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# Site specific endonuclease from Fusobacterium nucleatum

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## ABSTRACT

Four different isolates of Fusobacterium nucleatum (A,C, D and E) contain restriction endonucleases of differing specificity. Whilst many of the endonucleases are isochizomers of known enzymes, two novel activities are Fnu DII which recognizes and cleaves the sequence 5'-CG↓CG-3' and Fnu EI which recognizes and cleaves the sequence 5'-GC↓GC-5' / 3'-GC↑GC-5' / 5'-G↓ATC-3' / 3'-CTA↑G-5' irrespective of the extent of methylation of the adenine residues.

## INTRODUCTION

Following the isolation of the specific endonuclease from Hemeophilus influenzae Rd (1), a large number of aerobic bacteria have been surveyed for new restriction enzyme activities (2). In comparison, anaerobic bacteria have received very little attention. We report here that a non-pathogenic obligate anaerobic bacterium, Fusobacterium nucleatum, isolated from the human oral cavity, contains restriction endonucleases. Particularly interesting is the finding that isolates from different individuals contain different endonucleases.

## MATERIALS AND METHODS

(a) Growth conditions for bacteria

Fusobacterium nucleatum isolates were grown at 37°C on 1.7% (w/v) Trypticase (BBL, Becton, Dickinson and Co.), 0.3% (w/v) yeast extract (Difco), 0.5% (w/v) NaCl, 0.25% $K_2HPO_4$, 0.25% (w/v) glucose, 5 µg/ml hemin, pH 7.0 in an anaerobic chamber filled with 87% $N_2$, 5% $CO_2$ and 8% $H_2$. The cells were grown to stationary phase and were harvested by centrifugation

and stored at -20°C. Yields per litre of culture were, isolate A, 3 g; isolate C, 1.5 g; isolate D, 3 g and isolate E, 3 g.

(b) Purification of restriction endonucleases

The purification of Fnu DI, Fnu DII and Fnu DIII is described; essentially the same protocol was used for the enzymes isolated from Fusobacterium nucleatum A, C and E.

A total of 8 g of frozen Fusobacterium nucleatum D cells were resuspended in 16 ml of 0.01 M Tris-HCl pH 7.5, 0.01 M 2-mercaptoethanol and sonicated until most cells were broken, as determined by microscopy. The sonicate was centrifuged at 100,000 x g for 90 min in a Beckman Type 30 rotor. The supernatant was adjusted to 1 M NaCl and applied to a 24 x 500 mm Biogel A 0.5 m (BioRad, 100-200 mesh) column pre-equilibrated with buffer A (1.0 M NaCl, 0.01 M Tris-HCl pH 7.5, 0.01 M 2-mercaptoethanol). The column was eluted with buffer A at a flow rate of approximately 1 ml/min. Sixty 5 ml fractions were collected and 5 µl of selected fractions assayed for site-specific endonucleolytic activity using bacteriophage lambda DNA (1.5 µg) as the substrate as described by Sharp et al. (3). All restriction enzyme digestions were carried out in buffer B (6 mM Tris pH 7.9, 6 mM $MgCl_2$ and 6 mM 2-mercaptoethanol). Digested DNA fragments were separated by electrophoresis on 1.4% agarose in TBE buffer (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, pH 8.3) containing 1 µg/ml of ethidium bromide (4). Partial separation of Fnu DI, Fnu DII and Fnu DIII activities was achieved by the Biogel column (Fig. 1). All fractions containing enzyme activity were pooled and dialyzed against PC buffer (10% glycerol, 0.01 M $KHPO_4$, pH 7.4, 0.01 M 2-mercaptoethanol, $10^{-4}$ M EDTA). Dialyzed fractions were applied to a 24 x 150 mm phosphocellulose (Schleicher and Schuell) column pre-equilibrated with PC buffer and eluted with 200 ml of PC buffer containing a linear gradient of KCl (0 to 1.0 M) at a flow rate of approximately 1 ml/min. Five ml fractions were collected and assayed as described above. Fnu DI and Fnu DII eluted with approximately 0.06 M KCl (Fig. 2). Fnu DIII eluted with approximately 0.35 M KCl, Fnu DIII fractions were pooled, dialyzed against PC buffer containing 50% glycerol and stored at -20°C. These

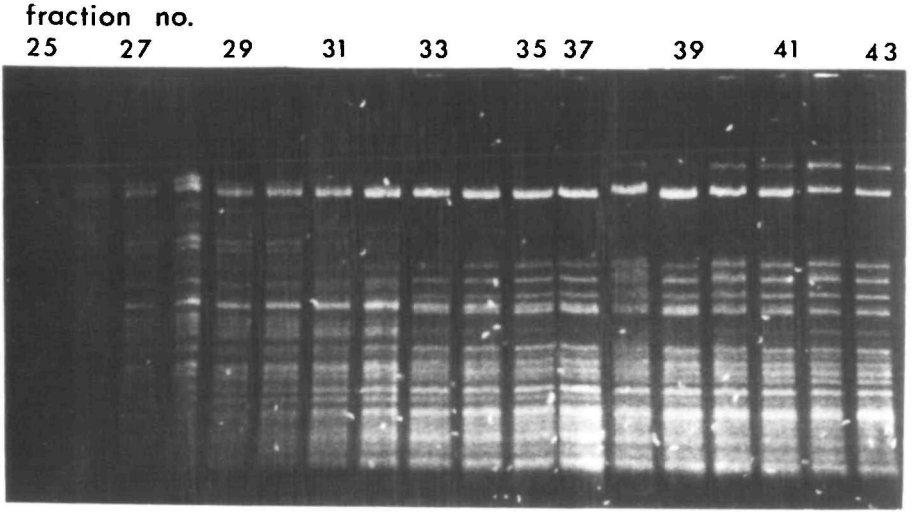


Fig. 1. Isolation of restriction enzymes from F. nucleatum D, Biogel A 0.5 column profile. Aliquots (5 µl) of column fractions (5 ml) were incubated in a 20 µl reaction mixture containing 1.5 µg λ DNA, and buffer B (6 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol) at 37°C for 2 hours. The stop mix (5 µl) containing 40% (w/v) sucrose, 0.025% (w/v) bromophenol blue, 0.025 M EDTA, pH 7.4 was added and the mixture loaded onto a 10 cm (length) 1.4% agarose horizontal slab gel in TBE buffer (90 mM Tris-borate pH 8.3, 90 mM boric acid, 25 mM EDTA) containing 1 µg/ml ethidium bromide and electrophoresed at 400 V for 1 h. The gel was photographed under ultraviolet light on Polaroid film (type 57) using an orange filter.

fractions were free from Fnu DI, Fnu DII and non-specific nuclease activity since no change in the band pattern was observed after 1 µg of lambda DNA was incubated at 37°C with more than 1 unit of enzyme for 12-16 hours. One unit of activity is defined as the amount required to completely digest 1 µg lambda DNA in one hour at 37°C. Fnu DI and Fnu DII containing fractions were pooled and dialyzed against PC buffer. The dialysate was applied to a 12 x 100 mm DEAE-Sephacel (Pharmacia) column pre-equilibrated with PC buffer and eluted with 200 ml of PC buffer containing a linear gradient of KCl (0 to 0.3 M) at a flow rate of approximately 0.5 ml/min. Five ml fractions were collected and assayed as described above.

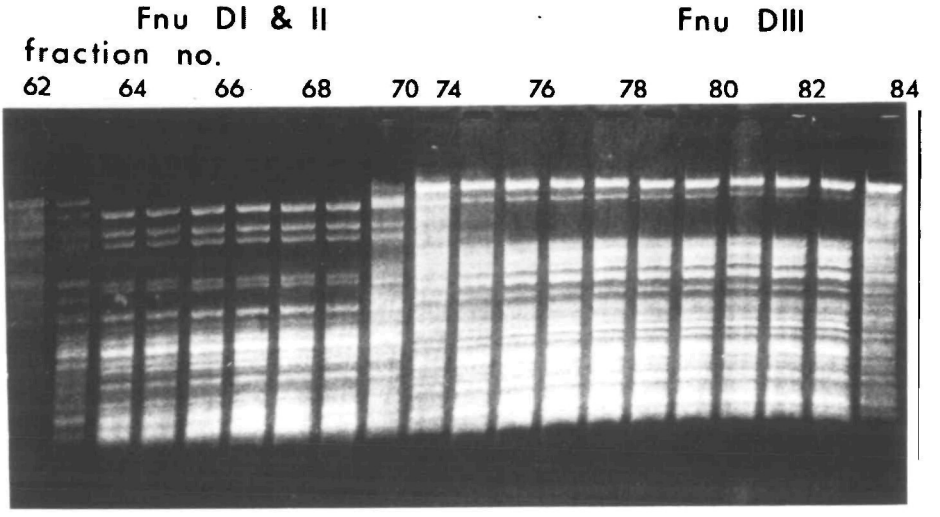


Fig. 2. Isolation of restriction enzymes from F. nucleatum D, phosphocellulose column profile. The gradient was started at fraction 40, and aliquots were assayed as described in Fig. 1. The first peak is a mixture of Fnu DI and Fnu DII and the second peak is Fnu DIII.

Fnu DI eluted at approximately 0.05 M KCl and Fnu DII at approximately 0.1 M KCl. Fractions containing each activity were pooled, dialyzed in PC buffer containing 50% glycerol and stored at -20°C. When this fractionation was carried out on DEAE-cellulose, there was very little separation of the two activities. DEAE-Sephadex A-50 (Pharmacia) yielded pure Fnu DI and Fnu DII contaminated with a small amount of Fnu DI (Fig. 3). All three enzymes were stable after 12 months at -20°C. This preparation yielded more than 1000 units of each of the specific endonucleases.

All the Fnu D restriction enzymes were inhibited by NaCl concentrations above 300 mM in buffer B. Fnu DI appeared to be most active in the absence of NaCl, Fnu DII at 50 to 150 mM NaCl and Fnu DIII at 50 mM NaCl.

Reactions of 1 to 8 μg of λ DNA incubated for 12 hours with 1 unit of Fnu DII in Buffer B containing 100 mM NaCl

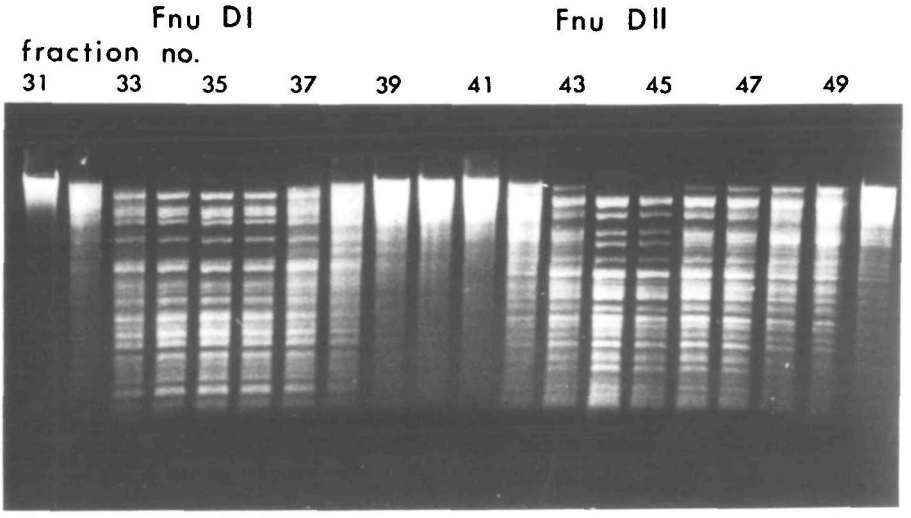


Fig. 3. Isolation of restriction enzymes from F. nucleatum D, DEAE-Sephadex column profile. Aliquots (5 µl) of column fractions (5 ml) were assayed as described in Fig. 1. The gradient was started at fraction 20. The first peak is Fnu DI and the second peak is Fnu DII.

showed that the enzyme was active for up to 8 hours at 37°C.

(c) Purification of restriction endonucleases from F. nucleatum A, C and E

The same procedure as above was used, except that DEAE-cellulose was used in place of DEAE-Sephacel. In the case of isolate A, two peaks of endonuclease activity were eluted from the phosphocellulose column at approximately 0.2 M KCl (Fnu AI) and 0.4 M KCl (Fnu AII). Fnu AI was eluted from DEAE-cellulose at 0.05 M KCl. Fnu CI eluted from phosphocellulose at 0.23 M KCl and was not retained by DEAE-cellulose. Fnu EI was eluted from phosphocellulose at 0.39 M KCl and from DEAE-cellulose at 0.03 M KCl. In each case 1,000-2,000 units of enzyme activity were obtained from 10 g of cells.

(d) Concentration of restriction enzymes

In the course of isolating restriction enzymes it was noted that a significant amount of activity was lost during

concentration by dialysis against 50% glycerol. Similar loss in activity has been reported for other restriction enzymes (5). Concentration of the enzymes by adsorption to a 12 x 60 mm column of DEAE-cellulose (Fnu DI, Fnu DII and Fnu AI) or phosphocellulose (Fnu DIII and Fnu CI) pre-equilibrated with PC buffer, then eluting with PC buffer containing 1 M KCl, overcame this problem. Fractions containing the restriction enzyme activity were pooled and dialyzed against PC buffer. Glycerol was added to the dialyzed fractions to a final concentration of 50% (v/v). Enzymes were concentrated at least five-fold with little loss in endonucleolytic activity.

(e) Other restriction enzymes and ØX174am3 RF fragments were obtained by methods described previously (6).

## RESULTS AND DISCUSSION

### (a) Characterization of Fnu DI, Fnu DII and Fnu DIII

Digestion of ØX174 RF DNA and S13 RF DNA with Fnu DI and Fnu DII produced sets of fragments identical with those produced by Hae III and Hha I, respectively. The method of Brown and Smith (6) which aligns the cleavage sites in both DNA strands alongside a "ladder" sequence pattern was used to confirm the identity of the recognition sites and to demonstrate that Fnu DI cleaves in the same manner as Hae III and that Fnu DIII cleaves like Hha I.

A new fragment pattern was obtained when ØX174 RF DNA was cleaved with Fnu DII (Fig. 4). The fragment sizes were estimated using standards of ØX RF DNA digested with Hae III and Alu I. The band patterns of Fnu DII in the presence or absence of the endonucleases Pst I and Xho I were in agreement with computer predictions (7) based on ØX174 DNA sequence data (8,9) if the Fnu DII recognition sequence were 5'-CGCG-3'. Thus, Pst I cleaved fragment 7 (259 base pairs) generating two new fragments of approximately 230 and 34 base pairs in length. Similarly, fragment 7 was cleaved by Xho I and fragments of about 200 and 58 base pairs in length were obtained (Fig. 4).

To show that the recognition sequence was unequivocally CGCG, the fragments from ØX174 DNA digested with Fnu DII were ordered on the genome by nearest neighbour analysis (10) as shown in Figure 5. The cleavage map determined from this

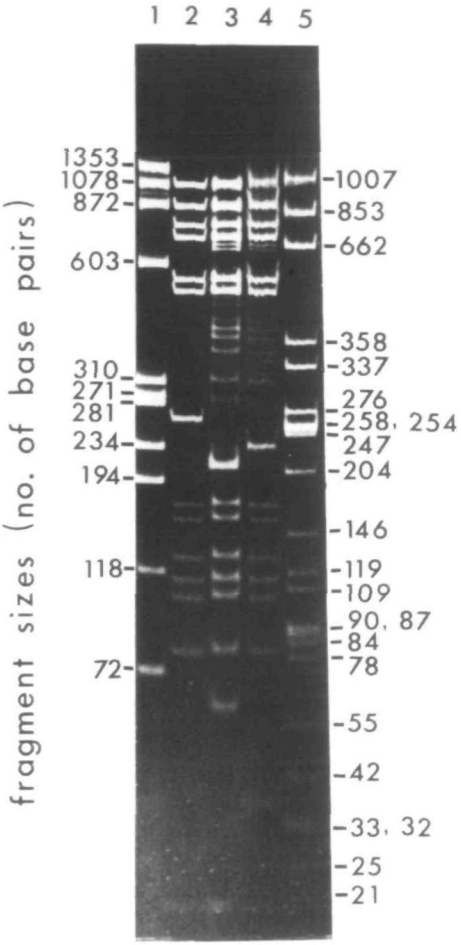


Fig. 4. Specific endonuclease digest patterns in 5% polyacrylamide gels. Reaction mixtures (20 µl) containing 2 µg ØX174 DNA and buffer B were incubated at 37° for 12 hours with various specific endonucleases as indicated below. Five µl of stop mix was added and the samples were loaded onto a 200 mm (length) x 1.5 mm (thick) 5% acrylamide slab gel in TBE buffer and electrophoresed at 200 V for 4 h. Slot 1, Hae III; 2, Fnu DII; 3, Fnu DII and Xho I; 4, Fnu DII and Pst I; 5, Alu I.

experiment was consistent with the computer predictions (Table 1).

The cleavage site for Fnu DII was determined by the method of Brown and Smith (6), to be at the center of the 5'-CGCG-3'

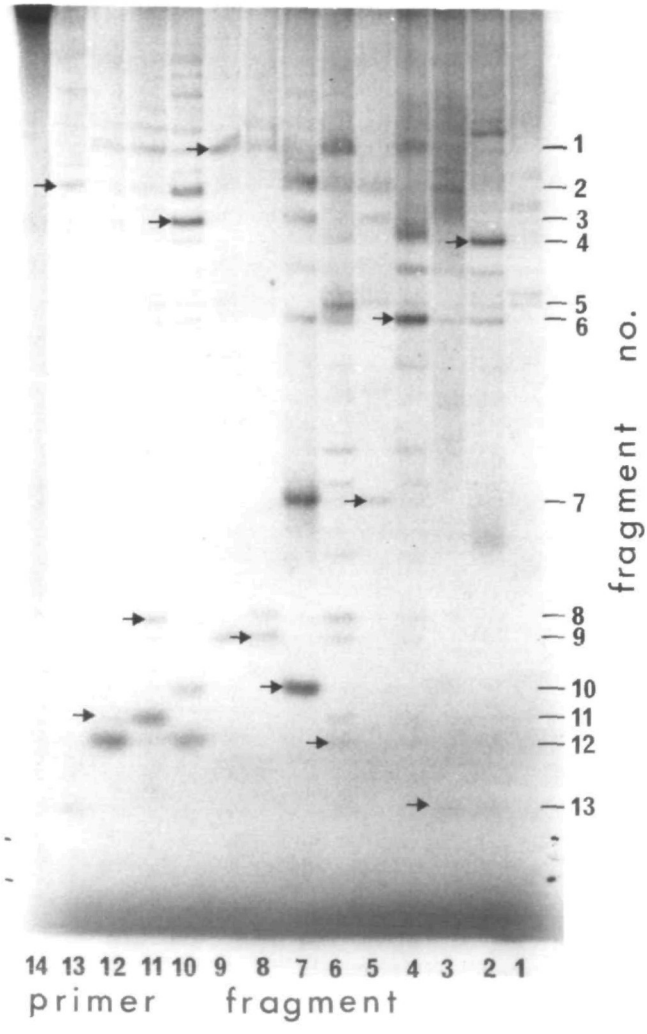


Fig. 5. Autoradiograph of nearest neighbour analysis for determining the cleavage map of Fnu DII on ØX DNA. Viral ØX DNA annealed with the indicated Fnu DII fragments in buffer C was incubated at 4° for 1 min with 0.9 units of E. coli DNA polymerase I (Klenow) in the presence of [$\alpha^{32}$P]-dATP (approximately 2 pmole, 341.9 Ci/mmole) 50 µM dCTP, dGTP, dTTP in a final volume of 16 µl. Excess dATP (2.5 µl of 0.5 mM) was added to chase the [$\alpha^{32}$P]-dATP. Fnu DII was added and the reaction mixture was incubated for a further 30 min at 37°. Five µl stop mix was added and the mixture was loaded onto a 200 mm (length) 5% acrylamide gel and electrophoresed at 100 V for 16 h. The X-ray film was exposed for 2 days to obtain the autoradiograph. The arrows indicate the nearest neighbour fragment.

Table 1. Results of nearest neighbour analysis (Fig. 6) compared with computer predictions (see Materials and Methods for experimental details).

| Fragment number[1] | Size[2] | Nearest neighbour fragment | |
|---|---|---|---|
| | | Predicted[2] | Found (Fig. 7) |
| 1 | 1050 | 14 | 5[3] |
| 2 | 820 | 4 | 4 |
| 3 | 718 | 13 | 13 |
| 4 | 695 | 6 | 6 |
| 5 | 530 | 7 | 7 |
| 6 | 496 | 12 | 1,5,6,9,11,12[3] |
| 7 | 259 | 10 | 10 |
| 8 | 170 | 9 | 9 |
| 9 | 156 | 1 | 1 |
| 10 | 127 | 3 | 2,3,12[3] |
| 11 | 114 | 8 | 8 |
| 12 | 103 | 11 | 11 |
| 13 | 79 | 2 | 2 |
| 14 | 19 | 5 | - |

[1]Fragments were numbered from their position on 5% polyacrylamide gels.

[2]Obtained from computer predictions based on ØX DNA sequence data (9-11). Size is given in number of base pairs.

[3]The apparent discrepancy between predicted and found nearest neighbours in these instances was caused by uncontrolled pulse-labelling resulting in labelling of more than the immediate neighbour. Fragment 14 was not located on the gel.

sequence. A similar activity, Tha I, has been reported recently (11).

(b) Characterization of Fnu EI

Fnu EI did not cleave ØX174 RF DNA. The gel pattern of polyoma DNA digested with Fnu EI was identical to that for Mbo I (Fig. 6, slots 2 and 3), which recognizes the sequence 5'-↓GATC-3' cleaving at the sites indicated by the arrows (12).
3'-CTAG↑-5'
No additional bands were generated when polyoma DNA was incubated with both these enzymes indicating that Fnu EI was an

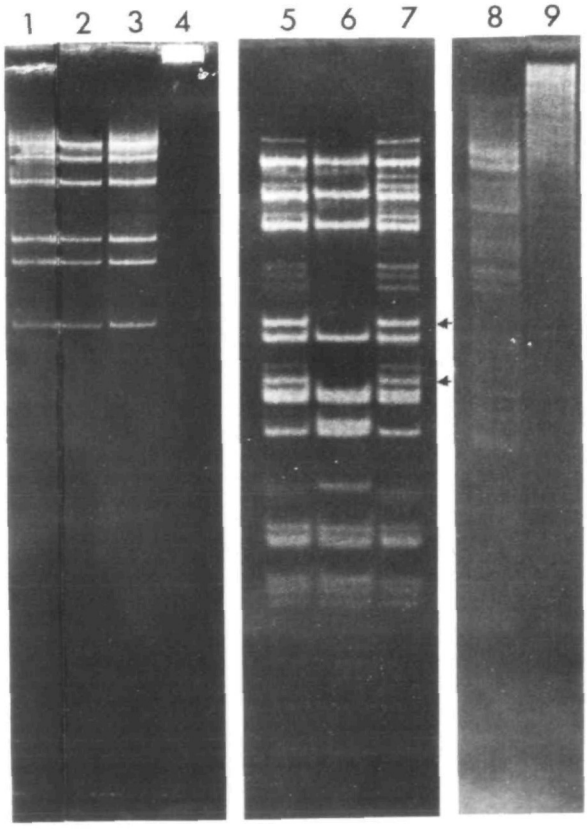


Fig. 6. Comparison of Fnu EI with Mbo I digestion patterns of various DNAs on a 5% acrylamide gel. Reaction mixtures (20 µl) containing 2 µg DNA, buffer A and 1 unit of each restriction enzyme, were incubated at 37°C for 12 h, then loaded onto a 5% acrylamide gel and electrophoresed at 200 V for 4 h. Polyoma DNA was used in slots 1 to 4, S13 RF DNA in 4-7, and λ DNA in 8 and 9. Restriction enzymes used were as follows: 1, Fnu EI and Mbo I; 2, Mbo I; 3, Fnu EI; 4, no enzyme added; 5, Mbo I and Alu I; 6, Fnu EI and Alu I; 7, Alu I; 8, Fnu EI; 9, Mbo I. The arrows indicate the two bands of Alu I digested S13 DNA that were cleaved by Fnu EI.

isoschizomer of Mbo I (Fig. 6, slot 1). However, S13 RF DNA digested with Alu I and Fnu EI gave a cleavage pattern different from that found with Alu I and Mbo I (Fig. 6, slots 5 to 7). The results show that S13 DNA contains two Fnu EI sites. Digestion of λ DNA with Fnu EI and Mbo I gave the patterns

shown in Fig. 6 slots 8 and 9.

The recognition sequence of Fnu EI was determined by 2-D fractionation of oligodeoxyribonucleotides from 5'-labelled λ DNA fragments. The 5'-terminal nucleotide was identified by degrading the labelled restriction fragments to mononucleotides with excess P1 endonuclease, separating the hydrolyzed products by paper electrophoresis and comparing them to standards. The labelled spot comigrated with the G standard. Starting from the 5'-terminal G, the recognition sequence is 5'-$\overset{\downarrow}{G}$ATC-3' from the mobility shifts on the 2-D fractionation of the $^{32}$P-labelled oligonucleotides obtained in the partial P1 digest (Fig. 7). Fnu EI is therefore identical to Mbo I in both sequence recognition and cleavage site.

The discrepancy found in the gel patterns of Fnu EI and Mbo I digested λ and S13 DNAs (Fig. 6) was resolved in the results obtained with phage f1 RFI DNA isolated from E. coli deficient in DNA methylase. The methylase present in dam$^+$ strains, methylates f1 RF DNA and renders it resistant to Mbo I, but susceptible to its isochizomer Dpn I (from Diplococcus pneumonia). Phage f1 RF DNA from a dam$^-$ host is not methylated and is digested by Mbo I but not by Dpn I (13,14). Fnu EI, however, completely digested both methylated and unmethylated f1 DNA generating a pattern identical to Mbo I digested dam$^-$ f1 DNA and Dpn I digested dam$^+$ f1 DNA (Fig. 7). It should be noted that unlike Fnu EI, Dpn I gave persistent partial fragments on dam$^+$ f1 RFI DNA due to incomplete methylation of the DNA.

(c) Characterization of Fnu AI, Fnu AII and Fnu CI

Analogous techniques to those used above established that Fnu AI is identical to Hin FI in recognition and cleavage sites. P. Myers and R.J. Roberts (personal communication) have shown that Fnu AII recognizes the same sequence as Mbo I. Fnu CI also recognizes the same sequence as Mbo I and cleaves in the same sites.

(d) Conclusions

This investigation establishes that isolates of F. nucleatum provide a very convenient source of a variety of stable and useful restriction endonucleases which are easily

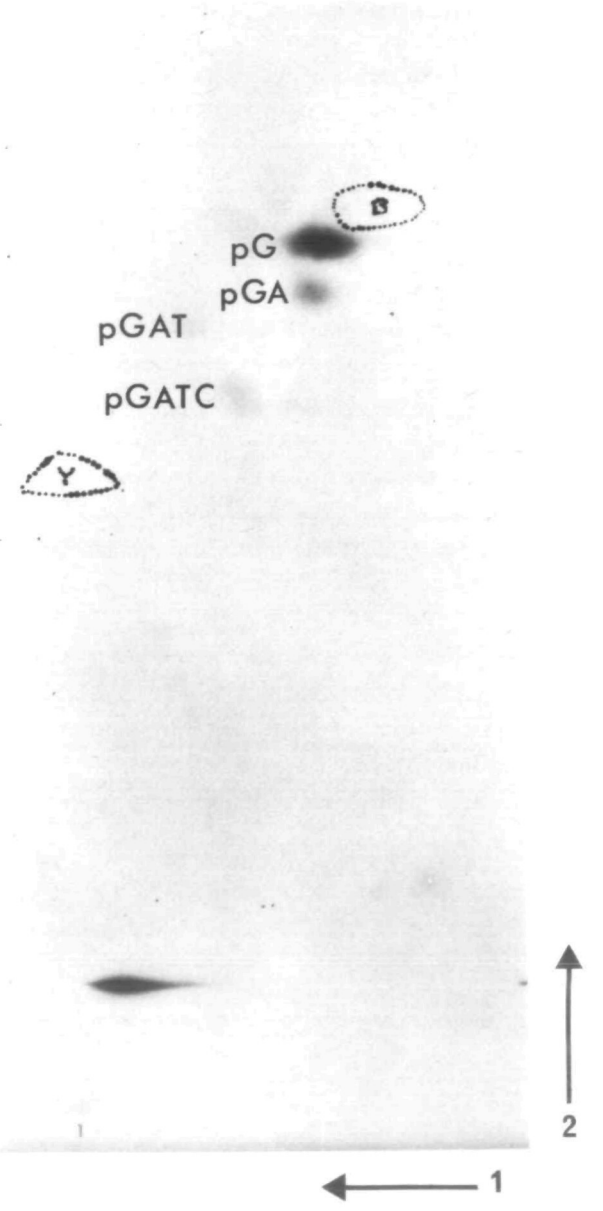


Fig. 7. Autoradiograph of 2-D fractionation of λ DNA digested with Fnu EI. The products from partial hydrolysis with P1 endonuclease of the 5'-end labelled restriction fragments were fractionated by electrophoresis on cellulose acetate at pH 3.5 in 7 M in the first dimension (← 1) followed by homochromatography in homomix V in the second dimension (↑ 2).

purified from all extracts. Of particular interest are Fnu DII and Fnu EI. The former enzyme recognizes and cleaves at 5'-CG↓CG-3' and completes the set of four enzymes which recog-
3'-GC↑GC-5'
nize guanine- and cytosine-containing symmetrical tetranucleotides, the other members being 5'-GGCC-3' (Hae III), 5'-CCGG-3'
3'-CCGG-5' 3'-GGCC-5'
(Hpa II), and 5'-GCGC-3' (Hha I). The interesting feature of
3'-CGCG-5'
Fnu EI is that it is a member of the set of nucleases whose ability to cleave at 5'-↓GATC-3' depends on the extent of
3'-CTAG-↑5'
methylation of A residues. The sequence is cleaved by Dpn I only when it contains 6-methyl adenine and by Mbo I and Dpn II only when the adenine is not methylated (13,14). The novel feature of Fnu EI is that it cleaves when the methyl group is present or when it is absent. This type of specificity also is possessed by Sau 3A I (E. Beck, personal communication). The inability of Fnu EI to discriminate between unmethylated and methylated sequences indicates a different sequence recognition mechanism. A possible explanation is that Fnu EI may bind to the minor groove of DNA whereas Mbo I, Dpn I and Dpn II may bind to the major groove which contains the site of methylation. In this context it is of interest that Fnu EI will digest phage SPO1 DNA in which the methyl of thymine is replaced by the hydroxymethyl group (E.P. Geiduschek, personal communication). Because an enzyme recognizing and cleaving 5'-↓GATC-3' is a very useful tool for dissecting DNA, λ DNA
3'-CTAG-↑5'
recombinants and the plasmid pBR322 have been grown in methylase deficient hosts in order to provide substrates for Mbo I (15,16). Fnu EI obviates the need for this strategy.

The present studies on polyoma DNA confirm that it, like SV40 DNA (13,14), is not methylated in the sequence 5'-GATC-3'. The resistance of ØX174 RF DNA to Fnu EI confirms that the DNA does not contain the sequence 5'-GATC-3' (8,9) whereas the present study shows that the DNA of the closely related bacteriophage S13 contains this sequence at two sites.

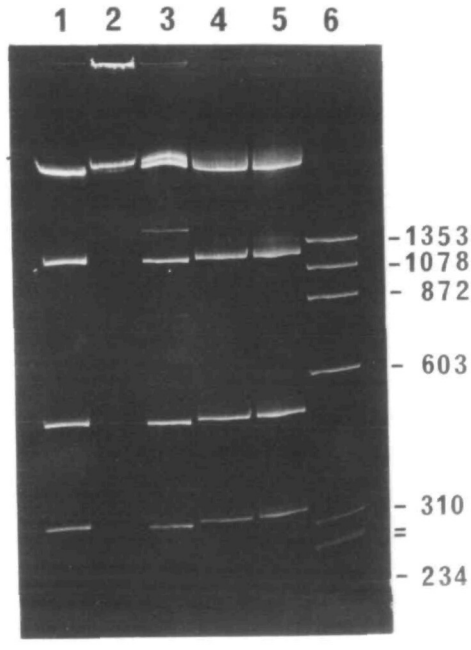


Fig. 8. Gel electrophoresis of f1 RF DNA digested with Fnu EI, Mbo I and Dpn I. Digestion and electrophoresis conditions were as described in the legend to Figure 3. 1, Mbo I digested dam$^-$ f1 RF DNA. 2, Mbo I digested dam$^+$ f1 RF DNA. 3, Dpn I digested dam$^+$ f1 RF DNA. 4, Fnu EI digested dam$^-$ f1 RF DNA. 5, Fnu EI digested dam$^+$ f1 RF DNA. 6, Hae III digested ØX174 RF DNA used as a size standard. The fragment sizes on the right hand side of the gel are given in number of nucleotides.

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