Enzymatic cleavage of a bacterial chromosome at a transposon-inserted rare site

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ABSTRACT

The sequential use of the methylase M·*Xba*I (5′-TCTAG^{m6}A) and the methylation-dependent endonuclease *Dpn*I (5′-G^{m6}A↓TC) results in cleavage at 5′-TCTAGA↓TCTAGA. This recognition sequence was introduced into a transposon derived from the Mu bacteriophage and transposed into the genome of the bacterium *Salmonella typhimurium*. M·*Xba*I methylation was provided *in vivo* by a plasmid containing the M·*Xba*I gene and the *S. typhimurium* genome was cleaved to completion by *Dpn*I at one or more sites, depending on the number of transposon insertions. The resulting genomic fragments were resolved by pulsed-field electrophoresis. The potential use of single M·*Xba*I/*Dpn*I cleavage sites as reference positions to map rare restriction sites is discussed.

INTRODUCTION

Cleavage of DNA at infrequent sites can be used for physical and genetic mapping of chromosomes by allowing investigators to utilize pulsed-field electrophoresis (PFE)(1) to separate large DNA fragments. A number of methods for cutting DNA at rare or unique sites have been proposed(2–11). We present a method that gives complete cleavage at a rare site and discuss its potential usefulness for physical mapping of a bacterial chromosome.

M·*Xba*I (5′-TCTAG^{m6}A) methylation at sites in tandem create, at their junction, a cleavage site for the methylation-dependent endonuclease *Dpn*I:

5′-T C T A G A T C T A G A-3′
3′-A G A T C T A G A T C T-5′

Methylation ↓ M·*Xba*I (5′-TCTAG^{m6}A-3′) + S-adenosylmethionine (methyl donor)

5′- T C T A G^{m6}A T C T A G^{m6}A-3′
3′-^{m6}A G A T C T^{m6}A G A T C T-5′

Cleavage ↓ *Dpn*I (5′ G^{m6}A↓T C 3′) + Mg^{2+}

5′- T C T A G^{m6}A-3′ 5′- T C T A G^{m6}A-3′
3′-^{m6}A G A T C T-5′ 3′-^{m6}A G A T C T-5′

The sequence 5′-TCTAGATCTAGA should occur once every 4^{12} (16,000,000) bp, on average, in DNA consisting of a random sequence of T,C,G, and A(12). It has recently been found that *Dpn*I will also cleave hemi-methylated GATC sites at approximately a 10- to 100-fold reduced rate (Nelson and McClelland, unpub., Ira Schildkraut, personal comm.). This implies that *Dpn*I will also cleave slowly at 5′-TCTAG^{m6}A↓TC. This sequence should occur about once every 32,000 bp in 'random' DNA.

To test the M·*Xba*I/*Dpn*I cleavage system in megabase mapping, we used as a model the chromosome of the enterobacterium *S. typhimurium* into which we inserted a transposon containing the 12-bp sequence 5′-TCTAGATC-TAGA. The *S. typhimurium* genome is about 5 million bp in size(13). Because the sequence 5′-CTAG is underrepresented in enterobacteria(14,15), 5′-TCTAGATCTAGA was unlikely to occur naturally in the genome. The putative slower hemi-methylated cleavage site, 5′-TCTAGATC, has an expected frequency of less than one in 300,000 bp in such genomes(12).

MATERIALS AND METHODS

Bacterial strains and plasmids

Salmonella typhimurium TT11692 *dam102* was provided by John Roth, University of Utah. Bacteriophage Muc*t*63 and pPR3(16), used to construct pPR3(MXD), were from Malcolm Casadaban, University of Chicago. The M·*Bsp*RI clone pBspRI-M(17) was obtained from Mario Noyer-Weidner. *E. coli* XL-1 blue and pBluescript SK(+) were purchased from Stratagene Inc., La Jolla, CA.

Oligonucleotides

Two complementary oligonucleotides 5′-AATTCTAGATCTA-GATCTAG and 5′-AGCTCTAGATCTATATCTAGATCTAG were purchased from Genetic Designs Inc., Dallas, TX.

DNA Preparation

Genomic DNA in agarose plugs was prepared from *S. typhimurium* using published procedures(11,14,15).

Enzymatic reactions

Restriction endonucleases were from Boehringer Mannheim, Indianapolis, IN. M·*Bsp*RI was prepared as previously

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described(18). Methylase-limited partial *Not*I digestion using M·*Bsp*RI and *Not*I was performed in modified 1.5× KGB (no 2-mercaptoethanol)(19) as previously described(20) except that these reactions also included *Dpn*I for complete cleavage at M·*Xba*I/*Dpn*I sites.

DNA Electrophoresis

Conventional electrophoresis through 1.0% agarose gels in TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0) was used to analyze plasmid constructs and to separate *Hin*dIII digested genomic DNA. PFE was performed on a Geneline transverse alternating field electrophoresis system(21) provided by Beckman Instruments. The 0.8% agarose gels were cooled during electrophoresis to 10°C by circulating 1/4×TAE buffer (10 mM Tris-acetate, 0.25 mM EDTA, pH 8.0) with a RM-20 Lauda refrigerating circulator. Pulse times and voltages are noted in the figure legends. Yeast markers were purchased from FMC. The molecular weights were those assigned by the vendor.

Southern Blotting

Gels were capillary blotted to Nytran membranes (Schleicher and Schuell) in 10× SSC and hybridized with an 800 bp *Eco*RI fragment of pPR3. This fragment is located in the transposon on one side of the M·*Xba*I/*Dpn*I sites. This *Eco*RI fragment was isolated from a 1% agarose TAE gel by GeneClean (BIO101, La Jolla, CA). The probe was then labelled by random priming(22) using the Genius™ non-radioactive method (Boehringer Mannheim) and the manufacturer's protocol.

RESULTS AND DISCUSSION

A transposon containing two tandem M·*Xba*I/*Dpn*I sites was constructed as follows. Two complementary oligonucleotides were annealed to produce the double stranded sequence referred to as MXD:

5′-AATTCTAGATCTAGATCTAG
GATCTAGATCTAGATCTCGA-5′

The MXD oligonucleotide was ligated into the *Eco*RI (E) to *Hin*dIII (H) sites of the plasmid pBS (SK+). Transformants of *E. coli* XL-1 blue were selected on ampicillin (Ap) (50 μg/ml). The *Sal*I (S) to *Bam*HI (B) fragment of the resulting plasmid designated, pBS(SK+)(MXD), was cloned into the (S) to (B) site of the plasmid pPR3(16). This plasmid carries a gene for Ap^R and a mini-Mu transposon, MudII that is chloramphenicol resistant (Cm^R). Clones were selected on 12.5 μg/ml Cm and 50 μg/ml Ap. The correct plasmid construct, designated pPR3(MXD) (Fig. 1), was determined by restriction analysis (data not shown).

The resulting derivative transposon, Mud(MXD), which contained two overlapping M·*Xba*I/*Dpn*I sites, was transduced from *E. coli* into *S. typhimurium* using bacteriophage Mu as the helper phage(23). A transducing lysate was made by infecting *E. coli* XL-1 blue [pPR3(MXD)] with bacteriophage Mu*cts*62. This donor lysate was absorbed to the recipient strain TT11692, a *dam102* Kanamycin resistant (Kn^R) derivative of *S. typhimurium*, and transductants with Mud(MXD) insertions were selected on 12.5 μg/ml Cm, 50 μg/ml Kanamycin (Kn) and screened for Ap^S on 50 μg/ml Ap. Mud(MXD) insertions into the chromosome should be stable because the transposase enzyme is required *in trans* from bacteriophage Mu for transposition of this defective transposon.

The *dam* gene of *S. typhimurium* encodes a methylase with the specificity 5′-G^{m6}ATC which is also the recognition specificity of *Dpn*I(24,25). A *dam*$^-$ *S. typhimurium* mutant was employed in our experiments so as to avoid *Dpn*I cleavage at *dam* sites.

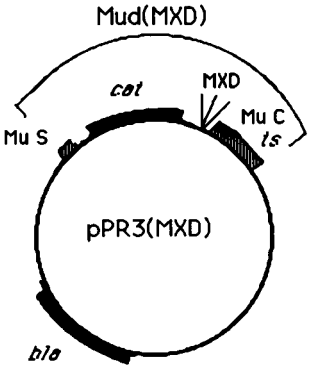

Figure 1. The pPR3(MXD) plasmid carrying a mini-Mu transposon with the M·*Xba*I/*Dpn*I cleavage sequence 5′-TCTAGATCTAGA.
Abbreviations: *bla*, beta-lactamase (ApR); *cat*, chloramphenicol acetyltransferase (Cm^R); MuS and Mu*Cts*, the ends of the defective Mu bacteriophage Mud(MXD) that are required for transposition; MXD, the inserted oligonucleotide containing the M·*Xba*I/*Dpn*I cleavage sequence 5′-TCTAGATCTAGATCTA-GA; Mud(MXD), the defective transposon that can be mobilized by bacteriophage Mu infections of cells carrying pPR3(Mud(MXD)).

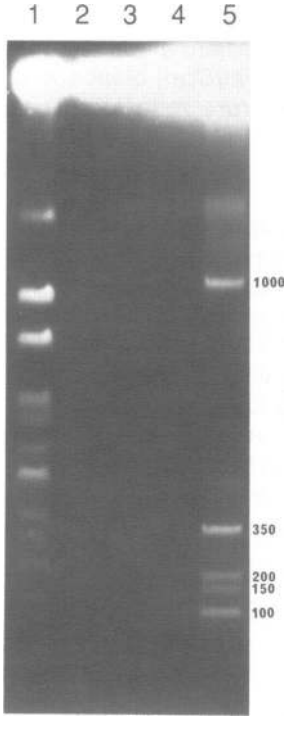

Figure 2. PFE gel showing *in vivo* M·*Xba*I methylation and *in vitro* *Dpn*I cleavage at 5′-TCTAGA$^{\downarrow}$TCTAGA. Strain TT11692 [pXbaI-M] with no Mud(MXD) insertions and a strain designated 'J' [pXbaI-M] with six insertions were each digested by 10 units of *Dpn*I at room temperature for 12 hr. The gel was run at 10V/cm with a pulse time of 60 seconds for 21 hr to resolve fragments in the 50 to 1,800 kb range. Lane 1; *Saccharomyces cerevisiae* chromosome molecular weight markers. Lane 2; uncut *S. typhimurium* [pXbaI-M] DNA; lane 3; *S. typhimurium* [pXbaI-M] DNA cut with *Dpn*I; lane 4; uncut *S. typhimurium*::Mud(MXD) [pXbaI-M] DNA strain J. Lane 5; Strain J DNA cut with *Dpn*I.

The number of Mud(MXD) insertions in each *S. typhimurium* transductant was determined as follows. Genomic DNA(26) (2 μg of each transductant) was digested with 10 units of *Hin*dIII. The resulting DNA fragments were separated on a 1% agarose gel, capillary blotted, and hybridized with an *Eco*RI fragment of pPR3 (See Methods). The 800 bp *Eco*RI fragment is internal to the transposon and flanks the new M·*Xba*I/*Dpn*I site. There is one HindIII site in Mud(MXD) but no *Hin*dIII site in the *Eco*RI fragment, so the number of *Hin*dIII fragments of different sizes that hybridize to the probe should reflect the number of Mud(MXD) insertions into the genome. Twenty six of 30 Cm^R Kn^R Ap^S clones had single insertions. Unexpectedly, one strain had six independent insertions (data not shown).

S. typhimurium strains that methylate Mud(MXD) insertions at M·*Xba*I sites *in vivo* were constructed. Purified M·*Xba*I enzyme can be used to methylate the M·*Xba*I/*Dpn*I site *in vitro*(12), but it is technically much easier to methylate the M·*Xba*I/*Dpn*I site using M·*Xba*I expressed by the cloned M·*Xba*I gene *in vivo*. *S. typhimurium* strains with Mud(MXD) insertions were transformed with a pUC19 (Ap^R) clone of the M·*Xba*I gene, pXbaI-M(27), and selected for Cm^R, Kn^R, Ap^R on 12.5 μg/ml Cm, 50 μg/ml Kn, 50 μg/ml Ap. The efficiency of transformation by pXbaI-M was similar to that of pBR322, indicating that this strain of *S. typhimurium* does not have a strong methyl-adenine dependent restriction system (*mrr*)(28,29) directed at $TCTAG^{m6}A$.

Agarose plugs containing genomic DNA from a *S. typhimurium* [pXbaI-M] strain which carries six insertions of Mud(MXD) was cleaved *in vitro* with *Dpn*I and the resulting fragments were separated by PFE (Fig. 2). *Dpn*I cleaved the genome at a number of sites, presumably at the 5'-TCTAGATCTAGA insertions. The fragments resolvable are about 1.0, 0.35, 0.2, 0.15 and 0.1 megabases, with a less intense fragment at about 0.5 megabases which could be a product of partial digestion. At least one hemi-methylated 5'-$TCTAG^{m6}ATC$ sequence may occur in the genome and is cut slowly to produce the partial band. The other fragments add up to 2.2 megabases. It is likely that a larger fragment of about 2 to 3 megabases is unresolved in the compression zone.

No *Dpn*I dependent cleavage was seen when M·*Xba*I methylation occurred in the absence of transposon integrations (Fig 2, lane 3). The hemi-methylated sites may not be observed because they are more slowly cleaved than the transposons that contain a tandem repeat of the 100-fold faster site and/or they may also generate fragments too large to see in this gel.

Genomic DNA from *S. typhimurium* [pXbaI-M] strains which carry one insertion of Mud(MXD) were cleaved to completion by *Dpn*I. The resulting linearized genomes were partially digested either with *Spe*I (5'-ACTAGT) using limiting amounts of enzyme or with *Not*I (5'-GCGGCCGC) in competition with a DNA methylase(18,20). *Spe*I and *Not*I recognition sites are among the least abundant of the conventional restriction sites in *S. typhimurium*, occurring in the genome about 30 and 50 times respectively(12). The resulting DNA fragments were separated by PFE and hybridized with one end of the transposon. The same *Eco*RI fragment, internal to the transposon and flanking the M·*Xba*I/*Dpn*I sequences, was used. This indirect end-labelling assay(30) displays the position of a series of *Not*I and *Spe*I sites

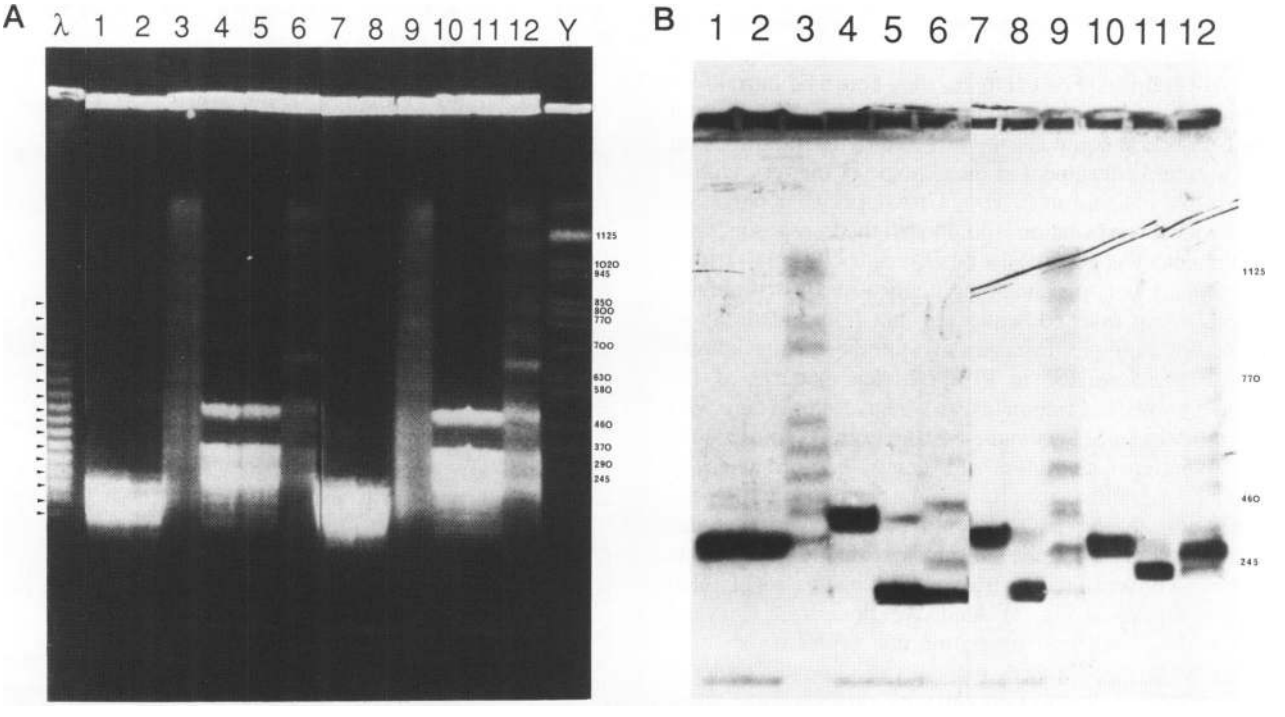

Figure 3. Mapping *Not*I sites relative to unique transposon-inserted M·*Xba*I/*Dpn*I sites. Two independent *S. typhimurium* [pXbaI-M] strains with single Mud(MXD) insertions were used to prepare genomic DNA in agarose plugs. Lanes 1 to 6 and lanes 7 to 12 contain genomic DNA from the two different strains, respectively. Plugs were treated as follows; lanes 1 and 7, *Not*I; lanes 2 and 8, *Not*I + *Dpn*I; lanes 3 and 9, *Dpn*I + *Not*I/M·*Bsp*RI (GG^mCC) partial; lanes 4, and 10, *Spe*I; lanes 5 and 11, *Spe*I + *Dpn*I; lanes 6 and 12, *Dpn*I + *Spe*I partial. Digestions were all performed in 1.5X modified KGB at room temperature. Complete cleavage reactions employed 10 units of *Dpn*I, 10 units of *Not*I, or 10 units of *Spe*I for 12 hr. Partial digests with *Not*I employed 10 units of *Not*I, 10 units of M·*Bsp*RI (GG^mCC) and 160 μM SAM for 12 hr. M·*Bsp*RI competes for *Not*I cleavage sites(18,20). Partial digests with *Spe*I employed 0.01 units of *Spe*I for 12 hr. The gel was run at 10V/cm with a pulse time of 60 seconds for 21 hr to resolve fragments in the 50 to 1,800 kb range. Panel **A** is an ethidium stained gel. Panel **B** is a Southern blot probed with an internal *Eco*RI fragment of pPR3(MudII).

relative to the site of insertion (Fig. 3). Note that cleavage was complete or almost complete at the transposon-inserted M·*Xba*I/*Dpn*I sites in these experiments (Fig. 3, lanes 4, 5 and 11). In lane 2 there was no change in the apparent size of the *Not*I fragment after *Dpn*I cleavage. However, comparing the same DNA cut with *Spe*I (lanes 5 and 6) indicates that this DNA is substantially cleaved. This data indicates that the transposon is located very close to the end of a *Not*I fragment. An *Spe*I and *Not*I restriction map of the whole genome is under construction using the independent Mud(MXD) insertions we have generated. We have also begun to genetically map some of the integrations (Wong and McClelland, manuscript in preparation).

Given our success in generating cleavage sites for *Dpn*I with M·*Xba*I supplied *in trans* on a plasmid, it should also be possible to construct transposons in which the M·*Xba*I gene is in the transposon and expressed *in cis* using a broad host range promoter.

A concern in all methylase/*Dpn*I strategies is that the amount of *Dpn*I will need to be carefully controlled so as to avoid significant digestion at the more slowly cleaved hemi-methylated *Dpn*I sites. In the experiments we present here we have not had any problems with the few M·*Xba*I/*Dpn*I hemi-methylated overlaps that almost certainly exist in the *S. typhimurium* genome. However, one method to further improve the ratio of cleavage at the fully methylated targets in the transposon versus hemi-methylated targets scattered throughout the host genome will be to place multiple methylase/*Dpn*I sequences in the transposon. These sequences need not all be in one long tandem repeat as that may affect their genetic stability. Less *Dpn*I will be needed for effective cleavage of the transposon and cleavage at the slower hemi-methylated sites will be correspondingly less.

The transduction experiments described here represent only one of many possible strategies for introducing rare cleavage sites into bacterial genomes. For example, they could be introduced directly into the genome on a broad host range transposon such as Tn5(31). Insertion could also be targeted by first introducing the transposon into a fragment of the genome of interest, cloned on a mobilizable plasmid in *E. coli*. Cross-species conjugation and homologous recombination would insert the transposon, with the rare site, into the genome to be mapped(32,33).

There are many potential mapping applications for strategies that cleave DNA at inserted sequences, not found naturally in the genome. For example, insertion of a number of sites allows a genome to be visualized in PFE cleavage patterns as the corresponding number of fragments, as we have shown here with M·*Xba*I/*Dpn*I sites in *S. typhimurium*. In contrast, transposons carrying conventional restriction sites cannot be used as easily because the host genome usually contains a number of these sites in addition to the new site (14,34,35). For example, *S. typhimurium* cannot be cleaved into less than 20 fragments by any known conventional restriction endonuclease (11; also *Not*I and *Spe*I digests in Fig. 3). Unconventional rare cleavage sites, such as M·*Xba*I/*Dpn*I, overcome this problem.

A strategy for physical/genetic mapping utilizing this protocol can be envisioned. A transposon carrying rare cleavage sites, such as multiple M·*Xba*I/*Dpn*I sequences, when integrated into a circular bacterial genome could define a 'type strain' that generates a characteristic cleavage site. A second integration with the rare sites could be introduced, either by random or targeted insertion. The resulting strain, that carries two sets of rare sites at two different locations on the chromosome, could be digested at the rare sites. A DNA fragment corresponding to the distance between the insertions would 'drop out' into the PFE gel. The size of the DNA fragment would determine the physical distance of the second integration relative to the first site. If the second integration results in a mutant phenotype the gene responsible is also mapped. This strategy could be of particular use when other ways of genetic mapping are difficult or non-existent, a situation that pertains for a large number of species.

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