Volume 13 Number 24 1985 Nucleic Acids Research

*Acc*III, a new restriction endonuclease from *Acinetobacter calcoaceticus*

Keiko Kita, Nobutsugu Hiraoka, Atsushi Oshima, Satoko Kadonishi and Akira Obayashi

Central Research Laboratories, Takara Shuzo Co. Ltd., Seta 3-4-1, Otsu-shi, Shiga 520-21, Japan

Received 6 November 1985; Accepted 26 November 1985

ABSTRACT

A new site-specific restriction endonuclease, AccIII, was isolated from Acinetobacter calcoaceticus. AccIII recognizes T↓CCGGA and cleaves at the position shown by the arrow. AccIII activity was inhibited by adenine methylation at the overlapping dam methylase recognition sequence.

INTRODUCTION

Although many site-specific restriction endonucleases have been isolated from various kinds of bacteria, some palindromic sequences, which are not yet found to be recognized by known restriction endonucleases, still exist (1). The two restriction endonucleases, AccI and AccII, in Acinetobacter calcoaceticus have been isolated and their recognition sequences and cleavage sites have been reported (2,3). The presence of a third activity was suggested by Roberts but its details were unknown (2). However, upon reexamining A. calcoaceticus, we found a new endonuclease, AccIII, which recognized a new palindromic sequence and some of its properties were investigated.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

A. calcoaceticus (kindly donated from M. Takanami) was used. Cells were aerobically incubated at 37°C in L-broth (10 g bacto-tryptone, 5 g yeast extract, 1 g glucose and 5 g NaCl per liter, pH 7.2) and harvested by centrifugation when it reached the late logarithmic phase. The yield of cells per liter culture was about 8 g (wet weight).

DNA and Enzymes

λ phage DNA (Dam$^+$λ DNA) was prepared from Escherichia coli K-12

W3350 (λ cI857 S7) lysogen by phenol treatment of phage particles banded in a CsCl gradient according to the procedure of Thomas and Davis (4). N^6-methyl-adenine-free λ DNA (Dam^- λ DNA) was purchased from New England Biolabs. Dam^+ and Dam^- pBR322 DNAs were isolated from E. coli C600 and GM33 (CGSC strain kindly supplied by A. Oka) cells by the procedure of Guerry et al. (5), respectively. ϕX174 RFI DNA was isolated from E. coli Cn infected with ϕX174 am3 by a modification of the method of Ueda et al. (6). Decanucleotide d(GTTCCGGAAC) was synthesized by the solid phase method (7). Adenovirus-2 (Ad2) and SV40 DNA were purchased from Bethesda Research Laboratories, Inc. Restriction endonucleases EcoRI, HindIII, PstI, SalI and HincII were prepared from E. coli RY13 (8), Haemophilus influenzae Rd(9), E. coli ED8654 carrying pBR322 with a PstI gene insertion (10), Streptomyces albus G (11), and Haemophilus influenzae Rc (12), respectively.

Assay of AccIII endonuclease

Enzyme activity was measured in a reaction mixture (50 µl) containing 10 mM Tris-HCl, pH 7.5, 7 mM $MgCl_2$, 60 mM NaCl, 7 mM 2-mercaptoethanol, 0.01% bovine serum albumin, and 1 µg of DNA. One unit was defined as the amount of enzyme required to digest 1 µg of λ DNA completely in 60 min at 37°C.

Purification of AccIII

349 g of cells were suspended in 10 mM potassium phosphate, pH 7.5, and 10 mM 2-mercaptothanol, and disrupted by sonication. Debris was removed by centrifugation (10^5 x g for 1 hr). The supernatant was treated with 50% (w/v) ammonium sulfate and the precipitate was collected and suspended in KP buffer (10 mM potassium phosphate, pH 7.5, 10 mM 2-mercaptoethanol, and 5% glycerol). After dialysis against this buffer, the enzyme solution was applied on to a phosphocellulose (Whatman P11) column (bed volume 300 ml), and eluted with a linear gradient of 0 - 1.0 M KCl in KP buffer. The AccIII was eluted at 0.65 - 0.70 M KCl, and was separated from the other two endonucleases, AccI and AccII. The AccIII fractions were pooled, dialyzed, and applied on to an Affi-Gel Blue agarose (BioRad) column (bed volume 4 ml). The AccIII fraction was passed through the column. The active fractions were applied on to a

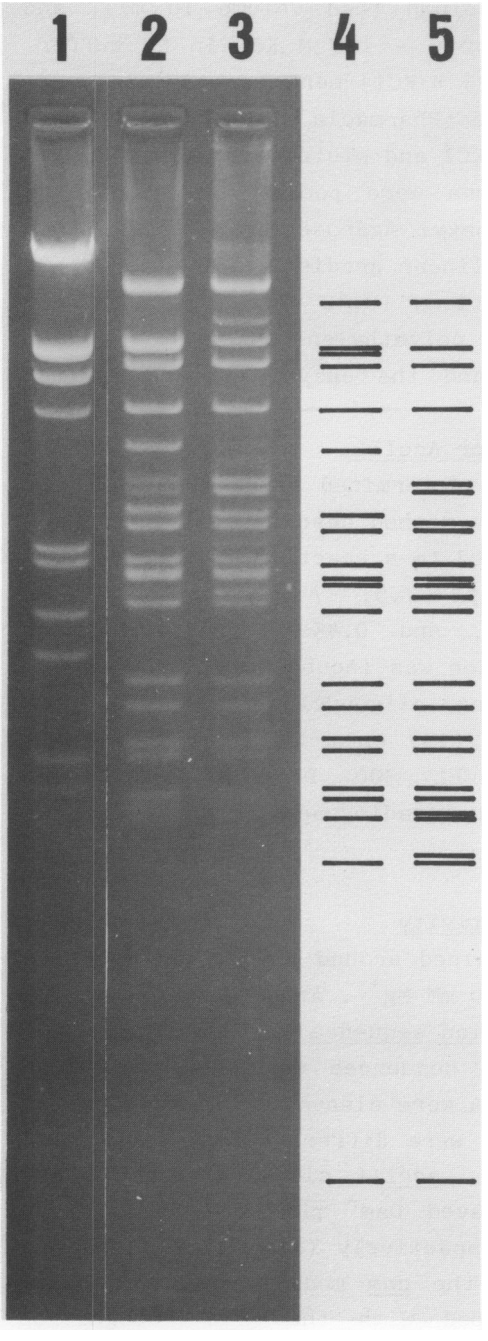

Fig. 1. Agarose gel electrophoresis of Dam^+ and Dam^- λ DNA cleaved with AccIII. Lane 1 is molecular weight marker (λ-HindIII·EcoRI fragments). AccIII was incubated with Dam^+ (lane 2) and Dam^- (lane 3) DNA. Lane 4 (Dam^+) and lane 5 (Dam^-) are computed patterns of AccIII cleaved λ DNA.

DEAE-cellulose (Whatman DE52) column (bed volume 10 ml), and eluted with a linear gradient of 0 - 1.0 M KCl in KP buffer. The active fractions (0.20 - 0.25 M KCl) were pooled, dialyzed, applied on to a Heparin-sepharose (Pharmacia CL-6B) column (bed volume 4 ml), washed with 0.7 M KCl and eluted with 1.0 M KCl in KP buffer. The active fractions were pooled, dialyzed, and finally applied on to an Aminohexyl Agarose (BRL) column (bed volume 4 ml), and eluted with a linear gradient of 0 - 1.5 M KCl in KP buffer. The active fractions (0.32 - 0.65 M KCl) were pooled, and concentrated with polyethyleneglycol. An equal volume of glycerol was added and the enzyme preparation was stored at -20°C.

Determination of cleavage site for AccIII

The cleavage site of AccIII was determined using the synthetic decanucleotide d(GTTCCGGAAC) by a method described elsewhere (3). AccIII (0.4 units) was added to a reaction mixture (20 µl) containing 10 mM Tris-HCl, pH 7.5, 7 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 60 mM NaCl, and 0.45 µM 5'-^{32}P-labeled oligonucleotides, and the solution was incubated at 37°C for 30 to 120 minutes. The resulting oligonucleotides were then separated by homochromatography (13) on a DEAE-cellulose thin layer plate (Masherey-Nagel CEL 300 DEAE/HR-2/15) using Homomixture III and detected by autoradiography.

RESULTS

Optimal conditions for AccIII activity

Maximum AccIII activity was obtained around 60-65°C in a buffer containing 150 mM KCl or NaCl, 20 mM Mg^{++}, at pH 8.5.

Determination of AccIII recognition sequence

Phage and plasmid DNA of known sequences were incubated with AccIII. Both Dam^+ and Dam^- λ DNA were cleaved with AccIII at 20 or more sites, but the patterns were different from each other at several fragments (Fig. 1). AccIII did not cleave SV40, φX174 or Dam^+ pBR322, but cleaved Dam^- pBR322 and Ad2 at a unique site and eight sites, respectively (Fig. 2). As we had described for MflI (14), when the dam modification sequence, GATC (15), completely overlapped with the MflI recognition sequence, Dam^+ DNA was not cleaved at all. However, when the

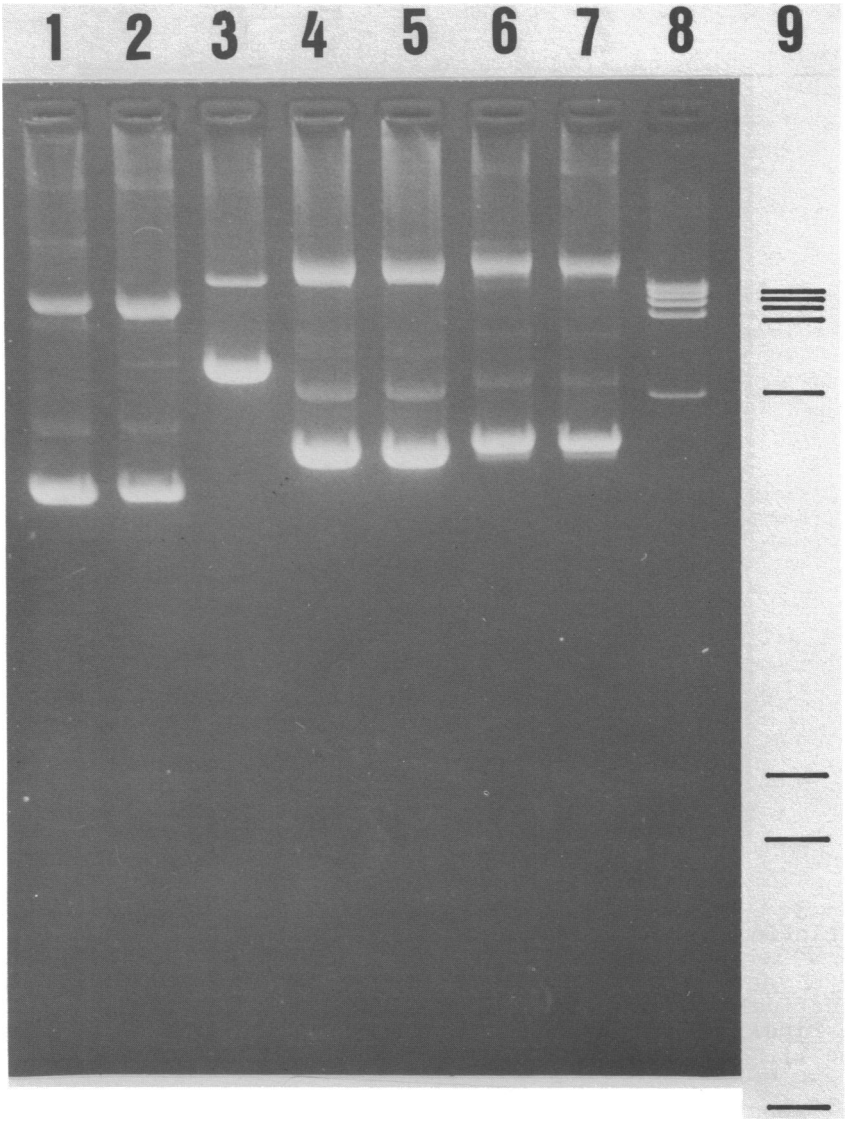

Fig. 2. Agarose gel electrophoresis of DNA cleaved with AccIII.
Various DNA were incubated with AccIII. Lane 1, Dam^+ pBR322; lane 2, Dam^+ pBR322+AccIII; lane 3, Dam^- pBR322+AccIII; lane 4, SV40; lane 5, SV40+AccIII; lane 6, ϕX174; lane 7, ϕX174+AccIII; lane 8, Ad2+AccIII (The small molecular bands of lane 8 were too small to be visible.); lane 9, computed pattern of lane 8.

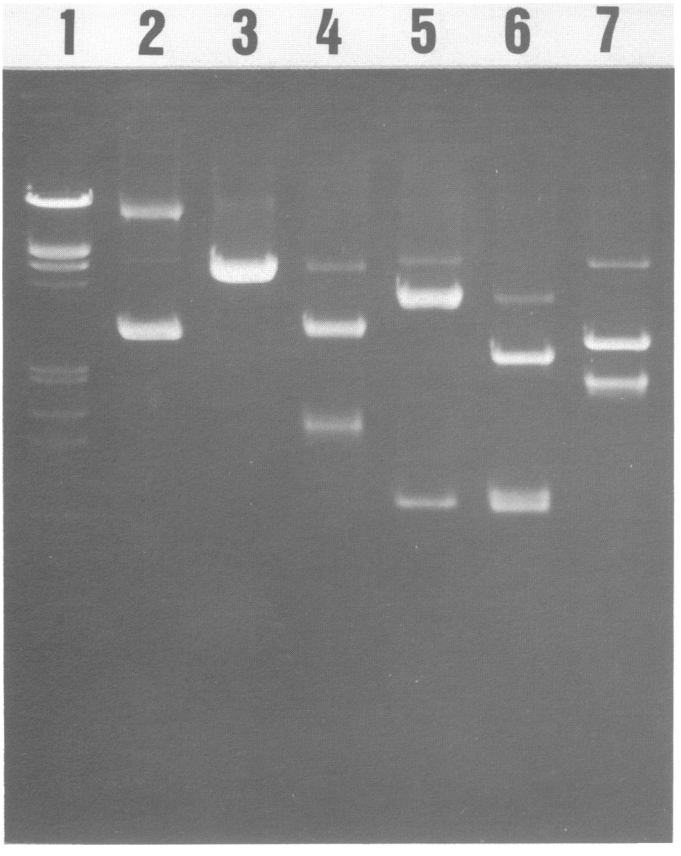

Fig. 3. Double digestion patterns of AccIII and other restriction endonucleases on Dam⁻ pBR322 DNA.
Lane 1 is molecular weight marker (λ-HinIII·EcoRI fragments). Lane 2 and lane 3 are Dam⁻ pBR322 and its AccIII digests, respectively. Dam⁻ pBR322 AccIII digests were further cleaved with HindIII (lane 4), SalI (lane 5), HincII (lane 6), and PstI (lane 7).

dam modification sequence partly overlapped, Dam⁺ DNA was cleaved but the patterns were different from those of Dam⁻ DNA, e.g. ClaI (16). We presumed that GATC partly overlapped at some of the AccIII recognition sites. Double digestion of Dam⁻ pBR322 with AccIII and with each of HindIII, SalI, HincII, and PstI, showed that a unique cleavege site was assigned around 1700 bp (Fig. 3). Furthermore, the computer search of the GATC sequence

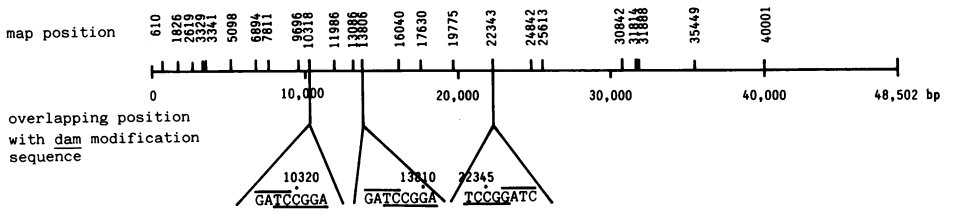

Fig. 4. Mapping of AccIII restriction sites in λ DNA. Numbering of the nucleotide sequence begins with the first base of the left end. The map positions are shown as the number of the first 5′ base in the AccIII recognition sequence. The positions partly overlapped with dam methylase modification sequence are indicated below the line.

around the 1700 bp region indicates that GATC appeared three times in the 1459 to 3040 bp region (17,18). However, since the TCCGGATC which is present at 1664 to 1671 was closest to the unique cleavage site which was assigned around 1700 bp as mentioned above, it is suggested that AccIII recognizes the palindromic sequence TCCGGA, which is present at the 1664 to 1669 bp region.

A computer search of TCCGGA through the sequence of λ, pBR322, SV40, ϕX174, and Ad2 indicates that this sequence should respectively occur at 24, 1, 0, 0, and 8 different sites (19,17,18,20,21,22). These are compatible with the digestion patterns of these DNA with AccIII, as shown in Fig. 1 and 2.

The recognition sequence was confirmed on a complete AccIII map of λ DNA (19). As shown in Fig. 4, the AccIII recognition sequence and the dam methylase recognition sequence are partially overlapped at three positions, 10320, 13810, and 22345 bp. Knowing that AccIII activity was inhibited at the three positions, the fragment sizes of both Dam^+ and Dam^- λ DNA were computed and are shown in Fig. 1 (lanes 4 and 5). As expected, the patterns produced by AccIII cleavage were the same as those computed from the nucleotide sequence.

Determination of AccIII cleavage site

The cleavage site of AccIII was identified using a synthetic oligonucleotide d(GTTCCGGAAC). The oligonucleotide was labeled with ^{32}P at the 5'-terminus, and then digested with AccIII. The resulting labelled oligonucleotides were then separated and

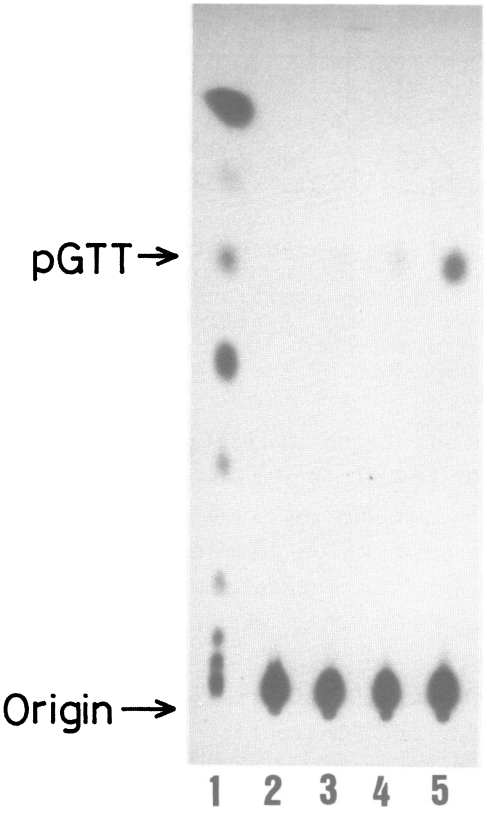

Fig. 5. Identification of oligonucleotides produced from synthetic decanucleotide by digestion with AccIII.
Lane 1 is of authentic markers obtained by digestion with venom phosphodiesterase. The decanucleotide was digested with AccIII for different times (lane 2, 0 min; lane 3, 30 min; lane 4, 60 min; lane 5, 120 min).

detected by autoradiography. The trinucleotide ^{32}P-GTT was found to be the product of AccIII digestion (Fig. 5). This shows that AccIII cleaves the substrate at GTT↓CCGGAAC, where indicated by the arrow.

DISCUSSION

This is the first report of a type II restriction endonuclease whose recognition sequence is TCCGGA. AccIII required different conditions for maximum activity from those of the other two

endonucleases in A. calcoaceticus. The optimum salt concentration for AccIII activity was 150 mM, at which concentration AccI and II activities were considerably inhibited (unpublished observation). Although the optimum temperature for AccI and II's maximum activity and the growth of A. calcoaceticus was around 30-37°C (23), AccIII showed maximum activity around 60-65°C. These differences show that in A. calcoaceticus, AccIII may play a distinct role from those of the other two enzymes.

Inhibition of cleavage due to overlapping E. coli dam or dcm modification sites have been observed for several restriction endonucleases (1). Partly overlapping with dam modification resulted in resistance to cleavage by ClaI (GATCGAT), HphI (GGTGATC), MboII (GAAGATC), NruI (GATCGCGA), TaqI (GATCGA), and XbaI (GATCTAGA) (24). Here, AccIII was found to be inhibited by such a modification.

AccIII cleaves between T and C in the recognition sequence, TCCGGA, and produces 5′protruding tetranucleotides, CCGG. The AccIII digestion products could be ligated directly with Cfr10I ((A/G)↓CCGG(T/C)), Cfr9I, XcyI, and XmaI (C↓CCGGG) fragments, which all have the CCGG sequence at the 5′termini (1).

ACKNOWLEDGMENT

We are grateful to Drs. M. Takanami and A. Oka, Institute of Chemical Research, Kyoto University, for supplying bacterial strains, and to Mr F. Kimizuka for synthesizing oligonucleotides.

REFERENCES

1. Kessler, C., Neumaier, P.S., and Wolf, W. (1985) Gene, 33, 1-102.
2. Roberts, R.J. (1985) Nucleic Acids Research, 13, r165-r200.
3. Kita, K., Hiraoka, N., Kimizuka, F., and Obayashi, A. (1984) Agric. Biol. Chem., 48, 531-532.
4. Thomas, M., and Davis, R.W. (1975) J. Mol. Biol., 91, 315-328.
5. Guerry, P., LeBlanc, D.J., and Falkow, S. (1973) J. Bacteriol., 116, 1064-1066.
6. Ueda, K. Morita, J., and Komano, T. (1981) J. Antibiot., 34, 317-322.
7. Ito, H., Ike, Y., Ikuta, S., and Itakura, K. (1982) Nucleic Acids Research, 10, 1755-1769.

8. Greene, P.J., Betlach, M.C., Goodman, H.M., and Boyer, H.W. (1974) Methods in Molecular Biology, 7, 87-111.
9. Old, R., Murray, K., and Roizes, G. (1975) J. Mol. Biol., 92, 331-339.
10. Walder, R.Y., Hartley, J.L., Donelson, J.E., and Walder, J.A. (1981) Proc. Natl. Acad. Sci. USA, 78, 1503-1507.
11. Arrand, J.R., Myers, P.A., and Roberts, R.J. (1978) J. Mol. Biol., 118, 127-135.
12. Landy, A., Ruedisueli, E., Robinson, L. Foeller, C., and Ross, W. (1974) Biochemistry, 13, 2134-2142.
13. Jay, E., Bambara, R., Padmanabhan, R., and Wu, R. (1974) Nucleic Acids Research, 1, 331-353.
14. Hiraoka, N., Kita, K., Nakajima, H., and Obayashi, A. (1984) J. Ferment. Technol., 62, 583-588.
15. Geier, G.E., and Modrich, P. (1979) J. Biol. Chem., 254, 1408-1413.
16. Mayer, H., Grosschedl, R., Schütte, H., and Hobom, G. (1981) Nucleic Acids Research, 9, 4833-4845.
17. Sutcliffe, J.G. (1979) Cold Spring Harbor Symp. Quant. Biol., 43, 77-90.
18. Peden, K.W.C. (1983) Gene, 22, 277-280.
19. Sanger, F., Coulson, A.R., Hong, G.F., and Petersen, G.B. (1982) J. Mol. Biol., 162, 729-773.
20. Fiers, W., Contreras, R., Haegeman, G., Rogiers, R., van de Voorde, A., van Heuverswyn, H., van Herreweghe, J., Volckaert, G., and Ysebaert, M. (1978) Nature, 273,113-120.
21. Sanger, F., Coulson, A.R., Friedman, T., Air, G.M., Barrell, B.G., Brown, N.L., Fiddes, J.C., Hutchison III, C.A., Slocombe, P.M., and Smith, M. (1978) J. Mol. Biol., 125, 225-246.
22. EMBL nucleotide sequence data library, release 5.0 (April, 1985)
23. Lautop, H. (1974) in Bergey's Manual of Determinative Bacteriology, Eighth edition, Buchanan, R.E., and Gibbons, N.E. Eds, pp.436-438, The Williams and Wilkins Company, Baltimore.
24. Nelson, M., Christ, C., and Schildkraut, I. (1984) Nucleic Acids Research, 12, 5165-5173.