Cloning and Expression of *Bacillus subtilis* GabR and ΔGabR

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Thank you also to Julia Klutkowsi and the Michael Wolfe Lab at Brigham and Women's Hospital for providing me with a space to perform some of my research. Julia deserves my sincerest thanks for assisting me with the research and teaching me many things along the way.

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Cloning and Expression of Bacillus subtilis GabR and ΔGabR

A thesis presented to the Biology Department

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ABSTRACT

Bacillus subtilis GabR is a transcriptional regulator involved in nitrogen fixation. Previous studies showed that GabR contains a short N-terminal helix-turn-helix (HTH) DNA binding domain and a long C-terminal aminotransferase domain. The aminotransferase domain has been studied extensively and has been shown to activate the gabTD operon, allowing for the utilization of γ-aminobutyric acid to produce succinate. The HTH domain is believed to repress this pathway when excess nitrogen is present.

Even though the domain organization of GabR is known, it is not possible to understand the precise details of how GabR functions as a transcriptional regulator until the crystal structure of GabR is determined. In this study, expression constructs of GabR and ΔGabR, a modified version of GabR lacking the N-terminal HTH-domain, were created and small-scale expression tests were performed to determine the criteria for optimizing the amount of protein produced. Large-scale expression and purification of GabR was then performed to obtain reasonably large amounts of purified protein. The future goal of this study is to crystallize and determine the atomic structures of GabR and ΔGabR to understand the structural basis of transcriptional regulation by GabR.
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LIST OF ABBREVIATIONS

AT = aminotransferase
CV = column volumes
GABA = γ-aminobutyric acid
GABA AT = GABA aminotransferase
GS = glutamate synthetase
HPAT = histidinol-phosphate aminotransferase
HTH = helix-turn-helix
LB = Luria Broth
Ntr = nitrogen regulatory system
PCR = Polymerase Chain Reaction
PLP = pyridoxal 5’-phosphate
PMP = pyridoxamine 5’-phosphate
SDS-PAGE = Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SS = succinic semialdehyde
SSDH = SS dehydrogenase
INTRODUCTION

1. Background on Bacillus subtilis GabR

*Bacillus subtilis* GabR is a member of the MocR/GabR family of bacterial proteins, which includes approximately 200 similar proteins from Gram-negative and Gram-positive bacteria (Belitsky 2004), including bacteria of the genus *Clostridium*, *Dorea*, *Clostridiales*, *Ruminococcus*, *Roseburia*, *Exiguobacterium*, *Butyrivibrio*, *Blautia*, *Coprococcus*, and *Staphylococcus* (Altschul et al. 1997). Proteins of this family are chimeric, created by the fusion of two or more genes that originally coded for separate proteins. While not much is known about this family of proteins, it has been determined that GabR is a transcriptional regulator, which activates the *gabTD* operon (Figure 1) in the presence of γ-aminobutyric acid (GABA). The operon encodes catabolic enzymes of GABA, converting GABA to succinic semialdehyde. The succinic semialdehyde (SS) is then converted into succinate (Belitsky 2004).

![Figure 1. The genetic map of the *gabRTD* segment of the *B. subtilis* GABA pathway. The region is 1.1 kb and is not drawn to scale (Belitsky and Sonenshein 2002).](image)

2. GabR Nitrogen Metabolism

While most intestinal bacteria have a nitrogen regulatory (Ntr) system used to regulate nitrogen intake and degradation, this system appears to be absent in *B. subtilis*. 
Nitrogen metabolism is instead controlled by different, independent, regulatory factors. Glutamate synthetase (GS) expression is regulated in response to the availability of nitrogen. The highest levels of expression occur during nitrogen-limited growth (Ferson et al. 1996). What makes GabR interesting is that GABA is the only nitrogen source for B. subtilis, and as mentioned above, GABA is catabolized by the gene products of the gabTD operon, which are in turn regulated by GabR. GABA can be obtained by most bacteria in two ways; it can be taken from the soil or it can be produced in the cell from glutamate or putrescine. B. subtilis, however, is unusual because it can only use extracellular GABA. It has no known mechanism for producing GABA from glutamate or putrescine (Atkinson and Fisher 1991). Arginine, the putrescine precursor, is also unable to stimulate the expression of gabT. B. subtilis utilizes GABA in the following way (see reaction scheme below): the gabT gene product (GABA AT) converts GABA and 2-ketoglutarate to SS and glutamate. The gabD gene product (SSDH) further converts the products of the first reaction to succinate.

\[
gabT \quad \text{GABA} + \text{2-ketoglutarate} \rightarrow \text{succinimide} + \text{glutamate} \quad \text{gabD} \rightarrow \text{succinate}
\]

High levels of GABA have also been shown to be toxic to many microorganisms, including B. subtilis, and so the gabTD operon may also be used as a way to keep the intercellular levels of GABA low (Belitsky and Sonenshein 2002). When gabD is mutated, GABA can no longer be used as the only nitrogen source, even though the conversion of GABA to SS and glutamate occurs in these mutants. It is believed that the accumulation of SS in gabD mutants could possibly be a negative feedback mechanism.
preventing the formation of glutamate and inhibiting cell growth. Also, in the absence of GABA, B. subtilis is unable to grow. This same result is seen when GabR is mutated; gabT no longer functions and cell growth is inhibited (Belitsky and Soncnshein 2002).

3. Domain Organization of GabR

It has been found that proteins of the GabR family have a short, 60-120 residue amino-terminal helix-turn-helix (HTH) domain, which is a DNA-binding domain, and a long, 330 amino acid, conserved carboxy-terminal domain (Figure 2) that is remarkably similar to aminotransferases, including their pyridoxal 5'-phosphate (PLP) binding domains (Belitsky 2004).

![GabR protein domain structure](image)

**Figure 2.** Domain structure of GabR protein.

4. GabR HTH-Domain and Repression of gabR and gabT Promoters

GabR represses transcription of the gabTD operon in the presence of excess nitrogen (Ferson et al. 1996) and has been shown to be a DNA-binding protein that acts by itself as a negative transcriptional auto-regulator. It has also been thought that the binding of the HTH-domain to a specific ATACCA double-repeat sequence, found at the two ends of a minimal GabR DNA-binding element within the gabR-gabT region, is necessary for gabR repression (Figure 3A and 3B). Belitsky hypothesized that the only
way GabR by itself is able to repress its synthesis is by being in a conformation that allows for DNA binding without PLP and/or GABA being present.

Figure 3. Model of gabR and gabT expression due to GabR-mediated regulation. The actual number of bound GabR molecules is not known (Belitsky 2004).

5. GabR Aminotransferase Domain and Activation of gabTD Transcription

Under conditions when both GABA and PLP (or SS and PMP) are present, it is believed that GabR is able to gain the conformation necessary for transcriptional activation at the gabT promoter (Figure 3C) (Belitsky 2004). This raises the question of the precise role of the aminotransferase domain, and whether it is catalytically active.
Aminotransferases (AT) catalyze the transfer of amino groups from amino acids to oxoacids. This reversible transfer is typically carried out by vitamin-B₆-dependent enzymes (Mehta et al. 1993), and is dependent on PLP as a co-factor (John 1994). Enzymes dependent on PLP have been found to be extremely important functionally because they catalyze a much wider assortment of reactions than enzymes containing any other cofactor (John 1994). The AT domain of GabR belongs to family I of the AT superfamily (Belitsky and Sonenshein 2002) and is classified as a histidinol-phosphate type aminotransferase (HPAT), which can accept a versatile assortment of substrates: alanine, carboxylic acids, and aromatic amino acids (Mehta et al. 1993).

The AT domain of GabR has been shown to have catalytic activity. Boris Belitsky and colleagues studied the AT domain of B. subtilis GabR and found that mutations in the AT domain, which is thought to be necessary for folding, PLP binding, and catalysis, abolished the activation of gabTD by the mutant GabR, but did not affect the ability of the mutant to function as an autorepressor. Based on these results, Belitsky speculated that GabR is able to bind to DNA even in the absence of co-regulators, but requires interaction with co-factors GABA and PLP to activate gabTD transcription. These results also suggest the catalytic activity by the AT domain of GabR is necessary for transcriptional activation of gabTD (Belitsky 2004).

6. Overview of the Project

Important clues to the mechanism of how GabR functions as an autorepressor and as a transcriptional activator of gabTD will be revealed by the high-resolution crystal structure of GabR. Knowing how the protein folds and where the active site is located
will be the key to uncovering how GabR interacts with DNA and how the enzymatic activity of the AT domain of GabR (in the presence of GABA and PLP) regulates the gabTD operon.

Previously, Cheryl Kreinbrinck and collaborators in the Petsko-Ringe lab at Brandeis attempted to solve the crystal structure of GabR. Briefly, bacterially expressed C-terminally His₆-tagged GabR protein eluted from a Nickel affinity column was crystallized, and crystals diffracting to ~3.5 Å were successfully obtained. Two complete X-ray datasets were collected from these GabR crystals but attempts to solve the crystal structure using molecular replacement did not work (Kreinbrinck, personal communication).

Upon inspection of the diffraction pattern produced from these above-described GabR crystals, Dr. Raji Edayathamangalam noted that the diffraction pattern suggests the presence of multiple lattices within the crystal. X-ray data collected from such crystals often do not produce good-quality data and are often unusable, particularly for phasing strategies like molecular replacement that are sensitive to the quality of the diffraction data. Dr. Raji Edayathamangalam also attempted to perform molecular replacement using two different programs, but both programs were unsuccessful at producing molecular replacement solutions. Since the diffraction data from these crystals seems to be of poor quality, the immediate alternative is to focus on strategies to obtain high-quality GabR crystals. Since any one of several factors could potentially impact the quality of GabR crystals obtained, the alternative strategies include: (1) modification of GabR construct, (2) modification of GabR protein purification protocol, and (3) crystallization of GabR–ligand complexes with ligands such as PLP, DNA etc.
Towards this end, this study describes the cloning of two different constructs of GabR and subsequent protein expression studies with full-length GabR protein. (At the time at which Dr. Raji Edayathamangalam inherited the project, the GabR construct was not available in a bacterial pET expression vector.) Two constructs were cloned in this study: (1) full-length GabR and (2) ΔGabR, a construct in which the N-terminal HTH-domain has been deleted. A deletion construct of GabR was generated in the event that full-length GabR does not yield crystals of high diffraction quality. Also, even if the crystal structures for both GabR and ΔGabR were successfully determined, it would be interesting to compare the two structures to gain insights on conformational changes in the GabR protein with and without the HTH domain. Two different cloning protocols were used to clone GabR and ΔGabR into pET vectors (Stratagene). One of the cloning protocols was successful at producing positive clones. Once clones were obtained, GabR and ΔGabR were expressed in bacteria. Furthermore, six-liter cultures of GabR were grown and a large-scale purification protocol was established to produce reasonable yields of GabR.

MATERIALS AND METHODS

1. Construction of C-terminal His-Tagged GabR and ΔGabR Expression Clones

Two different protocols were used to clone GabR and ΔGabR containing a C-terminus His$_6$ tag. The first protocol uses a modified pET28 vector along with a cloning enhancer
to help clean up the PCR product and an enzyme that allows for homologous recombination of the PCR product with the plasmid. The second protocol is a ligation-independent cloning strategy, which uses a pETite vector and does not require treatment of the PCR.

*Protocol 1: Cloning into pET28 vector*

GabR and ΔGabR were amplified using a 50μL PCR reaction mixture containing 5.0μL 10X Pfu buffer, 1.5μL of each primer (10μM), 1.0μL of GabR or ΔGabR template DNA, 0.5μL dNTPs, and 1.0μL Pfu polymerase. The PCR is a touch-down scheme from 65°C to 45°C at 0.5°C intervals with a 2 minute extension time (Figure 4).

![Diagram of touch-down PCR scheme](image)

*Figure 4. Diagram of the touch-down PCR scheme used for cloning of GabR and ΔGabR.*

The PCR was then treated with a cloning enhancer (5μL PCR + 2μL cloning enhancer) and incubated at 37°C for 20 minutes and then at 80°C for 15 minutes. A cloning enzyme was then used according to the following protocol making a 10μL reaction: 2μL
5X enzyme reaction buffer, 1μL enzyme, 2μL NcoI digested and linearized pET28
vector, and 2μL pretreated PCR. This reaction was then incubated at 37°C for 15
minutes and then at 50°C for 15 minutes. The 10μL cloning reaction was then diluted to
a total volume of 50μL using TE, and 10μL of this diluted mixture was transformed into
50μL of high efficiency DH5α competent cells. The transformation mixture was plated
on Luria broth (LB)-agar plates supplemented with Kanamycin (Figure 5).

**Figure 5.** Schematic of Protocol I (pET28 vector). It is a five-step process involving
treatment of the PCR products with cloning enhancer and cloning enzyme to catalyze
recombination of the vector with the GabR or ΔGabR PCR product. The purple and red
refer to the primers used for the PCR reaction.
Protocol II: Cloning into pETite vector

GabR and ΔGabR were amplified using a 50μL PCR reaction as before. The amount of PCR transformed is dependent on the amount of product produced as seen on an agarose gel. 2μL pETite vector and 1-3μL PCR product were added to 50μL of 10G competent cells and transformed. The transformation was plated on LB-agar plates containing Kanamycin.

**Figure 6.** Schematic of Protocol II (pETite vector). It is a two-step protocol which only requires amplification of GabR and ΔGabR followed by the transformation step. The purple and red refer to the primers used in the cloning process.
Diagnostic colony PCR reactions (25μL) were performed for transformants obtained from both cloning protocols using Taq DNA polymerase and T7 (forward and reverse) primers. Colony PCRs of positive clones yielded PCR products corresponding to the sizes of 1.4kb and 1.3kb for GabR and ΔGabR, respectively. Once positive clones were confirmed by colony PCR, miniprep DNA was prepared from the positive clones and the DNA was then sent for sequencing. Correct clones verified by DNA sequencing were used for protein expression tests (see below).

2. GabR and ΔGabR Small-Scale Overexpression Tests

The expression clones for GabR and ΔGabR (pETite-GabR and pETite- ΔGabR) were transformed into E. coli BL21(DE3) cells and plated on LB-Kanamycin agar plates. 40mL LB-broth cultures for GabR and ΔGabR were grown at 37°C to an O.D. of 0.6 followed by protein induction at 37°C for 2 hours with isopropyl-thio-β-D-galactoside (IPTG) at varying concentrations (0.2mM, 0.4mM, and 1mM). The cultures were spun down, the pellets were resuspended in lysis buffer (see below) and then lysed by sonication. The supernatant was bound to Talon His-affinity beads for affinity purification using the His6 tag at the C-terminus of the protein. The samples were analyzed by SDS-PAGE.
3. **GabR Large-Scale Overexpression and Purification**

Six-liter LB-broth cultures for GabR were initially grown at 37°C to an A$_{600}$ value of around 0.6 followed by protein induction by addition of 1M IPTG at 37°C for 2 hours. The cells were harvested and pelleted by centrifugation at 4000Xg for 30 minutes at 4°C. The cell pellets were then resuspended with 60mL Lysis Buffer (20mM Tris-HCL (pH 8.0), 0.5M NaCl, 20% sucrose, 1mM β-mercaptoethanol and 20mM imidazole), flash-cooled in liquid nitrogen and stored as cell pellets at -80°C until further use.

The cell pellets were thawed to room temperature followed by incubation in the presence of protease inhibitor cocktail, DNAse I and lysozyme at room temperature on a rotator for 30 minutes. The cell resuspension was disrupted by sonication. The cell lysate was subject to two rounds of centrifugation at 12,000Xg for 40 minutes at 4°C. The clarified supernatant was bound to Talon His-affinity beads, washed with 10 bead or column volumes (CV) of Wash Buffer (Lysis Buffer containing 40mM imidazole) and eluted with 2 CV of Elution Buffer (Lysis Buffer containing 250mM imidazole). The eluate was then loaded on to a Superdex200 gel-filtration column for further purification by size exclusion chromatography. Protein fractions collected during the gel-filtration run were analyzed by SDS-PAGE and fractions corresponding to pure protein were pooled, concentrated to 2.2 mg/mL and stored on ice until further use.
RESULTS

1. Construction of GabR and ΔGabR Expression Clones

The cloning of GabR and ΔGabR was attempted using two different protocols: pET28 vector (protocol I) and pETite vector (protocol II). Protocol II involving the pETite vector and the following cloning scheme produced successful clones:

1. PCR amplify GabR and ΔGabR using primers that have an 18-base-pair overlap with the vector ends. The reverse primer is the same for both GabR and ΔGabR:

(GTGATGGTGTTGATGATGATCCCCTGAACGGGGATTATTATG), while the forward primers differ:

(GAAGGAGATATACATATGAGATCAGTTACACTCGATCGTTG C for GabR and
GAAGGAGATATACATATGGAGCTGGCTGAAATCTCAAGG for ΔGabR). The GabR and ΔGabR genes were amplified using Pfu Ultra II Fusion HS DNA polymerase with the following PCR components:

1. 39.5 μL H2O
2. 5.0 μL 10X Pfu Buffer
3. 1.5 μL of 10μM forward primer
4. 1.5 μL of 10μM reverse primer
5. 1.0 μL GabR or ΔGabR template DNA
6. 0.5 μL of 25mM dNTP mix
7. $1.0 \mu L$ Pfu Ultra II Fusion HS DNA polymerase

2. Analyze PCR products by agarose gel electrophoresis.

3. Mix together:
   1. 1-3 $\mu L$ of the unpurified PCR product
   2. 2 $\mu L$ pETite vector
   3. 50 $\mu L$ 10G Competent cells

4. Transform and select for positive clones on LB-Kanamycin agar plates.

Protocol I (pET28 vector) was attempted many times, with varying concentrations of DNA and vector, never producing any positive clones. Protocol II (pETite vector) was successful on the second try once the yields of PCR product was increased significantly by switching from Pfu Turbo DNA polymerase to Pfu Ultra II Fusion HS polymerase (Figure 7).

![Figure 7. Agarose gel analysis of PCR products obtained using two different Pfu DNA polymerases. Pfu Turbo was used for the reactions in lanes 1 and 2 and Pfu Ultra II Fusion HS was used for the reactions in lanes 3 and 4. Lanes 1 and 3 are GabR and lanes 2 and 4 are ΔGabR. 5 $\mu L$ of PCR product was loaded in each lane.]
By comparing the GabR samples (lanes 1 and 3), it can be observed that the PCR performed using Pfu Ultra II Fusion HS polymerase has a higher yield based on the thicker and brighter band seen in lane 3. The same can be noted for the ΔGabR samples (lanes 2 and 4).

All 20 of the colonies/transformants that were screened by colony PCR yielded colony PCR products of the expected size (~1.4kb for GabR; 1.3 kb for ΔGabR) on an agarose gel (Figure 8). As expected, ΔGabR runs a little faster on the gel due to its smaller size relative to GabR.

Figure 8. Agarose gel of colony PCR samples. Lanes 1-10 represent GabR and lanes 11-20 represent ΔGabR. All 20 samples are positive.

Miniprep DNA samples were prepared for two colonies each of GabR and ΔGabR and were sent for sequencing by Dr. Raji Edayathumangalam. Both clones for each GabR
and ΔGabR were positive as verified by the DNA sequencing results (Appendix I and Appendix II).

2. Small-Scale Overexpression Tests for GabR and ΔGabR

Small-scale expression tests were conducted using the clones produced from the pETite cloning protocol (protocol II). GabR and ΔGabR are expressed in HI-Control BL21(DE3) cells. The cells were grown to an O.D. of 0.6-0.7. Protein inductions were performed at different concentrations of IPTG (0.2mM, 0.4mM and 1mM) to determine which produced the highest expression levels. It was found that using 1mM IPTG gave the best result when expressing GabR (Figure 9A). There does not appear to be a distinct difference in expression levels when inducing ΔGabR cultures with varying IPTG concentrations (Figure 9B).

<table>
<thead>
<tr>
<th></th>
<th>Gab R</th>
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<th>ΔGab R</th>
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<tbody>
<tr>
<td>0.2 mM</td>
<td>T S E</td>
<td>0.4 mM</td>
<td>T S E</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>T S E</td>
<td>0.2 mM</td>
<td>T S E</td>
</tr>
<tr>
<td>0.4 mM</td>
<td>T S E</td>
<td>1.0 mM</td>
<td>T S E</td>
</tr>
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Figure 9. SDS-PAGE gel of small-scale expression tests of GabR and ΔGabR. The millimolar concentrations refer to the concentration of IPTG used to induce the culture. On both gels, the “T” lane is total cell lysate after sonication. The “S” lane is the supernatant after centrifuging the lysed cells. The “E” lane is the eluted protein from the His-Affinity column.
The expression levels for GabR (Figure 9A) under similar expression conditions is significantly higher than that for ΔGabR (Figure 9B).

3. Large-Scale Expression and Purification of GabR

Six-liter cultures of GabR were grown to an O.D. of 0.6 and induced with 1mM IPTG at 37°C for two hours. The cell pellets were harvested and processed as described in Materials and Methods. The supernatant for the cell lysate was bound and purified by affinity purification using Talon His-Affinity resin (Figure 10). Large amounts of soluble GabR protein were obtained following the Talon affinity purification step, as seen on the SDS-PAGE gel (Figure 10, lane ‘E’).

![SDS-PAGE gel](image)

**Figure 10.** SDS-PAGE analysis of Talon affinity purification of GabR. The “T” lane is total cell lysate after sonication. The “S” lane is the supernatant after centrifuging the lysed cells. The “FT” lane is the flow through from the Talon His-Affinity column. The “E” lane is the eluted protein from the His-Affinity column.
The GabR sample eluted from the Talon resin was purified further by size-exclusion chromatography on a Superdex 200 column. A single, large peak was seen at 13.85mL, which corresponds to GabR (Figure 11). SDS-PAGE analysis of the fractions showed that the majority of the protein eluted between fractions 7 and 9, which correspond to a volume of 12-15mL (Figure 12). Following gel filtration and SDS-PAGE analysis, GabR fractions corresponding to 13-17 mL (fractions 8-11) were pooled and concentrated to a final concentration of around 2.2 mg/mL. The total estimated yield of GabR for 6L of culture was 6.6 mg. This calculates to approximately 1 mg of GabR per liter of culture.
Figure 11. Gel filtration chromatogram of GabR run on a 24 mL Superdex 200 10/300 GL column. The numbers at the bottom of the chromatogram refer to the different fractions and the volumes at which they occurred.
<table>
<thead>
<tr>
<th>MW</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
<th>F6</th>
<th>F7</th>
<th>F8</th>
<th>F9</th>
<th>F10</th>
<th>F11</th>
</tr>
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</table>

Figure 12. SDS-PAGE analysis of GabR fractions collected following gel filtration chromatography on a Superdex 200 10/300 GL column. Fractions 1 (F1) through 11 (F11) were run on the gel.

DISCUSSION

1. GabR and ΔGabR Background Information

GabR regulation of the gabTD operon is a common pathway found in many bacteria. The pathway utilizes GABA, which can be found as debris in the soil or made intercellularly from glutamate and putrescine. *B. subtilis*, however, is only able to obtain GABA from extracellular sources, and does not have the genes to produce GABA from glutamate or putrescine. GABA has also shown to be toxic to *B. subtilis*, indicating that
they must maintain low intercellular concentrations. GABA is also the sole nitrogen source for *B. subtilis* (Belitsky and Sonenshein 2002).

GabR is a transcriptional regulator belonging to the MocR/GabR family of chimeric proteins. It contains a short N-terminal HTH domain and a long C-terminal PLP-binding AT domain (Belitsky and Sonenshein 2002). The modified ΔGabR protein contains a deletion of the N-terminal HTH domain.

2. Evaluation of Protocol I (pET28 vector) and Protocol II (pETite vector) in Producing Positive Expression Constructs of GabR and ΔGabR

Two different protocols were used to form the GabR and ΔGabR expression constructs. Protocol I (pET28 vector) was not successful in producing clones. The failure of Protocol I (pET28 vector) could be due to a number of reasons including low ligation efficiency or sub-optimal pET28 vector to PCR insert ratios. Protocol II (pETite vector), however, yielded 100% positive clones once the PCR polymerase was changed from Pfu Turbo polymerase to Pfu Ultra II Fusion HS polymerase (Figures 7 and 8).

3. Large-Scale Expression and Purification Optimization of GabR

Expression constructs of GabR and ΔGabR were transformed into BL21(DE3) cells. Initially, small-scale expression tests were performed to optimize expression conditions. Subsequently, large-scale 6L cultures of GabR were grown and a purification strategy involving Talon His-affinity chromatography and size-exclusion chromatography over a Superdex 200 gel filtration column was established. The
purification protocol successfully produced reasonably high yields of purified GabR protein.

4. Summary

In this study, two constructs of GabR, namely full-length GabR and ΔGabR were successfully cloned into a pET expression vector. The expression constructs were tested for recombinant protein expression in *E. coli* BL21(DE3) cells. Both GabR and ΔGabR were successfully expressed in BL21(DE3) cells under the conditions tested. The expression levels of GabR protein were found to be significantly higher than for ΔGabR under the expression conditions examined. Large-scale six-liter cultures were grown for GabR and a purification scheme was established for full-length GabR.

The next steps of this study would include the following: (a) optimization of ΔGabR protein expression conditions, (b) large-scale expression and purification of ΔGabR, and (c) optimization of GabR and ΔGabR purification protocols to obtain maximum yields of purified GabR and ΔGabR proteins for crystallization and X-ray crystallographic studies.

5. Future Directions

In order to elucidate how GabR functions as a transcriptional activator and as an autorepressor, it is important to determine the crystal structure of GabR so as to understand how the HTH and AT domains of the protein are organized, how the protein is folded, where the active site is, and how the domains interact with each other. In the long term, it would also be interesting to understand the structure of GabR in the presence
of DNA and the structures of both the apo protein and the DNA complex in the presence of GABA and PLP to reveal what conformational changes are brought about in the GabR protein upon DNA binding or upon binding of substrate and essential cofactor. This knowledge would be essential to understanding the role of GabR as a transcriptional regulator. The structure of GabR may eventually open up avenues for structure-based drug design aimed at GabR homologues in human bacterial infections, especially those with antibiotic resistance.
REFERENCES


Appendix I: GabR-His6 sequence

ATGGATATTCAGATTTACACACTCGATCGTTTCAGAAACAAAGCAGATTATATCTA
TCAGCAAAATTTATCAAAAGCTGAAAAAAGAAATCCTCAGCCGCAATCTGC
TGCCGCACTCGAAGGTTCCTCTTCAAAGCGGAGCTGGGCTGAATAATCTCAAG
GTCAGCGTAAATTCAGTGAATTCAGCCTATTCAGCAGCTGGCTGGCTGAGGG
GTATTTGTACGTCATTTTACGAAACGAGGGTTTCTTCTCGTGGGAGGAAGTACA
TGTTTTCCGCGGAGAGCAACCCTCCATTTGCACTGGCCGAGTGCCTAAAA
GAGATTGACATCTGAGCCAGAGCGATTGGATATCCTTTACACATGACTGTTCC
CGATACAGGCGAATTTCGATCAAAAGCTGGTTCCGGCTGCGAGCAAAAAAG
CGGCCTTCGGCTCATACCCGACGCTCGCGGAGTATGGCAGATCATTCCGCAAGGG
ATATATGAGAGAGAGCGGGCATATTAGGAAGGCTCATTTTGCAGAGAGGGG
TGTAAAAATGCGAGGGCAGGAAACAAATGACTATAGGGGAGGACAGCAGCTGTC
TCATGACGACTGTTGACTGAGCTTTTAACAAAGGAAGCCTGTTGATCGAGTG
GAGGAGGCTGGCTACAGGCGCATGATACAGCTTTTGAAGAATGCGGAAA
AACAAGTAAAGAGACGATCATGCTGGATGAAAAAGGCATGTGCGATTGGCTGAAA
TCACCGACAGCAGCAGGATGTGCTGGTGGTGACACCACCCCCGTCGCTATCGTTT
CCGGCGGAAACAGATATTGCTGTATCCAGAAGAAATCAGCTGCTGAACTG
GGCGAGCCGAGGACGCGCGCCGATATATCATTGAGGAGGATTATGATAGTG
AATTTACATATGATGATGACAGTATTTCCGGCGCTGCAAGGGCCTCGACGGT
TTTAAAAATGTACATCTATATGGGAAACCTTTTCCAAGTCCCTTCTCCCGG
CTTACGGATACGCTATATGTTGCGCTGCTGAGCGCTGGTGGAGGCGATACA
AACACGCGGGGGCTATGATCTGCAGACTTGCTCATCATTCCACAGCAGCTCACC
CTGCAAGGATTTATCGTTGGTGAATATCAGAAGCATAAAAAAAAT
GAAGCAGCATATTAAAAAGAAAAAGGAGAGACGCGCAGTACCGGCTTTAGAAG
CAGAGTTCCAGCGGAGAGGGTACCTGGAATAGGGCAATGCGGGGGCTGCAT
TTTTTACCAAGTTGATACCCGCCACGAGAACACGATCCCTGTCACA
TGCTGAGGAGGGCAGTGGTTTGAATATTCCGGAATGAGCCGAATTTAACTGGA
AGGAAAAAACGCGGCAAAAGGGGAGGCGCTGCTCTCTATTATCGGGCTTTGCA
GGGCTGAAGGAAGAGATTATTTAAGGAGGGTGCTGAGCGGGCTTTTCAAGGC
GGTTTACCGGACATAAAAATCCCCGGTTACAGGGGATcaccacacccacaccac
caccacTGA
Appendix II: ΔGabR-His6 sequence

ATGGAGCTGGCCTGAAAAATCTCAAGGGTCAGCGTAAATTCAATGGAATTCCAGC
CTATCAGCAGCTGCTGGCTGAGGGGTATTTGTACGCCATTTGAACGAAAGG
GTTTCTCTGGAGGAGAATGACATGTGTTTCCGCAGGGAGGACCCCTCCA
TTTGCACCTGGCAGATGACCTAAAGAAGAGATTCACATCGACCAGAGCGATTG
GATATCGTTTTCACACATGAGTTCCGATACAGACCAATTTCGATCAAAA
GCTGGTTCGGTCTGCAGAACAAGGCAGGCTCTCCGCTCATTACGACGACGCTC
GGCGATATGTCACTCCGCAAGGGGATATATGAAAGTGAGAGCGGCCATTAC
GAGGCTCATTTCCCTGAGACGAGGCGGTGAATAATGCAGGCAGGAAACAAATGA
TCATAGGGGCGACACGAGGTGCTCATGCAAGCCTGTTGACTGAGCTTTTA
CCCAAGGGAAGCCGTGTATAGCGATGGAGGAGCCTGGCTACAAGGGCGCATGTA
TCAGCTTTGAAAGAATCGGCGGAAAACAAAGTAAGGACGATCATGCTGGATG
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GTGACCACCCCGTCGCACTACGTTTTCGCTGGGAAAGCTATTATGCTGATTC
CAGAAGAATTTCAGCTGCTGAAGTGGGCAGCGAGCCGAGCCCGCCCGATATA
TCATTGAGGACGATTTATGATAGTGAAATTCCACATATGATGTAGACAGATT
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CTTTCCAAAGTCCCTTCTCCCCGGCCTACGGATCAGCTTATATGGTGTTCGC
CGCCTCTAGCTGTTGAGGGCATACAACACAGCGGGGGCTATGTGATCTCGAGAICT
TGCTCATACTCACAACAGCTCACCCTCCTGCGAGAATTATCAGGATCTGCTGGTG
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AACGCCGATCACCCTTGTAAAGCAGAGATTCAAGCGGAGGTTACCGTA
AAAGGCGAAATGCAGGGCTCAATTGGTTACCAGATTGGTACACACGGCG
CACCGAACAAGCATCCTGTCACTAGCTGCGGGGCTGAGCAGCTGGAAATAT
TCGGAATGACGCCTATTAACATGGAAAGGAAACAGCGGGAAAAACGGGCAGG
CCTGCTCTCATTATCGTCTTTGCACGGCTGGAAGGAAAGAAAGATATTCAGGA
GGGTGTGCAAGCGGCTTTTTCAAAGCGGTTTACGGAACATAAAAATACTCCCCG
TTACAGGGGATcaccaccaccaccaccacTGA