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Some amino acids of the *Pseudomonas aeruginosa* MutL D(Q/M)HA$(X)_2$E$(X)_4$E conserved motif are essential for the *in vivo* function of the protein but not for the *in vitro* endonuclease activity

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ABSTRACT

Human and *Saccharomyces cerevisiae* MutLα, and some bacterial MutL proteins, possess a metal ion-dependent endonuclease activity which is important for the *in vivo* function of these proteins. Conserved amino acids of the C-terminal region of human PMS2, *S. cerevisiae* PMS1 and of some bacterial MutL proteins have been implicated in the metal-binding/endonuclease activity. However, the contribution of individual amino acids to these activities has not yet been fully elucidated. In this work we show that *Pseudomonas aeruginosa* MutL protein possess an *in vitro* metal ion-dependent endonuclease activity. In agreement with previous published results, we observed that mutation of the aspartic acid, the first histidine or the first glutamic acid of the conserved C-terminal DMHAAHERITYE region results in nonfunctional *in vivo* proteins. We also determined that the arginine residue is essential for the *in vivo* function of this protein. However, we unexpectedly observed that although the first glutamic acid mutant derivative is not functional *in vivo*, its *in vitro* endonuclease activity is even higher than that of the wild-type protein.

1. Introduction

The DNA mismatch repair system (MRS) is evolutionary conserved, and corrects replication errors which have escaped the proofreading activity of the replicative DNA polymerase [1]. In *Escherichia coli*, this repair pathway has been well characterized and it is initiated by binding of MutS to a mismatch. After the recruitment of MutL, this complex activates the strand discriminating endonuclease MutH, which cleaves the newly synthesized, unmethylated daughter strand at an hemimethylated d(GATC) site, and thereby marks it for removal and a repair–synthesis process that involves a variety of other proteins [1]. The delay in methylation of the newly replicated d(GATC) sequences by the *E. coli* DNA adenine methyltransferase (Dam) provides the strand discrimination signal [1].

However, the *E. coli* MutH/d(GATC) strand discrimination mechanism is only found in a small group of proteobacteria from the γ-subdivision [1]. For the rest of bacterial and eukaryotic organisms with an active MRS it was proposed that a latent endonuclease activity present in some MutL homologues generates the strand cleavage that directs the repair system [2]. In this sense it has been reported that human MutLα (hMutLα), *Saccharomyces cerevisiae* MutLα (yMutLα) and some bacterial MutL proteins possess a metal ion-dependent nicking endonuclease activity [2–6]. This endonuclease activity seems to be dependent on the integrity of three conserved sequence motifs of the C-terminal region, which are conserved in some eukaryotic MutL homologues and in most MutL homologues from bacteria that do not relay on the endonuclease activity of MutH for strand discrimination [2–10].

The presumed endonuclease/metal-binding site of human PMS2 (hPMS2) C-terminal domain has been modeled [9]. This model and the crystal structure of the C-terminal region of *Bacillus subtilis* MutL (BsMutL-CTD) and *Neisseria gonorrhoeae* (NgMutL-CTD) [6,13] revealed that the most highly conserved residues from the three motifs [DQHA$(X)_2$E$(X)_4$E, ACR, and C(P/N)HGRP] are spatially close, suggesting that they are also functionally linked. In this sense, mutation of some amino acids of these conserved sequence motifs in the hPMS2 subunit of hMutLα, *S. cerevisiae* PMS1 (yPMS1) subunit of yMutLα, and in MutL from *N. gonorrhoeae* (NgMutL), *Thermus thermophilus* (TtMutL), *Aquifex aeolicus* (AaMutL), and *B. subtilis* MutL (BsMutL) affected their *in vitro* endonuclease activity [2–6,8,9] or resulted in proteins inactive for *in vivo* mismatch repair [3,4,6,7,10,11]. In the case of hPMS2, mutation of some of

Abbreviations: LB, lysogeny broth medium; MRS, mismatch repair system; Rif^R, rifampicin resistant.

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these conserved residues also affected the *in vitro* mismatch repair function of MutLα [2,9,10].

Although bacterial and eukaryotic MutL proteins displaying an endonuclease activity share conserved amino acids motifs in the C-terminal region, the endonuclease activity of these proteins have some particularities. While hMutLα and yMutLα proteins display an endonuclease activity in the presence of Mn^{2+} but not in the presence of Mg^{2+} [2,3], bacterial MutL homologues have more heterogeneous ion requirements. For instance, TtMutL relaxed supercoiled plasmid in a Mn^{2+}, Ni^{2+} or Co^{2+} dependent manner, but not in the presence of Mg^{2+}, Zn^{2+} or Ca^{2+} [4]. AaMutL relaxed supercoiled plasmid in a Mn^{2+}, Ni^{2+}, Zn^{2+} or Co^{2+} dependent manner, but not in the presence of Mg^{2+} or Ca^{2+} [4,12]. NgMutL and NgMutL-CTD relaxed supercoiled plasmid in a Mn^{2+} or Mg^{2+} dependent manner, but not in the presence of Ca^{2+} [5]. Finally, BsMutL relaxed supercoiled plasmid in a Mn^{2+} dependent manner, but not in the presence of Mg^{2+}, Zn^{2+} or Cd^{2+} [6].

The effect of ATP binding/hydrolysis on MutL endonuclease activity also showed different effects. While the nicking activity of hMutLα and yMutLα was significantly stimulated by ATP [2,3], the endonuclease activity of TtMutL and NgMutL protein was down-regulated [4,5]. It was reported that the endonuclease activity of AaMutL protein can be stimulated [8] and also repressed [4] by ATP, effects that probably depend on protein, ATP and/or Mn^{2+} concentration. In addition, BsMutL endonuclease activity was stimulated at low concentration of ATP but inhibited at high concentration of ATP, probably due to excess nucleotide chelating ions away [6]. Curiously, addition of Mg^{2+}, Mg^{2+} and ATP, Zn^{2+} or Co^{2+} to BsMutL, in the presence of Mn^{2+}, stimulated a second cut on the nicked DNA to yield a linear product [6].

Although some biochemical, mutational, bioinformatics and crystallographic analyses have been performed with different MutL homologues, based on the data published so far, the contribution of conserved amino acids of the C-terminal region to the endonuclease activity, its regulation, and metal ion binding, has not yet been fully elucidated.

Pseudomonas spp. MutL proteins contain most of the conserved amino acids in the motifs described to be important for the endonuclease/metal-binding activity found in some MutL homologues [5,9,13]. In this work we show that *Pseudomonas aeruginosa* MutL (PaMutL) protein possess a metal ion-dependent endonuclease activity and characterized the sequence–function relationship of the D(Q/M)HA$(X)_2$E$(X)_4$E conserved motif by combining *in vivo* and *in vitro* experimental analyses.

2. Materials and methods

2.1. Bacterial strains and chemicals

E. coli BL21(DE3) pLysS was from Novagen (Madison, WI, U.S.A.); *E. coli* ER2566 was from New England BioLabs (Beverly, MA, U.S.A.); *P. aeruginosa* MPAO1 wild-type strain and mutants MPA46306 and MPA2537 (*mutL*::IS*lacZ*/hah-Tc^R) were gently provided by Dr Michael Jacobs from the University of Washington Genome Center (USA) [14]. Plasmids were purified using the Wizard Plus SV Miniprep DNA purification system (Promega, Madison, WI, U.S.A.). DNA extractions from agarose gels were performed using the QIAEX II Gel Extraction kit (Qiagen, Chatsworth, CA, U.S.A.). Restriction enzymes were from Promega and New England Biolabs.

2.2. Cloning of *P. aeruginosa* full length *mutL* gene and a derivative devoid of the ATP binding region

For expression of *P. aeruginosa* MutL protein, the full length *mutL* coding sequence was cloned in the T7 polymerase-driven expression vector pET-15b (Novagen) to generate plasmid pET-PaMutL. For complementation analysis, *P. aeruginosa mutL* gene was cloned in the pBBRIMCS-5 (p5) plasmid [15] to generate plasmid p5-PaMutL. For expression of a MutL derivative devoid of most of the N-terminal ATP binding region, a portion of the *P. aeruginosa mutL* gene (codifying for amino acids 225 to 633) was cloned in plasmid pET-15b to generate plasmid pET-PaMutLΔ1–224, and in plasmid pTYB12 (New England Biolabs) to generate plasmid pTYB12-PaMutLΔ1–224. The pET derivative plasmid produces an N-terminal His-tagged protein (twenty extra amino acids). The pTYB12 derivative plasmid also expresses an N-terminal fusion protein, which after cleavage and elution results in a fusion protein with 15 extra N-terminal amino acids but no His-tag. For a detailed explanation of plasmid constructions see "plasmid construction" in Supplementary data.

2.3. Site directed mutagenesis

P. aeruginosa MutL D467N, M468A, H469A, H472A, E473A, E473K, R474A, Y477A and E478A single mutants were generated by PCR using primers designed to introduce the desired mutations (for details see "plasmid constructions" in Supplementary data). All plasmid constructions were verified by DNA sequencing.

2.4. Complementation assay

Complementation assays were performed by transforming a *P. aeruginosa* MutL-deficient strain with plasmid p5 (no insert), p5-PaMutL, p5-PaMutL-D467N, p5-PaMutL-M468A, p5-PaMutL-H469A, p5-PaMutL-H472A, p5-PaMutL-E473A, p5-PaMutL-E473K, p5-PaMutL-R474A, p5-PaMutL-Y477A or p5-PaMutL-E478A, carrying the wild-type *P. aeruginosa mutL* gene or a single mutant derivative (see "plasmid constructions" in Supplementary data).

Mutation rates were determined by fluctuation analysis. Three to five parallel cultures, inoculated with a small number of cells in order to avoid introducing in the culture any preexisting mutants, were grown overnight in LB medium containing gentamicin (30 μg/ml). Appropriate dilutions of the overnight cultures were plated onto LB-agar to determine the total number of viable cells, and aliquots were plate onto LB-agar containing rifampicin (100 μg/ml) to score for rifampicin resistant cells, following incubation overnight at 37 °C. The mutation rate was determined from the distribution of the number of mutants in the cultures by the MSS maximum-likelihood method [16] using SALVADOR 2.3 [17].

2.5. Proteins expression and purification

His-tagged wild-type and mutants *P. aeruginosa* MutL full length proteins and derivatives devoid of the ATP binding region were isolated after overexpression of the corresponding pET derivative plasmid in an *E. coli* BL21(DE3) strain, as recommended by the pET system manual (Novagen). The pTYB12 derivative plasmid was overexpressed in an *E. coli* ER2566 strain, as recommended by the IMPACT-CN system manual (New England BioLabs). All proteins were purified from soluble cell extracts. Transformed *E. coli* cells were grown at 37 °C with shaking in LB medium supplemented with ampicillin (200 μg/ml) and glucose (0.5%, w/v) to an OD_{600} of 0.6–0.8. Protein expression was induced by addition of 0.15 mM IPTG and was then incubated for 16 h at 18 °C with shaking. After induction, cells were collected, resuspended in binding buffer (50 mM Tris–HCl pH 7.5, 500 mM NaCl, 15% glicerol, and 5 mM PMSF) and lysed by high pressure homogenization (C3 Emulsiflex, Avestin). The supernatants of the centrifuged lysates were incubated with 0.5 ml (bed volume) of ProBond™ Resin (Invitrogen), at 4 °C with continuous mixing. Unbound proteins were washed off with 40 column bed volumes of binding buffer containing increased concentrations of imidazole (0, 30, 60 and 80 mM). Proteins were eluted using 200–400 mM imidazole and analyzed on 10% SDS/PAGE. Fractions containing His-tagged proteins as determined by SDS–PAGE were pooled. Purified proteins were concentrated using centriprep filters (Ultracel YM 10-10000) and the buffer was changed to storage buffer (20 mM Tris–HCl pH 7.5, 50 mM NaCl and 5% glicerol). Purified proteins were >95% pure as judged by Coomassie Brilliant Blue-stained SDS/PAGE (not shown). When cited in molar terms, protein concentrations are expressed as monomer equivalents.

2.6. Endonuclease activity test

Proteins were incubated with 10 nM of supercoiled pBKS plasmid (Stratagene), in 20 mM Tris/HCl (pH 7.5), 50 mM NaCl, 5 mM divalent ion (unless otherwise indicated in the figure legend) at 37 °C, in a final volume of 30 μl. Reactions were stopped by adding 5 μl of a 7× loading buffer (100 mM EDTA, 0.7% SDS, and 70% glycerol) and analyzed by 1% agarose gel electrophoresis. DNA was visualized by ethidium bromide staining. At least three independent experiments were performed for each protein, obtaining similar results. One representative experiment is shown in Figs. 2–5 and 7. Quantification of nicked or supercoiled plasmid DNA was done using the Gel-Pro program (MediaCybernetics, Silver Spring, MD, USA).

2.7. Far western assays

Different amounts (2, 9 and 17 pmol) of non-tagged purified *P. aeruginosa* MutS protein were spotted onto Protran nitrocellulose membranes (0.2 μm; BioSciences). Positive (His_6-MutS; 2 pmol) and negative (BSA; 10 pmol) spots were included. The membranes were blocked for 1 h at room temperature with buffer A (10 mM Tris–HCl, pH 8.0, 0.15 M NaCl and 1 mM EDTA) supplemented with 0.1% Triton X-100 and 5% (P/V) of fat-free dried milk and then incubated with His_6-tagged wild-type PaMutL, PaMutL-D467N, PaMutL-E473K or PaMutL-R474A (0.6 μM) in buffer A overnight at 4 °C. After wash, the membranes were incubated with rabbit anti-His_6 antibody (1/500; Santa Cruz Biotechnology) for 3 h at room temperature, washed, and then incubated for 1 h with IRDye 800CW-conjugated goat anti-rabbit antibody