Tsp*8E I were used to completely digest 1 μg of the substrates λphage DNA, pBR322, φX174, pUC18 and M13mp18, at 65 °C. The reaction products were separated by electrophoresis in 1.4% agarose gels and the sizes of the DNA fragments were determined by reference to the markers, i.e. a *Hin*dIII digest of λ DNA and a 123 bp ladder. Digest patterns produced by the two enzymes for each of the substrates were compared with computer-generated patterns for 147 different restriction endonucleases [14]. When no obvious match with any of these enzymes could be detected, possible recognition sites were identified using computer-generated tables listing the frequencies, distances between, and locations of all possible tetra-, penta- and hexanucleotide palindromes [15]. Many, but by no means all, restriction endonucleases recognize continuous or interrupted palindromic sequences of nucleotides. By comparing the numbers and sizes of DNA fragments predicted for each palindromic recognition site with those observed experimentally, recognition sites were eliminated until only a few putative sites remained. These sites were entered in the PC/GENE restriction site editor and simulated digestions of a number of substrates were carried out using the program RESTRI. The fragment sizes were then compared with those that had been obtained experimentally.

Determination of the cut site

The precise position of the cut sites within double-stranded DNA substrates for the enzymes *Tsp*4C I and *Tsp*8E I was determined using the procedure described by Brown and Smith [16]. Once recognition sites had been determined, synthetic 24-mer primers were designed that would initiate DNA synthesis on the single-stranded M13 template, 50–150 nucleotides 3′ of an isolated putative recognition site. The ^{35}S-labelled product of primer extension through this site was isolated by extraction with phenol, desalted by chromatography on Sephadex G-25, digested with the respective restriction endonuclease and electrophoresed on a buffer gradient DNA sequencing gel alongside a sequencing ladder. A sample of the restriction digest was incubated with 4 units of T4 DNA polymerase and the four nucleoside triphosphates (0.2 mM) for 30 min at 37 °C and electrophoresed on the DNA sequencing gel alongside the sequencing ladder for M13mp18 generated from the same 24-mer primer. The procedure of Brown and Smith [16] not only makes it possible to

Table 1 Partial purification of restriction endonucleases *Tsp*4C I and *Tsp*8E I from 3 g of cells

Stage	Volume (ml)	Protein (mg)	Enzyme activity (units)	Specfic activity (units/mg)	Yield (%)	Purification (fold)
*Tsp*4C I						
Cell-free extract	10.0	125	15000	120	100	–
$(NH_4)_2SO_4$ ppt.	4.2	98	14500	148	97	1.2
Phosphocellulose	6.0	14	9000	678	63	5.7
DEAE-Sephacel	5.8	1.8	3800	2100	25	17.5
Sephadex G-150	1.1	0.45	1600	3555	10.7	29.6
*Tsp*8E I						
Cell-free extract	10.0	142	18500	130	100	–
$(NH_4)_2SO_4$ ppt.	3.6	102	171500	171	95	1.3
Phosphocellulose	6.0	18	10200	566	55	4.4
DEAE-Sephacel	5.8	2.6	4000	1538	22	11.8
Sephadex G-150	0.9	0.51	1300	2450	11.5	18.8

identify the position of the cut site but it also serves to confirm the sequence of the recognition site.

RESULTS AND DISCUSSION

Cell-free extracts from 16 isolates of the genus *Thermus*, collected from neutral and alkaline hot water springs at different locations in the south-west region of Iceland, were tested at two dilutions for ability to digest unmethylated λphage DNA. Extracts from 11 of the isolates showed either no evidence of restriction endonuclease activity, since agarose gel electrophoresis revealed that the substrate had not been digested, or that the substrate had been cut into many small molecular mass products, indicative of non-specific nuclease activity. Extracts from the remaining five isolates digested λDNA, producing discrete DNA fragments. Isolates 4C and 8E contained high levels of restriction endonuclease activity, such that even at high dilutions (2 μl of a 1 in 30 dilution) 0.25 μg of λDNA was totally digested in 1 h at 65 °C. The fragment patterns generated by 4C and 8E were significantly different, indicating the presence of different restriction endonuclease activities within these two *Thermus* isolates. The restriction endonuclease activity in the other extracts was at least one order of magnitude lower than that in 4C and 8E. This present study describes the isolation and characterization of the restriction endonucleases from the *Thermus* isolates 4C and 8E.

Restriction endonuclease from *Thermus* isolate 4C, *Tsp*4C I

*Tsp*4C I was partially purified following the protocol described in the Experimental section. The enzyme was eluted from the phosphocellulose column by 0.48 M KCl and from the DEAE-Sephacel column by 0.2 M NaCl. The final yield from a 3 g cell pellet was 1600 units (Table 1). The results from the experiments designed to establish the optimum pH, Mg, NaCl and KCl requirements and heat stability are given in Table 2.

The fragment patterns produced by *Tsp*4C I, resulting from the digestion of five different DNA substrates, are shown in Figure 1. When λphage DNA (49000 bp unmethylated) was digested by *Tsp*4C I, a complex mixture of more than 50 fragments was produced, the largest of which was only 1500 bp. This result suggests that the recognition site for *Tsp*4C I occurs many times within λDNA and is therefore probably small, possibly consisting of only four bases. pBR322 was digested by *Tsp*4C I to reaction products containing no fragments of 1000 bp or larger, whereas M13mp18 and φX174 digested with *Tsp*4C I

Table 2 Conditions for optimum activity of restriction endonucleases *Tsp*4C I and *Tsp*8E I and the heat stabilities of the enzymes

	*Tsp*4C I	*Tsp*8E I
pH optima (50 mM Tris/HCl)	9.0	9.4
$MgCl_2$ (mM)	4	5
NaCl (mM)	None required	None required
KCl (mM)	None required	None required
Residual activity (%) after		
10 min incubation at: 76 °C	100	100
78 °C	90	100
80 °C	65	80
82 °C	0	0

produced just one fragment greater than 1000 bp in each case. From the Fuchs' tables [15] only 19 of all the possible 4, 5 and 6 nucleotide palindromes would generate no fragments in pBR322 above 1000 bp. Of these 19, only eight would give a single fragment greater than 1000 bp from M13, and of these eight, only two would generate a single fragment greater than 1000 bp from φX174. Therefore, by elimination, the only two possible palindromic sequences of 6 nucleotides or less that could be the recognition site for *Tsp*4C I are ACNGT or CANTG. Neither of these are the recognition sequences for any of the presently known restriction endonucleases listed in the latest edition of REBASE [2]. The final substrate used was pUC18, for which data on the occurrence of palindromic sequences are not provided by Fuchs et al. [15]. However, using the RESTRI computer program of PC/GENE, we searched the sequence of pUC18 for the incidence and locations of the sequences ACNGT and CANTG. CANTG was eliminated as a possible recognition site of *Tsp*4C I since the expected fragment sizes were significantly different from those observed experimentally. However, for the interrupted palindrome ACNGT, the agreement between observed and expected fragment sizes for the substrates pBR322, M13mp18, φX174 and pUC18 was found to be extremely close. As a result of these observations we conclude that *Tsp*4C I recognizes the sequence ACNGT and therefore represents an example of a previously unreported Type II restriction endonuclease.

The recognition site for *Tsp*4C I was confirmed by the experiment used to determine the precise location of the cut site.

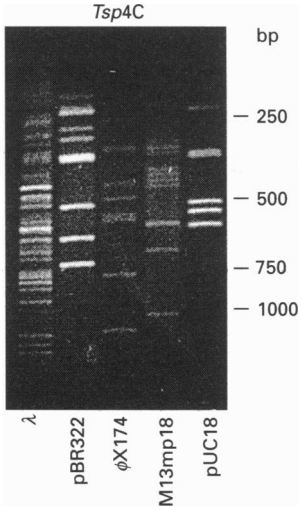

Figure 1 Digestion of five DNA substrates by *Tsp*4C I

A 1 μg amount of each substrate was incubated with 20 units of *Tsp*4C I for 4 h at 65 °C. Reaction products were separated by electrophoresis in a 1.4% agarose gel. The sizes of the DNA fragments (bp) were measured by reference to calibration markers.

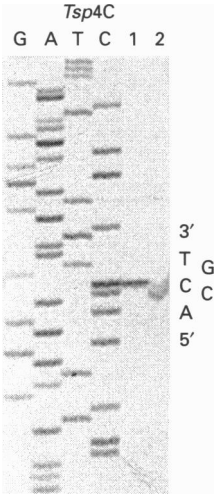

Figure 2 DNA sequencing gel to determine the location of the cut site of *Tsp*4C I

Full details are given in the Experimental and Results sections. The dideoxy sequencing ladder can be seen in lanes G, A, T and C). *Tsp*4C I digest of the labelled primer extension before (lane 1) and after (lane 2) incubation with T4 DNA polymerase.

Using a synthetic 24-mer nucleotide primer (see Materials section) designed to bind to the single-stranded M13mp18 template, 80 nucleotides from a putative recognition site (ACGGT) for *Tsp*4C I within M13 (2515-102519), the procedure of Brown and Smith [16] was carried out to determine the position of the cut site. The results are shown in Figure 2. The ^{35}S-labelled product from the primer extension has been cut by *Tsp*4C I to give a polynucleotide that migrates in the sequencing gel to a position level with the second C base in the sequence 5′-ACCGT. This would produce a single 3′-base (C) overhang on the labelled strand of the double-stranded product, which should subsequently be removed by the action of T4 polymerase. Figure 3 confirms such a predicted result.

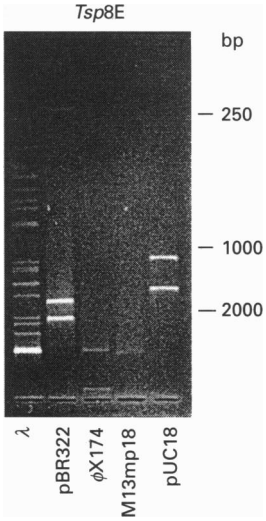

Figure 3 Digestion of 5 DNA substrates by *Tsp*8E I

A 1 μg amount of each substrate was incubated with 20 units of *Tsp*8E I for 4 h at 65 °C. Reaction products were separated by electrophoresis in a 1.4% agarose gel. The sizes of the DNA fragments (bp) were measured by reference to calibration markers.

From the results of these experiments we deduce that the recognition site for the restriction endonuclease *Tsp*4C I is ACNGT (where N can be any of of the four bases in DNA), and that the enzyme cuts both strands of the DNA substrate between the third and fourth bases within the recognition sequence:

5′-A C N↓G T-3′

3′-T G↑N C A-5′

*Tsp*4C I is a novel Type II restriction endonuclease, present in an isolate of the genus *Thermus* from Iceland and possessing high thermal stability.

Restriction endonuclease from *Thermus* isolate 8E, *Tsp*8E I

*Tsp*8E I was partially purified following the protocol described in the Experimental section. The enzyme was eluted from the phosphocellulose column by 0.85 M KCl and from the DEAE-Sephacel column by 0.2 M NaCl. The yield from 3 g of cell pellet was 1300 units (Table 1).

The results of experiments designed to establish the optimum pH, Mg, NaCl and KCl requirements and heat stability are given in Table 2. The fragment patterns generated by *Tsp*8E I with five DNA substrates are shown in Figure 3. It is apparent from Figure 3 that *Tsp*8E I has produced fragments that are both fewer in number and larger in size than those produced by *Tsp*4C I (Figure 1). In particular, *Tsp*8E I failed to cut φX174 and only cut the circular M13mp18 DNA at one site, generating a single linear polynucleotide of 7250 bp. These results indicate that the recognition site for *Tsp*8E I occurs far less frequently within the five substrates tested than does the site for *Tsp*4C I. Reference to Fuchs' tables [15] failed to identify any possible 4, 5 or 6 base palindromes that correspond to the recognition site of *Tsp*8E I. However, when the fragment patterns produced by *Tsp*8E I were compared with the computer-generated patterns of 147 known restriction endonucleases [14] there was found to be very close

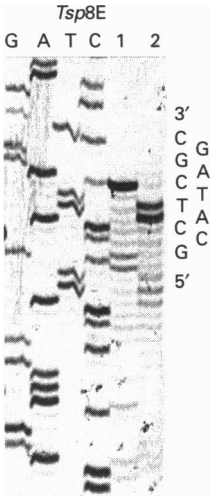

Figure 4 DNA sequencing gel to determine the location of the cut site of *Tsp*8E I

Full details are given in the Experimental and Results sections. The dideoxy sequencing ladder can be seen in lanes G, A, T and C. *Tsp*8E I digest of the labelled primer extension before (lane 1) and after (lane 2) incubation with T4 DNA polymerase.

agreement between the patterns observed for *Tsp*8E and those predicted for the well-characterized restriction endonuclease *Bgl* I [17,18], a commercially available restriction endonuclease from the mesophilic bacterium *Bacillus globigii*. This enzyme recognizes the sequence GCCNNNNNGGC, cutting the DNA between the 7th and 8th bases of the 11 nucleotide sequence. When the experimentally observed sizes of the major fragments produced by *Tsp*8E I acting on the four substrates, pBR322, pUC18, M13mp18 and ϕX174, were compared with those predicted for a restriction endonuclease recognizing the sequence GCCNNNNNGGC, the agreement was found to be extremely close.

The recognition site for *Tsp*8E I was confirmed during the course of the experiment designed to locate the position of the cut site using the method of Brown and Smith [16], using a synthetic 24-mer primer (see Experimental section) designed to bind to the single-stranded M13mp18 template 73 nucleotides 3′ of an isolated putative *Tsp*8E I site GCCTGAATGGC within M13 (6330–6340). The results are shown in Figure 4. The labelled product has been cut by *Tsp*8E to produce a single polynucleotide level with the 7th base of the recognition sequence in the sequencing ladder (C). This would produce a double-stranded restriction endonuclease product with a 3-base 3′ overhang on the labelled strand, which should subsequently be trimmed back by three bases due to the 3 → 5 nuclease activity of T4 polymerase. This prediction is confirmed by the result shown in Figure 4, although it can be seen from lane 2 that under the conditions used in this experiment, T4 DNA polymerase has not fully trimmed all three bases from every molecule of the *Tsp*8E I digest (lane 1), there being a small proportion of the digest in which the reaction has not gone to completion. We conclude from these results that *Tsp*8E I is a Type II restriction endonuclease recognizing the sequence GCCNNNNNGGC and cutting both strands of DNA substrates thus:

5′-G C C N N N N↓N G G C-3′

3′-C G G N↑N N N N C C G-5′

Such a specificity is identical with that of *Bgl* I. *Tsp*8E I differs from *Bgl* I in that it has the property of extreme thermal stability and can be used to digest DNA at temperatures that would irreversibly denature *Bgl* I.

One advantage of restriction endonucleases that retain full activity at high temperatures is that they can be used to cut DNA under conditions that inhibit the formation of secondary structures within the DNA molecule. An additional potential use for thermally stable restriction endonucleases is in conjunction with thermally stable DNA ligases in the ligase chain reaction [19].

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