Asia Pacific Journal of Molecular Biology and Biotechnology
Volume 2, No. 2, June, 1994, pp. 78-85

MINI REVIEW

Pulsed-Field Gel Electrophoresis: A Revolution in Microbial Genetics

ANTONIUS SUWANTO*

Department of Biology, Faculty of Mathematics and Natural Science, and
Inter University Center for Biotechnology,
Bogor Agricultural University,
Bogor 16143, Indonesia

(Received 16 April 1994/Accepted 23 April 1994)

Abstract. Gel electrophoresis is a relatively simple but reliable technique for the analysis of DNA and RNA molecules. However, this method, in practical terms, cannot resolve the separation of DNA fragments more than 50 kilo base pairs (kbp). Pulsed-Field Gel Electrophoresis (PFGE) was able to overcome this problem by forcing the DNA molecules to change their orientation periodically from one electric field configuration to the other. PFGE analysis of large sized DNA molecules requires a technique to isolate intact genomic DNA as well as the availability of rare-cutting restriction endonucleases and the appropriate mega-base molecular size markers. Under optimized condition, PFGE can efficiently separate DNA fragments of 100 to 10,000 kbp to distinguish strains of microorganisms which otherwise exhibit similar or identical physiological and morphological characteristics. This technique has also been employed to study the occurrence of genomic rearrangements, plasmid fingerprinting, mutant analysis, cloning, as well as the construction of physical maps. This review is intended to introduce the principles of PFGE and its applications in the genetic analysis of microorganisms.

INTRODUCTION

Electrophoresis employing an agarose or polyacrylamide matrix has been a standard method to separate, identify and purify DNA fragments. This simple and rapid technique is able to separate DNA fragments which cannot be separated by other means such as density gradient centrifugation. Moreover, the position of DNA fragments inside the gel can be directly determined by staining with ethidium bromide and subsequent exposure to UV light. The observed DNA bands might also be isolated from the gel for further molecular work such as cloning, construction of subgenomic libraries, and generation of DNA probes.

Agarose gel has lower capacity of resolution in comparison with polyacrylamide gel, but it has a wider separation range of size of DNA fragments. DNA fragments ranging from 200 base pairs to approximately 50 kbp can be separated well through appropriate concentrations of agarose gel. Several types of agaroses have also been available for particular purposes. For example, low-melting point agarose and other special agarose for the optimal separation of DNA fragments with size less than 500 base pairs. Agarose gel electrophoresis is usually conducted in a horizontal position in a constant electrical field. Although theoretically one might be able to separate larger DNA fragments by

*Mailing address: Biologi, FMIPA, IPB, Bogor, 16143, Indonesia. Telephone/Fax No.: 62-251 21724.

lowering the concentration of agarose, in practice it is difficult to achieve useful resolution for DNA molecules larger than 50 kbp (1 - 3). Separation of large DNA molecules (>50 kbp) was not possible until the development of pulsed-field gel electrophoresis.

PULSED-FIELD GEL ELECTROPHORESIS

In conventional gel electrophoresis, all double-stranded, linear DNA molecules larger than a certain size (usually more than 50 kbp) will move at the same speed. This limit of separation will be achieved if the radial gyration of the DNA molecules exceeds the pore size of the gel (4).

Under these conditions, the DNA molecules cannot be sieved through the gel according to their size, but instead they move by deforming their shapes and size to match the gel pores. Therefore, in an electrical field in a conventional gel, large DNA molecules appear to behave like highly extended coils, oriented with their long axis of coil parallel to the electrical field. This kind of mechanism of DNA movement is called reptation (5). The larger the pore size, the larger the size of DNA molecules which can pass through without reptation. Thus, a 0.1- 0.2 % agarose gel can accommodate separation of larger DNA molecules. However, these are not satisfactory experimental conditions due to the long run times required and the mechanical difficulties involved in handling very dilute gels. Moreover, this very dilute gel is also unable to separate DNA molecules more than 700 kbp. The importance of this limit of separation will be significant if we realize that all of the physical maps of bacterial and some eucaryotic genomes documented to date were constructed through the analysis of large DNA fragments generated by rare-cutting restriction endonucleases (6, 7).

This separation problem was partially resolved in 1984 when Schwartz and Cantor at Columbia University reported the development of PFGE. This new technique takes advantage of the elongated and oriented configuration of large DNA molecules in agarose gels at finite field strengths. Unlike virtually all other separation methods, PFGE relies on the ability of the applied electrical forces to perturb molecular shape such that it overcomes the reptation effect (8). To migrate through the gel matrix, the DNA molecules must change their direction of motion in response to the changing electrical field. Separation is achieved because the time required to change direction is dependent on the size of the DNA molecules. In most cases the apparent reorientation time increases with DNA size; large molecules move more slowly than smaller ones. The critical experimental variable is the pulse time, which is the time an electrical field is applied in one direction before it is switched abruptly to another direction (9). DNA molecules with orientation time less than the given pulse times will be able to be separated according to their size (9). There is only a certain range of pulse times, however, which will give optimal separation of a particular set of DNA fragment size (10). DNA molecules up to 10,000 kbp in size can be separated well in most PFGE apparatus available to date (11 - 13).

INTACT GENOMIC DNA ISOLATION : A PREREQUISITE FOR PFGE ANALYSIS

Preparation of genomic DNA is generally conducted in solution (2, 14). Firstly, the target cells are made into spheroplasts or protoplasts by enzymatic treatment. The resulting protoplasts or spheroplasts are lysed by detergent treatment together with a chelating agent, such as EDTA. The result of this treatment is mainly a complex mixture of DNA, RNA and protein. The protease and ribonuclease treatments are intended to remove most of the protein and RNA in the mixture. The remaining protein can be further removed through phenol extraction or by the exclusion chromatography technique. DNA-containing solution is concentrated and precipitated by the addition of ethanol. To obtain DNA solution which is free from contaminants, which might interfere with subsequent analysis, sometimes it is necessary to repurify the DNA in the solution.

For genomic DNA analysis employing PFGE, it is important to prepare intact genomic DNA (7). An isolation method in solution as described above will generate fragmented DNA with an average size of less than 700 kbp. This fragmentation is due to the mechanical shearing which is intrinsic in the handling of a solution. Since the size of a living cell genome is generally more than 1000 kbp (6), DNA isolation must be performed carefully to avoid any mechanical shearing or contamination by DNA-degrading enzymes (7, 15).

A simple method to purify, handle, and store large genomic DNA in its intact state was introduced in 1984. This technique is required for PFGE analysis in general (8, 15). In addition, this technique is easily adapted for practical applications in the laboratory as well as in the field since it only requires simple equipment and allows the analysis of a large number of samples in a relatively short period of time. In this technique, DNA molecules are isolated from cells embedded in an agarose matrix. Cell lysis and DNA purification are conducted *in situ* to minimize DNA damage due to hydrodynamic shearing. This embedded DNA (which is also called gel insert or gel plug) is stable at 50°C for days, at room temperature for months, or even longer at 4°C. Thus DNA samples can be sent directly without a cooling system (A. Suwanto, unpublished result). DNA inside the gel insert is easily manipulated by simply treating them with the appropriate solutions (10, 16). An alternative method to the formation of agarose gel insert has been devised, which involves the embedding of cells in small agarose beads (17, 18).

APPLICATIONS OF PFGE TECHNIQUE

Eucaryotic microorganisms such as yeasts, molds, and protozoa possess linear chromosomal DNA with sizes which are amenable for genomic DNA analysis employing PFGE (8, 11, 12, 13, 19). Therefore, by using this new electrophoresis technique, it is possible to analyse directly the chromosomal profiles or chromosomal length polymorphisms (CLP) in these organisms (11, 13, 19-22). Most bacteria, however posses circular chromosomes (6) which behave differently from their linear counterparts (23 - 25). Therefore, a circular DNA molecule should be linearized to accurately determine its size (10, 26 - 29). Individual chromosome size as well as the total genomic DNA content can be directly determined from the bands generated by PFGE. Genetic markers, whether homologous or heterologous, can be localized on a particular chromosome by Southern hybridization analysis (10, 11). Moreover, subgenomic DNA libraries from individual chromosomes can be constructed through isolation of each chromosome followed by fragmentation and cloning into the appropriate vector (30). In addition to its value in routine static genome analysis, such as chromosome sizing (6), schizotyping (31), karyotyping (20, 21, 22), and genomic organization (26, 32 - 35), PFGE has been very useful in monitoring and analysing the dynamics of genomic DNA. Studies on chromosomal rearrangement and transposition employing PFGE have been conducted for bacteria, yeast, molds and a variety of protozoa. For example, by employing PFGE it was demonstrated that variation in the expression of outer membrane glycoprotein genes in trypanosomes was controlled by local genomic rearrangement or translocation (22, 36). Genomic rearrangement which includes deletion, insertion, translocation, inversion, and amplification, is frequently observed in the microbial world and has been part of the mechanism underlying the life cycle of several organisms. Generation of RNA polymerase sigma factor K (σ^k or σ^{27}) during sporulation in *B. subtilis* and changes in the expression of nitrogen fixation during heterocyst differentiation in *Anabaena* are but few examples of genomic rearrangement involving deletion (37, 38). Genomic rearrangement also plays a key role in certain variations in physiological, serological or pathogenic characteristics as well as fitness and survival in some bacteria (39, 40 - 44). The importance of this process in studying microbial genetics has prompted a general method for detecting genomic rearrangements (45). Due to its capability in examining the entire genome, PFGE will contribute significantly in this field of study.

Further detailed analysis of the genome, especially in bacteria, requires the digestion of total genomic DNA by rare-cutting restriction endonucleases. The type of restriction endonucleases required for such analysis may be predicted from the G + C content of the organism or from a knowledge of the frequency of the oligonucleotide sequences in the genome if such data are available (46). However, the bias for A + T or G + C rich recognition sites might not necessarily yield the smallest number of DNA fragments relative to other "unbiased enzymes" (10). The resulting DNA fragments can be ordered to generate a physical map of the chromosome and to unravel the organization of the genome. A physical map will be a foundation in the construction of a genetic map through Southern hybridization analysis or schizotyping analysis which is very useful for microorganisms with little or no known classical genetic system. Moreover, a high resolution physical map such as the Kohara map of the *E. coli* chromosome (47) will facilitate gene cloning and gene localization. Changes in the genome such as insertion, inversion and deletion can provide important data which, under systematic examination, will facilitate the construction of genomic maps. Physical maps can also be constructed by the "overlapping" method frequently used in plasmid mapping. Sites for the occurrence of genomic rearrangement (Hot Spots) could be localized in similar manner in conjunction with Southern hybridization analysis. PFGE has also been employed to perform DNA fingerprinting analysis which is applied in many areas of microbiology such as clinical (48 - 52), agriculture, food and industry (53 - 57). This technique has been very useful in characterizing strains within a species, in distinguishing strains which otherwise exhibit similar morphological and physiological properties, as well as in the construction of phylogenetic trees which will increase accuracy and stability in microbial systematics (58, 59). Thus far, PFGE has demonstrated greater sensitivity and reliability in strain differentiation than other commonly used typing techniques, such as auxotyping, serotyping, restriction endonuclease analysis (REA), ribotyping, and random amplified polymorphic DNA (RAPD) assays (48 -52). Although genome analysis can be performed through many molecular biological approaches, PFGE offers at least three unique characteristics as follows: (*i*) most of the results from gel electrophoresis (i.e. PFGE banding patterns) can be analysed directly, (*ii*) it gives the overall picture of the genomic profile, and (*iii*) it allows the construction of the physical map of a genome. Since the availability of a genome map is essential in understanding microbial diversity, microbial genome analysis has become routine work in many microbial genetics laboratories since its development in 1984. Genome size, schizotyping and physical map have been common keywords for many of the reports dealing with molecular microbiology. It will not take too long for us to realize that the availability of genomic maps will facilitate further research in this field as well as other explorative activities.

ORGANIZATION OF THE BACTERIAL GENOME: A SURPRISING SIDE OF BIODIVERSITY

One of the paramount applications of PFGE is to estimate genome size and to construct physical and genetic maps. In fact, PFGE has been regarded as the most definitive of currently available techniques to estimate sizes of bacterial chromosomes (7). Restriction fragments produced by rare-cutters can be used to determine the shape or geometry of the chromosome. In some instances, the chromosome geometry can be inferred from the difference in the mobility of linear molecules from their circular counterparts in PFGE (23, 26, 32, 60). Almost all of the bacterial chromosomes which have been physically mapped indicated endless set of ordered fragments or circular geometry (6). Sizes of chromosomes vary from 500 kbp in many strains of *Mycoplasma* to nearly 10 Mbp in *Myxococcus xanthus*.

It has been a general view in molecular biology that plasmids and bacterial chomosomes are topologically circular. However, in 1987 Kinashi and Shimaji had demonstrated physically that strains of *Streptomyces* carry giant linear plasmids with sizes up to 590 kbp (61). In 1989, Ferdows and Barbour reported the existence of small linear plasmids as well as a 950 kbp linear DNA molecule in *Borrelia burgdoferi* (60). This megabase-sized linear DNA molecule was shown to be a chromosome and its physical map was completed in 1992 (62).

Physical and genetic mapping of the *Rhodobacter sphaeroides* 2.4.1 genome showed that this bacterium carries seven different replicons (10, 26). The existence of these two chromosomal replicons was further verified employing a genetically-engineered Hfr-like system (31). Subsequent work on *Brucella melitensis* and *Leptospira interrogans* also demonstrated the presence of two unique circular chromosomes in each of these bacteria (33, 63). The genome of *Rhizobium meliloti*

1021 has been physically mapped and the location of the three *rrn* operons has been determined. This bacterium carries a single circular chromosome (3540 kbp) and two circular megaplasmids (1700 and 1340 kbp). These large plasmids carry genes involved in nodulation and nitrogen fixation (35). However, in *Bradyrhizobium japonicum*, the genes for nodulation and N_2-fixation are present in a single circular chromosome approximately 8700 kbp in size (34). Very recently Allardet-Servent *et al.* (32) reported that *Agrobacterium tumefaciens* C58 genome consist of one linear (2100 kbp) and one circular (3000 kbp) chromosomes as well as its cryptic 450 kbp plasmid and the famous 200 kbp Ti plasmid.

These studies have led us to realize that it is not possible to draw a clear-cut line between chromosome and plasmid based on their sizes or DNA topology. The accumulated data on genome organization will also widen our perspective on the issue of horizontal gene transfer and the concept of species in bacteria as well as other microorganisms.

For almost a decade after its development in 1984, PFGE has been very useful in dissecting genome structure and organization of various microorganisms which are crucial in our full understanding of microbial genetics. Due to its optimal separation range, today's PFGE apparatus are well suited and readily exploited for microbial genetics analyses. An extremely large body of physical maps of prokaryotes have become available in the last five years (*J. Bacteriology*, 1989-1993). A "revolution" has been going on in our understanding of diverse microbial genetic systems.

Acknowledgement. This study was supported in part by a grant from Riset Unggulan Terpadu (RUT I-1) to AS through the Indonesian Institute of Sciences (LIPI) with contract number 60/SK/PRT/93. I thank Martinus R. Tjahjadi for his excellent assistance in typing this manuscript.

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