ISOLATION AND CHARACTERIZATION OF
SITE-SPECIFIC ENDONUCLEASES FROM ORAL BACTERIA

by

COLIN FRASER YARROW
B.Sc., McGill University, 1978

A Thesis Submitted In Partial Fulfilment
For the Degree of Master of Science
in
THE FACULTY OF GRADUATE STUDIES
DEPARTMENT OF BIOCHEMISTRY
THE UNIVERSITY OF BRITISH COLUMBIA

We accept this thesis as conforming to
the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
February, 1981

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Department of [Block Industries]

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date [April, 1981]
Abstract

Twelve strains of oral bacteria were screened for the presence of site-specific endonucleases. These screenings yielded the following results: recognition specificity, 5'-GATC-3', was identified from the enzymes FnuF793 I, FnuF794 I, and FnuBC6 I of the respective strains of Fusobacterium nucleatum F793, F794 and BC6, and, it was also identified in the Val3 I enzyme from Veillonella alcalescens 3; recognition sequence 5'-GATC-3' (both modified and unmodified recognized) was identified in the Val8 I enzyme of strain Veillonella alcalescens 8; recognition sequence 5'-GGCC-3' was identified in the FnuBC5 I enzyme of strain F. nucleatum BC5 and in the Smu I enzyme of strain Streptococcus mutans; recognition sequence 5'-GCGC-3' was identified in the FnuBC5 II enzyme of strain F. nucleatum BC5.
In spite of Ruth
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Acknowledgements

I would like to extend special thanks to Carol for her unending patience and help towards this thesis; Shirley for her cakes, encouragement and advice; David for his midnight philosophy and discussions; Diane for the typing; Barry, Heather and Mary for all the good bugs; and most importantly to Mike Smith, without whom all of this would not have been possible, for his time, understanding and inspiration.
Abbreviations

ATP  5'-triphosphate ribonucleoside of adenine
\textit{d}ATP  5'-triphosphate deoxyribonucleoside of adenine
DEAE  diethylaminoethyl
DNA  deoxyribonucleic acid
EDTA  ethylenediaminetetraacetate
\textit{g}  gram
M, mM, \(\mu\text{M}\)  molar (moles per litre), millimolar, micromolar
min  minutes
rpm  revolutions per minute
Tris  tris(hydroxymethyl)aminomethane
1.

Introduction

In the last 10 years our approach to modern genetics has been fundamentally altered by the development of new approaches and methodologies for probing the structure and function of DNA. The basic components for these techniques are the site-specific type II restriction endonucleases. These bacterial endonucleases recognize specific nucleotide sequences in duplex DNA and produce defined double stranded fragments by cleavage at a defined site in, or near, the recognition sequence. A collection of these enzymes, each with its own particular sequence specificity, can be used to cleave DNA molecules into unique sets of fragments for DNA sequencing, chromosome analysis, gene isolation, and for the construction of recombinant DNA.

Observations leading to the discovery of restriction enzymes span a period of nearly two decades and constitute a prime example of how basic research on an apparently inconsequential bacteriological phenomenon has had far-reaching implications. In the early 1950's studies by Luria and Human (1) and Bertani and Weigle (2) concerning the behaviour of bacteriophage grown on two different strains of bacteria marked the beginning of this research. A bacteriophage propagated on one strain of *E. coli* was found to grow poorly (that is to say, was 'restricted') on a second strain, and vice versa. However, a few bacteriophage, which had apparently acquired some type of host-specific modification that protected them from the effects of restriction, grew on the new host. The biochemical basis of this phenomenon was elucidated in the early 1960's when Arber and co-workers demonstrated that host specific modification was carried on
the bacteriophage DNA (3) and that restriction was associated with the
degradation of the bacteriophage DNA (4). In 1965 Arber speculated
about the existence of site-specific restriction endonucleases and
suggested that modification might be produced by host specific DNA
methylases (5). Thus, the idea became established that each
restriction and modification (R-M) system in bacteria consisted of two
enzymes with identical specificity. Specifically, each (R-M) system
consists of a modification enzyme that recognizes the same DNA
sequence that the restriction enzyme recognizes and thus allows
protection against cleavage. In this way, the host cell DNA was
protected, but foreign DNA entering from outside without modification
would be degraded.

In 1968 Linn and Arber (6) found in extracts of Escherichia coli B
an activity which had properties of a restriction endonuclease
conforming to those previously predicted by Arber. At the same time,
Meselson and Yuan (7) reported experiments with a highly purified
endonuclease from E. coli K. They demonstrated that the enzyme
cleaved unmodified bacteriophage λDNA into large fragments while
modified DNA remained undegraded. An unusual feature of the enzyme
was its requirement for the co-factors s-adenosylmethionine, ATP, and
Mg$^{2+}$. From Arber's work it was incorrectly assumed that the enzyme
cleaved λDNA at fixed sites.

It is now known that these endonucleases of E. coli B and K are
examples of a class of restriction enzymes that do not cleave DNA at
specific sites. Such class I enzymes are complex, multimeric proteins
and are non-specific in their cleavage patterns. Because of this
property, they have not proved useful as enzymatic tools for DNA
analysis.
By 1970, Wilcox and Smith (8) had succeeded in obtaining a purified preparation of a new and highly active restriction enzyme from *Haemophilus influenzae*, strain Rd. The fact that this restriction enzyme appeared to require only $\text{Mg}^{+2}$ as a cofactor suggested that it would prove to be a simpler enzyme than those from either *E. coli* B or K.

Using sucrose gradient centrifugation, the purified enzyme was shown to selectively degrade duplex, but not single-stranded bacteriophage T7 DNA to large fragments. No nucleotides were detected during the reaction, therefore, the enzyme was clearly an endonuclease that produced double-strand breaks and was specific for non-host DNA. Kelly and Smith (9) were able to show that the cleavages were sequence-specific by identifying the termini of the cleavage fragments.

Clearly, the most important result of these studies was the identification of cleavage specificity. The cleavage specificity of this enzyme distinguishes it from the *E. coli* B and K enzymes, which are randomly cleaving class I enzymes. The *Haemophilus* enzyme belonged to a different, and more important class of restriction endonucleases. Such class II enzymes (10) are cleavage site-specific and require only $\text{Mg}^{+2}$ as a cofactor. Studies revealed that they are relatively simple proteins, existing typically as dimers or tetramers of a single polypeptide chain (11,12). The modification methylases which correspond to these class II enzymes are separate proteins that exist as monomers (13).

More recently, Nathans and colleagues conducted experiments applying cleavage site-specific restriction endonucleases in their
analysis of the Simian tumour virus 40 (SV40) genome. The success of these experiments provided further evidence of the value of cleavage site-specific enzymes as tools for the physical analysis of DNA. The sequence specific property of these enzymes enables them to cleave a particular DNA into a unique subset of DNA fragments which can be analyzed individually. This fragmentation of the DNA enables chromosomes, which are largely inaccessible to molecular genetic methods to be analyzed piece by piece in chemical detail.

The central role of restriction endonucleases in the development of recombinant DNA technology has stimulated a search for new enzymes of differing specificities. A consequence of the work with the _Haemophilus_ site-specific endonuclease was the realization that such enzymes could be readily detected in bacteria through purely biochemical procedures. The efficacy of these biochemical procedures was enhanced by the introduction of gel electrophoresis, which facilitated the analysis of DNA restriction cleavage fragments (14), and by the introduction of ethidium bromide as a fluorescent stain for DNA (15). Using these simple and specific assays, many laboratories have reported isolation of new site-specific endonucleases.

On the current list, known site-specific endonuclease cleavage specificities are grouped according to recognition sequence (16). In many cases, several enzymes are known which recognize the same sequence. These enzymes have been called isoschizomers by Roberts (16). Further, the site within the recognition sequence at which isoschizomers cleave may vary. For example, Sma I cleaves (5')-C-C-C-G-G-G-(3') while Xma I cleaves (5')-C-C-C-G-G-G-(3').
Nucleotide sequences of recognition sites have, in most cases, been determined by analysis of the oligonucleotides which are released from the 3' or 5' labelled termini of cleavage fragments. A simple method for detecting recognition sequences (very often palindromic sequences) has been devised. Recognition sequences can now be detected by comparing digest fragments of ϕX174 and SV40 DNA produced by a given site-specific endonuclease, with a table of possible fragments which are predicted by computer analysis of all tetra, penta and hexanucleotide sequences in these DNA's (18). Unique assignment of specificity is usually possible.

Sites are classified according to whether they show two-fold rotational symmetry (palindromes) such as Hae III, which recognizes the sequence (5')-GGCC-(3'), or are asymmetric as in Mbo II, which (3')-CCGG-(5') recognizes the sequence (5')-GAAGANNNNNNNN-(3') (3')-GTTCTNNNNNNN-(5') .

Duplex structure is necessary for recognition in most site-specific endonucleases. However, in several instances cleavages are found in single stranded DNA. Although the existence of cleavage suggests direct recognition of single-stranded sequences (19), these cleavages may, in fact, be due to the presence of duplex regions generated by secondary structures in the single stranded DNA's (20).

Three main cleavage modes are observed in enzymes with symmetric recognition sites. These modes include even breaks (flush ends), staggered breaks which generate 3'-single-stranded cohesive termini (3'-sticky ends), and staggered breaks which generate 5'-single-stranded cohesive termini (5'-sticky ends). Each of these
types of terminus has found special use in recombinant DNA work. So far, all of the enzymes examined cleave to produce 3'-hydroxyl, and 5'-phosphoryl termini.

Many aerobic bacteria have been surveyed for restriction endonuclease activities, whereas relatively few anaerobes have been examined. This thesis describes studies on various isolates of the anaerobes *Fusobacterium nucleatum*, *Veillonellae alcalescens*, *Streptococcus faecalis*, and the facultative anaerobe *Streptococcus mutans*. These anaerobes were all isolated from the human oral cavity, and only the genus *Streptococcus faecalis* failed to demonstrate the existence of any site-specific endonucleases.

For this thesis, the screening of oral bacteria for site-specific endonucleases conforms to the screening procedure used by Smith and Wilcox (21) in their purification of the *Hind* enzyme from *Haemophilus influenza*. Enzymes that are used as tools for studying DNA must be free from non-specific endo- and exo-nucleases, from phosphatases and from nucleic acids. Further, these enzymes must be separated from other site-specific endonuclease activities.

The screening procedure involves the removal of nucleic acids from the cell extract. Either of the following methods can be used: gel filtration chromatography, or precipitation with ammonium sulfate, streptomycin sulfate, or polyethyleneimine (PEI) (22-25). In the experiments reported in this thesis, a Biogel A 0.5 m column was used to remove nucleic acids and to effect some size separation, thus allowing detection of site-specific endonuclease activity in crude extracts after a single chromatographic step. For most site-specific
endonucleases further purification was achieved by one or more steps of column chromatography. A phosphocellulose column, followed by a diethylaminoethyl (DEAE)-Sephacel column, was used for preparations in which the site-specific endonuclease was difficult to free from non-specific nuclease contamination.

Eight isolates of *Fusobacterium nucleatum* were screened for the presence of site-specific endonucleases in Biogel A 0.5 m column eluents. Endonucleolytic activities were found in four of these strains. Two isolates of *Veillonellae alcalescens* and an isolate of *Streptococcus mutans* were also screened, and yielded presence of endonucleolytic enzyme activity. Further, a strain of *Streptococcus faecalis* H.B. was screened but failed to demonstrate the presence of any such endonucleolytic activity.
Nomenclature

Restriction endonucleases derive their names from a restriction-modification (R-M) system nomenclature (17). An italicized three-letter abbreviation is used for the host organism followed by a fourth letter for strain when necessary. For example, the enzyme from *Haemophilus influenzae*, strain D, is called **Hind**. Roman numerals are used when more than one enzyme is found in the same organism. Thus, there are **Hind I**, **Hind II**, and **Hind III** enzymes. Similarly, an enzyme from *Streptococcus mutans* is called **Smu I**.
Methods and Materials

The bacteria

All the bacterial strains used in this thesis were isolated and characterized in the laboratory of Dr. B.C. McBride (Department of Microbiology, University of British Columbia) as described by Lui et al. (26).

(a) Growth conditions for bacteria

Fusobacterium nucleatum isolated were grown at 37° 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% K2HPO4, 0.25% (w/v) glucose, 5 µg/ml hemin, pH 7.0 in an anaerobic chamber filled with 87% N2, 5% CO2 and 8% H2. Streptococcus mutans, Streptococcus faecalis and Veillonellae alcalescens isolates were grown at 37° on Trypticase Soy Broth (BBL, Becton, Dickinson and Co.). The cells were grown to stationary phase and were harvested by centrifugation and stored at -20°. Between 2-3 g of cells per litre of media were obtained.

Purification of site-specific endonucleases

1. Crude extracts

Between 4 to 10 g of frozen cells were used for the isolation of site-specific endonucleases. All purification procedures were done at 4°. Cells were allowed to thaw in 10 ml of buffer A (0.01 M Tris-HCl, pH 7.4, 0.01 M 2-mercaptoethanol) and sonicated (10 x 20 s) until most cells were broken, as determined by microscopy. In cases where disruption of cells was poor, the cells were ground with HCl-washed glass beads (149-210 µM, Potter Industries) in a mini-mill homogenizer (Gifford-Wood Inc.) at 3/4 speed for 60 min. The broken cell preparation was centrifuged at 30,000 rpm for 90 min in a Beckman Type 30 rotor to remove cellular debris.
2. Biogel A 0.5 m column

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 B (1.0 M NaCl, 0.1 M Tris-HCl, pH 7.4, and 0.01 M 2-mercaptoethanol). The column was washed with buffer B at a flow rate of approximately 0.6 ml/min. Sixty 5 ml fractions were collected and 5 μl of selected fractions were assayed for site-specific endonuclease activity (see section on assays). Fractions containing potential endonuclease activity were pooled and dialyzed against buffer C (10% (v/v) glycerol, 0.01 M KHPO₄, pH 7.4, 0.01 M 2-mercaptoethanol, 10⁻⁴ M K₂EDTA).

3. Phosphocellulose column

The dialyzed enzyme fraction from the Biogel column was applied to a 25 x 150 mm phosphocellulose (Whatman P11) column pre-equilibrated with buffer C. The column was washed with 50 ml of buffer C, and eluted with 200 ml of buffer C, which contained a linear gradient of KCl (0 to 1.0 M). Five ml fractions were collected at a flow rate of approximately 2 ml/min, and 5 μl of selected fractions were assayed as above. Fractions containing activity were pooled and dialyzed against buffer C.

4. DEAE-Sephacel column

The dialyzed enzyme fraction from the phosphocellulose column was applied to a 12 x 100 mm DEAE-Sephacel (Pharmacia) column pre-equilibrated with buffer C. It was then eluted with 200 ml of buffer C which contained a linear gradient of KCl (0 to 0.4 M) set at a flow rate of approximately 0.3 ml/min. Five ml fractions were collected and 5 μl aliquots were assayed.
5. Concentration

The purified fractions were concentrated either by dialysis in a buffer C solution containing 50% glycerol (v/v), or by adsorption onto an ion exchange column after being eluted from either the phosphocellulose or DEAE-Sephacel column. Concentration using the chromatography method involves elution with M KCl in buffer C from a 8 x 50 mm DEAE-Sephacel column pre-equilibrated with buffer C. This eluent was then dialyzed against 50% glycerol (v/v) in buffer C. The final enzyme preparation was then stored at -20° where it remained active for 6 months.

6. Assay

Column eluents were assayed for site-specific endonuclease activity using both methylated λ DNA (a gift from Dr. P. Dennis) and unmethylated λ DNA (New England Biolabs) as the substrate. The assay method was essentially that described by Sharp et al. (15). Aliquots of the column fractions were incubated in a 20 μl reaction mixture containing buffer D (final concentration; 6 mM Tris-HCl, pH 7.4, 6 mM 2-mercaptoethanol, 6 mM MgCl₂, 1.0 mg/ml gelatin) and either 0.6 μg of methylated or 0.25 μg of unmethylated λ DNA at 37° for one hour. The incubation period of some samples were as long as 16 hours, and were set at 37° either to check for non-specific nuclease activity, or to determine whether weak endonuclease activity was present. The reaction was stopped by the addition of 5 μl of stop mix A (40% sucrose (w/v), 25 mM K₂EDTA, pH 7.4, 0.02% bromophenol blue, and 0.02% xylene cyanol). The resulting mixture was loaded onto a 1.4% agarose (Sigma type 1) horizontal slab gel (100 mm long and approximately 3 mm thick) in TBE buffer (90 mM Tris-borate,
pH 8.3, 90 mM boric acid, 25 mM K$_2$EDTA) (27) containing 1.0 µg/ml ethidium bromide. The mixture was then electrophoresed at 400 volts for two hours. The gel was then photographed under ultraviolet light using Polaroid (Type 57) film and an orange filter.

7. Specific Activity

The concentrated enzyme preparations were assayed, as above, using varying amounts of enzyme with 1.0 µg of λDNA to determine the specific activity of the preparation. One unit of enzyme activity was defined as the amount of enzyme required to completely digest 1.0 µg of λDNA in one hour at 37°.

Characterization of site-specific endonucleases

One µg of methylated λ, unmethylated λ, φX174 am 3 replicative form (courtesy of Miss A.C.P. Lui), and SV40 (gift from Dr. J. Colter) DNA was separately digested, as before, with one unit of a purified enzyme, and with one unit of Fnu DI (A. Lui), Fnu DII (A. Lui), Fnu DIII (A. Lui), Fnu EI (A. Lui) and Mbo I (M. Smith). Digestion of the λ DNAs was conducted for two hours at 37°, while digestion of the φX174 am 3 and SV40 DNAs entailed incubation for 16 hours in order to check for complete cleavages, and for the presence of non-specific nucleases. The reaction was stopped as before, and the mixtures were loaded onto a 5% acrylamide (Eastman) vertical slab gel, 1.5 x 200 x 200 mm. The gel was prepared from a deionized stock solution of acrylamide (43.5%) and from bis-acrylamide (1.5%, w/v in H$_2$O) and 0.05% ammonium persulfate in TBE buffer. The gel was polymerized by adding N'N'N'N'-Tetramethylethylenediamine (TEMED) (final concentration; 1 µl/ml solution). After 70 min of
electrophoresis at 300 volts, the gel was stained for approximately 30 min in an aqueous solution of ethidium bromide (1.0 ug/ml). The stained gel was then photographed in the manner described previously. The recognition sequence of the purified enzyme was determined by comparing the different fragment patterns which were produced.
Results

The Bacteria

The bacteria used in this thesis were isolated from the human oral cavity. The species that was most productive in site-specific endonucleases was the *Fusobacterium nucleatum*, a gram negative obligate anaerobe that was easily identified by its typical needle-shaped morphology.

The *F. nucleatum* species isolated from various individuals were designated as different strains although they were similar in properties (McBride, B.C., personal communication). The anaerobe *Veillonella alcalescens* ATCC 17744 was obtained from the American Type Culture Collection.
Purification and Identification of FnuBC5 I and FnuBC5 II

The endonuclease activity in the extract from F. nucleatum BC5 was eluted from the Biogel A 0.5 m column as shown in Fig. 1. Fractions that degraded λDNA to discrete fragments were pooled, dialyzed and applied to a phosphocellulose column. The FnuBC5 I enzyme was eluted by 0.05 M KCl, and the FnuBC5 II enzyme by 0.35 M KCl (Fig. 2).

The FnuBC5 I and FnuBC5 II enzymes were further purified, separately, by eluting the enzyme from a DEAE-Sephacel column with a linear gradient of KCl (0 to 0.4 M) and fractions were assayed at 37° for 16 hours to check for non-specific nuclease activity (Fig. 3). Both enzymes contained very little non-specific nuclease contamination, as evidenced by the constancy of the band pattern which remained after the λDNA was digested with the enzyme for 10 hours at 37° (Fig. 4). The FnuBC5 I and FnuBC5 II enzymes were concentrated by dialysis against 50% glycerol (v/v) in buffer C and both exhibited little loss in specific endonucleolytic activity. Comparison of the observed banding patterns with catalogued banding (29), and with FnuD I, FnuD II, and FnuD III controls, showed that the banding pattern for FnuBC5 I was similar to FnuD I (Fig. 5), and that FnuBC5 II had similar banding patterns to those of FnuD III (Fig. 6). Studies by Lui (28) have shown that FnuD I and FnuD III recognize respectively, 5'GGCC-3' and 5'-GCGC-3' sequences for cleavage specificity, and that these enzymes are isoschizomers of the prototype enzymes Hae III and Hha I (29,30).
Fig. 1. Gel electrophoretic patterns of methylated \( \lambda \)DNA digested by the fractions from the Biogel A 0.5 m column for the assay of endonucleolytic activity from \( F. \) nucleatum BC5

Aliquots (5 \( \mu l \)) from column fractions (5 ml) were assayed as described in methods and materials and subjected to electrophoresis on a 1.4% agarose gel containing 1 \( \mu g/ml \) ethidium bromide. The resulting electrophoretic patterns were photographed under ultraviolet light. The appearance of low molecular weight fragments in fractions 35-38 are indicative of endonucleolytic activity. Assay results of all column fractions in subsequent figures were determined in the same manner. Fraction number c was the control, no enzyme present in the assay mix. Incubation conditions were 15 min at 37\(^{\circ}\).
Fig. 2. Gel electrophoretic patterns of methylated λDNA digested by the fractions from the phosphocellulose column for the assay of FnuBC5 I and FnuBC5 II endonucleolytic activity.

Incubation conditions were 9 hours at 37°.
fraction no.

FNUBC 5 I   FNUBC 5 II

15  17  19  21  23  25  27  29  31  33
Fig. 3. Gel electrophoretic patterns of methylated λDNA digested by the fractions from the DEAE-Sephacel column for the assay of FnuBC5 I endonucleolytic activity

Incubation conditions were 16 hours at 37°.
fraction no.

18  20  22  24  26  28  30  32  34
Fig. 4. Gel electrophoretic patterns of methylated λDNA digested by the fractions from the DEAE-Sephacel column for the assay of FnuBC5 I and FnuBC5 II endonucleolytic activity

Low molecular weight DNA fragments were separated on a 5% acrylamide gel as described in methods and materials. Incubation conditions were 10 hours at 37°. The samples were as follows: slot 1, FnuD III standard; slot 2, FnuD II standard; slot 3, FnuD I standard; slots 4-9, FnuBC5 II; slots 10-13, FnuBC5 I.
Fig. 5. Endonucleolytic cleavage patterns of methylated λDNA and unmethylated λDNA, φX174 RF DNA, SV40 DNA

Incubation conditions were 14 hours at 37°. Low molecular weight DNA fragments were separated on a 5% acrylamide gel. Slots 1-8 were the FnuBC5 I enzyme digest samples, slots 9, 10, and 11 were the FnuD I, FnuD II and FnuD III enzyme samples, respectively. Slots 1, 2, 12 and 13 were the digest patterns of methylated λDNA, slots 3, 4, 9, 10 and 11 were the digest patterns of unmethylated λDNA, slots 5 and 6 were the digest patterns of φX174 RF DNA and slots 7 and 8 were the digest patterns of SV40 DNA.
Fig. 6. Gel electrophoretic patterns of digested \( \Phi X174 \) DNA

One unit of restriction enzymes were incubated with 1 \( \mu \)g \( \Phi X174 \) RF DNA for 1 hour at 37°. Low molecular weight DNA fragments were separated on a 5% acrylamide gel. The samples were as follows: slot 1, \( \text{FnuBC5} \) I; slots 2-5, \( \text{FnuBC5} \) II; slot 6, \( \text{FnuD} \) I standard; slot 7, \( \text{FnuD} \) II standard; slot 8, \( \text{FnuD} \) III standard; slot 9, no enzyme.
slot no.

1  3  5  7  9
Purification and Identification of FnuF794 I

Endonuclease activity was isolated from F. nucleatum F794. This activity, eluted at 0.4 M KCl from the phosphocellulose column (Fig. 7) in which the linear gradient of buffer C was 0-1.0 M KCl. This enzyme eluted at 0.05 M KCl from the DEAE-Sephacel column (Fig. 8) in which the linear gradient of buffer C was 0-0.2 M KCl. The enzyme yielded about 600 units of the site-specific endonuclease FnuF794 I from 4 g of frozen cells (Fig. 9). The final preparation had a small amount of contaminating non-specific nuclease. The FnuF794 I and Mbo I digestion patterns, which appeared on a series of different DNAs (methylated λ, φX174 RF, SV40 and pBR322 DNAs, all incubated at 37° for 90 min), were compared to allow identification of the FnuF794 I endonucleolytic cleavage sequence. The FnuF794 I enzyme was identified as an isoschizomer of Mbo I recognizing the unmethylated sequence 5'-GATC-3' (Fig. 10) (31).
Fig. 7. Gel electrophoretic patterns of λDNA digested by the fractions from the phosphocellulose column for FnuF794 I endonucleolytic activity

Slot 2 was the control, Fnu4H.
fraction no.

31C 33 35 37 39 41 43 45 47 49
Fig. 8. Gel electrophoretic patterns of \(\lambda\)DNA digested by the fractions from the DEAE-Sephacel column for \textit{FnuF794} I endonucleolytic activity.
Fig. 9. Specific activity of FnuF794 I on SV40 DNA

The assay was performed under standard conditions using 1.0 μg of SV40 DNA and varying amounts of FnuF794 I. Incubation conditions were 1 hour at 37°C. Digested DNA fragments were separated by gel electrophoresis on 5% acrylamide gel. The control (C) was a FnuD I standard digest.
μl of enzyme used - F794I

C10.08.06.0 4.0 2.0 1.0 0.5
Fig. 10. Endonucleolytic cleavage patterns of methylated λ, φX174, SV40, pBR322 DNAs digested with restriction enzymes

Incubation conditions were 90 min at 37°. Low molecular weight DNA fragments were separated by gel electrophoresis on a 5% acrylamide gel. Slots 1, 3, 5 and 7 were the $\text{Mbo I}$ enzyme digest samples, slots 2, 4, 6 and 8 were the $\text{FnuF794 I}$ enzyme digest samples and slot 9 the control enzyme sample, $\text{FnuD I}$. Slots 1, 2, 11 were the digest patterns of SV40 DNA (1 μg), slots 3, 4, 9 and 10 were the digest patterns of φX174 DNA (1 μg), slots 5, 6 and 12 were the digest patterns of pBR322 (1 μg) and slots 7, 8 and 13 were the digest patterns of methylated λDNA (1 μg).
The restriction endonucleases FnuF793 I, FnuBC6 I, and Val3 I (isolated, respectively, from F. nucleatum F793, F. nucleatum BC6, and Veillonella alcalenscens 3 ATCC 17744) were also found to be isoschizomers of Mbo I.

The FnuF793 I, FnuBC6 I, and Val3 I enzymes were eluted at approximately 0.24 M KCl from the phosphocellulose column (Fig. 11, 12, 13). Overnight digests (16 hours) were performed on methylated λDNA with all three enzymes. Fractions containing enzyme activity and no detectable non-specific nuclease were concentrated by dialysis against 50% glycerol (v/v) in buffer C. About 500 units of activity were obtained from 6 g of frozen cells.

By comparing the resultant banding patterns to both Mbo I controls and the Mbo I catalogued banding pattern (New England Biolabs), FnuF793 I, FnuBC6 I, and Val3 I were identified to be isoschizomers of Mbo I, recognizing the sequence 5'-GATC-3' in unmethylated DNA (Fig. 11).
Fig. 11. Gel electrophoretic patterns of λDNA digested by the fractions from the phosphocellulose column for FnuF793 I endonucleolytic activity.

Incubation conditions were 60 min at 37°. Slots 1 and 2 were Mbo I standard and FnuF794 I standard, respectively.
Fig. 12. Gel electrophoretic patterns of λDNA digested by the fractions from the phosphocellulose column for FnuBC6 I endonucleolytic activity

Incubation conditions were 60 min at 37°.
fraction no.

21  23  25  27  29  31  33  35  27  39  41
Fig. 13. Gel electrophoretic patterns of λDNA digested by the fractions from the phosphocellulose column for Val3 I endonucleolytic activity.

Incubation conditions were 75 min at 37°.
Val8 I

Endonucleolytic activity with a Mbo I-like digestion pattern was isolated from Veillonella alcalescens and was eluted at 0.36 M KCl from the phosphocellulose column (Fig. 14). Fractions with endonucleolytic activity were incubated for 10 hours in order to check for non-specific nuclease contamination.

Comparison of the observed digestion pattern of Val8 I with the digestion pattern of methylated and unmethylated λDNA indicated that Val8 I was able to recognize and cleave both, methylated and unmethylated DNA (Fig. 15). This indiscriminate quality is not exhibited by Mbo I which can cleave only unmethylated DNA. The Val8 I enzyme was identified as an isoschizomer of Sau3A and FnuE I enzymes which recognize the sequence 5'-GATC-3', and which cleave methylated and unmethylated sequences (31,28).
Fig. 14. Gel electrophoretic patterns of λDNA digested by the fractions from the phosphocellulose column for Val8 I endonucleolytic activity

Incubation conditions were 90 min at 37°.
Fig. 15. Endonucleolytic cleavage patterns of methylated λDNA and unmethylated λDNA digested with Val8 I enzyme.

Incubation conditions were 8 hours at 37°. Slots 1-6 were the methylated λDNA samples and slots 7-12 were the unmethylated λDNA samples.
Smu I

Endonucleolytic activity from *Streptococcus mutans*, was eluted from the Biogel A 0.5 m column (Fig. 16). The activity which followed the phosphocellulose (Fig. 17) was eluted at 0.67 M KCl in buffer C. Fractions were incubated 10 hours along side FnuD I, FnuD II, and FnuD III controls to check for non-specific nuclease contaminants and to allow identification of Smu I's recognition specificity (Fig. 18).

Fractions which exhibited endonucleolytic activity were concentrated by dialysis against 50% glycerol (v/v) in buffer C. About 3500 units of activity were obtained from 8 g of cells. By comparing the observed banding patterns of Smu I with those of the controls, Smu I was identified as an isoschizomer of FnuD I, recognizing the sequence 5'-GGCC-3'.
Fig. 16. Gel electrophoretic patterns of λDNA digested by the fractions from the Biogel A 0.5 m column for Smu I endonucleolytic activity

Incubation conditions were 60 min at 37°.
fraction no.

21  23  25  27  29  31  33  35  37  39  41
Fig. 17. Gel electrophoretic patterns of λDNA digested by the fractions from the phosphocellulose column for Smu I endonucleolytic activity.

Incubation conditions were 60 min at 37°.
Fig. 18. Endonucleolytic cleavage patterns of methylated λDNA digested with the enzyme, Smu I

Incubation conditions were 10 hours at 37°. Slots 1-13 were the DNA samples digested with Smu I; slots 14, 15 and 16 were the DNA samples digested with FnuD I, FnuD II and FnuD III, respectively.
Anaerobes With No Apparent Site-specific Endonuclease.

A series of strains of *F. nucleatum*: including BC2, BC7, BC11, BC12, and a *Streptococcus faecalis* H.B., were examined for the presence of site-specific endonucleases. No endonucleolytic activity was detected in any of these bacteria. When each bacterial strain was assayed, at least two types of DNA (λ and SV40) were used as substrates for digestion. This strategy was used to investigate the possibility that an endonuclease was indeed present, despite the absence of a recognition specificity.
Discussion

The wide variety of site-specific endonucleases in *Fusobacterium*, and the ease with which they were isolated by Lui et al. (26) warranted a more thorough search in this genus for 'new' site-specific endonucleases. These 'new' site-specific endonucleases could be enzymes with novel sequence recognition, or could act as substitute sources of the isoschizomer which is found only in small amounts in other bacteria or pathogenic strains. The initial analysis of *Fusobacterium nucleatum* suggested a high probability of finding different activities in various isolates in this species (28).

This thesis reports the screening of eight new isolates of *F. nucleatum*, four of which produced detectable site-specific endonucleolytic activity. Other oral bacteria species were also screened for activity. A list of the identified enzymes and their recognition sequences can be found in Table 1.

The detection of endonucleases is dependent on many factors including: (i) the presence of at least 50 units of (stable) enzyme activity per g of cells; (ii) the adequate breakage of cells and release of enzymes into the supernatant; (iii) the presence of the NaCl (250 mM) in the assays of the Biogel A 0.5 m column eluents which inhibit endonucleolytic activity; (iv) the enzyme being activated in Tris buffer (6 mM, pH 7.4) by the single cofactor Mg (6 mM); (v) the use of substrate DNA which has a sufficient number of recognition sequences for the detection of endonucleolytic activity on agarose gels, (for example, *Xma I* cuts λDNA three times but the DNA fragments are unresolvable as the assay is run) and (vi), the absence of highly active non-specific nucleases which obscure defined endonucleolytic cleavage patterns.
Table 1. List of site-specific endonucleases isolated from a series of anaerobic bacteria

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>GATC</th>
<th>GATCa</th>
<th>GGCC</th>
<th>GCGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>FnuF793 I</td>
<td></td>
<td>Val8 I</td>
<td></td>
<td>FnuBC5 I</td>
</tr>
<tr>
<td>FnuF794 I</td>
<td></td>
<td></td>
<td></td>
<td>Smu I</td>
</tr>
<tr>
<td>FnuBC6 I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val3 I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) The recognition sequence may have a methyl group in the N-6 position of the adenine residue.
Of the reported four enzymes that yielded site-specific endonucleolytic activity from *Fusobacterium nucleatum*, three have been identified as isoschizomers of *Mbo* I (GATC). This identification was achieved through comparison of the cleavage patterns which they generated in their digestion of λDNA. Lui (28) and D. Leung (personal communication) have also reported isolates with similar activity. It is possible that these isolates are the same strain. Further studies on screening *F. nucleatum* for site-specific endonucleases may require a better classification of isolates in order to enhance the probability that new strains of this bacterium will be screened. This poor classification may also account for the high number of isolates (four of eight) screened from *F. nucleatum* that yielded no evidence of site-specific endonucleases.

Like *Fnu* F793 I, F794 I, and BC6 I, *Val* 3 I has a recognition sequence identical to that of *Mbo* I. *FnuBC5* I and *Smu* I recognize the same sequence as *Hae* III, (GGCC), while *FnuBC5* II and *Val* 8 I are identical in recognition to *Hha* I (GCGC) and *FnuE* I (GATC) respectively. This thesis has shown that in the screening of oral bacteria new isoschizomers can replace low producing, or pathogenic strains of bacteria as sources of site-specific endonucleases. Unfortunately, some isoschizomers are difficult to obtain in a nuclease-free form. One example is *FnuE* I, which is invaluable for the digestion of DNA's containing methylated adenine residues and also for the analysis of methylation patterns in eucaryotic genomes (32).
In this laboratory, FnuE I has routinely been isolated with trace amounts of non-specific nucleases. While Fusobacterium nucleatum strains are generally low in non-specific nucleases, and hence are good sources of site-specific endonucleases, their trace contamination can be difficult to remove. The bacterium Veillonella alcalescens yielded Val8 I, an izoschizomer of FnuE I which, under standard purification procedures gave a nuclease-free preparation.

Detailed studies of restriction and modification enzymes require substantial quantities of these enzymes in a purified state. However, pure enzymes are not easily obtained in large amounts. Because of this deficiency, genetic engineers are exploring the possibility of cloning various R-M systems as a means to achieve enzyme overproduction. Mann et al. (33) have cloned the Hha II system from H. haemolyticus in the E. coli-pBR322 host-vector system using a 'shotgun' approach. After transfection into an r-m E. coli host (HB101), recombinant clones were tested, using bacteriophage λ, for the presence of a new restriction phenotype. A recombinant plasmid was found that exhibited classical restriction and modification behaviour with bacteriophage λ.

To increase plasmid copy number and consequent enzyme overproduction, a fragment of the recombinant plasmid DNA which contained the cloned gene was recloned into a second plasmid vector. This vector was a hybrid of pBR322 and the thermally inducible λ replication region (λC857 region) of bacteriophage λ (34). The recloning yielded a new hybrid plasmid. This plasmid when incubated at 42° for 20 min, raised the plasmid copy number and the enzyme
yield to several times the amount that was obtained from the original plasmid. At the same time, the new plasmids' enzymes were found to be subject to simple one-step purification methods. In crude extracts, single-stranded DNA agarose affinity chromatography was used to purify both the restriction and modification methylase.

There is a great advantage to having a R-M systems' genes on a small segment of DNA that can be propagated and expressed in E. coli. In the new host the genes are easily accessible for genetic studies. Also, the DNA segment is now small enough to be readily sequenced, thus providing direct information on gene arrangement, regulatory sequences, and protein amino acid sequences. Finally, it is evident that future studies of restriction and modification enzymes will be dependent upon gene cloning. Only this practice can induce the enzyme overproduction which is so indispensable for further genetic studies on the mechanism of action, and biological role of these enzymes.
References

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