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Review

Field alternation gel electrophoresis — status quo

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ABSTRACT

Since the description of the original technique of field alternation gel electrophoresis (FAGE) about ten years ago there have been significant developments in the area. Between 1983 and early 1987 dramatic improvements in the technique and apparatus resulted in a 500- to 600-fold increase in the functional separation capacity of conventional agarose gel electrophoresis. Details of the improvements in technique and equipment was the subject of an earlier review [H. J. S. Dawkins, *J. Chromatogr.*, 492 (1989) 615]. This review concentrates on the application of FAGE technology. The FAGE technique is no longer restricted to simply separating large DNA fragments. This method is presently being used for electrophoretic karyotyping, long-range genomic mapping, cloning of large DNA fragments into new vectors, the study of pathogenic chromosomal alterations and the structural analysis of chromosomes. The applications of FAGE in molecular biology and genetics is constantly expanding, with the full potential of this technique still to be realised.

CONTENTS

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LIST OF ABBREVIATIONS

AHC	Adrenal hypoplasia
ALL	Acute lymphoblastic leukaemia
bcr	Breakpoint cluster region
CHEF	Contour-clamped homogeneous electric field
CF	Cystic fibrosis
CML	Chronic myelogenous leukaemia
CMV	Cytomegalovirus
CpG	Cytosine guanine
DMD	Duchenne muscular dystrophy
dsb	Double-strand break
EBV	Epstein Barr virus
FAGE	Field alternation gel electrophoresis
FCR-3	A non-clonal Gambian *Plasmodium falciparum* isolate
FIGE	Field inversion gel electrophoresis
Gk	Glycerol kinase
HLA	Human leukocyte antigen
HS	Haemorrhagic septicaemia
HTF	*Hpa* II tiny fragment
KAHRP	Knob-associated histidine-rich protein
kb	Kilobase pairs of nucleic acid
Mb	Megabase pairs of nucleic acid
MDV	Marek's disease virus
MHC	Major histocompatibility complex
MRSA	Methicillin-resistant *Staphylococcus aureus*
PCR	Polymerase chain reaction
OFAGE	Orthogonal field alternation gel electrophoresis
Ph^1	Philadelphia chromosome
RFLP	Restriction fragment length polymorphism
TAFE	Transverse alternating field electrophoresis
TNF	Tumour necrosis factor
TS DHFR	Thymidylate synthetase dihydrofolate reductase
VSG	Variant surface glycoproteins (of Trypanosomes)
YAC	Yeast artificial chromosome

1. INTRODUCTION

The ultimate test of a technique is acceptance in the scientific community and breadth of application. With these criteria in mind, two techniques stand out in recent scientific advancement for their widespread acceptance and almost universal applicability. The first is the large DNA separation technique of pulsed field gradient electrophoresis, now more accurately referred to as field alternation gel electrophoresis (FAGE), and the second, the more recent development of polymerase chain reaction (PCR) technology.

Since the description of the original FAGE technique ten years ago there have been dramatic developments in the area. Between 1983 and early 1987 attention was focused on the technique, electrophoresis tanks and electrode arrays used to separate large DNA molecules. Improvements in the techniques yielded a 5- to 10-fold increase in the size of molecules that could be separated when compared with the original FAGE techniques [1] but, more significantly, resulted in a 500- to 600-fold increase in the functional separation capacity of conventional agarose gel electrophoresis. Since 1986, improvements in the equipment and techniques have been relatively minor, but the application of the technique has been continuously expanding as have the fields in which FAGE has found application. An earlier review of the field alternation technique by Dawkins [2] detailed improvements made in the technique and apparatus. It is our intention in this review to concentrate on the application of FAGE technology. Before embarking on FAGE applications it would seem appropriate to briefly review the theoretical aspects of DNA separation and some of the current theories on the mechanisms by which FAGE is able to separate large DNA fragments and intact chromosomes which can be up to 10 million base pairs (megabase pairs, Mb) in length.

2. ELECTROPHORETIC THEORY

Conventional application of electrophoresis employs a uniform electric field in one direction generated by a single pair of electrodes. Nucleic acids, at neutral pH, carry a negative net charge that is proportional to their length. The charge is carried by the phosphate groups of the polynucleotide backbone [3]. In an electric field, these macromolecules migrate through the solution at a rate proportional to the net charge (an inverse measure of the size of the molecule). Optimal separation of such macromolecules is largely dependent upon the applied field, choice of buffer and the pore size of the gel matrix. The mobility of macromolecules during electrophoresis can be significantly influenced by any alteration in these parameters [4]. High-resolution size fractionation can be achieved in a gel because of the sieving properties of the gel matrix [5].

Macromolecules can penetrate a gel if their overall size is equal to or smaller than the average pore size of the gel matrix [6]. The radius of the pores in a typical 1.0% agarose gel are estimated to be about 100 nm [7]. Under the influence of an electric field, small DNA molecules will move through the gel in a relatively straight path [5]. Large DNA molecules, on the other hand, must migrate in a more indirect pattern to find gel pores large enough to accommodate it. When the radius of gyration of a linear double-stranded DNA molecule exceeds the gel pore size, the limit of resolution is reached. The DNA must migrate "end-on" through the matrix, instead of being sieved by the gel, with the remainder of the molecule following in a snake-like fashion, often referred to as "reptation". Thus, the sieving nature of the gel matrix becomes irrelevant once a molecule is larger than the largest pore present in the gel. In agarose gels, sieving properties are lost when the DNA exceeds about 30–40 kilobases (kb) in size, at which point the molecules co-migrate. At this size, gel electrophoretic mobility is independent of molecular mass and strongly depends on the strength of the electric field and the gel concentration [7]. Conventional agarose gels are applicable for DNA molecules in the 0.2–20 kb size range.

The actual mechanism whereby DNA molecules are separated according to their size is not fully understood, and at best the description

above is oversimplified. In order to clarify the basis of macromolecule sieving in agarose gels a number of models have been developed. The simplistic model of De Gennes describes the molecule moving down a "tube" among the gel fibres with the molecule "head" migrating in the direction of the electric field [8]. Deutsch and Madden [9] used a computer simulation of the DNA migration in an agarose matrix which they constructed by observing individual, fluorescently stained DNA molecules as they migrated through the gel. In this instance, the molecules were visualised using a microscope mounted above a miniature electrophoresis system. Recurring cycles of elongation, resolution and relaxation were observed and the conformational changes recorded. Results showed an unexpected transition of a normally migrating polymer chain between a coiled conformation and one in which the chain was extended in the direction of the field [8,10].

Schwartz and Koval [11] used an experimental model to describe the migration of DNA. Their results were in agreement with proposed DNA migration suggested by Deutsch and Madden [9], who also showed that the mobility of long chains in high electric fields was independent of chain length. They concluded that size separation of molecules was usually restricted within upper and lower molecular mass ranges resulting from the limitations of the system in question. Conventional agarose gel electrophoresis is capable of separating molecules within the 1–40 kb range. Any DNA molecules longer than 30–40 kb cannot be resolved using this system, and co-migrates in the gel matrix [12]. Attempts have been made to separate larger molecules using lower gel concentrations (*e.g.* 0.1% agarose) [13]. Despite separations of up to 500 kb, resolution in low-agarose-concentration gels is poor, long running times are required, and there is increased fragility of the gels or the gels cannot be manipulated at all.

3. FIELD ALTERNATION GEL ELECTROPHORESIS

The technique of FAGE, first described in 1983 by Schwartz *et al.* [1], allowed the resolution of DNA molecules up to 2 Mb. The size limitations of conventional gel electrophoresis were overcome by forcing DNA molecules to reorientate by periodically changing the direction of the electric field. Each time the field direction was altered, the DNA fragments were forced to reorientate along the new field before they could begin migrating forwards again. Periodically controlled electric field alternation prevented the DNA from establishing reptation, and separated the molecules on the basis of the sieving capacity of the gel and the size-related turning time of the DNA. Smaller molecules have a capacity to turn and reorientate more quickly than larger molecules, thus having a higher mobility. Size-dependent DNA separation by field alternation allowed resolution of molecules 100 times larger than with a unidirectional electric field. The original apparatus consisted of a complex electrode array in conjunction with an electrical switching unit [1,2]. Non-uniform fields in both directions were found to provide the best resolution, but in order to achieve narrow bands, one homogeneous and one non-homogeneous field was used. The electrode array of the homogeneous field usually consisted of 16–20 vertical, diode-isolated platinum electrodes at both the cathode and the anode, while the non-homogeneous field consisted of a series of 16–20 electrodes at the cathode and a single-wire anode. However, the non-uniform nature of the field meant that the speed and direction of migration was influenced by the position at which the DNA was loaded, resulting in a very skewed migration pattern [1,2]. This makes comparison of samples and accurate size estimation extremely difficult.

Modifications to the original field alternation gradient unit were made by Carle and Olson [14]. This system, termed orthogonal field alternation gel electrophoresis (OFAGE), consisted of two non-homogeneous electric fields generated by a continuous cathode and a single vertical diode-isolated anode [2,14]. OFAGE resulted in the DNA migration through the gel being in an "hour-glass" shape. Although more symmetrical, the hour-glass pattern still restricted accurate

fragment size comparisons between samples to the small number of central lanes on each gel.

Transverse alternating field electrophoresis (TAFE), first described by Gardiner *et al.* [15], was based on OFAGE but reoriented the gel in the vertical plane. The vertical gel resulted in all locations in the gel being symmetrically affected by the fields from the two pairs of electrodes. Thus, all lanes experienced identical electric fields, resulting in uniform, straight-lane migration of the DNA, regardless of position across the gel. Several electrode configurations were examined before being optimised at an angle of 115°. The applied alternating fields induce DNA molecules to migrate in a "zig–zag" motion through the thickness of the gel and progressively downwards [2,16].

The technique of field inversion gel electrophoresis (FIGE), designed by Carle and Olson [17], abandoned the orthogonal arrangement of two electric fields in favour of periodic inversion of a single electric field through an angle of 180° [2]. This system utilised conventional submarine agarose gel electrophoresis equipment in conjunction with a switching unit controlling the electric field. It essentially enabled large DNA separation to be undertaken in any laboratory. FIGE used a homogeneous electric field, with each switch cycle reversing the polarity of the electrodes. Net forward movement was achieved by having a programmed longer forward pulse than the reverse pulse, resulting in DNA movement along straight tracks. A "ramped" switching routine, *i.e.* one with a gradual increase in both forward and reverse pulse times, was required to circumvent the anomalously low mobility of larger DNA fragments and to allow a molecular mass-dependent separation of the DNA [2,13].

The topology of DNA is known to influence its migrational rate in agarose, and can be determined by comparing its FAGE mobility with those of linear or circular size standards at different pulse times [18]. The migration of supercoiled and relaxed circular DNA in agarose gels is considerably slower than that of linear DNA of similar size [7]. Relaxed circular DNA larger than about 15 kb will only migrate in agarose when pulsed fields are 180° apart, *i.e.* in the FIGE system as the DNA was thought to become impaled on projections in the agarose matrix. The "shuffling" movement of FIGE by field reversal appears to free entrapped circular molecules thus enabling such DNA to migrate through the agarose. Lower voltages or transverse pulsing can also prevent this migrational hindrance. FIGE is the only field alternation system that allows the resolution of *Giardia intestinalis* chromosomes [19]. Upcroft *et al.* [19] observed that minor alterations in running conditions resulted in significant consequences for separation of the chromosomes. It was thought that the structure or confirmation of the chromosomal DNA from varying species may influence these differences. Separation of DNA molecules by FIGE appears to be limited to a maximum threshold size of 2 Mb [2,20].

The development of contour-clamped homogeneous electric field (CHEF) overcame nearly all the limitations of previous FAGE systems [21]. The electric field is alternately pulsed through electrode arrays at 120° angle using voltages applied along opposing sides of a hexagonal array of electrodes clamped to predetermined electric potential [22]. The hexagonal array and the clamped field resulted in a homogeneous electric field being applied at all points within the contour of the hexagon. It was shown that a combination of low field strengths, long switching intervals and low agarose gel concentrations could resolve molecules greater than 5 Mb in size [23]. Bio-Rad Labs. further modified the CHEF system, commercially producing the CHEF-DRII system that is capable of resolving molecules of sizes up to 12 Mb [22].

Excellent resolution can be achieved using the CHEF-DRII system. After electrophoresis of *Saccharomyces cerevisiae* under optimised conditions, 16 bands are visible as compared to 11–13 bands with other field alternation techniques [22]. The programmable switching unit provides precise control over the electrical field, and allows a continuously adjustable range of switch times useful for enhancing resolution within a certain DNA size range. The field switcher also permits

division of the run into two separate switch times giving better separation over a wider size range [2]. Secondary DNA structures may also be identified by CHEF, in a similar fashion to that by FIGE [21]. A square electrode can be used to generate a CHEF with differing field strengths in the two orientations. Such DNA structures will appear as spots distinct from the arc of the linear molecules. It is this versatility that has made the CHEF technique a major force in large DNA separation. Further development and modification have resulted in the release of the CHEF-DRIII and the CHEF Mapper (Bio-Rad Labs., Richmond, CA, USA). These new-generation units have the capacity to alter the angle of separation by manipulating the electrode array clamping. The CHEF Mapper incorporates an in-built algorithm which automatically sets the CHEF parameters for optimal separation of specific-sized fragments. The advantages offered by these new generation systems have improved the separation capacity and also improved speed of separation. The electrophoresis systems described by Anand [13] and Southern *et al.* [24] known as the Hula Gel (Hoeffer Scientific Instruments, San Francisco, CA, USA) and ST/RIDE (Stratagene, La Jolla, CA, USA) and GeneLineII (Beckman Instruments, Fullerton, CA, USA) vertical gel electrophoresis units all have the capabilities to alter the vector angle of separation, but do not have the integrated algorithm for automatic control of separation conditions. The functional qualities of all these units and their prices are comparable.

All the above systems have brought significant improvements to the large DNA separation technique, thus broadening the range of applications in which FAGE is now employed. The spectrum of work now possible using FAGE is staggering, hence a detailed review of every application is not realistically possible. However, in collecting literature for this review, it was noted that there have been several areas in which the application of FAGE has been of major importance. FAGE has revolutionised microbial genetics, with particular reference to the study of bacterial and parasitic genomes. It has also been of major importance in studying chromosomal abnormalities or alterations that correlate with pathogenesis in man. The following sections discuss the impact field alternation electrophoresis has made in molecular genetics.

4. BACTERIOLOGY

4.1. Gram-positive bacteria

4.1.1. Enterococcus

Enterococcus is one of the gram-positive bacterial species that has been well documented by FAGE analysis. Enterococci are known to have distinct biochemical properties and multiple antibiotic resistances that distinguish them from other gram-positive cocci [25]. Traditional methodologies, such as antibiotic resistance patterns, biochemical reactions, phage typing, plasmid isolation and serology, have been used to type these bacteria. Many of these methods, however, are not sufficiently sensitive to distinguish between strains. FAGE has recently been used in epidemiological analyses of clinical enterococcal isolates. Murray *et al.* [26] digested agarose-embedded isolates of *E. faecalis*, which has an approximate G+C content of 40%, with *Sma* I. They estimated that the G+C-rich restriction site of *Sma* I should produce approximately 15–20 bands. Analysis of isolates demonstrated a considerable degree of polymorphism among enterococcal isolates and thus demonstrated the value of FAGE for epidemiological studies. More recently another group [25] used FAGE to compare chromosomal restriction patterns of *Sma* I-digested *E. faecium*, and determined that this technique is sufficiently discriminatory to be of value in comparison of these isolates. They were able to confirm the degree of polymorphism seen by Murray *et al.* [26]. They were also able to show polymorphisms among isolates from the same location in addition to those from distinct geographic sites [25]. Murray *et al.* [27] continued to use FAGE as an epidemiological tool, and were able to show in a later study, by detection of identical restriction patterns, that there had been inter-hospital spread of a single strain of *E. faecalis* in six hospi-

tals in five states of the USA. These reports clearly demonstrate the capability and potential use of field alternation electrophoresis, and the advantages of this technique in the field of epidemiology and in the study of virulence characteristics.

4.1.2. Staphylococcus

Of the number of gram-positive organisms that have been examined by field alternation electrophoresis, the *Staphylococcus* species has been studied in greatest detail. Goering and Duensing [28] used the FIGE technique to epidemiologically evaluate difficult staphylococcal groups. They used antibiotic-susceptible *S. aureus* containing no detectable plasmids which were unreactive to bacteriophage typing, and also methicillin-resistant *S. epidermidis* carrying a single 30-kb plasmid. FIGE analysis demonstrated restriction fragment length polymorphism (RFLP) differences in strains that were indistinguishable by the more traditional epidemiological methods such as antibiograms (antibiotic susceptibilities) and plasmid isolation. Hybridisation with an rRNA probe confirmed the relationships demonstrated by FIGE analysis. Although this was only a preliminary study evaluating the potential value of this technique in epidemiological analyses of these organisms, it can be clearly seen that it is a rapid, reproducible method capable of separating isolates that lack phenotypically or biologically distinguishing characteristics. The *S. aureus* genome, in particular methicillin-resistant *S. aureus* (MRSA), has been examined by field alternation electrophoresis, as a tool for both epidemiological analysis and typing among isolates. Methods such as enzyme analysis, protein profiles and RFLP analysis, in addition to those described above for *Enterococcus*, have been used to type MRSA. Wei *et al.* [29] reported the use of the CHEF system to type these isolates (Fig. 1), with results confirming previous designations. This study also demonstrated that isolates indistinguishable by tradional typing systems could be differentiated by this technique. Prevost *et al.* [30], in a study comparing the effectiveness of ribotyping and large DNA restriction enzyme analysis for distinguishing MRSA isolates, suggested that FAGE (the TAFE system in this case) provided greater discrimination than ribotyping. This finding is understandable when related to the method of detection. Ribotyping involves the detection of a single chromosomal region (rRNA gene copies), whereas FAGE analysis is based on restriction site spread throughout the entire chromosome. FAGE analysis can detect polymorphisms due to ancestral strain-to-strain mutational differences or even variation in gene content. In contrast, the ribotyping technique only detects variation in or around the rRNA genes, therefore, FAGE analysis has greater opportunity of reflecting differences in the structural organisation of the bacterial chromosome.

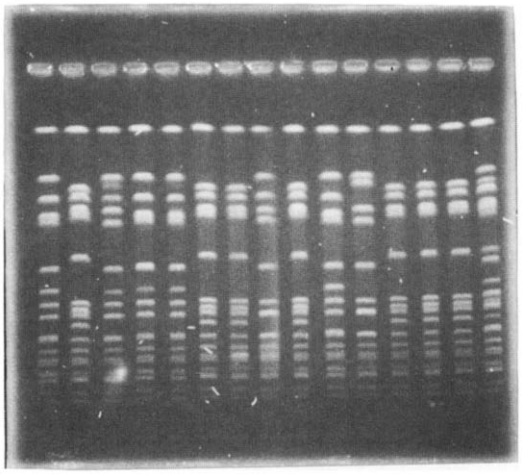

Fig. 1. Field alternation gel electrophoresis of methicillin-resistant *Staphylococcus aureus* (MRSA). Isolates from an outbreak of MRSA in a large teaching hospital were treated and electrophoresed on a Bio-Rad CHEF DR II by the method of Wei *et al.* [29] as modified by Udo and Grubb [191]. In this outbreak there were two epidemic types represented by the isolates in tracks 2, 6, 7, 9, 12, 13 and 14, and those in tracks 1, 4, 5, and 10. The other isolates have varying degrees of relatedness to the two types and to each other and were not regarded as being epidemic strains. (Reproduced from Wei *et al.* [29].)

4.2. Gram-negative bacteria

4.2.1. Escherichia coli

The genome of *Escherichia coli* was one of the first bacterial chromosomes to be analysed and mapped using FAGE. Smith *et al.* [31] reported

the physical mapping of the *E. coli* K12 genome using the enzymes *Not* I and *Sfi* I, and size fractionation using the Pulsaphor system. *Not* I cleavage of the K12 genome resulted in 22 DNA fragments sized between 20 and 1000 kb. The order of the fragments in the genome was determined from detailed information on the genetic map of the *E. coli* available at the time. The five fragments unable to be located and ordered by this technique were mapped using two other methods: the use of linking clones (clones containing uncommon restriction enzyme cleavage sites) or by the analysis of partial *Not* I digestion patterns. Linkage clones are useful in the identification of DNA fragments from adjacent positions in the genome. When a clone containing a particular rare site is used to probe a blot of FAGE-separated DNA digested with the same enzyme, the probe will hybridise to two adjacent DNA fragments. This technique enables information to be gathered without the need of pre-existing genetic information.

Detailed mapping of the *E. coli* genome will permit the identification of any significant rearrangements between strains, facilitating epidemiology studies and strain identification. Arbeit *et al.* [32] utilised FAGE, in particular the field inversion technique, to analyse evolutionary divergence among *E. coli* strains from related lineages. FAGE is increasingly being used in the field of molecular epidemiology, particularly in cases such as MRSA in which traditional epidemiological methods cannot discriminate between strains [28]. FAGE analysis is able to distinguish between isolates which are epidemiologically independent but evolutionally related and isolates which were unable to be differentiated by phenotypic or genotypic techniques such as serotyping or RFLP analysis [32]. It was also demonstrated that distinct restriction patterns could be identified for both genetically distinct and closely related isolates. This degree of genetic diversity has only been shown previously by time-consuming techniques such as detailed sequencing analysis.

Analysis of virulence patterns and virulence determinants have also been performed by field alternation electrophoresis. Ott *et al.* [33] described the use of FAGE in the study of the virulence pattern of extraintestinal *E. coli* K1, K5, and K100 isolates. *Xba* I-digested DNA was hybridised with probes specific for the genes of various adhesins. This analysis revealed highly heterogeneous restriction and hybridisation patterns, even within strains of the same serotype. FAGE analysis was also able to determine the location of virulence genes in the genome and genetic relatedness of strains [33]. Blum *et al.* [34], in a similar study, used OFAGE combined with hybridisation of virulence-associated gene probes to examine the genomic structure and virulence patterns of extraintestinal *E. coli* O6 strains. This report demonstrated that haemolysin genes and P fimbrial determinants were in close linkage in three isolates. The results of the FAGE analysis indicated that although these strains are all of the same serotype, they are very heterogeneous with regards to virulence and restriction patterns [34]. The extremely valuable nature of FAGE in the genetic examination and epidemiological evaluation of *E. coli*, and indeed all pathogenic bacteria, will ensure its increasing role in the study of virulence and strain identification.

4.2.2. Pasteurella multocida

Specific serotypes of the bacterium *Pasteurella multocida* are responsible for haemorrhagic septicaemia (HS), one of the most economically important livestock diseases of South East Asia. HS is a peracute disease of cattle and buffalo, which is endemic to most parts of tropical Asia, Africa and India [35]. HS is one of a number of diseases produced by infection with *P. multocida*, an organism capable of producing illness in a wide range of mammalian and avian hosts. Numerous attempts have been made to correlate specific *P. multocida* serotypes with a specific disease entity, and to classify the various types. Restriction enzyme patterns produced by the rare-cutting endonucleases *Not* I and *Sma* I, in conjunction with FAGE, were used to examine 17 isolates of *P. multocida* (Figs. 2 and 3) [36]. The isolates analysed included isolates from animals with HS, reference strains and non-HS causing strains. After restriction enzyme digestion, *P. multocida* iso-

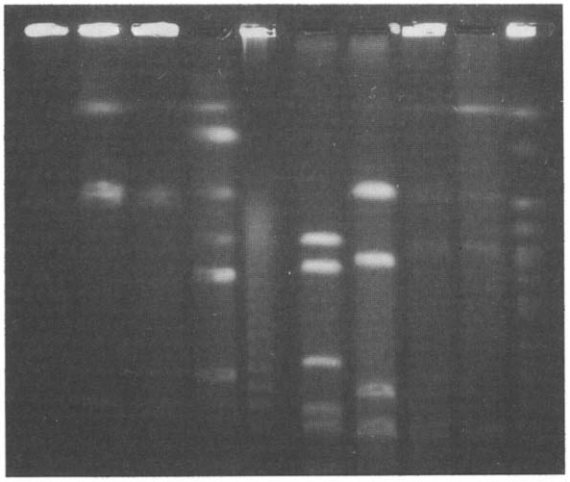

Fig. 2. Examples of *Not* I-digested chromosomal DNA from *Pasteurella multocida* isolates analysed by FAGE. This study included isolates that were obtained from animals with haemorrhagic septicaemia (HS), as well as reference strains and non-HS isolates covering a broad representation of the *P. multocida* species. The origin of these isolates varies greatly with geographic location, including Asia, Africa and North America. Electrophoresis was performed for 24 h at 180 V and 14°C, and separated on a 1% agarose gel in 0.5 X TAE with a 10–150 s switch time. (Reproduced from Townsend *et al.* [36].)

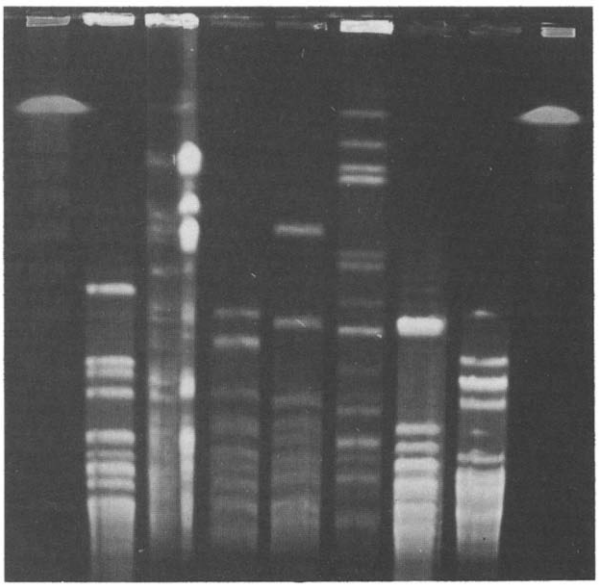

Fig. 3. FAGE gel showing *Sma* I chromosomal restriction patterns generated by cleavage of DNA from a broad spectrum of *Pasteurella multocida* isolates. This gel was subjected to electrophoresis for 24 h at 180 V and 14°C with pulse times of 5–35 s in a 1% agarose gel in 0.5 X TAE. (Reproduced from Townsend *et al.* [36].)

lates showed relatively complex RFLP patterns with *Sma* I, yet a remarkable degree of homogeneity among the field isolates from Asia with both enzymes. FAGE separation of *Sma* I-digested DNA from three isolates, previously indistinguishable by serotyping and protein studies, clearly demonstrated that these isolates were distinct. It was suggested that restriction patterns produced after *Not* I digestion rather than *Sma* I may be more useful as a typing system as the patterns are less complex and some serotypes can be clearly differentiated. Although it was found that neither restriction enzyme-generated pattern was indicative of pathogenesis, FAGE analysis was valuable in distinghuishing haemorrhagic septicaemia-causing *P. multocida* organisms [36].

4.2.3. Mycoplasma

The mycoplasma genome has been examined using FAGE since 1988. Pyle *et al.* [37] utilised the FIGE technique in conjunction with restriction endonuclease digestion to estimate the genomic size of a number of mycoplasmas. Restriction enzymes were chosen on the basis of their frequency of cleavage in the genome. The generation of one fragment was preferred for ease of estimation of genomic size. Pyle *et al.* [37] used eight different restriction enzymes to cleave the mycoplasma genomes: *Apa* I, *Bam* HI, *Bgl* I, *Bss* HI, *Kpn* I, *Sal.* I, *Sma* I and *Xho*, I. The estimated sizes obtained by FAGE fractionation varied among the mycoplasma species, but were found to be considerably larger than estimates previously reported by other techniques. The sizes ranged from approximately 900 kb to 1330 kb, as compared with previous estimates of 750 kb. Chen and Finch [38] reported the use of FAGE in analysing the arrangement of rRNA genes in *M. gallisepticum*. Large restriction fragments from *M. gallisepticum* were fractionated by the FIGE system. Southern blots of these fragments were subsequently hybridised with the plasmid pMC5 which contained the entire 23S and 5S rRNA genes and most of the 16S rRNA gene. Results from this study indicated that the genomes con-

tained three widely separated rRNA loci. Multiple hybridisations with various sub-fragments of the plasmid pMC5 determined that one locus contained genes for all of the three rRNA genes, another contained 23S and probably 5S rRNA genes, and the third locus appeared to have only a 16S rRNA gene. This study demonstrated the value of field alternation electrophoresis in the examination of rRNA gene organisation in bacterial genomes.

In recent years, there have been a number of studies into the generation of physical and genomic maps of some *Mycoplasma* strains [39–41]. Miyata *et al.* [40] constructed a physical map of the *M. capricolum* genome using separation of large DNA restriction fragments by FAGE. The order of the DNA fragments in the genome was determined by two-dimensional FIGE of partial and complete single enzyme digests and complete double enzyme digests, and by Southern hybridisation analysis. Cleavage sites for seven restriction endonucleases were included in the map of the estimated 1160-kb genome. Ladefoged and Christiansen [41] constructed a physical and genetic map of five *M. hominis* genomes by FAGE. Linkage order of the fragments was determined primarily by analysis of double digests, and secondly by the use of linkage clones. The gene order for these strains was found to be identical to that of *Clostridium perfringens*, an organism known to be phylogenetically related to *M. hominis*.

The FAGE techniques have been used in the analysis of a broad spectrum of bacteria and in a wide variety of applications. Space prevents us talking about all the innovative methods and the extremely broad spectrum of organisms analysed by FAGE. Table 1 illustrates a summary of publications using FAGE analysis in the study of bacterial genomes. Not only is FAGE an extremely useful tool in the field of molecular epidemiology, but it has proved to be effective in the construction of physical and genetic maps of bacterial genomes. It has also shown to be beneficial in the identification and differentiation of bacterial species. FAGE despite the work to date still has enormous, as yet, comparatively untapped potential in bacteriology.

TABLE 1

LIST OF ORGANISMS ANALYSED BY FAGE

Bacterium	Reference(s)	Bacterium	Reference(s)
Bacillus	142	*Mycoplasma*	37–41
Borrelia	143	*Myxococcus*	162, 163
Campylobacter	144–147	*Neisseria*	164
Caulobacter	148, 149	*Pseudomonas*	165–168
Clostridium	150	*Pasteurella*	169
Enterococcus	25, 27	*Rhizobium*	170
Escherichia	31–34, 151, 152	*Salmonella*	171, 172
Halobacterium	153	*Shigella*	173
Legionella	154, 155	*Staphylococcus*	28–30, 174–176
Leptospira	156, 157	*Streptococcus*	177
Methanococcus	158	*Streptomyces*	178, 179
Mycobacterium	159–161	*Yersinia*	180, 181

5. VIROLOGY

FAGE is now being used as a novel technique for the separation of large viral genomes from host cell chromosomes. Molecular analysis of some viral genomes has been hindered by the cell-associated *in vitro* growth characteristics of the virus [42]. Isolation of DNA from certain classes of herpes viruses is difficult and often produces very low yields as the virions are cell-associated and are not shed to any significant extent [43]. Standard electrophoresis techniques are unable to separate large viral DNA from chromosomal DNA due to size limitations of the technique. Density gradient separation has also proved to be difficult, particularly in the case of Marek's disease virus (MDV), as the guanine and cytosine content makes the viral DNA density similar to that of chicken DNA, its natural host. Thus, an alternative procedure needed to be developed in order to obtain purified, intact viral DNA.

Isolation of viral DNA using FAGE has been used for Chlorella virus [44], poxvirus [45], Epstein Barr virus (EBV) [46], cytomegalovirus (CMV) [47] and more frequently, MDV [42,43]. Chlorella and MDV were isolated using the CHEF system, whereas CMV was isolated and analysed with the FIGE apparatus. The presence of viruses that replicate in tissues or cells but are

deficient in packaging or shedding functions can also be isolated by FAGE by a virus-specific band. Viruses that are no longer able to be isolated as shed virion due to mutation or other causes can now be examined by molecular analysis after FAGE isolation. Until recently, infectivity of FAGE-isolated viral DNA had not been demonstrated in any viral system. However, Wilson and Coussens [42] have recently described procedures for the recovery of purified, infectious viral DNA from field alternation gels. The transfection efficiency from electroporation of cell-free MDV DNA, however, was 5 times lower than that obtained by electroporation of total DNA isolated from MDV-infected cells.

FAGE isolation of Chlorella viruses has enabled structural analysis of the viral genome to be carried out. Results obtained by Rohozinsky *et al.* [44] indicate that PBCV-1 (the most studied Chlorella virus), and presumably other Chlorella viruses, consists of linear, non-permuted double-stranded DNA molecules lacking free 5′ and 3′ ends. Several observations also indicate that PBCV-1 DNA contained covalently closed hairpin ends, and was thus similar to the pox viruses in this respect.

Studies by Bostock [45] introduced FAGE, in particular the FIGE system, as a routine method for sizing and mapping large viral genomes of the pox viruses. Analysis was carried out on wild-type strain WR and recombinant 997D vaccinia virus genomic DNA, with the estimated genomic size of vaccinia virus strain WR being about 190 kb. Bostock [45] showed that, in conjunction with the ability to probe Southern blots with terminus-specific sequences, FIGE provides a rapid method for directly producing linear maps of poxvirus genomes. Episomal EBV has also been separated from host cell DNA using field alternation gel electrophoresis in Burkitt's lymphoma-derived cell lines [46]. It has been estimated that approximately 35 ng of EBV genome can be isolated using this technique which is sufficient for molecular analysis.

6. PARASITOLOGY

The technique of FAGE, although initially applied to yeast by Schwartz *et al.* [1], was successfully used to characterise chromosomal DNA and DNA rearrangements in parasitic protozoa. Molecular karyotyping has been conducted on a number of protozoa, including *Giardia, Leishmania, Plasmodium, Pneumocystis* and *Trypanosoma*. Table 2 illustrates the vast array of studies on parasitic genomes using FAGE analysis that have been published to date. Initial studies have also been done on *Cryptosporidium* using FIGE separation to examine potential isolate and species chromosome size variations [48]. This novel technique has been successful for species differentiation of *Cryptosporidium* with the detected differences probably relating to variation in cell surface proteins. Previous attempts of karyotypic characterisations of many unicellular organisms, prior to the development of FAGE, were unsuccessful as such organisms (*e.g.* yeasts and protozoa) failed to show condensed metaphase chromosomes during the cell cycle [19]. With the advent of large-scale DNA analytical techniques, it is now possible to separate chromosome-sized DNA molecules.

Upcroft *et al.* [19] analysed chromosomal DNA from stocks of *Giardia duodenalis* by FIGE. These isolates are morphologically indistinguishable and have a broad host range. Attempts have been made to characterise these stocks on the basis of host species and geographical origin, however, techniques such as ribotyping [49] and isoenzyme studies [50] have proved

TABLE 2

LIST OF PROTOZOA ANALYSED BY FAGE

Parasite	Reference(s)
Giardia	19, 51, 52, 182, 183
Leishmania	53–57, 184–187
Plasmodium	58 68, 71, 188
Pneumocystis	69
Trypanosoma	72–75, 189, 190

complex and failed to definitively classify these isolates. Chromosomal patterns observed after FIGE analysis of *Giardia* isolates showed an apparent geographical distribution, with North American stocks being of one karyotype and isolates from Australia are of a second. Genetic variation between *Giardia* isolates was seen to be much less than that reported in different isolates and species of *Plasmodia*, *Trypanosoma* and *Leishmania* [51]. Results of molecular karyotyping of *Giardia* using the OFAGE system [52] appear to be consistent with those obtained by Upcroft *et al.* [51]. Adam *et al.* [52] described the separation of five major chromosomes and several minor chromosomes ranging in size from 1 to 4 Mb. Southern hybridisation of field alternation gels with total chromosomal DNA, in addition to chromosome-specific probes, indicate cross-hybridisation between minor and major bands. This may suggest that the minor and major bands represent homologous chromosomes of different sizes. The relevance of this to virulence and the life cycle of the organism is yet to be determined.

Extensive studies have been done using FAGE to karyotype the *Leishmania* species. This technique not only allows classification of the species, but also presents an opportunity to characterise the genomic organisation of *Leishmania* [53]. Molecular karyotyping by FAGE (utilising both OFAGE and CHEF systems) shows that *Leishmania* isolates have karyotypes distinct from chromosomal DNA profiles of other complex protozoan parasites. After analysis by FAGE, molecular karyotypes of various *Leishmania* species were shown to be quite diverse with respect to chromosome size and number [54]. Present studies indicate a minimum of 26 distinct chromosomes, a result consistent with the diploid nature of the promastigotes [53]. It has also been shown that FAGE karyotypes of *Leishmania* isolates from different species or subspecies indicate low-level homology between their chromosomes. Diversity was also seen in the chromosomal patterns of stocks of different aetiological agents. Following genomic DNA separation by FAGE, analysis of gene locations and chromosomal rearrangements can be done. Gene localisation studies suggest that similar genetic information is contained on chromosomes of a similar size among isolates, and may represent homologous chromosomes [53].

Specific gene probes have been used to analyse the degree of polymorphism with regard to chromosomal gene location. Studies of genomic organisation of housekeeping genes, such as α- and β-tubulin, thymidylate synthetase–dihydrofolate reductase (TS–DHFR) and heat shock proteins, have indicated that these genes are not clustered but found on at least seven bands in *L. major* [54]. A number of studies have examined the genomic arrangement of the tubulin genes of *Leishmania*. Consistent observations report that the α- and β-tubulin genes were found in unlinked tandem repeat clusters in *L. enriettii* and *L. major* with a β-tubulin cluster characterised in *L. tropica* in two chromosomal locations [55]. This novel arrangement is in contrast to *Trypanosoma brucei* tubulin genes in which the genes exist as a tandem array of alternating α- and β-tubulin gene pairs. It has also been reported that β-tubulin genes are dispersed among several chromosomal loci in *Leishmania* species [53]. This finding could suggest that these extra isogenes may encode functionally different tubulin proteins.

Two genes showing homology to eukaryotic 70-kD heat shock proteins have been isolated and characterised in *Leishmania* [56]. Sudden temperature increases induce heat shock protein expression which is believed to be essential in precursor protein protection and protein complex disaggregation. FAGE was used in these studies to aid in mapping the chromosomal location of each hsp70 gene. These genes, although related by sequence homology to the tandemly repeated hsp70 genes of trypanosomatids, are found dispersed on different chromosomes within the *Leishmania* genome.

FAGE has also been useful to examine the structure and function of amplified DNA in laboratory stocks of *Leishmania tarentolae* [57]. FAGE was used to determine whether the amplified region of DNA was contiguous sequences in the genome or whether it was extra-chromosomal circular DNA. Properties characteristic of supercoiled circular DNA were present after electrophoresis in the cell lines exhibiting amplification.

The introduction of γ-irradiation produced a spectrum of structural lesions into the DNA, which provided information as to the nature of the DNA topology. Irradiated linear molecules yielded a heterogeneous array of fragments of varying lengths in contrast to irradiation of circular molecules which produced discrete linear molecules whose length equalled that of the original circular molecule. Therefore, the structural nature of the DNA can be determined using this technique combined with FAGE. This study provided a simple model for the analysis of gene amplification in tumours and mammalian cell lines.

Extensive studies have been done on the *Plasmodium* species with respect to karyotype, chromosomal abnormalities and drug resistance. Cytogenetics of this parasite, as with many other protozoa, has not been successful due to the inability of the chromosomes to condense during meiosis. FAGE was useful in providing a technique for the genetic analysis of these organisms. FAGE separation of *P. falciparum* DNA indicated that homologous chromosomes in independent isolates exhibit size polymorphisms [58–60] (Fig. 4). It has been argued that this chromosomal variation occurred during both meiosis and mitosis as the polymorphism was consistent in both fresh clinical samples and cultured isolates. Corcoran *et al.* [58] suggested that the size polymorphism between homologous chromosomes was due to deletion/duplication of chromosomal sequences. Additional studies by this group showed a degree of polymorphism in subtelomeric regions regularly associated with fragments bearing rep20 repeats [61]. rep20 is a *P. falciparum*-specific repetitive DNA element reported to reside exclusively within the subtelomeric region on all falciparum chromosomes. Corcoran *et al.* [61] proposed that the chromosome size polymorphism was a result of recombination within the rep20 repeats leading to deletions of rep20 including the end of the chromosome. Larger deletions may remove telomere-proximal genes as well. Genetic linkage maps of *P. falciparum* chromosomes have been produced by probing Southern blots of FAGE gels with specific chromosomal probes. A number of cloned antigens, enzymes and other *Plasmodium* genes have been mapped to particular chromosomal locations [62]. Many antigenic genes have been localised near the ends of the chromosomes, and it is postulated that this allows for the observed antigenic diversity through frequent recombination of rep20 sequences [61,62]. Deletions of the subtelomeric regions of these chromosomes, as described earlier, result in the loss of several of these antigenic genes.

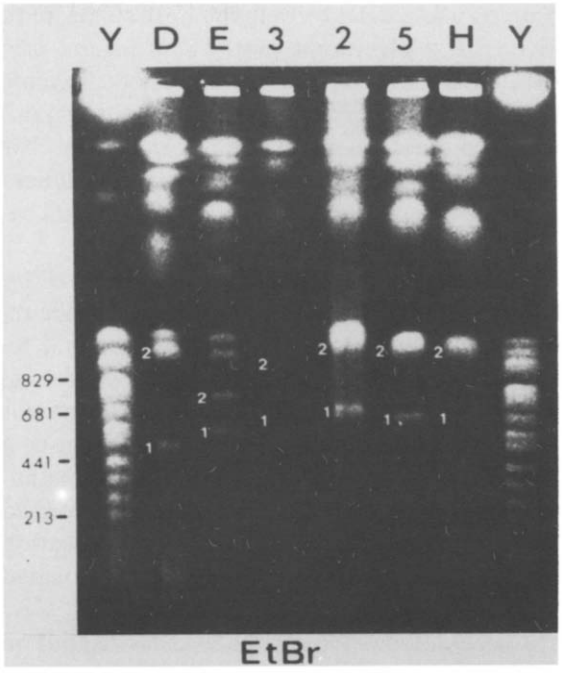

Fig. 4. Contour-clamped homogeneous electric field (CHEF) separation of the smaller *Plasmodium falciparum* chromosomes (lanes 2, 3, 4, 5, 6 and 7) demonstrating chromosomal rearrangements in these organisms. Lanes 1 and 8 contain the resolved chromosomes of *Saccharomyces cerevisiae*. Separation conditions were 1% agarose, 120 V with a 150-s pulse time over a 22 h period at 12°C. (Reproduced from Kemp *et al.* [59].)

These *P. falciparum* antigens include the knob-associated histidine-rich protein (KAHRP) and the ring-infected erythrocyte surface antigen gene of the *P. falciparum* isolate FCR-3 [64]. KAHRP is required for knob formation which mediates the binding of the infected erythrocyte to capillary endothelial cell surfaces [63]. Studies by Biggs *et al.* [64] located the cytoadherence gene (KAHRP) to one end of chromosome 2 as the

chromosome 2 deletion was the only correlate to the knobless phenotype [64]. These results support earlier findings by Pologe and Ravetch [63,65]. Biggs *et al.* [64] used two-dimensional-FAGE to detect the subtelomeric deletions. The chromosomes were separated in the first dimension, with the sizes of the telomeric fragments being determined in the second.

Other studies of *P. falciparum* using FAGE include those which are mainly interested in the relationship between the TS–DHFR gene and resistance to pyrimethamine [66–68]. Most of these reports indicate that a genetic alteration of this gene, being either a single amino acid change or a point mutation, confers resistance to pyrimethamine in falciparum malaria. All studies have used FAGE to determine the chromosomal location of the TS–DHFR gene, unambiguously located to chromosome 4.

Molecular karyotyping has been carried out on the opportunistic AIDS pathogen, *Pneumocystis carinii* [69]. Results of these studies, using OFAGE and CHEF, indicate sixteen or twenty chromosome bands respectively. The total size of the *P. carinii* genome has been estimated at 8–16 Mb. Fishman *et al.* [70] demonstrated that *P. carinii* contained fourteen chromosomes, ranging in size from 295 to 710 kb with chromosomal variation between isolates. The TAFE system was used to separate the chromosomes, and the filter replicate was hybridised with a *P. carinii* DHFR probe [71]. DHFR inhibitors have been the most thoroughly characterised of the anti-*P-carinii* agents. These techniques were used to extend studies of the basic biology of this organism and to facilitate the development of new anti-*P. carinii* agents. Edman *et al.* [71] were able to isolate *P. carinii* DHFR cDNA sequences, and clearly demonstrated that there is no genetic linkage between DHFR and TS unlike *P. falciparum* in which DHFR is found as a bifunctional enzyme with TS [66].

Studies of trypanosomes using FAGE have been carried out since 1984, shortly after the introduction of the technique. Antigenic variation in *Trypanosoma brucei* chromosomes have been demonstrated, and FAGE techniques have been used to resolve trypanosome DNA into four fractions [72]: (1) a number of mini-chromosomes estimated to range in size from 50 to 150 kb; (2) about six discrete chromosomes of 200–700 kb; (3) several chromosomes of 2 Mb or more; (4) large DNA fraction remaining close to the gel slot.

Hybridisation of field alternation gel blots with variant surface glycoprotein (VSG, antigenic determinants) probes demonstrate that the VSG genes are widely spread throughout the *T. brucei* genome. Van der Ploeg *et al.* [72] also showed that the mini-chromosomes hybridise strongly with the VSG probe and postulated that their main function was to provide a large pool of telomeric VSG genes. Majiwa *et al.* [73] demonstrated the presence of two different VSG genes on mini-chromosomes of *Trypanosoma (Nannomonas) congolense* with activation of these genes by duplicative and non-duplicative activation. Bernards *et al.* [74] used FAGE to characterise the duplicative activation of the VSG 1.3 gene in *T. brucei*. Johnson and Borst [75] mapped the VSG genes of *T. brucei* on chromosomes separated by FAGE in addition to analysing the mechanism of activation. It was shown that duplicatively activated genes are invariably translocated to the same chromosomal band. During non-duplicative activation, the chromosomal location of the VSG gene is constant in its active and inactive state. This research group further identified the second largest chromosome band as being preferentially used for VSG gene expression during chronic infections. Van der Ploeg *et al.* [76] demonstrated that the large chromosome class (> 1 Mb) contains a number of housekeeping genes and several VSG genes, whereas VSG genes are the only structural genes coded for on the intermediate (200–1000 kb) and mini-chromosomes (50–200 kb).

It is quite clear that the advent of FAGE has had a dramatic impact on the genetic analysis of protozoa. Prior to the introduction of FAGE, genetic studies were impeded by the complexity of the protozoan life cycle and the inability to carry out chromosome condensation during meiosis. FAGE has allowed separation of chromosome-

sized molecules up to at least 10 Mb in length. It has also proven useful for chromosomal studies such as molecular karyotyping, construction of linkage and restriction maps enabling chromosomal localisation of genes.

7. GENETIC ANALYSIS BY FAGE

7.1. Mammalian genetics

FAGE has become an important technique in unravelling the structure and function of the major histocompatibility complex (MHC), the multigene family essential in antigen presentation and cell cooperation during the immune response. Geneticists are now able to directly determine genomic structure and gene linkage within large contiguous regions [77]. Dunham *et al.* [78], using FAGE and Southern blotting, established a map of approximately 4 Mb of DNA encompassing the human MHC (*HLA*, human leukocyte antigen). This study was able to orient the complement and the steroid 21-hydroxylase gene loci relative to the class I and class II regions, and also determine that the distance between the *C2* gene and the *HLA-B* (a class I gene) locus is approximately 650 kb. Dunham *et al.* [78] demonstrated that, while the organisation of the human MHC is very similar to that of the mouse, the estimated size of the *HLA* regions was twice that of the mouse. It is thought that establishment of a physical linkage map of the *HLA* will aid in a further understanding of *HLA* disease associations.

A more specific analysis of the human MHC was carried out by analysis of multiple HTF (*Hpa* II tiny fragment) island-associated genes in the class III region [79]. It is known that these CpG-rich sequences (HTF islands) usually occur flanking housekeeping genes and some tissue-specific genes [80]. It was therefore hypothesised that the location of such regions would lead to the discovery of novel class III loci. Identification of new genes within the *HLA* is of major importance as this knowledge may aid in a better understanding of genomic evolution and autoimmune and non-immune disease pathogenesis [81]. Genomic Southern blot analysis of digested DNA fractionated by crossed field gel electrophoresis [24] allowed determination of the methylation status of the DNA restriction sites. Eleven potential HTF islands were found by Sargent *et al.* [79] in the region between the *TNF-α* and *C2* genes, and two were mapped between the *Factor B* and *C4A* genes. Sargent *et al.* [79] were able to identify transcripts (between 0.6 and 6 kb) corresponding to the products of twelve novel single copy genes. These potential new genes are of unknown function, although it has been suggested that they may be important in *HLA* association with the development of autoimmune disease. A further search for novel MHC-associated genes in humans was undertaken by Janatipour *et al.* [81]. This research group was able to identify five new genes that mapped centromeric of the class II loci. The discovery of these genes required the use of mouse genes as hybridisation probes, which are known to be expressed in specific tissues. Expression and function of these novel human homologues have yet to be determined.

FAGE has also been useful in evaluating differences in gene copy number [82] in the assessment of different ancestral haplotypes. The ancestral haplotypes examined were conserved MHC haplotypes that appear identical between unrelated subjects. The gene copy number of certain ancestral haplotypes have already been established at the *C4* and *CYP21* loci [83]. In the study by Zhang *et al.* [82], gene copy number at other loci were determined by densitometric comparison of different ancestral haplotypes after DNA separation by FAGE. Results showed that there was at least 2-fold differences between ancestral haplotypes with respect to tumour necrosis factor (TNF). This difference may affect the degree of TNF production. Analysis of *HLA-DRB* showed that gene copy number was a function of the particular ancestral haplotype and serologically, specificity of certain ancestral haplotypes has been correlated with the number of *DRB* genes present. Therefore, immunological reactions involving the DRB proteins may be influenced by the gene copy number as well as the protein sequence. Conclusions made by Zhang *et*

al. [82] were that differences in ancestral haplotype gene copy number may influence autoimmune disease susceptibility.

Using the technique of FAGE, many genes of the class II, class I and central MHC regions have been mapped. Müller *et al.* [84] generated long-range restriction maps of the murine MHC complex (*H-2*) surrounding the three gene clusters located in the *K*, *I*, *S* and *D* regions. As a result, this group were able to orient the complement gene cluster, mapping it to 170 kb distal to the *I* region gene cluster and at least 350 kb from the *D* region cluster. These findings suggested that there may, in fact, be more genes located within this region of the MHC than have yet been cloned. FAGE analysis of the BALB/c mouse *H-2* has also led to a minimum size estimate of 2.3 Mb for this region. Lafuse *et al.* [85] established a large-scale genomic restriction map of the *S/D* region of the murine *H-2* complex using FAGE, confirming previous results [84]. This study also reported that the *BAT* gene, recently mapped to the human *HLA* complex between *C2* and *HLA-B*, demonstrated a similar order and position in the mouse. Woolf *et al.* [86] utilised the novel two-dimensional gel electrophoresis technique to determine the organisation of the murine T-cell receptor γ locus. Large restriction fragments were fractionated by FIGE in the first dimension, followed by a second restriction enzyme digestion and conventional agarose gel electrophoresis. FIGE permitted the determination of the maximum distance between the genes in a multigene family after size estimation of the hybridising fragments. Electrophoresis in the second dimension allowed mapping of different cross-hybridising genes to the large fragments. This report indicated that the predominant rearrangements in the organisation of the murine T-cell receptor γ chain involve gene segments separated by no more than 60-70 kb. It was also seen that the γ chain differs from the α and β chains in that the V gene segments are interspersed between several C genes. The murine γ gene family, however, appeared to exhibit a very different gene organisation compared to that of the human loci.

Vardimon *et al.* [87] developed a physical map of the rat MHC complex and the MHC-linked growth and reproduction complex (*grc*) with the use of FAGE. Alterations in this *grc* region correlate with susceptibility to chemical carcinogens and defects in growth and reproduction. This report demonstrated that the MHC-*grc* region contained five fragments of DNA of approximately 3–3.2 Mb and indicated that the rat MHC is closer in size to the human *HLA* than the murine *H 2*. Analysis of grc^+ and grc^- strains has demonstrated that a deletion of 3–4 kb in grc^- strains and a loss of genes normally present in this region may cause the phenotypic defects observed in these strains [88]. A susceptibility to cancer in grc^- strains suggested that this deleted region may contain tumour suppressor genes [89,90].

Studies have also been aimed at analysing the organisation of the central MHC (class I and class II) of the goat [77]. The interest in the caprine MHC stemmed from the recent observation that susceptibility to a lentivirus-induced polyarthritis (caprine arthritis that resembles human rheumatoid arthritis in many aspects) segregates with serologically defined MHC class I antigens. RFLP analysis coupled with FAGE allowed determination of polymorphism and linkage of central MHC genes to class I and class II genes. This study by Cameron *et al.* [77] demonstrated that the class I and class II genes were highly polymorphic, with *C4* and *C2* genes exhibiting moderate levels of polymorphism as well. *TNF-α*, however, appeared relatively conserved with respect to the sequence data of mouse, rabbit, pig and man. FAGE analysis established linkage of *C4*, *CYP21* and *C2* genes on a single cluster of less than 200 kb in length. *HSP70* (a heat shock protein of 70 kD) was found to be within 380 kb of the *C4* genes, and linkage was shown of *HSP70* to *TNF* and class I genes. These findings suggest a total distance of less than 800 kb from *C4* to class I.

To determine whether trans-species evolution of *Mhc-DRB* haplotype polymorphism in primates has occurred, FAGE, in combination with chromosome walking and sequencing, allowed organisation of the *DRB* genes in the chimpanzee [91]. In man, this region of the MHC displays

considerable polymorphism, as five major haplotypes have been identified, each differing in the number and type of genes present. It has been found that the *DRB* region in the chimpanzee also contains length polymorphism, with some haplotypes being longer than the longest known human *DRB* haplotypes. This report also demonstrated that at least a portion of the haplotype polymorphism in the human population is conserved in the chimpanzee.

7.2. Plant genetics

A novel approach to isolating and analysing plant genomes came to fruition following the development of FAGE techniques. FAGE has enabled genetic analysis of plants and provides the capability of generating physical maps of large plant genomes, for example cereals and tomatoes. It has been shown that intact DNA molecules from protoplasts of wheat, barley and rye were 6 Mb or more in length [92]. High-molecular-mass DNA from tomato leaf protoplasts have also been examined using FAGE [93]. Tomato DNA was shown to be at least 2 Mb in size, which was then subjected to restriction enzyme digestion and fractionation by FIGE [93]. It was also reported that intact chloroplast DNA, after release from the agarose-embedded protoplasts, was not mobile under these electrophoretic conditions. It is known that large circular molecules, either supercoiled or relaxed, are able to migrate through the gel matrix when subjected to FIGE. Therefore, tomato chloroplast DNA was expected to be mobile during FIGE. This, however, was not the outcome and raises the question as to the unique packaging and tertiary structure of chloroplast DNA.

Examination of the nuclear genes coding for 5S ribosomal RNA (5S DNA) in wheat by FAGE has shown a high degree of polymorphism [94]. This study provided an insight into how polymorphisms such as this can be used to identify genotypes that were previously difficult to characterise. This technique has proved extremely useful in fingerprinting bacterial and parasitic species in particular. The 5S DNA patterns obtained by Röder *et al.* [94] were reproducible and found to be inherited through generations, therefore could be used as single locus markers for genetic and breeding studies. Broun *et al* [95] reported the use of FAGE to examine the long-range structure of tomato telomeres in closely related plant varieties. Genetic analysis of these telomeric arrays indicates high levels of heritable polymorphism. Therefore, these can be similarly used to the 5S patterns obtained by Röder *et al.* [94] to identify unique DNA fingerprints for self-pollinated crops such as soybean, wheat and tomato.

Genetic mapping of plant chromosomes using FAGE has generated information on the chromosomal location of many disease-resistant loci and some plant proteins [93,96]. In addition, knowledge of linkage of these loci to molecular markers has also been obtained in some cases. Siedler and Graner [96] used CHEF to fractionate high-molecular-mass DNA fragments from two barley cultivars in order to construct physical maps of the *Hor 1* locus. Hordeins are barley storage proteins that are deposited in the endosperm. Five different loci on chromosome 1H code for different hordein families [97]. Little was known of the chromosomal organisation of the *Hor 1* locus prior to this study. Siedler and Graner [96] reported an estimated size of 135 kb for the *Hor 1* locus in both cultivars and the presence of a CpG island at the left end of the locus, similar to those found in mammalian genomes. It is generally thought that these CpG-rich regions are found flanking transcribed genes in mammalian systems [98]. Therefore, it may be reasonable to suggest that this may also be the case in plant genomes, that CpG islands flank genes such as disease resistance genes. FAGE is already used extensively in detection of HTF islands and hence may permit the identification of resistance loci.

8. CLINICAL APPLICATIONS OF FAGE

8.1. Oncology

The genetic instability typical of cancer cells is frequently manifested by gene amplification, *i.e.*

the acquisition of extra copies of a specific gene [99]. Amplification is frequently associated with gene overexpression [100] and is thought to provide a selective growth advantage, hence the ability to survive in suboptimal growth conditions. This increase in gene copy number is generally thought to be initiated by the production of acentric, circular, extrachromosomal DNA molecules which replicate autonomously [101]. The formation of these abnormal chromosmal structures appear to be linked with chromosomal deletions. It has been established that most types of human neoplasia are associated with particular chromosomal defects [102]. It is also known that some genomic alterations induce the activation of an oncogene, a recurring chromosomal translocation, for example in leukaemias and lymphomas. Increased transcription of an oncogene as a result of integration of a viral promoter in the vicinity of the oncogene can also contribute to the progression of malignancy. Oncogenic amplification has been well documented, usually with single cellular oncogenes amplified in any individual tumour [103]. Several types of neoplasms are consistently associated with oncogenic amplification, for example N-*myc* amplification in human neuroblastomas, *ERBB2* amplification of human mammary cancers and amplification of *ABL* in chronic myelogenous leukaemia.

Another aspect of FAGE application in oncology is in the detection of "episomes" in human tumours. Episomes are a class of DNA amplification structures that have recently been described in several mammalian tumour cell line cultures. These structures are autonomously replicating circular DNA elements, that appear to share many characteristics of double-minute chromosomes. However, these DNA elements cannot be visualised by light microscopy at metaphase, as they consist of fewer than one million base pairs of DNA. The limitations of cytogenetic analysis prevent questions with clinical relevance from being answered. Primarily, what is the frequency of such chromosomal amplification structures in human tumour cells? FAGE analysis is now capable of providing some insight into this question, as episomes and other circular extra-chromosomal structures can be detected by this technique. Circular extra-chromosomal DNA structures that contained amplified N-*myc* oncogenes in three primary neuroblastomas and three metastatic lesions have been recently reported [104]. One tumour was shown to contain amplified N-*myc* genes on both double-minute chromosomes and an episome. Different amplification structures were identified by γ irradiation followed by electrophoresis using the CHEF apparatus. This method is dependent on the differential electrophoretic behaviour of chromosomal and extra-chromosomal DNA sequences following irradiation.

Results from this study by Van Devanter *et al.* [104] indicated that extra-chromosomal amplification of N-*myc* was present in the majority of neuroblastomas examined. It was suggested that this technique combining DNA irradiation and FAGE can complement traditional cytogenetic approaches in the study of DNA amplification in human neoplasia. The presence of an episome and a double-minute chromosome in one tumour sample supports the hypothesis that episomes are precursor amplification structures of double-minute chromosomes [101]. Although this study was unable to provide any information as to the mechanism by which extra-chromosomal circular structures arise in these neuroblastomas, it was shown that extra-chromosomal amplification structures predominate *in vivo*. Amler and Schwab [105] reported that human neuroblastoma cells exhibit a regular pattern of arrangement of amplified DNA, and that the coding region of N-*myc* was not affected by recombination despite numerous rearrangements of the amplified DNA. These findings suggest that N-*myc* itself provides the selective advantage for the retention of the amplified DNA. Detection of DNA amplification structures would play an essential role in the investigation of gene amplification within tumour cells, which has great prognostic value.

There has been a great deal of interest in the q13 region of the human chromosome 11 in recent years. Reports indicate that genes and DNA markers mapping to this band are involved in the pathogenesis of a number of distinct malignan-

cies [106]. A reciprocal chromosomal translocation between a locus on 11q13 termed *BCL-1* and the immunoglobulin heavy chain locus on 14q32 has been documented in some B-cell malignancies. It has also been reported that the 11q13 region is amplified in major epithelial neoplasms including breast and oesophageal carcinomas, and squamous cell carcinomas of the head and neck. FAGE studies by Lammie *et al.* [106] demonstrated that D11S287, an oncogene implicated in the pathogenesis of parathyroid adenomas, is located within 250 kb of the *BCL-1* locus on 11q13. Physical linkage was also established between *BCL-1* and D11S287. It was shown that D11S287 is co-amplified with *INT-2* (a known oncogene at least 1000 kb distant to *BCL-1*) in 36 primary breast tumours and was also overexpressed in tumours and cell lines containing 11q13 amplification. It was suggested by Lammie *et al.* [106] that the D11S287 gene may be activated by both translocation and amplification, and in a variety of distinct neoplasms. Rosenberg *et al.* [107] reported that rearrangement of the *BCL-1* locus may induce deregulation of the D11S287 gene (now designated as *PRAD-1*, for parathyroid adenomatosis). This study examined the 11q13 region using FAGE. Results obtained from this analysis demonstrated physical linkage between *PRAD-1* and *BCL-1*, with a distance of no more than 130 kb between the two breakpoint loci. *PRAD-1* amplification was also shown in all centrocytic lymphomas examined and in two unusual chronic lymphocytic leukaemias with a t(11;14) translocation and *BCL-1* rearrangement. Suggestions have been made implicating amplification of *PRAD-1* in centrocytic lymphoma and also as a major consequence of *BCL-1* rearrangements in B-cell neoplasms.

Field alternation analysis has proved significantly useful in detection and localisation of chromosomal translocations implicated in particular neoplasms. This technique has been shown to readily detect t(14;18) translocations in malignant lymphoma [108], with an enhanced detection rate when compared to Southern blotting, PCR and cytogenetic analysis. Of the 29 cases examined in this study, FAGE successfully detected translocation in 25 cases, conventional electrophoresis and cytogenetic analysis each detected 21 translocations, and PCR detected 19. It is interesting to note that in one case, FAGE was the only technique able to detect the chromosomal rearrangement. Detection of the t(14;18) translocation by conventional electrophoresis followed by Southern blotting requires hybridisation of two molecular probes prepared for the major breakpoint region and the minor cluster region. The advantage of using FAGE as opposed to conventional electrophoresis and subsequent hybridisation is that by generation of large DNA fragments there is less likelihood of a restriction enzyme site occurring between the probes either side of the breakpoint. Therefore, the results presented by Zelenetz *et al.* [108] conclusively demonstrate that FAGE is the most effective and comprehensive technique available for detection of the t(14;18) translocation in malignant lymphoma.

Specific chromosomal translocations are known to be implicated in the pathogenesis of several types of leukaemia. The most predominant alteration results in the formation of the Philadelphia chromosome (Ph^1), which is reported to be present in over 90% of patients with chronic myelogenous leukaemia (CML) [109]. The translocation resulting in the Ph^1 has been characterised cytogenetically as being between chromosomes 9 and 22, t(9;22)(q34;q11) [110]. It was later discovered that this translocation involves sequences of the *ABL* protooncogene and the breakpoint cluster region (*bcr*) gene on chromosomes 9 and 22, respectively, combining to produce a *bcr-ABL* fusion gene on the Ph^1 [111]. Although the presence of the Ph^1 is known to be highly specific to CML, it has also been observed in a minority of acute lymphoblastic leukaemia (ALL) patients [112]. FAGE analysis, as previously stated, has been successfully applied to the detection of translocation breakpoints in a number of neoplasms. Detection of the Ph^1 has been well documented in CML, and more recently in ALL [113–115], with Ph^1 identification in ALL patients being a strong negative prognostic factor. Interestingly, molecular analysis of the Ph^1

in ALL indicated that only half of ALL patients had alterations within the *bcr* similar to CML. The remaining ALL patients were found to have a translocation breakpoint upstream of the *bcr*. It is in detection of these Ph^1 (+) ALL patients with an unrearranged *bcr* that FAGE has proved most useful. Analysis of the translocation breakpoints in this group has shown that the rearrangements are scattered on both chromosomes 9 and 22 over large regions of up to 200 kb. These regions are too large to be resolved using conventional electrophoresis in conjunction with Southern blotting. However, FAGE is capable of detecting rearrangements over a large genomic distance in clinical samples, and is therefore currently being evaluated as a diagnostic test in detecting the *bcr*-unrearranged subtype of ALL patients.

FAGE has also been used to facilitate large-scale physical mapping of chromosome 22, which until recently has been limited by the availability of useful polymorphic probes. One of the advantages of FAGE is that significantly fewer probes would be required to map the entire chromosome in comparison to genetic mapping by RFLPs. Using the CHEF system, Herzog *et al.* [116] were able to establish a physical map for the regions surrounding the platelet derived growth factor (*PDGFB*) gene and the *D22S16* marker in the meningioma chromosomal region. *PDGFB* is an oncogene known to be the most proximal marker to be lost in meningioma with a (46,22q-) karyotype [117]. Results indicated that both loci are localised to the same band region 22q12.3–13.1. Linkage analysis by FAGE demonstrated the *D22S16* is situated within 900 kb of the *PDGFB* gene. However, probes directed against these two loci were unable to detect any structural rearrangements of these sequences in meningioma. This study by Herzog *et al.* [116] should contribute to the construction of a complete physical map of chromosome 22 and may aid in localising the putative suppressor gene for meningioma.

8.2. Duchenne muscular dystrophy

The disease locus for Duchenne muscular dystrophy (DMD) has been mapped within the Xp21 band of the human X-chromosome, with the DMD gene sized at 2.3 Mb [118]. A number of other X-chromosome diseases have been localised to the same region of the genome, including the disease loci for adrenal hypoplasia (AHC) and glycerol kinase (GK) deficiency. Most of these X-chromosome deletions are cytogenetically detectable in extended chromosome spreads indicating that these deletions are large (>2–3 Mb) and in some cases non-overlapping. Analysis of deletions in patients suffering from various combinations of the three syndromes has established the order of the disease loci, Xpter–AHC–GK–DMD–Xcen [119]. Early mapping studies using the original FAGE technique described by Schwartz and Cantor [120] analysed the positions of several cloned probes closely linked to and flanking the DMD gene [121]. Love *et al.* [119] reported a region of at least 4 Mb separating the 3′ end of the dystrophin gene and the closest distal marker to AHC, DXS28. A new locus, designated JC-1, was also described which maps between GK and AHC in Xp21.2–21.3. This locus, after physical mapping, failed to be linked with either the 3′ end of the dystrophin gene or with DXS28 (distal to AHC). Results from FAGE analysis also show a lack of HTF islands, indicating the presence of few genes in the Xp21 region. This study should assist in further genetic and physical analysis of the GK–AHC region in patients with clinical phenotypes relevant to the Xp21 region.

Den Dunnen *et al.* [122] used FIGE to analyse mutations in the DMD locus, a common cause of DMD. The detection of such deletions is of clinical importance, resulting in reliable prenatal diagnosis and carrier status. Previous detection of structural rearrangements was through the use of linked RFLP markers. FIGE analysis of DMD mutations has a significantly higher detection rate than conventional methods. This finding is particularly apparent in cases where DMD deletions occur at large distances from a given probe. Using conventional Southern blotting techniques, such deletions would not be detected as the distance between the deletion and probe is too great. Den Dunnen *et al.* [118] recently re-

ported 61% detection of DMD deletions without linkage analysis, illustrating the diagnostic potential of FAGE. The FIGE technique has also shown great importance in the analysis of carrier females. In comparison to the FIGE technique, cDNA analysis of carrier detection relies on densitometric analysis of the hybridising fragments. Results obtained in this study clearly show two easily distinguishable sets of hybridising FIGE fragments, the normal and mutated chromosome. Southern analysis of carrier DNA, however, shows similar patterns to the control with differing hybridising intensities.

8.3. Cystic fibrosis

FAGE has been applied to many aspects in the search for knowledge regarding the cystic fibrosis (CF) gene. CF is a lethal autosomal recessive disorder in the Caucasian population, with a disease frequency of about 1:2000 and a 5% carrier rate [123]. Linkage analysis and high-resolution cytogenetics has allowed the CF locus to be mapped to the long arm of chromosome 7, band q31, with a number of flanking markers, including the *met* oncogene, exhibiting linkage to the CF locus [124]. Estivill *et al.* [125] identified a genomic sequence with the characteristics of an HTF island, in high linkage disequilibrium with CF, using a "rare-cutter cosmid library". The location of this putative HTF island, particularly in relation to the *met* locus, was determined using FAGE analysis of *Not* I-digested human DNA and subsequent hybridisation. Results demonstrated that the putative HTF island is 650–750 kb from the *met* locus. Julier and White [126] described a *Not* I polymorphism detected by a cloned fragment of the *met* protooncogene, designated p*met*H. This polymorphism was detected in both normal and CF-affected individuals, and was shown by FAGE analysis to be 200–370 kb distant from the *met* locus, thus defining a new polymorphic locus in the CF region.

Rommens *et al.* [127] constructed a long-range physical map of the CF region by FAGE and determined the location of two DNA markers that are closely linked to the CF locus. These markers, D7S122 and D7S340, have been shown to be located between *met* and D7S8 (two markers thought to flank the CF locus). The combination of FAGE and cloning studies has permitted an accurate estimation of the size of the CF region and the distances separating linked DNA markers. Fulton *et al.* [128] have mapped a region of DNA spanning 12 Mb that contains the CF locus. This field alternation restriction map includes recognition sites for eight infrequent-cutting restriction enzymes and the location of seven DNA markers within the CF region. This is quite possibly the largest region of human genomic DNA that has been restriction-mapped by FAGE. Others include the DMD locus (3–4 Mb) [121,129] and the HLA complex (3 Mb) [130]. Thirteen putative HTF islands were also identified within this restriction map, spaced at intervals of 0.3–3.2 Mb. These CpG-rich sequences were determined by the presence of highly clustered restriction sites for several enzymes with a high C + G content in their recognition sequences. The CF locus was genetically mapped between the *met* and D7S8 locus, with a minimum distance between these markers of approximately 1.4 Mb.

Chromosome jumping libraries were constructed using FAGE by Collins *et al.* [131] and Rommens *et al.* [132], and applied to cystic fibrosis. Collins *et al.* [131] used this technique to derive cloned DNA sequences located approximately 100 kb downstream from the *met* oncogene. Chromosome jumping involves circularisation of size-selected DNA (fractionated by FAGE), bringing genomic fragments together that were originally 100 kb apart. The position of joining between the two fragments is marked by a suppressor transfer RNA or *supF* gene allowing fragment selection after the circles are *Eco* RI-digested and ligated into a phage vector. One fragment generated by this technique by Collins *et al.* [131], denoted CF63, was a 0.7-kb single-copy sequence located downstream to the 3′ end of the *met* transcription unit. This sequence was consequently mapped to chromosome 7, and then used as a probe to examine OFAGE-fractionated *Not* I-digested human DNA. Localisation of the cloned regions generated by Rom-

mens *et al.* [132] allowed a more detailed field alternation restriction map than the one previously published [133], indicating that the CF locus is approximately 250 kb in size. Chromosome jumping and walking has had a significant impact on genetics of diseases in which there is no prior knowledge of the basic defect [132]. It has allowed cloning of the locus responsible for cystic fibrosis, a gene that is several hundreds of kilobases away from the closest linked DNA markers. Cloning of a large, contiguous DNA segment, such as the CF region, has permitted a more thorough analysis of the region for candidate gene sequences.

9. OTHER APPLICATIONS OF FAGE

Intact chromosomes can be resolved with FAGE thus enabling full chromosomal analysis to be carried out. *Candida albicans* was one of the first eukaryotic genomes to be analysed by FAGE [134]. It has been demonstrated, by analysis of the genetics and molecular biology of *C. albicans*, that the organism is diploid and naturally heterozygous. The diploid nature of the organism complicates genetic analysis, as homozygous chromosomes may migrate separately or together as a single band, depending on the presence or absence of deletions, translocations or breakages in the DNA [135]. FAGE has been shown to be more sensitive in typing *C. albicans* isolates than conventional restriction endonuclease analysis, and in addition, more useful in the strain differentiation of *C. albicans*. FAGE has also been successful in karyotyping other yeasts including *S. cerevisiae* and *S. pombe* (Fig. 5), as well as a number of unicellular organisms such as *Plasmodia*, *Trypanosoma* and *Leishmania*. Genetic maps have been shown to be extremely useful in locating particular genes on the chromosomes. As a result, it is now possible to work on specific chromosomes, large intact genes or gene clusters in a region of interest, by locating the gene in specific restriction fragments.

FAGE has initiated further interest in DNA cloning, with the development of novel cloning techniques enabling the cloning of large DNA fragments into new vectors such as the phage P1 system and yeast artificial chromosomes (YAC) [12,13]. The size capacity of cloning has increased at least 10-fold with the use of FAGE [12]. As a result, long-range physical mapping of genomes

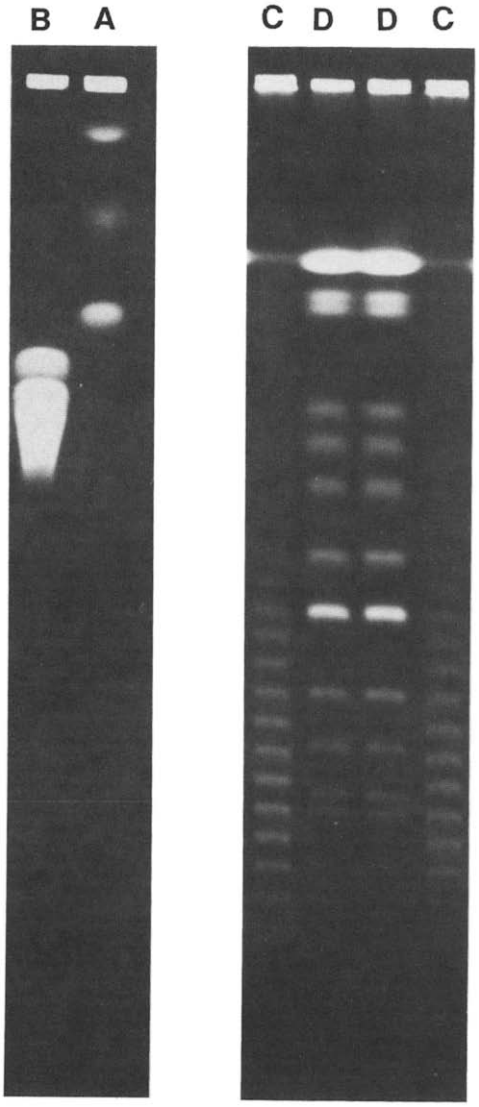

Fig. 5. Rotating gel electrophoretic (Waltzer) separation in the first gel of (A) the three chromosomes of *Schizosaccharomyces pombe* of 3, 6 and 9 Mb and (B) *Saccharomyces cerevisiae* exhibiting compression of the larger chromosomes. Separation conditions were a 60-min switch time over seven days, 1.2 V cm^{-1} and 0.7% agarose at 5°C. The second gel demonstrates separation of (C) lambda concatemers and (D) the chromosomes of *Saccharomyces cerevisiae*. The separation conditions for this gel were 1.5% agarose, 6 V cm^{-1} with a 60-s switching time over a 33-h period at 20°C. (Reproduced from Anand [13].)

is now possible. Previously, physical mapping had been limited by DNA fragments size threshold, usually DNA fragments smaller than 200 kb, after which progress was blocked by uncloned segments of DNA [136]. In the YAC system, cleavage of genomic DNA results in large DNA fragments that are ligated to vector sequences, each of which ends in a telomere and other indispensable chromosomal elements, as well as markers selectable in the yeast host, enabling their propagation as linear artificial chromosomes [136,137]. Individual YACs have been shown to be capable of containing inserts of up to 1 Mb or more [136]. YACs have already been made for plant and bacterial species, and libraries have been organised for *Drosophila* and human DNA. YAC libraries are constructed by preparing and fractionating large DNA fragments in agarose by FAGE, with the advantage of requiring fewer clones for physical mapping with *E. coli* vectors [138]. Thus, YACs may permit the use of intact genes for further medical and scientific studies, and also generate comprehensive libraries of the genomes of higher eukaryotes. The YAC cloning system may also provide a bridge between the data obtained from the two different maps of complex genomes: linkage data from genetic maps and data from physical or cytogenetic maps [136,137], none of which would have been possible without the development of FAGE systems. A model genome mapping is that of *Drosophila melanogaster*, in which YACs have been used to organise contiguous clones (contigs), which are in turn used to bridge to the physical and genetic maps of the organism [136].

Chromosomal structure is being analysed by using FAGE to examine variations in methylation patterns along a chromosome [139]. Variations that occur during development and differentiation in specific regions can also be determined utilising the methylation sensitivity of the restriction enzymes used in the FAGE digestion. Long-range FAGE mapping has been able to identify large clusters of under-methylated CpG dinucleotides associated with the 5′ end of some genes [80,139,140]. These clusters are known as HTF islands. The non-methylated clusters of CpG are rich in cleavable sites for mCpG-sensitive restriction enzymes. Inter-island restriction sites are highly methylated, thus resistant to cleavage. Therefore, any site that is cleaved by a rare-cutting enzyme identifies a methylation-free region in the genome. HTF islands have now been associated with many genes at the origin of their transcription. This association in conjunction with the technique of FAGE will permit the construction of long-range maps which may lead directly to new genes from the identification of possible HTF-islands.

FAGE has also been described as one of the newest strand-breakage assays of radiation-induced DNA damage [141]. Damage to the DNA following ionising radiation can be of many types. Such damage requires measurement in order to understand the molecular processes involved in the cellular response to radiation. Amongst the different types of DNA damage, DNA double-strand breaks (dsb) has been shown to be the most significant lesion induced by ionising radiation. Quantification of dsb induction is by the fraction and distribution of DNA migrating into the gel. This use of FAGE is sensitive to doses as low as 1 *Gy*, with *in vitro* studies by Ahn *et al.* [192] measuring dsb rejoining in mammalian cells irradiated with doses as low as 5 *Gy*. It is important to note that other strand-breakage assays require supra-lethal doses of X-irradiation to follow dsb rejoining. At lower doses, measurable by FAGE, there is a higher probability of cell survival and thus is more likely to reflect significant lesions for cell killing or tumourigenesis. There is potential using this technique for more detailed studies of the nature and distribution of radiation-induced damage.

10. CONCLUSION

So, what can be said in conclusion? Less than ten years after the development of Schwartz and Cantor's FAGE technique, a highly theoretical approach to DNA separation, a technique has emerged which has permeated every aspect of biological science. This technique, in the guise of two or three main electrophoretic configurations,

has made a major impact on our understanding of DNA structure, size, mapping, genotyping and a host of other applications. It would appear that the limits of the application of this technique are the investigators imagination.

In collecting the literature for this review, one cannot help but be overawed by the spectrum and volume of the work that has been made possible with FAGE. It can be seen that FAGE is no longer restricted to simply separating large DNA fragments. This technique is presently being used for electrophoretic karyotyping, long-range genomic mapping, cloning of large DNA fragments into new vectors, the study of pathogenic chromosomal alterations and the structural analysis of chromosomes. The applications of FAGE in molecular biology and genetics is constantly expanding, with the full potential of this technique still to be realised. As to its status quo — its development and innovative modification occurred at an alarming rate. One doubts whether Schwartz and Cantor could have suspected the impact of their technique on biological science. The developments which occurred in three years were mind-numbing. But the application to which this technique has been employed is the greatest testament to the sheer force of this now simple and universally accepted technique.

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