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In vitro affinity of Deinococcus radiodurans MutS towards mismatched DNA exceeds that of its orthologues from Escherichia coli and Thermus thermophilus

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ABSTRACT

The mismatch binding protein MutS is responsible for the recognition of mispaired and unpaired bases, which is the initial step in DNA repair. Among the MutS proteins most extensively studied in vitro are those derived from Thermus thermophilus, Thermus aquaticus and Escherichia coli. Here, we present the first report on the in vitro examination of DNA mismatch binding activity of MutS protein from Deinococcus radiodurans and confront this with the properties of those from E. coli and T. thermophilus. The analyses which included mobility gel-shift assay, colorimetric and qPCR estimation of MutS-bound DNA clearly showed that D. radiodurans MutS exhibited much higher affinity towards mismatched DNA in vitro than its counterparts from E. coli and T. thermophilus. In addition, D. radiodurans MutS displayed a significantly higher specificity of DNA mismatch binding than the two other orthologues. The specificity expressed as the ratio of mismatched to fully complementary DNA bound reached over 4 and 20-fold higher values for D. radiodurans than for T. thermophilus and E. coli MutS, respectively. The results demonstrate mainly the biotechnological potential of D. radiodurans MutS but the in vitro characteristics of the MutS orthologues could reflect substantial differences in DNA mismatch binding activities existing in vivo.

Keywords: DNA-protein binding MutS, DNA mismatch, Nickel-coated microplate, Deinococcus radiodurans, DNA mismatch enrichment

1. Introduction

MutS is a component of MMR system (Mismatch Repair system) the role of which is to recognize and remove mispaired and unpaired bases resulting from DNA damage and replication errors. MutS does not recognize mutations, but the disturbed conformation of misaligned nucleotides, also called pre-mutational changes (Jiricny, 2013). The mismatch repair protein is also designated as MutS1, while additional prokaryotic MutS homologues, MutS2, MutS3, MutS4 and MutS5, are not involved in DNA mismatch repair (Sachadyn, 2010). Dissimilar to prokaryotic MutS proteins which form homodimers (structural heterodimers upon DNA binding), the eukaryotic ones act as heterodimers: MSH2-MSH6 and MSH2-MSH3 (Kunkel and Erie, 2005).

MutS proteins from different bacteria show diversity in DNA binding properties. To date, most of the studies on MutS proteins have been conducted using recombinant proteins derived from *Escherichia coli* (Blackwell et al., 2001; Lamers et al., 2000), *Thermus aquaticus* (Biswas and Hsieh, 1996; Obmolova et al., 2000; Sixma, 2001) and *Thermus thermophilus* (Fukui et al., 2013; Joshi and Rao, 2001; Stanislawska-Sachadyn et al., 2005, 2006, 2003; Whitehouse et al., 1997). *In vivo* examination of *Deinococcus radiodurans* MutS was focused on the consequences of *mutS* gene deletion (Mennecier et al., 2004). Although the canonical properties of MutS proteins in terms of DNA binding activity and specificity are similar, a number of differences distinguishing MutS orthologues from different species have been reported. The *E. coli* MutS protein does not recognize C/C mismatches *in vitro,* whereas the protein derived from *T. thermophilus* does. Not surprisingly, *T. thermophilus* MutS is more stable at higher temperatures than that of *E. coli* (Whitehouse et al., 1997), yet it shows DNA mismatch binding activity in a range of temperatures, including 25 °C, 35 °C, and 60 °C (Takamatsu et al., 1996; Whitehouse et al., 1997). *T. aquaticus* MutS exhibits a lower binding affinity towards fully matched DNA comparing to that of *E. coli*, but its affinity towards mismatched DNA is also lower than that of the *E. coli* counterpart (Brown et al., 2001; Cho et al., 2007). What is more, MutS could be sensitive to the sequence context (Joshi and Rao, 2001; Stanislawska-Sachadyn et al., 2005), binding conditions and the presence of adenine nucleotides (Blackwell et al., 2001), so the characteristics of DNA mismatch binding properties reported in different studies reflect not only the natural properties of the examined proteins but they strongly depend on the experimental setup.

In vitro DNA binding properties of MutS from *D. radiodurans* have not been characterized to date. *D. radiodurans* has been the most deeply

Abbreviations: DrMutS, *Deinococcus radiodurans* MutS protein; EcMutS, *Escherichia coli* MutS protein; TthMutS, *Thermus thermophilus* MutS protein

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studied among the microorganisms belonging to the *Deinococcaceae* family, containing non-sporulating cocci, that are able to endure the effects of ionizing radiation doses which are lethal to most other known organisms (Minton, 1994). It is stated that *D. radiodurans* cells can survive an acute dose of 5000 Gy of gamma radiation without significant loss of viability as compared to the lethal dose of 2000 Gy for *E. coli* (Moseley and Mattingly, 1971). Moreover, some surviving cells can also be isolated from the cultures exposed to even 20,000 Gy of gamma radiation (Ito et al., 1983). It was shown that MutS-mediated removal of DNA replication errors and the inhibition of recombination between partially divergent sequences is crucial for the maintenance of genome stability in *D. radiodurans*. The extreme resistance of *D. radiodurans* to gamma radiation is explained by exceptional features of its genome and DNA repair system, based on the two, consequent mechanisms: extended synthesis-dependent single-strand annealing (ESDSA) and ensuing RecA-mediated double-strand break repair (Cox and Battista, 2005; Minton, 1994). However, the MutS and/or MutL deficient mutant cells have been reported to be equally radioresistant as the wild type ones, thus indicating that these mismatch repair proteins are not essential for the radiation resistance (Battista et al., 1999).

The unusual properties of DNA mismatch repair system in *D. radiodurans* has encouraged us to evaluate the application potential of MutS from this organism. Several MutS based methods of DNA analysis have been reported so far, including employing MutS for mutation detection (Lishanski et al., 1994; Stanislawska-Sachadyn et al., 2005), suppression of non-specific PCR amplification (Fukui et al., 2013), removal of erroneous DNA in gene synthesis (Binkowski et al., 2005), enrichment of mutated DNA fraction in directed evolution "*in vitro*" (Zhong et al., 2011). Notwithstanding its interesting properties and multiple application trials, MutS has not become a standard tool in molecular diagnostics and MutS binding to fully complementary DNA appears to be the main impediment. The formation of MutS complexes with fully complementary DNA could be decreased (Blackwell et al., 2001), but it rather cannot be eliminated without the loss of DNA mismatch binding or at least reduced to the degree at which it would not interfere with the detection of DNA mismatches. Successful biotechnological application of a protein requires the selection of a proper orthologue. Proteins with useful properties are often found in extremophiles. Therefore, it is worth interrogating as to whether the DNA mismatch repair protein in *D. radiodurans* exhibits exceptional features useful in diagnostic applications. This is the first report describing the *D. radiodurans* MutS protein in terms of its binding affinity and specificity towards mismatched DNA *in vitro*, juxtaposed with two other, prokaryotic MutS proteins from *E. coli* and *T. thermophilus*.

2. Materials and methods

2.1. Cloning

The *D. radiodurans mutS* gene was PCR amplified from the genomic DNA template extracted with Genomic Mini AX Bacteria Spin kit (A & A Biotechnology, cat. no. 060-100S Poland) from the *D. radiodurans* R1 strain purchased from the DSMZ collection (DSM No. 20539). The forward and reverse primers used for the amplification of the *D. radiodurans mutS* gene have the following sequences: **FDrmutS1 –** TTTTAAGGTACCCATA TGCGAGCAGTTTCCCCCACCAAAGCG, **RDrmutS1** – TTATAAGCTTC GTCTCGTACCGACAGGATCCAACCGTCTCTTACCGGGATTCATGGCCCTCC CCC (Genomed, Poland) and they contained engineered KpnI and HindIII restriction sites (underlined) at their 5′ ends. The *E. coli mutS* gene was PCR amplified using the genomic DNA template extracted as above from *E. coli* Top10F'. The nucleotide sequences of the primers for the *E. coli mutS* gene amplification were: **FecMutS-** ACGTAGGTACCATGAGTGCAATAGA AAATTTCGAC, **RecMutS**- GCACAGAATTCTTACACCAGGCTCTTCAAGC (Genomed, Poland) and they included engineered KpnI and EcoRI restriction sites (underlined). The construction of recombinant plasmid bearing the *Thermus thermophilus* MutS protein (TthMutS) coding sequence (pUET1-*Tth-mutS* (5266 bp) was described previously (Stanislawska-Sachadyn et al., 2003).

The PCR amplified *E. coli* and *D. radiodurans mutS* genes were cloned in-frame with the 6 x histidine tag sequence downstream from the T7 promoter of the pUET3 vector (Olchowy et al., 2006) into the KpnI and HindIII, and the KpnI and EcoRI sites, respectively. The obtained recombinant plasmids of 5460 and 5455 bp were designated as pUET3Dr-MutS and pUET3EcMutS, respectively. The correctness of plasmid constructs was confirmed by DNA sequencing (Genomed, Poland) and compared with the reference nucleotide sequences of *mutS* genes (GI:1026245073, GI:556503834). The maps and nucleotide sequences of the recombinant plasmids are presented in Supplementary Figs. S2–S4.

2.2. Protein expression and purification

The competent *Escherichia coli* BL21(DE3)pLysS cells (Promega, cat. No. L1195, USA) were transformed with the recombinant plasmids pUET1-*Tth-mutS*, pUET3DrMutS, and pUET3EcMutS, thus obtaining three strains producing MutS proteins containing 6 x histidine tags at the N-termini. The amino acid sequences of the fusion his-tagged MuS proteins are shown in Supplementary Figs. S2–S4.

Five hundred ml of LB medium supplemented with ampicillin (0.1 mg/ml) were inoculated with 20 ml of overnight cell culture for each of the three expression *E. coli* strains. The inoculated cultures were grown with agitation at 37 °C to OD_{600} = 0.5 and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) to the final concentration of 1 mM, following cultivation at 30 °C for an additional 8 h. Then, the cultures were harvested by 10 min centrifugation at 6000*g*, at 4 °C and stored for further use at −20 °C.

Next, the recombinant MutS proteins of *D. radiodurans* (DrMutS), *T. thermophilus* (TthMutS) and *E. coli* (EcMutS) were purified using Immobilized Metal Affinity Chromatography (IMAC). The pellets obtained from 250 ml of the bacterial cell cultures expressing the above mentioned recombinant proteins were suspended in 30 ml of buffer A (50 mM phosphate buffer, pH 8.0/25 °C; 500 mM NaCl; 10% glycerol (v/v)) and disrupted by sonication on ice at an amplitude of 7 μm, ten times for 30 s with intervals of 30 s using MSE Soniprep 150 (Sanyo, Japan). The crude lysates were centrifuged at 10,000*g* for 40 min, the supernatant was collected and applied to 1 ml of the cOmplete His-Tag Purification Resin containing immobilized Ni^{2+} ions (Roche, cat. no. 05893682001, Switzerland) equilibrated with ten volumes of buffer A. The column was washed with 10 ml of the buffer A, followed by washing with 10 ml of buffer W1 (50 mM phosphate buffer, pH 8.0/25 °C; 500 mM NaCl; 10% glycerol (v/v); 10 mM imidazole). The his-tagged MutS proteins were eluted with 10 1-ml portions of elution buffer E (50 mM phosphate buffer, pH 8.0/25 °C; 500 mM NaCl; 10% glycerol (v/v); 500 mM imidazole) (Supplementary Fig. S8A). Subsequently, the collected eluates were dialyzed against buffer S (50 mM phosphate buffer, pH 8.0/25 °C; 100 mM NaCl; 10% glycerol (v/v)) to remove imidazole. The dialyzed samples were concentrated by centrifugation using the Vivaspin device with 100 kDa cut-off limit (Viva Science, cat. no. Z614661, UK) to the final volume of 500 μl (Supplementary Fig. S8B). The concentrations of purified proteins were determined by densitometric analysis of SDS-PAGE electropherograms stained with Coomassie brilliant blue, using bovine serum albumin (BSA) as the reference (Supplementary Fig. S8B). The concentrations of proteins were adjusted to 1.0 μg/μl using buffer S diluted with pure glycerol (50% glycerol v/v).

2.3. Mismatched DNA and fully complementary DNA controls

The DNA fragments were obtained from pairs of HPLC purified synthetic oligodeoxyribonucleotides (Genomed, Poland), either perfectly complementary, or forming a single mismatch, as shown in Supplementary Table S1. The oligodeoxyribonucleotides were sus-

pended in a buffer containing 10 mM Tris–HCl (pH 8.8/25 °C), 50 mM KCl, 0.08% (v/v) Nonidet P40, 5 mM $MgCl_2$ to the concentration of 10 μM in a final volume of 100 μl, following heating (92 °C/120 s) and two-stage cooling (65 °C/120 s and 25 °C/120 s). For the colorimetric assays, one of the oligodeoxyribonucleotides was biotin-labelled at the 5′ end.

2.4. MutS microplate immobilization

The his-tagged MutS proteins (DrMutS, EcMutS and TthMutS) were immobilized in the wells of a Ni^{2+}-coated ELISA microplates (Pierce, Thermoscientific, cat. no. 15442, USA). Prior to protein loading the microplate wells were activated by triple washing with 100 μl of PBS buffer (Sigma® Life Science, cat. no 79383, USA) supplemented with 1% (v/v) of Tween® 20 (Sigma – Aldrich, cat. no. P7949, USA) and 5 mM $MgCl_2$. One μg of his-tagged-MutS, suspended in 100 μl of the buffer mentioned above was applied per well, following 60 min incubation in a thermomixer at room temperature with shaking at 300 rpm. Next, the wells were emptied and the unbound protein was removed by triple washing with the same buffer.

2.5. DNA mismatch binding – assessment using colorimetric assay

The DNA mismatch binding properties were assessed using 49 bp DNA fragments biotinylated at one of 5′ ends (Supplementary Table S1). The experiment involved parallel examination of mismatched DNA (TG49, GA49, CT49, or a single insertion/deletion loop (unpaired T), ΔT49/48), perfectly complementary DNA (TA49), and the mock control (without DNA added) in three microplate wells (Supplementary Fig. S1). Ten pmoles of 49 bp biotinylated DNA suspended in 100 μl of PBS buffer (Sigma® Life Science, cat. no 79383, USA) supplemented with 1% (v/v) of Tween® 20 (Sigma-Aldrich, cat. no. P7949, USA) and 5 mM $MgCl_2$ was loaded per one microplate well containing his-tagged-MutS immobilized as described above. After 15 min incubation at the temperature of 37 °C, the wells were emptied and the unbound DNA was removed by triple washing with the same buffer at room temperature. Colorimetric detection of biotinylated DNA captured by MutS was carried out at room temperature using horseradish peroxidase conjugated with extravidin (Extravidin, Sigma, cat. no. E2886) and TMB (3,3′,5,5′-Tetramethylbenzidine, Sigma, cat. no T0440) as the substrate. The details of the colorimetric assay are described in our previous paper (Banasik and Sachadyn, 2016).

2.6. DNA mismatch binding – qPCR quantitation

Equal amounts of mismatched (TG69) and perfectly complementary (TA69) DNA fragments were loaded into the same microplate well containing his-tagged-MutS immobilized as described above. Mismatched DNA and complementary DNA controls, either 10^{-14} or 10^{-13} mol each, were suspended in 100 of the PBS buffer (Sigma® Life Science, cat. no 79383, USA) supplemented with 1% (v/v) of Tween® 20 (Sigma – Aldrich, cat. no. P7949, USA) and $MgCl_2$ (5 mM) and fully complementary 48 bp competitive DNA (1 μM). The competitive DNA fragments had no sites for the primers used for TG69 and TA69 DNA amplification (Supplementary Table S1), and consequently were not detectable in the subsequent PCR quantitation. A parallel mock experiment, with the addition of neither TG69 nor TA69 DNA was performed to prepare the negative PCR controls.

After 60 min incubation at 37 °C in a thermomixer at 300 rpm, the wells were emptied, and washed five times with 200 μl portions of the same buffer to remove the unbound DNA. Next, 100 μl of 0.2 mg/ml subtilisin (cat no 19155; Qiagen, Germany) suspended in 50 mM Tris–HCl pH 8.8, 200 mM KCl, was applied in order to release DNA by MutS digestion. After 60 min incubation at 37° and 300 rpm, the samples were collected and heat-treated at 65 °C for 40 min to inactivate the protease. The samples were tenfold diluted and used as the templates for PCR quantitation (Fig. 1).

The nucleotide sequences of TG69 and TA69 amplicons were designed in such a manner to enable their discrimination in the same template. For this purpose, the terminal regions of TA69 and TG69 corresponding to the primer sites contained completely different sequences, while the central parts of the 69 bp amplicons were identical (Supplementary Table S1). Further, similar AT and GC contents were maintained in both amplicons and corresponding primers. Finally, the results of PCR with the standard templates of known concentrations were compared for both amplicons to ascertain that they were amplified with very similar efficiencies, which is to say, 1.883 in the case of mismatched TG69 DNA and 1.932 for the fully complementary TA69 DNA (Supplementary Fig. S9).

Real-Time PCR reactions were performed in triplicates in a 10 μl volume containing 5 μl of *Taq* DNA polymerase based SYBR Green Master mix (2x HS PCR Master Mix SYBR®A, A & A Biotechnology, Poland, cat. No. 2017-100A), the forward and reverse primers (0.1 μM each), and 1 μl of the DNA template. The PCR thermal profile was as follows: 95 °C/600 s (hot-start DNA polymerase activation), 95 °C/10 s (template denaturation step), 55 °C/10 s (primers annealing step), 72 °C/10 s (primers extension step), (40 cycles). The high resolution melting (HRM) was performed by gradually increasing the temperature from 60 °C (20 s) to 95 °C (20 s) at a rate of 0.05 °C/s. The PCR and HRM experiments were carried out using Nano LightCycler®Instrument (Roche Diagnostics). The C_t (cycle threshold) values were calculated using LightCycler® Nano Software version 1.1 supplied by the manufacturer. The standard curves for TG69 and TA69 were determined for the following DNA template final concentrations [μM]: 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, respectively. The standard curves were linear within the DNA concentrations range of the analysed templates (Supplementary Fig. S9a and b).

2.7. Enrichment of mismatched DNA fraction by immobilized MutS

The conditions of the DNA mismatch enrichment were the same as described above except that the amount of mismatched DNA fragment TG69 (10^{-16} mol) was a hundredfold lower than that of the fully complementary TA69 DNA (10^{-14} mol).

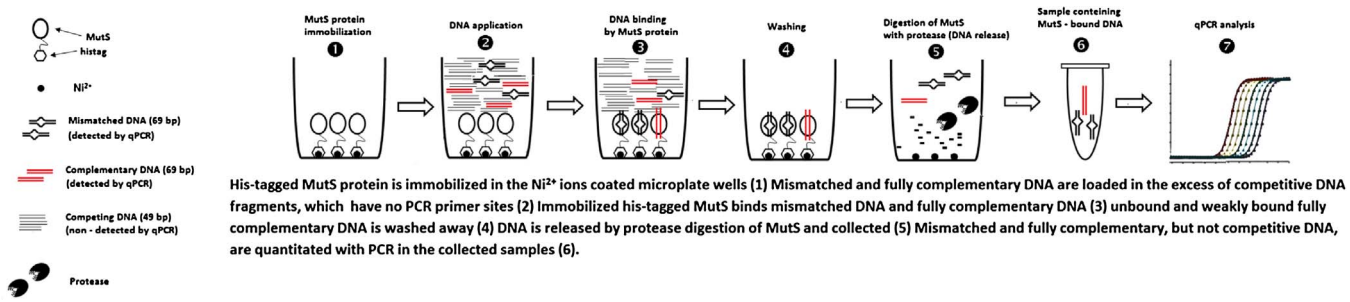

Fig. 1. The principle of PCR quantitation of MutS-captured DNA.

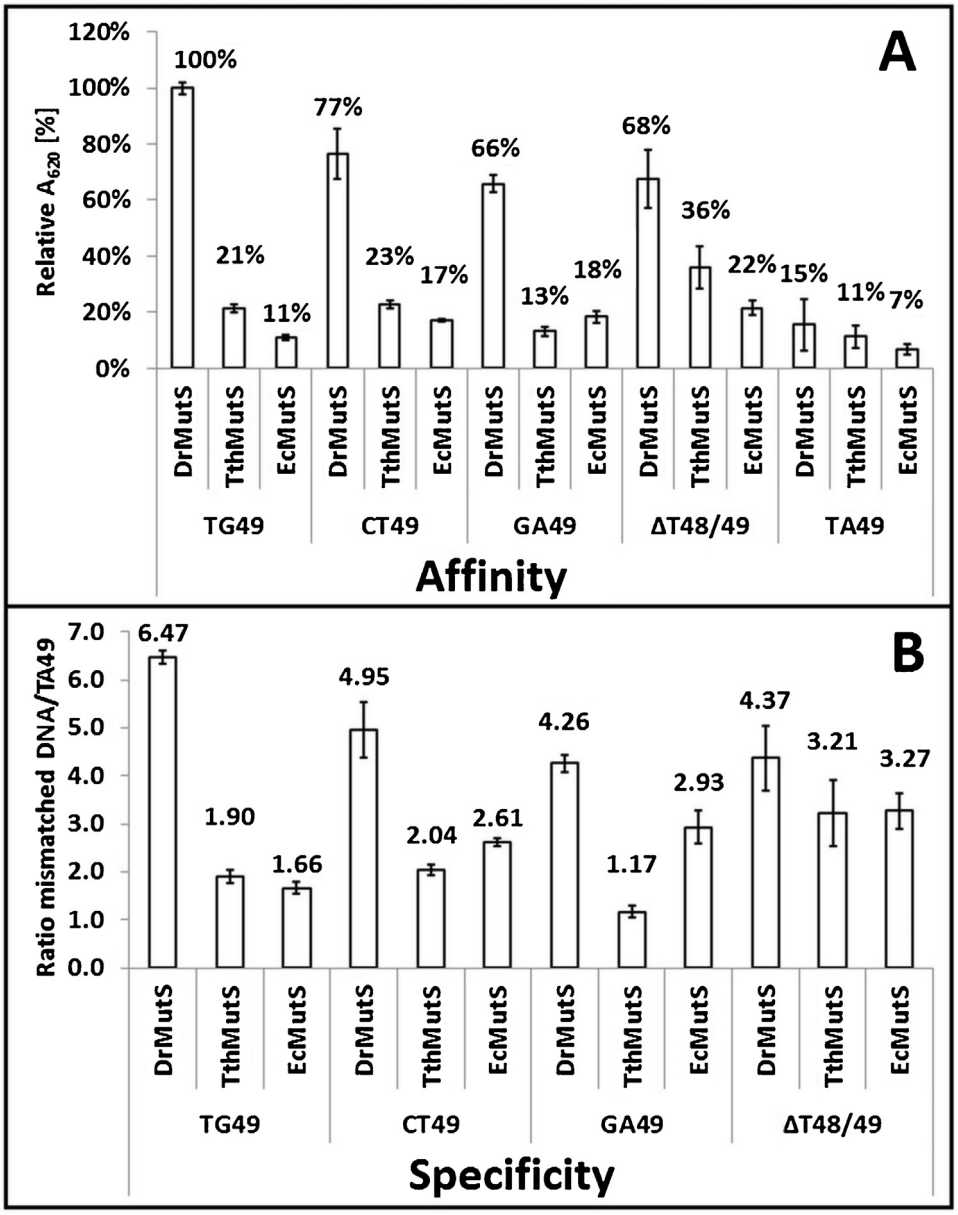

Fig. 2. Specificity of MutS proteins from three bacterial species towards DNA mismatches estimated using colorimetric assay. (A) The relative affinities for mismatched (TG49, CT49, GA49), unpaired T (ΔT49/48) and complementary (TA49) DNA shown as the percentage of the highest affinity value (DrMutS towards TG49 mismatched DNA, 100%). (B) The specificity towards mismatched DNA determined as arithmetic means of three mismatched DNA to fully complementary DNA control (TA49) ratio values obtained in three independent experiments.

2.8. Exclusion of non-specific DNA binding by nickel coated ELISA microplates

In order to rule out the possibility that the ELISA microplates with immobilized nickel ions were capable of binding the DNA fragments of interest, thus leading to false positive results, an additional control experiment was performed in the absence of MutS and in the presence of TG49 DNA. No colorimetric signal was observed for this control. A similar control experiment was prepared for the qPCR experiment where 10 pmoles of TG69 and TA69 DNA were loaded into the microplate wells without MutS (final concentration of 10^{-4} μM). Neither TG69 nor TA69 DNA was detected in thus obtained templates, which indicates that DNA is not retained in the nickel-coated plates in the absence of MutS after washing.

2.9. Gel mobility shift assay

DNA binding reactions were performed in a final volume of 20 μl containing 2.5, 5, 10, 15 and 30 pmoles of one of recombinant MutS proteins (DrMutS, TthMutS or EcMutS) and 0.5 pmole of 49 bp DNA fragments either containing a single TG mismatch (TG49) or fully complementary (TA49) suspended in PBSx1 buffer (Sigma® Life Science, cat. no 79383, USA), supplemented with 1% (v/v) of Tween® 20 (Sigma-Aldrich, cat. no. P7949, USA), and 5 mM $MgCl_2$. The samples were incubated at 37 °C for 20 min and next subjected to electrophoresis in a 6% non-denaturating polyacrylamide gel at room temperature, in 89 mM Tris-borate buffer, pH 8.3, at 12 V/cm for 1 h, followed by staining with GelRed dye (cat. no. 41003; Biotium, Inc., USA) suspended in 50 ml of 89 mM Tris-borate buffer.

2.10. Determination of MutS affinity for DNA and specificity towards DNA mismatches

We used two terms, affinity and specificity, in order to characterize the properties of examined MutS proteins. The affinity of the given MutS protein towards DNA either mismatched or fully complementary

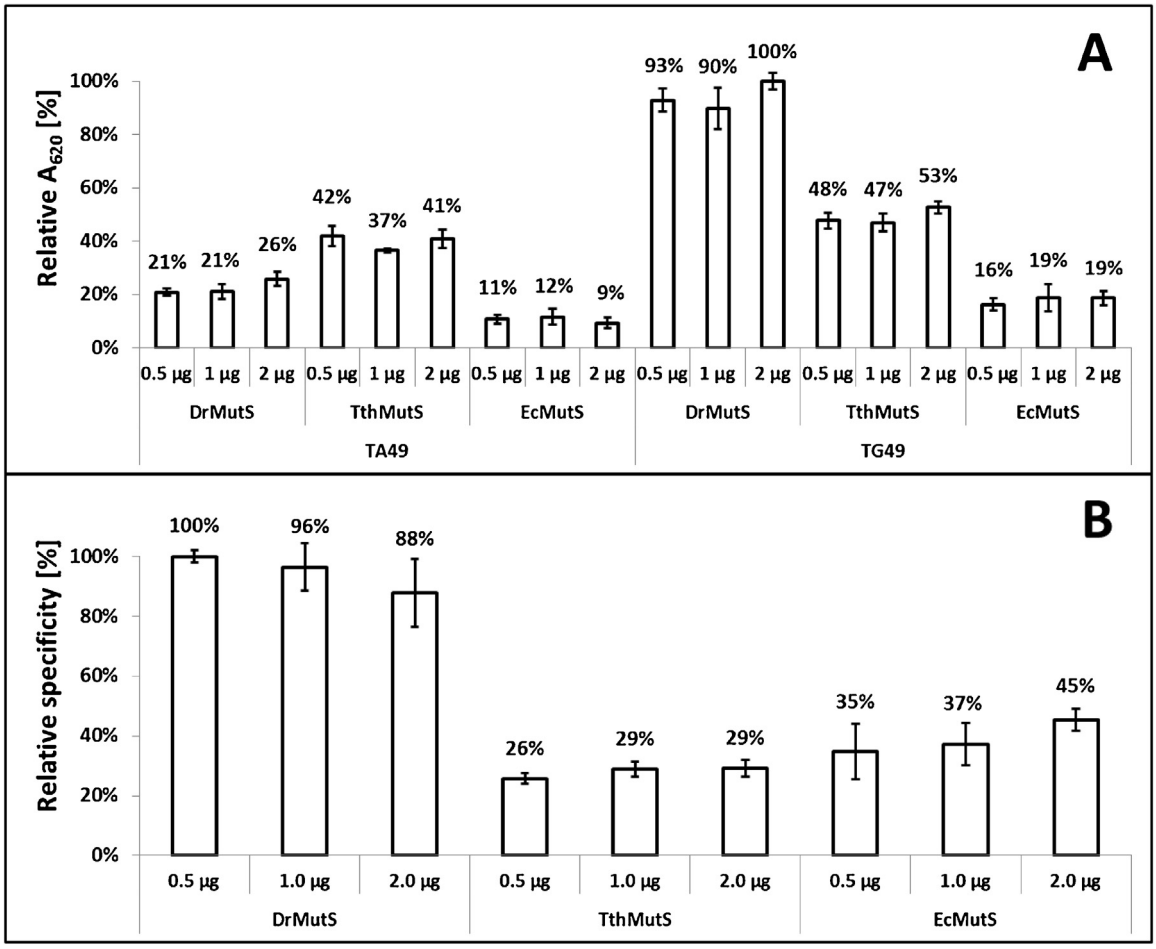

Fig. 3. The amount of histag-MutS loaded into nickel-coated microplate wells and the specificity of DNA mismatch binding. (A) Relative affinities obtained in the colorimetric assay for 0.5, 1.0, and 2.0 µg of loaded MutS using 49 bp mismatched (TG49) and fully complementary DNA (TA49) (B) Relative specificities calculated as arithmetic means of TG49 to TA49 ratio values determined for 0.5, 1.0, and 2.0 µg of loaded MutS. The experiments for each MutS species and each quantity of loaded MutS were performed in triplicates.

corresponds to the mean signal values calculated from the results of three independent experiments. The means were calculated from either A_{620} signals measured in the colorimetric assays or the determined amounts of captured DNA in qPCR. The specificity is defined as the ratio of the mean affinity for the mismatched to that for the control, perfectly complementary DNA. The statistical significance of results was calculated using two-tailed heteroscedastic Student's *t*-test.

3. Results

3.1. Comparison of MutS proteins from three different bacterial species by colorimetric estimation of MutS bound DNA

The his-tagged-MutS proteins from *D. radiodurans*, *E. coli* and *T. thermophilus* were purified from overproducing *E. coli* cell cultures using metal affinity chromatography (Supplementary Fig. S8). The specificities of MutS proteins towards various DNA mismatches (TG49, CT49, GA49) and the insertion-deletion loop containing unpaired T (ΔT49/48) were estimated using a colorimetric assay based on capturing DNA by MutS immobilized in solid phase on ELISA nickel-coated microplates. In the assay, 10 pmoles of MutS (approximately 1 µg) and 10 pmoles of DNA were loaded per microplate well. The examination revealed significant differences in both DNA binding affinities and the specificities towards mismatched DNA between the three MutS species (Fig. 2). The affinity towards mismatched DNA (TG49, GA49, CT49, ΔT49/48) was higher than towards that for fully complementary fragments (TA49) for each of the examined MutS orthologues, though the affinities for different types of mismatches markedly varied. The highest affinity and specificity towards mismatched DNA, strongly exceeding those determined for *E. coli* (EcMutS) and *T. thermophilus* (TThMutS), was shown by that of *D. radiodurans* MutS (DrMutS) towards all types of examined DNA mismatches. The differences were statistically significant, except for those between the specificities towards the single insertion-deletion loop, ΔT49/48, where satisfactory values were observed for the three MutS proteins. The differences in fully complementary DNA binding were not significant, if any (Fig. 2A).

3.2. Moderate changes in the amounts of loaded MutS do not affect the specificity of DNA mismatch binding

In order to rule out the possibility that the obtained differences in the specificity values resulted from incorrect determination of MutS concentrations, the assay was tested for doubly decreased and doubly increased amounts of loaded MutS. The experiments confirmed that DrMutS showed superior specificity of DNA mismatch binding, while the amounts of loaded MutS had very little consequence. Significant changes in neither affinity (Fig. 3a) nor specificity (Fig. 3b) towards DNA mismatch were observed in the experiments with increasing amount of loaded MutS (0.5, 1.0 and 2.0 µg). The colorimetric assay with immobilized MutS was to some extent insensitive to potential errors resulting from inaccurate determination of recombinant MutS proteins. As we showed in our previous paper, even tenfold changes in the amount of DrMutS protein applied into the microplate well had no significant impact on the measured specificities (Banasik and Sachadyn, 2016). In this work, we made similar observations for DrMutS, TthMutS and EcMutS. The experiment demonstrated that even a 4-fold over- or

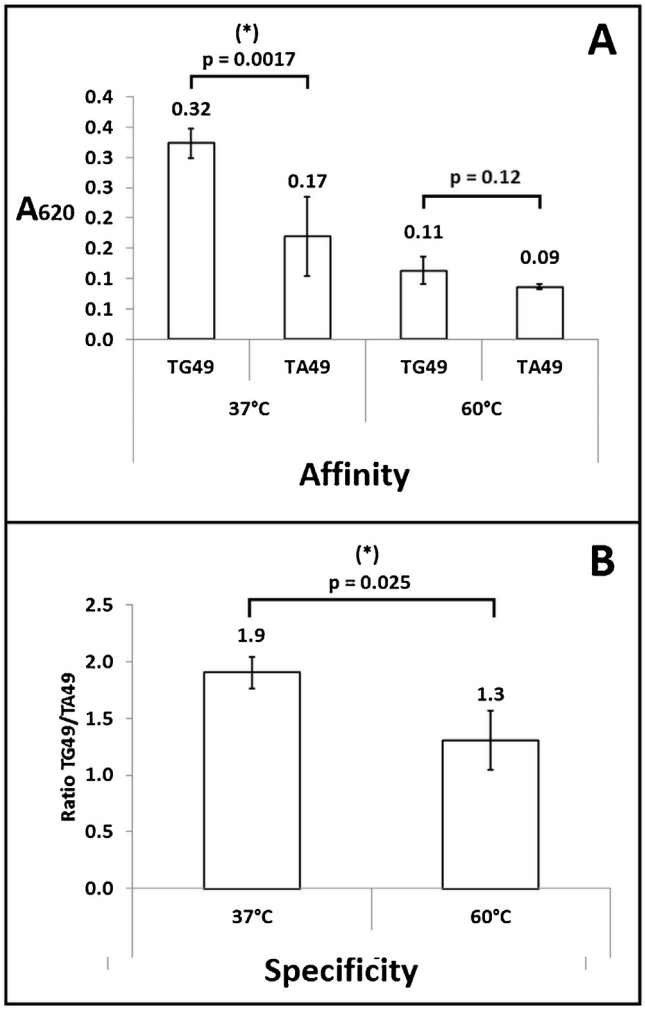

Fig. 4. The impact of temperature on DNA mismatch binding of TthMutS. TthMutS binding to mismatched (TG49) and fully complementary (TA49) DNA was performed at either 37 °C or 60 °C. (A) The affinity for mismatched (TG49) and fully complementary (TA49) DNA (B) The specificities towards mismatched DNA expressed as arithmetic means of three TG49/TA49 ratio values obtained in three independent experiments. The statistical significance was indicated with an *asterisk* (*).

underestimation of MutS loaded, which is very unlikely to occur, could not have a consequential effect on MutS specificity in the colorimetric assays. However, it should be stressed that the amounts of loaded MutS could have critical impact on the specificity in other methods, such as *e.g.* gel mobility shift assays.

3.3. Impact of temperature on T. thermophilus MutS DNA binding

As *T. thermophilus* is a thermophilic organism showing optimum growth at 65–75 °C, it could be expected to demonstrate a higher activity at temperatures above 37 °C. In our microplate assay, the rise of MutS-DNA binding temperature from 37 °C to 60 °C led to a decrease of TthMutS affinity towards both mismatched and fully complementary DNA (Fig. 4a) and practically the loss of specificity towards mismatched DNA (Fig. 4b).

3.4. Comparison of the specificities towards mismatched DNA of three different MutS orthologues using real-time PCR quantitation

The results of colorimetric assessment of MutS-DNA were verified using a more sensitive, yet a more demanding, PCR-based approach. This method involves quantitation of DNA captured by immobilized MutS proteins using real-time PCR. In this experiment, we examined 69 bp DNA fragments, longer than those used in the colorimetric assay (Supplementary Table S1). The amounts of mismatched and fully complementary DNA bound by MutS, designated as TG69 and TA69, respectively, were precisely determined on the basis of standard curves depicted in Supplementary Fig. S9, panels A and B. As in the colorimetric assay, the experiments were performed for 10 pmoles of MutS, whereas the amounts of analysed DNA fragments were greatly lower. Equal molar ratios of TG69 and TA69 DNA fragments, either 0.01 or 0.1 pmole each, were applied. What is important, differently from the colorimetric assay, DNA mismatch (TG69) binding was carried out in the presence of the perfectly complementary DNA control (TA69) in the same well. Additionally, a 10^4–10^3 molar excess of competitor fully complementary DNA was applied.

The quantitation results are presented in Fig. 5. All three examined MutS orthologues showed strong preference for the binding of mismatched DNA for both DNA concentrations tested, yet better specificities towards the DNA mismatch were achieved at the lower one. The affinity (Fig. 5a) and specificity (Fig. 5b) towards mismatched DNA exhibited by DrMutS decidedly surpassed that of TthMutS and EcMutS. The specificity towards the DNA mismatch of DrMutS expressed as the ratio of TG69/TA69 was over 90, thus by almost one order of magnitude higher than that for EcMutS and TthMutS. However, we should note that the amounts of recovered DNA were very low, in the range from atto- to femtomoles (Fig. 5a). The immobilized DrMutS, which showed the best efficiency in DNA fishing captured less than 2% of mismatched DNA loaded (Fig. 5a). The affinity (Fig. 5a) and specificity (Fig. 5b) values determined in the qPCR assay were relevantly inconstant as it can be inferred from error bars, yet the differences determined between the examined MutS orthologues were statistically significant. The specificity values obtained using qPCR approach were markedly higher than those in the colorimetric assay (compare Figs. 2b and 5b). The qPCR tests confirmed that DrMutS exhibited the highest specificity towards mismatched DNA among the three examined proteins.

3.5. Enrichment of mismatched DNA fraction using immobilized MutS

As DrMutS showed remarkably higher affinity towards mismatched DNA over fully complementary DNA than the two other orthologues, we examined as to whether this protein could be applied for enrichment of mismatched DNA fraction if it constituted as little as *e.g.* 1% of analysed DNA fragments. For this purpose, we applied a mixture consisting of 10^{-16} mol of mismatched DNA (TG69) and 10^{-14} mol of fully complementary DNA (TA69) into the ELISA microplate wells with immobilized MutS proteins. As in the previously described experiment, we added the competitor DNA (at 10^6 molar excess relative to TG69). Three examined MutS orthologues showed the ability to enrich the fraction of mismatched DNA, as demonstrated by the increase of the initial ratio of TG69:TA69 from 1:100 in the loaded DNA to 57:100, 24:100 and 17:100, in the samples recovered from the ELISA microplate wells with immobilized DrMutS, TthMuts, and EcMutS, respectively (Fig. 6). Although the results of this experiment displayed a considerable variation between the replicates (78:100, 42:10, 51:100 for DrMutS: 22:100, 16:100, 33:100 for TthMutS; and 16:100, 29:100 and 7:100 for EcMutS), DrMutS invariably exhibited a significantly higher efficiency in the enrichment of mismatched DNA than EcMutS and TthMutS (Fig. 6). However, the differences between the MutS orthologues observed in this experiment were less accentuated than in those with equal molar ratios of mismatched and fully complementary DNA (Fig. 5).

3.6. Gel mobility shift assay

As an additional confirmation of the observed differences between the three MutS orthologues we decided to apply gel mobility shift assay, which is an established approach in the examination of DNA-protein

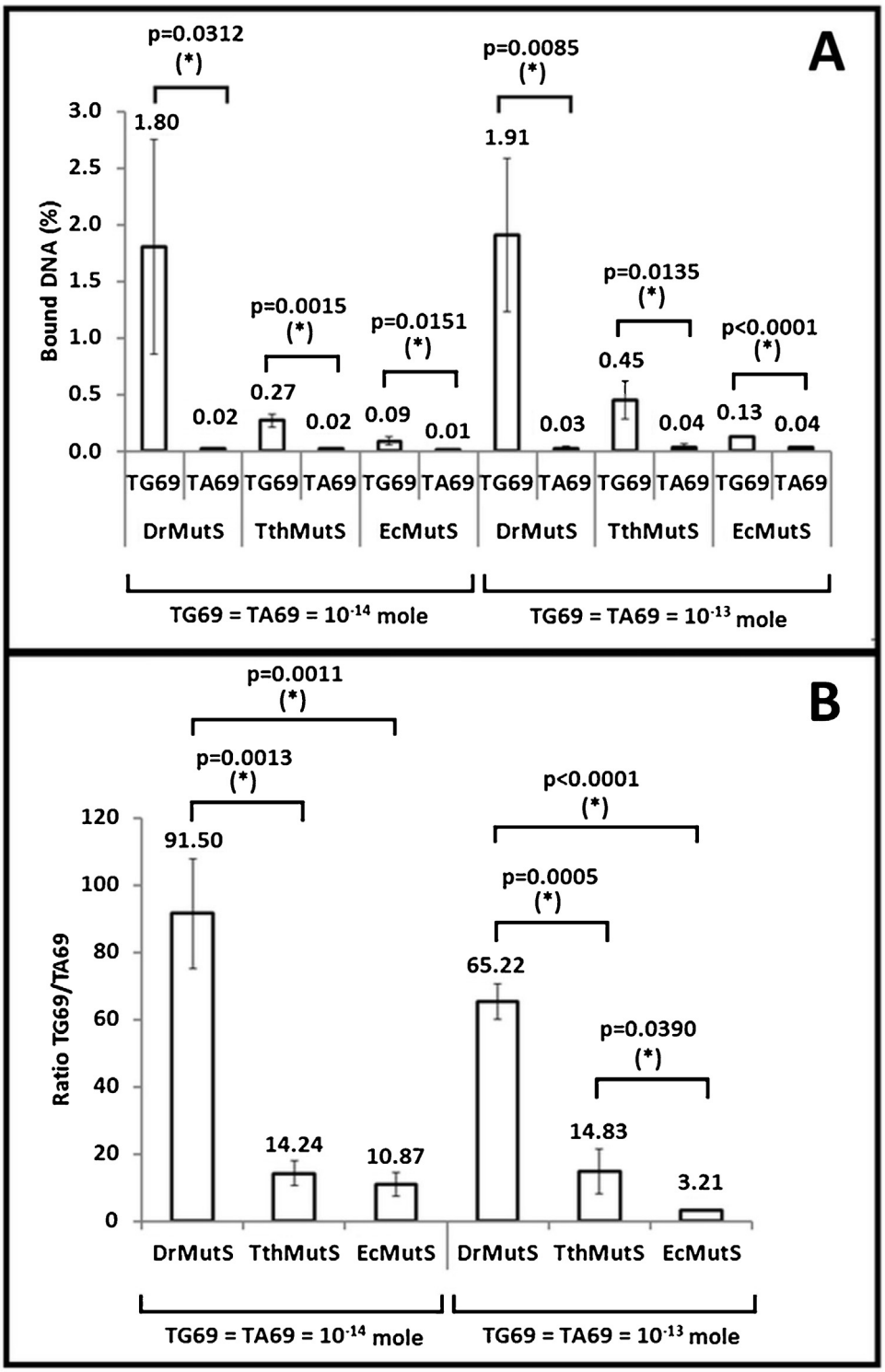

Fig. 5. The specificities of MutS proteins from three bacterial species towards mismatched DNA determined using qPCR. (A) Mean affinity values expressed as the percentages of initial DNA that was bound by immobilized MutS proteins (B) The specificities towards mismatched DNA expressed as arithmetic means of TG69/TA69 ratio values obtained in three independent experiments. The statistical significance was indicated with an *asterisk* (*).

interactions. The method, though less convenient for quantitative analysis than qPCR or colorimetric assays, involves direct DNA detection and it allows to discriminate between the MutS complexes with the mismatched and fully complementary DNA (Stanislawska-Sachadyn et al., 2003).

The experiments were performed for the three examined MutS species and 49 bp DNA fragments, either mismatched (TG49) or fully complementary (TA49). Increasing concentrations of MutS and the constant DNA amount (0.5 pmole) were applied. DNA retardation required a considerable MutS molar excess and the following MutS to DNA molar ratios were used 5, 10, 20, 30, 60. For the sake of simplicity, the non-retarded bands only are presented, so that the efficiency of DNA binding is manifested by a decreasing band intensity (Fig. 7). A threefold higher EcMutS than DrMutS concentration was necessary to achieve similar DNA mismatch binding levels (Fig. 7, DrMutS:DNA = 20:1, EcMutS:DNA = 60:1). DNA mismatch binding activities

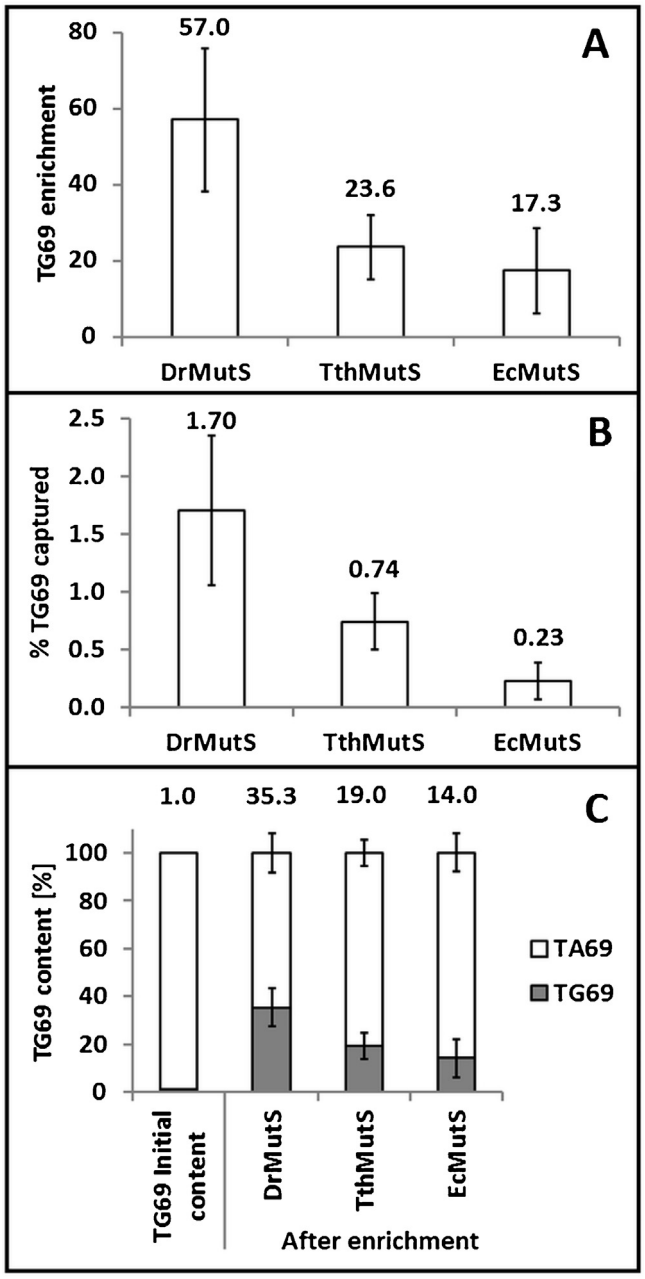

Fig. 6. DNA mismatch enrichment using immobilized MutS proteins. (A) DNA mismatch enrichment folds for three MutS orthologues. (B) Relative amounts of mismatched DNA bound expressed as the percentage of the initial amount of loaded TG69 DNA. (C) Proportional content of mismatched DNA (TG69) obtained after enrichment.

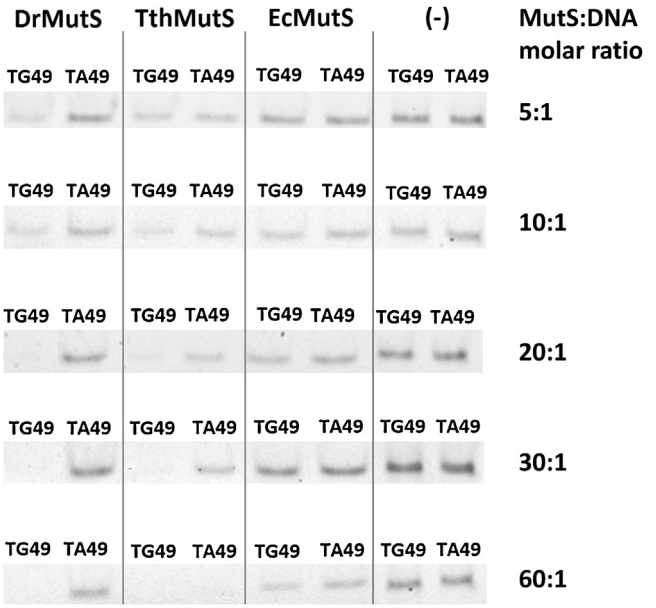

Fig. 7. DNA binding activities of MutS proteins from three bacterial species examined using mobility gel shift assay. Non-retarded bands only are shown. Electrophoresis was performed in a non-denaturing 6% polyacrylamide gel stained with GelRed. The assay was carried out for increasing MutS to DNA molar ratios (5:1, 10:1, 20:1, 30:1 and 60:1) and the constant amount of 0.5 pmole of either mismatched DNA (TG49) or fully complementary control (TA49). (M – DNA molecular weight ladder (500, 400, 300, 200, 100 bp; cat. No. MR71; DNA – Gdansk, Poland).

of DrMutS and TthMutS were comparable but the latter one displayed a markedly higher affinity for fully complementary DNA control. This is spectacular for the highest MutS concentrations, where TThMutS but not DrMutS bound and retarded the whole non-mismatch DNA. The comparison of DNA binding properties performed using a gel mobility shift assay indicated that DrMutS exhibited a noticeably stronger and more specific binding to mismatched DNA than EcMutS and TthMutS.

4. Discussion

The prokaryotic MMR system was reconstituted *in vitro* for the first time by Modrich et al. in 1983, (Lu et al., 1983). Ever since this achievement, numerous reports have been published, in both basic and applicative facets. Due to its inborn capacity of binding mismatched DNA, there were some efforts put to harness MutS proteins as a tool in molecular biology, however, with little success. The main obstacle in the application of MutS proteins in molecular biology and diagnostics is its affinity for fully complementary DNA, although lower than that to DNA mismatches (Stanislawska-Sachadyn et al., 2005), still strong enough to complicate and hinder the detection of mispaired bases. Among the most often studied MutS proteins are those from *E. coli*, *T. aquaticus* and *T. thermophilus*. Each of these proteins binds mismatched DNA with affinity significantly exceeding that for perfectly matched DNA, however, minor differences in binding different types of mismatched bases have been found (Brown et al., 2001; Whitehouse et al., 1997). Nevertheless, no direct comparison of MutS orthologues have been shown to date.

In this study, we contrasted DNA mismatch binding properties of the *D. radiodurans* MutS to those of two other prokaryotic MutS proteins from *E. coli* and *T. thermophilus*. We analysed the DNA mismatch binding properties of the three MutS orthologues using three different methods for the assessment of MutS bound DNA: a gel mobility shift assay, an assay based on colorimetric estimation and PCR quantitation (Banasik and Sachadyn, 2016). The results obtained using all three approaches clearly confirmed that *D. radiodurans* MutS (DrMutS) exhibited a markedly higher specificity towards mismatched DNA than two other orthologues. In the colorimetric assay, the highest specificity was observed for DrMutS, which in the case of TG mismatch exceeded that of TthMutS and EcMutS by 3–4-fold. The results determined with the qPCR based method were in agreement with those measured in the colorimetric assay but the differences in the specificity between DrMutS and the two other orthologues were even more conspicuous (Fig. 5b). The specificity of DrMutS towards mismatched DNA achieved in the qPCR-based experiments exceeded that of TthMutS and EcMutS by 4.4 and 20.3 times, respectively (Fig. 5b, 10^{-13} mol of TG49). These higher values of MutS specificity determined with qPCR could be explained by a 1000-fold lower concentration of the analysed DNA and the presence of the excess of DNA competitor during the DNA binding step. Lower DNA amounts could be accurately quantitated with qPCR thanks to the high sensitivity of the method, greatly surpassing that of colorimetric measurements. Another critical difference is that the use of PCR allows

convenient discrimination of mismatched and fully complementary DNA fragments collected from the same microplate well using two pairs of specific primers, which would not be feasible in the colorimetric assay. Additionally, DNA mismatch binding by MutS occurs in the excess of fully complementary DNA, thus resembling the *in vivo* conditions.

It is worth noting that the superior specificity of DrMutS (Figs. 2B and 5b) resulted from the enhanced affinity towards the mismatched DNA, while binding levels to that fully complementary were similar for the three examined MutS proteins (Figs. 2A and 5a).

The immobilized DrMutS showed significantly better efficiency in enrichment of mismatched DNA. In the enrichment experiment, the relative content of mismatched DNA was raised 57-fold with the use of DrMutS, and 23.6 and 17.3 times using TthMutS and EcMutS, respectively.

4.1. Critical remarks

It should be underlined that comparative characterization of MutS orthologues from three different bacteria depends on and could be biased by the experimental setup. It is worth considering that different MutS proteins may prefer different mismatch types, nucleotide sequence contexts, buffer and temperature conditions. In addition, MutS stability, purity, and the amount of immobilized MutS may impact the results. Nonetheless, it should be pointed out that DrMutS unfailingly exhibited a decidedly higher specificity towards the DNA mismatch than TthMutS and EcMutS in multiple experiments performed with three different methods. Therefore, it seems unlikely that the remarkable specificity of DrMutS we observed could be primarily the effect of the mismatch types or the buffer we chose for the experiments. The observed differences between the MutS orthologues are also unlikely to have resulted from potential errors in the determination of protein quantities, as neither two-fold increase nor decrease of MutS loaded had a significant impact on the specificity of DNA mismatch binding.

Another possibility that should be taken into consideration is unequal binding of three MutS species to microplate wells. If DrMutS superiority of DNA mismatch binding resulted from more efficient immobilisation alone, it would not have been confirmed with a gel-shift assay (Fig. 7), a direct method which does not involve immobilisation.

The impact of temperature could be essential in the case of *T. thermophilus* MutS, as the specificity towards mismatched DNA has been estimated as twice higher at 60 °C than 35 °C (Takamatsu et al., 1996). The comparison of MutS proteins from mesophilic *D. radiodurans* and thermophilic *T. thermophilus* with optimal temperature of growth at 65–75 °C was based on the experiments performed at 37 °C. It could be argued that TThMutS activity and specificity towards DNA mismatches might be enhanced at higher temperatures. Indeed, as showed by Stanislawska-Sachadyn et al., *T. thermophilus* MutS displayed the specificity towards mismatched DNA in gel retardation assays at room temperature after binding at 60 °C, at a significant molar excess of MutS (Stanislawska-Sachadyn et al., 2005). Takamatsu et al. demonstrated using a gel shift assay that *T. thermophilus* MutS exhibited higher specificity of DNA mismatch binding at 60 °C than at 35 °C but affinity to DNA was strongly decreased (Takamatsu et al., 1996). The stability of *T. thermophilus* MutS complexes with mismatched DNA decreased with temperature and efficient DNA binding required a huge molar excess of MutS to DNA at 60 °C. This observation corroborates with our results that showed a great reduction of DNA binding by immobilized TthMutS at 60 °C as contrasted to 37 °C. The use of elevated temperatures may not be practical in the experiments with MutS-mediated mismatched DNA enrichment because of limitations in the amounts of the immobilized protein.

It is also worth noting that all experiments presented in this study were performed using two sets of MutS samples obtained in independent bacterial cultures and purifications experiments. The analyses of the TG mismatch binding were performed for one set of MutS preparations, and those for the TC, GA and insertion-deletion loop were carried out using another one.

One more remark relates to the colorimetric assay, where DNA mismatch detection depends on extravidin binding to biotinylated DNA. It should be taken into account that this binding could be blocked by MutS in the case of mismatch situated in the vicinity of the biotin label.

5. Conclusions

On the basis of the observations presented herein, it can be stated that under applied experimental conditions *D. radiodurans* MutS protein exhibited remarkably higher affinity and specificity towards mismatched DNA in comparison with the *T. thermophilus* and *E. coli* proteins. The differences between the MutS orthologues determined *in vitro* may reflect their *in vivo* properties but the results of this study indicate predominantly the biotechnological potential of *D. radiodurans* MutS. Contrasted with the current technologies of DNA analysis, the use of MutS does not seem to be an attractive solution for direct mutation detection in PCR products. Our results show that MutS of *D. radiodurans* deserves further attention as a tool for mismatched DNA enrichment.

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