Protein-DNA interactions at the extreme

Final Report for the Biochemical Society's Summer Vacation Studentship (2007), awarded to Dr. Richard Bowater (Supervisor) and Hung M Bui (Undergraduate Student).

Background information

Genomic DNA is frequently damaged due to errors that occur during DNA metabolism and due to reactions with a variety of chemicals and radiation, which are likely to occur more frequently under extreme environmental conditions [1]. Unless repaired, such damage can kill an organism, either directly or indirectly due to induction of mutations.

Ferroplasma acidarmanus is an Archaeon that grows at around 40 °C in the most acidic environment on earth (pH <1) [2]. Recently, we identified that the genome of *F. acidarmanus* encodes 2 unusual but recognisable homologs to *mutS*, which we refer to as *famutS1* and *famutS2*. We have now expressed and purified recombinant forms of these proteins from *Escherichia coli* (unpublished data). Two different classes of MutS proteins have been well-characterised, which function to repair DNA replication errors (such as DNA mismatches) or influence recombination. Thus, these proteins have different enzymatic activities, although they always have a pronounced influence on genome stability. Bioinformatics analysis of FaMutS1 and FaMutS2 shows that they harbour characteristics that are common to both classes of MutS proteins, but they also lack key components of each class.

Aims and objectives

The primary objective of this proposal is to analyse the enzymatic activities of FaMutS proteins (i.e. FaMutS1 and FaMutS2). To biochemically characterise the properties of the proteins, mutations will be introduced that are predicted to alter their enzymatic activities. The main objectives are:

- Mutate the gene sequences of *F. acidarmanus* mutS1 and mutS2 (*faMutS1* and *faMutS2*) in ways that are predicted to alter the ATPase activities of their expressed proteins
- Express and purify from *E. coli* wild-type and mutated forms of FaMutS1 and FaMutS2
- Assess the ability of the proteins to hydrolyse ATP

Material and methods

➢ *Site-directed Mutagenesis and gene cloning:* Genes for *faMutS1* and *faMutS2* were cloned previously into expression vectors that incorporate a His-tag at the termini of the proteins (i.e. His_6 tag). The QuickChange™ Site-directed Mutagenesis Kit (Stratagene) was used to make mutations in the *faMutS* genes, details of which are shown in the Appendix. The mutated plasmids were then treated with an endonuclease, *Dpn*I, which only inactivated native plasmids with methylated restriction sites. Then, these expression vectors (pET-28a containing mutated *faMutS* genes) were transformed into *E. coli* DH5α. These DNA plasmids were purified using Wizard Plus SV Minipreps kit. Mutagenesis was confirmed by restriction enzyme analysis of the DNA plasmids.

➢ *Protein expression:* Plasmids containing the wild type and mutated genes were transformed into *E. coli* BL21 (Rosetta), which were grown and induced to express the FaMutS proteins. Expression of FaMutS proteins in soluble and insoluble fractions (from cultures grown at 37 ^{o}C) was examined by Coomassie Blue staining and Western blotting.

➢ *Protein purification:* 150ml cultures of *E .coli* BL21 Rosetta containing the pET-domain constructs were grown at 25 ^{o}C, harvested by centrifugation and lysed by sonication. Soluble FaMutS proteins were purified using His-Bind and PD10 columns. The purified proteins were then stored in buffer containing 20% glycerol at -80 ^{o}C.

➢ *ATPase assays:* The Enliten® rLuciferase/Luciferin Reagent (rL/L) was used to analyse the ATPase activity of the wild type and mutated proteins. This kit determined the ATP concentration of samples in the range of 10^{-12} to 10^{-17} moles. Comparison of the amount of ATP hydrolysed by the wild type and mutated proteins identified whether the mutagenesis had any effect on the functions of the proteins.

Results

Mutagenesis of faMutS genes

PCR amplification of *faMutS* genes was successful, resulting in 6 different mutated plasmids that were then transformed into *E. coli* (Figure 1). The success of the mutagenic PCR reactions was confirmed by the activity of restriction enzymes (data not shown).

Analysis of soluble and insoluble mutated FaMutS proteins at 37 ^oC

Bacterial cultures (10mL) were grown at 37 ^oC to express FaMutS proteins in both soluble and insoluble fractions. Samples were analysed by SDS-PAGE and analysed with Coomassie Blue staining (data not shown) and western blot (Figure 2) to identify the states in which the mutated proteins were made. Some soluble protein was observed for all samples, except for sample 1, for which no protein (soluble or insoluble) was observed; further experiments are required to determine why expression was unsuccessful for sample 1.

Purification of recombinant FaMutS proteins

Recombinant FaMutS was purified from a 150 ml culture of *E. coli* BL21 (Rosetta), using a column with affinity to the His-tag. The proteins eluted from the column were analysed by SDS-PAGE (Figure 3), which confirmed good purification of all recombinant FaMutS proteins (except sample 1). Purified proteins kept at -80 ^oC in 20% glycerol for further use. Due to the bands' intensity, only proteins expressed from samples pRB 307, pRB 312, sample 5 and sample 6 gave a good amount of protein to provide reliable analyse of the ATPase activity of the proteins.

Analysis of ATPase activity of recombinant FaMutS

The purified proteins (except for sample 1) were incubated for 35 minutes in the ATPase activity reaction mixture before being analysed by a luminescence spectrophotometer. Good ATPase activity was found when two wild-type proteins were together. In the case that 2 proteins were simultaneously expressed, mutation in FaMutS1 reduced the activity significantly, whereas mutations in FaMutS2 had much less effect.

Conclusion

- The mutations of FaMutS genes (*faMutS1* and *faMutS2*) were made and transformed into bacterial expression vector.
- FaMutS proteins were over-expressed in *E. coli* BL21 (Rosetta) and purified successfully using a column with affinity to the His-tag, as well as analysing soluble and insoluble proteins.
- Analysis of the ATPase activity confirmed that the ATP is mainly hydrolysed by FaMutS1 only when FaMutS2 is also present.

Perspectives

Although several mutations of FaMutS proteins have been prepared, further studies are required to confirm their activities. Experiments should be performed as follows:

- Optimisation of expression and purification of the protein to increase their yield
- Analysis of DNA binding by mutated proteins and comparison with that of the wild-type proteins
- Various mutagenesis reactions that would assess the functional structure of the FaMutS proteins.

Student's statement

I highly appreciate the opportunity given to me to work on the summer studentship, which was not only interesting but also priceless to me. I have gained valuable laboratory experience, which will definitely be useful to my 3rd year project. Moreover, all the knowledge, as well as experience, that I have earned also inspired me with a great enthusiasm to direct my career intention towards research involving Biochemistry and Molecular Biology. As a result, I would wish to achieve a PhD studentship in this field. In the end, I am very thankful to the Biochemical Society for this most enjoyable time.

Acknowledgements

We thank Brian Jackson and Andrew Cobb for their theoretical and practical assistance.

References

1. Friedberg, Walker, and Siede, "*DNA repair and mutagenesis*". 1995, Washington, DC: American Society for Microbiology.
2. Edwards, et al., "*An archaeal iron-oxidizing extreme acidophile important in acid mine drainage*". Science, 2000. **287**: 1796-1799.

The above report is an accurate representation of work performed during the Biochemical Society Summer Vacation Studentship (2007), awarded to Hung M Bui.

Signed: Hung Bui   Richard Bowater   Date: 1st Oct. 2007

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Appendix

Table 1: Primers used for mutagenesis reactions

Mutagenesis sample (+ original plasmid)	Genes Expressed & Nature of Mutation	PCR primers (forward sequence shown only)
Sample 1 (pRB307)	FaMutS2 Truncate	*5′-GGT-GTG-AAA-TTG-GGT-TCT-AGA-TAT-CAT-CAA-TTA-ACC-TTC-TGG-3′*
Sample 2 (pRB312)	FaMutS1 Walker A mutation-FaMutS2	*5′-CGG-GCC-AAA-TAA-TGG-AGC-TCA-AAC-AAC-ATT-TGC-CAG-GTC-ATT-CG-3′*
Sample 3 (pRB312)	FaMutS1-FaMutS2 ATG mutation	*5′-CAT-ATG-AAA-GGG-AGG-ATA-TCT-TGA-TAA-AAA-CAT-ATT-TGC-TTG-ACA-G-3′*
Sample 4 (pRB306)	FaMutS1 Walker A mutation	Same as sample 2
Sample 5 (pRB322)	FaMutS1 Walker A mutation -FaMutS2 Walker A mutation	Same as sample 2
Sample 6 (pRB322)	FaMutS1-FaMutS2 Walker A mutation/Walker B mutation	*5′-GAA-ATA-GTT-TGA-TAC-TGT-TTA-ACG-CGT-CAT-TTT-CAT-CGA-CCA-ACA-CAC-G-3′*

Table 2: Plasmids provided at the start of this project

Plasmid	Vector and Gene(s) Expressed
pRB306	pET28a + FaMutS1
pRB307	pET28a + FaMutS2
pRB312	pET28a + FaMutS1- FaMutS2
pRB322	pET28a + FaMutS1- FaMutS2 Walker A mutation

Figures:

Figure 1. Mutagenesis of FaMutS genes. The mutated plasmids were prepared using the QuickChange™ Site-directed Mutagenesis Kit and purified by Wizard Plus SV Minipreps. Analysis on a 1% agarose gel confirmed the success of the PCR reactions. The marker used was the 1kb DNA standard ladder while the numbers (1-6) showed their corresponding mutated plasmids

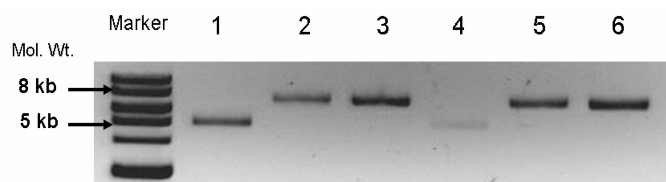
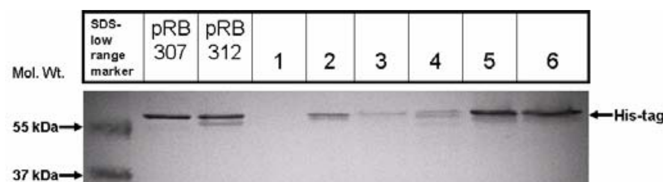
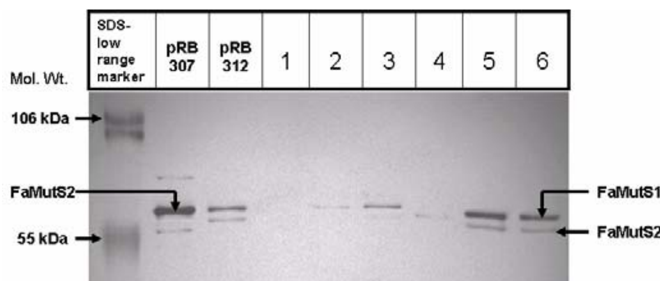


Figure 2. Analysis of soluble mutated FaMutS proteins at 37 °C. *E. coli* BL21 (Rosetta) containing mutated plasmids was grown overnight in 10 mL LB + kanamycin + chlorophenicol cultures at 37 °C. The cells were harvested and the soluble proteins were collected and analysed on a 10% SDS-PAGE. Recombinant proteins were detected by western blotting with an antibody to the His-tag.





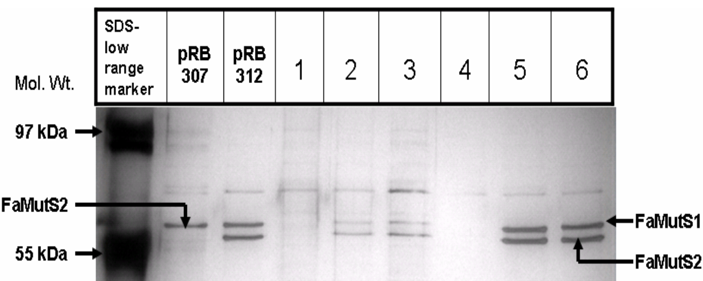


Figure 3. Purification of recombinant FaMutS proteins. The proteins were induced to be expressed in 150mL cultures of *E. coli* Rosetta at 25 °C (0.4 mM IPTG was added when OD_{600} ~ 0.5). Recombinant proteins were purified using His-bind columns (Novagen), followed by PD10 columns. Aliquots of samples washed from the column were analysed on a 10% SDS-PAGE and detected by western blotting (left) and Silver staining (right).

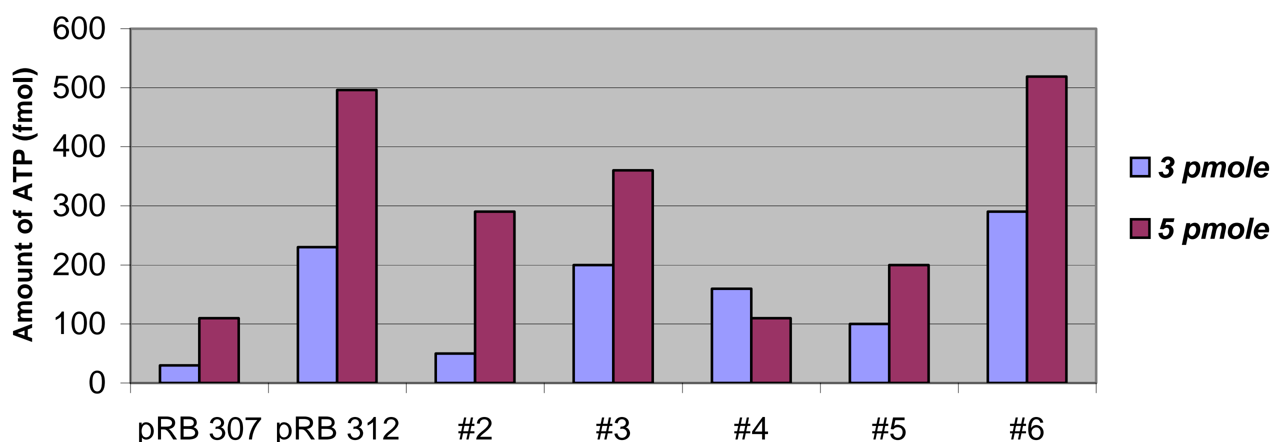


Figure 4. Analysis of ATPase activity of recombinant FaMutS proteins. A luminescence spectrophotometer was used to detect the amount of ATP hydrolysed by each protein (except for sample 1). The amount of ATP remaining was determined after reactions with each protein at two different amounts, i.e. 3 pmol and 5 pmol. Note that sample 5 (mutation in FaMutS1) has a significantly reduced ATPase activity compared to wild-type (pRB312) and sample 6 (mutations in FaMutS2).