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Architecture of Bacterial Promoters; The case of the *Escherichia coli ogt* Promoter

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Abstract

All bacteria utilize RNA polymerase enzyme and transcription activator proteins to regulate gene expression in response to internal or external stress. Some bacterial promoters are regulated with only one transcription factor whilst two or more transcription activators regulate some other promoters. NarL is a transcription activator protein that activates the *E. coli yeaR* and *ogt* promoters in response to nitrate and nitrite induction in absence of oxygen. In the present study we have studied *ogt1052* promoter, which is a derivative of *ogt* promoter containing only one NarL binding site very close to -35 element. Therefore, it is considered as class II activator dependent promoter just as *yeaR* promoter. A molecular structure of *ogt1052* promoter was proposed which suggests that NarL binding site is located in opposite face of DNA that contains α-CTD and sigma domain 4 of RNA polymerase enzyme required for promoter recognition. The aim of the present study was to study and test the suggested molecular model by creating point mutations at -35 element and deletion of one base pair in spacer region, to test whether sigma domain 4 is necessary to bind -35 hexamer in order to start transcription initiation, and to test whether NarL activates the promoter by interaction with α-CTD in the opposite face of the DNA. Based on the result achieved, *ogt1052* promoter is a class I promoter "dressed" like a class II promoter.

Keywords: bacterial gene expression regulation, RNA polymerase enzyme, *ogt* promoter, two- component regulatory systems

1. Introduction

1.1 RNA Polymerase Recognition of Bacterial Promoters

All bacteria have DNA dependent multi subunit RNA polymerase enzyme that is essential for bacteria to be able to do transcription when necessary (Naryshkin et al., 2000). RNA polymerase (RNAP) is present within the bacteria in two different compositions, core enzyme and holoenzyme. The core enzyme that is not able to start transcription has a conserved subunit composition of (α_2 β β′ ω) with a molecular mass of approximately 400 KDa. However, when bacterial specific initiation factor, σ, binds to the core enzyme, it converts the core enzyme to holoenzyme which is capable to recognize specific sequences of DNA called promoters, and therefore it starts transcription in response to specific stress (Borukhov & Lee, 2005). The α subunit of RNAP is composed of two independently folded domains, α-CTD and α-NTD, that are linked by a flexible linker that is 20 amino acids long. The β and β′ subunits are large subunits that are assembled by amino terminal domain of α subunit (α-NTD) and are known as the active site of RNAP enzyme that has locations for DNA templates. The ω subunit is a small subunit that seems to assist the folding of the β′ subunit and it has no direct role in transcription (Browning et al., 2004).

The σ subunit or factor consists of four different domains (σ1, σ2, σ3, and σ4) joint by linkers that are responsible for promoter recognition (Murakami et al., 2003; Browning et al., 2004). RNAP recognizes promoters from four different sequences. Two principal sequences are the -35 hexamer (consensus sequence of TTGACA) and -10 hexamer (with consensus sequence of TATAAT), which are located at position 35 and 10 base pairs upstream (before) from the transcription start site respectively. The other two sequences are the UP element (20 base pare long) and extended -10 element (3-4 base per) that are located upstream of -35 hexamer and -10 hexamer, respectively. The hexamers are separated by spacer region, which is a nonspecific DNA sequence and is usually 16-18 base pairs long (Browning, et al., 2004; Borukhov and Lee, 2005). The α subunit of RNAP is responsible to recognize the UP element while Domain 3 of σ is responsible to recognize and bind to the extended -10 element; -10 element is recognized by σ domain 2 and -35 element is recognized by σ domain 4.

In prokaryotes, there are more than one σ factor. For instance, *E. coli* has one main σ factor known as σ^{70} and six other σ factors responsible to bind to RNAP to response to specific stress. Two of these specific response σ factors are σ^{H} and σ^{E} that bind to RNAP in response to heat shock stress in cytoplasm and periplasm respectively. The σ subunit ensures the recognition of promoter sequences and locates the RNAP holoenzyme to the recognized sequences and enhances the unwinding of the DNA duplex (Ishihama, 2000; Yura et al., 1999; Browning and Busby, 2004).

1.2 Regulation of Bacterial Gene Expression

Most of the bacteria are versatile organisms that are able to prosper in different environmental conditions. These organisms contain large genetic information that encodes mechanisms necessary for bacterium to cope with variety of challenges. Gene expression in bacteria is expensive, in terms of ATP consumption; therefore, the process is controlled in a way that prevents wasteful synthesis of unnecessary materials. This can be an explanation of why different bacteria produce different numbers of proteins in order to survive in variety of conditions (Dale & Park, 2004). Bacteria produce proteins by making messenger RNA (mRNA) copies using RNAP. mRNA carries the message that is translated into amino acids for producing specific proteins. Transcription is the first step in gene expression, which has three main stages, initiation, elongation and termination. In the initiation stage, RNAP holoenzyme is bound to -35 and -10 hexamers and forms the closed RNAP- promoter complex. Next, the DNA becomes unwound, forming a bubble, by isomerization in which the non-template DNA binds to σ2 of RNAP. This results in formation of a stable open complex. Short RNA products are synthesized and released in presence of NTPs and after, elongation stage starts. In elongation stage, the conformational change of RNAP-promoter open complex leads to loss of RNAP-promoter contact (σ factor dissociates).

Transcription factors are involved in regulation of gene expression at initiation stage. In some promoters, one transcription factor is required in order to activate the promoter (simple promoters), whereas, some promoters are complex and need two or more activators to start transcription initiation. There are three types of promoters being activated by three different mechanisms; Class I, class II and class III activator dependent promoters. In class I activator dependent promoters, there is only one binding site for the activator, upstream of -35 element and therefore, only one transcription factor interacts directly with α-CTD, recruiting RNAP to promoter. A good example of this type of promoter is the *E. coli lac* promoter that is activated by CRP (CAMP receptor protein) in response to lactose availability. In class II activator dependent promoters, there is only one binding site for the activator but located at roughly position -41.5 very close to -35 element and in some promoters, binding site overlaps with -35 element. In such promoters the activator binds to the sequence that overlaps with -35 and can contact to σ4, resulting the recruitment of RNAP to the promoter. For instance, bacteriophage λ PRM promoter is activated by λ CI protein that binds to σ4 of RNAP. In some of the class II activator dependent promoters, activator contacts to α-NTD, while binding to the sequence that overlaps the -35 element. In class III activator dependent promoters, there are two binding sites for the transcription factors, which activate the promoter in response to multiple signals. One site is located upstream of -35 element and the other binding site is positioned very close to -35 element and sometimes overlaps with the element. There are two mechanisms by which promoter is activated by class III mechanism: the first one is when one activator functions as class II and the other one functions as class I. the best example can be the *proP* P2 promoter in which CRP and fis activate the promoter as class I and class II respectively. The second mechanism is when both activators function as class I promoter in both activate promoter-binding sites. For instance, *acs* P2 in which CRP activates the promoter by binding to α-CTD and functions as class I (Busby et al., 1996; Scott et al., 1995; McLeod et al., 2002; Beatty et al., 2003; Browning & Busby, 2004).

1.3 Responses to Nitrate and Accompanying Stress (NarL; NarP; NsrR; NorR)

In pathway of nitrate reduction, reactive nitrogen species (RNS) are produced from which the bacteria need to be protected. Transcription regulators such as NarL, NarP, NsrR and NorR are involved in gene regulation in *E. coli* in response to RNS (Lin & Stewart, 2010; Hartig et al., 1999). A two-component system named the Nar system in *E. coli* is composed of four regulatory proteins, NarX and NarQ which are receptors located in the cell membrane and NarL and NarP which are the response regulators located in the cytoplasm of the *E. coli*. Together, these proteins activate the transcription of genes that are needed in anaerobic respiration condition and presence of nitrate and nitrite that are electron acceptors in the absence of oxygen. NarX and NarQ are activated by extracellular nitrate or nitrite and create a signal by phosphorylation. In turn, phosphorylation causes conformational change in NarL and NarP respond regulators that causes the interaction of NarL and NarP with Nar-regulated promoters to regulate transcription of genes positioned downstream of promoter (Zhang et al., 2003; Lin & Stewart, 2010; Egan & Stewart, 1991; Noriega et al., 2010). NsrR and NorR are activated in response to cytoplasmic nitric oxide (NO) in order to repress transcription. NsrR is a global regulator while NorR is a specific regulator that interacts only by σ^{54} and controls the transcription of *norVW* gene. The product of this gene is an enzyme, which is required in

cell for NO detoxification. Another enzyme which is produced by *hmp* gene that is activated by NsrR has a key role in cytoplasmic NO detoxification as well (Patridge et al., 2009). NarL and NarP sense the extracellular RNS and anticipate the DNA or cellular damage. Therefore, they are activated to protect the cell from any upcoming damage. In contrast, NsrR and NorR sense the cytoplasmic NO and act when NO is inside the cell and cell is partially damaged and needs detoxification (Lin & Stewart, 2010; Patridge et al., 2009).

1.4 The ogt Promoter and the ogt1052 Model

The *E. coli ogt* promoter encodes O^6 alkylguanine DNA alkyltransferase (a DNA repair protein) in response to DNA damage caused by reactive nitrate species. This promoter is activated by NarL and NarP transcription factors in absence of oxygen and in presence of nitrate and nitrite (Squire et al., 2009). Most of the promoters regulated by NarL and NarP are regulated by FNR "the master anaerobic regulator" as well. However, studies have shown that NarL and NarP can activate *ogt* promoter independent of FNR (Constantinidou et al., 2006). *ogt* promoter contains two 7-2-7 NarL binding sites centered at positions -78.5 and -45.5. Both locations of NarL in *ogt* promoter must be occupied with NarL proteins in order to activate the promoter. According to this fact, *ogt* promoter is known as a class III activator dependent promoter (Squire et al., 2009).

It was reported that *yeaR* promoter which is a class II dependent promoter and contains only one NarL binding site centered at position -43.5, was expressed 5 folds greater than *ogt* promoter. This indicates that NarL binding site in *yeaR* promoter corresponds better to the consensus sequence (Squire et al., 2009). Based on this finding, it was suggested that *ogt* promoter with single NarL binding site, which corresponds better to the consensus sequence, can have a higher activity compare to the *ogt* promoter with two weak NarL binding sites. Therefore, two *ogt* promoter mutants were constructed by disrupting one NarL binding site and optimizing the other one thus it corresponds better to the consensus sequence. The derivatives were introduced each containing single NarL binding site, one centered at position -78.5 named *ogt1041* and the other one centered at position - 45.5 named *ogt1052*. *ogt1041* functions as class I activator dependent promoter whilst *ogt1052* is known to function as class II activator dependent promoter. It was reported that the activity of *ogt1052* is much higher than the activity of *ogt1041*, which confirms the fact that an activator-binding site located close to the core promoter functions more effectively (Chismon, 2010).

In the present study we have tested this model in order to investigate whether σ4 of RNAP is essential for promoter recognition in *ogt1052* promoter and if NarL dimer interacts with α-CTD in order to activate the promoter in the opposite face of DNA. We have done point mutations at -35 element to test the interaction of σ4 with -35. We have created a mutant of *ogt1052* containing 17bp spacer and compared it with the *yeaR100* promoter that also contains 17bp spacer to test if the mutant has the same promoter activity as the *yeaR100* promoter has. *yeaR* promoter is a class II activator dependent promoter. The NarL binding site in *yeaR* promoter overlaps with -35 element. By deleting 1bp of *ogt1052* spacer, in case *ogt1052* is a class II activator, binding site should overlap -35 element and the activity of both promoters will be nearly the same.

2. Methods

2.1 Bacterial Strains, Plasmids and Promoter Fragments

Escherichia coli K-12 strains and promoter fragments used in this study. Each strain was streaked on an agar plate containing lactose and relevant antibiotic, and was incubated in 37°C with 200rpm shaking in a conical flask containing 2XLB and relevant antibiotics. At all stages of the bacterial growth, aseptic technique was applied. Plasmid used in this study is pRW50, a low-copy number lac expression vector that clones the EcoRI-HindIII fragments as transcriptional fusion to lacZ. The plasmid encodes tetracycline resistance and was used in all β-galactosidase assays.

2.2 Bacterial Media, Solutions, Buffers, Reagents and Antibiotics

Specified amount of bacterial media as described below were dissolved in distilled water and autoclaved for 20 minutes at 120 C, 1 atmosphere.

LB (Luria Broth)**:** It was purchased from Sigma. It contained 10g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl. 1X LB was prepared by dissolving 20 g powder in 1 L of distilled water.

LB agar**:** It was purchased from Oxoid. LB agar was prepared by dissolving 28 g powder in 1 L of distilled water.

MacConkey lactose agar**:** MacConkey lactose agar was prepared by dissolving 50 g powder in 1 L of distilled water. It was purchased from Difco.

Table 1 Bacterial strains and promoter fragments used in the study

Strain	Genotype
JCB387	*nir*, *lac* (Page et al., 1990)
JCB3875	*narP* derivative of JCB387 (Page et al., 1990)
JCB3883	*narL* derivative of JCB387 (Page et al., 1990)
JCB3884	*narLP* derivative of JCB387 (Page et al., 1990)
Promoter fragment	Description
Ogt1052	Derivative of the *ogt100* promoter fragment carrying mutations at -85,-63,-51,-48,-44 and -42.
Ogt1052A1	Derivative of the *ogt1052* promoter fragment carrying mutations at position-36.
Ogt1052A2	Derivative of the *ogt1052* promoter fragment carrying mutations at position -35.
Ogt1052A3	Derivative of the *ogt1052* promoter fragment carrying mutations at position -28.
yeaR100	*E. coli yeaR* promoter fragment carrying nucleotide sequences from -294 to +96.

MS (Minimal Salts) medium: 1 l MS medium contained 4.5 g KH_2PO_4, 10.5 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 0.5 g Tri-Sodium Citrate, 0.05 g $MgSO_4.7H_2O$, 1 ml *E.Coli* Sulphur-free salt (82 g $MgCl_2.7H_2O$, 10 g $MnCl.4H_2O$, 4 g $FeCl_2.6H_2O$, 1 g $CaCl_2.6H_2O$, 2o ml HCL per 1 l of distilled water), 1 ml sodium selenate (1mM), 1 ml ammonium molybdate (1 mM), 0.1 g caseine digest, 0.05 g yeast digest, 0.32 g sodium fumarate, 8 ml 50% glycerol.

SOC medium: It was purchased from Sigma. It contained 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 8.6 mM NaCl, 3.5 mM KCl, 20mM $MgSO_4$, 20 mM glucose.

All the solid and liquid autoclaved bacterial media were supplemented by tetracycline during the study. Antibiotic was added to the media after it had cooled down enough. Tetracycline was prepared by dissolving 20 mg of tetracycline stock in 1 ml of 100% methanol.

2.3 Measurements of Promoter Activity

β-galactosidase activity assays: β-galactosidase was measured using the Miller protocol. β-galactosidase assay was used to observe the activity of promoter in *vivo* by measuring the amount of β-galactosidase within cell extract. The promoter of interest was cloned in to pRW50 containing *lacZ* gene and was prepared for the assay. Assay was done with two sets of capped tubes containing minimal media only and minimal media supplemented with 20 mM sodium nitrate as final concentration in anaerobic condition. Bacteria containing the cloned promoter were left in 37°C and optical density (OD) was checked until it reached to 0.5 in 650 nm. Tubes were kept in ice in case of having rapid growth compare to other tubes and when all the tubes reached to the OD between 0.480-0.500, 2 ml of each cultures were vortexed with 30 l 1% sodium deoxycholate and 30 l toluene in order to lysate. While prepared tubes were shaking for 20 minutes, assay tubes containing z buffer were warmed at 30 C. After that, 100 l of the lysate was added and mixed in the assay tubes. The reaction was stopped by adding 1M sodium carbonate after yellow color developed. Then, OD 420 was measured and used in the formula to calculate promoter activity.

2.4 molecular Biology Methods

Agarose gel electrophoresis: TBE buffer (0.5X) containing agarose powder was boiled in microwave oven until totally dissolved. The agarose concentration was 0.8% for separating large fragments like plasmids and 1%-1.5% concentration for separating small fragments like Polymerase Chain Reaction (PCR) products. Sybrsafe™ 10,000X (Invitrogen) was added to make the final concentration of 1X before the gel was poured. DNA samples were mixed with 6X Green DNA loading dye (Fermentas) to make a final concentration of 1X. The DNA samples were run together with DNA ladder such as 100 bp ladder or 2-log ladder (NEB). Electrophoresis was done at 100V in 0.5X TBE buffer for about 30 minutes or when fragments separated adequately for observation.

Plasmid isolation: pRW50 is the plasmid used in this study and was isolated through QIA prep Mini-prep Spin Kit following provided protocol from the manufacturer. The day before isolation, a 5 ml 2x LB containing tetracycline culture was inoculated by a single colony containing pRW50 and was shake overnight in 37°C and before isolation cell pellet was collected by centrifugation at 4,000 rpm for 1minute.

DNA purification: In order to gain concentrated plasmid DNAs, the equal volume of phenol/chloroform was added to the sample. The mixture was vortexed until the emulsion was formed then it was centrifuged at 13,000 rpm for 1 minute to separate organic components from the aqueous phase. The aqueous phase was taken to a new micro

centrifuge tube. In order to obtain more DNA, TE buffer was added to the organic phase followed by vortex and re-centrifugation. The upper aqueous phase was taken and combined with the previous one (final volume ~400 µl). The next step was alcohol precipitation. The process removes chloroform traces and precipitates concentrated DNA. 44 µl of 3M sodium acetate (pH 5.2), 4 µl of 1M magnesium chloride and 888 µl of iced cold (-20°C) 100% ethanol was added. The DNA sample was incubated for 15 minutes at -80°C. The sample was centrifuged at 14,000 rpm (4°C) for 15 minutes. The supernatant was removed and the pellet was suspended again by 70% iced cold ethanol before re-centrifugation. Supernatant was discarded and the DNA pellet was vacuum-dried for 10 minutes to get rid of ethanol. The last step was to re-suspend the pellet with 50 µλ TE Buffer. The more convenient way to clean up DNA was using QIAquick PCR purification kit. In appropriate pH and high salt condition, the DNA bound to the silica membrane of the spin-column while proteins and free nucleotides could not be absorbed thus passed through the column. The same protocol as described by the manufacturer was applied when DNA gel extraction was done by QIAquick gel extraction kit.

Ligation for DNA recombination: DNA ligation was done to insert the desired DNA fragment into the pRW50 vector. T4 DNA ligase (NEB) was used to connect 5′ phosphate and 3′ hydroxyl groups by forming phosphodiester bonds. The protocol involves 2 µl of dephosphorylated plasmid vector, 1 l of T4 DNA ligase, 2 µl of 10X T4 ligase buffer and 10 to 15 µl of inserted DNA. The total volume was made to 20 µl using sterile water. The mixture was left incubate at room temperature for at least 2 hours until transformation. Also, the control was done using the same protocol but without inserted DNA.

Transformation of DNA: In order to do transformation, competent cells were prepared according to the protocol below:

Five ml of 2X LB media containing tetracycline was inoculated with a single colony picked from the agar plate. The culture was incubated shaking overnight at 37°C. The next day, 100 ml of 2X LB media within 500 ml conical flask was inoculated with 1 ml of the overnight culture. The flask was incubated shaking until the optical density at 650 nm (OD 650) reached around 0.4-0.5. The flask was incubated on ice for another 10 minutes then the content was transferred to two 50 ml centrifuge tubes in order to centrifuge at 4,000 rpm at 4°C for 9 minutes to collect the cell pellet. The supernatants in both tubes were discarded. Thirty ml of TFB1 buffer was added to one tube to re-suspend the pellet then the whole content was transferred to the second tube. After re-suspension, the tube was placed in ice for at least 90 minutes. Centrifugation was done as before and the supernatant was discarded. The pellet was re-suspended with 4 ml of TFB2 buffer. Aliquots of 300 µl were prepared and immediately frozen at 80°C.

Transformation method: Plasmids from mini-prep or ligation reaction were transformed to competent cells. Started by taking competent cells from -80°C and placing them in ice to allow cells to defrost, 40 µl of thawed cells were transferred to the prepared micro-centrifuge tubes containing 5- 10 µl of ligation mix or 1 µl in case of plasmids gained from mini-prep. The tubes were placed in ice for 30 minutes. Heat-shock was done by incubating the tubes in 42°C water bath for 42 seconds before immediately placing the tubes back in ice. Heat-shock made cell walls permeable for plasmids to enter to the cells easily. After 5 minutes, 250 µl of room temperature SOC broth (Sigma) was added to the transformed cells. The tubes were incubated shaking at 37°C for 30 minutes. Finally, 100 µl of the transformation reaction was plated on appropriate media agar. Plates were incubated overnight at 37°C.

DNA restriction: Digestion of DNA was done using <5% (v/v) restriction enzyme with 10X restriction buffer provided with the enzyme. Plasmid DNA was digested at 37°C for at least 3 hours to get plasmid totally cut while PCR product digestion was done for less than 1 hour. After complete digestion, DNA purification was done using PCR purification kit (QIAquick). Then, the required DNA fragments were collected by gel extraction after analyzing by gel electrophoresis. Multiple enzymatic cuts at the same time needed a control digestion in order to check for complete digestion. Prior to digestion, 2µl of DNA-buffer was taken out. The DNA-buffer mixing was separated to two tubes and enzyme was added to each tube. 2 µl of each was taken out. Then, the two tubes were mixed together. After digestion, another 2 µl of DNA-buffer was taken. All control samples were run by gel electrophoresis to compare and ensure that the DNA was cut completely.

DNA vector dephosphorylation: During preparation of the plasmid vector, calf intestinal phosphatase (CIP, from NEB) was additionally added after restriction digestion to prevent re-ligation by removing 5′ phosphates. DNA vector without 5′ phosphates cannot perform re-circularization, thus increasing cloning efficiency and decreasing background problems. CIP is active in NEBuffer 3, which gives the condition of 100mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$ (pH 7.9 at 25°C). It also works well in NEBuffer 2, 4, NEBuffer for EcoRI and BamHI. Preparation of plasmid vector was done by growing 6 cell cultures of JCB387 containing the pRW50 plasmid overnight in 5 ml rich media. After centrifugation at 4,000 rpm for harvesting, the plasmid was extracted separately by mini-prep

(QIA-prep mini-prep Spin Kit). EB buffer was used to elute the plasmid to get final 50 l volume of each, thus total 300 µl was obtained when added altogether. Digestion with appropriate restriction enzymes was done followed by adding 5 µl of CIP to the mixture. The plasmid vector was purified by QIA-quick PCR purification kit after incubating at 37°C for at least 30 minute.

Polymerase Chain Reaction: PCR is a method using DNA polymerase and two oligonucleotides as primers to amplify DNA template. All primers used in this study were synthesized by Alta Bioscience, University of Birmingham; UK. The enzyme used was polymerase (NEB), which contains 5′-3′ DNA polymerase activity and 3′-5′ exonuclease activity. If proof-reading was not required for PCR, Taq polymerase (Bioline) was used instead. The protocol of 50 µl of PCR reaction using Phusion as DNA polymerase contained 10 µl of 5X reaction buffer, 0.1 µM of each primers, 1µl of the template (e.g. plasmid DNA), 4 µl of 2.5 mM dNTP mix, 0.5 µl of Phusion and 15.5 µl SDW. The program was set at 98 °C, 1.30 minutes for initial denaturation. The 30 cycles were set at 98°C, 10 seconds for denaturation; 40-60°C, 20 seconds for annealing depending on annealing temperature of primers used; 72°C, 15 seconds per kb for extension. The final stage was set at 72°C for 5 minutes then immediately cooled down to 4 °C.

2.5 Site-Directed Mutagenesis

In this study, *ogt1052A1* and *ogt1052A2* fragments were constructed by point mutation using mega-primer PCR method. Fragments were cloned to pRW50 plasmid and were synthesized in the first-round PCR. In the first-round PCR, D10527 flanking primer was used with the mutagenic primer. For the second-round PCR, 10-15 µl of mega-primer products of the first-round PCR purified from gel were used as a primer of the second-round PCR together with primer D10520. This step created a full length fragment with mutation at the desired point. After PCR purification, the PCR products were digested with EcoRI and HindIII, and then cloned into pRW50 vector by ligation method that is explained above.

Deletion of DNA using overlapping PCR product: In this study, *ogt1052A3* fragment was constructed with overlapping PCR. Two PCR products were generated separately in the first-round PCR. One PCR used the upstream D10520 flanking primer while the downstream primer (f1052A6) was designed to carry sequence-carrying deletion. Another PCR used the downstream D10527 primer with the upstream primer (e1052A6) carrying deletion.

DNA sequencing: Functional Genomics and Proteomics Laboratory, University of Birmingham sequenced Plasmids. Plasmids were mini-prep and stored as stock. Three µl of plasmids and 3.2 µl of D10527 plasmid and 3.8 µl of distilled water were mixed for sequencing.

3. Results

3.1 Activity of the ogt1052 Promoter

The result clearly indicates that in presence of NarL and NarP activators, the *ogt1052* promoter is highly expressed while in absence of these activators the expression of promoter is highly reduced. It can be concluded from the result that presence of NarL and NarP activators is necessary for activating the *ogt1052* promoter. It can be clearly seen from the Figure 1 that *ogt1052* promoter in JCB387 cells is more activated in growth medial supplemented with nitrate in compare to the promoter activity in growth media without nitrate induction. The expression level of the *ogt1052* promoter in JCB3884 cells with or without nitrate induction is the same as the control vector pRW50. Therefore, comparing the activity of *ogt1052* promoter in JCB387 with promoter activity in JCB3884, it can be concluded that nitrate induction results in high expression level of the *ogt*1052 promoter only in the case that NarL and NarP are present in the cell. Based on this result, *ogt*1052 is an activator dependent promoter that only is activated in response to extracellular nitrate to protect DNA from any damage caused by RNS.

3.2 Mutations in the ogt1052 Promoter -35 Hexamer

An experiment was planned to see the effect of different mutations in RNA polymerase recognition of sigma domain 4 and to check whether sigma domain 4 is necessary for the promoter activation or to see if C- terminal domain of RNA polymerase alpha subunit (α-CTD) can bind to NarL and activate the promoter *ogt1052* instead. Two point mutations were introduced in -35 element of *ogt1052* promoter fragment. The sequence of the -35 element in this promoter is TGGCTG and the consensus sequence for -35 hexamer is TTGACA (Browning & Busby, 2004). The first mutation was done in order to replace one base in -36 position to make the sequence of the hexamer to be more like the consensus sequence (G◊T) and was named *ogt1052A1*. The second mutation was done to replace one base of the -35 hexamer at position -35 to have mutant, which has the sequence that is less like the consensus sequence and is named *ogt1052A2* (G◊T). The mutants were constructed by mega primer PCR method. Based on the results achieved, -35 element plays an important role in promoter activation with or without presence

of NarL activator protein. In summary, recognition of -35 hexamer by sigma domain 4 of RNA polymerase is necessary for transcription activation. NarL is not the only factor for promoter activation and *ogt* promoter can be activated even in the absence of NarL with a sequence that corresponds to the consensus sequence of -35 element.

3.3 Spacer Mutation

NarL can activate both ogt and *yeaR* promoters in response to RNS. Therefore, an experiment was planned to create a mutation in spacer region of ogt1052 promoter to measure the activity of both promoters in the presence and absence of NarL. By deletion of one base pair in spacer region of ogt1052 the NarL, binding site was expected to overlap the -35 element and function like *yeaR* promoter. In order to do this experiment, spacer mutation was done by overlapping PCR and the mutant was named ogt1052A3. The *yeaR* 100 promoter fragment was used along in this experiment to compare the activity of ogt1052A3 promoter. The activity of *yeaR* promoter in the presence of NarL is much higher compare to its activity when NarL is not present in the cell. Activity of promoter when NarL is present is higher with nitrate induction compare to the growth condition that is not supplemented by nitrate. This confirms that *yeaR* promoter is activated with NarL in response to RNS. There is a slight difference between the expression level of *yeaR* with nitrate induction and without nitrate induction in JCB3884 (NarL is not present). This can be due to regulation of NsrR that was discussed previously in section 1.3. Following the experiment, the suggested model of *ogt1052* was tested by *ogt1052A3* mutant. The promoter fragment was cloned into pRW50 vector as promoter::*lacZ* fusion and was transformed into JCB387 and JCB 3884 cells. Cells were grown anaerobically in minimal media only and minimal media supplemented with nitrate to the final concentration of 20 Mm. Based on the results achieved, the spacer mutation does not play an important role in the presence of NarL. Also, the results show that the activation of *ogt1052A3* promoter is more like the *yeaR* promoter. This indicated that the spacer mutation only is important and causes activation of *ogt1052A3* promoter more like the *yeaR* promoter only in absence of NarL activator.

4. Discussion

This experiment shows that *ogt* promoter does not contain FNR binding site. Therefore, it is not activated by FNR. Two weak NarL binding sites identified by Squire et al, makes the promoter to function as class III and each derivative of this promoter with only a single NarL binding site, *ogt*1042 and *ogt*1052, to function as class I and class II promoters, respectively. These characteristics of ogt promoter make it a good model for studying promoter regulation in three different classes. Generally, we call a promoter class I when the activator binding site is located upstream of -35 element and the activator directly interacts with α-CTD of RNAP subunit and recruits RNAP to start transcription. In a class II promoter, the activator binding site overlaps with -35 element and it contacts sigma domain 4 of RNAP and enhances RNAP promoter recognition. A good example of class II promoter is the *yeaR* promoter in which there is only one NarL binding site overlapping with -35 element. In *yeaR* promoter, there is another sequence that overlaps with NarL binding that belongs to NsrR repressor. NsrR regulates the promoter by sensing the intracellular nitrate or RNS and activating the transcription of genes, which encodes the enzyme necessary for detoxification. This mechanism protects cell from further DNA damage. Whereas in case of *ogt* promoter, NarL/P involves in two-component regulatory system called Nar as response regulator. The system contains two receptors in cell membrane, NarX and NarQ that sense the RNS and conformational change results in NarL binding to its target sequence at promoter. So we can conclude that *ogt* promoter anticipates the DNA damage by RNS while NsrR in *yeaR* promoter is activated when RNS are in the cell and causing cellular or DNA damages to cell.

In conclusion, if we consider *ogt*1052 promoter as a class II promoter, the NarL binding site should overlap with -35 element. In the present study we obtained results that confirms that binding site is not overlapping with -35 element as it showed different activity compare to *yeaR* promoter. Therefore, the *ogt* 1052 is a class I promoter but seems to be class II at first sight. The NarL binding site is located at position -45.5 but in the other face of the DNA. This explains why even though it is close to -35 element but it's not contacting with sigma domain 4. So if it is a class I promoter NarL should directly bind to α-CTD and activate the promoter. The residues involved in this interaction were identified recently. Two important residues in α-CTD are 288/273 and 293 and one, interacting with these residues in NarL dime is 178/179.

It should be mentioned that the result about the sigma domain 4 experiment clearly showed that RNAP is not able to recognize promoter without sigma domain four, Whereas, the promoter can be activated if containing the -35 hexamer that corresponds better to consensus sequence.

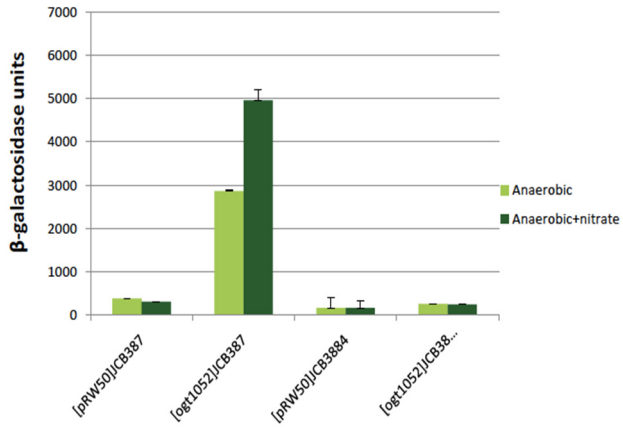

Figure 1. Measured β-galactosidase activity in different E.coli strains

Note. The figure illustrates measured β-galactosidase activity in JCB387 (both NarL and NarP activators are present in the cell) and JCB3884 (neither NarL nor NarP activators are present in the cell) cells carrying pRW50 as control vector and pRW50 containing ogt1052 promoter fragment. Cells were grown in minimal salt media in anaerobic condition and where indicated, sodium nitrate was added to the final concentration of 20 mM. Data shown in figure are averages of three biological repeat for each plasmid. Error bars were put to show the standard deviation from the mean. The activity of ogt1052 promoter in JCB387 is much higher than the activity of the promoter in JCB3884. It shows the necessity of NarL and NarP presence for the activation of ogt1052 promoter. In JCB387 cells, the promoter is highly expressed with nitrate induction while in JCB3884, even though growth media is supplemented by nitrate, the activity is as low as the control vector pRW50. Therefore, the result shows the promoter is highly expressed through nitrate induction only in the presence of NarL/NarP.

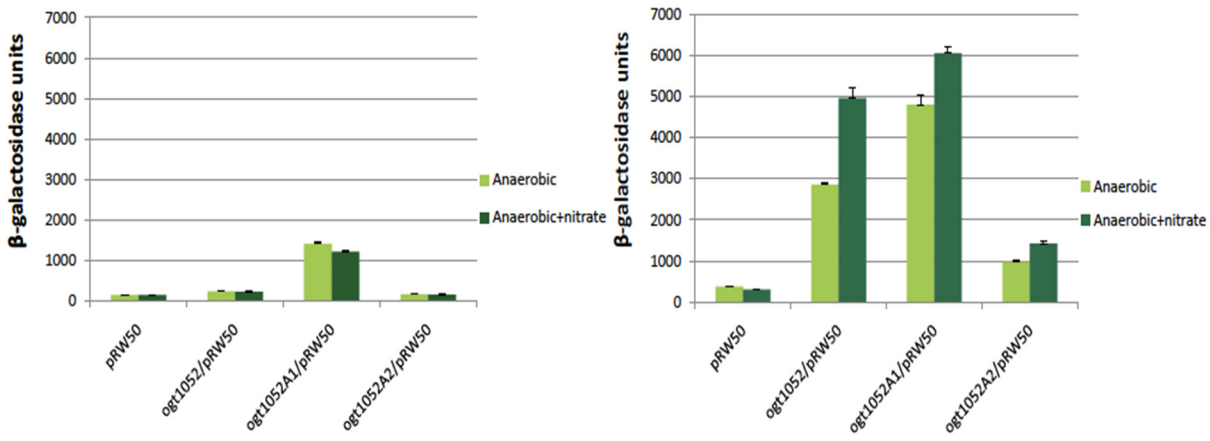

Figure 2. Measured β-galactosidase activity in different E.coli strains containing mutation in -35 element.

Note. The left chart reveals measured β-galactosidase activity in JCB387 (both NarL and NarP activators are present in the cell) cells carrying pRW50 as control vector and pRW50 containing ogt1052, ogt1052A1, *ogt1052A2* promoter fragment respectively. Cells were grown in minimal salt media in anaerobic condition and where indicated, sodium nitrate was added to the final concentration of 20 mM. Data shown in the figure are averages of three biological repeat for each plasmid. Error bars were put to show the standard deviation from the mean. Ogt1052A1 is highly expressed in compare to ogt1052 and ogt1052A2 which shows that -35 hexamer with sequence similar to the consensus sequence can be recognized by RNA polymerase and can activate the promoter. The activation of the promoter in mutant Ogt1052A2 is highly reduced compare to *ogt1052A1* that means NarL is not the only factor, which can activate the promoter.

The right chart shows measured β-galactosidase activity in JCB3884 (neither NarL nor NarP activators are present in the cell) cells carrying pRW50 as control vector and pRW50 containing *ogt1052, ogt1052A1, ogt1052A2* promoter fragment respectively. Cells were grown in minimal salt media in anaerobic condition and where indicated, sodium nitrate was added to the final concentration of 20 mM. Data shown in the figure are averages of three biological repeat for each plasmid. Error bars were put to show the standard deviation from the mean. *ogt1052A1* still is expressed in absence of NarL but the expression is not as high as when NarL is present. The result shows that promoter can be activated by -35 hexamer with near consensus sequence independent from NarL.

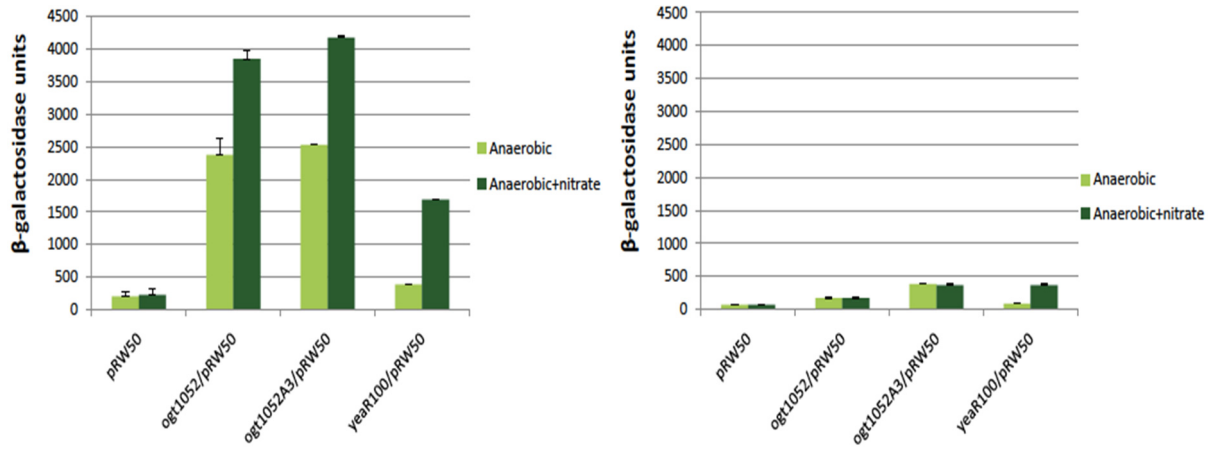

Figure 3. Measured β-galactosidase activity in different E.coli strains containing spacer mutation

Note. The left chart reveals measured β-galactosidase activity in JCB387 (both NarL, and NarP activators are present in the cell) cells carrying pRW50 as control vector and pRW50 containing *ogt1052, ogt1052A3*, yeaR100 promoter fragments, respectively. Cells were grown in minimal salt media in anaerobic condition and where indicated, sodium nitrate was added to the final concentration of 20 mM. Data shown in the figure are averages of three biological repeats for each plasmid. Error bars were put to show the standard deviation from the mean. The activation of *ogt1052A3* promoter is similar to the activation of *ogt1052* promoter, which means that with spacer mutation it still acts like the *ogt1052* promoter than like *yeaR* promoter.

The right chart shows measured β-galactosidase activity in JCB3884 (neither NarL, nor N NarP activators are present in the cell) cells carrying pRW50 as control vector and pRW50 containing *ogt1052, ogt1052A3*, *yeaR100* promoter fragments, respectively. Cells were grown in minimal salt media in anaerobic condition and where indicated, sodium nitrate was added to the final concentration of 20 mM. Data shown in the figure are averages of three biological repeats for each plasmid. Error bars were put to show the standard deviation from the mean. The activation of *ogt1052A3* promoter is more like the *yeaR* promoter. This indicated that the spacer mutation only is important and causes activation of *ogt1052A3* promoter more like the *yeaR* promoter only in absence of NarL activator.

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