Ligation overcomes terminal underrepresentation in multiple displacement amplification of linear DNA

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The amount of DNA available for informative genomic studies is often limiting. Thus, several methods have been developed to achieve a whole genome amplification (WGA). Particularly promising is a variant, called multiple displacement amplification (MDA) (1,2), which exploits the high processivity of Φ29 phage DNA polymerase and suffers an amplification bias significantly lower than previous, PCR-based WGA (3).

Even though MDA-DNA has been successfully used for many applications (1,4–7), some problems have been reported. For example, sequences near the ends of linear chromosomes appear underrepresented after MDA (6,8,9). Both defective priming events and abortive chain terminations could contribute to this problem, which limits the applications of MDA and jeopardizes its use on short linear DNA (e.g., cDNA and degraded genomes from forensic, archeological, and fetal origin) (10). In these cases, the terminal underrepresentation would cause the loss of substantial information.

We studied this problem using λ phage DNA (48.5 kb; Promega Biosciences, San Luis Obispo, CA, USA). MDA was performed using the Genomiphi® kit (Amersham Biosciences, Little Chalfont, England, UK) first on the uncut λ DNA (1 ng) and then on the mixture of its seven *Hin*dIII fragments (1 ng). Reactions were incubated overnight at 30°C using the conditions suggested by the manufacturer. We then compared the same amount of amplified samples and unamplified native DNA to assess the representation of the various restriction fragments after MDA. To achieve this aim, we used frequent cutters (*Rsa*I: 113 sites on λ; *Pst*I: 28 sites) in combination with *Hin*dIII (Promega Biosciences). Restriction digests with *Hin*dIII alone were also performed. Digestions were visualized on either agarose (Eppendorf, Hamburg, Germany) or polyacrylamide gels (Sigma, St. Louis, MO, USA) for discriminating both high and low molecular weight bands, respectively. *Hin*dIII and *Hin*dIII+*Pst*I digestions were visualized on 1% agarose gels, and *Hin*dIII+*Rsa*I digestions were visualized on 1.4% gels. The electrophoresis was run at 80 V for 2 h in 1× TAE buffer (40 mM Tris-base, 20 mM acetic acid, 1 mM EDTA, pH 8.0) and visualized using ethidium bromide (Sigma). Four-percent polyacrylamide gels (acrylamide:bisacrylamide 37.5:1) were run in 0.5× TBE buffer (45 mM Tris-base, 45 mM boric acid, 1 mM EDTA, pH 8.0; Sigma) at 120 V for 30 min and 200 V for 3 h. Patterns were visualized using VistraGreen™ (Amersham Biosciences).

The restriction patterns are shown in Figure 1. Dean et al. (1) observed that, in Southern blot experiments, DNA fragments longer than 5 kb appear substantially underrepresented after MDA and their electrophoretic resolution was impaired. The same problem emerges from our electrophoretic patterns, especially with high molecular weight fragments (Figure 1). Dean et al. (1) ascribed this to DNA degradation due to the initial denaturation step required for MDA, the effects of which are supposedly stronger on longer fragments. However, the structure proposed for MDA products could suggest another explanation. It was hypothesized (8) that they are characterized by networks of hyperbranched DNA. These structures might compound the electrophoretic problems. In particular, since hyperbranched DNA products are expected to be more frequent in longer amplified fragments, their mobility would be reduced proportionally to their molecular weight. Thus, the quality difference of gels in Figure 1A (high molecular weight bands) and Figure 1B (low molecular weight bands) could be due not only to the different type of gel (agarose versus polyacrylamide) but also to the hyperbranched structure of the products.

Figure 1 shows the restriction patterns of the unamplified native λ DNA (lanes 1) and of the MDA products generated from the intact λ genome (lanes 2). Patterns are easily recognizable in amplified samples, suggesting that MDA produces double-stranded DNA that represents nearly the whole λ genome. The disappearance of a terminal band (the 3′ 4.4-kb band in the *Hin*dIII digest) may be observed in the MDA sample (Figure 1A, lane 2, signaled with an asterisk to the right of the gel). Other bands present diminished intensity. This is more easily visible in the *Pst*I+*Hin*dIII pattern (Figure 1B, bands marked with circles). In accordance with the terminal underrepresentation phenomenon, all of these fragments map in the first 5 kb at the 5′ end of the genome (Figure 2A).

We then subjected the λ/*Hin*dIII fragments to MDA. Their subsequent *Hin*dIII digestion did not produce recognizable patterns (Figure 1, lanes 3 of the *Hin*dIII panels) as neither intact *Hin*dIII fragments nor reconstituted sites are present in this sample. Concerning the other restriction digests, recognizable patterns are still present, but some fragments are no longer detectable (Figure 1B, indicated by arrowheads). These are *Rsa*I or *Pst*I fragments containing, or close to, *Hin*dIII sites (Figure 2), again in concordance with terminal underrepresentation.

Thus, our analysis shows that after MDA, the sequences in proximity of the template termini are lost or underrepresented. We have defined that in the λ genome this phenomenon affects regions within about 5 kb of the ends. The behavior of the *Hin*dIII+*Pst*I 5′ terminal fragments (Figures 1B

and 2A, circles) makes it likely that premature detachment of the growing chain contributes to the terminal underrepresentation. The relevant bands are much less intense when amplifying the entire λ genome, while their intensities become comparable to those of the unamplified native λ after MDA of the *Hin*dIII fragments.

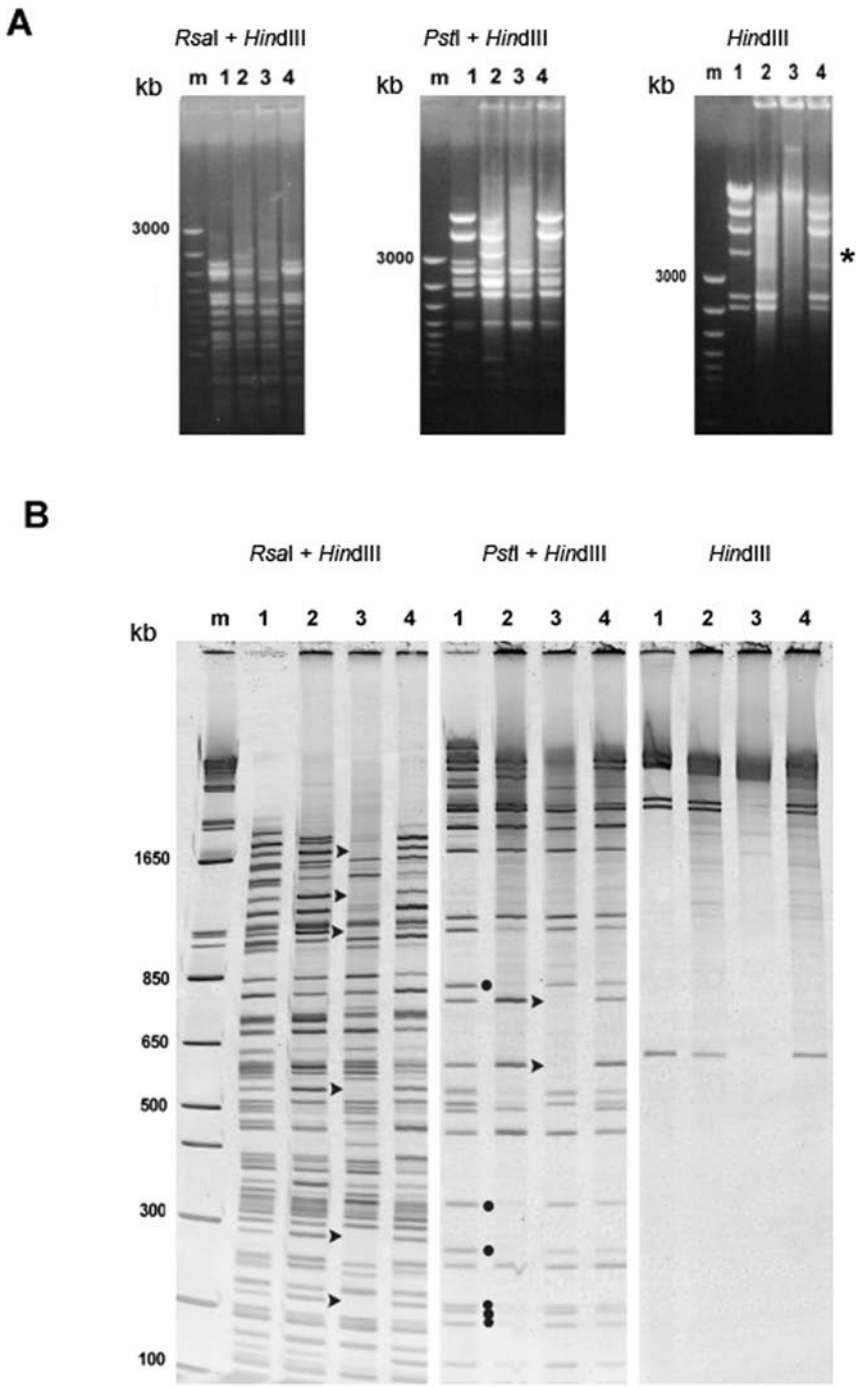

Figure 1. Restriction patterns resolved on (A) agarose and (B) polyacrylamide gels. In all cases, lanes are the following: 1, native λ DNA; 2, uncut λ DNA+MDA; 3, λ/*Hin*dIII+MDA; 4, λ/*Hin*dIII+ligase+MDA. (A) Digested unamplified DNA (2 µg) and the same amounts of digested MDA samples were loaded. A band not detectable in the *Hin*dIII digestion (lane 2) is signaled with an asterisk to the right of the gel. Lane m, GeneRuler™ 100-bp DNA Ladder Plus (Fermentas Life Sciences, Hanover, MD, USA) (B) Digested unamplified DNA (500 ng) and the same amounts of digested MDA samples were loaded. Bands not detectable in lanes 2 and 3 are signaled with circles and arrowheads, respectively. Lane m, 1-kb Plus DNA Ladder (Invitrogen). MDA, multiple displacement amplification.

In order to overcome the termini problem, we developed a simple protocol based on ligation of linear templates before MDA to obtain a random permutation of the original fragments and possibly the circularization of the resulting products. T4 DNA ligase (3 Weiss units; Invitrogen, Carlsbad, CA, USA) was used to ligate the cohesive ends of λ/*Hin*dIII fragments (7.5 ng/µL). Other restriction enzymes have been tried with comparable results (*Pst*I and *Bgl*II, not shown). Reactions were incubated overnight at 30°C and were analyzed on 1% agarose gels (11). The ligated sample (750 pg) was subjected to amplification. For all MDA reactions, we obtained 10–20 µg product.

The product was then compared with the unamplified native DNA as described above. Lanes 4 (Figure 1) show the ligated samples. Each digestion was repeated at least three times using different λ/*Hin*dIII+ligase and MDA preparations. We always observed the same patterns, and the resultant bands were reproducible. The patterns shown were obtained from representative experiments. The restriction patterns were the most similar to those of the unamplified DNA. Nearly all the bands not detectable in Figure 1, lanes 2–3, were recovered, even if their intensities did not always perfectly match those of the unamplified DNA, indicating slight differences in the ligation and/or amplification efficiency of different fragments.

Special consideration was given to the terminal sequences of λ DNA, cosL and cosR, which after *Hin*dIII digestion are borne respectively by the 23- and 4.4-kb fragments. Once ligated, they are resolvable only by specialized enzymes. The λ/*Hin*dIII 3′ band (4.4 kb) is not rescued after ligation (Figure 1A). In this sample, the 4.4-kb fragment, after cosL/R joining, forms a 27.4-kb band, indistinguishable from the 23-kb band, as they both lie in the nonresolving region of the gel.

In both gels of Figure 1, A and B, there are some faint bands that do not correspond to the expected restriction patterns. The clearest example is a band of about 4.0 kb, in lanes 2 and 4 of the

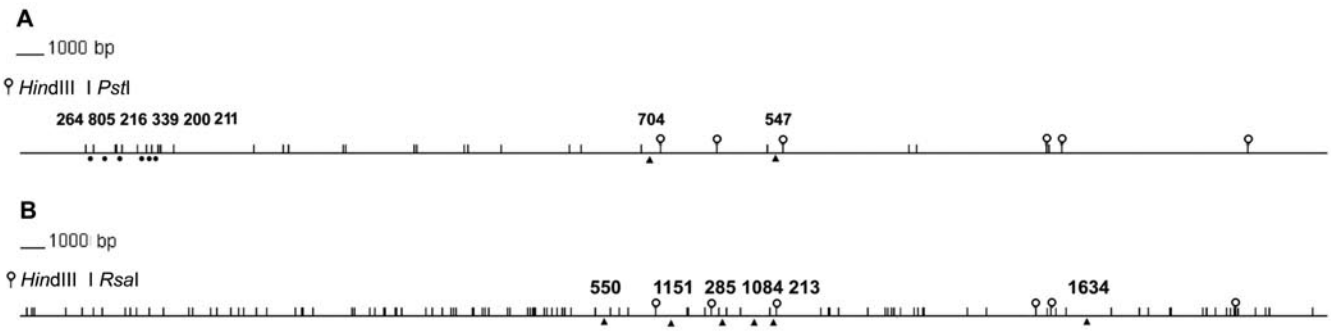

Figure 2. Map positions of fragments underrepresented after standard multiple displacement amplification (MDA) as determined through (A) *Pst*I+*Hin*dIII or (B) *Rsa*I+*Hin*dIII digestion. Numbers in bold above the maps refer to the molecular weight of the lost or underrepresented fragments. Bands not detectable when subjecting the uncut λ DNA to MDA are indicated with circles. Bands lost when amplifying λ/*Hin*dIII fragments are signaled with arrowheads. In the first case (circles), *Pst*I fragments confined within 5 kb from the 5′ end present a very diminished intensity after MDA (A). In the second case (arrowheads), the λ/*Hin*dIII+MDA sample subjected to *Pst*I+*Hin*dIII digestion loses the 704- and 547-bp fragments (A). Both derive from *Pst*I fragments containing internal *Hin*dIII sites. The same sample, after *Rsa*I+*Hin*dIII digestion, loses several fragments (B), all close to *Hin*dIII sites. Other fragments, including those at the termini, could not be unambiguously identified because of co-migration and gel resolution problems. They were thus excluded from our analysis.

*Hin*dIII digestion (Figure 1A). The unexpected bands are not produced by the ligase treatment, since they are also present in lane 2. Moreover, they generally disappear in lane 3 (i.e., when digesting the unligated λ/*Hin*dIII fragments). In the light of data on the enzymatic properties of the Φ29 DNA polymerase (12), a reasonable hypothesis is that the unexpected bands are due to the presence of hairpins in the DNA template. These structures are known to cause polymerase detachment from the template, template switching, or specific endonucleolytic activity (13,14). The result is the accumulation of discrete small DNA molecules. We found a palindromic sequence, GGTTGATATCAACC, starting at nucleotide 41269 (GenBank® accession no. NC_001416) and located 3810 bp downstream of the 37459 λ/*Hin*dIII site. It is related to the sequence TGTTTCACGTGGAACA, which Murthy et al. (12) call a dyad symmetry element (DSE) and show to be specifically responsible for the replication block of the Φ29 DNA polymerase. This hairpin may thus be responsible for the production of the cited band in lanes 2 and 4 of the *Hin*dIII digestion (Figure 1A). This hypothesis also explains why this band disappears in lane 3. In fact, when subjecting the unligated λ/*Hin*dIII fragments to MDA, the relevant region is underrepresented in the MDA product since it is located near a terminus.

In conclusion, the ligation of templates helps to overcome underrepresentation of the termini and expands the potential for the application of MDA. It is useful to obtain faithful amplifications of small, linear templates that otherwise could not be successfully amplified. Both cohesive and blunt ends can be satisfactorily ligated before MDA. For a wider range of applications, and especially for the study of degraded genomic DNA, one could consider blunt-ending (mediated, for example, by T4 DNA polymerase) and ligation, mediated by adaptors containing restriction recognition sequences. Our protocol provides a simpler alternative to the OmniPlex® technology (Rubicon Genomics, Ann Arbor, MI, USA) (15) for performing WGA on such samples.

Recently, a method has been described for isothermal in vitro amplification of specific regions (16). It exploits DNA helicase to generate a single-stranded template for primer hybridization and subsequent extension. However, its application could be problematic if the template is limited or degraded. Our procedure, through the fill-in and blunt-end ligation of linear DNA before MDA, could substitute DNA extraction as the first step of a totally isothermal process for amplifying specific DNA sequences regardless of the template quantity and quality.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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