Microbiology (1998), **144**, 167–175 Printed in Great Britain

Isoschizomers of the restriction endonuclease *Taq*I (T/CGA) requiring different metal ion concentrations and having a range of thermal stabilities from *Thermus* species from different continents

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One-hundred-and-fifty-two isolates of the genus *Thermus*, collected from hot springs on four continents, were screened for evidence of the presence of the thermophilic Type II restriction endonuclease *Taq*I (T/CGA). The presence of isoschizomers of *Taq*I in 27 of the isolates, originating from hot springs in New Zealand, Iceland, USA, Japan, mainland Portugal and the island of São Miguel in the Azores, is reported. Six of the *Taq*I-containing isolates from diverse geographical locations, identified by means of DNA/DNA homology and 16S rRNA sequence alignment as belonging to the *Thermus* species *T. aquaticus*, *T. filiformis*, *T. thermophilus*, *T. scotoductus* and *T. brockianus*, were selected for comparative studies. The *Taq*I isoschizomers from each of the six isolates were partially purified. They differed in their magnesium ion requirements, isoelectric points, subunit molecular masses and thermal stability.

Keywords: *Taq*I, *Thermus*, thermostable, restriction endonuclease

INTRODUCTION

Since the discovery of type II restriction endonuclease activity in *Escherichia coli* (Meselson & Yuan, 1968) and *Haemophilus influenzae* (Smith & Wilcox, 1970) thousands of microbial strains have been screened for other site-specific endonucleases. Restriction enzymes have provided the molecular biologist with tools for the analysis, rearrangement, cloning and sequencing of DNA. At least 2900 bacterial restriction endonucleases have been described and this number is continually increasing (Rebase release 708, July 1997). Out of these, only some 250 have different and unique specificities and there are many short nucleotide sequences for which no site-specific enzyme has yet been discovered. Many of the enzymes described are isoschizomers, although they may be from different genera or species than the prototypes. Enzymes that share the same recognition sequence, but cleave differently in relation to it (neoschizomers), are more uncommon. The search for restriction endonucleases from a wide range of genera and species continues to produce enzymes with novel recognition and cleavage sites, thereby expanding the repertoire of the molecular biologist's tool kit. Most of these enzymes have been isolated from mesophilic bacteria, but although stable below 45 °C, they are usually denatured quickly at higher temperatures. Some restriction endonucleases have high thermal stability and activity at high temperatures which are potentially useful properties for applications that require restriction endonucleases to be incorporated into PCR. Thermostable site-specific endonucleases have been discovered, particularly amongst strains of *Thermus* and thermophilic bacilli, and more recently a few restriction enzymes have been discovered amongst the thermophilic Archaea (Rebase release 708, July 1997).

Strains of the genus *Thermus* (Brock & Freeze, 1969) are aerobic, non-sporulating heterotrophic rods with optimum growth temperatures in the region of 70 °C that occur in neutral and alkaline hot springs world-wide (Williams & da Costa, 1992). Valid species of the genus include *T. aquaticus* (Brock & Freeze, 1969), *T. thermophilus* (Oshima & Imahori, 1974; Williams *et al.*, 1995), *T. filiformis* (Hudson *et al.*, 1987; Georganta *et al.*, 1993), *T. brockianus* (Williams *et al.*, 1995), *T. scotoductus* (Kristjansson *et al.*, 1994) and *T. oshimai* (Williams *et al.*, 1996), but the variability of phenotype amongst strains often renders the identification of species problematical (Williams & Sharp, 1995). Strains

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of *Thermus ruber* are quite different to the yellow- and fawn-coloured strains (Williams & da Costa, 1992; Williams & Sharp, 1995) and have now been reclassified as *Meiothermus ruber* (Nobre *et al.*, 1996). *Taq*I activity has not been reported among these red strains, which are therefore not considered further here.

The first thermostable restriction endonuclease to be reported was *Taq*I from the North American *T. aquaticus* type strain YT1 (Sato *et al.*, 1977). This enzyme recognizes the tetranucleotide TCGA and cleaves dsDNA between the T and C (T/CGA), producing staggered ends with a 5′ 2-base overhang. The corresponding enzyme *Tth*HB8I from the Japanese isolate *T. thermophilus* HB8 (Sato & Shinomiya, 1978) is similar in its structure, properties and gene organization to *Taq*I. We have found that isoschizomers of *Taq*I occur in most *Thermus* species, but do not occur in more than a minority of strains of any given species. The *Taq*I gene sequence (Barany *et al.*, 1992b) comprises 789 bp, translating to give a polypeptide of 263 aa (M_r 31600). The active form of the enzyme is a dimer composed of two identical polypeptide subunits. The mean base composition of the *Taq*I and *Tth*HB8I genes (53 and 51% G+C, respectively) are over 10% lower than estimates of the mean base composition of the rest of the genome (Barany *et al.*, 1992a), which may indicate that these genes originated in another taxon, but isoschizomers are yet to be discovered outside the genus.

Since the discovery of *Taq*I, a further 22 Type II restriction endonucleases with different recognition sequences have been discovered in the genus *Thermus* (Rebase release 708, July 1997). Many of these are thermostable isoschizomers of enzymes previously discovered in mesophilic bacteria, but several are novel (Table 1). We have screened 152 isolates of the genus *Thermus* collected from hot springs on four continents for isoschizomers of *Taq*I. We report here the partial purification and comparison of six such isoschizomers from isolates obtained from geographic sites as far apart as possible. This enzyme is presumably inessential to the bacterium and may represent an example of horizontal gene transfer in strains of *Thermus*. If so it should have been able to diverge without the constraints placed on enzymes that are essential to the economy of the cell. Comparison of the sequences of such diverse variants of the same enzyme will be useful in the engineering of these enzymes towards even greater thermal stability.

METHODS

Materials. DNA substrates λcI857Sam7 phage DNA (unmethylated), pBR322, pUC18, M13mp18 and φX174 were all from NBL Gene Sciences. SV40 virus DNA, *Hin*dIII-digested λ DNA and the 123-bp DNA ladder were from Sigma and adenovirus Type 2 DNA was from Gibco-BRL.

Bacterial cultures. Cells were grown overnight at 65 °C in 500 ml shake-flask cultures containing 0·3% tryptone (BDH), 0·1% yeast extract (BDH) and 10% (v/v) Castenholtz mineral salts (Ramaley & Hixson, 1970), centrifuged at 6000 ***g*** for 30 min and stored at −20 °C.

Screening bacterial isolates for restriction endonuclease activity. Cell pellets (3 g wet wt) were thawed, resuspended in 10 ml 20 mM Tris/HCl, 0·1 mM EDTA, 2 mM DTT, 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 1:5 and 1:30, respectively. Each dilution (2 μl) was incubated with 12 μl ‘One-Phor-All’ buffer (Pharmacia) containing 0·25 μg λ DNA at 65 °C for 1 h. Reactions were terminated by the addition of 3 μl stop buffer (4 g sucrose, 10 mg bromophenol blue and 740 mg EDTA in 10 ml 0·25 M Tris/HCl, pH 8·0). Samples were electrophoresed in 1·4% agarose gels for 2 h in TBE buffer, pH 8·3 (Sambrook *et al.*, 1989) containing ethidium bromide (0·5 μg ml^{-1}). DNA fragments were detected by UV transillumination and photographed.

Partial purification of *Taq*I isoschizomers. Cell-free extracts (60 ml) from 8·0–14·0 g wet weight of frozen cells were adjusted to 0·2 M NaCl, 1·8 ml 10% (v/v) polyethyleneimine (pH 7·5) was added to each extract with stirring and the suspensions were centrifuged for 1 h at 16000 ***g***. The supernatants were adjusted to 70% saturation by the addition of solid ammonium sulphate and the precipitates were collected by centrifugation at 16000 **g** for 1 h. The pellets were resuspended in 5 ml 20 mM K_2HPO_4, 1 mM EDTA, 5 mM 2-mercaptoethanol, 4% (v/v) glycerol, pH 7·5, and dialysed against 800 ml of the same buffer for 16 h. The samples were applied to phosphocellulose (P11, Whatman) columns (10×1·5 cm) and eluted in a step-wise fashion with 20 ml aliquots of the same buffer containing 0·1, 0·2, 0·35, 0·5, 0·6, 0·75 or 0·9 M NaCl. Fractions were tested for restriction endonuclease activity and those containing the peak of enzyme activity were pooled and dialysed for 16 h against 10 mM Tris/HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, 5% glycerol, pH 7·5. The samples were applied to DEAE-Sephacel columns (10×1·5 cm) and eluted in a step-wise fashion with 20 ml aliquots of the same buffer containing 0·05, 0·1, 0·25, 0·4, 0·55 or 0·7 M NaCl. Fractions containing the peak of each restriction endonuclease activity were pooled, concentrated by pressure filtration, applied to gel filtration columns (Sephadex G-150, 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^{-1}. The fractions containing the peak of enzyme activity were pooled, concentrated by pressure filtration to 0·5 ml and 50% glycerol was added prior to storage at −20 °C. One unit of enzyme activity is defined as the amount of restriction endonuclease required to totally digest 1 μg phage λ DNA in 1 h at 65 °C in a reaction volume of 50 μl.

Purification of *Taq*I (*T. aquaticus* YT1) restriction endonuclease cloned in *E. coli*. Plasmid pTIRM116 was used to transform competent cells of *E. coli* strain XL1-MlRF (both supplied by Dr P. Eastlake, NBL Gene Sciences), colonies of which were selected from LB plates containing ampicillin and tetracycline and grown in LB broth containing both antibiotics. A cell-free extract was prepared from 50 g *E. coli* cells resuspended in 100 ml 50 mM Tris/HCl, pH 8·0, 200 mM NaCl, 1 mM EDTA, 7 mM 2-mercaptoethanol, 0·2 mM PMSF containing 0·1 mg lysozyme ml^{-1} at 4 °C and the suspension was sonicated 6×1 min with a Branson 10 mm probe at at least 60% maximum power in an ice bath. Following centrifugation, the cell extract was adjusted to 0·5 M NaCl and polyethyleneimine added to 0·2%, stirred for 30 min and centrifuged at 10000 ***g*** for 1 h. The supernatant was adjusted to 75% saturation with ammonium sulphate and the precipitate was collected by centrifugation at 10000 ***g*** for 1 h. The pellet was resuspended in 50 ml 20 mM potassium phosphate, pH 7·5, 5 mM 2-mercaptoethanol, 1 mM EDTA, 4% glycerol

Table 1. Restriction endonucleases isolated from yellow- and fawn-pigmented strains of *Thermus*

Enzyme	Recognition sequence and cleavage site	*Thermus* species or strain	Mesophilic isoschizomer
*Taq*I	T/CGA	*T. aquaticus* YT1	None
*Tsp*E1	/AATT	*Thermus* sp.	None
*Taq*52I	G/CWGC	*T. aquaticus*	None
*Tau*I	GCSGC	*T. aquaticus*	None
*Tfi*I	G/AWTC	*T. filiformis*	None
*Tsp*II	ACTGGN/	*Thermus* sp.	None
*Tat*I	WGTACW	*T. aquaticus*	None
*Tsp*45I	/GTSAC	*T. brockianus*	None
*Tth*111I	CAARCA(17,–9)	*T. thermophilus*	None
*Tsp*4C1	ACN/GT	*Thermus* sp.	None
*Taq*II	GACCGA or CACCCA	*T. aquaticus* YT1	None
*Tth*111II	GACN/NNGTC	*T. thermophilus*	None
*Tsp*R1	CASTGNN/	*Thermus* sp.	None
*Tsp*133I	GATC	*Thermus* sp.	*Mbo*I
*Tsp*11	ACTGG(1/–1)	*Thermus* sp.	*Bsr*I
*Taq*X1	CC/WGG	*T. scotoductus*	*Eco*RII
*Tsp*301I	GGWCC	*Thermus* sp.	*Ava*II
*Tsp*560I	GGCC	*Thermus* sp.	*Hae*III
*Tsp*49I	ACGT/	*Thermus* sp.	*Mae*II (neoschizomer)
*Tse*A1	GDGCHC	*Thermus* sp.	*Bsp*1286I
*Ttm*II	GCGCGC	*T. thermophilus*	*Pau*I
*Tsp*504I	CGGCCG	*Thermus* sp.	*Eag*I
*Tsp*507I	TCCGGA	*Thermus* sp.	*Bsp*E1
*Tsp*8E1	GCCNNNN/NGGC	*Thermus* sp.	*Bgl*I
*Tse*D1	RCCGGY	*Thermus* sp.	*Cfr*10I
*Tsp*273I	GATATC	*Thermus* sp.	*Eco*RV
*Tse*A1	GDGCHC	*Thermus* sp.	*Sdu*I

(PCB) and heated for 8 min at 60 °C to denature the bulk of the soluble *E. coli* proteins. Precipitated protein was removed by centrifugation and the supernatant chromatographed on phosphocellulose (P11 Whatman) in PCB containing 50 mM KCl. The column was developed with a KCl gradient (0·05–1·0 M) and fractions containing *Taq*I activity were pooled and dialysed against 20 mM Tris/HCl, pH 7·5, 1 mM EDTA, 5 mM 2-mercaptoethanol, 5 % glycerol, 50 mM NaCl. The sample was then chromatographed on DEAE-Sephacel and eluted with a NaCl gradient (0·05–1·0 M). Active fractions were pooled, concentrated by pressure filtration and chromatographed on Sephadex G-200. Fractions containing *Taq*I activity were pooled, concentrated and stored at −20 °C in 50 mM Tris/HCl, pH 7·5, 0·5 mM 2-mercaptoethanol, 50 % glycerol.

Raising an antiserum to cloned *Taq*I. A polyclonal antiserum to the purified cloned *Taq*I (from strain YT1) was raised in an adult New Zealand White rabbit. On days 1 and 12, 0·15 mg protein in Freund's incomplete adjuvant was injected subcutaneously at four sites. On day 34, 0·15 mg protein in 0·15 M NaCl was injected intravenously. On days 43 and 60, 20 ml blood was collected and on day 70 the rabbit was exsanguinated by cardiac puncture. The serum was pooled and the IgG fraction purified by Na_2SO_4 fractionation and DEAE-Sephacel chromatography. SDS-PAGE, non-denaturing PAGE, dot blotting and Western blotting were carried out using standard procedures (Sambrook *et al.*, 1989). Methods used to determine the molecular masses, pH optima, thermal stability and requirements for magnesium, sodium and potassium ions for the six *Taq*I isoschizomers are described in Results and Discussion.

Isolation of DNA. DNA was purified from frozen wet cells as described by Williams *et al.* (1995).

PCR of 16S rDNA. Each amplification comprised 10–100 ng template DNA, 20 pmol each primer (6F, GAGTTTGATC-CTGGCTCAG; 1541R, AAGGAGGTGATCCAGCC), 125 µmol each dNTP l^{-1}, 10 µl buffer (10 × PCR buffer with magnesium: 200 mM Tris/HCl, pH 8·4, 500 mM KCl, 15 mM $MgCl_2$), 1 unit of *Taq* DNA polymerase and sterile water to 100 µl final volume. The thermal programme comprised 30 cycles of 95 °C for 40 s, 55 °C for 40 s and 72 °C for 2 min. The last cycle was 6 min at 72 °C.

Determination of sequence of 16S rDNA. The PCR products (5 µl) were treated with 1 µl each of exonuclease I and shrimp alkaline phosphatase and incubated at 37 °C for 15 min, then at 80 °C for 15 min. The sequence was determined using dITP termination master mix and [α-^{33}P]ddNTPs of the thermo-Sequenase radiolabelled terminator cycle sequencing kit (Amersham) with 1·3 µl DNA template (50–500 ng) and 2 pmol 6F primer. The thermocycler (Appligene) was programmed for 45 cycles of 95 °C for 30 s, 50 °C for 30 s and extension at 60 °C for 8 min. Stop solution (4 µl) was added to the tubes which were heated to 70 °C for 10 min immediately

before loading. Nucleotide fragments were separated on 21 × 50 cm gradient polyacrylamide gels at 55 °C in a Bio-Rad SequiGen Sequencing Cell at 2500 V per gel for 2–4 h.

RESULTS AND DISCUSSION

Survey of restriction endonuclease activity in *Thermus* isolates

Of the 152 *Thermus* isolates tested, 62 (40 %) showed restriction endonuclease activity in either or both of the cell-free extract dilutions incubated with phage λ DNA. Nine of the isolates generated restriction fragment patterns (with multiple substrates) that have not been identified with any previously reported Type II restriction endonuclease and the identification of the recognition and cleavage sites of these enzymes is in progress. A further 26 samples were found to contain restriction endonuclease activity that was identified as either enzyme specificities previously found in *Thermus* or as novel thermophilic restriction endonucleases that have been isolated in our laboratory previously (Raven *et al*., 1993a, b; Welch & Williams, 1995a, b, c, 1996). The remaining 27 isolates had activity like *Taq*I as demonstrated by the specific fragment patterns generated from bacteriophage λ DNA, pBR322 and M13mp18RF. The 27 *Taq*I-containing *Thermus* isolates originated from hot springs in New Zealand (3), Iceland (1), Yellowstone National Park, USA (12), Japan (2), mainland Portugal (6) and the island of São Miguel in the Azores (3). We selected one isolate from each of these geographical locations (Table 2) for the purpose of this comparative study.

Partially purified *Taq*I isoschizomers from *Thermus* isolates

The *Taq*I isoschizomers were partially purified from the selected *Thermus* isolates but the enzyme yields and specific activities varied considerably (Table 2). Stocks comprising 90 % of each of the partially purified *Taq*I isoschizomers were stored at −20 °C in the presence of 50 % glycerol and 500 μg BSA ml^{-1} to preserve catalytic activity for subsequent enzyme characterization and comparative studies. The remaining 10 % of each enzyme was dialysed to remove all traces of glycerol and salt ions and used for electrophoretic and immunological studies.

Electrophoretic, chromatographic and immunological studies of the enzyme proteins

When the partially purified *Taq*I isoschizomers were subjected to SDS-PAGE (12 %) and stained with Coomassie blue (Fig. 1), none of the preparations showed just a single protein band. This is not surprising considering the relatively simple purification protocols that are required to produce functionally pure restriction enzymes suitable for experimental use and commercial production. Nevertheless, the isoschizomer preparations (particularly D and E) with the least complex patterns of total protein were the ones that had the highest specific activities (Table 2). To confirm the location of the *Taq*I isoschizomers of samples D and E in the gels and to locate the *Taq*I polypeptide amongst the more complex SDS-PAGE patterns of samples A, B, C and F, immunoblotting was used employing the polyclonal IgG to *Taq*I. Dot blotting confirmed that the antibody reacted with all six isoschizomers of *Taq*I, but not with a range of mesophilic restriction endonucleases (*Bam*HI, *Eco*RI, *Hin*dIII, *Mse*I and *Sty*I) or a selection of thermostable restriction endonucleases from other *Thermus* isolates (*Tsp*EI, *Tsp*4CI, *Tsp*49I and *Tsp*52I) (data not shown). In view of this degree of specificity of the IgG raised against *Taq*I, we subjected the six isoschizomers to SDS-PAGE followed by Western blotting using a peroxidase-conjugated goat anti-rabbit IgG as the second antibody. The results indicated that all six isoschizomers have similar, but not identical, subunit molecular masses in the range 29–31 kDa (Fig. 1). The *Taq*I isoschizomer C from the Japanese isolate GK24 showed an additional immunoreactive polypeptide at 25 kDa. We do not have direct evidence that both these polypeptides, in the absence of SDS, have enzyme activity, although the results of the pH optima experiments suggest that this might be the case. The smaller polypeptide may represent a hydrolysis product of the 29–31 kDa protein.

The molecular masses of the native enzymes were estimated by gel filtration chromatography on a 100 × 1·5 cm Sephadex G-150 column calibrated with protein markers and eluted with 50 mM Tris/HCl, 50 mM

Table 2. Strain, species, geographic origin and specific activity of the isoschizomers

*Taq*I code	*Thermus* isolate	*Thermus* species	Geographic origin	Recovery after Sephadex G-150		Specific activity (units mg^{-1})
				Protein (mg)	Activity at 65 °C (units)	
A	YSS20	*T. aquaticus*	USA	0·21	3500	16600
B	Ork2A3	*T. filiformis*	New Zealand	0·53	1750	3300
C	GK24	*T. thermophilus*	Japan	0·24	700	2900
D	Vi4a	*T. scotoductus*	Portugal	0·05	3500	70000
E	JK51	*T. brockianus*	Iceland	0·07	7000	100000
F	RQ1	*T. thermophilus*	Azores	0·49	700	1430

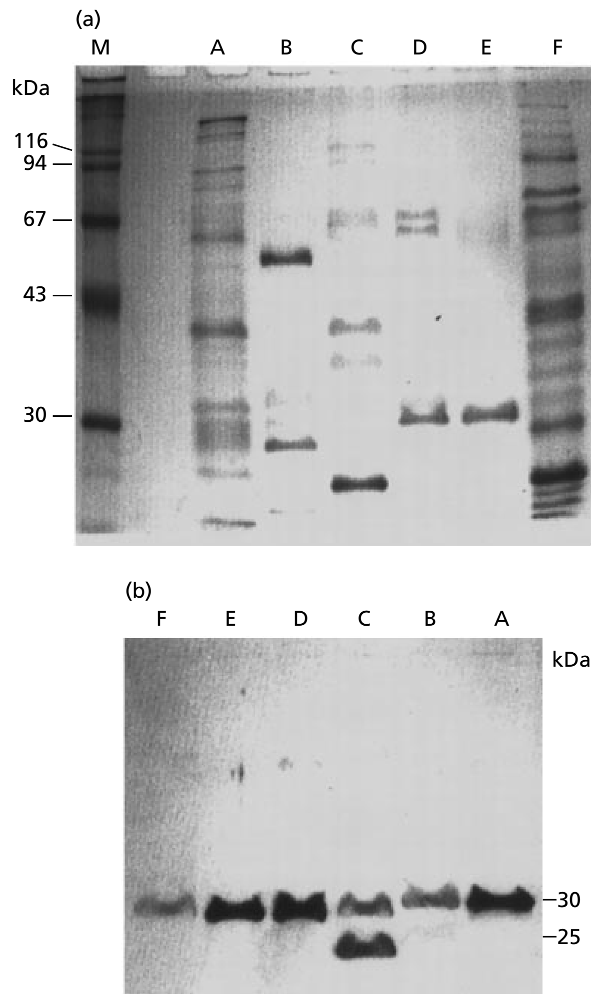

Fig. 1. 1. SDS-PAGE (14 %) electrophoresis of partially purified *Taq*I isoschizomers A–F. (a) Gel stained with Coomassie blue. Lane M, molecular mass markers. (b) Western blot using rabbit anti-*Taq*I IgG primary antibody and peroxidase-conjugated secondary antibody.

NaCl, 5 % glycerol, pH 7·5. All six isoschizomers were found to have molecular masses within the range 55–60 kDa, corresponding to active dimers of the subunits detected on SDS-PAGE. We have not determined the isoelectric points of the partially pure *Taq*I isoschizomers directly, but indirect evidence from ion-exchange chromatography indicates significant differences. The ionic strengths of the salts required to elute the isoschizomers from phosphocellulose and DEAE-Sephacel varied widely (Table 3). They do not correspond for each enzyme, presumably because the DEAE acts as an anion exchange column but the phosphocellulose may act partly as a cation exchanger and partly as an affinity matrix.

Comparison of the optimum conditions for enzyme activity

For all experiments the six *Taq*I isoschizomer solutions were diluted or concentrated, as appropriate, to produce solutions of approximately equal enzyme activity (2·0 units μl^{-1}). To determine the optimum conditions for activity, 0·8 units of enzyme were incubated with 1·0 μg bacteriophage λ DNA for 1 h at 65 °C. This was calculated to be insufficient enzyme to cleave at all the TCGA sites and so give extensive but recognizably incomplete digests on agarose gel electrophoresis. The use of slightly insufficient enzyme for complete digestion allowed the recognition of both increased and decreased hydrolysis as the reaction components were altered. When the approximate optimum conditions were identified for each isoschizomer, the experiments were repeated with enzyme carefully titrated to 0·8 units at those conditions.

To determine the optimum Mg^{2+} ion concentration, incubations were carried out in 50 mM Tris/HCl, 50 mM NaCl, pH 9·0, at $MgCl_2$ concentrations from 0 to 12 mM. It was possible to estimate the minimum Mg^{2+} ion concentration required for maximum activity

Table 3. Optimum activity criteria, elution conditions and thermal stability of the six *Taq*I isoschizomers

*Taq*I code	pH optimum	Optimum Mg^{2+} (mM)	Optimum Na^+ (mM)	Thermal stability (°C)*	KCl concn required to elute the enzyme from the P11 column (M)	NaCl concn required to elute the enzyme from the DEAE-Sephacel column (M)
A	9·5–10·0	1·0†	75	73	0·35	0·25
B	9·5–10·0	2·5	75	84	0·5	0
C	9·5–10·0 and 8·0–8·5	1·0†	75	68	0·35	0·25
D	9·5–10·0	2·5	75	73	0·6	0·1
E	9·5–10·0	2·0	75	75	0·5	0·1
F	9·5–10·0	2·0	75	78	0·35	0·25

* Maximum temperature that the enzyme can stand for 5 min without loss of activity.

† Isoschizomers A and C show evidence of partial inhibition of activity at Mg^{2+} concentrations above 5 mM.

Table 4. Sequences of 16S rRNA used to identify species of *Thermus*

Upper case letters indicate base-paired nucleotides, lower case letters indicate nucleotides that are not base-paired. Spaces are used to emphasize the various segments of the sequences and '-' represents a missing nucleotide requiring a space to maximize the alignment. Above the sequences, nucleotides with the same numeral are generally base-paired with one another although individual sequences may have bases that do not pair. Loops at the ends of helices are indicated by '/'.

(a)	Region 5		Helix 6		Bulge 7
		222222222222222222	///	222222222222222222	
T. filiformis					
Wai33A1	uaagacaugcaag	UCGUGCgGGCUGCGGGGU	uuu	ACUCCGUGGUCaGCGgCGG	acggguga
Ork2A3	uaagacaugcaag	UCGUGCgGGCUGCGGGGU	uuu	ACUUCGCGGUCaGCGgCGG	acggguga
T. brockianus					
YS38	uaagacaugcaag	UCGGGCgGGCCAUGGGGU	uuu	ACUCCGUGGUCaGCGgCGG	acggguga
SM32	uaagacaugcaag	UCGGGCgGGCCACGGGGU	uuu	ACUCAGUGGUCaGCGgCGG	acggguga
JK51	uaagacaugcaag	UCGGGCgGGCCUCGGGGU	uuu	ACUCUGUGGUCaGCGgCGG	acggguga
T. scotoductus					
Vi7	uaagacaugcaag	UCGaGCgGGGCA---GGU	uuau	GCC--GUgUCCaGCggCGG	acggguga
Vi4a	uaagacaugcaag	UCGUGCgGGGCa---GGU	uuau	ACC---UGUCCaGCGgCGG	acggguga
T. aquaticus					
YT1	uaagacaugcaag	UCGUGCgGGCCGUgGGG-	uau	-UCUCACGGUCaGCGgCGG	acggguga
YS20	uaagacauggaag	UCGUGCgGGCCGUgGGU-	uau	-UCUCACGGUCaGCGgCGG	acggguga
T. thermophilus					
HB8	uaagacaugcaag	UCGUGCgGGCCGCGGGGU	uuu	ACUCCGUGGUCaGCGgCGG	acggguga
T. oshimai					
SPS14	uaagacaugcaag	UCGUGCgGGG--UGG---	uucg	---CCA--CCCaGCGgCGG	acggguga

(b)	Stem 8				Helix 9				Bulge
		111111	---	222222	////	222222	----	111111	
T. filiformis									
Wai33A1	cuggaaga	GGGGGA	caa	CCUGGG	gaaa	CUCGGG	cuaa	UCCCCC	aug
Ork2A3	ccggaaga	GGGGGA	caa	CCUGGG	gaaa	CUCGGG	cuaa	UCCCCC	aug
T. brockianus									
YS38	ccggaagu	GUGGGA	caa	CCCGGG	gaaa	CUCGGG	cuaa	UCCCGC	aug
SM32	ccggaagu	GUGGGA	caa	CCCGGG	gaaa	CUCGGG	cuaa	UCCCGC	aug
JK51	ccggaagu	GUGGGA	caa	CCCGGG	gaaa	CUCGGG	cuaa	UCCCGC	aug
T. scotoductus									
Vi7	ccggaaga	GGCGGA	caa	CCUGGG	gaaa	CCCAGG	cuaa	UCCGCC	aug
Vi4a	ccggaaga	GGCGGA	caa	CCUGGG	gaaa	CCCAGG	cuaa	UCCGCC	aug
T. aquaticus									
YT1	ccggaaga	GGGGGA	caa	CAUGGG	gaaa	CCCAGG	cuaa	UCCCCC	aug
YS20	ccggaaga	GGGGGA	caa	CCUGGG	gaaa	CCCAGG	cuaa	UCCCCC	aug
T. thermophilus									
HB8	ccggaaga	GGGGGA	caa	CCCGGG	gaaa	CUCGGG	cuaa	UCCCCC	aug
T. oshimai									
SPS14	ccggaagu	GGGGGA	caa	CCCGGG	gaaa	CCCGGG	cuaa	UCCCCC	aug

(c)	Stem 8		Helix 10		Bulge		Helix 11			Opposite stem 8
	3333	222222222	//	222222222		1111	/////	1111		3333
T. filiformis										
Wai33A1	GuGg	UCGUGCCCU	uu	GGGGUGCGA	uuaaa	GGGU	gaaga	GCCC	g	gUuC
Ork2A3	GuGG	UCGUGCCCU	uu	GGGGUGCGA	uuaaa	GGGU	gaaga	GCCC	g	CUuC
T. brockianus										
YS38	GuGG	UCAUGUCCU	gu	GGGGCAUGA	uuaaa	GGGC	gaaa-	GUCC	g	CUuC

Table 4 (cont.)

(c)	Stem 8	Helix 10			Bulge	Helix 11				Opposite stem 8
	3333	222222222	//	222222222		1111	/////	1111		3333
SM32	GuGG	UCAUGUCCU	gu	GGGGCAUGA	uuaaa	GGGC	cagg-	GUCU	C	gUuC
JK51	GuGG	UCAUGUCCU	gu	GGGGCAUGA	uuaaa	GGGC	gagg-	GUCU	g	CUuC
T. scotoductus										
Vi7	GuGG	UCCUGUCCU	gu	GGGGUAGGA	cuaaa	GGGU	gaaua	GCCC	g	CUuC
Vi4a	GuGG	UCCUGUCCU	gu	GGGGCAGGA	cuaaa	GGGU	gaaua	GCCC	g	CUuC
T. aquaticus										
YT1	GuGG	ACACAUCCU	gu	GGGGUGUGU	uuaaa	GGGU	-uuu-	GCCC	g	CUuC
YS20	GuGG	ACACAUCCU	gu	GGGGUGUGU	uuaaa	GGGU	-uuu-	GCCC	g	CUuC
T. thermophilus										
HB8	GuGG	ACCCGCCCC	uu	GGGGUGUGU	ccaaa	GGGC	-uuu-	GCCC	g	CUuC
T. oshimai										
SPS14	GuGG	UCCGGCCCC	-u	GGGCCGUGA	cuaaa	GGcC	-aaaa	G-CC	g	CUuC

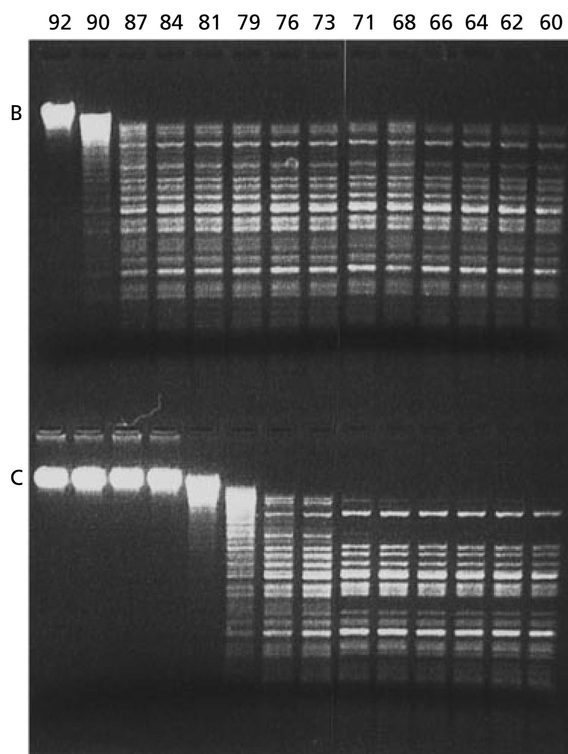

Fig. 2. Thermal stability of isoschizomers B and C. Each enzyme (0·8 units) was incubated in reaction buffer for 5 min at the temperatures (°C) shown. Residual enzyme activity was estimated following incubation of the enzyme with 1·0 µg bacteriophage λ DNA for 1 h at 65 °C and agarose gel electrophoresis of the resulting restriction enzyme digest.

and also the maximum concentration of Mg^{2+} ions that did not inhibit the enzyme (Table 3). In the absence of Mg^{2+} no hydrolysis was detected. All six isoschizomers required similar surprisingly low concentrations of Mg^{2+} for full activity and isoschizomers A and C were sensitive to inhibition at Mg^{2+} concentrations greater than 5 mM.

The optimum pH for catalytic activity was estimated by incubating bacteriophage λ DNA in 50 mM Tris/HCl, 50 mM NaCl, 3 mM $MgCl_2$ at pH 7·5–10·5. All six isoschizomers showed broad pH optima with maximum activity in the range pH 9·5–10·0 (Table 3). Enzyme C also showed evidence of a second pH optimum in the region of pH 8·0. In view of the SDS-PAGE Western blot results for this isoschizomer (two bands at 30 and 25 kDa), the double pH optima support the possibility of two active isoenzymes of *Taq*I from this Japanese *Thermus* isolate.

Na^+ ion requirements were determined with bacteriophage λ DNA in 50 mM Tris/HCl, 3 mM $MgCl_2$, pH 9·5, at 25 °C, at NaCl concentrations from 0 to 300 mM. All six enzymes were indistinguishable, showing about 20 % maximum activity in the absence of NaCl, and requiring 75 mM NaCl for full activity. At NaCl concentrations above 250 mM all six isoschizomers were totally inhibited. The requirement was for Na^+ rather than Cl^- ions since either 37·5 mM Na_2SO_4 or 75 mM $NaNO_3$ could replace NaCl. Using other salts at 75 mM with respect to Cl^-, the activity of all six isoschizomers was as follows: NaCl, 100 %; KCl, 100 %; LiCl, 100 %; $BaCl_2$, 80 % and $CaCl_2$, 40 %.

Comparison of thermal stability and substrate specificity for the isoschizomers

The ability of the six isoschizomers to withstand heat inactivation was determined by incubating 0·8 units of enzyme in 20 µl 50 mM Tris/HCl, 75 mM NaCl, 3 mM $MgCl_2$, pH 9·5, for 5 min in a precisely thermostatted water bath at 60–92 °C. The solutions were rapidly cooled in melting ice and following the addition of 1·0 µg phage λ DNA, incubated for 1 h at 65 °C. The restriction digests were examined by agarose gel electrophoresis. Thermal stability was estimated as the maximum

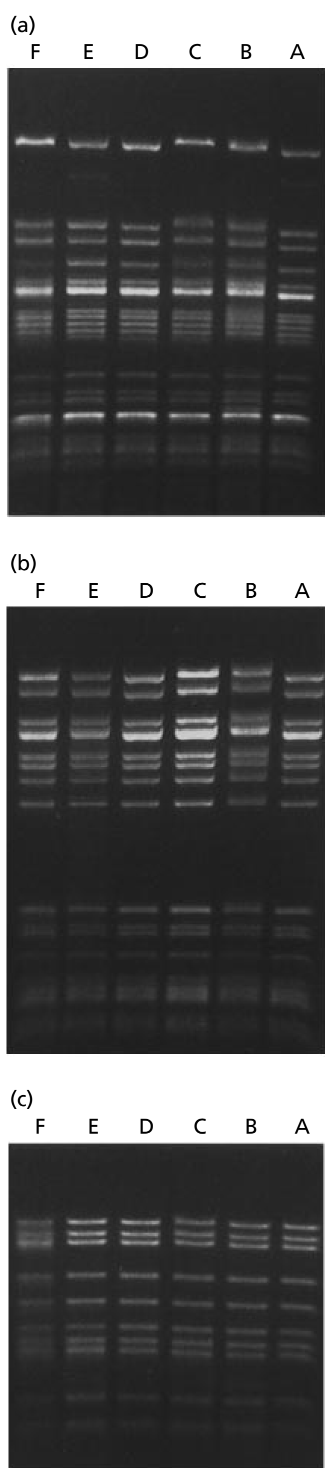

Fig. 3. Agarose gel electrophoresis of restriction digests by isoschizomers A–F of bacteriophage λ DNA (a), adenovirus Type 2 DNA (b) and M13mp18 (c).

temperature each isoschizomer could withstand for 5 min without any reduction in activity (Table 3). The *Taq*I isoschizomers showed a wide range of thermal stabilities from 68 °C (C) to 84 °C (B) (Fig. 2). Isoschizomer B, from a *Thermus* isolate from New Zealand, is significantly more thermostable than the prototype *Taq*I restriction endonuclease from *T. aquaticus* YT1.

To determine whether any of the many *Taq*I cleavage sites (T/CGA) present in the DNA substrates used (bacteriophage λ, pBR322, pUC18, M13mp18, ϕX174, adenovirus Type 2 and SV40 virus) were less effectively cut by the isoschizomers, optimum buffers were prepared for each enzyme on the basis of the previous results. Each substrate (0·5 μg) was over-digested by incubation for 4 h at 65 °C with 5 units of each isoschizomer. The restriction fragment patterns determined by agarose gel electrophoresis showed no differences between the isoschizomers from the various strains (Fig. 3).

Taxonomic position of the *Thermus* strains

All the strains had a DNA base composition in the range appropriate for the genus *Thermus* (62–67 % G + C) and previous DNA : DNA homology data indicate that these strains represent most of the described species of yellow-pigmented *Thermus*. Strain Ork2A3 belongs to *T. filiformis*, like all the other New Zealand isolates we have tested (Georganta *et al*., 1993). Both RQ1 from the Azores and GK24 from Japan (invalidly described as *T. caldophilus*) have high DNA : DNA homology with *T. thermophilus* and belong to that valid species (Williams *et al*., 1995). Strain Vi4a belongs to a DNA : DNA homology group of strains from Portugal and the Azores (Williams *et al*., 1996) that has been identified with *T. scotoductus* (Tenreiro *et al*., 1995). The species of *Thermus* show particular variation in the sequence of 16S rRNA in the region of helices 6, 9, 10 and 11 (Williams *et al*., 1995, 1996), all of which are accessible by sequencing from the 6F primer. The 16S rRNA sequences of strain YS20 from Yellowstone National Park and JK51 from Iceland were aligned with other sequences for the genus (Williams *et al*., 1995) and these strains were allocated to *T. aquaticus* and *T. brockianus*, respectively (Table 4). The strain of *Thermus* from which we have previously isolated isoschizomers of *Taq*I (SM32 from the Azores, Welch & Williams, 1995b) was also identified as *T. brockianus*.

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Received 1 July 1997; revised 26 August 1997; accepted 29 August 1997.