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Characterization of a Site-Specific Restriction Endonuclease from *Streptomyces aureofaciens*

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A new type II sequence-specific restriction endonuclease, *Sau*I, was isolated from *Streptomyces aureofaciens* IKA18/4. The purified enzyme was free of contaminating exonuclease and phosphatase activities. *Sau*I cleaved lambda DNA at two sites, but did not cleave pBR322, simian virus 40, or ϕX174 DNA. *Sau*I recognized the septanucleotide sequence 5'-CC↓TNAGG-3' and cleaved at the position indicated by the arrow, producing a trinucleotide 5'-terminal extension.

Restriction enzymes are endodeoxyribonucleases which recognize specific nucleotide sequences that are usually 4 to 7 base pairs (bp) in length and contain a twofold axis of symmetry (8). The type II restriction enzymes, which require only magnesium as a cofactor, have become an indispensible tool for analysis of the structure and function of DNA.

Restriction enzymes have been isolated from a great variety of bacteria, including the actinomycetes (8). We have found restriction endonuclease activities in two strains of *Streptomyces aureofaciens* (12). This report describes the purification and characterization of a new type II site-specific restriction endonuclease, *Sau*I, from *S. aureofaciens* IKA18/4. *Sau*I recognizes the septanucleotide sequence 5'-CC↓TNAGG-3' and cleaves at the position indicated by the arrow, producing a trinucleotide 5'-terminal extension.

MATERIALS AND METHODS

Strains and culture conditions. *S. aureofaciens* IKA18/4 was grown as described previously (12). Cells were harvested after 35 h of incubation and stored at −20°C.

Preparation of *Sau*I. Five grams of washed frozen cells was thawed and suspended in 10 ml of buffer A (0.01 M Tris-hydrochloride, pH 7.9, 0.01 M 2-mercaptoethanol, and 0.1 mM EDTA). The cells were placed on ice and disrupted with 20 30-s pulses from the 0.5-cm-diameter probe of an MSE sonicator. The cell debris was removed by centrifugation at 105,000 × *g* for 2 h, and 5 ml of a 5% (wt/vol) streptomycin sulfate solution was added to the supernatant. The nucleic acids were removed by centrifugation at 12,000 × *g* for 30 min, and protein in the supernatant was precipitated by ammonium sulfate at 50% saturation. The precipitate was sedimented by centrifugation at 12,000 × *g* for 30 min, and the pellet was dissolved in 15 ml of PC buffer (0.01 M phosphate, pH 7.6, 0.1 M EDTA, 0.01 M 2-mercaptoethanol, and 10% [vol/vol] glycerol). The resulting solution was applied to a DEAE-cellulose (Whatman DE-52) column (1.5 by 16 cm) which had been equilibrated in PC buffer. The column was washed with 100 ml of PC buffer. A linear gradient made with 150 ml of PC buffer; then 150 ml of PC buffer with 1.0 M NaCl was applied to the column, and 5-ml fractions were collected. Fractions containing restriction endonuclease activity were pooled and dialyzed against PC buffer. The dialysate was applied to a DNA-agarose column (1 by 4 cm) equilibrated in PC buffer. The column was washed with 25 ml of PC buffer and successively eluted with 20-ml volumes of PC buffer containing 0.1, 0.3, and 0.5 M NaCl, respectively. Two-milliliter fractions were collected, and the active fractions were pooled and dialyzed against storage buffer (PC buffer with 50% glycerol). The preparation was stored at −20°C.

Enzyme assays and electrophoresis conditions. *Hin*dIII, *Bam*I, *Sal*I and *Taq*I were assayed by previously described procedures (2, 4, 10). Reaction mixtures for detecting *Sau*I activity contained 10 mM Tris-hydrochloride, pH 7.5, 10 mM $MgCl_2$, 75 mM NaCl, 0.5 μg of λ *c*I857 DNA, and 1–2 μl of material to be assayed in a total volume of 10 μl. Samples were incubated for 1 h at 37°C, and the reaction was stopped by the addition of 2.0 μl of 0.01 M EDTA. One unit of *Sau*I activity is defined as that amount of enzyme required to digest 1 μg of λ *c*I857 DNA to completion in 1 h at 37°C. Electrophoresis was performed on 1 or 0.8% (wt/vol) agarose gels (11) or 5% polyacrylamide gels (1). Phosphatase activity was assayed by adding 1 μg of a 5' end-labeled DNA fragment (100,000 cpm/μg) to 5 U of *Sau*I in 20 μl of *Sau*I buffer and incubating at 37°C. Portions were removed at 1 and 2 h and counted after precipitation with cold 10% trichloroacetic acid, 1% $Na_2P_2O_7$, and 1% NaH_2PO_4.

DNA sequence analysis. Restriction fragments were 5' end labeled with [γ-^{32}P]ATP and polynucleotide kinase essentially as described by Maxam and Gilbert (6). DNA fragments were labeled at the 3' end with [α-^{32}P]dCTP and reverse transcriptase as described elsewhere (7a). Single-end-labeled fragments were obtained after restriction with a second enzyme. The fragments were isolated from the gel matrix by electroelution. Single-end-labeled fragments were subjected to the base-specific chemical cleavage reactions of Maxam and Gilbert (6). The guanosine (G), guanosine plus adenosine (G + A), A > C, cytosine (C),

and cytosine plus thymidine (C + T) reactions were used. The products were analyzed on 0.4-mm-thick gels (9), using acrylamide concentrations of 16 or 8%. The gels were developed at −70°C with or without intensifying screens.

Identification of 2'-deoxynucleoside 5'-phosphate after cleavage with *Sau*I. Bacteriophage lambda DNA was restricted with *Sau*I, and fragment C (see Fig. 1) was isolated and labeled at the 5' ends with [γ-^{32}P]ATP and polynucleotide kinase. The fragment was digested to completion with a combination of pancreatic DNase I and snake venom phosphodiesterase and then chromatographed adjacent to standard 2'-deoxynucleoside 5'-phosphates as described previously (13).

Enzymes and chemicals. *Bam*I and *Sal*I were purified as described previously (4). *Taq*I was purified by the methods of Sato et al. (10). *Hin*dIII was purchased from Boehringer-Mannheim Biochemicals, Indianapolis, Ind. Bacterial alkaline phosphatase was purchased from Worthington Biochemicals Corp., Freehold, N.J. Polynucleotide kinase was purchased from New England Biolabs, Beverly, Mass. Reverse transcriptase was obtained from the National Cancer Institute, Bethesda, Md. [γ-^{32}P]ATP was prepared as described by Johnson and Walseth (3). [α-^{32}P]dCTP was purchased from Amersham Corp., Arlington Heights, Ill.

RESULTS

Isolation of *Sau*I. The isolation of *Sau*I used a DEAE-cellulose column and a DNA agarose column (see Materials and Methods). *Sau*I activity was eluted between 0.35 and 0.4 M NaCl from the DEAE-cellulose resin and at 0.5 M NaCl from the DNA agarose matrix. The yield of enzyme was approximately 1,000 U of *Sau*I per g (wet weight) of cells. No significant 3'-exonuclease or 5'-exonuclease activities were detected since digestion of a *Bam*I fragment labeled at the 5' or the 3' end with *Sau*I gave only a single band on a DNA sequencing gel (see Fig. 3). There was no detectable phosphatase activity present since greater than 90% of the label in an end-labeled DNA fragment could be precipitated after incubation for 2 h with *Sau*I as described in Materials and Methods. The enzyme was stable in storage at −20°C for at least 1 year with little or no loss in activity.

Specificity of *Sau*I. There were no *Sau*I cleavage sites on pBR322, simian virus 40 (SV40), or ϕX174 DNA (data not shown). Three restriction fragments were produced when λ *c*I857 DNA was restricted with *Sau*I alone: fragment A was 27.1 kilobases (kb), fragment B was 14.3 kb, and fragment C was 7.6 kb (Fig. 1A, lane c). The *Sau*I cleavage sites were mapped relative to the known *Hin*dIII and *Bam*I restriction sites of λ *c*I857 DNA. The two *Sau*I sites were found at positions corresponding to 55.3 and 70.9% of the lambda genome (Fig. 1B). The *Sau*I site at 55.3 resulted in the appearance of two new fragments, 1.59 and 0.65 kb, when λ *c*I857 DNA was digested with *Hin*dIII and *Sau*I (Fig. 1A, lane b); restriction with *Bam*I and *Sau*I resulted in the appearance of two new fragments of 1.38 and 4.16 kb (Fig. 1A, lane d). The *Sau*I site at 70.9 kb resulted in the appearance of two new fragments of 6.86 and 2.6 kb when digested with *Hin*dIII and *Sau*I (Fig. 1A, lane b). However, the *Bam*I plus *Sau*I restriction of λ *c*I857 DNA did not result in a significant change in the mobility of the 6.5-kb *Bam*I fragment. Further analysis on 5% polyacrylamide gels revealed that a 180-bp *Bam*I-*Sau*I fragment had been generated (data not shown). Thus, it seemed likely that the DNA sequence of the *Sau*I site at 70.9 could be determined by labeling the *Bam*I site at 71.3.

Determination of the *Sau*I cleavage site. The λ *c*I857 DNA was digested with *Bam*I and 5' end labeled as described in Materials and Methods. A single-end-labeled 1.2-kb DNA fragment containing the *Sau*I site at 70.9 was produced by restriction with *Sal*I. The fragment was isolated by electrophoresis on a 5% polyacrylamide gel and sequenced by the method of Maxam and Gilbert (6). The 1.2-kb *Bam*I-*Sal*I fragment labeled at the *Bam*I site was restricted with *Sau*I and subjected to electrophoresis adjacent to the sequencing lanes on an 8% polyacrylamide gel (7). The mobility of the *Bam*I-*Sau*I fragment corresponded with T in the sequence 5'-CCTCAGG-3' (Fig. 2). Thus, *Sau*I cleaved between a C and a T on the 5' strand.

The site of cleavage on the opposite strand of the DNA at the 70.9 *Sau*I site was also determined. In this experiment, λ *c*I857 DNA was digested with *Bam*I and 3' end labeled with [α-^{32}P]dCTP. The 1.2-kb *Bam*I-*Sal*I fragment was isolated as above. A portion of the purified fragment was digested with *Sau*I, and the remaining portion was used to prepare a set of size standards. A comparison of the mobilities of the 3' and 5' end-labeled *Bam*I-*Sau*I fragments showed that the distance between the two fragments was consistent with a size difference of 3 bp (Fig. 3). Thus, *Sau*I cleaved between a C and T on the 3' strand within the sequence 3'-GGAGTCC-5'.

If *Sau*I cleaves all sites in λ *c*I857 DNA between a C and T residue, then the 5'-terminal nucleotides should be T. The *Sau*I C fragment was labeled at the 5' end with [γ-^{32}P]ATP and digested to 2'-deoxynucleoside 5'-phosphates with a combination of pancreatic DNase I and snake venom phosphodiesterase as described by Wells et al. (13). Ninety percent of the radioactivity appeared at dTMP (Table 1). That means that the first nucleotide is dTMP and that *Sau*I cleaves between C and T of the sequence 5'-

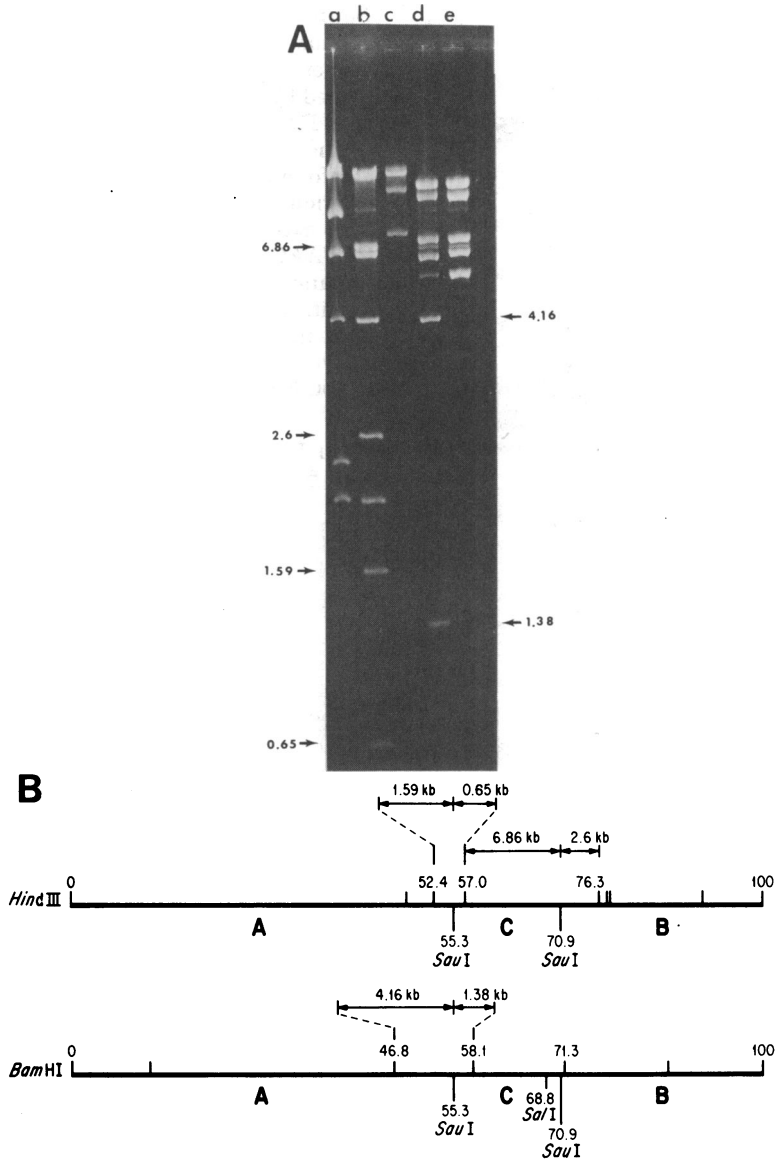

FIG. 1. *SauI restriction map of phage lambda DNA. (A) λ cI857 DNA was incubated with the indicated enzymes and electrophoresed on a 0.8% agarose gel. (a) HindIII, (b) HindIII and SauI, (c) SauI, (d) BamI and SauI, and (e) BamI. (B) Physical map of λ cI857 showing the SauI sites. The locations of the HindIII, BamI, and SalI sites were determined previously (2).*

CCTCAGG-3′ at the 70.9 *Sau*I site. The 5′-terminal nucleotide of the *Sau*I site at 55.3 must also be T since 90% of the radioactivity appeared as dTMP.

A partial sequence of the *Sau*I site at 55.3 was obtained. The *Sau*I-generated fragment C was isolated by preparative electrophoresis from a 0.8% agarose gel and labeled at the 5′ end with [γ-^{32}P]ATP. The labeled fragment was digested with *Taq*I, producing two 5′ end-labeled fragments of 200 and 1,500 bp which were isolated from a 5% polyacrylamide gel. The DNA sequence at the 5′ terminus of each fragment was determined on 16% polyacrylamide gels calibrated with fragments of known sequence (data not shown). The 200-bp fragment had the sequence 5′-TCAGGATGC ... -3′, which corresponded to the sequence at the 70.9 *Sau*I site. The 1,500-bp fragment had the sequence 5′-TAAGGAAA ... -3′. Thus, the penultimate nu-

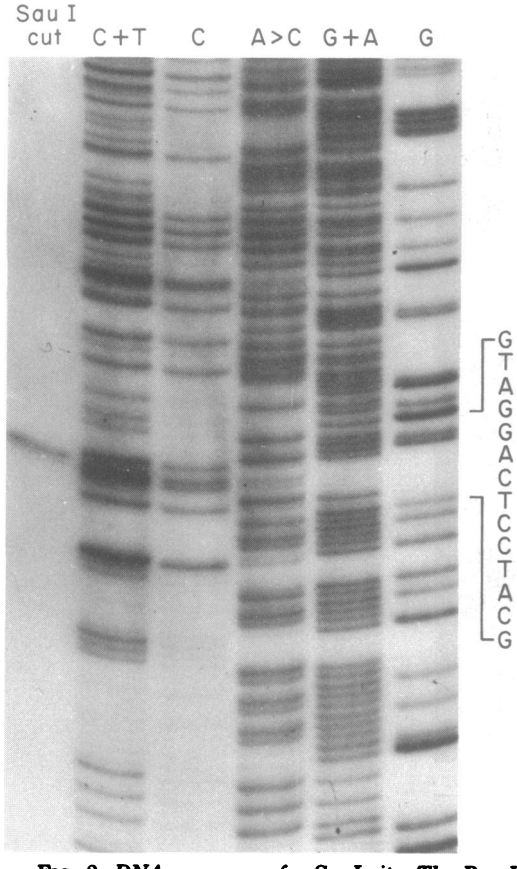

FIG. 2. *DNA sequence of a SauI site. The BamI-SalI fragment described in the text was labeled at the 5′ end and sequenced as described in Materials and Methods. The 5′ end-labeled BamI fragment was cleaved with SauI and run in an adjacent lane to indicate the cleavage site.*

cleotide on the 5′ strand of the *Sau*I site at 55.3 is A, whereas it is C for the *Sau*I site at 70.9.

DISCUSSION

The preparation of the *Sau*I restriction endonuclease was relatively easy, involving a streptomycin sulfate fractionation and two column chromatography steps. The yield was typically 1,000 U of enzyme per g (wet weight) of cells. The enzyme is relatively free of exonuclease and phosphatase activities. Since the enzyme is completely inhibited by the presence of 0.01 M KCl, NaCl was used in all buffers for purification and assay of *Sau*I.

We have shown that *Sau*I cleaves between the C and T of the sequence 5′-CCTGAGG-3′ at position 70.9 in λ cI857 DNA. This was inferred from direct DNA sequence analysis across a *Sau*I site by comparison of the mobility of the *Sau*I-cleaved restriction fragment with the chemically cleaved fragments used in the DNA sequence analysis. The site of cleavage was confirmed by determining the 5′ end after digestion with DNase I and spleen phosphodiesterase.

The *Sau*I site at 55.3 was partially sequenced and found to contain an A rather than a C in the position corresponding to the center of the two-fold axis of symmetry in the sequence 5′-CCTAAGG-3′. The sequences adjacent to the septanucleotide found at 55.3 and 70.9 are different, suggesting that the recognition site for *Sau*I might be 5′-CCTNAGG-3′. *Sau*I did not cleave pBR322, SV40, or φX174 DNA. Indeed, the putative recognition sequence was not found

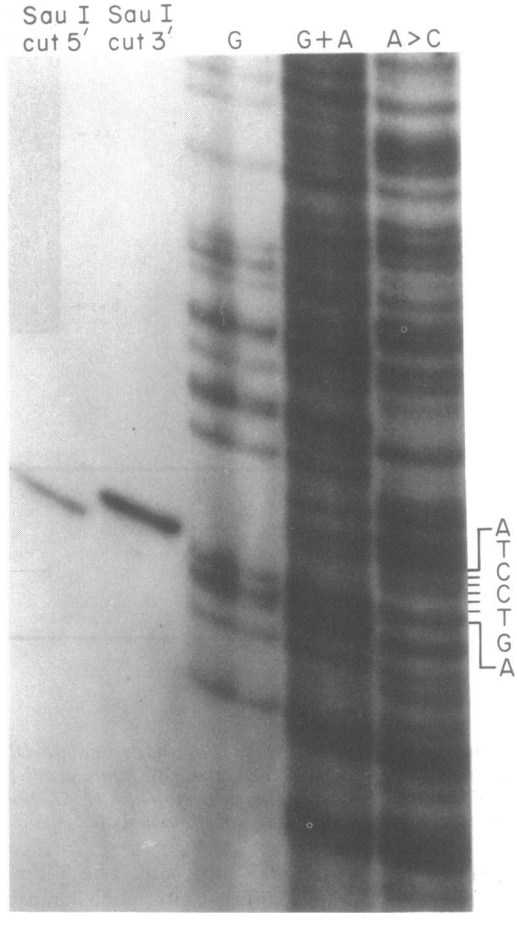

FIG. 3. *Determination of the cleavage site of SauI. λ cI857 DNA was restricted with BamI and either 3′ or 5′ end labeled as described in Materials and Methods. The fragments were then restricted with SauI, and their size was determined by comparison with the sequenced BamI-SalI fragment labeled on the 3′ strand. The gel was interpreted as described by McConnell et al. (7).*

TABLE 1. *Identification of 5'-terminal 2'-deoxynucleoside 5'-phosphate (dNMP) generated after cleavage with SauI*[a]

dNMP	cpm (%)
dTMP	90.4
dAMP	2.4
dCMP	0.6
dGMP	6.4

[a] Determination of the dNMP's after digestion with pancreatic DNase I and snake venom phosphodiesterase was as described in Materials and Methods.

in a computer search of these sequenced DNA molecules (T. Gingeras, personal communication). In conclusion, our data are consistent with the hypothesis that *SauI* recognizes the sequence 5'-CCTNAGG-3' and cleaves between nucleotides C and T on both strands of the DNA, producing a trinucleotide 5'-terminal extension.

The central pentanucleotide of the *SauI* recognition sequence, 5'CTNAG-3', is recognized by the enzyme *DdeI* from *Desulfovibrio desulfuricans* (5). The enzyme *DdeI* cleaves at the same position in the palindrome as *SauI*. *SauI* should be a useful enzyme for restriction analysis and further manipulation of DNA molecules cloned into pBR322 or SV40 since these vectors do not contain a *SauI* site.

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