

A type II restriction endonuclease of *Streptococcus thermophilus* ST117

1. SUMMARY

Streptococcus thermophilus strain 117 produces a type II restriction endonuclease designated as *Sth117I*. This enzyme was isolated from cell extracts by membrane filtration and ammonium sulfate fractionation. Anion exchange chromatography on a DE52 column yielded an enzyme preparation free of nonspecific nucleases. The optimal reaction conditions for *Sth117I* are: (1) $[\text{MgCl}_2] \geq 5$ mM; (2) pH range of 6.5–7; (3) incubation temperature between 37 and 50 °C; and (4) $[\text{NaCl}]$ or $[\text{KCl}] \leq 50$ mM. The results of single- and double-digestion experiments indicated that the *Sth117I* was an isoschizomer of *Bst*NI and *Eco*RII with the recognition sequence of 5'-CCWGG-3'. The cleavage site of *Sth117I* was identified as 5'-CC^WGG-3' by 5'-end analysis. This was supported by the results of ligation experiments with *Sth117I*-restricted DNAs and the *Bst*NI- or *Eco*RII-generated fragments.

2. INTRODUCTION

Lactococci represent a group of industrial microbes used extensively in the dairy foods industry. Various metabolic activities of these microorganisms contribute to the characteristic aroma, flavor and texture to a variety of fermented dairy products. Because of their commercial importance, there has been continued research interest in improving the existing traits of these bacteria. More recently, increased attention has been focused on the application of genetic engineering methodology on strain improvement of these lactococci. As a result, a number of gene cloning vectors and DNA transfer protocols have been developed for this group of microorganisms [1–5].

A functionally related species, *Streptococcus thermophilus*, is important as a starter culture in the production of yoghurt and certain cheese varieties. Earlier molecular biological studies in our laboratory had resulted in the development of methodologies that facilitated the genetic manipulation of this microbe [6,7]. Several strains used in these studies, however, responded poorly to electrotransformation and yielded genetic transfor-

nants at consistently low frequencies. Since the restriction-modification (R-M) systems of host organisms are known to influence the stability of transforming DNA, we initiated a study to characterize the R-M systems of *S. thermophilus* that had led to the isolation of *Sth134I* [8]. We report in this communication the isolation and characterization of a second restriction endonuclease (R-ENase) from *S. thermophilus*. In accordance with the conventional nomenclature [9], this type II R-ENase from *S. thermophilus* strain 117 was designated as *Sth117I*.

3. MATERIALS AND METHODS

3.1. Bacterial culture

Streptococcus thermophilus strain 117 was from our laboratory culture collection. Cell cultures were grown in lactose media as previously described [10].

3.2. Preparation of cell extracts

S. thermophilus cultures (400 ml) were grown without agitation in a 5%-CO₂ atmosphere at 37°C for 16.5 h. The medium was supplemented with 20 mM DL-threonine to facilitate subsequent cell breakage [11]. Cells were harvested by centrifugation (5000 × g; 10 min; 4°C) and washed with 50 ml of TG₅B buffer (10 mM Tris-HCl, pH 7.6; 50 glycerol; 20 mM 2-mercaptoethanol) containing 0.2 mM phenylmethylsulfonylfluoride (PMSF). The cell pellet was resuspended in 5–6 ml of the same buffer. Cell breakage was effected by sonication (Sonicator Model W-225; Heat Systems-Ultrasonics, Farmingdale, NY) with a stepped microtip probe (6.5 power setting; 50% pulse duty cycle; 18 × 20-s bursts; 4°C). Cell debris was removed by centrifugation (11 000 × g; 1.5 h; 4°C), and the supernatant constituted the crude cell extracts.

Alternatively, cells were lysed by first incubating the washed cells with lysozyme (15 mg/ml) and mutanolysin (500 units/ml) in TG₅B buffer at ambient room temperature (R.T.) for 60 min. Both lytic enzymes were purchased from Sigma (St. Louis, MO). Sodium deoxycholate (0.05%, w/v; Sigma), EDTA (10 mM) and NaCl (0.5 M) were

then added. After further incubation at R.T. for 15 min, the lysis mixture was centrifuged at high speed (38,000 × g; 1.5 h; 4°C) to remove cell debris. The supernatant was dialysed against TG₅B buffer; any precipitates were removed by centrifugation. The clear dialysate constituted the crude extracts used in the subsequent isolation steps.

3.3. Isolation of *Sth117I*

The cell extracts were filtered through Centri-con-100 microconcentrators (Amicon, Beverly, MA). After washing with 2 ml of TG₅B containing 0.2 M NaCl, the retained R-ENase activity was eluted with TG₅B + 0.5 M NaCl. The enzyme present in the eluates was further purified by (NH₄)₂SO₄ fractionation. Saturated (NH₄)₂SO₄ stock solution (pH 7; filtered through 0.2-μm membrane filter) was added to the eluates to a final 80% saturation. EDTA (10 mM) was included in this fractionation mixture to prevent possible enzyme inactivation by heavy metal ions. After an incubation on ice for 30 min, the precipitates were sedimented by centrifugation (38 000 × g; 50 min; 4°C). The supernatant which contained the R-ENase activity, was dialysed against TG₅B.

Anion exchange chromatography was performed on a 1 × 2 cm DE52 (Whatman LabSales, Hillsboro, OR) column equilibrated with TG₅B + 0.02 M NaCl. Following sample loading, the column was washed with the same buffer until *A*_{280nm} of the eluate returned to baseline. Elution was with a 15-ml NaCl linear gradient (0.02–0.8 M NaCl) in TG₅B.

At each step of the isolation, the relative purity of *Sth117I* preparations was assessed by agarose gel electrophoresis.

3.4. Restriction, ligation, and analysis of DNA

PhiX174 RF-1 DNA, SV40 RF-1 DNA, pBR322, *Eco*RII, T4 DNA ligase and PhiX174 RF-1/*Hae*III fragments were purchased from BRL Life Technologies (Gaithersburg, MD). T4 polynucleotide kinase, pancreatic DNaseI, venom phosphodiesterase I (type II), T7 phage DNA and λ DNA/*Hind*III fragments were from Sigma. pBR322 amenable to *Eco*RII restriction was isolated from *dcm*⁻*dam*⁻*E. coli* strain ATCC 47013

(ATCC, Rockville, MD). Calf intestinal alkaline phosphatase (CIP) was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). *Bsr*NI was from New England Biolabs (Beverly, MA). All molecular biology procedures were performed as previously described [8].

Sth117I activity was assayed using T7 phage DNA (0.1–0.4 μ g) as substrate, unless specified otherwise. Typically, reaction mixtures containing 20 mM Tris-HCl (pH 7), 20 mM $MgCl_2$ and 0–50 mM NaCl were incubated at 37°C for 0.5–2.0 h.

3.5. Determination of 5'-terminal mononucleotide

The 5'-terminal mononucleotide analysis of the *Sth117I*-restricted DNA was performed as described by Wu et al. [12]. Briefly, 2.3 μ g λ DNA (BRL Life Technologies) were digested for 1 h at 37°C with 26 μ g of *Sth117I* purified from DE52 column. The 5' ends of the DNA fragments were then labelled with ^{32}P by sequential treatment with CIP (1.9 units) and T4 polynucleotide kinase (20 units) as recommended by Ausubel et al. [13]. The radioactively labelled DNAs were hydrolysed completely with DNaseI (10 μ g) and venom phosphodiesterase I (20 μ g) for 3 h at 37°C. After adding 50 μ g each of dAMP, dGMP, dCMP and TMP as carriers, the reaction mixture was spotted on a cellulose thin-layer-chromatography (TLC) sheet (Eastman Kodak, Rochester, NY). The chromatogram was developed with a 3.2 M $(NH_4)_2SO_4$ + 0.2 M Na-acetate (pH 7.2) buffer system [12] by ascending chromatography. The separated mononucleotides were visualized and marked under UV light for determination of R_f values. The TLC sheet was then cut into 1-cm strips, and the ^{32}P radioactivity was determined by Cerenkov radiation measurement using a Beckman LS-8100 liquid scintillation counter (Fullerton, CA).

4. RESULTS AND DISCUSSION

4.1. Isolation of *Sth117I*

Cell breakage and the subsequent release of *Sth117* activity was achieved as expected by the sonication procedure [8]. We found that this procedure also yielded discretely fragmented chromosomal DNA (< 2 kbp in size). In the subse-

quent purification step with Centricon-100, these DNA fragments, together with the desired *Sth117I* and the unwanted nonspecific DNase activities, were retained on the membrane of the apparatus under low salt conditions. Washing the membrane with medium-salt buffer (TG_5B + 0.2 M NaCl) removed the bulk of the contaminating nonspecific nucleases. The *Sth117I* activity with only trace amounts of nonspecific DNases, was eluted from the Centricon-100 with a high-salt buffer (TG_5B + 0.5 M NaCl). The subsequent $(NH_4)_2SO_4$ fractionation step at 80% saturation effectively precipitated the residual contaminating nucleases, leaving the desired *Sth117I* activity in the soluble fraction.

Equally efficient isolation of *Sth117I* was achieved by cell lysis with lytic enzymes and sodium deoxycholate. Since 0.5 M NaCl was included in the lysis mixture, the R-ENase activity was recovered in the supernatant following sedimentation of the cell debris and DNA by high speed centrifugation. As expected with this DNA-free *Sth117I* preparation, filtration of the supernatant through Centricon-100 now yielded a filtrate that contained the R-ENase activity. Traces of nonspecific nucleases in this Centricon-100 eluate were further removed by anion exchange chromatography on a DE52 column. Fig. 1 shows that the *Sth117I* activity, as indicated by the digestion of T7 DNA into discrete fragments, was eluted from the DE52 column at ca. 50 mM NaCl. The nonspecific nucleases, shown by diminished intensities of all DNA bands, did not desorb from the column until [NaCl] reached ca. 110 mM. Fig. 1 also shows that the *Sth117I* activity was reasonably separated from the bulk of the other cellular proteins.

4.2. Optimal reaction conditions of *Sth117I*

The reaction conditions of *Sth117I* were optimized using T7 phage DNA as substrate. An absolute requirement for $MgCl_2$ in the reaction mixture was demonstrated by results depicted in Fig. 2. *Sth117I* activity was not observed in the absence of $MgCl_2$, nor was it detected when the added $Mg(II)$ (20 mM) was chelated by excess EDTA (30 mM). The enzyme exhibited optimal activity in the temperature range of 37–50°C.

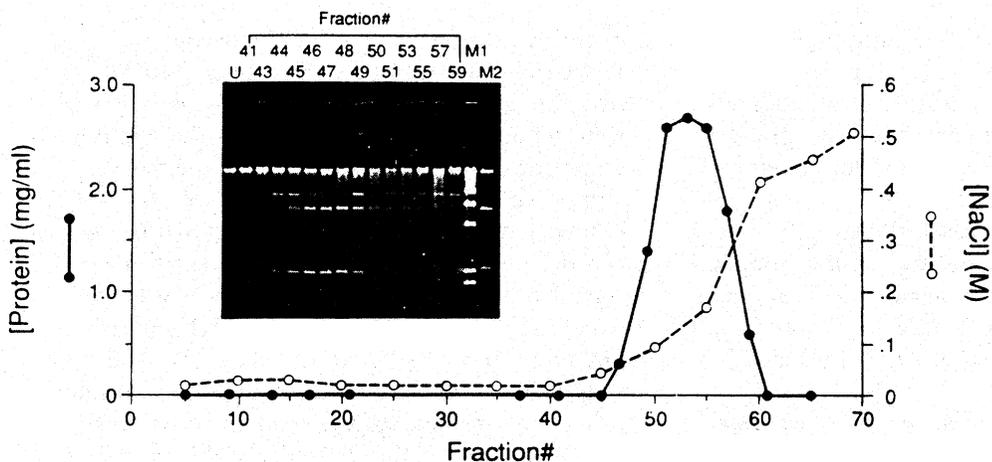


Fig. 1. Isolation of *Sth1171* by DE52 anion exchange chromatography. Gel bed dimensions: 1.0×2.0 cm; flow rate: 6.6 ml/h; fraction volume: 0.4 ml. [NaCl] and [Protein] were determined by conductivity and dye-binding assay [17], respectively. Inset: *Sth1171* assay of column fractions. 20 μ l of fraction were added to 80 μ l of assay mixture (25 mM Tris-HCl, pH 7; 25 mM MgCl₂; 1.6 μ g/ml T7 DNA) and incubated at 37°C for 2 h. DNA was precipitated and analysed on a 0.8% agarose gel as previously described [8]. U, assay mixture alone; M1, λ DNA/*Hind*III fragments; M2, assay mixture + 10 units *Bst*NI (60°C, 2 h).

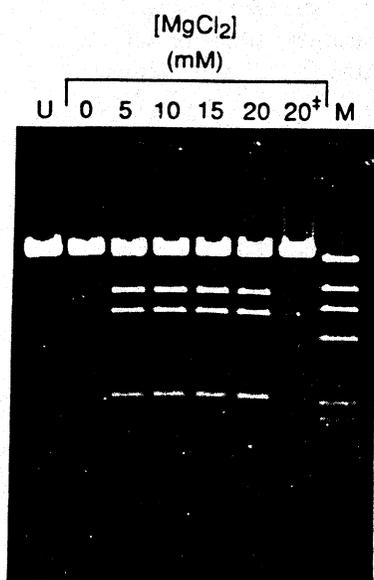


Fig. 2. [MgCl₂] dependence of *Sth1171*. *Sth1171* (4.9 μ g) obtained after (NH₄)₂SO₄ fractionation step, was incubated (37°C, 1 h) with T7 DNA (0.32 μ g) in a reaction mixture (15 μ l) containing 6.2 mM Tris-HCl (pH 7.6), 3% (v/v) glycerol, 12.5 mM 2-ME, 50 mM NaCl and MgCl₂ at the indicated concentrations. Electrophoretic analysis was performed on a 0.7% agarose gel. U, T7 DNA alone (0.32 μ g); M, λ DNA/*Hind*III fragments (0.18 μ g); 30 mM EDTA were included in the reaction mixture.

Reduced reactivity was observed at 22°C, and almost complete inactivation occurred at $\geq 56^\circ\text{C}$. This R-ENase did not seem to be sensitive to pH variation. Although the optimal pH range for the reaction of *Sth1171* was 6.5–7.0, considerable DNA restriction still occurred at acidic pH of 5.4 and alkaline pH of 8.3. *Sth1171* did not require NaCl or KCl for activity; the enzyme cleaved DNA substrates with equal avidity at [NaCl] or [KCl] ≤ 50 mM. Increasingly higher salt concentrations reduced the enzyme activity proportionately; the enzyme became inactive at ≥ 200 mM NaCl or KCl.

4.3. DNA cleavage specificity of *Sth1171*

Sth1171 cleaved T7 phage DNA into 31.8-, 5.8- and 2.4-kbp fragments, along with a conspicuous 8.2-kbp partial digest (Figs. 1 and 2). PhiX174 RF-1 DNA was restricted into 2.6- and 2.8-kbp DNA pieces. pBR322 restricted with *Sth1171* yielded notably 1.9, 1.1, 0.9, 0.4 and ≥ 0.1 kbp fragments, as well as various partial digests. Cleavage of SV40 RF-1 viral DNA or λ phage DNA by *Sth1171* resulted in numerous DNA fragments. By comparing these data to the DNA digestion patterns of known type II R-ENases

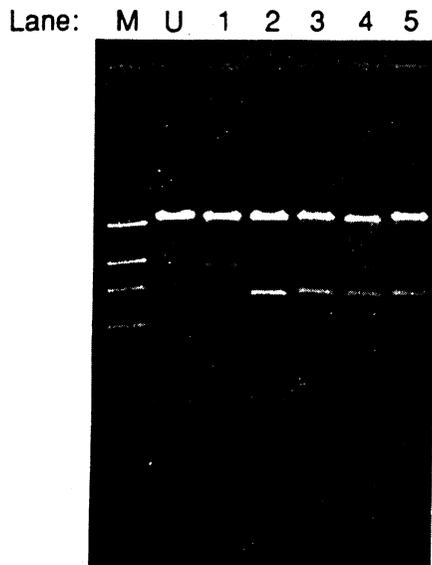


Fig. 3. *Sth117I* and *BstNI* double digestion of T7 DNA. Reaction mixtures (50 μ l) containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 mM NaCl, 1% (v/v) glycerol, 4 mM 2-ME, 2.5 μ g/ml T7 DNA and the indicated R-ENase(s) were incubated at 50°C for 1 h, except nos. 4 and 5 where reactions proceeded for another hour after the addition of second R-ENase. Reaction products were precipitated and analysed on a 0.7% agarose gel. Lanes: 1, *Sth117I*; 2, *BstNI*; 3, *Sth117I* + *BstNI*, simultaneously; 4, *Sth117I*, then *BstNI*; 5, *BstNI*, then *Sth117I*; M, λ DNA/*HindIII* fragments (0.18 μ g); U, reaction mixture alone.

[14], *Sth117I* appeared to be an isoschizomer of *BstNI* and *EcoRII* with the recognition sequence of 5'-CCWGG-3'. This conclusion was supported by the results of a double-digestion experiment. Fig. 3 shows that when T7 DNA was digested either simultaneously or sequentially with *Sth117I* and *BstNI*, the digestion patterns were indistinguishable from those obtained from single digestions with individual enzymes. If *Sth117I* were to cleave DNA at sites other than the recognition sequence of *BstNI* and *EcoRII*, different digestion patterns would have been obtained in the double-digestion experiment.

The cleavage site of *Sth117I* was deduced as 5'-CC-WGG-3' based on the results of the 5'-end determination. When the 5'-[³²P]-labelled λ DNA/*Sth117I* fragments were hydrolysed and chromatographed on TLC, the percent distribution of the ³²P radioactivity among the four mono-

nucleotides was as follows: dpA, 43%; pT, 37%; dpG, 15%; and dpC, 5%. These results indicated that the 5'-terminal mononucleotides of the *Sth117I*-generated DNA fragments were dpA and pT. The relatively high labelling of the dpG (15%) seen in this experiment could be attributed to the fact that the 5' end of the linear λ DNA substrate was a G residue [15]. This assignment of 5'-CC-WGG-3' as the cleavage site of *Sth117I* was supported by the results of ligation experiments with pBR322/*EcoRII* and pBR322/*Sth117I* fragments. In those experiments, attempts to ligate a 1.1 kbp pBR322/*Sth117I* fragment with the CIP treated 0.4 kbp pBR322/*EcoRII* piece failed to yield an expected 1.5 kbp ligation product; under similar reaction conditions, a 1.1 kbp BR322/*Sth117I* fragment ligated successfully with the CIP-treated 0.4 kbp pBR322/*EcoRII* fragment (data not shown). These results suggested that the cleavage sites of *Sth117I* and *EcoRII* were different and thus incompatible. In a separate experiment, attempts at ligating the 2.6- and 2.8-kbp PhiX174 RF-1/*Sth117I* fragments failed to produce the expected 5.4-kbp product (data not shown). This result agreed with the reported observation [16] that ligation efficiencies were extremely low with DNA fragments containing one-base overhang termini. Data from these studies conclusively showed that the cleavage site of *Sth117I* was similar to that of *BstNI* at 5'-CC-WGG-3'.

Although an isoschizomer of *BstNI* and *EcoRII*, the restriction endonuclease *Sth117I* is nevertheless a unique enzyme with potential research applications. This enzyme functions optimally between 37 and 50°C and is insensitive to the Dcm-methylation system of *E. coli*. These parameters distinguish *Sth117I* from the *BstNI* which has a 60°C optimal reaction temperature, and *EcoRII* which is sensitive to Dcm-methylation.

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