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Analysis of DNA structure and sequence requirements for *Pseudomonas aeruginosa* MutL endonuclease activity

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The hallmark of the mismatch repair system in bacterial and eukaryotic organisms devoid of MutH is the presence of a MutL homologue with endonuclease activity. The aim of this study was to analyse whether different DNA structures affect *Pseudomonas aeruginosa* MutL (PaMutL) endonuclease activity and to determine if a specific nucleotide sequence is required for this activity. Our results showed that PaMutL was able to nick covalently closed circular plasmids but not linear DNA at high ionic strengths, while the activity on linear DNA was only found below 60 mM salt. In addition, single strand DNA, ss/ds DNA boundaries and negatively supercoiling degree were not required for PaMutL nicking activity. Finally, the analysis of the incision sites revealed that PaMutL, as well as *Bacillus thuringiensis* MutL homologue, did not show DNA sequence specificity.

Keywords: DNA sequence or structure/endonuclease activity/mismatch repair/MutL/*Pseudomonas aeruginosa*.

Abbreviations: BtMutL, *Bacillus thuringiensis* MutL; MMR, mismatch repair; PaMutL, *Pseudomonas aeruginosa* MutL; pBKS, pBluescript II KS plasmid; rTdT, terminal deoxynucleotidyl transferase, recombinant.

In the mismatch repair (MMR) system of *Escherichia coli* and related organisms, the cleavage site of MutH endonuclease activity is well characterized. In these organisms, MutH recognizes the transitory hemi-methylated state of GATC sequences (in a mismatch and MutS independent manner), and generates an incision in the non-methylated DNA strand immediately 5′ to a GATC sequence (*1–4*) triggering the MMR process. On the other hand, it was proposed that the strand cleavage that directs this repair system in eukaryotic organisms and bacterial species devoid of MutH depends on the endonuclease activity present in some MutL homologues (*5*, *6*). In particular, the endonuclease activity of bacterial MutL homologues has been evidenced by the ability of MutL proteins to incise and relax negatively supercoiled plasmids (in the absence of mismatch and other proteins of the MMR system) (*7–10*). This activity displayed different behaviours in the presence of different metal ions. While MutL from *Thermus thermophilus*, *Aquifex aeolicus*, *Neisseria gonorrhoeae*, *Bacillus subtilis* and *Pseudomonas aeruginosa* (PaMutL) showed an endonuclease activity in the presence of Mn^{2+}, only some of these bacterial MutL proteins were also active in the presence of Mg^{2+}, Ni^{2+}, Zn^{2+} or Co^{2+} (*7–11*). In addition, ATP binding/hydrolysis also showed different effects on the endonuclease activity of different MutL proteins, as the nicking activity could be stimulated or downregulated, depending on the MutL homologue analysed and the experimental conditions (*7–11*). In general, the experiments reported in the bibliography related with MutL endonuclease activity were performed using supercoiled plasmid as DNA substrate, and the product of the reaction was mostly nicked plasmid. In one case, a complete degradation of supercoiled DNA was found after incubation with MutL from *A. aeolicus* (*11*).

To our knowledge there is no evidence of DNA sequence/structure requirement for MutL endonuclease activity from bacteria that lack a MutH homologue. On the other hand, it was described that eukaryotic MutL proteins generated several DNA fragments when incubated with a linear homoduplex DNA or nicked circular molecules (*5*, *12*, *13*), suggesting that human and yeast MutLα do not depend on a particular DNA sequence to make incisions.

The aim of this investigation was to study DNA sequence dependence and the DNA structural requirements of *P. aeruginosa* MutL (PaMutL) endonuclease activity, in a simplified *in vitro* system (in the absence of other MMR proteins). It is expected that this activity is regulated by other partners of the MMR system, as in the case of MutH, which is activated by MutL and MutS. However, the DNA sequence/structure dependence should be conserved, as MutH has a mismatch-independent *in vitro* endonuclease activity which is the same activity responsible for DNA strand discrimination during methyl-direct MMR.

In addition, as there is no consensus regarding the ionic strength condition among different works in which MutL endonuclease activity was studied (20–200 mM salt), the effect of ionic strength on MutL endonuclease activity was also investigated.

Therefore, this study could shed light on the mode of MutL DNA incision involved in the MMR system.

Materials and Methods

Plasmid and chemicals

pBluescript II KS plasmid (pBKS) (Stratagene) was purified using the GenElute HP Plasmid Midiprep Kit (Sigma-Aldrich). DNA extractions from agarose gels were carried out using the QIAEX II Gel Extraction Kit (QIAGEN) or Wizard SV Gel and PCR Clean-Up System (Promega). Restriction enzymes were obtained from Promega and New England Biolabs. *Aspergillus oryzae* S1 nuclease was from Fermentas, *E. coli* Topoisomerase I was from New England Biolabs and terminal deoxynucleotidyl transferase, recombinant (rTdT) was from Promega. dATP [α-^{32}P] was from Perkin Elmer, and 1 kb DNA ladder was from Fermentas.

Protein expression and purification

The full-length *Bacillus thuringiensis mutL* coding sequence was PCR amplified from genomic DNA using the primers BTLs (5′-GCATATGGGGAAAATTCGCAAACTCGATG-3′) and BTLa (5′-CTACATAACCCTCTTAAACATCTTCTCC-3′). Primer BTLs contains four extra nucleotides (underlined) to generate a NdeI restriction site at the ATG initiation codon. The PCR fragment was cloned in the pGEM-T Easy cloning vector (Promega). Then, the NdeI–EcoRI fragment was cloned in the T7 polymerase-driven expression vector pET-15 b (Novagen) to generate plasmid pET-BtMutL. The PaMutL and *B. thuringiensis* MutL (BtMutL) proteins were expressed from the plasmids pET-PaMutL (*10*) and pET-BtMutL, respectively.

Protein purification was performed as previously described (*10*). Briefly, His-tagged proteins were isolated from soluble cell extracts after expression of the corresponding pET-15 b derivative plasmid in an *E. coli* BL21 (DE3) pLysS strain (Novagen). The supernatants of the centrifuged lysates were incubated with ProBond Resin (Invitrogen), and after washing with increasing concentrations of imidazole the proteins were eluted using 200–400 mM imidazole and analysed on 1% SDS/10% PAGE gels. The purified proteins were concentrated using Amicon ultra-15 centrifugal filters (Merck Millipore, 10,000 MWCO) and stored in 20 mM Tris–HCl pH 7.5, 50 mM NaCl and 10% glycerol at −20°C. More than 95% purity was obtained as determined by Coomassie-stained SDS–PAGE gels (data not shown). When cited in molar terms, the protein concentrations were expressed as monomer equivalents.

DNA substrates

Supercoiled and linear DNA. pBKS plasmid (2,961 bp) was used as the supercoiled substrate, and two linear DNA substrates were also used: pBKS linearized with XbaI, and a 478 bp fragment obtained by PCR amplification of a pBKS region using primers pB2-S (5′-GCGAACGTGGCGAGAAAGGA-3′) and pB2-A (5′-CCATGATTACGCCAAGCTCG-3′).

DNA molecules containing a bulge or a bubble (heteroduplexes). Linear DNA molecules with looped-out bases (bulge) were synthesized by annealing a pBKS region of 478 bp obtained by PCR amplification [primers pB2-S (5′-GCGAACGTGGCGAGAAAGGA-3′) and pB2-A (5′-CCATGATTACGCCAAGCTCG-3′)] with the same region containing a deletion of 6, 18 or 39 nucleotides (generated by digestion and subsequent ligation of pBKS with SalI/XhoI, SmaI/EcoRV or SmaI/HincII, respectively). The positions of the bulges from the 5′ end of pB2-S primer were 400, 363 and 363 bp, respectively. Likewise, a linear DNA with multiple mismatches (bubble) was synthesized by annealing two PCR fragments (512 bp) amplified from two pBKS plasmids, using primers pB2-S and pB2-A, with both containing a non-complementary region of 60 nucleotides (cloned between the HindIII and ApaI restriction sites) beginning at position 389 bp from the 5′ end of pB2-S primer. In all cases, DNA annealing was made in a thermocycler. All samples were heated for 10 min at 99°C, and then the temperature was reduced from 99 to 90°C at 1°C/12 s and from 90 to 40°C at 1°C/30 s. The hybridized samples were composed of two homoduplexes and two heteroduplexes. As described earlier (*14*), heteroduplexes migrate like a higher molecular weight molecule than the corresponding homoduplex.

pBKS topoisomers. Samples with different distributions of pBKS topoisomers were generated by the incubation of pBKS with Topoisomerase I (0.4–20 mU/ml) using the conditions recommended by the supplier. The presence of topoisomers was confirmed by 0.8% agarose gel electrophoresis (1.5 V/cm, 12 h) followed by ethidium bromide staining.

PaMutL endonuclease activity assay

PaMutL (1.3 μM) was incubated with DNA substrates for 2 h at 37°C in endonuclease buffer [20 mM Tris/HCl (pH 7.5), 5 mM divalent ion mixture of Mn^{2+}/Mg^{2+}] containing NaCl (30–150 mM) as indicated. The reactions were stopped (by adding 14 mM EDTA, 0.1% SDS and 10% glycerol), and the PaMutL endonuclease activity was revealed depending on the DNA substrate used as follows: (i) supercoiled DNA: 1% agarose gels containing 0.5 μg/ml of ethidium bromide. (ii) Linear DNA: denaturing 6% polyacrylamide/urea 7 M gels followed by ethidium bromide staining. Alternatively, the 3′ ends were ^{32}P-labelled before using denaturing polyacrylamide electrophoresis. (iii) Heteroduplex DNA: 6% polyacrylamide gels followed by ethidium bromide staining. (iv) pBKS topoisomers: 0.8% agarose gels, either containing 0.5 μg/ml of ethidium bromide before electrophoresis or followed by ethidium bromide staining. At least three independent experiments were performed. The quantification of nicked and supercoiled plasmid was carried out using ImageJ software (*15*). We have previously shown that a His-tagged MutL protein from *E. coli* purified in the same manner has no detectable *in vitro* endonuclease activity (*10*).

S1 nuclease assay

Supercoiled plasmid or linear heteroduplex DNA was incubated with 0.33 U/μl of S1 nuclease for 30 min at room temperature, as recommended by the supplier. These reactions were stopped by adding 30 mM EDTA and heating at 70°C for 10 min. At 0.33 U/μl of S1, the activity on supercoiled DNA consisted of mainly nicking activity.

Characterization of PaMutL incision site(s)

Supercoiled DNA was incubated with PaMutL (1.3 μM) at 37°C for 2 h in endonuclease buffer containing 100 mM NaCl ('Material and Methods' section). The protein was removed by phenol : chloroform extraction and ethanol precipitation, and a second digestion with XbaI was performed to linearize the plasmid. The restriction enzyme was removed by a second step of phenol : chloroform extraction and ethanol precipitation, and the 3′ ends were ^{32}P-labelled by rTdT using the conditions recommended by the supplier. The samples were resolved using denaturing 6% polyacrylamide/7 M urea gel electrophoresis. Radioactive signals were analysed using a Fuji FLA-3000 phosphor-imager.

Results

Dependence of PaMutL endonuclease activity on ionic strength

As previously described by our group, PaMutL harbours endonuclease activity, demonstrated by its ability to introduce nick(s) on a negatively supercoiled plasmid (*10*). Several studies evidenced the activity of MutL on supercoiled plasmid using different NaCl or KCl concentrations, with no consensus in ionic strength between them (*5*, *7–13*, *16*). However, using linear DNA as substrate, it was recently reported that hMutLα has endonuclease activity at low ionic strengths (60 mM or lower) but not at high salt

concentrations (90 mM or higher) (*13*). Considering this result and other studies in *E. coli* demonstrating that ionic strength also regulates other MutL functions (*i.e.* MutL-ssDNA binding and MutL ATPase activity) (*17*, *18*), we analysed the effect of ionic strength on PaMutL endonuclease activity in supercoiled plasmid and linear DNA. When supercoiled plasmid was used as substrate, the endonuclease activity was revealed by native agarose gels. In the case of homoduplex linear DNA, denaturing polyacrylamide gels were performed to detect nicking activity. Figure 1A shows that PaMutL was able to nick supercoiled plasmid at all NaCl concentrations tested (30–150 mM), having the highest activity at 30 mM. The same trend was obtained using a linear DNA substrate (PCR fragment, 478 bp), although a complete inhibition was observed at salt concentrations higher than 100 mM (Fig. 1B and C). Similar results were obtained using KCl (not shown). In agreement, a complete inhibition was also found when linearized pBKS was used as substrate at high ionic strength conditions (100 mM, Fig. 1D). Since both, the closed and linearized pBKS plasmids have the same nucleotide sequence, cleavage should has been observed on linearized pBKS if the endonuclease activity of PaMutL was to depend solely on a particular DNA sequence (not present in the PCR DNA fragment substrate). However, our results clearly show that PaMutL was able to incise supercoiled plasmid, but not linear DNA substrates, at physiological ionic strength conditions (~100 mM salt concentration).

PaMutL endonuclease activity on linear DNA molecules containing a single-strand DNA region

As shown in Fig. 1, at physiological ionic strength, PaMutL is still able to exert its endonuclease activity on supercoiled, but not on a linear DNA plasmid substrate. Considering that the nucleotide sequence of the supercoiled and linear DNA plasmid substrate is the same, it could be proposed that a structural characteristic of the supercoiled plasmid, absent in linear DNA, is essential for PaMutL endonuclease activity. One structural characteristic of plasmids with negative supercoiling is the transient presence of single-strand regions (*19*). Therefore, we analysed if a single-strand DNA region or a ss/ds DNA boundary are requirements for PaMutL endonuclease activity at 100 mM NaCl. To perform this analysis, several DNA substrates containing a bulge of different sizes (6, 18 or 39) or a bubble (60 bases) were generated (Fig. 2A), and incubated with PaMutL in endonuclease buffer (100 mM NaCl). Figure 2B shows that PaMutL was not able to digest any of the heteroduplex substrates analysed, indicating that single-strand DNA regions and ss/ds DNA boundaries present on negative supercoiled plasmids were not essential for PaMutL endonuclease activity. The digestion by S1 nuclease of all

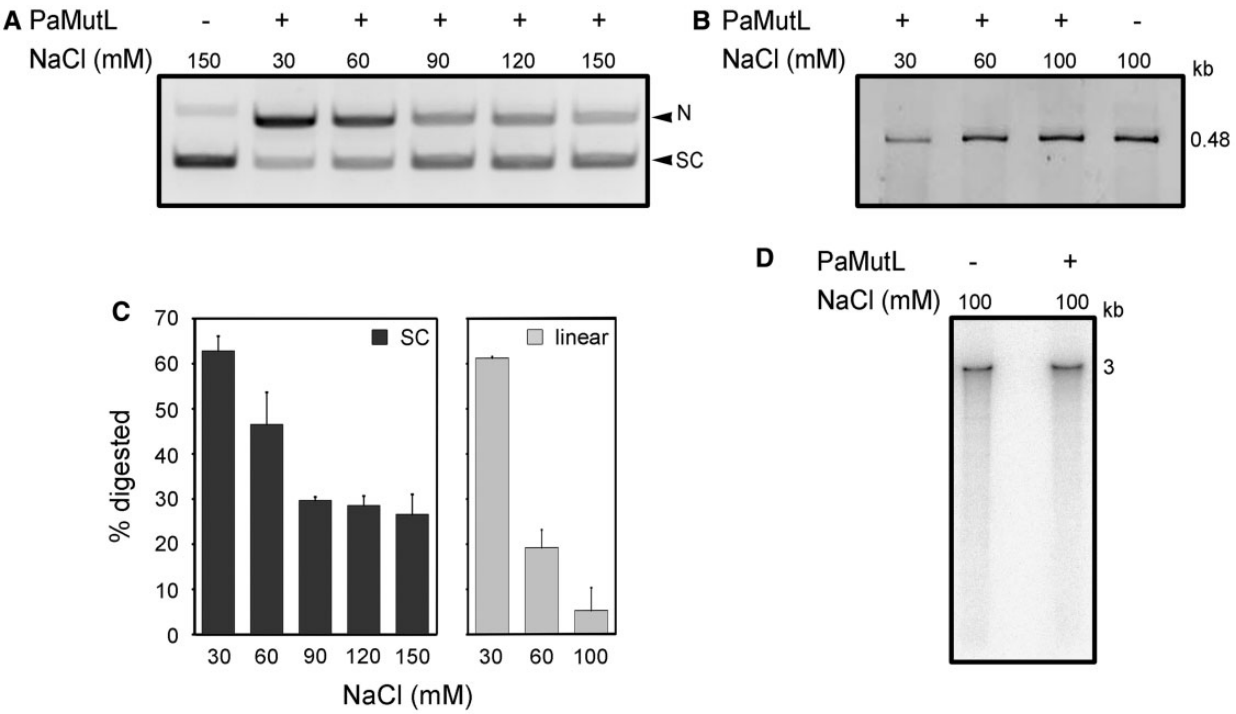

Fig. 1 Effect of ionic strength on PaMutL endonuclease activity. (**A**) PaMutL (1.3 μM) endonuclease activity on supercoiled DNA determined as a function of NaCl concentration. A negative photograph of a 0.8% agarose/0.5 μg/ml ethidium bromide gel is shown. Migration of supercoiled (SC) and nicked (N) DNA is indicated. (**B**) PaMutL (1.3 μM) endonuclease activity on linear DNA (478 bp) determined as a function of NaCl concentration. A negative photograph of a denaturing 6% polyacrylamide/urea 7 M gel ethidium bromide staining is shown. (**C**) Quantification of PaMutL endonuclease activity is shown in Fig. 1A and B. Error bars represent the SEM. (**D**) PaMutL (1.3 μM) endonuclease activity on linearized pBKS at 100 mM NaCl. After the incubation, the 3′ ends were ^{32}P-labelled and analysed using denaturing 6% polyacrylamide/urea 7 M gel electrophoresis. At least three independent experiments were performed.

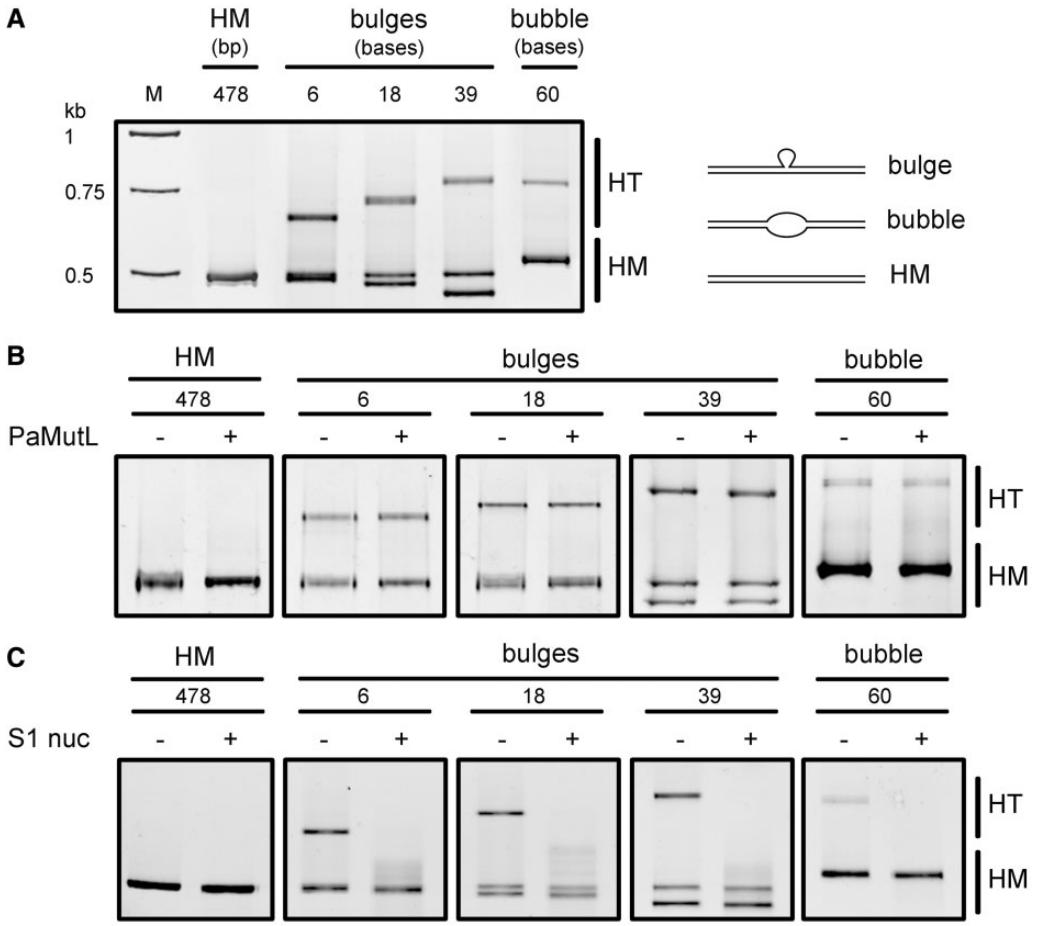

Fig. 2 PaMutL endonuclease activity on linear DNA molecules containing a single-strand DNA region. (**A**) Electrophoretic migration of the homoduplex (478 bp) and all homoduplex/heteroduplex substrates. Homoduplex/heteroduplex samples contain dsDNA with a bulge of 6, 18 or 39 bases or a bubble of 60 bases, and also the homoduplexes used to generate the heteroduplex molecules. As expected, heteroduplexes migrate like a higher molecular weight molecule than the corresponding homoduplex. On the right, the schematic representation of a bulge, a bubble and a linear homoduplex DNA. (B and C) DNA substrates shown in Fig. 3A after incubation with (**B**) PaMutL (1.3 μM) in 100 mM NaCl or (**C**) S1 nuclease (S1 nuc, 0.33 U/μl). (A–C) 6% polyacrylamide gel electrophoresis, followed by ethidium bromide staining. Negatives of the ethidium bromide-stained photographs are shown.

the heteroduplexes generated was used as a positive control of single-strand presence (Fig. 2C).

Effect of negative supercoiling degree on PaMutL endonuclease activity

To evaluate whether the negative supercoiling is necessary for PaMutL endonuclease activity at physiological ionic strength, plasmid topoisomers were generated by the incubation of pBKS with Topoisomerase I (Fig. 3A), and were then incubated with PaMutL. Figure 3A shows pBKS topoisomers distribution generated after the incubation of supercoiled plasmid with different amounts of Topoisomerase I (analysed by 0.8% agarose gel electrophoresis followed by ethidium bromide staining). Samples containing mainly supercoiled plasmid (Fig. 3A, line 1), a distribution of topoisomers (Fig. 3A, line 3) and an almost relaxed plasmid (Fig. 3A, line 6) were then incubated with PaMutL in endonuclease buffer (100 mM NaCl) and analysed using agarose gels containing ethidium bromide (in the presence of ethidium bromide, relaxed molecules became supercoiled and could be distinguished from nicked molecules). Figure 3B shows that PaMutL generated similar relative amounts of nicked plasmid when a supercoiled or a relaxed plasmid was used as substrate (Fig. 3B, lines 3–5). These results indicated that PaMutL incised plasmids independently of their negative supercoiling degrees. As a control, the same samples were incubated with S1 nuclease, and in this case the nicked plasmid generated decreased as the plasmid became more relaxed (Fig. 3C). Figure 3D shows the quantification of PaMutL, and S1 enzymatic activity was shown in Fig. 3B and C.

PaMutL incision site(s) on pBKS

Figure 1 shows that PaMutL endonuclease activity generated mainly nicked plasmid, as previously described (*10*). To determine if this nicking activity depended on a particular DNA sequence, supercoiled plasmid was incubated with PaMutL in endonuclease buffer (100 mM NaCl) and then linearized with XbaI. As a control, supercoiled plasmid was digested only with XbaI. Then, the 3′ ends of DNA products were ^{32}P-labelled and resolved by using denaturing polyacrylamide gels (Fig. 4). The results showed that PaMutL generated DNA fragments that ranged from

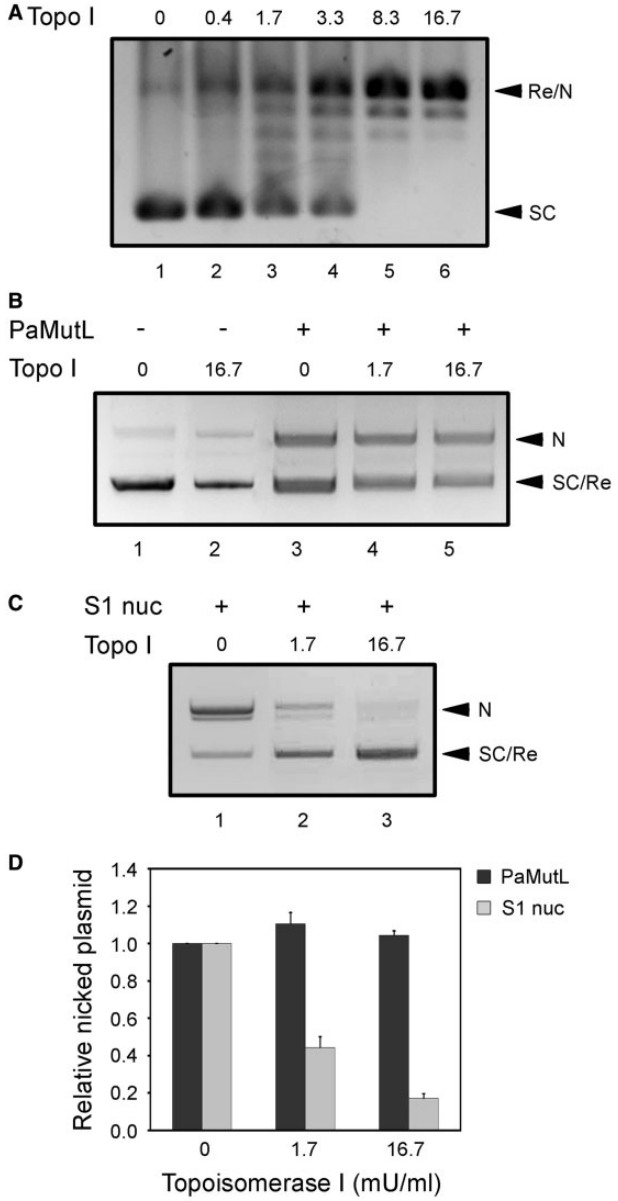

Fig. 3 Effect of negative supercoiling degree on PaMutL endonuclease activity. (**A**) pBKS topoisomer distribution generated after incubation of supercoiled plasmid with Topoisomerase I (Topo I), analysed by 0.8% agarose gel electrophoresis followed by ethidium bromide staining. (**B**) Migration of supercoiled plasmid and topoisomers generated with Topo I (1.7 and 16.7 mU/ml) after no further treatment (lines 1–2) or after incubation with PaMutL (1.3 μM, lines 3–5) or (**C**) after incubation with S1 nuclease (S1 nuc, 0.33 U/μl). (B–C) 0.8% agarose/0.5 μg/ml ethidium bromide gels. (A–C) Negatives of the ethidium bromide stained photographs are shown. Migration of supercoiled (SC), nicked (N) and relaxed (Re) DNA is indicated. (**D**) Quantification of endonuclease activity of PaMutL and S1 nuclease is shown in (B) and (C). Data are presented as endonuclease activity, relative to that obtained using the supercoiled plasmid as substrate. Error bars represent the SEM. At least three independent experiments were performed.

the complete size of the plasmid (3 kb) down to the migration front of the gel (~0.1 kb), with some weak discrete DNA bands also being observed (similar results were obtained by Southern blotting analysis, data not shown). Note that each new incision on DNA generates a new 3′end, so the total radioactive label in

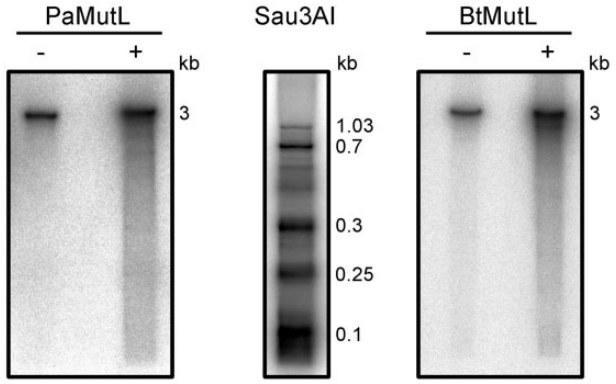

Fig. 4 MutL cleavage site(s). After incubation of pBKS with PaMutL (1.3 μM) or with BtMutL (1.3 μM) at 100 mM NaCl, the DNA was linearized with XbaI, the 3′ ends were ^{32}P-labelled and the products analysed by denaturing 6% polyacrylamide/urea 7 M gel electrophoresis. As a control of an endonuclease enzyme that recognized a specific DNA sequence, the pBKS was digested with Sau3AI and analysed similarly.

DNA samples treated with MutL is higher than non-treated plasmid although the same amount of DNA is loaded in the gel. The same result was obtained when the experiment was performed at low ionic strengths (30 mM salt, data not shown) suggesting that, although the ionic strength regulated the enzymatic activity (Fig. 1), it did not affect the incision site(s) specificity.

To establish whether this nicking activity behaviour was specific for PaMutL or shared with other bacterial MutL proteins with endonuclease activity, BtMutL incision site(s) were also analysed, as BtMutL cation-dependent endonuclease activity was recently demonstrated by our group (Correa *et al.*, unpublished results). Figure 4 shows that BtMutL, as in the case of PaMutL, also generated products that ranged in size from 3 kb down to the migration front of the gel (0.1 kb or less), with some weak DNA bands also being observed. These results showed that PaMutL and BtMutL were able to generate incisions anywhere within the pBKS plasmid, suggesting that their endonuclease activities did not possess sequence specificity.

Discussion

In the MMR system of *E. coli* and related organisms, MutH cleaves specifically the newly synthesized DNA strand on hemimethylated GATC sequences (*1*). This activity can be considerably stimulated by MutL in a mismatch-independent manner, or by MutS and MutL in a mismatch-dependent manner (*4*). Although the MutH incision site is well characterized in *E. coli*, this protein is absent in most prokaryotes and in all eukaryotes (*6*). However, a distinctive feature of the MMR system in these organisms is the presence of a MutL homologue with an endonuclease activity (*5*, *7–10*, *12*, *13*). In this investigation, we analysed the effect of ionic strength and DNA structure/sequence on the endonuclease activity of PaMutL.

In agreement with other studies (*7*, *12*, *13*), our results showed that the ionic strength regulated the

endonuclease activity of PaMutL. At high ionic strength (similar to physiological conditions), the endonuclease activity was partially inhibited on supercoiled plasmid, but was completely inhibited on linear DNA. Moreover, PaMutL was able to generate nicks on plasmid molecules independently of their supercoiling degree. These results suggest that DNA molecules with restriction of rotation about its helix axis (closed circular DNA) appear to be a more favourable substrate for PaMutL endonuclease activity than free-ends linear DNA molecules at physiological ionic strength.

Regarding the DNA structure, our results showed that ssDNA (6–60 bases) or ss/ds boundaries are not substrates for PaMutL endonuclease activity. Consequently, the PaMutL endonuclease activity observed at high ionic strength on supercoiled plasmid would not be due to the presence of a single strand DNA region or ss/ds boundaries.

Concerning cleavage site(s), we showed that PaMutL and BtMutL endonucleases were able to generate products that ranged from the complete size of the plasmid (3 kb) down to the migration front of the gel (0.1 kb or less). This indicated that none of these endonucleases possess sequence specificity in this experimental system. Although some weak DNA bands were observed among the products of both proteins, these may correspond to some double-strand digestions or be indicative of some weak sequence-context preferences. These results agree with others found for hMutLα and yMutLα (*5*, *12*), where it was shown that these proteins generated a wide range of DNA product sizes (in the presence of other MMR proteins and a mismatch), suggesting that neither of these endonucleases have sequence specificity.

Our results show that contrary to MutH, which specifically recognizes and cleaves GATC sequences (by itself or in the presence of a mismatch, MutS and MutL), MutL homologues with endonuclease activity do not require a specific sequence to make incisions. As a consequence, the MMR system in these organisms might be more efficient than in those containing MutH, considering that GATC distribution can affect the MMR function (*20*).

On the other hand, the finding that under physiological ionic strength conditions, MutL preferred terminal-less DNA instead of linear DNA is consistent with the fact that linear DNA is rarely found in normal cells during DNA replication. This finding suggests that DNA lesions could have repair priorities depending on their potential damaging consequences to the cell. Hypothetically, mismatches and double-strand breaks/ends could appear at the same time within the DNA molecule. In this case double-strand discontinuities (the single most potent inducer of genomic instability in cells) will be probably repaired first by the corresponding repair system and the mismatches will be repaired later since MutL does not show a preference for linear DNA.

Summing up, according to our results PaMutL endonuclease activity was regulated by DNA structure and ionic strength and did not show DNA sequence specificity neither under high nor low ionic strength conditions. It remains to determine how these features influence the MMR process. To achieve this, further experiments have to be performed using a reconstituted *in vitro* system to evaluate the nature of DNA substrate and the ionic strength conditions, suitable for stimulation of the PaMutL endonuclease activity.

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Conflict of Interest

None declared.

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