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ORIGINAL PAPER

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Characterisation of the novel restriction endonuclease *Sui*I from *Sulfolobus islandicus*

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Abstract A restriction endonuclease activity from *Sulfolobus islandicus* REN2H1 was purified by phosphocellulose and cation exchange chromatography. The enzyme cuts DNA at the recognition site GCwGC as could be shown by restriction analysis of plasmids and short synthetic duplex DNA. The cleavage occurs after the first guanosine base and is inhibited by 5-methylcytosine methylation. The restriction activity is salt-sensitive and has an optimal activity around 70°C.

Keywords *Sulfolobus islandicus* · Restriction endonuclease type II · *Sui*I · *Tse*I · Methylation

Introduction

Restriction endonucleases recognize specific DNA sequences and are able to cleave double-stranded DNA either within the recognition site or outside. Their physiological function is to restrict foreign DNA which has a different methylation pattern than the host DNA. Therefore, restriction endonucleases and methylases are the two principal components of restriction-modification (R-M) systems.

Four types of restriction endonucleases can be distinguished. Type I restriction endonucleases form a multimeric complex with three different subunits which are capable of sequence recognition, modification and restriction, respectively. The enzyme complex can translocate along DNA fuelled by the hydrolysis of ATP. Cleavage usually occurs distant to the recognition site. A typical example is *Eco*KI.

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Restriction endonucleases of type II are the most abundant type. They recognize short DNA sequences of 4–8 bases length. Most recognition sites are palindromic. The DNA is cut on both strands either within the recognition site or in its close neighbourhood. In many cases compatible ends are produced which result in the efficient ligation of DNA fragments produced by restriction with type II enzymes.

Type III restriction endonucleases consist of two subunits with recognition/restriction and modification activity. Like type I enzymes they may also translocate along DNA in an ATP-dependant manner. Cleavage occurs close to the recognition site. A typical example is *Eco*PI.

Type IV restriction endonucleases are not a component of a restriction-modification system. These enzymes cleave DNA between two recognition sites containing a methylated or hydroxymethylated cytosine. The best studied examples is *Mcr*BC from *Escherichia coli*.

Most restriction enzymes have been isolated from bacteria. However an increasing number of restriction enzymes can be recognized in sequenced archaeal genomes. According to the current release of Rebase (Roberts et al. 2005) *Haloarcula marismortui* appears to have a R-M type IV system and three methyltransferases of type II. *Methanosarcina acetivorans* C2A seems to have two complete RM systems of type I and one complete R-M system of type IV as well as several additional methylase and restriction endonuclease genes. *Pyrococcus furiosus* has two methyltransferase genes. *Sulfolobus acidocaldarius* has a complete R-M system of type II and additional restriction and methylase genes.

For most of these predicted archaeal R-M enzymes no biochemical studies have been undertaken. Therefore it is not known whether the genes are expressed and whether the enzymes are functional in vivo. Also the sequence specificity is not known for the majority of these enzymes. So far only very few archaeal restriction enzymes have been investigated. To our knowledge the first archaeal restriction enzyme, *Tha*I, was purified from *Thermplasma acidophilum* in 1978 (McConnell et al.

1978). From *Pyrococcus* sp. strain GI-H the type II restriction endonuclease *Psp*GI has been isolated and characterized (Morgan et al. 1998; Pingoud et al. 2003) and from *Pyrococcus abyssi* the restriction endonuclease *Pab*I has recently been identified and its cleavage site has been determined (Ishikawa et al. 2005). Three restriction endonucleases were found in *Methanococcus aeolicus* (Ishikawa et al. 2005) and a R-M system was identified on the plasmid pFV1 from *Methanobacterium thermoformicicum* (Nolling et al. 1992). Among the *Crenarchaeota*, one of the two major branches of archaea, so far only the restriction endonuclease *Sua*I from *S. acidocaldarius* has been studied (Prangishvili et al. 1985) and the *Ape*KI restriction endonuclease from *Aeropyrum pernix* K1 is commercially available. For the former enzyme its methylation sensitivity has been analysed. The enzyme is inactive on DNA substrate where the inner cytosine of the recognition sequence GGCC is methylated at the N4 position. However the enzyme is active on substrates with methylation at the C5 position of the cytosine base (Grogan 2003). This suggests that in the thermophile *S. acidocaldarius* the cytosines are modified at the N4 position and not at the C5 position. Spontaneous deamination of 5-methyl-cytosine leads to the formation of thymidine in a GT mismatch which might be less well repaired than an uridine base which is produced when N4-methyl-cytosine deaminates. Since deamination is favoured at higher temperatures it might be advantageous for thermophiles to methylate cytosine at the N4 position.

In an ongoing project to develop a *Sulfolobus–E. coli* shuttle vector we studied whether several *Sulfolobus islandicus* strains contain restriction endonucleases which could hinder the transformation of *Sulfolobus* with plasmids prepared in *E. coli*. In this report we describe an endonuclease activity which we isolated from *S. islandicus* strain REN2H1 (Zillig et al. 1994). The palindromic recognition sequence of the enzyme is GCwGC (w = A or T) and the enzyme cleaves after the first guanosine leaving a 5′ overhang of three bases. We suggest to name this enzyme *Sui*I, to differentiate against *Sis*I, a type II restriction endonuclease from *Salmonella isangi*. The enzyme is an isoschizomer of *Tse*I isolated from the thermophilic bacterium *Thermus* sp. and of *Ape*KI from the crenarchaeote *A. pernix* K1.

Experimental

Purification of *Sui*I

Sulfolobus cells were grown in Brock medium (Grogan 1989) with 0.1% tryptone and 0.2% arabinose at 75°C up to an OD_{600} of 1. The cells (1 g wet weight) were harvested by centrifugation. The frozen cell pellet was resuspended in 50 mM sodium phosphate, pH 7.0 and the cells were lysed by seven cycles of freezing in liquid nitrogen and thawing at 60°C. The cell debris was pelleted by centrifugation for 30 min at 14,000*g* at 4°C. 2.5 ml of the crude extract was adjusted to 100 mM NaCl, supplemented with 1 mM β-mercaptoethanol and 2 mM $MgCl_2$ and loaded onto 4 ml P1 phosphocellulose (Whatman) column equilibrated with 50 mM Tris–HCl pH 7.9, 2 mM $MgCl_2$, 1 mM β-mercaptoethanol, 100 mM NaCl (buffer T + 100 mM NaCl). The column was developed with a 40 ml linear salt gradient up to 1 M NaCl. *Sui*I restriction activity eluted at about 500 mM NaCl. The fractions were dialysed against 20 mM Tris–acetate pH 7.0, 10 mM $MgCl_2$, 1 mM β-mercaptoethanol (buffer TA). Activity containing fractions were combined and loaded onto a 1 ml EMD–sulphate (Merck) column equilibrated with buffer T + 100 mM NaCl. The column was eluted with a 10 ml salt gradient. *Sui*I eluted at about 400 mM NaCl. The fractions were dialysed against buffer TA. Protein concentrations were determined by UV spectroscopy.

Restriction assay

Five-hundred nanograms of plasmid pUC–pRN1 (Keeling et al. 1996) was incubated with *Sui*I in a 10 µl reaction in 20 mM Tris–acetate pH 7.0, 50 mM potassium acetate, 10 mM $MgCl_2$, 1 mM β-mercaptoethanol (NEB 4) for 1 h at 65°C. After the restriction the samples were eventually treated with proteinase K in order to digest further DNA binding proteins present in the samples. Then the digests were analysed on a 1% agarose gel and stained with ethidium bromide. The activity of the enzyme was estimated to be 0.5 U when the plasmid was completed restricted.

Cloning of restriction fragments

A restriction digest of pUC–pRN1 was purified over a silica membrane (Promega), incubated with 5 U Klenow fragment (NEB) in the presence of 40 µM dCTP/dGTP/dTTP and 200 µM dATP in order to remove potential 3′ overhangs or to fill up potential 5′ overhangs. The Klenow fragment was heat inactivated and then the sample was incubated with 5 U *Taq* DNA polymerase (Peqlab). The latter incubation will generate a single base 3′ overhang with adenine which can now be ligated into the pGEM-T vector (Promega). A clone containing a restriction fragment was sequenced and allowed to identify the cleavage site of *Sui*I.

Restriction of methylated DNA and genomic DNA

To investigate the methylation sensitivity of *Sui*I we prepared synthetic duplex DNA by hybridising two complementary 33 base long oligodeoxynucleotides (5′-ACGCGTCGGCCTGGCACGTCGGCCGCTGCGGCC-3 and 5′-GGCCGCAGCGGCCGTCGTGCCAGGCCGACGCGT-3′, recognition site underlined). The

latter oligodeoxynucleotide was labelled with γ-[^{32}P]-ATP at the 5′ end with T4 polynucleotide kinase. As substrates we used the unmodified DNA as well as DNA symmetrically methylated at the inner cytosine or the outer cytosine of the recognition sequence. The digests were analysed on a denaturing polyacrylamide gel.

Genomic DNA from the *S. islandicus* strains was prepared by phenol/chloroform extraction of crude extracts followed by ethanol precipitation.

Results and discussion

Purification

When we screened several *S. islandicus* strains for restriction endonuclease activity we found that the strain REN2H1 has a restriction endonuclease activity. Preliminary experiments showed that the plasmid pUC–pRN1 (8036 bases) is cut into three larger fragments of the sizes 3, 1.6 and 0.9 kb by a crude extract prepared from *S. islandicus* REN2H1. A comparable activity was not seen for the strains *S. islandicus* REN1H1 nor HVE10/4 (Zillig et al. 1994) nor in *Sulfolobus solfataricus* P1 (ATCC 35091). As substrate in these initial screening test we used the plasmid pUC–pRN1. This

Table 1 Purification of *Sui*I

	Protein (mg)	Spec. activity (U/μg)	Total activity (kU)
Crude extract	27	~0.05	~1.4
Phosphocellulose	2	1.25	2.5
Cation exchange	0.4	4.1	1.6

The restriction activity was purified approximately 80-fold by phosphocellulose and cation exchange chromatography. Total yield from 1 g of cells was 1,600 U after cation exchange chromatography

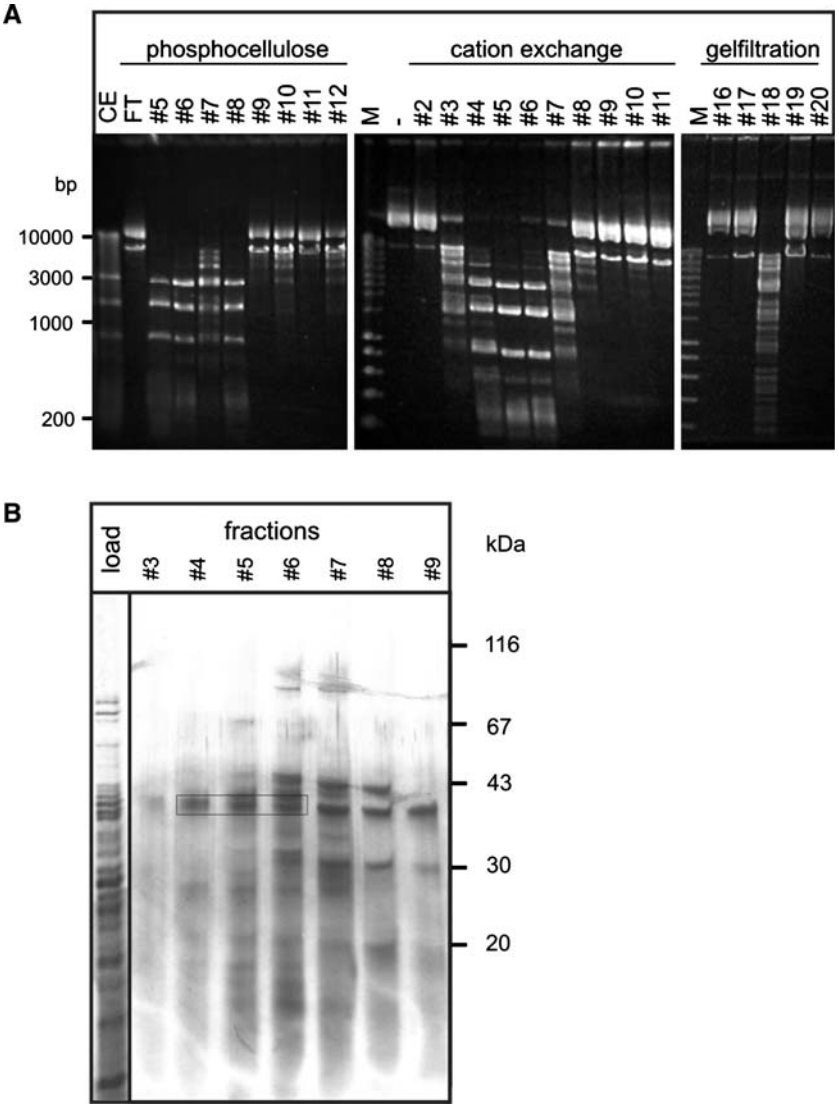

Fig. 1 Purification. **a** The restriction enzyme *Sui*I was purified by phosphocellulose, cation exchange and gelfiltration chromatography. The fractions of the respective columns were assayed by digestion of pUC–pRN1 plasmid DNA. The digests contained about 11 μg protein (*CE* crude extract), 0.4 μg (phosphocellulose) and 0.2 μg (cation exchange), respectively. The recovery of activity after the gelfiltration was rather low and only an incomplete restriction was obtained. We therefore used the cation exchange fractions to characterize the restriction activity of *Sui*I. **b** Silverstained SDS-PAGE protein gel from the cation exchange chromatography. The pool from the phosphocellulose column (load) and the fractions of the salt gradient were analysed. The boxed region highlights a protein which coelutes with the restriction activity

pUC18 derivative contained the complete sequence of the plasmid pRN1 isolated from *S. islandicus* (Keeling et al. 1996). The GC content of the plasmid pRN1 (37%) was rather low and comparable to the GC content of the *Sulfolobus* chromosomes. We used this substrate as we reasoned that a restriction endonuclease isolated from *Sulfolobus* is less likely to contain an AT-rich recognition sequence.

In order to characterize the restriction endonuclease activity from *S. islandicus* strain REN2H1 in more detail we purified the enzyme through several chromatographic steps. The enzymatic activity could be enriched about 80-fold by purification over phosphocellulose and cation exchange chromatography (Table 1). SDS-PAGE analysis showed that several polypeptides are present in the activity containing fractions after the ion exchange chromatography (Fig. 1). In an attempt to further purify the activity we fractioned the protein mixture on a gel-filtration column. We were able to find restriction endonuclease activity in one single fraction. In this fraction proteins of about 40 kDa would elute. However the remaining restriction endonuclease activity was very low and we were not able to identify a protein band in the corresponding SDS-PAGE. We therefore used the material purified by phosphocellulose and cation exchange chromatography for studying the enzyme's properties.

*Sui*I is a thermophilic restriction endonuclease with the recognition site GCwGC

During our studies we found that we cannot detect the restriction endonuclease activity in the fractions from both columns prior to dialysis. The enzyme is inhibited by the salt present in these eluates. We found that *Sui*I is inhibited by sodium chloride and ammonium sulphate (data not shown). Consequently *Sui*I is not active in NEB3 which contains 100 mM sodium chloride (Fig. 2a). Furthermore *Sui*I is most active at a pH around 8 (Fig. 2b). Next we investigated in which temperature range *Sui*I is active. *Sui*I is isolated from a thermophilic archaeon which grows around 75°C. When assaying the activity of *Sui*I at different temperatures we clearly find that *Sui*I is thermophilic enzyme which works best around 65–75°C (Fig. 2c). The kinetics of the plasmid digestion shows that 0.5 µg of plasmid are completely digested in about 30 min at 65°C. At a three fold overdigestion (time point 90 min) we did not observe additional restriction fragments indicative of star

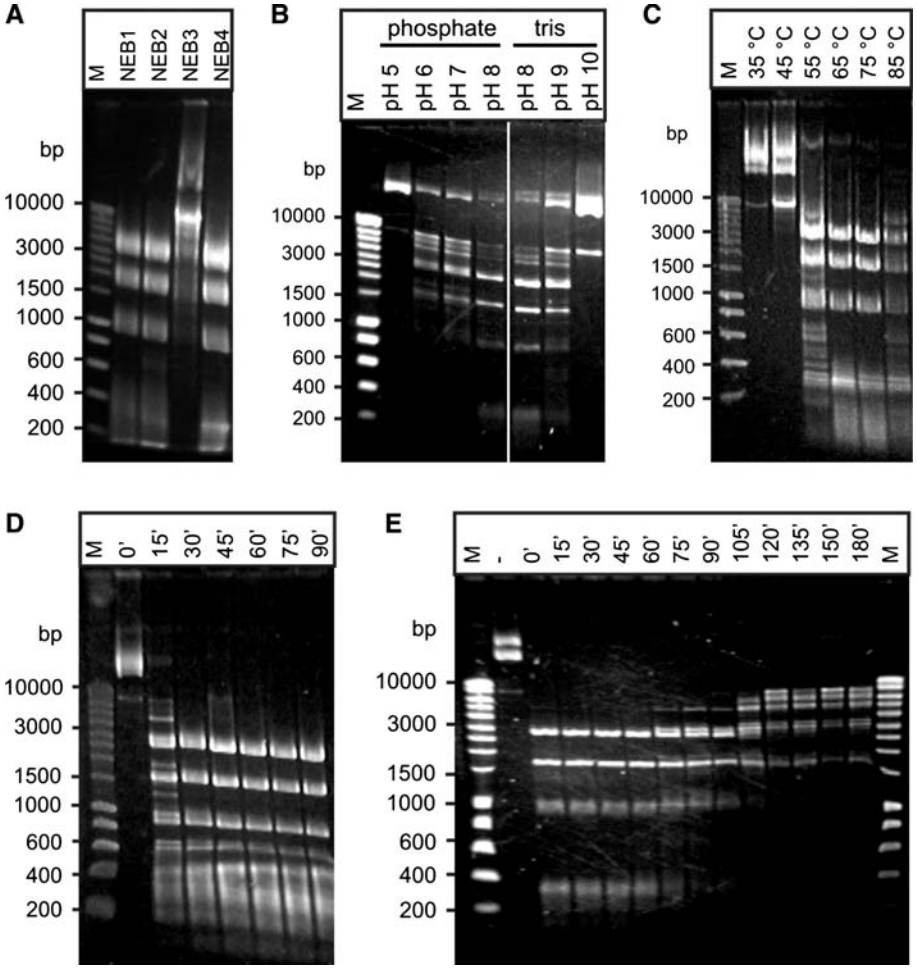

Fig. 2 Restriction endonuclease activity of *Sui*I. **a** *Sui*I is a salt-sensitive restriction enzyme. *Sui*I is active in the buffers NEB1,2 and 4, but is not active in a buffer containing 100 mM NaCl (NEB3). The digests contained 0.24 µg protein (1 U) and were incubated for 1 h at 65°C. **b** DNA substrate and 0.18 µg protein (0.7 U) were incubated in phosphate buffer (pH 5–8) and Tris buffer (pH 8–10) for 1 h at 65°C. **c** *Sui*I is a thermophilic enzyme with a temperature optimum around 70°C. 1 U of *Sui*I was incubated with plasmid DNA for 30 min in the temperature range from 35 to 85°C. **d** Time course of the plasmid digestion at 65°C. The digests contained 0.24 µg (1 U) and were performed in NEB4 buffer. **e** Heat inactivation of *Sui*I. 0.36 µg of *Sui*I (1.5 U) were incubated for various time spans at 95°C. Then the DNA substrate was added and the reaction was incubated for 30 min at 65°C. The half-life of the enzyme is about 1 h at 95°C (compare *lane* 60′ with *lane* 120′). *Lane*—did not contain any enzyme

activity of *Sui*I (Fig. 2d). The enzyme appears to be quite thermostable as we did not find any inactivation after 3 h at 75°C (data not shown). The half-life of the enzyme at 95°C is approximately 1 h (Fig. 2e). Furthermore these and other partial digestions do not indicate that some sites on this DNA substrate are cut more slowly than others.

In order to find the recognition site of *Sui*I we digested pUC–pRN1 and pMJ05 (22 kb). The latter plasmid is a pBluescript derivative containing the complete sequence of the *Sulfolobus shibatae* virus SSV1 (Jonuscheit et al. 2003) and has also a rather low GC content of 39%. The main fragments of pUC–pRN1 are 3.000, 1.600, 950 and 600 base pairs long. The restriction of pMJ05 by *Sui*I yielded fragments of 10.000, 5.000, 1.600, 1.400 and 550 base pairs (Fig. 3b). The observed restriction fragments are compatible with the restriction recognition site of *Tse*I (GCwGC). *Tse*I would produce

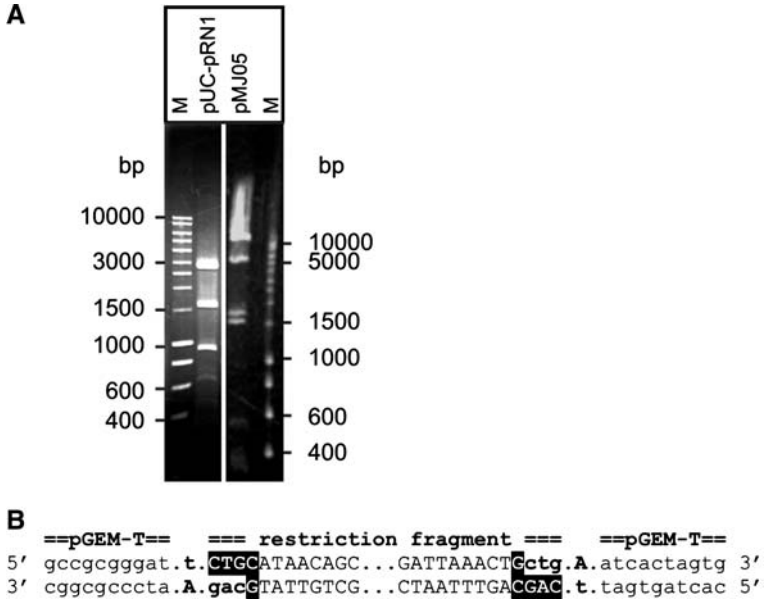

Fig. 3 Determination of the recognition sequence and cutting site of *Sui*I. **a** *Sui*I was incubated with pUC–pRN1 and pMJ05 and the digestion products were separated in an agarose gel. The obtained restriction fragments (see text for details) suggest that *Sui*I recognizes the sequence GCwGC. **b** The restriction fragments were cloned and one clone was sequenced. Shown are the sequences at both vector-insert borders. The sequence in capitals is the sequence of the restriction fragment with the recognition sequence highlighted with *black background*. Before ligation the DNA polymerase filled in the 5′ overhang generated by *Sui*I and added an extra adenine base (shown in *bold letters*) at the 3′ ends of the fragment. The first G of the recognition sequence is lost since the enzyme cuts after the first G. The sequence indicates that *Sui*I cuts within the recognition sequence GCwGC after the first G symmetrically on both strands

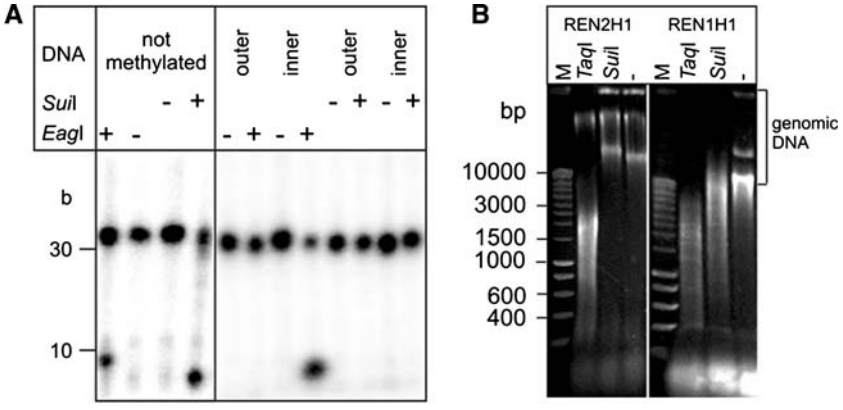

Fig. 4 Restriction of methylated DNA and genomic DNA. **a** Synthetic duplex radioactively labelled DNA was incubated with *Sui*I and *Eag*I and analysed in a denaturing polyacrylamide gel. Unmethylated DNA yields restriction fragments of 5 and 9 bases respectively. As expected DNA methylated at the C5 position of the outer cytosine of the recognition sequence is not cut by *Eag*I, as the recognition sites overlap. *Sui*I is not able to restrict either of the methylated DNA substrates. **b** Genomic DNA of *S. islandicus* strain REN2H1 is not cut by *Sui*I. In contrast genomic DNA isolated from strain REN1H1 does not appear to be appropriately methylated and is digested by *Sui*I. Control digestions were performed with *Taq*I

fragments of 2,843, 1,555, 895 and 611 bp as well as smaller fragments from pUC–pRN1 and the major fragments of 10,911, 4,876, 1,591, 1,441 and 611 bp from pMJ05. The observed fragments are in good agreement with the fragments expected from *Tse*I. We can exclude the more relaxed recognition site GCnGC of *Fnu*4HI, since this enzyme would produce more fragments. This analysis suggests that *Sui*I has the same recognition site as *Tse*I. In order to identify the exact cleavage site of *Sui*I we cloned the restriction fragments of a pUC–pRN1 digestion and sequenced one clone. Before cloning we treated the fragments with Klenow fragment and *Taq* DNA polymerase to polish the ends and to create a single base adenosine 3′ overhang. The sequence we obtained is in perfect agreement with a restriction of *Sui*I after the first guanosine of the recognition sequence GCwGC (Fig. 3b). The palindromic sequence is cut symmetrically leaving a three base 5′ overhang. The cloned restriction fragment does not contain a further internal recognition site.

Next we investigated the methylation sensitivity of *Sui*I. For this purpose we used complementary deoxynucleotides which had a C5 methylation at the inner and outer cytosine of the recognition sequence. Whereas *Sui*I is able to restrict the unmodified DNA, no restriction is seen with the DNA methylated at the inner nor the outer cytosine. This assay does not allow to conclude whether the corresponding methylase carries out a methylation at the C5 position of the cytosine. In contrast Grogan and coworkers (Grogan 2003) found that *Sua*I is able to restrict C5 methylated cytosine but not N4 methylated cytosine suggesting that the respective methylase does not methylate the C5 position of cytosine.

We were neither able to detect a restriction endonuclease activity in the crude extract of *S. islandicus* strain REN1H1 nor after an attempted purification over phosphocellulose (data not shown). This strain is the host of the plasmids pRN1 and pRN2 which are suitable starting points to construct a *Sulfolobus–E. coli* shuttle vector. As a further indication that this strain does not contain *Sui*I restriction activity we could show that genomic DNA of this strain is restricted by *Sui*I (Fig. 4).

In summary, we report the purification and characterisation of the restriction endonuclease *Sui*I from *S. islandicus*. The properties of the enzyme suggest that *Sui*I is a restriction endonuclease of type II. The enzyme is an isoschizomer of *Tse*I and *Ape*KI and shares with these enzymes a high temperature optimum. In contrast *Sui*I is rather salt-sensitive whereas *Tse*I and *Ape*KI have the highest activity in high-salt restriction buffers.

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