Methylase-limited partial *Not*I cleavage for physical mapping of genomic DNA

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ABSTRACT

Partial cleavage of DNA with the restriction endonuclease *Not*I (5'...GC/GGCCGC...3') is an important technique for genomic mapping. However, partial genomic cleavage with this enzyme is impaired by the agarose matrix in which the DNA must be suspended. To solve this problem we have purified the blocking methylase M. *Bsp*RI (5'...GG^{m}CC...3') for competition digests with *Not*I. The resulting methylase-limited partial DNA cleavage is shown to be superior to standard techniques on bacterial genomic DNA.

Abbreviations: bp, base-pair; kb, one thousand base-pairs; Mb, one million base-pairs; Tris, Tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic; β-ME, β-mercaptoethanol; PMSF, phenyl methyl-sulfonyl fluoride; PEG, polyethyleneglycol (MW = 8000); ^{3}H, tritium; SAM, S-adenosylmethionine; KGB, potassium glutamate buffer; DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; BSA, bovine serum albumin.

INTRODUCTION

Partial restriction enzyme digestion is necessary to map regions of genomic DNA when endonucleases cleave too frequently. The resulting large fragments are used to determine orientation and distance between adjacent probes (1). They can also be used to determine distances from the end of a chromosome with a telomeric probe (2–5) or from a unique restriction site. *Not*I (5'...GC/GGCCGC...3') is a widely used restriction enzyme in genomic mapping. It cleaves infrequently due to its eight bp recognition site, and partial digestion of DNA yields fragments that are some of the largest produced for many genomes.

Partial cleavage of genomic DNA with *Not*I (and other endonucleases) is hindered by the agarose matrix in which large DNA must be embedded to prevent degradation. This matrix interferes with the restriction enzymes' accessibility to the DNA. Attempts at partial cleavage, by using less enzyme than required for complete digestion or limiting concentrations of Mg^{++}(6), result in the outer molecules being completely digested and interior DNA left intact. The proportion of the DNA molecules that are partially cleaved is small, creating a problem for investigators working at the limits of detection with hybridization protocols.

A methylase and restriction endonuclease, with the same site specificity, can be used in a competition reaction to overcome this problem (7). The enzymes are used in a specific ratio, in excess of that required to completely methylate or cleave the sample, resulting in a uniform partial digest as they penetrate the agarose matrix. The major obstacle to applying this technique to genomic DNA has been that most commercially prepared methylases are contaminated with co-purified, non-specific endonucleases that cleave DNA in the presence of Mg^{++}. We have purified M. *Bsp*RI (5'...GG^{m}CC...3') to a level sufficient for methylase-limited *Not*I partial cleavage. We demonstrate this method on *E. coli* and *Salmonella* genomic DNA.

MATERIALS AND METHODS

Purification of M. *Bsp*RI

An HB101 strain carrying pPB132 with the M. *Bsp*RI gene was kindly provided by Mario Noyer-Weidner (Max Plank Institute; 8,9). Expression of methylase was induced with IPTG when cells were in early log phase. The cells were pelleted, resuspended in five volumes of 20 mM Tris-Cl pH 7.2, 50 mM KCl, 0.1 mM EDTA, 10 mM β-ME (buffer A) with 20 μg/ml PMSF and broken with a French press. KCl was added to 500 mM and the lysate stored on ice for 10 min followed by centrifugation at 13,000 g for 10 min at 4°C. PEG was added to 7.5% and the solution was stirred overnight on ice.

The overnight suspension was centrifuged at 13,000 g for 30 min at 4°C. The supernatant was diluted three-fold with buffer A and loaded onto a phosphocellulose column. The methylase was eluted with a 50 mM-1 M KCl gradient and two peaks were collected at 50–200 mM and 450–600 mM KCl. Column fractions were assayed for methylase activity by measuring ^{3}H-methyl incorporation in an oligomer containing the M. *Bsp*RI recognition site using ^{3}H-SAM (Amersham) as the methyl donor (10). The peak fractions were then diluted three-fold with buffer A and placed onto a heparin column. The M. *Bsp*RI peak eluted at 200–400 mM KCl and was subsequently run on a hydroxylapatite column (10 mM phosphate pH 7.2, 50 mM KCl, 0.1 mM EDTA, 10 mM β-ME). The methylase was eluted with a 10–500 mM phosphate gradient at 50–130 mM phosphate.

Peak fractions were then loaded on a Sephadex G-75 molecular weight sizing column (20 mM Tris-Cl pH 7.5, 1 mM EDTA, 0.5 M KCl, 10 mM β-ME). The peak was concentrated on a

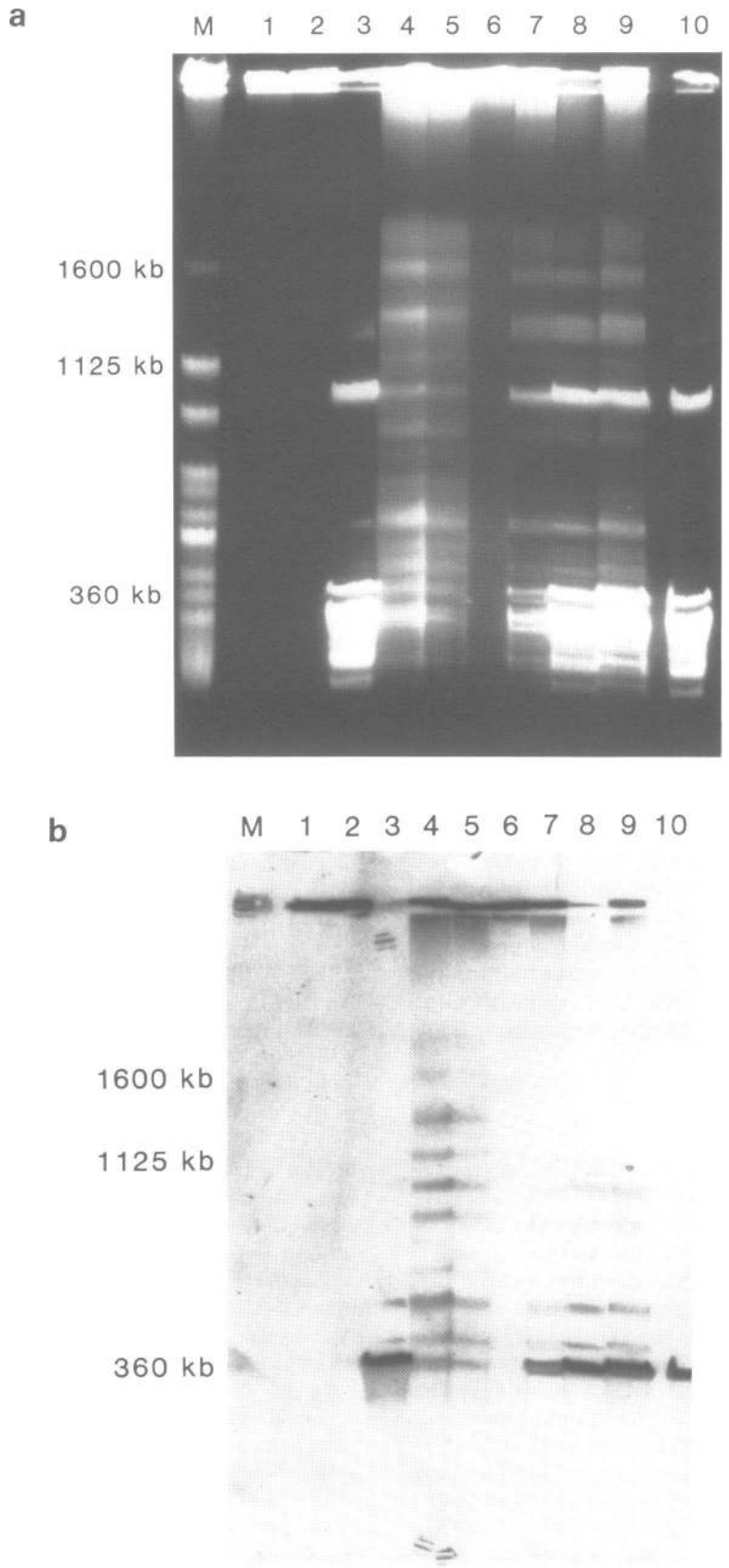

Figure 1. M. *Bsp*RI methylase-limited partial *Not*I cleavage of *E. coli* genomic DNA (2 μg per lane). Panel (a): Ethidium-stained pulsed-field gel run at 10 V/cm with a pulse time of 60 sec for 18 hr. Panel (b): Southern blot of this gel hybridized with a portion of the *E. coli lon* gene. Lane M- *Saccharomyces cerevisiae* chromosomes. Lane 1- uncut *E. coli* genomic DNA. Lane 2- 10 units M. *Bsp*RI. Lane 3- 8 units *Not*I. Lane 4- 3 units M. *Bsp*RI/8 units *Not*I. Lane 5- 4 units M. *Bsp*RI/8 units *Not*I. Lane 6- 10 units M. *Bsp*RI/8 units *Not*I. Lane 7- 0.5 units *Not*I. Lane 8- 1 unit *Not*I. Lane 9- 2 units *Not*I. Lane 10- 5 units *Not*I.

Mono-Q-Sepharose column (20 mM Tris-Cl pH 8.0, 100 mM KCl, 0.5 mM EDTA, 10 mM β-ME, 5% glycerol) and eluted with a 50−600 mM KCl gradient at 250−300 mM KCl. Peak fractions from each column were analyzed for contaminating nucleases by monitoring degradation of *E. coli* DNA in the presence of Mg^{++} to establish purity for genomic DNA applications. The methylase peak from the Mono-Q-Sepharose column gave no degradation in overnight nuclease assays as visualized on ethidium-stained pulsed-field gels. The protein was stored by dialyzing with 20 mM Tris-Cl pH 7.5, 50 mM KCl, 0.1 mM EDTA, 0.1 mM DTT, 50% glycerol and adding BSA to 50 μg/ml. From 20 grams of cells we obtained approximately 125,000 units of M. *Bsp*RI as determined by protection assays on lambda DNA. A detailed purification protocol will be provided upon request.

*Not*I was purchased from Boehringer Mannheim. Sea-Plaque Agarose (for suspension of genomic DNA) and LE Agarose (for pulsed-field gels) were purchased from FMC BioProducts. Genomic DNA was prepared from *E. coli* LE392 and *Salmonella* TT11692 (Chris Conner and John Roth, University of Utah) by a previously described method (11) with the exception that lysostaphin was not necessary. Methylase-limited partial *Not*I digestion of genomic DNA was performed by using M. *Bsp*RI and *Not*I in a specific ratio on one-fifth of an agarose insert (20 μl) in 100 μl 1.5× modified KGB (no β-ME) (12) with 160 μM SAM for 8 hr at room temperature. Controls were performed with varying amounts of *Not*I.

Pulsed-field electrophoresis was performed on a Gene-Line transverse alternating field electrophoresis system (13) purchased from Beckman Instruments. Electrophoresis was performed by using 0.8% agarose gels in 0.25× TAE buffer (10 mM Tris acetate, 0.25 mM EDTA). The gels were cooled to 10°C during electrophoresis by circulating the buffer with an RM-20 Lauda refrigerating circulator. Specific pulse times were as noted in the figure legends and were varied depending on the size of the fragments to be separated.

The separated DNA fragments were transferred to Nytran membranes (Schliecher and Schuell, Inc.) by Southern hybridization (14). A portion of the *E. coli lon* gene (Alvin Markovitz, University of Chicago; 15) was used as a unique-sequence probe for the *E. coli* blots while an internal portion of the Mu-transposable element (Malcolm Casadaban, University of Chicago; 16) was used in the *Salmonella* blots. Preparation of non-radioactive probes and subsequent hybridization and analysis were carried out using the Boehringer Mannheim Genius kit following the manufacturer's protocol.

RESULTS AND DISCUSSION

Methylase-limited partial *Not*I digestion of genomic DNA was performed using M. *Bsp*RI to compete for sites with *Not*I. The two enzymes, in a specific ratio, produce a uniform partial digest. Using 3 or 4 units of M. *Bsp*RI in competition with 8 units of *Not*I resulted in excellent partial digestion of *E. coli* genomic DNA (Figure 1a). Lanes 4 and 5 have an array of bands of equal intensity while lanes 7−10, in which less than 8 units of *Not*I were used, show incomplete cleavage as the majority of the sample is uncut or in bands representing complete cleavage.

The results are more clearly demonstrated when the gel is blotted and hybridized with a unique-sequence probe of the *E. coli lon* gene (Figure 1b). The *lon* gene is contained on the 360 kb *Not*I fragment (17) in lane 3. Detection of partial bands is

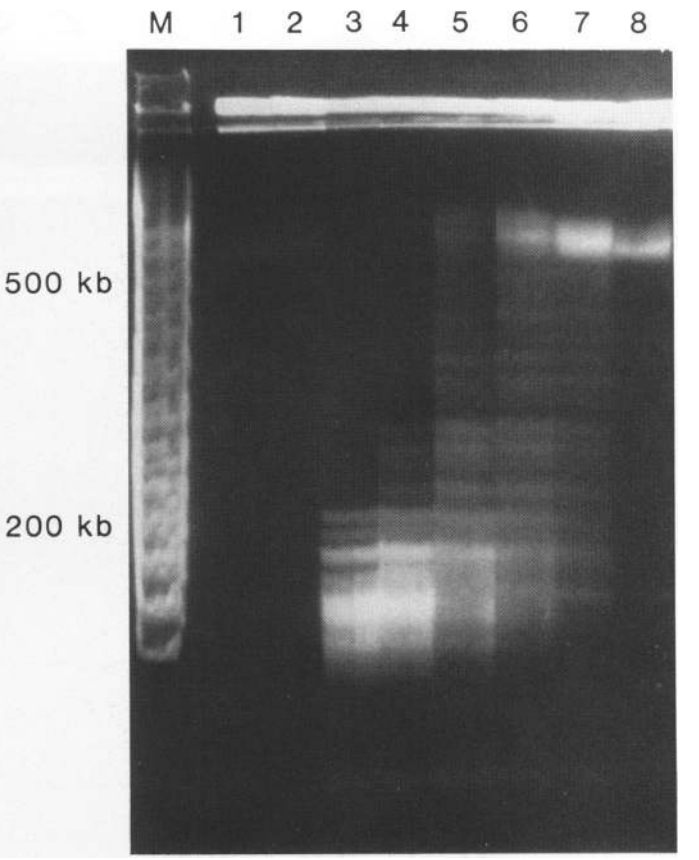

Figure 2. Ethidium-stained pulsed-field gel run at 10 V/cm with a pulse time of 20 sec for 12 hr. M. *Bsp*RI methylase-limited partial *Not*I digestion of *Salmonella* genomic DNA (2 μg per lane). Lane M- Lambda phage DNA multimers. Lane 1- uncut *Salmonella* genomic DNA. Lane 2- 10 units M. *Bsp*RI. Lane 3- 8 units *Not*I. Lane 4- 1 unit M. *Bsp*RI/8 units *Not*I. Lane 5- 3 units M. *Bsp*RI/8 units *Not*I. Lane 6- 5 units M. *Bsp*RI/8 units *Not*I. Lane 7- 7 units M. *Bsp*RI/8 units *Not*I. Lane 8- 10 units M. *Bsp*RI/8 units *Not*I.

greater with methylase-limited partial digestion (lanes 4,5) than with the standard method (lanes 7−10).

By changing the ratio of methylase to endonuclease, it is possible to choose the size of the partially cleaved fragments. Figure 2 demonstrates the effect of going from 1 to 10 units M. *Bsp*RI against 8 units *Not*I on *Salmonella* genomic DNA. The best partial in the range of 200−500 kb is given with 3−5 units of M. *Bsp*RI (lanes 5,6) but more methylase would give partial fragments greater than 500 kb (lanes 7,8).

We are currently using methylase-limited *Not*I partials for the construction of a long-range physical map of the *Salmonella* genome. In brief, partial digests are used to map distances of restriction sites from a unique M. *Xba*I/*Dpn*I (5′...TCTAG^{m}A/TCTAG^{m}A...3′) (11) cleavage site that has been inserted with a Mu transposon. We use probes for the transposon located on either side of the M. *Xba*I/*Dpn*I site. Figure 3 shows a blot of such a pulsed-field mapping gel. Lane 1 shows a 100 kb *Not*I fragment of the *Salmonella* genome. Lane 2 shows the 75 kb band resulting when the genome is cleaved with M. *Xba*I/*Dpn*I and *Not*I and lane 3 shows bands resulting from M. *Xba*I/*Dpn*I complete cleavage and M. *Bsp*RI methylase-limited partial *Not*I digestion. We are able to determine the distance of *Not*I sites up to 1 Mb upstream from the M. *Xba*I/*Dpn*I unique cleavage site on this particular gel. By adjusting the amount of

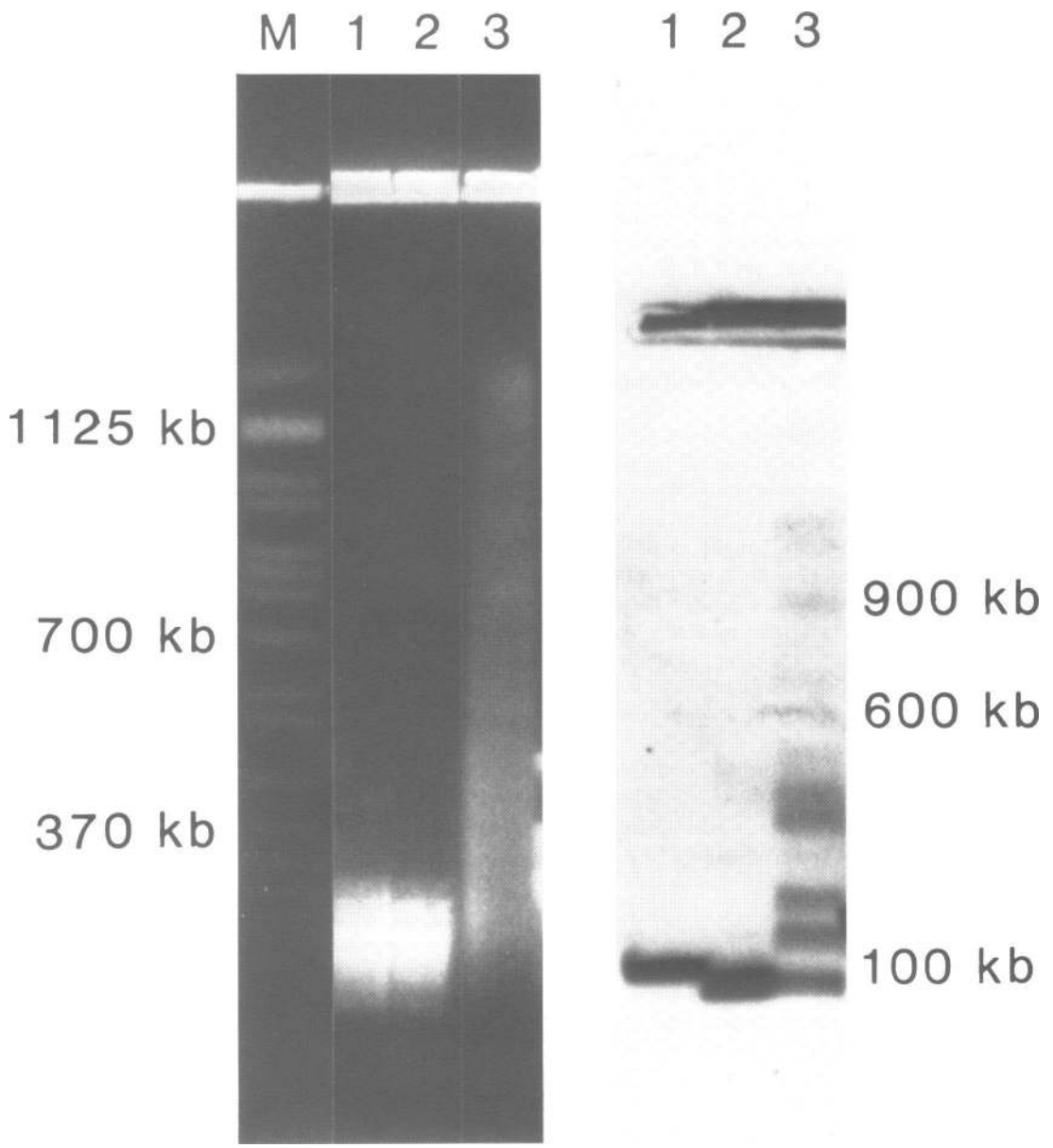

Figure 3. Pulsed-field physical mapping experiment of *Salmonella* genomic DNA (4 μg per lane). Panel (a): Ethidium-stained pulsed-field gel run at 10 V/cm with a pulse time of 60 sec for 18 hr. Panel (b): Southern blot of this gel hybridized with internal fragment of the Mu transposon. Lane M- *Saccaromyces cerevisiae* chromosomes. Lane 1- *Not*I complete digest. Lane 2- M. *Xba*I/*Dpn*I and *Not*I complete double digest. Lane 3- M. *Xba*I/*Dpn*I complete and M. *Bsp*RI methylase-limited partial *Not*I digestion (using 15 units M. *Bsp*RI/10 units *Not*I).

methylase used and pulse times of the gels, one can adjust the mapping range. Another advantage of this method is that it allows other endonucleases to cleave to completion in simultaneous reactions while methods relying on interference with enzyme activity do not (18).

It is our view that most DNA methylases could be purified to the level needed for these experiments. We are currently purifying M. *Fnu*DII (5′...CG^{m}CG...3′) which competes with *Nru*I (5′...TCG/CGA...3′) and *Mlu*I (5′...A/CGCGT...3′). Preliminary results indicate that it can be made pure enough for methylase-limited partial digestion. High purity methylases have other potential uses for physical mapping. An example is the creation of a unique cleavage site by using a methylase to block all of its sites except for one that has been covered by a DNA-binding protein (19) or synthetic triple helix (20,21). Removal of the blocking agent allows subsequent cleavage with the corresponding restriction endonuclease.

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