Volume 5 Number 4 April 1978 Nucleic Acids Research

A second site-specific restriction endonuclease from Staphylococcus aureus

J.S. Sussenbach, P.H. Steenbergh, J.A. Rost[X], W.J. van Leeuwen[X] and J.D.A. van Embden[X]

Laboratory for Physiological Chemistry, State University of Utrecht, Utrecht, and [X]National Institute of Public Health, P.O. Box 1, Bilthoven, Netherlands

Received 6 February 1978

ABSTRACT

A site-specific restriction endonuclease has been isolated from Staphylococcus aureus PS 96. This enzyme, Sau96 I, recognizes the DNA sequence 5'--G↓G-N-C-C--3' and cleaves as indicated by the arrows. The enzyme
3'--C-C-N↑G-G--5'
cleaves adenovirus type 5 and λ DNA many times, SV40 DNA 10 times and ØX174 RF DNA 2 times. Evidence is presented that the enzyme is involved in biological restriction-modification.

INTRODUCTION

Host-controlled restriction and modification of bacteriophage DNA is a common phenomenon in Staphylococci and the differentiation between different strains of these bacteria by phage typing depends at least partially on the presence of restriction-modification systems (1,2).
Recently, Sussenbach et al. (3) identified a restriction endonuclease in Staphylococcus aureus PS 3A, which recognizes the sequence 5'-↓GATC-3' and
3'-CTAG-↑5'
cleaves as indicated by the arrows. This enzyme, Sau3A I, was found in six other strains of a particular lytic group, while strains of several other groups were devoid of this enzyme (4). After extension of our search for class II restriction endonucleases to other lytic groups we found a new endonuclease activity in strains of the lytic group XIII. In this communication we describe the purification and the determination of the recognition site of this enzyme that will be designated as Sau96 I according to the nomenclature proposed by Smith and Nathans (5).

MATERIALS AND METHODS

Materials and Strains

Lysostaphin obtained from Schwarz-Mann, New York was dissolved in water (2 mg/ml) and stored at -20 °C. The restriction endonucleases Pst I

and Hae II were obtained from Biolabs, Beverly. Hae III and Asu I were kindly provided by drs. J.Vereyken and A. de Waard, respectively. The preparation of nutrient broth has been described previously (6).

Staphylococcus aureus PS 96 (7) was used for the isolation of the endonuclease Sau96 I. The restriction-deficient PS 96 mutants R_5 and $R_{5.16}$ (1) were obtained from dr. M.J. de Saxe. S. aureus PS 16 belongs to the same lytic group as PS 96. This lytic group has been designated as group XIII (6). S. aureus PS 71 and the phage 71 are from the International Phage Typing System (7). Phage 16 is identical to phage 71 except that it is propagated on PS 16 and is modified by the group XIII modification system (8). Escherichia coli K12 strain HB 129 which is lysogenic for $\lambda_{CI\ 857.S7}$(obtained from dr. J.Maat) was used as source for λ DNA.

DNA preparations

ØX 174 RF DNA and SV40 DNA were kindly provided by Miss J.Zandberg and dr. J. ter Schegget, respectively.

Staphylococcal phage DNA preparations were prepared as follows: 250 ml nutrient broth supplemented with 0.01% $CaCl_2$ was inoculated with 1.25 ml of an overnight culture of staphylococci and a phage suspension of a final concentration of 1 x RTD. After 5 hours of incubation at 37 °C under vigorous agitation cells were removed by two cycles of low speed centrifugation. The phage particles were concentrated by centrifugation for 4 hours at 27,000 rpm in a Spinco SW 27 rotor and the pellet was resuspended in 1 ml nutrient broth. The phages were purified by CsCl gradient centrifugation (3 hours at 40,000 rpm in a Spinco SW 50.1 rotor) and the phage band was dialyzed against TES buffer (0.01 M Tris-HCl, 0.001 M EDTA, 0.01 M NaCl, pH 7.0) and extracted three times with phenol. The phage DNA was dialyzed against TES buffer. λ was prepared from thermally induced E. coli cultures and phages and phage DNA were purified as described for the staphylococcal phages. DNA from adenovirus type 5 was isolated as described previously (9).

Extraction of Sau96 I

One liter of nutrient broth was inoculated with 10 ml of an overnight culture of PS 96 and shaken for 6 hours at 37 °C. Cells were harvested and washed three times with 50 ml 0.01 M Tris-HCl, 0.015 M sodium citrate, pH 7.4 and resuspended in 0.01 M Tris-HCl, 0.145 M NaCl, pH 7.4. Lysostaphin was added to a final concentration of 20 µg/ml and the mixture was incubated for 5 min. at 37 °C. The partially lysed cells and cell debris were washed two times with 50 ml TM buffer (0.01 M Tris-HCl, 0.01 M

β-mercaptoethanol, pH 7.4) and resuspended in 5 ml of the same buffer. This suspension was sonicated at 0°C for 9 x 1 minute at 62 Watt with a Branson sonifier (type B12 equiped with a 3 mm tip) and centrifuged for 60 min. at 35,000 rpm in an 8 x 25 ml angle rotor (MSE). Streptomycin sulphate (10% in TM buffer) was added to a final concentration of 1.4% and the mixture was gently stirred for 15 min. at 0°C. The DNA precipitate was removed by centrifugation and the supernatant was dialyzed against TM buffer, again centrifuged and the supernatant was stored at 0°C. This material is designated as crude extract.

Digestion of DNA and gel electrophoresis

About 0.4 to 1.0 µg of DNA was incubated with Sau96 I in a volume of 20 µl during 1 to 2 hours at 30°C. The incubation buffer consisted of 15 mM $MgCl_2$, 6 mM Tris-HCl, 6 mM β-mercaptoethanol, 60 mM NaCl, pH 7.4. One unit of enzyme activity was defined as the amount of enzyme required for complete digestion of 1 µg of λ DNA in 1 hour at 30°C. Digestions with the restriction enzymes Pst I, Hae III, Asu I and double digestions with Sau96 I were carried out in 6 mM Tris-HCl, 10 mM NaCl, 10mM KCl, 7 mM $MgCl_2$, 6 mM dithiothreitol, pH 7.5 at 37°C.

Gel electrophoresis was performed on slab gels as described by Sugden et al. (10) using 1.4% agarose in 0.04 M Tris-HCl, 0.02 M sodium acetate, 0.002 M EDTA, pH 7.7. Gels were stained with ethidiumbromide and photographed under UV light illumination.

Analysis of the recognition site of Sau96 I

DNA fragments were obtained by digestion of 5 µg of adenovirus type 5 DNA with Sau96 I. After deproteinization by extraction with chloroform-isoamylalcohol (24:1) and ethanol precipitation of the DNA, the fragmented DNA was brought in 100 µl 50 mM Tris-HCl, pH 8.0. Then 25 µg (0.12 U) of bacterial alkaline phosphatase (Worthington, Freehold,NJ) was added and the DNA was incubated for 1 hour at 37°C. Subsequently the fragmented DNA was extracted three times with equal volumes of freshly distilled phenol and once with chloroform-isoamylalcohol. After alcohol precipitation the fragments were taken up in 42.5 µl 5 mM Tris-HCl, 0.01 M EDTA, 0.1 mM spermidine, pH 9.5, denatured by heating at 100°C for 3 min. and rapidly cooled in ice. Then 5 µl 0.5 M Tris-HCl, 0.1 M $MgCl_2$, 0.05 M dithiothreitol, pH 9.5 was added, the mixture was transferred onto dried γ^{32}P-ATP (25 µCi, 750 Ci/mmole) (The Radiochemical Centre, Amersham) and after addition of 10 U of T_4 polynucleotide kinase (Boehringer, Mannheim) incubated for 45 min. at 37°C to label the 5'-ends of

the fragments. After stopping the reaction by addition of 6 µl 0.25 M EDTA and heating for 2 min. at 100 °C the mixture was eluted over a Sephadex G-100 column in 2.5 mM Tris-HCl, 0.5 mM EDTA, pH 7.5. The DNA fragments eluting with the void volume of the column were precipitated with ethanol and dissolved in 100 µl 2.5 mM Tris-HCl, 0.5 mM EDTA, pH 7.5. For the analysis of the 5'-terminal nucleotide 0.5 µg ^{32}P-phosphorylated DNA was digested with 1 µg of snake venom phosphodiesterase (Boehringer, Mannheim) for 1 hour at 37 °C. Under these conditions the DNA fragments are completely digested to 5'-mononucleotides. Separation of the nucleotides was performed by electrophoresis on Whatman 540 paper and DEAE paper at pH 3.5. The labeled 5'-terminal nucleotides were identified by autoradiography.

For the analysis of the recognition site ^{32}P-phosphorylated DNA was partially digested with snake venom phosphodiesterase. The products were fractionated according to a standard two-dimensional fractionation procedure developed by Brownlee and Sanger (11) in a modification according to Volckaert, Min Jou and Fiers (12). In the first dimension fractionation was performed by electrophoresis at pH 3.5 on cellulose acetate and in the second dimension by homochromatography on PEI-cellulose using an RNA homomix, prepared by hydrolysis of a 3% solution (yeast RNA, sodium salt, BDH, Poole) at pH 12.8 for 30 min. at room temperature.

RESULTS

Isolation and purification of Sau96 I

Crude extracts were made from PS 96 and the restriction endonuclease activity was monitored by incubation with λ DNA. Fig. 1 shows that λ DNA is cleaved many times. From 4 grams of packed cells crude enzyme (6000 units) was prepared as indicated in Materials and Methods. Exonuclease activity could not be detected even after incubation for 18 hours with 10 units of enzyme. The crude extract was fractionated by DEAE-cellulose chromatography as described by Sussenbach et al. (3). The column (29 x 1.8 cm) was eluted with a KCl gradient (400 ml, 0.0-0.7 M KCl) and 6 ml fractions were collected. One peak of endonuclease activity was found in the fractions containing 0.20-0.25 M KCl (Fig. 1). After dialysis against PME buffer (0.01 M KPO_4, 0.01 M β-mercaptoethanol, 0.1 mM EDTA, pH 7.4) the endonuclease containing fractions were applied to a phosphocellulose column (18 x 1.1 cm) and eluted with 200 ml 0.0-0.7 M KPO_4 gradient. The restriction endonuclease activity was found in three fractions at 0.16-0.20 M phosphate. These fractions were pooled and dialyzed against PME buffer and concentrated by dialysis against 65% poly ethy-

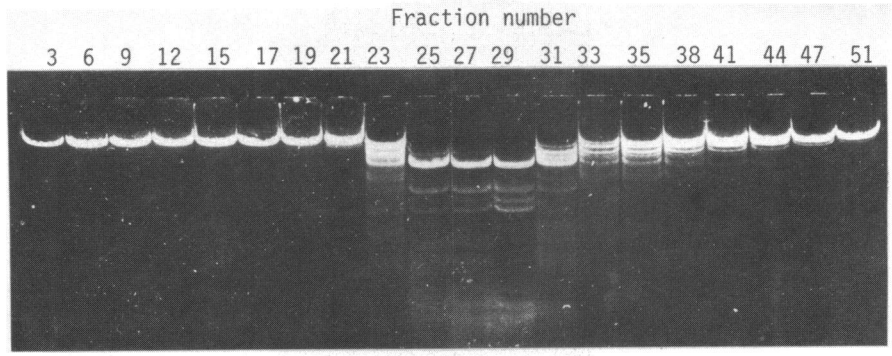

Fig. 1. Endonuclease activity in column fractions after DEAE-cellulose chromatography of a crude extract of S. aureus PS 96. Elution was performed with a KCl gradient (0.0-0.7 M). 10 µl of each fraction was incubated with 0.5 µg of λ DNA and the digested DNA was subjected to electrophoresis on 1.4% agarose gels.

leneglycol and 50% glycerol in PME buffer, consecutively. A yield of about 1000 units of purified enzyme was obtained. The purified enzyme had the same cleavage specificity as the crude extract on DNA's of λ , ØX 174 and several staphylococcal phages. No endonucleolytic activities with other specificities than that of Sau96 I were found during the purification procedure. The activities of Sau96 I in the crude extracts from the restriction-deficient PS 96 mutants R_5 and $R_{5.16}$ were at least 200 times lower than the activity in wild type PS 96 extracts.

Characterization of Sau96 I

For a further characterization of the substrate specificity of Sau96 I various DNA preparations were incubated with purified enzyme (Fig. 2). It appears that ØX 174 RF DNA is cleaved into 2 fragments, adenovirus type 5 and λ DNA into many fragments and phage 71 DNA into 3 fragments. Phage 16 DNA is not cleaved by Sau96 I. This staphylococcal phage is identical to phage 71 except that this phage has been propagated on S. aureus PS 16. The latter strain belongs to the same lytic group (group XIII) as PS 96 and also contains a restriction endonuclease with the specificity of Sau96 I. Apparently, phage 16 is modified by passage on PS 16 and is protected against the endonucleolytic attack of Sau96 I.

Determination of the recognition site of Sau96 I

The recognition site of Sau96 I was characterized by determination of the

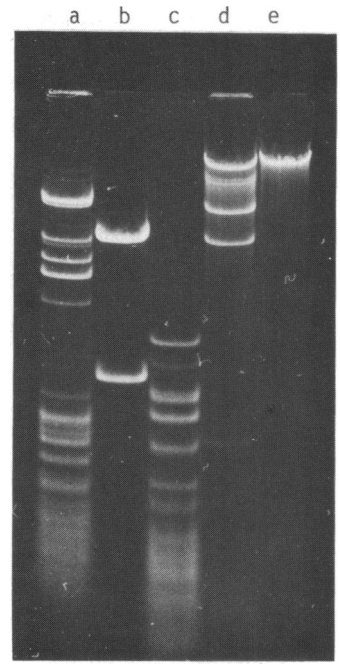

Fig. 2. Substrate specificity of Sau96 I purified by DEAE-cellulose and phosphocellulose chromatography. Sau96 I was incubated with 1 µg of the following DNA's: a, phage λ; b, ØX 174 RF; c, adenovirus type 5; d, staphylococcal phage 71; e, staphylococcal phage 16. Electrophoresis was performed on 1.4% agarose gels.

5'-nucleotide sequence of the DNA fragments produced by this enzyme. Furthermore, a number of DNA's were cleaved of which the complete nucleotide sequence is known viz. ØX 174 DNA (13), SV40 DNA (personal communication, S.M.Weissman) and the adenovirus type 5 Sma I-K fragment (personal communication, H. van Ormondt and J. Maat).

For the analysis of the 5'-sequences adenovirus type 5 DNA was cleaved with Sau96 I, digested with bacterial alkaline phosphatase to remove 5'-phosphate groups and subsequently labeled at the 5'-ends with ^{32}P-phosphate using T_4 polynucleotide kinase and γ-^{32}P-ATP (see Materials and Methods). Complete digestion of this ^{32}P-labeled DNA to 5'-mononucleotides with snake venom phosphodiesterase followed by electrophoresis on Whatman 540 and DEAE paper of the mononucleotides showed that more than 99% of the radioactivity was present in pG, while traces of radioactivity were found in the three other nucleotides. For a further analysis of the 5'-terminal nucleotide se-

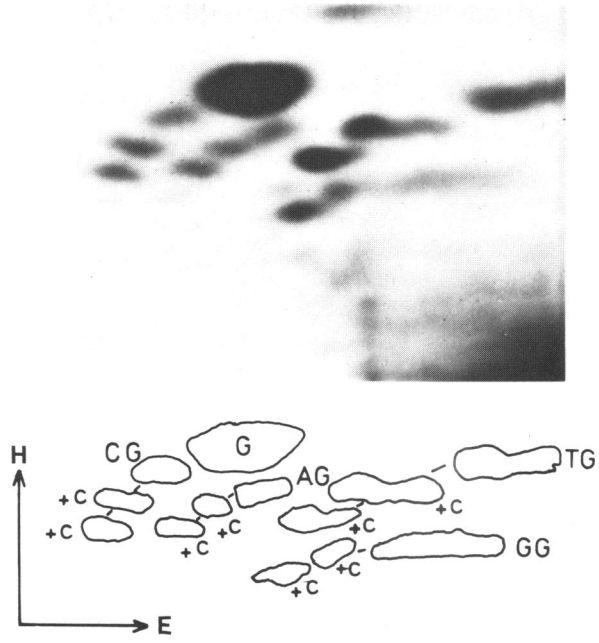

Fig. 3. Autoradiogram and schematic representation of the two-dimensional fractionation of oligonucleotides obtained after partial snake venom phosphodiesterase digestion of adenovirus type 5 DNA fragments produced by Sau96 I. E and H refer to the directions of electrophoresis and homochromatography.

quences of the cleavage sites the 5'-^{32}P-labeled DNA fragments were partially digested with snake venom phosphodiesterase. The digestion products were separated two-dimensionally by electrophoresis on cellulose acetate (1st dimension) followed by homochromatography on PEI-cellulose (2nd dimension). The autoradiogram showed that the fragment strands terminate specifically with the sequence 5'-G-N-C-C---- inwhich N stands for any one of the four nucleotides (Fig. 3).

This sequence suggests that Sau96 I recognizes the palindromic sequence 5'-G↓G-N-C-C-3' and cleaves as indicated by the arrows. This suggestion was
3'-C-C-N↓G-G-5'
confirmed by determination of the sizes of the fragments produced by cleavage of DNA's of SV40, ØX 174 RF and the Sma I-K fragment of adenovirus type 5 DNA with Sau96 I. Table I shows that the observed lengths for the Sau96 I fragments are in very good agreement with those expected to be generated by a restriction enzyme recognizing the sequence mentioned above.

Table I Cleavage of various DNA's by Sau96 I[a]

DNA	Position of GGNCC	Sequence around the position of GGNCC	Predicted size of fragment	Observed size of fragment
SV40	457	AGGTCCA		
			31	-
	506	GGGACCT		
			430	410
	936	GGGTCCA		
			489	460
	1425	TGGCCCC		
			506	520
	1931	AGGACCA		
			245	260
	2176	GGGCCCT		
			540	560
	2716	GGGCCCT		
			373	395
	3089	TGGCCCT		
			385	395
	3474	TGGTCCT		
			1582	1625
	5056	AGGTCCT		
			664	690
ØX 174	977	CGGCCCT		
			4054	4050
	5031	AGGACCT		
			1321	1325
Ad5 Sma I-K			351	370
	351	AGGGCCG		
			265	260
	616	TGGACCA		
			96	90
	712	CGGCCCC		
			166	160
	878	GGGTCCG		
			129	115

[a]The sizes of the fragments were determined by polyacrylamide gelelectrophoresis in the presence of markers of known sizes. The position of the first G residue in the sequence GGNCC is indicated and refers to the DNA sequences of ØX 174 RF DNA (13), SV40 DNA (personal communication, S.M.Weissman) and adenovirus type 5 (Ad5) Sma I-K fragment (personal communication, H. van Ormondt and J.Maat) respectively.

The cleavage sites in ØX 174 RF DNA were localized more precisely by double digestions of this DNA with Sau96 I plus Hae II, Sau96 I plus Hae III and Sau96 I plus Hae II and Pst I, respectively (results not shown). From these double digestions it was derived that the two Sau96 I cleavage sites in ØX 174 RF DNA are located at position 990 $\pm$ 40 basepairs and at position 5040 $\pm$ 20 basepairs in the Sanger sequence (13), respectively. This is in agreement with the sequences GGACC at position 5031-5035 and GGCCC at position 977-982 in this map, respectively.

Finally, SV40 DNA was digested with Sau96 I and Asu I, respectively. It has been reported that the latter enzyme also recognizes and cleaves the

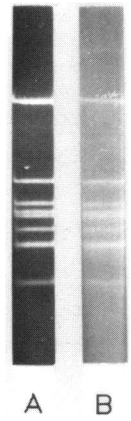

Fig. 4. Electrophoresis in 1.4% agarose gels of SV40 DNA digested with Sau96 I (A) and Asu I (B).

sequence 5'-G↓G-N-C-C-3' (personal communication S.G.Hughes, T.Bruce and
3'-C-C-N↑G-G-5'
K.Murray). Fig. 4 shows that both enzymes yield an identical cleavage pattern of SV40 DNA which supports the notion that both enzymes recognize and cleave the above sequence.

DISCUSSION

S. aureus strains belonging to the lytic group XIII carry a characteristic restriction-modification system (1,8, van Leeuwen, unpublished results). A representative strain of this group, PS 96, was shown to produce a restriction endonuclease, which we have designated Sau96 I. This enzyme is part of a biological restriction-modification system, because modified staphylococcal phage 16 DNA is not cleaved by Sau96 I, whereas the unmodified DNA of the homologous phage 71 is cut into three fragments. Furthermore the Sau96 I activity in two restriction-deficient mutants of PS 96 was highly reduced. These results indicate that Sau96 I plays a role in the restriction of staphylococcal bacteriophages. Preliminary studies on restriction activities in strains of other lytic groups have shown that the presence of Sau96 I is characteristic for strains of group XIII.

Sau96 I was purified from PS 96 by DEAE-cellulose and phosphocellulose chromatography. The enzyme is very stable for several months in purified form at -20°C in 50% glycerol.

Analysis of the 5'-termini of DNA fragments produced by Sau96 I has

revealed that the enzyme recognizes the sequence 5'-G↓G-N-C-C-3' and cleaves
3'-C-C-N↓G-G-5'
as indicated by the arrows. A restriction enzyme with an identical specificity has been isolated from Anabaena subcylindrica (personal communication, S.G.Hughes, T.Bruce and K.Murray).

Genetic studies suggested that in PS 96 more than one restriction-modification system is present (unpublished observations). However, no other class II restriction enzyme than Sau96 I was found. It is well conceivable that in the other system a class I restriction enzyme is involved which is more difficult to detect and requires other cofactors than those used in our assay (14).

ACKNOWLEDGEMENTS

The authors thank Miss M.G.Kuijk for technical assistance, dr. J. ter Schegget and Miss J.Zandberg for gifts of DNA, dr. J.Vereyken and dr. A. de Waard for restriction enzymes and drs. S.M.Weissman, H. van Ormondt and J.Maat for communicating nucleotide sequence data prior to publication. This investigation was supported in part by the Netherlands Foundation for Chemical Research with financial aid from the Netherlands Organization for the Advancement of Pure Research.

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