Structural and Biochemical studies of SofG, a GTPase involved in bacterial cell motility

A thesis submitted towards partial fulfillment of the requirements of BS-MS Dual Degree Program

By

Sonal Gulab Lagad

20121072

Under the guidance of

Dr. Gayathri Pananghat

Department of Biology, Indian Institute of Science Education and Research (IISER) Pune

Thesis Advisor

Dr. Sunando Datta, IISER Bhopal

Certificate

This is to certify that this dissertation entitled "Structural and Biochemical studies of SofG, a GTPase involved in bacterial cell motility" towards the partial fulfillment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents study/work carried out by " Sonal Gulab Lagad at the Indian Institute of Science Education and Research (IISER) Pune" under the supervision of "Dr. Gayathri Pananghat, Assistant Professor, Biology Department, IISER Pune" during the academic year 2016-2017.

Sonal Gulab Lagad

20121072

BS-MS Student

IISER Pune

Dr. Gayathri Pananghat

Assistant Professor

Biology Department

IISER Pune

Date: 20th March 2017

Declaration

I hereby declare that the matter embodied in the report entitled "Structural and Biochemical studies of SofG, a GTPase involved in bacterial cell motility" are the results of the work carried out by me at the Department of Biology, The Indian Institute of Science Education and Research (IISER) Pune, under the supervision of Dr. Gayathri Pananghat and the same has not been submitted elsewhere for any other degree.

Sonal Gulab Lagad

20121072

BS-MS Student

IISER Pune

Dr. Gayathri Pananghat

Assistant Professor

Biology Department

IISER Pune

Date: 20th March 2017

Abstract

Cell motility is one of the important functions performed by the bacteria in order to survive. *Myxococcus xanthus*, a rod shaped bacteria exhibits two different types of motility namely Adventurous Gliding motility characterized by the use of complexes similar to Focal adhesion Complexes and Social motility using the type IV Pili machinery. Recent studies have shown that small proteins namely GTPases help in establishing cell polarity in *Myxococcus xanthus* which help the bacteria in deciding the direction. It was found that SofG is one such GTPase which with the help of BacP sorts the motor ATPases PilB and PilT to the opposite poles thus maintaining the cell polarity. To investigate the molecular mechanisms of the action of SofG in *Myxococcus xanthus*, in vitro studies were carried out using cloning and protein over-expression in *E.coli.* The protein was purified and used for biochemical assays like Malachite Green Assay to get insights into the activity of the protein. With the help of such structural and biochemical studies on SofG and other contributing proteins, it would be possible to determine the molecular mechanism of cell polarity in *Myxococcus xanthus.*

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List of Abbreviations

GAP- GTPase Activating Protein

GDP- Guanosine Di Phosphate

GTP- Guanosine Tri Phosphate

LB- Luria Bertini

Mant-GDP - (2'-(or-3')-O-(N-Methylanthraniloyl) Guanosine 5'-Diphosphate

MglA- Mutual gliding protein A

MglB- Mutual gliding protein B

MWCO- Molecular Weight Cut Off

Ni-NTA- Nickel- Nitrilotriacetic acid

OD_{600}- optical Density at 600nm wavelength

PAGE- Poly Acrylamide Gel Electrophoresis

PCR- Polymerase Chain Reaction

RPM- Rotations per Minute

SofG- S motility function GTPase

SDS- Sodium Dodecyl Sulphate

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Chapter 1: Introduction

Cell motility is an important aspect for bacterial survival and function. It helps the bacteria to change the direction, move towards the food source, spread during infection, etc (Josenhans and Suerbaum, 2002). Hence studying bacterial motility in detail would help in getting more insights into the underlying mechanisms governing its function in pathogenic and other bacteria.

1.1 Model system- *Myxococcus xanthus* and its motility

The model organism used here for studying the regulation of cell polarity and motility in bacteria was *Myxococcus xanthus.* It is a gram negative rod shaped bacterium which mostly lives in soil habitats and feeds on the dissolved nutrients (O 'connor and Zusman, 1991). In nutrient rich conditions or the vegetative phase, these bacteria move by forming swarms (Fig. 1.1). The mechanism used for movement is gliding motility since they do not possess flagella (Burchard, 1981). But on nutrient depletion, they undergo a complex developmental life cycle wherein the cells aggregate and form multicellular fruiting bodies containing the myxospores. *Myxococcus xanthus* exhibits two different independent motility systems (Hodgkin and Kaiser, 1979) – A-motility or the Adventurous gliding motility where the cells move individually on a solid surface due to absence of any cellular appendages and S motility or the Social motility where co-ordinate cell movement is required (Ward and Zusman, 1997). A-motility is characterized by the presence of protein complexes similar to focal adhesion complexes (Mauriello and Zusman, 2007), whereas S motility is dependent on the Type-IV Pili machinery which includes the different pilin proteins and is characterized by the extension and retraction of the pilus (Li et al., 2003). Type IV Pili are cell surface structures used for locomotion by the bacteria (Pelicic, 2008). In *Myxococcus xanthus*, the motor ATPases PilB and PilT that regulate the extension and retraction of the pilus respectively, localize to opposite poles of the cell and change their localization during reversals (Bulyha et al., 2009).

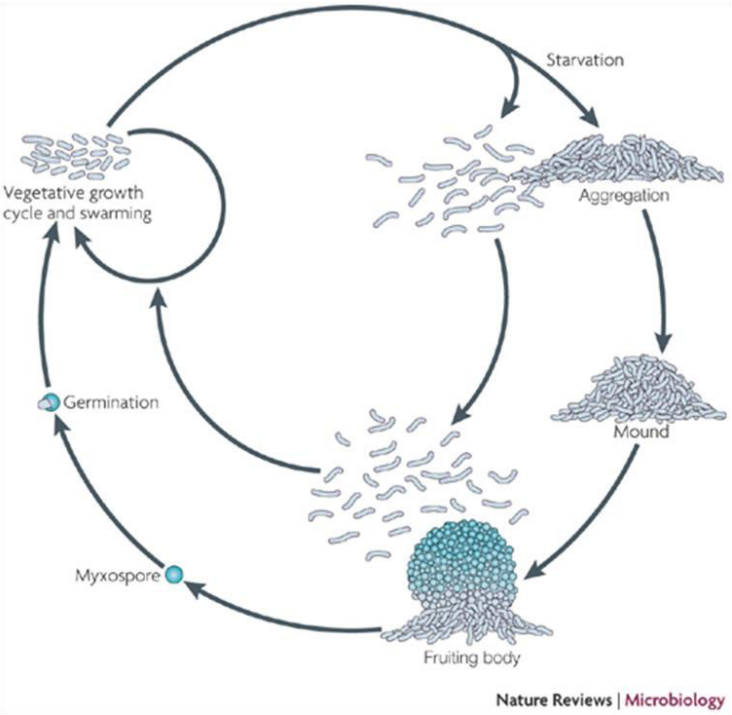


Fig. 1.1: Life cycle of *Myxococcus xanthus*. Adapted from (Zusman et al., 2007)

1.2 Cell Polarity and reversals in *Myxococcus xanthus*

As mentioned earlier, the localization of PilB and PilT to the opposite poles of the cell determines the cell polarity (Fig. 1.2). PilB localizes to the leading pole of the cell where the pilus is formed. During reversals, PilB starts to build up at the lagging poles and after the reversal, PilB relocates to the new leading pole. PilT predominantly localizes to the lagging pole and is relocalized by the Frz system during reversals. Both PilB and PilT require ATPase activity and are called the motor ATPases (Bulyha et al., 2009).

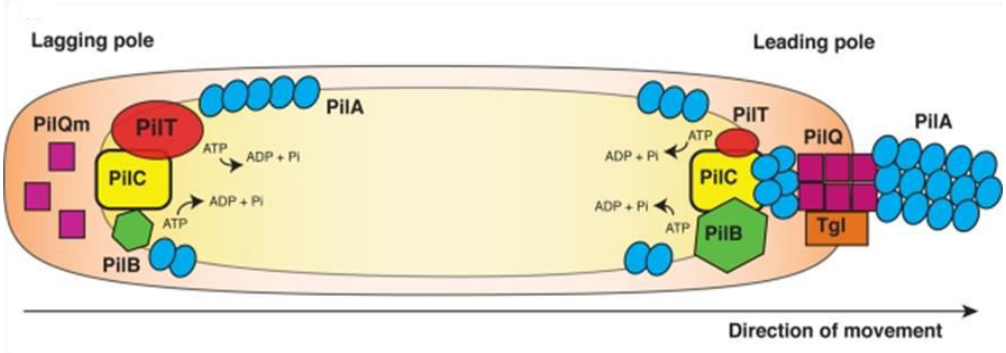


Fig. 1.2: Components of the S- motility system. Adapted from (Mauriello et al., 2010)

The cells of *Myxococcus xanthus* periodically reverse their direction of motion which is required for directed motility. The reversals are controlled by the Frz signal transduction system (Sun et al., 2000) as shown in Fig. 1.3.

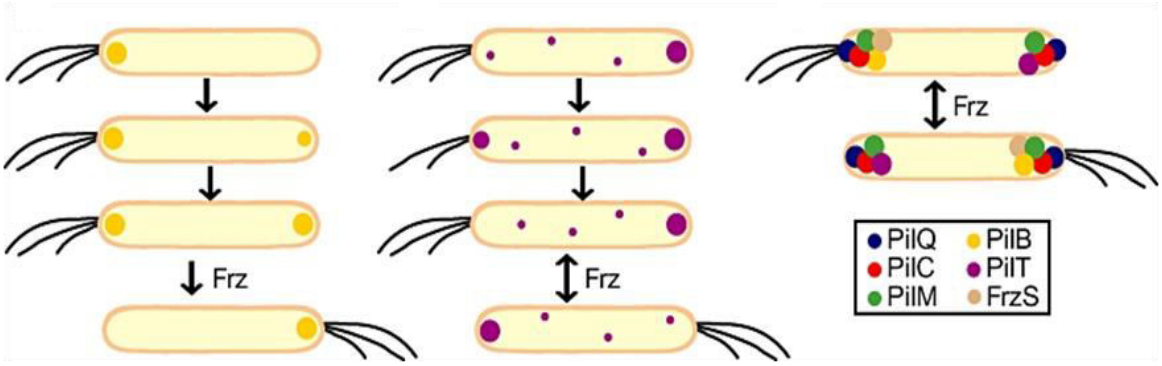


Fig 1.3: Model of PilB and PilT localization and the pole switching of T4P. Adapted from (Bulyha et al., 2009).

1.3 The role of SofG in motility of *Myxococcus xanthus*

A recent study determined the role of a Ras like GTPase which was found similar to MglA. This novel GTPase was named SofG (S motility function GTPase) since it helped in regulating Type 4 dependent motility. Based on its function to sort the two

motor ATPases PilB and PilT to the opposite poles of the cell, a model was proposed to show how the two GTPases SofG and MglA and the bactofilin cytoskeleton protein, BacP interact and help in polar localization of PilB and PilT. Initially BacP filaments localize to both the sub polar regions of the cell. Later one of the patches of BacP directly recruits SofG. SofG performs its function by shuttling back and forth on BacP in a GTPase-dependent manner and thus localizes PilB and PilT to the same pole. Later MglA sorts PilB and PilT to opposite poles. Thus SofG and MglA work in a cascade like manner to establish the cell polarity in *Myxococcus xanthus* (Fig. 1.4). During cellular reversals introduced by the Frz chemosensory system, MglA and MglB switch their localization due to which the polarity of PilB and PilT also changes enabling the cell to change its direction of motility (Bulyha et al., 2013).

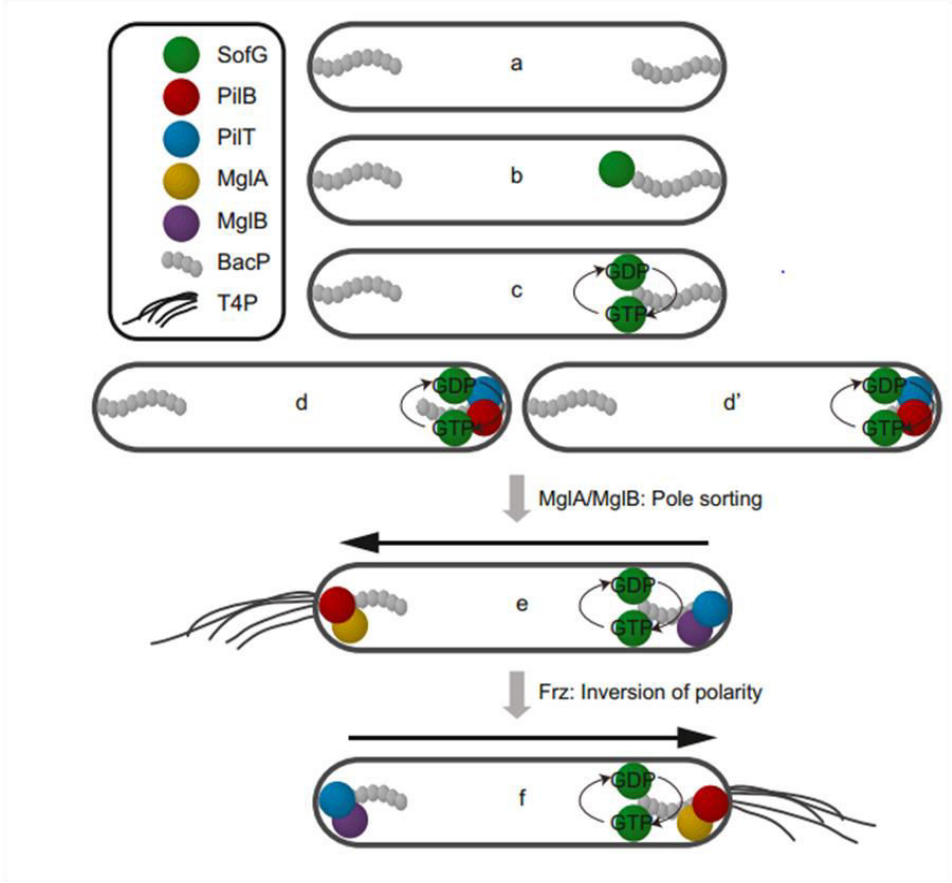


Fig 1.4: Proposed model for step-wise regulation of PilB and PilT polarity. Adapted from (Bulyha et al., 2013).

1.4 Small Ras like GTPases and their structural characteristics

GTPases are enzymes which bind to and hydrolyze GTP. Moreover three decades of research have shown the importance of small Ras like GTPases. They are involved in a variety of biological functions like cell growth and differentiation, survival, vesicular and nuclear transport, signaling to the cytoskeleton, etc (Cherfils and Zeghouf, 2013).

The small Ras like GTPases (20-35 KDa) work as molecular switches by cycling between the ON and OFF state. They alternate between the GTP bound active state which affects downstream signaling and GDP bound inactive state (Fig. 1.5) which can also be distinguished by the conformational changes in Switch I and Switch 2 regions of the GTPase (Vetter and Wittinghofer, 2001).

Generally small GTPases hydrolyze very slowly. Also they have higher affinity to GDP. Hence in order to enhance the GTP hydrolysis, they require the help of some regulators. These regulators are Guanine Nucleotide Exchange Factors (GEF) which help in dissociating the GDP and exchange it with GTP to get the active form. The other being GTPase Activating Protein (GAP) which binds to the active form and help in the hydrolysis of GTP. Together they form the molecular switch and undergo the GTPase cycle (Bos et al., 2007).

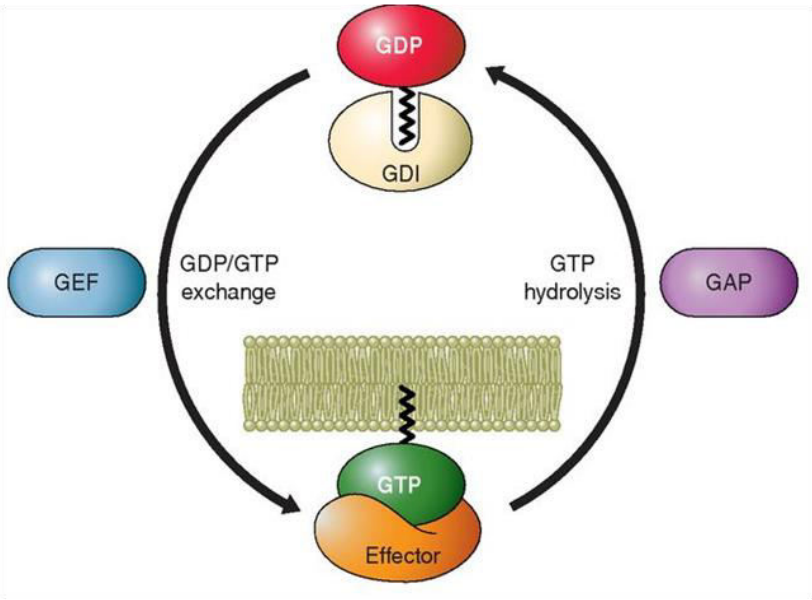


Fig 1.5: GTPase Cycle of small Ras like GTPases. Adapted from (Cherfils and Zeghouf, 2013).

The structural features of the small Ras like GTPases include different conserved motifs (Fig. 1.6). Small GTPases are made of a conserved domain called the G domain. The G domain is made of six β strands surrounded by 5 α helices. The G domain consists of different motifs required for the nucleotide binding and hydrolysis. The (N/TKxD) motif (where x can be any amino acid) is conserved and recognizes the Guanine base in the nucleotide. The P loop i.e. the Phosphate Binding loop has the signature motif of (Gx_4GKS/T) which recognizes the β, γ phosphate of the nucleotide and the magnesium ion. The (DxxG) motif helps in providing specificity for the Guanine base through its Asp side chain from Alanine (in Fig, Ala146) in the (SAK) motif (Vetter and Wittinghofer, 2001). The other motifs which also sense the nature of the bound nucleotide and help in GTP hydrolysis are the Switch 1 and Switch 2 regions which are not conserved.. They are variable since they contribute differently to binding of the nucleotide in different GTPases. When the GTPase is in active state i.e. the GTP bound state, Switch 1 binds to magnesium and the γ phosphate of GTP through its conserved threonine. Whereas Switch 2 binds to the γ phosphate of GTP through Glycine from its conserved

(DTAGQ/T/H) motif (Biou and Cherfils, 2004). Thus they undergo conformational changes upon nucleotide binding and convey the nature of nucleotide to the GTPases.

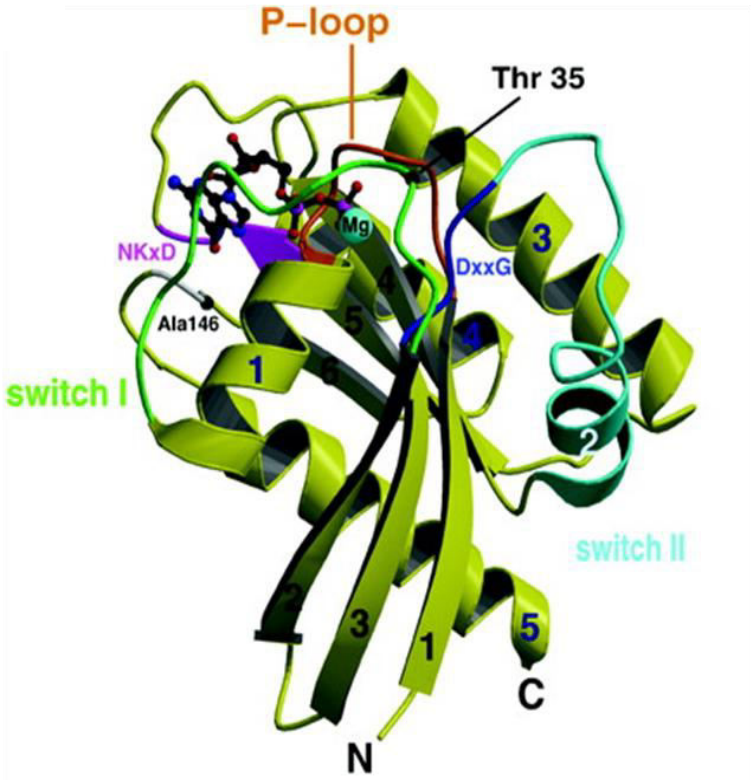


Fig 1.6: Structure of Guanine Nucleotide Binding Protein (GNBP). Adapted from (Vetter and Wittinghofer, 2001).

1.5 Conformational changes in active site residues of small Ras like GTPases upon GTP hydrolysis

In small Ras like GTPases similar to MglA, conformational changes upon GTP binding are mostly observed in the Switch 1 and Switch 2 regions (Fig. 1.7). The most significant change observed upon GTP binding is the movement of β strand from Switch 1 towards the nucleotide. This movement affects the positioning of the conserved active site residues like Arginine and Glutamine. Arginine in Switch 1 is shifted to a position where it can contact the γ phosphate of GTP. Not much conformational changes occur in Switch 2 but the conserved catalytic Glutamine is

positioned towards the γ phosphate and the nucleophilic water molecule (Miertzschke et al., 2011). Thus Arg and Glu are important for GTPase activity and mutations in these residues(R<->A, Q<->L) affect the GTPase activity and keep the GTPase mostly in the active state (Miertzschke et al., 2011).

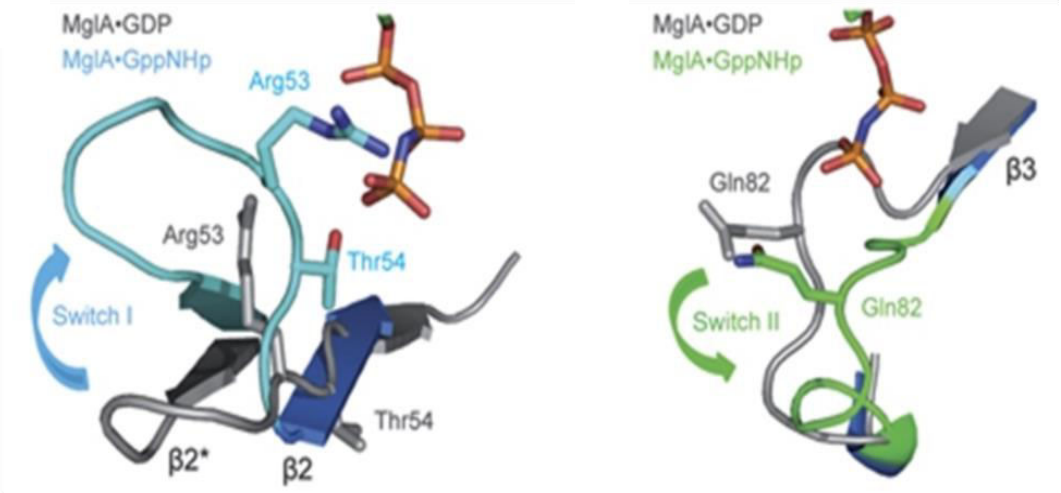


Fig 1.7: Structural changes in Switch 1 and Switch 2 regions upon GTP binding. Adapted from (Miertzschke et al., 2011).

1.6 Similarity between SofG and MglA

Multiple Sequence Alignment between SofG and MglA showed that SofG is similar to MglA but still is distinct from MglA. Figure 1.8 below shows the alignment between full length MglA (1-195 amino acids) and SofG (59-298 amino acids) excluding the N-terminal part of SofG since it wasn't present in other homologs of SofG. It also shows the conserved motifs present in small Ras-like GTPases along with the conserved active site residues, arginine in Switch I region and glutamine in Switch II region (Bulyha et al., 2013).

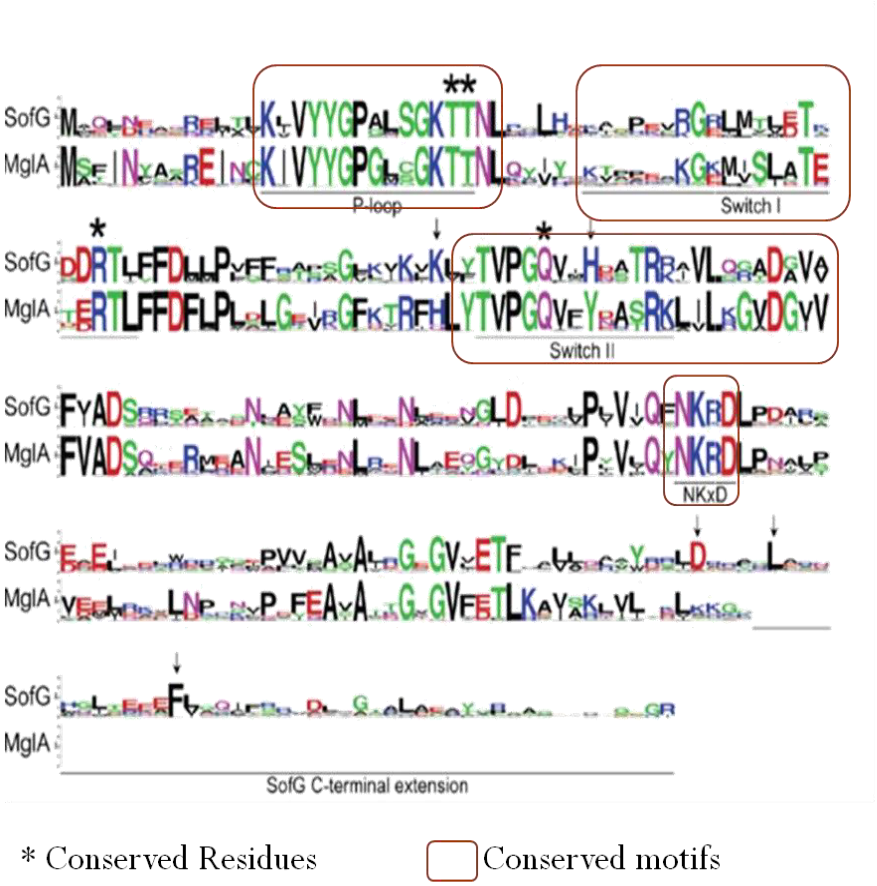


Fig 1.8: Sequence Alignment between SofG and MglA. Adapted from (Bulyha et al., 2013).

1.7 Objectives

Currently the molecular basis of how the two GTPases SofG and MglA act in concert with each other and contribute to the cell polarity in *Myxococcus xanthus* is not known in detail. Hence a structural and biochemical approach towards the study of these proteins would help to provide an insight of how these proteins interact with each other and also with other motility proteins to help establish the cell polarity and help in bacterial motility.

The principal objectives were-

i) Further optimization of SG-60purification to get higher protein yield.

ii) Standardization of expression and purification of other constructs of SofG.

iii) Cloning, optimization of expression and purification of active site mutants of SG-60C $(His)_6$.

iv) Biochemical assays to compare the activity of wild type SG-60C $(His)_6$ and the active site mutants.

Chapter 2: Materials and Methods

The following chapter describes the different techniques used for the study of SofG.

2.1. Design of SofG constructs.

The SofG constructs already available in the lab are listed in Table 2.1 In addition; new SofG constructs were made which included the active site mutants and N-terminal $(His)_6$ tagged constructs (listed in Table 2.2).

Table 2.1 Formerly cloned constructs of SofG.

Construct Name	Construct description
SG-U	Full Length Untagged
SG-H6	C-terminal $(His)_6$Full length
SG-18	18-298 $(His)_6$
SG-60	60-298 $(His)_6$
SG-60-277	60-277 $(His)_6$
SG-277	1-277 $(His)_6$

Table 2.2 Newly cloned constructs of SofG.

Construct Name	Construct description
SG60-R111A	Active site mutation in SG-60
SG60-Q140L	Active site mutation in SG-60
SG60-N$(His)_6$	SG60- N terminal $(Histidine)_6$ tagged
SG18-N$(His)_6$	SG60- N terminal $(Histidine)_6$ tagged

2.2 Cloning

Cloning is a technique used to insert our gene of interest into a suitable vector. Different constructs of SofG were cloned in pHis17 vector using the specific primers mentioned in Table 2.3.

Table 2.3 Primers used for cloning different SofG constructs.

Primer Name	Primer Sequence (5'-3')	Length of primer (bp)
SG60-R111A Forward	CCCACGACGACGCCACGCTCTTCTTCGACC	30
SG-C$(His)_6$ Reverse	GCTTTTAATGATGATGATGATGATGGGATCC TCGCCCTTCTCCGCTG	47
SG60 Forward	GTTTAACTTTAAGAAGGAGATATACATATGT TGCAACTCAACCATGCCC	49
SG60-Q140L Reverse	GGCGTTGTGGATGACCAGGCCCGGCACGG	29
SG60- N$(His)_6$ Forward	CTTTAAGAAGGAGATATACATATGCGTCACC ACCACCACCACCACTTGCAACTCAACCATG CCC	64
SG18- N$(His)_6$ Forward	CTTTAAGAAGGAGATATACATATGCGTCACC ACCACCACCACCACAGCATCGCCTCCACGC CTG	64
SG Reverse	GCTTTTAATGATGATGATGATGATGGGATCC TCATCGCCCTTCTCCGCTG	50

2.2.1 PCR (Polymerase Chain Reaction)

For cloning, initially the gene of interest was amplified using PCR. The following reaction mix (Table 2.4) was used to amplify the gene of interest.

Table 2.4 Reaction Mix used for PCR

Components	Volume	Final Concentration
DNA Template	1μl	100ng/ul
Forward primer	1μl	400nM
Reverse primer	1μl	400nM
Reaction buffer (5X)	10 μl	1X
dNTPs	5 μl	0.2mM
Prime Star GXL DNA Polymerase	0.5 μl	
MilliQ	31.5 μl	
Total	50 μl	

Table 2.5 PCR Cycle Conditions

Temperature	Time	
95°C	5 minutes	
95°C	30 seconds	
55°C	30 seconds	30 cycles
70°C	20 seconds	
70°C	5 minutes	
4°C	∞	

Following PCR, the PCR product was then gel extracted or PCR purified using Qiagen Gel Extraction Kit or PCR Purification Kit in order to be used for further reactions. In case if the yield of the product after gel extraction or PCR purification was not sufficient, it was further amplified following the same procedure. The amplified gene was then inserted into the vector using Restriction Free Cloning.

2.2.2 RF (Restriction Free) Cloning

RF Cloning is a technique used to insert the gene of interest into a suitable vector without any restriction sites or ligation. In this technique, the amplified gene of interest acts as a pair of primers for the RF PCR (Fig. 2.1). The primer when annealed to the vector extends linearly around the vector thus producing the plasmid with the gene of interest (van den Ent and Löwe, 2006). The following reaction mix was used for RF PCR.

Table 2.6 Reaction Mix used for RF PCR

Components	Volume	
	Test	Control
DNA Template	~ 500 ng	~ 500 ng
Primer	~ 1000 ng	-
Reaction buffer (5X)	10μl	10 μl
dNTPs	5 μl	5 μl
Prime Star GXL DNA Polymerase	0.5 μl	0.5 μl
MilliQ		
Total	50 μl	50 μl

Table 2.7 RF PCR Cycle Conditions

Temperature	Time	
95°C	5 minutes	
95°C	30 seconds	30 cycles
55°C	30 seconds	
70°C	According to 1kbp/min	
70°C	5 minutes	
4°C	∞	

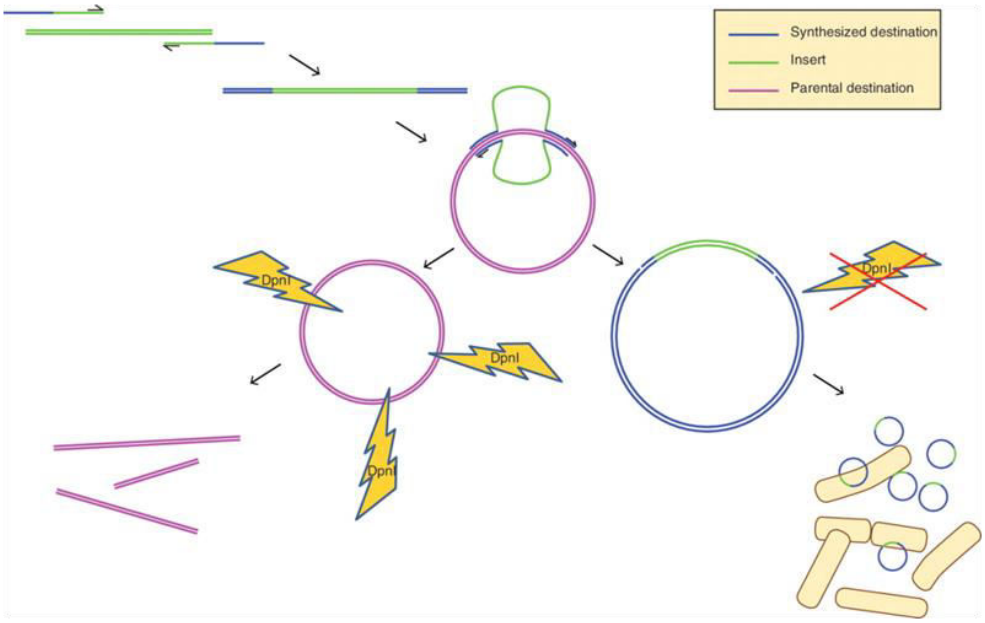


Fig 2.1: Schematic of RF Cloning protocol. Adapted from (Bond and Naus, 2017)

After RF PCR, the parental plasmid was then digested using DpnI which cleaves the methylated DNA. Following DpnI digestion, the plasmid were then transformed into NEB Turbo Electro Competent *E.coli* cells using electroporation and plated on LB Agar plates containing ampicillin (100μg/ml). Few of the single colonies obtained were then screened for positive clones, following plasmid extraction.

To check whether the clones thus obtained contained the gene of interest, the plasmids were double digested with NdeI and BamHI enzymes since the genes were introduced between these restriction enzymes and the reaction was incubated at 37° C for about 3 hours and then checked on 0.8% or 1% agarose gel. The positive clones were further confirmed using sequencing.

2.3 Expression and Solubility check of different SofG constructs.

Once the positive clones were obtained, the plasmids were further chemically transformed into over-expression strains of *E.coli* like BL21AI and then plated on a LB

Agar plate containing 100µg/ml Ampicillin. A small patch of colonies was then inoculated in sterile 1X LB and incubated at 37° C till a specific O.D. was reached. The culture was then induced with 0.2% Arabinose and further incubated for induction at 15° C for 16-18 hours. The cells were then pelleted down at 15000 rpm at 4° C using a bench top centrifuge. The pellet was then resuspended in lysis buffer (50mM Tris pH 8, 200mM NaCl and 10% Glycerol). The cells were sonicated using a sonicator for 1 minute (Pulse-1sec ON, 3 sec OFF) at 60% amplitude. An aliquot of 15µl was taken out as the total lysate and the remaining sample was again centrifuged at 15000 rpm for 15 minutes at 4° C to obtain the supernatant. The total lysate indicated the expression of the protein whereas the supernatant indicated the soluble fraction of the protein. The samples were then treated with 2X SDS Loading Dye and heated at 99° C for 10 minutes and then loaded on a 12% resolving SDS PAGE Gel.

2.4 Protein Purification

Following the standardization of protein expression, the same protocol was followed and larger volumes of culture were grown (6 litres). In some optimization trials, the Constant Cell Disruptor was used for lysing the cells instead of Sonicator. After Sonication, the lysate was then spun at 18000 rpm for 55 minutes using High Speed Centrifuge (Beckman Coulter) at 4° C and the soluble fraction was then used for further purification process. All the purification steps were carried out using The AKTA Prime Chromatography Systems (GE Life Sciences).

2.4.1 Affinity Chromatography- Ni-NTA

Since SofG contains Hexa Histidine Tag at the C terminal, the first step of purification used was using Ni-NTA based affinity chromatography.

Ni-NTA is an affinity chromatography technique used for purifying recombinant proteins containing a polyhistidine tag. The Histidine residues in the tag bind to the nickel (Ni^{2+}) ions with high affinity and specificity. Cell lysates are loaded onto the matrix. Histidine tagged proteins are bound to the matrix whereas the other nonspecific proteins pass through the matrix and are removed by washing the column. Imidazole is used for eluting the protein since Imidazole has similar structure to that of Histidine and it

competes with Histidine to bind to the Ni^{2+} ions. Thus the protein is eluted using varying concentrations of Imidazole.

The standardized protocol used for the Ni-NTA purification is mentioned below. The 6L culture pellet was resuspended in (180ml-200ml) lysis buffer (50mM Tris pH 8, 200mM NaCl and 10% Glycerol) and sonicated using a sonicator for a cycle of 6 minutes (1 sec ON, 3 sec OFF) at 60% amplitude. Following sonication, the lysate was then centrifuged in High Speed Centrifuge using a JA25.50 rotor at 18000 rpm for 55 minutes at 4° C. Meanwhile, the 5ml Ni-NTA column was equilibrated with Buffer A (50mM Tris pH 8, 200mM NaCl). The supernatant was then loaded on this pre-equilibrated column. The column was then washed using 8 Column Volumes of 2%, 5%, 10% Buffer B (50mM Tris pH 8, 200mM NaCl, 500mM Imidazole) to remove the bound impurities. The protein was then eluted using 20%, 50%, 100% Buffer B which was collected as fractions of 5ml each. Alternate fractions were then treated with 2X SDS Loading dye and loaded on a 12% resolving SDS PAGE Gel after heating at 99° C for 10 minutes.

The fractions containing the protein were supplemented with 0.25 mM GDP and 0.5mM $MgCl_2$ to avoid precipitation. They were then pooled together and spun at high speed to remove the precipitate if any was present and later concentrated using top to bottom Pall10K MWCO centricon. When the amount of precipitation was high, bottom to top centricon was used. The protein was concentrated to a volume of about 1ml or less so as to be further used for Size Exclusion Chromatography.

2.4.2 Size Exclusion Chromatography (SEC)

Size Exclusion Chromatography also called as Gel Filtration Chromatography (SEC) is a technique used to separate the molecules based on their size by filtration through a gel. The gel consists of beads of specific size. Larger molecules cannot penetrate the pores and are excluded from the beads and eluted in the column's void volume. The smaller molecules can access most of the pores and hence are eluted later. Thus separation occurs based on the molecule's size as they pass through the column and is eluted in decreasing order of their molecular weight.

SEC was done to remove the Imidazole present after Ni-NTA purification and was carried out in AKTA Prime as well as AKTA Purifier systems (GE Life Sciences). The protocol used is as follows. The 24 ml Superdex 75 (10/300 GL) column was first equilibrated with Buffer A50 (50mM Tris pH 8, 50mM NaCl, and 0.1mM GDP). GDP was added to the buffer in order to prevent the protein from precipitating. The protein was then loaded onto the column and later collected as fractions of 0.5ml. The fractions were then treated with 2X SDS Loading dye and loaded on a 12% resolving SDS PAGE Gel after heating at 99° C for 10 minutes. The fractions containing the protein were then pooled together and concentrated using top to bottom 3K MWCO (2 ml) Centricon. Bottom to top centricon (centricon in which the membrane was oriented above the sample, and concentration took place by the buffer getting collected in a floating reservoir) was used when more precipitation was observed. After concentrating, small aliquots of the protein were made which were flash freezed and stored in -80° C.

Dialysis:

An alternative method of purification to remove Imidazole was Dialysis. It is a process which separates molecules in a solution based on their diffusion through a semi permeable membrane. The Ni-NTA fractions containing the protein were spun at high speed to remove precipitation and transferred to 10 K MWCO Snake Skin Dialysis Bag (Thermo Fisher) and dialyzed against Buffer A50 (50mM Tris pH 8, 50mM NaCl) for 2 hours. The protein was again spun at high speed to remove precipitation if present and concentrated further using bottom to top centricon.

2.5 Biochemical Assay

2.5.1 Malachite Green Assay

Since SofG is a GTPase, to study the activity of the protein, GTPase Assay using Malachite Green was carried out which determined the phosphate released as a measure of its GTPase activity. The amount of inorganic phosphate released is measured after the formation of the complex of malachite green oxalate and phosphomolybdate under acidic conditions. Here, the assay was carried out for the

proteins SG60 and Q140L. The concentration of the proteins used was 5μM each. The30-μl reaction mix consisted of buffer (50mM Tris pH 8, 50mM NaCl, 5mM $MgCl_2$), protein and GTP. The GTP concentrations used were 0.5mM and 1mM GTP. Each of the reaction was done in triplicates. The reaction was then incubated at 30° C for different time durations and then stopped by heating at 65° C for 10 minutes. The samples were then spun at 4° C for 10 minutes. 50μl of freshly prepared malachite green solution (Malachite Green, Ammonium Molybdate and 11 % Tween 20) was added to 25μl of the reaction and the absorbance was measured at 630nmusing Varioskan Flash Micro plate Reader.

2.5.2 Binding Assay using mant-labeled nucleotides

To study the binding of the nucleotide to the protein, fluorescence based assay with mant-labeled nucleotides was carried out. The reaction mixture consisted of buffer (50mM Tris pH 8, 50mM NaCl, 5mM $MgCl_2$), protein and mant-GDP. The concentration of mant-GDP used was 0.4μM and was titrated with different concentrations of the protein. Anisotropy and Polarization of the sample were measured at 25° C using FluroMax4 Spectrofluorometer.

Chapter 3: Results and Discussion

This chapter includes results of the different experiments carried out in studying SofG. The chapter is mainly divided in four parts explaining the results of the four main objectives mentioned above.

3.1 Standardization of the Purification of SG60

Earlier work on SofG showed that among the different constructs of SofG, the construct SG60 gave the best expression and solubility. Hence purification trials were carried out according to the previously standardized protocol in the lab. However, the yield of the protein obtained after purification wasn't sufficient enough so as to get better quality crystals after the crystallization set-up. Hence the main objective was to optimize the purification of SG60 further to get higher yield of the protein. Table 3.1 summarizes the different optimization trials done for the purification of SG60. It gives an overview of the different conditions tried during the purification steps and the corresponding yield of the protein obtained. The Ni-NTA and SEC gel profiles of the trial which gave the best yield of protein are shown in Figures 3.1 and 3.2.

Table 3.1 Summary of different conditions used for standardization of SG60

	Conditions			
Trial	**For Ni-NTA**	**For Superdex75**	**Yield**	**Remarks**
1	Buffer A, Buffer B	Buffer A50 + 0.1mM GDP	**1.8mg/ml (6L) (120µl)**	-
2	Buffer A, Buffer B(Lysis-Cell Disruptor)	Buffer A50 + 0.1mM GDP	**1.3mg/ml (6L) (120µl)**	Solubility enhanced but most protein in FT
3	Buffer A, Buffer B	Buffer A50 + 0.1mM GDP + 2mM $MgCl_2$	**3.5mg/ml (2L) (100µl)**	-
4	Buffer A, Buffer B (5mM $MgCl_2$ in all buffers)	Buffer A50 + 0.1mM GDP + 5mM $MgCl_2$	**- (2L)**	Protein had impurities even after Superdex
5	Buffer A, Buffer B (DNase in Lysis Buffer)	Buffer A50 + 0.1mM GDP + 5mM $MgCl_2$	**- (2L)**	Most protein in void while doing Superdex
6	Buffer A, Buffer B (DNase in Lysis Buffer + 5mM $MgCl_2$ in all buffers)	Buffer A50 + 0.1mM GDP + 5mM $MgCl_2$	**- (2L)**	Very less protein in Ni-NTA fractions
7	Buffer A, Buffer B	Buffer A50 + 0.1mM GDP + 5mM $MgCl_2$	**- (2L)**	Most protein precipitated. Less input to Superdex
8	Buffer A, Buffer B	Buffer A50	**2.35mg/ml (6L) (80µl)**	-

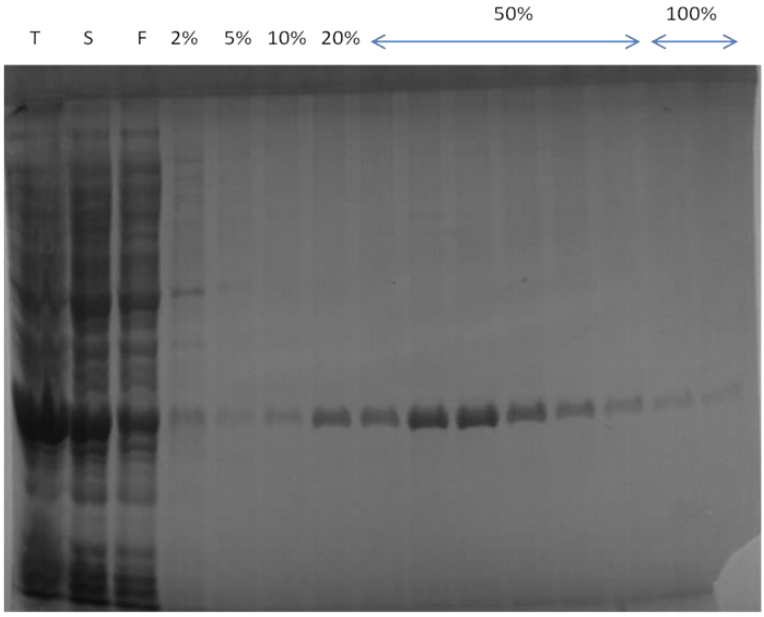


Fig. 3.1: Ni-NTA purification profile of SG60 trial #3. T- Total Cell Lysate, S- Soluble Lysate, F- Flow through, 2%, 5%, 10%, 20%, 50%, 100%- Buffer B washes given to remove nonspecific impurities and elute the protein. Protein (27.5 KDa) was observed in the fractions eluted using 20%, 50%, 100% Buffer B.

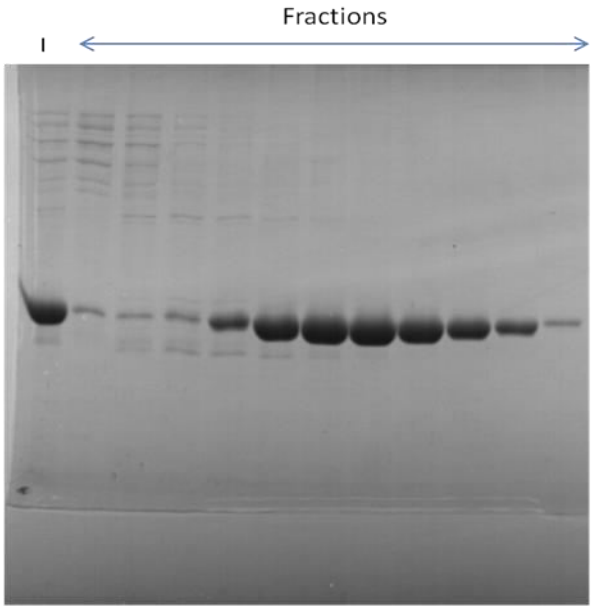


Fig. 3.2: SEC purification profile of SG60 trial #3. I- Input. Pure protein was obtained in the fractions.

Despite different trials of purification as mentioned above (Table 3.1), the protein yield could not be obtained more than 3.5mg/ml. Hence may be more optimization is required. Screening of conditions having different combinations of buffer, pH of the buffer, salt concentration, presence of additives could be done as an optimization step to enhance the stability of the protein and to reduce precipitation.

3.2 Standardization of protein expression and purification of other SofG constructs.

Besides SG60, the standardization of protein expression of other constructs of SofG which were cloned earlier was carried out. Standardization of protein expression was done for the constructs- SG-H6, SG-18, SG-277 and SG-60-277 (Refer Table 2.1 in Chapter 2 for details). For SG-H6, SG-18, SG-277, the cell strain used was BL21AI and the culture was induced when the $O.D_{600}$had reached1-1.2. For SG-60-277, the culture was induced at different$O.D_{600}$ of 0.6, 0.8 and 1.1. Figures 3.3 and 3.4 show the expression and solubility profiles for the constructs.

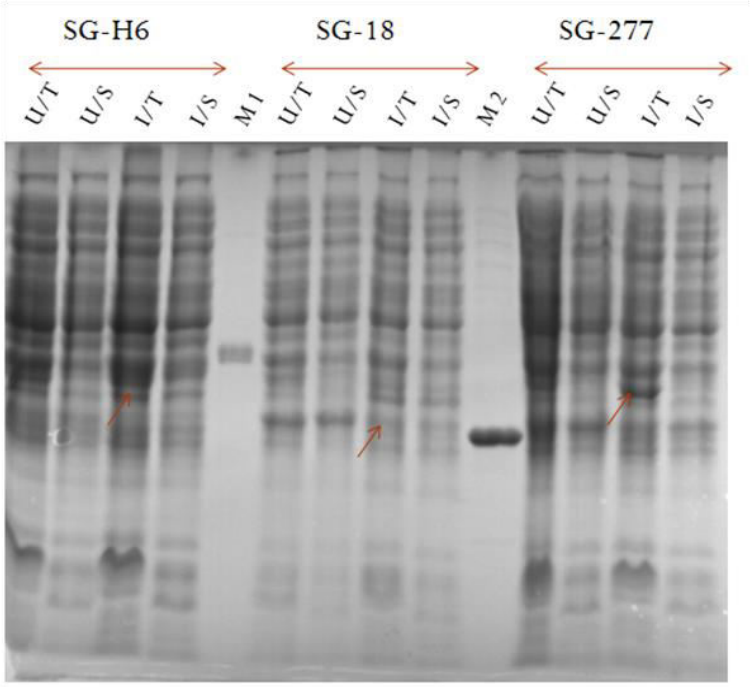


Fig. 3.3: Protein Expression check of SG-H6, SG-18, SG-277. U/T- Uninduced Total Cell Lysate, U/S- Uninduced Soluble Lysate, I/T- Induced Total Cell Lysate, I/S- Induced Soluble Lysate.M1- Marker (38 KDa), M2- Marker (27.5 KDa). The proteins are shown by the arrow -SG-H6- (33.5 KDa), SG-18 (31.5 KDa), SG-277 (31.5 KDa).

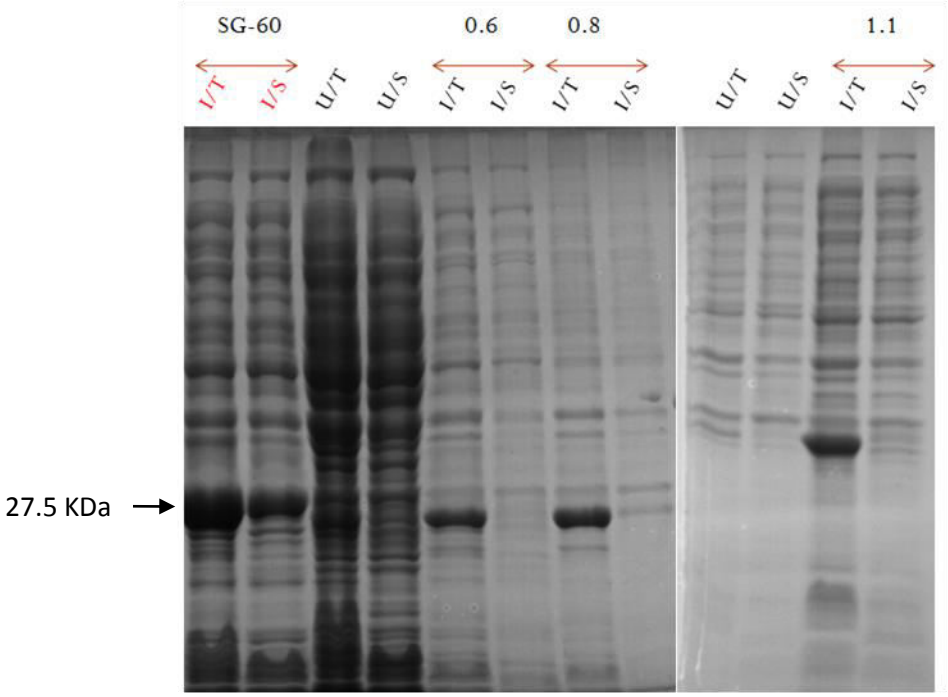


Fig. 3.4: Protein Expression check of SG-60-277. U/T- Uninduced Total Cell Lysate, U/S- Uninduced Soluble Lysate, I/T- Induced Total Cell Lysate, I/S- Induced Soluble Lysate. SG60 is used as a marker (27.5 KDa). The protein SG-60-277 (25 KDa) is shown by the arrow for the samples induced at $O.D_{600}$0.6, 0.8, 1.1.

In an attempt to increase the solubility and stability of SG60 protein, N terminal $(His)_6$ tagged construct was designed. The cloning and expression results of SG60-N $(His)_6$ construct are given below. Along with SG60, SG18-N $(His)_6$construct was also designed and its cloning results are included in Fig. 3.5.

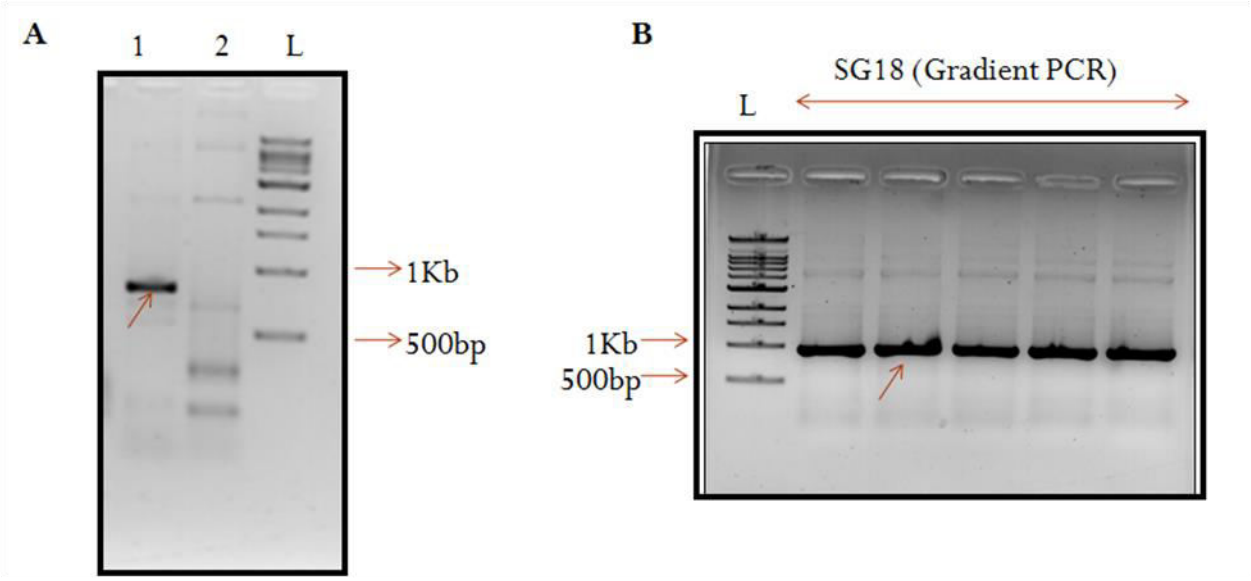


Fig. 3.5: Amplification of the N-Terminal $(His)_6$ tagged SofG constructs using PCR. (A)- PCR result of SG60-N $(His)_6$ – lane 1 and SG18-N $(His)_6$– lane 2. L- Supermix DNA Ladder. For SG60-N $(His)_6$ expected band around 730bp was observed.(B)- PCR (Gradient) result of SG18-N $(His)_6$ – lane 2-6. L- Supermix DNA Ladder. Expected band around 860bp was observed.

The confirmation of the clones was done by double digestion using the enzymes NdeI and BamHI (Fig. 3.6). Clone confirmation check of only SG60-N $(His)_6$is shown. SG18-N $(His)_6$ cloning didn't work and still needs further optimization of the RF cloning PCR conditions.

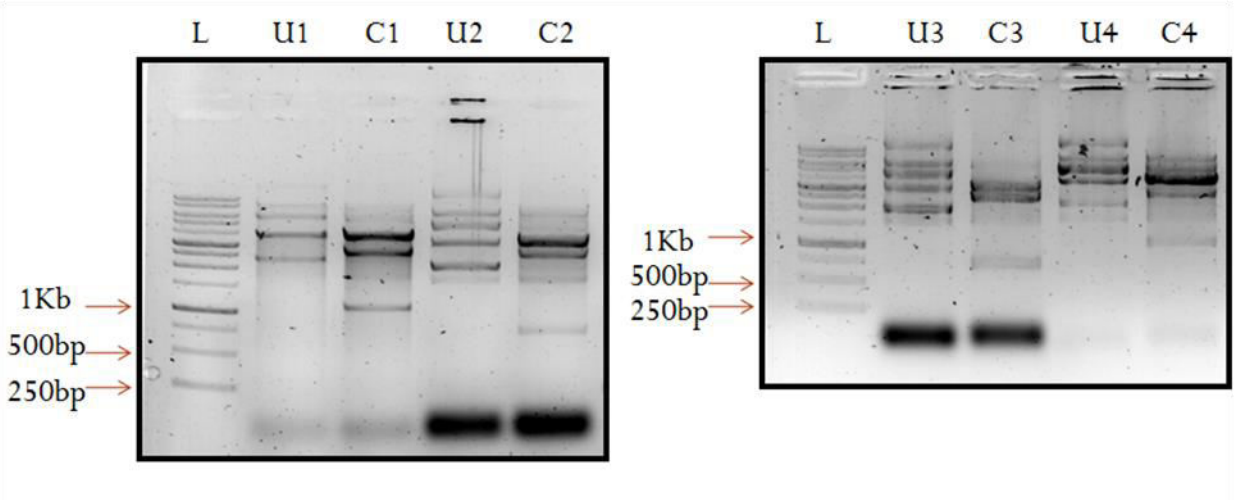


Fig. 3.6: Restriction digestion ofSG60-N $(His)_6$ clones using NdeI and BamHI. L- Niegen 1 Kb Ladder, U1-U4- Uncut plasmid, C1-C4- Double digested plasmid. Clones 2 and 3 seemed to be positive as the expected band around 750bp was observed.

The positive clones were further confirmed by sequencing and the protein expression was checked for the clones. Figure 3.7 represents the expression results of one of the clones.

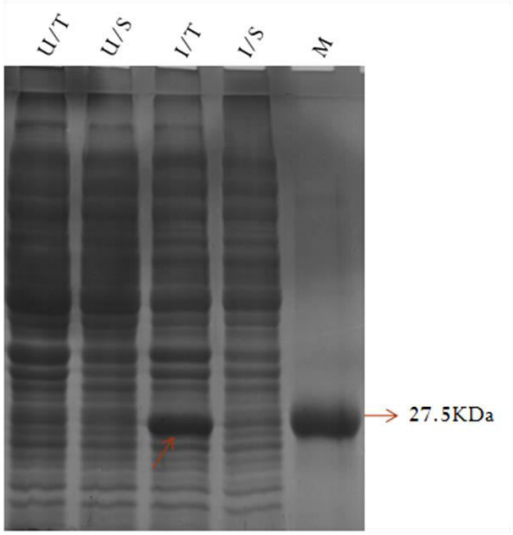


Fig. 3.7: Protein Expression check of SG60-N $(His)_6$. U/T- Uninduced Total Cell Lysate, U/S- Uninduced Soluble Lysate, I/T- Induced Total Cell Lysate, I/S- Induced Soluble Lysate. M- Marker (27.5 KDa). The protein (27.5 KDa) indicated by the arrow for the sample induced at $O.D_{600}$1.1 showed good expression but the solubility was poor.

3.3 Cloning, optimization of expression and purification of active site mutants of SG-60 C $(His)_6$.

To study more about the activity of the protein, active site mutants of wild type SG-60 C $(His)_6$were cloned, purified and used for the biochemical assays. This section shows the cloning, expression check and purification results of the mutants SG60-R111A and SG60-Q140L.

3.3.1 Cloning:

SG60-R111A and SG60-Q140L were cloned through site directed mutagenesis protocols and the positive clones confirmed by sequencing (Figs. 3.8 and 3.9).

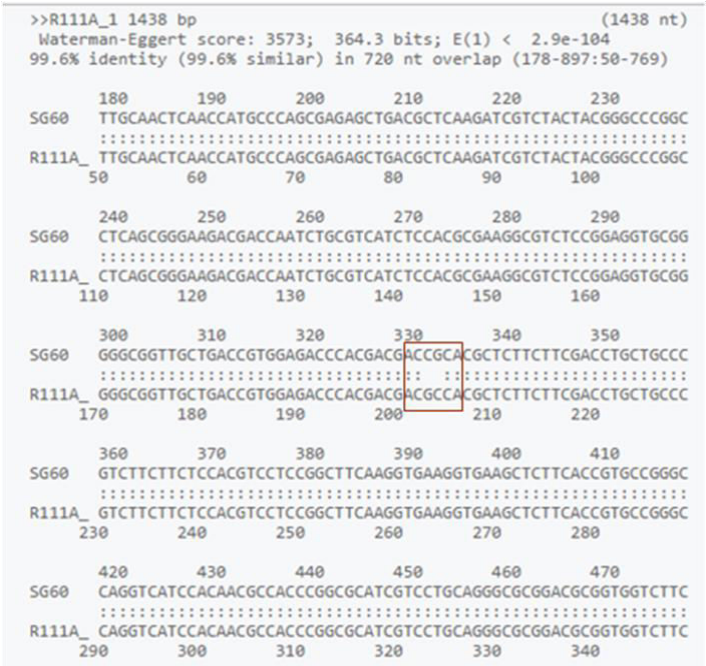


Fig. 3.8: Sequencing result of SG60-R111A. The box highlights the mutation in SG60-R111A as compared to wild-type SG60.

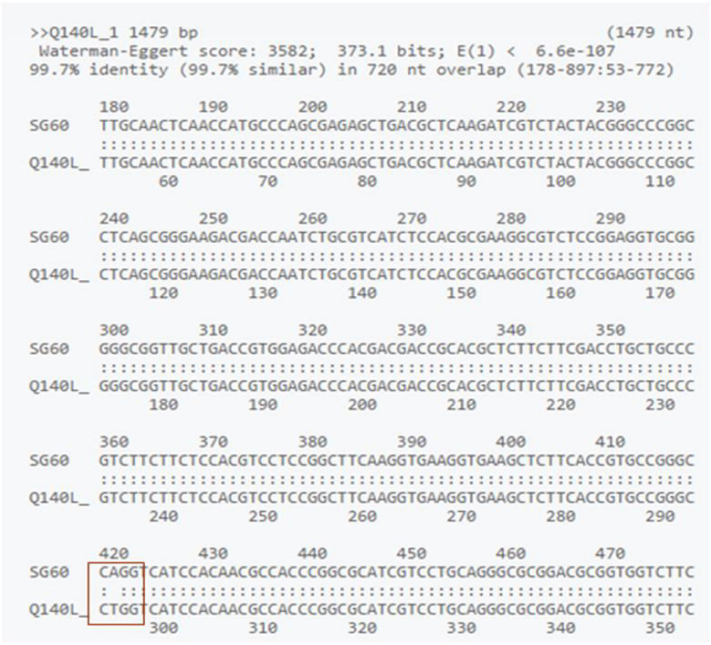


Fig. 3.9: Sequencing result of SG60-Q140L. The box highlights the mutation in SG60-Q140L as compared to wild-type SG60.

3.3.2 Expression Check:

The protein expression was optimized for the mutants SG60-Q140L and SG60-R111A (as shown in figure 3.10) using the same cell strain (BL21AI) and same induction temperature (15°C) but at different $O.D_{600}$.

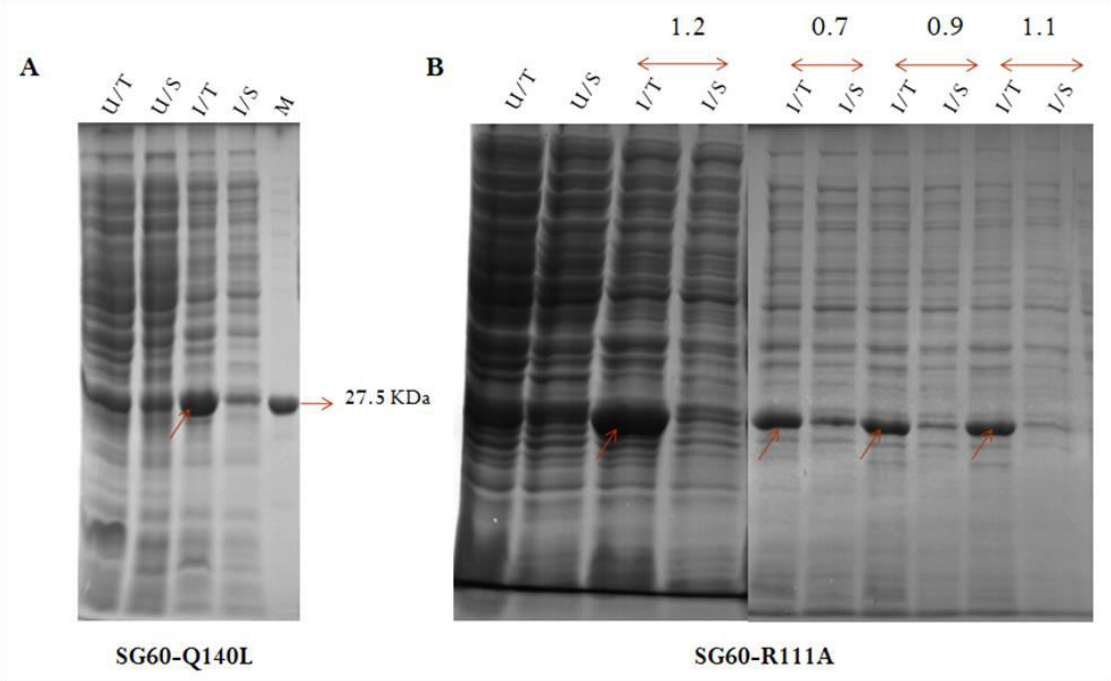


Fig. 3.10: Protein Expression check of (A) SG60-Q140L and (B) SG60-R111A. U/T- Uninduced Total Cell Lysate, U/S- Uninduced Soluble Lysate, I/T- Induced Total Cell Lysate, I/S- Induced Soluble Lysate. M- Marker (27.5 KDa). The protein (27.5 KDa) indicated by the arrow for the sample of SG60-Q140L induced at $O.D_{600}$1.2 and for the sample of SG60-R111A induced at $O.D_{600}$ 0.7, 0.9, 1.1, 1.2 showed good expression but the solubility wasn't best for both.

3.3.3 Purification:

Table 3.2 summarizes the optimization trials carried out for the purification of SG60-R111A and SG60-Q140L. Similar conditions that were used for SG60 were used for the purification of SG60-R111A and SG60-Q140L. The Ni-NTA and SEC purification profiles of SG60-Q140L and SG60-R111A are shown in Figures 3.11, 3.12, and 3.13 respectively.

Table 3.2 Summary of different conditions used for standardization of SG60-Q140L

	Conditions			
Trial	**For Ni-NTA**	**For Superdex75**	**Yield**	**Remarks**
1	Buffer A, Buffer B	Buffer A + 0.1mM GDP + 5mM $MgCl_2$	**0.45mg/ml (2L) (60µl)**	Less precipitation compared to other trials
2	Buffer A, Buffer B	Buffer A50 + 0.1mM GDP + 2mM $MgCl_2$	**0.75mg/ml (6L) (220µl)**	Precipitation observed.
3	Buffer A, Buffer B	Buffer A50	**0.98mg/ml (6L) (350µl)**	Precipitation observed.

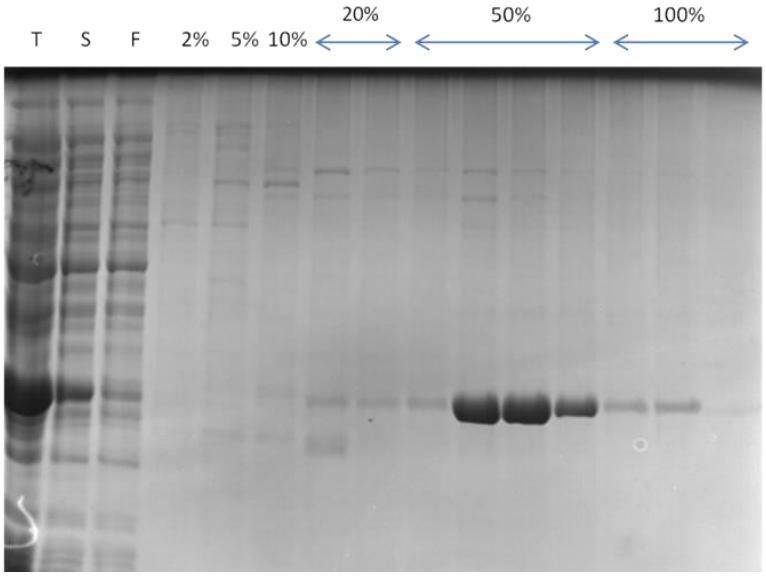


Fig. 3.11: Ni-NTA purification profile ofSG60-Q140L trial #2. T- Total Cell Lysate, S- Soluble Lysate, F- Flow through, 2%, 5%, 10%, 20%, 50%, 100%- Buffer B washes. Protein (27.5 KDa) was mostly observed in the fractions eluted using 50% Buffer B.

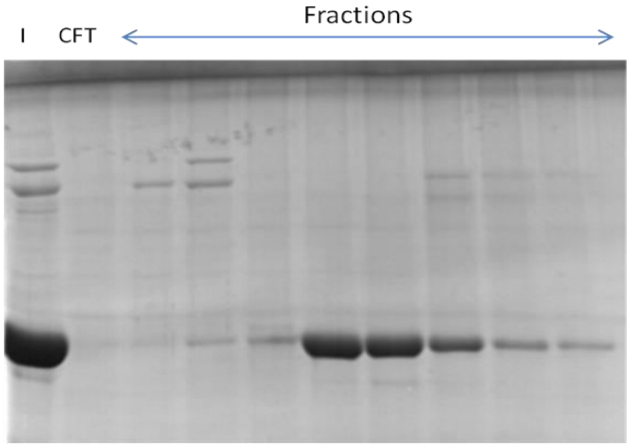


Fig. 3.12: SEC purification profile of SG60-Q140L trial #2. I- Input, CFT- Centricon Flow through. Pure protein was obtained in the fractions.

For SG60-R111A, only one purification trial was done using the conditions similar to trial #2 used for SG60-Q140L given in the above table. Since no protein was observed in the Ni-NTA fractions, the further optimization wasn't done immediately.

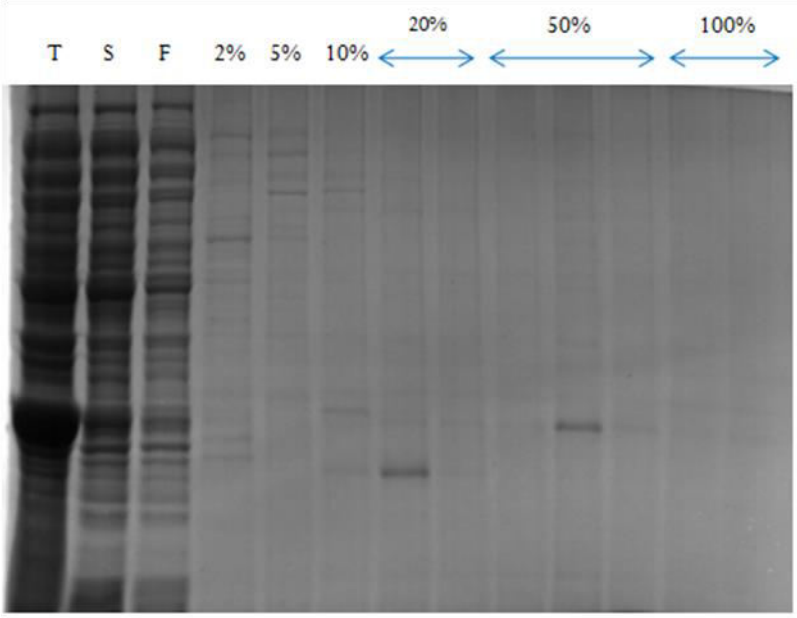


Fig. 3.13: Ni-NTA purification profile ofSG60-R111A. T- Total Cell Lysate, S- Soluble Lysate, F- Flow through, 2%, 5%, 10%, 20%, 50%, 100%- Buffer B washes. Protein (27.5 KDa) wasn't observed in any of the fractions.

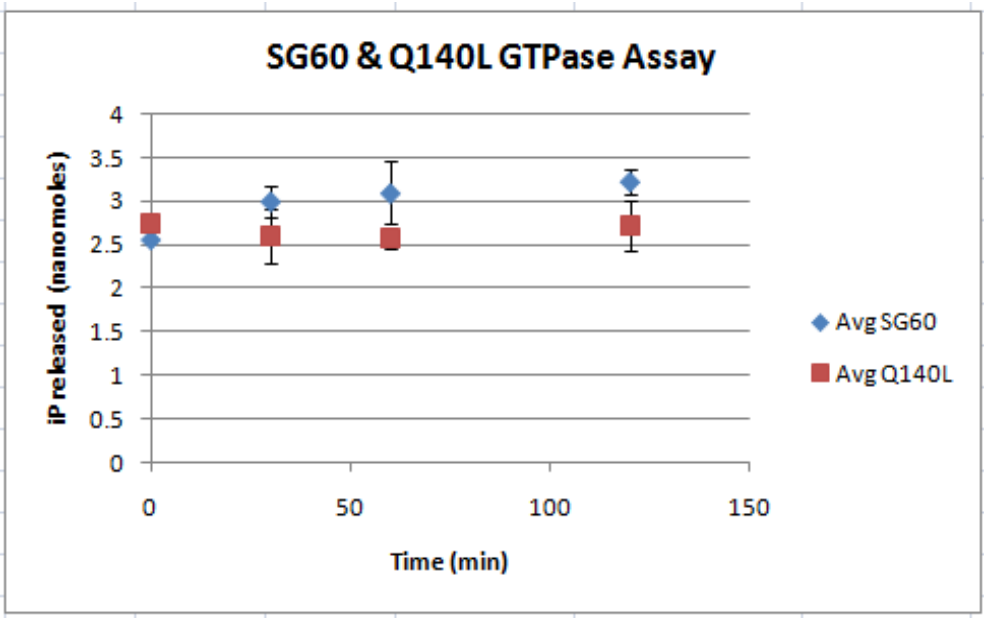
3.4 Biochemical assays to compare the activity of wild type SG-60 C $(His)_6$ and the active site mutants.

Earlier experiments in order to study the GTPase activity of SofG were carried out using wild-type SofG. The results obtained were not much conclusive, since whatever GTPase activity was observed could have been due to some artifact. Hence to confirm further whether the activity is indeed because of the protein itself, the activity of the wild type was compared to that of the active site mutants.

3.4.1 GTPase Assay- Malachite Green Assay.

Earlier biochemical assay (Malachite Green Assay) performed on wild type SG-60 C $(His)_6$ didn't give much conclusive results about the activity of the protein since not many trials were performed. Hence further optimization was done varying the protein and GTP concentrations. The assay was done using the wild type and the active site mutants of SG60 (here only SG60-Q140L, since SG60-R111A couldn't be purified) so as to determine whether the activity of the wild type protein was indeed because of the presence of the active site residues and not due to some artifact. Figure 3.15 shows the results of these assays.

A



B

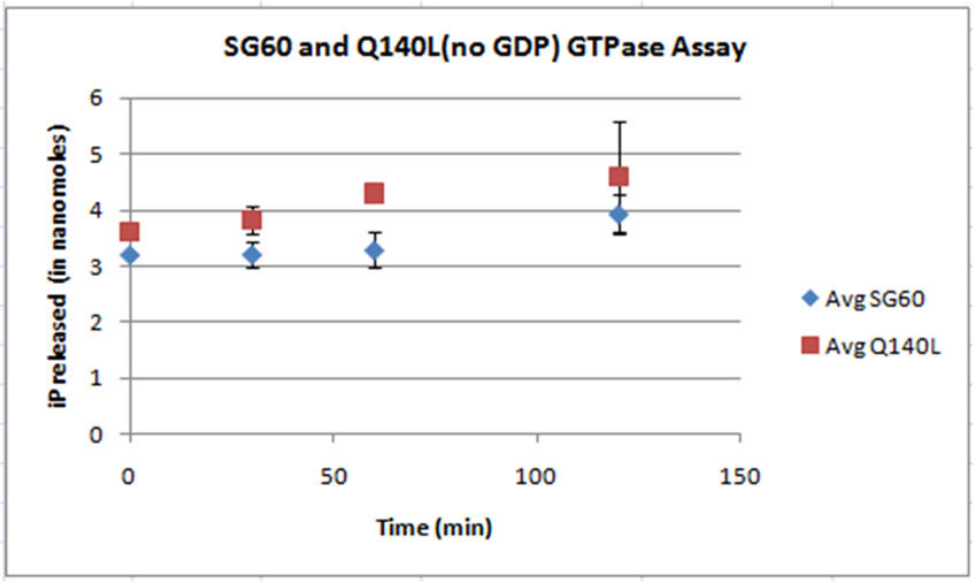


Fig. 3.14: Malachite Green Assay of SG60 and Q140L - Comparison between the released inorganic Phosphate between wild-type SG60 and active site mutant SG60-Q140L at (A) 5μM concentration of each of the protein containing externally added GDP while purification steps for stability and 0.5mM concentration of GTP and (B) 5μM concentration of each of the protein without any external GDP added and 1mM concentration of GTP. Number of trials performed for experiments in (A) were 3 and for those in (B) was 1. The error bars denote the standard deviation

In theory, mutation in the active site residues of the protein would affect the activity of the protein (here the GTPase activity) to some extent. But from the above plots, it can be seen that no significant difference is observed between the activity of the wild type and the mutant. Also no difference was seen when the protein used for the assay contained extra GDP in the surrounding and when it didn't. Hence further optimization is required to get more conclusive results.

3.4.2 Binding Assay using mant-labeled nucleotide.

Fluorescence based binding assays using mant-GDP and mant-GTP didn't show conclusive results. Hence further optimization was needed with respect to protein and nucleotide concentration to obtain significant information. Initial trial was done using mant-GDP titrating it with different concentrations of SG60 protein (no external GDP was added to the protein during purification steps). The volume of the reaction (buffer, protein, mant GDP) used for the experiment should be around 200-210μl, since the protein used was already diluted, after titrating the reaction mix with the protein, the

volume exceeded 300µl. This affected the final concentration of the protein and also mant-GDP used.

Fig. 3.15 shows the plot of Anisotropy vs. protein concentration. Anisotropy of the fluorescently labeled nucleotide increases as the nucleotide binds to the protein. Hence when the concentration of protein is increased, anisotropy also increases which can also be seen in the following plot (but the increase was very less indicating weak binding of the protein to the nucleotide).

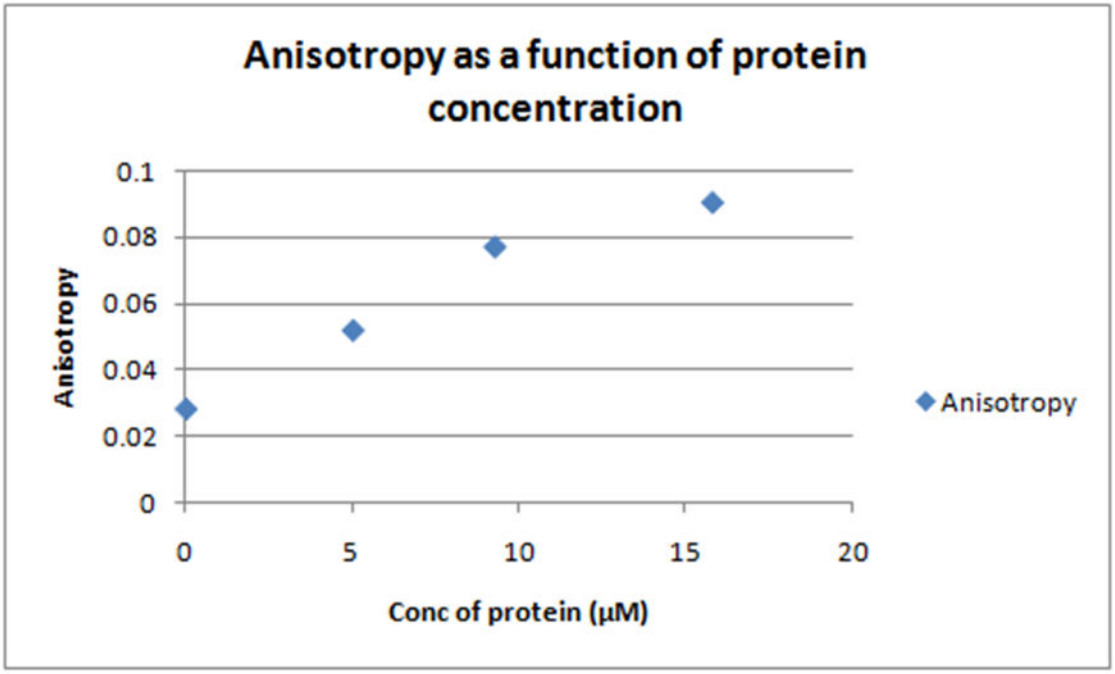


Fig. 3.15: Fluorescence based binding assay of SG60 using mant-GDP. Anisotropy measured for mant-GDP (0.4µM concentration) titrated with different concentrations of SG60 protein.

Chapter 4: Conclusion and Future Prospects:

The different experiments carried out so far helped in studying some aspects of the protein SofG. Earlier studies showed that the protein could be purified but the concentration wasn't sufficient enough to get good quality crystals. Though the protein folded properly as shown by Circular Dichorism (CD) and gel filtration experiments done earlier in lab, the GTPase activity observed was weak. Hence the project was continued further trying to optimize further the purification and biochemical assays with the hope of getting higher concentration of protein so as to set up crystallization and obtain better quality crystals and getting some conclusive insights about the activity of the protein.

The purification of the best soluble construct of SofG was optimized but higher concentration of the protein couldn't be obtained so that crystallization could be set up. It was a tough protein to work with since many difficulties were encountered in the course of the project. Initially there were problems with solubility of the protein, binding of the protein to the column (may be due to column's issue). Later there were problems with concentrating the protein since it was seen that there was protein loss during concentrating the protein (may be due to blocked and damaged centricon). But the main and consistent problem faced was precipitation of the protein. This might be because of higher concentration of the protein which may also lead to the formation of protein aggregates. Also sometimes, the change in buffer compositions might also trigger precipitation. Earlier optimization trials done in lab to reduce precipitation had shown that precipitation was reduced by externally adding GDP to the Ni-NTA fractions and also having 0.1mM GDP in the final buffer. Even after trying such methods to overcome it, the precipitation was still present thus giving lesser concentration of the protein.

The optimization of protein expression of other SofG constructs didn't give any better results in hope to continue them further. The active site mutants were successfully cloned and only one could be purified which too showed similar problems of high precipitation. Further standardization can be done to lower the precipitation like checking the stability of protein in different buffer conditions. Also further trials are

needed to purify the other active site mutant. The biochemical assays done with the wild type and mutant proteins didn't give any convincing results hence that too need to be repeated. Overall it was a tough task dealing with this protein and more efforts are required in the future to get better results so that it can be well characterized structurally and biochemically.

The future goals with respect to this protein would be standardizing the experiments further which didn't give conclusive results and further optimizing the experiments like purification which can give more better results. Also the long term goals can include studying the interaction of SofG with BacP and other motility proteins as hypothesized in the model to get more mechanistic insights about the proteins and also about the regulation of cell motility in *Myxococcus xanthus*.

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