Volume 16 Number 5 1988 Nucleic Acids Research

Preparation and FIGE separation of infrequent restriction fragments from *Mycoplasma mycoides* DNA

Louise E.Pyle and Lloyd R.Finch

The Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria 3052, Australia

Received November 16, 1987; Revised and Accepted February 12, 1988

ABSTRACT

Use of procedures for obtaining satisfactory preparation and digestion of intact DNA of Mycoplasma mycoides subsp. mycoides Y in agarose blocks is reported. The use of inverted field agarose gel electrophoresis (FIGE) for separation of the small number of fragments derived from the genome by several restriction endonuclease digestions is shown. An effect that fragments containing replication forks remain in the well during FIGE, distorting the representative yield of restriction fragments on the gels, is overcome by incubating cells with chloramphenicol for $1^1/_2$ h before harvest to allow rounds of replication to go to completion without new initiations of DNA synthesis.

INTRODUCTION

Recent developments in techniques for the electrophoretic separation of large DNA molecules in agarose gels by application of pulsed-fields in various configurations (1,2,3) have been applied particularly to the separation of the DNA of eukaryotic chromosomes. We are interested to use them for restriction endonuclease analysis of mycoplasma genomes which are reported to be small in size $^1/_5$ to $^1/_6$th that of Escherichia coli, and have a low G/C content (4). For reasons of convenience, economy and availability we chose to use field inversion gel electrophoresis (FIGE) (2). In this technique pulsing is effected by direct inversion of the field allowing multiple samples to be run in parallel lanes. The field in the reverse direction can use either a shorter pulse time or a lower voltage.

We report the procedures which we have developed to obtain satisfactory digests of the genome of Mycoplasma mycoides subsp. mycoides, strain Y as a small number of fragments and to give adequate separation of these fragments.

MATERIALS AND METHODS

Organism and culture.

Cultures of M.mycoides subsp. mycoides, strain Y were grown in PPLO broth (5), harvested and washed as described previously (6).

Chemicals, enzymes, etc.

Chemicals and biochemicals were from Sigma Chemical Co., St. Louis. Restriction endonucleases were from either Boehringer Mannheim Biochemicals or Pharmacia and Bovine Serum Albumin (BSA) of Molecular Biology Grade from Boehringer Mannheim Biochemicals. Agarose for DNA preparation was FMC Sea Plaque kindly supplied by Dr. Lynn Corcoran.

Preparation of DNA in agarose blocks.

Cells were incorporated into agarose blocks (at 4×10^9 cells/ml) and their DNA purified *in situ* as decribed by Schwartz and Cantor (1) with the modifications of Kemp *et al* (3).

Restriction endonuclease digestion of DNA in agarose blocks.

Blocks or sections of blocks were washed with 10 mM Tris/HCl pH 8.0, 1 mM EDTA, for 6 h at 4^oC, then incubated overnight with a 10-20 fold excess of restriction endonuclease using the appropriate buffer and temperature specified by the supplier. BSA was also added at 0.1mg/ml.

FIGE separation of restriction endonuclease fragments.

Sections of blocks containing suitable quantities of DNA were inserted into the gel slots of a $1.5^o/_o$ agarose gel (20cm x 21.5cm x 0.6cm) and electrophoresed using the FIGE technique of Carle *et al* (2). This uses a constant voltage with periodic inversions of the field with a shorter pulse in the reverse direction than in the forward direction. The procedure was greatly assisted by the use of a FIGET pulse programmer (Acronym Pty Ltd Boronia, Vic.).

Counting of radioactivity in DNA fragments separated by FIGE.

Agarose gels were stained in 0.5µg/ml ethidium bromide and DNA fragments visualized by exposure to UV light. Gel segments containing individual DNA fragments were excised from the gel and then further cut up into slices of approximately 0.25g. 0.75ml of 0.1M HCl was added to each slice in a scintillation vial and the agarose was dissolved by autoclaving. 10ml of scintillator (5 g of 2,5-diphenyloxazole per litre of toluene; Teric-10 [2:1, vol/vol] (7)) was added and the radioactivity in the DNA fragments was counted in an LKB Rack Beta 1215 liquid scintillation spectrometer.

RESULTS AND DISCUSSION

With FIGE separations it is known that the size of the DNA molecules showing least mobility varies with the pulsing conditions and this size increases with the pulse-time (2). Stepwise adjustments of the pulse-time are needed to separate molecules of a wide range of sizes. Periods at short pulse-times give effective separation of the smaller molecules and periods at

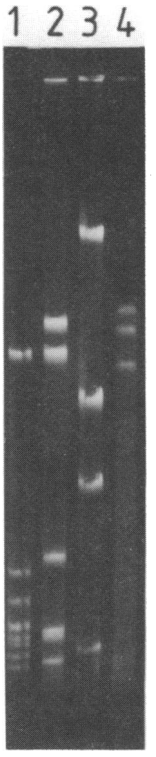

Figure 1. FIGE separation of fragments from digestion of M.mycoides DNA by various restriction endonucleases.
Electrophoresis was carried out in 44.5 mM Tris-borate, 44.5 mM boric acid, 1 mM EDTA pH at 8 volts/cm with a temperature maintained at 14°C by buffer circulation through a cooling coil. Pulsing was controlled by a FIGET Pulse Programmer set for forward to reverse pulse-times in a ratio of 5:2 with settings for the forward pulse being: 2 h at 4.3 sec; 24 h at 4.3 to 17.2 sec increasing linearly with time; 19.2 h at 21.4 to 34.3 sec increasing linearly with time. On completion of electrophoresis the gel was stained with ethidium bromide and photographed on a U.V. transilluminator. Restriction endonucleases used were: Lane 1, Bam HI; Lane 2, Bgl 1; Lane 3, Xho I; and Lane 4, Kpn I.

longer pulse times ensure the separation of the larger molecules and retard them strongly relative to the smaller molecules to eliminate the overlap of large and small which can arise at short pulse-times. Fig. 1 shows the separation of fragments from digestion of M.mycoides DNA with a number of endonucleases showing infrequent restriction sites in the genome. The DNA was prepared from cells incubated with chloramphenicol (80 ug/ml) for $1^1/_2$ hours before harvest. The gradation of intensity of the ethidium bromide

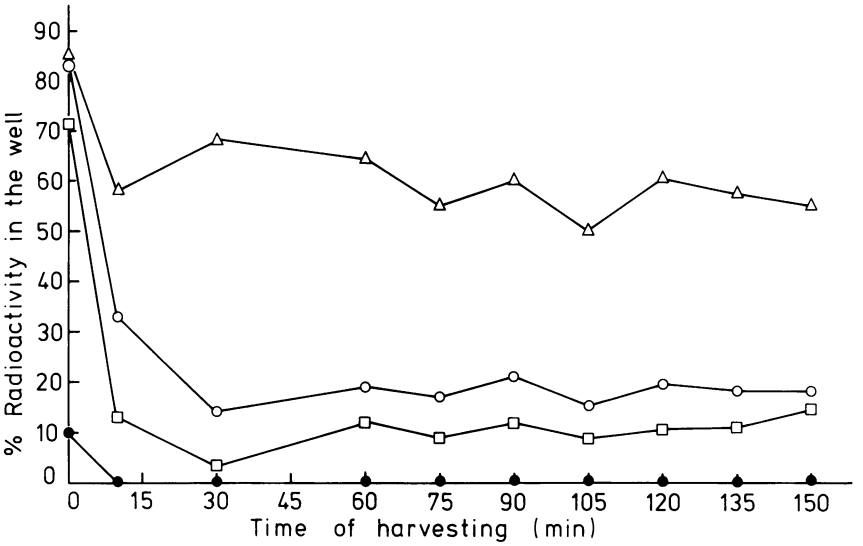

Figure 2. Effect of chase time on the retention of in vivo pulse-labelled DNA in the well during FIGE of restriction endonuclease digested M.mycoides DNA. Pulse-labelling of a mid-log phase culture of M.mycoides (7ml) was commenced at zero time by the addition of 14 uCi (330 pmoles) of [^{3}H]dTMP and terminated at 6 min by addition of 512 nmoles of unlabelled dTMP (final conc. 80 μM). Samples of 0.6 ml were taken at various times from 1 to 150 min after addition of label and cells harvested from them for incorporation into agarose blocks. Sections of the blocks were weighed and loaded into the well of a gel either untreated or after digestion with the indicated restriction endonuclease. Following FIGE, sections of the gel in and very near the wells were taken for counting of ^{3}H. The counts remaining in the well for the digested sample are expressed as a percentage of those remaining in well for the undigested sample. EcoRI ●—●, BamHI □—□, KpnI ○—○, ApaI △—△.

fluorescence with the position of bands on the gel shown in Fig. 1 indicates that the bands are distributed in order of decreasing size with the pulsing conditions used. This suggestion has been confirmed by comparison with digests in gels run for us by Dr. Lynn Corcoran, of the Walter and Eliza Hall Institute, Parkville, in a contour clamped homogeneous electric field (CHEF) apparatus (8) in which a linear relationship is shown between distance moved and molecular size over a considerable size range which can be varied by varying the pulse-time.

The use of stationary phase or chloramphenicol treated cultures of M.mycoides is desirable to obtain high and representative yields of all the restriction fragments generated from infrequently cut DNA. This requirement appears to result from an effect that replication forks remain in the well in

FIGE, leading to a preferential loss of large fragments which are the ones more likely to contain forks. This effect is illustrated in Fig. 2 which gives the distribution of label to the well on electrophoresis of various restriction digests of DNA from cells harvested at various times after pulse labelling of their DNA. To pulse label efficiently we used the capacity of M.mycoides to utilise deoxynucleoside monophosphates as preferred, but rapidly catabolised precursors of the corresponding nucleotide in DNA (9). The restriction enzymes used in the digestions for Fig. 2. were chosen to give: many small fragments, Eco RI; two ranges of intermediate size fragments, Bam HI giving 9 fragments and Kpn I giving 3 fragments of approximately equal size (see Fig. 1.); and a very large fragment approximating 90% of the genome from Apa I digestion which gives only 2 fragments.

For each of the digests shown in Fig. 2., the samples from zero time, when the labelling site and the fork have the greatest probability of being in the one restriction fragment, show the highest level of label in the well. The smaller the fragments, the less the fraction of label retained in the well for the zero time sample and the more rapidly the retention of label is decreased for later samples. These observations again reflect the probability that the labelled site and the fork may be associated in the same fragment.

In the case of the Apa I digests, in which one fragment is very large there is very high retention of label in the well for the zero time sample (85%), and only limited decrease in the retention for later samples. Thus most of the DNA labelled by the pulse is not available to appear as defined restriction fragments separated on the gel. This high level of retention would reflect the difficulty of having a very large fragment free of a replication fork. Assuming that the genome is circular and has bidirectional replication so that a replicating genome would have two forks, such a fragment might only be free of forks in non-replicating, completed genomes. The use of chloramphenicol to prevent reinitiations of replication while allowing existing replications to go to completion is one way around this difficulty. For example, for a culture incubated in chloramphenicol for 1 hour after labelling, the level of label retained in the well for a Bam HI digest was only 1.2% as compared with the level of approximately 12% without chloramphenicol as shown in Fig. 2. Allowing cells to reach stationary phase before harvest appears to be another way to ensure good yield of all fragments.

These observations on M.mycoides DNA restriction fragments in pulsed field electrophoresis have implications for similar studies on other procaryotes with small genomes. Another paper reports the use of pulsed field electrophoresis

of DNA restriction digests to estimate genome sizes for several mycoplasma species (10).

ACKNOWLEDGEMENTS

This work was supported by a grant from the Australian Research Grants Scheme.

REFERENCES

1. Schwartz, D.C. and Cantor, C.R. (1984). Cell 37: 67-75.
2. Carle, G.F., Frank, M. and Olson, M.V. (1986). Science 232: 65-68.
3. Kemp, D.J., Corcoran, L.M., Coppel, R.L., Stahl, H.D., Bianco, A.E., Brown, G.V. and Anders, R.F. (1985). Nature 315: 347-350.
4. Razin, S. (1985). Microbiol. Rev. 49: 419-455.
5. Rodwell, A.W., Peterson, J.E. and Rodwell, E.S. (1975). J.Bacteriol. 122: 1216-1229.
6. Mitchell, A., Sin, I.L. and Finch, L.R. (1978). J.Bacteriol. 134: 706-712.
7. Neale, G.A.M., Mitchell, A. and Finch, L.R. (1983) J. Bacteriol. 154: 17-22.
8. Chu, G., Vollrath, D. and Davis, R.W. (1986). Science 234: 1582-1585.
9. Neale, G.A.M., Mitchell, A. and Finch, L.R. (1984). J.Bacteriol. 158: 943-947.
10. Pyle, L.E., Corcoran, L.N., Cocks, B.G., Bergemann, A.D. and Finch, L.R. (1988) Nucleic Acids Res. (submitted).