Volume 10 Number 24 1982 Nucleic Acids Research

ScrFI: a new sequence-specific endonuclease from *Streptococcus cremoris*

G.F.Fitzgerlad, C.Daly[1], L.R.Brown and T.R.Gingeras*

Department of Dairy and Food Microbiology, University College, Cork, Ireland, and *Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA

Received 13 August 1982; Revised and Accepted 24 November 1982

ABSTRACT

A novel sequence-specific endonuclease has been isolated from *Streptococcus cremoris* F. ScrFI recognises the sequence:

5'CC↓NGG3'
3'GGN↑CC5'

and cleaves as indicated by the arrow (↓). It is the first enzyme to recognise this sequence and the first endonuclease reported from the lactic streptococci used in dairy fermentations.

INTRODUCTION

The contribution of type II restriction enzymes to recombinant DNA technology has stimulated extensive searches for these enzymes such that at the present time 355, with at least 86 different specificities, have been demonstrated in a variety of bacteria (2). However, in most cases, there is no direct genetic evidence for the presence of a restriction-modification (R-M) system (3, 4). A total of 6 type II restriction enzymes have been isolated from members of the genus *Streptococcus* as follows: *Str. faecalis* (SfaI, SfaGUI, SfaNI, Sfa91161), *Str. zooepedemicus* (SzoI) and *Str. agalactiae* (SagI) (2, 5). Enzyme activity was not detected in two *Str. lactis* strains examined (5). In our studies on the industrially important Group N lactic streptococci we have demonstrated several R-M systems operating on bacteriophage (6) in agreement with other workers (7, 8) but the presence of restriction enzymes in these bacteria has not been reported. Here we describe the isolation of a type II restriction enzyme, ScrFI, from *Streptococcus cremoris* F, a strain isolated from a mixed strain starter culture used in Cheddar cheese manufacture.

METHODS AND MATERIALS

Cultures Used:

Streptococcus cremoris strains F and KH were transferred routinely

© IRL Press Limited, Oxford, England.

0305-1048/82/1024-8171$2.00/0

at 30°C in Lactic Streptococcal Broth (LSB) a medium similar to M17 (9) containing (g/l); lactose 10g, tryptone 5g, tryptose 5g, yeast extract 5g, lab-lemco 10g and B-glycerophosphate 7.2g, pH 7.4. _E. coli_ λCI857S7 was grown in Luria Broth at 30°C.

Growth of Bacteriophage and Isolation of Bacteriophage DNA:

Bacteriophage λ was isolated by induction from _E. coli_ λCI857S7, concentrated by centrifugation (10) and purified on a caesium chloride block density gradient. Bacteriophage f.KH was propagated to high titre on _Str. cremoris_ KH and, after successive centrifugation at 10,000 rpm x 30 min and 20,000 x 2h on a Beckman J-21C centrifuge, purified on a CsCl block gradient. DNA from both λ and f.KH was isolated by extracting with phenol. CsCl was removed by dialysis against 2000 volumes of buffer (0.025M Tris-HCl, pH 7.5, 0.01M Na_2EDTA and 0.1% SDS) for 2 hours, while dialysis against 4000 volumes of TE buffer (0.01M Tris-HCl, pH 7.5, 0.001M EDTA) for 2 days at 4°C removed the phenol.

The other DNA substrates, SV40, ØX174 and pBR322 and the enzyme Sau 3A were obtained from Bethesda Research Laboratories (BRL) or New England Biolabs. Adenovirus-2 DNA and the enzymes EcoRII, BstNI and NciI were provided by R. Roberts, Cold Spring Harbor Laboratory. TaqI, HindIII and PstI were purchased from the Boehringer Corporation. Some ScrFI was also supplied by New England Biolabs.

Enzyme Assays and Agarose Gel Electrophoresis:

The reaction buffer used for EcoRII, BstNI and NciI was 0.006M Tris-HCl pH 8.0, 0.006M $MgCl_2$ and 0.006M $SHCH_2CH_2OH$. For other enzymes the buffers suggested by the supplier were used. The reaction mixtures (40 ul) were incubated at 37°C or at 65°C (in the case of TaqI and BstNI) and the reactions were stopped by the addition of 10 ul of a loading dye solution containing 4.0M urea, 0.05M Na_2EDTA, 50% sucrose and 0.1% bromophenol blue.

Samples were electrophoresed for 2½ - 3½h on 1.0%, 1.5% or 2.0% agarose (HGT Agarose, Miles, Stoke Poges, England) in Tris Borate (0.089M Tris-HCl, pH 8.0, 0.0025M EDTA, 0.089M boric acid and ethidium bromide, 0.05 ug/ml) as the running buffer. The gels were visualized under short-wave ultraviolet illumination (254 nm) and photographed.

Isolation and Purification of ScrFI:

Frozen cells (15g wet weight from 3 litres of stationary phase culture) were resuspended in 30 mls extraction buffer (0.05M Tris-HCl, pH 7.5, 0.001M Na_2EDTA and 0.007M $SHCH_2CH_2OH$) and sonicated (12 x 30 sec) at 7.5 u using a 1.0 cm probe (MSE model 100 w). After successive

centrifugation at 10,000 rpm x 30 min, the supernatant was made 30% and 70% with $(NH_4)_2 SO_4$. After a further centrifugation, the pellet from the 70% $(NH_4)_2 SO_4$ back-cut was resuspended in 10 ml of PC buffer (10% glycerol, 0.01M potassium phosphate, pH 7.4, 0.01M $SHCH_2CH_2OH$ and 10^{-4}M Na_2EDTA), and dialysed against 300 volumes of this buffer. This material (5 ul) was incubated with 1 ug DNA (in 0.02M potassium phosphate buffer, pH 7.0, containing 0.007M $MgCl_2$) at 37°C for 1 h. Electrophoresis on a 1% agarose gel showed specific restriction activity as well as nonspecific nuclease activity. The active sample was applied to a DEAE cellulose (DE 52, Whatman) column (8.0 x 2.5 cm diameter) which had been equilibrated with PC buffer. After washing with PC buffer, the column was eluted with a linear gradient (250 ml total volume) from 0 to 0.8M KCl in PC buffer. Active ScrFI enzyme eluted between 0.45 and 0.65M KCl in fraction numbers 25 - 34. Exonuclease activity was evident in fractions 11 - 15. The active fractions were pooled, dialysed against PC buffer and applied to a phosphocellulose (Whatman P11) column (8.0 x 2.8 cm diameter). After washing with PC buffer, fractions (5.0 ml) were eluted with an 0 to 1M KCl linear gradient (250 ml total volume). Enzyme activity was detected in the main protein peak at 0.6 - 0.7M KCl and was shown to be essentially free of nonspecific nuclease activity. The active fractions were pooled, dialysed against PC buffer and concentrated by dialysis against solid polyethylene glycol 6000. Further enzyme concentration was achieved using an hydroxylapatite (Bio-Gel HTP) column. After washing with 2 volumes of 0.01M and 0.1M KP buffer, pH 7.5, the enzyme was batch eluted with 2.0 ml of 0.25M KP buffer, pH 7.5, dialysed against 0.02M KP buffer, pH 7.0, 0.001M Na_2EDTA, 0.01M $SHCH_2CH_2OH$ and 80% glycerol for 1h. The enzyme yield was in excess of 7,000 units/gram wet weight of cells, based on the criterion that one unit of ScrF1 completely digested one ug of λ DNA in one hour at 37°C.

Factors Affecting Activity of ScrFI:

The effects of temperature, pH, $MgCl_2$, $MnCl_2$, NaCl and KCl on enzyme activity were examined. The concentrated enzyme preparation was diluted in buffer (0.01M potassium phosphate, pH 7.2, 0.005M Na_2EDTA, 0.005M $SHCH_2CH_2OH$, 500 ug/ml BSA, 50% glycerol and 0.2% Triton X) to a level which yielded partial digests of substrate (phage f.KH) DNA, chosen because the low number of bands generated made it easier to detect partials (6).

Determination of Recognition Sequence

ScrFI was used in single and double digests with PstI, EcoRII and BstNI on pBR322, SV40, Ad2 and ØX174 RF DNA's. The sized fragments from each of these digests were used as raw data for a computer program designed to predict recognition sequences of restriction endonucleases (11). The computer predicted recognition site was verified and the cleavage site determined using Ad2 DNA and procedures identical with those described previously (12). Ad2 DNA was digested with ScrFI, the 5'terminal phosphate removed with alkaline phosphatase (Boehringer) and replaced by using polynucleotide kinase (13) and {gamma-32P} ATP (14). A portion of the labeled fragments was digested with pancreatic DNase (Sigma) and snake venom phosphodiesterase (Sigma) and the resulting mononucleotides analyzed by electrophoresis on Whatman 540 paper at pH 3.5. Another portion of the labeled fragments was treated with exonuclease I (15) in order to determine the 5'terminal dinucleotide sequence. The remainder of the labeled fragments was treated with pancreatic DNase and fractionated by electrophoresis on cellulose acetate at pH 3.5 in the presence of 7M urea followed by homochromatography (16) using homomix VI (17). The resulting fingerprints were exposed to autoradiographs to determine the recognition sequence based on the mobility shifts of each succeedingly longer oligonucleotide (18).

RESULTS AND DISCUSSION

Isolation of ScrFI:

The Group N or lactic streptococci are important in dairy fermentations. Bacteriophage attack is a significant problem in commercial strains. Biological evidence of phage DNA restriction in some strains is available and is considered to be a factor in phage insensitivity (6, 7, 19, 20). In an attempt to determine the biochemical nature of the restriction activity present we examined Str. cremoris strains F, KH, C3, AM1 and SK11G and Str. lactis strains C10 and SK_3 and Str. lactis subsp. diacetylactis strain 18-16 using λ DNA as substrate. Specific endonuclease activity was detected only in the case of Str. cremoris F. This activity was present in the 70% $(NH_4)_2SO_4$ precipitate and was freed from non-specific exonuclease activity by the two-column procedure described in the methods section.

The effects of several parameters on the activity of ScrFI were examined. There was an absolute requriement for either Mg^{++} (range 1-40mM) or Mn^{++} (range 1-6mM), the optimum concentrations being 7mM and 4mM, respectively. The ability of Mn^{++} to substitute for Mg^{++} has been reported

for other restriction enzymes (21, 22). The divalent ion requirements of ScrFI were not satisfied by Ca^{++}. The presence of S-adenosylmethionine or ATP was not required for activity, nor did they stimulate activity, indicating that ScrFI is a classical type II restriction enzyme. Neither KCl (0-100mM) or NaCl (0-80mM) had any effect on enzyme activity but 100mM NaCl or greater did cause inhibition. ScrFI was active at temperatures between 21 and 48°C and pH's between 5.5 and 9.0, the optima being about 43°C and pH 7.0. Heating the enzyme at 40°C x 40 min had no effect but no activity was detected after heating at 58°C. No loss of activity was observed after storage of ScrFI in dilution buffer at -20°C for 8 months or at 4°C or room temperature for 2 months.

Determination of the Recognition Sequence of ScrFI

ScrFI was initially characterised by its action on Ad2 DNA (>50 sites), λDNA (>50 sites), pBR322 DNA (>10 sites), SV40 DNA (>10 sites) and ØX174 DNA (3 sites) (Fig. 1). Close examination of the digest pattern of ScrFI on SV40 DNA revealed that the sizes of the fragments detectable were identical (within experimental error) to the computer predicted pattern for EcoRII digestion of this DNA. Furthermore, a double digest of ScrFI and PstI on SV40 DNA gave a pattern identical to that predicted for an EcoRII / PstI double digest i.e. the largest ScrFI fragment was cleaved and the products comigrated with fragments 4 and 5 and of the original digest (data not shown). A direct comparison of the cleavage patterns of ScrFI and EcoRII on SV40 DNA and a double digest with these enzymes confirmed that they cleaved this substrate at the same recognition sequence i.e. CC^{A}/TGG (Fig. 2).

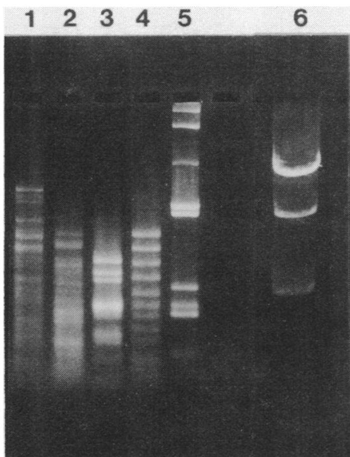

Figure 1. Agarose gel electrophoresis of ScrFI digests
The incubation and electrophoresis conditions are described in the Methods section.
Slot 1, ScrFI on λ DNA; Slot 2, ScrFI on Ad2 DNA; Slot 3, ScrFI on pBR322 DNA; Slot 4, ScrFI on SV40 DNA; Slot 5, TaqI on pBR322 DNA; Slot 6, ScrFI on ØX174 DNA.
The samples in slots 1-5 were fractionated on 1.5% Agarose. The sample in slot 6 was fractionated on 1% Agarose.

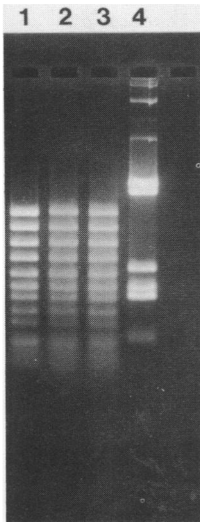

Figure 2. Agarose gel electrophoresis of ScrFI and EcoRII digests of SV40 DNA

The incubation and electrophoresis (1.5% Agarose) conditions are described in the Methods section. Slot 1, EcoRII on SV40 DNA; Slot 2, EcorII + ScrFI on SV40 DNA; Slot 3, ScrFI on SV40 DNA; Slot 4, control digest, TaqI on pBR322 DNA.

Examination of λ,pBR322 and Ad2 DNA's showed that ScrFI produced different cleavage patterns, with more fragments, than predicted for EcoRII. Detailed results for pBR322 are shown in Fig. 3. ScrFI produced more digest products than either EcoRII or BstNI (an isoschizomer of EcoRII, but less affected by methylation) but both EcoRII / ScrFI and BstNI / ScrFI double digests were identical to the ScrFI single digest. The findings suggest that ScrFI does cut CC^{A}/TGG but also an additional site, probably CC^{C}/GGG, the degenerate sequence.

The apparently conflicting results obtained with ScrFI digests of

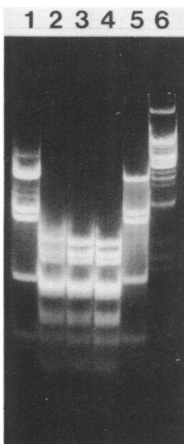

Figure 3. Agarose gel electrophoresis of ScrFI, EcoRII and BstNI digests of pBR322 DNA

The incubation and electrophoresis (1.5% Agarose) conditions are described in the Methods section. Slot 1, EcoRII; Slot 2, EcoRII + ScrFI; Slot 3, ScrFI; Slot 4, BstNI + ScrFI; Slot 5, BstNI; Slot 6, control digest, HindIII on SV40 DNA (incomplete).

SV40 DNA compared to those on pBR322 and Ad2 DNA's were reconciled with the aid of a computer programme (11). A computer scan of SV40 DNA showed that the sequence CC^{C}/GGG was not present. This would explain why ScrFI and EcoRII digests of this substrate were identical i.e. both cutting CC^{A}/TGG. The computer scan of pBR322 DNA showed the presence of 6 CC^{A}/TGG sequences and 10 CC^{C}/GGG sequences. In addition, the predicted sizes of the 9 largest fragments generated by an enzyme recognising these 16 sequences (i.e. recognition sequence CCNGG) were identical to those observed experimentally with ScrFI (Fig. 3). Further experimental evidence is shown in Fig. 4. A double digest of EcoRII and NciI (which recognise CC^{A}/TGG and CC^{C}/GG, respectively) was identical to the ScrFI digest. These data strongly suggest ScrFI as the first restriction enzyme to recognise the sequence CCNGG.

The recognition site of the new enzyme was confirmed using biochemical techniques. ScrFI fragments of Ad2 DNA were prepared and labeled at their 5'-ends with polynucleotide kinase and alpha-^{32}-P-ATP. The 5'terminal nucleotides released after digestion of these labeled fragments with a mixture of pancreatic DNase and snake venom phosphodiesterase were pG, pC, pA, pT, while the 5'terminal dinucleotides obtained with exonuclease I was pNG (Table 1). A two-dimensional anslysis of the oligonucleotides produced by digestion with pancreatic DNase alone indicated the unique sequence NGG

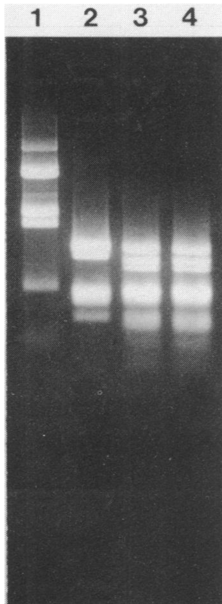

Figure 4. Agarose gel electrophoresis of ScrFI, EcoRII and NciI digests of pBR322 DNA

The incubation and electrophoresis (1.5% Agarose) conditions are described in the Methods section. Slot 1, EcoRII; Slot 2, NciI; Slot 3, EcoRII + NciI; Slot 4, ScrFI.

TABLE I
Identification of the 3'-dinucleotides present after digestion with ScrFI

Dinucleotides	R_B Standards	ScrFI
pC-G	0.86	0.91
pA-G	1.05	1.12
pG-G	1.30	1.39
pT-G	1.48	1.60

The standard dinucleotides were prepared by phosphorylating the corresponding dinucleoside monophosphates (Collaborative Research) with polynucleotide kinase and (alpha-^{32}P) ATP as described in Methods. Products were purified by electrophoresis on DEAE-cellulose paper in 7% (v/v) formic acid. Products from exonuclease I digestions were analyzed by electrophoresis on Whatman 540 paper at pH 3.5 (23) using purified dNG dinucleotide as markers. R_B refers to the electrophoretic mobility with respect to that of the blue dye xylene cyanol FF.

as being present on all fragments. After this sequence the following nucleotides become degenerate at the fourth nucleotide position. The simplest interpretation of these results is that ScrFI recognises the pentanucleotide sequence CC↓NGG with the cleavage site indicated by the arrow.

CONCLUSION

A new site-specific endodeoxyribonuclease has been isolated from *Streptococcus cremoris* F. The enzyme, designated ScrFI, was shown to recognise the sequence, 5'-C-C-↓N-G-G-3'. This is the first demonstration of a restriction enzyme in the industrially important lactic streptococci. The possible role of the enzyme in the *in vivo* restriction of phage DNA is being examined.

ACKNOWLEDGEMENTS

L.R. Brown was a visiting W.K. Kellogg Foundation Fellow from the Department of Microbiology, Oregon State University, Corvallis, OR 97331-3804 (U.S.A.). We wish to thank Patrick O'Reilly and Lourdes O'Connor for excellent technical assistance and Rich Roberts for guidance and facilities extended to G.F.F. while visiting Cold Spring Harbor.

REFERENCES

1. To whom correspondence should be addressed.

2. Roberts, R.J. (1982) Nucleic Acids Research 10, r 117 - r 144.
3. Roberts, R.J. (1976) CRC Critical Rev. Biochem. 4, 123 - 164.
4. Arber, W. (1974) Progress in Nucleic Acid Research and Molecular Biology 14, 1 - 37.
5. Wu, R., King, C.T. and Jay, E. (1978) Gene 4, 329 - 336.
6. Daly, C. and Fitzgerald, G.F. (1982) in Microbiology 1982. Schlessinger, D. Ed. pp. 213 - 216, American Society for Microbiology, Washington DC, U.S.A.
7. Pearce, L.E. (1974) N.Z. J. Dairy Sci. Technol. 13, 166 - 171.
8. Sanders, M.E. and Klaenhammer, T.R. (1980) Appl. Environ. Microbiol. 40, 500 - 506.
9. Terzaghi, B.E. and Sandine, W.E. (1975) Appl. Environ. Microbiol. 29, 807 - 813.
10. Davis, R.W., Botstein, D. and Roth, J.R. (1979) A Manual for Genetic Engineering : Advanced Bacterial Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
11. Gingeras, T.R., Milazzo, J.P. and Roberts, R.J. (1979) Nucleic Acid Research 5. 4105 - 4127.
12. Gelinas, R.E., Myers, P.A. and Roberts, R.J. (1977) J. Molecular Biology 114, 169 - 179.
13. Richardson, C.C. (1965) Proc. Natl. Acad. Sci. U.S.A. 54, 158 - 165.
14. Glynn, I.M. and Chappell, J.B. (1964) Biochem. J. 90, 147 - 149.
15. Lehman, I.R. and Nussbaum, A.L. (1964) J. Biol. Chem. 239, 2628 - 2636.
16. Brownlee, G.G. and Sanger, F. (1969) Eur. J. Biochem. 11, 395 - 399.
17. Jay, E., Bambara, R., Padmanabhan, R. and Wu, R. (1974) Nucleic Acids Research 1, 331 - 353.
18. Sanger, F., Donelson, J.E., Coulson, A.R., Kossel, H. and Fischer, D. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 1209 - 1213.
19. Limsowtin, G.K.Y., Heap, H.A. and Lawrence, R.C. (1978) N.Z. J. Dairy Sci. Technol. 13, 1 - 8.
20. Boussemaer, J.P., Schrauwen, P.P., Sourrouille, J.L. and Guy, P. (1980) J. Dairy Res. 47, 401 - 409.
21. Bickle, T.A., Pirrotta, V. and Imber, R. (1980) in Methods in Enzymology Grossman, L. and Moldave, K. Eds, Vol 65, Part 1, pp. 132 - 138, Academic Press Ltd., London.
22. Wilson, G.A. and Young, F.E. (1980) in Methods in Enzymology, Grossman, L. and Moldave, K. Eds, Vol 65, Part 1, pp. 147 - 153, Academic Press Ltd., London.
23. Barrell, B.G. (1971) in Procedures in Nucleic Acid Research, Cantoni, G.L. and Davies, D.R. Eds, Vol 2, pp. 751 - 779, Harper and Row, New York.