Architecture of bacterial promoters:

the case of the *E. coli ogt* promoter

By

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Synopsis

The *Escherichia coli ogt* gene encodes an O^6-alkylguanine DNA alkyltransferase, which is reported to repair DNA against methylation damage caused by reactive nitrogen species, which are generated during an anaerobic respiration in a presence of nitrate. NarL regulator is one part of a two component system that controls many genes, mostly related to nitrate respiration. This study examined transcription activation by NarL at the *ogt* promoter using biochemical techniques with various semi-synthetic *ogt* promoters.

The *ogt* promoter has two crucial DNA sites for NarL at positions -78.5 and -45.5 relative to the transcript start site. NarL binds as a dimer to the target binding sites and directly interacts with the C-terminal domain (CTD) of α subunit of RNA polymerase. One αCTD contacts with the NarL monomer located at the downstream half of the distal NarL site upstream of the -35 element. Another αCTD contacts with the NarL monomer located at the upstream half of the proximal NarL site adjacent to the -35 element.

An interaction between NarL and αCTD was investigated using site-specific mutagenesis. Locations and orientations of NarL and RNA polymerase in the transcript initiation complex were also confirmed in this study. It was found that NarL, at position -45.5, is located on the different DNA helical face from αCTD, which binds to the minor groove immediately upstream the -35 element. This unrecognized promoter architecture allows residue 273 of αCTD to interact with residue 178 of NarL.

Dedicated with love to my family

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Chapter 1: Introduction

1.1 *Escherichia coli*: how it survives in environment

Escherichia coli is a common facultatively anaerobic gram-negative bacterium. The ability to detect and respond to intra/extra-cellular stimuli via gene expression modulation allows *E. coli* to grow in a wide-range of environmental conditions including the mammalian gastrointestinal tract (Drasar *et al.,* 1966). *E. coli* has evolved various fundamental mechanisms to maintain survival under unfavorable environments. It can survive in both soil and water by adapting to nutrient abundance, carbon sources, oxygen, UV, water, acid conditions, etc (Jones *et al., 2*007).

Normally, bacteria stop growing in a sub-optimal habitat; however, they can use various strategies involving maintenance metabolism, protective substances and pathways of repair and detoxification in order to overcome antagonistic conditions. In many environments such as the gut of animals, *E. coli* must adapt to respire efficiently in the absence of oxygen by using alternative available electron accepters such as nitrate and nitrite, which are available in the gut (Richardson, 2000; Saul *et al.,* 1981). It has developed systems to protect itself from cytotoxic intermediates generated from nitrate/nitrite metabolism and host defence systems. The survival strategies involve detection and detoxification of harmful by-products, as well as repairing DNA damage (Nathan and Shiloh, 2000).

1.2 Anaerobic adaptation and nitrate respiration

E. coli can adapt to survive in both aerobic and anaerobic conditions (Cole, 1996; Drasar *et al.,* 1966). In aerobic environments, oxygen serves as the most powerful terminal electron acceptor, releasining energy in the process of electron transfer pathway (Richardson,

2000). Therefore, *E. coli* can grow rapidly in the presence of oxygen. In anaerobic environments, alternative electron accepters are utilized during anaerobic respiration. In the host's intestinal tract, nitrate (NO_3^-) and nitrite (NO_2^-) are available and serve as oxidants in redox reactions. In anaerobic conditions that lack alternative exogenous electron acceptors, *E. coli* is also able to use fermentation of carbon sources to generate ATP via substrate level phosphorylation (Klein *et al.*, 2006; Richardson, 2000; Unden and Bongaerts, 1997).

During respiratory growth, electrons, generated from an electron donor, are transferred through an electron transport chain to an electron acceptor, causing proton accumulation outside the cell membrane. This proton gradient creates the proton-motive force that drives the synthesis of metabolic energy in the form of ATP. In the absence of oxygen, alternative terminal electron acceptors are used. Among various alternatives, nitrate has a higher electronegativity and serves as the most preferable oxidant (Ferguson, 1998; Richardson, 2000).

Figure 1.1 illustrates how a transmembrane proton gradient is generated in coupling with electron transfer. Nitrate reduction in *E. coli* is driven by multiple forms of nitrate reductases under different circumstances and generates subsequent toxic intermediates, often known as reactive nitrogen species such as nitric oxide, nitrous oxide, and other toxic nitrogen compounds (Berks *et al.*, 1995; Corker and Poole, 2003; Lundberg *et al.*, 2004; Philippot and Hojberg, 1999).

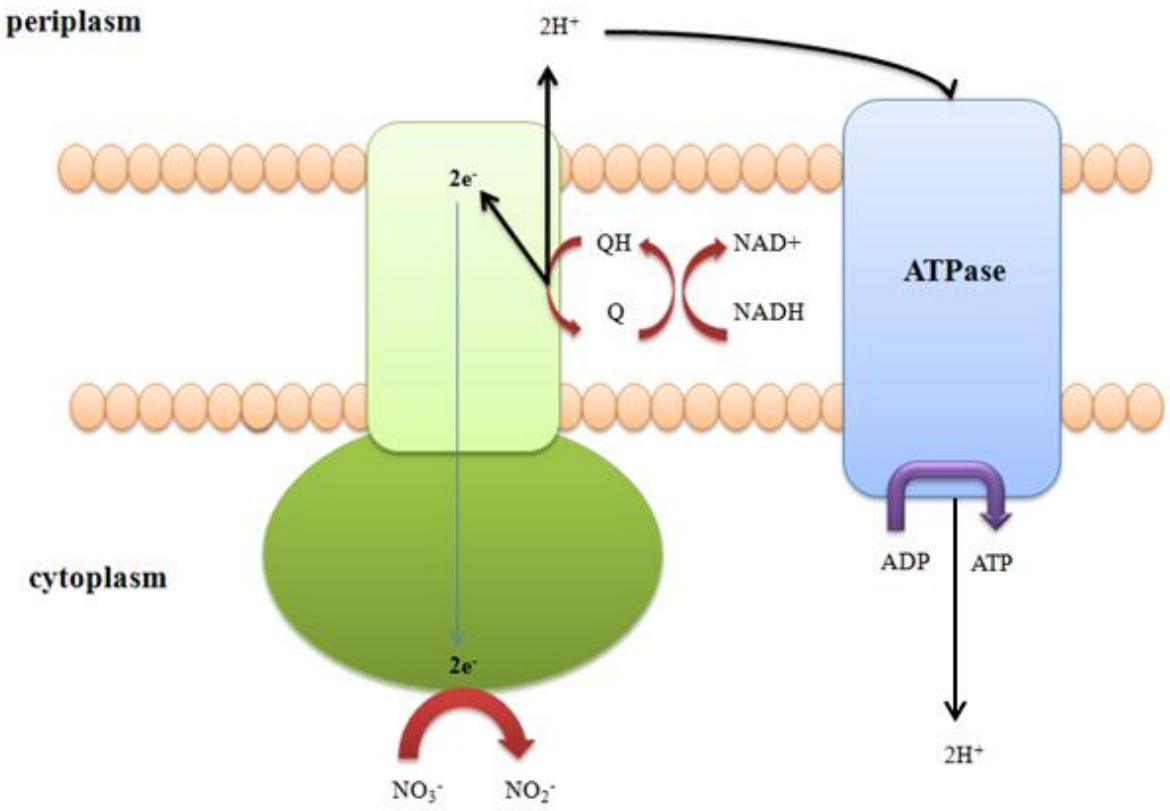


Figure 1.1 Electron flow during nitrate reduction by NarG

Electrons are generated from NADH and transferred through multiple subunits of NarG nitrate reductase until they reach the cytoplasm where nitrate is reduced to nitrite. The process is coupled with the release of $2H^+$ into the periplasm, creating the proton gradient that drives subsequent ATP synthesis via proton transfer across ATPase.

1.3 Reactive nitrogen species (RNS) and nitric oxide detoxification

Reactive nitrogen species (RNS) are nitric oxide (NO) and its various derived compounds. Reactions of NO with proteins that contain accessible metal clusters form nitrosyl complexes that cause proteins to become inactive, affecting bacterial growth. NO can react with superoxide to generate additional RNS that directly disrupt various biological targets such as lipids, amino acids, DNA bases, etc (Cruz-Ramos *et al.*, 2002; Justino *et al.*, 2007). The intermediates produced from the reactions of NO with other molecules can be toxic as well, e.g. formation of reactive NO-derived products such as $ONOO^-$ and NO^+ results in the conversion of nucleotides and thiols to nitroso-derivatives (Hughes, 1999; Pullan *et al.*, 2007). The nitosated derivatives of thiols, in turn, are able to transfer nitroso-groups to other cellular components. In addition, their decomposition under specific conditions generates toxic hydroxylamine and nitrous oxide (Arnelle and Stamler, 1995).

Amine nitrosation is thought as one among several sources of endogenous DNA methylation agent (Lutz, 1990). Nitrosated primary amines are unstable and can be hydrolyzed spontaneously (Hussain and Ehrenberg, 1974; Boido *et al.*, 1980). RNS such as NO^+ can react with amines, yielding nitrosamines that potentially cause methylating damage to DNA by forming covalent bond with DNA at 12 possible alkylated sites (Bartsch and Montesano, 1984; Hecht, 1997). Introducing methylation to DNA leads to the interruption of DNA replication, mutation and DNA double-strand breaks (Taverna and Sedgwick, 1996; Sedgwick, 1997; Noll *et al.*, 2006). Spek *et al.* (2002) revealed that NO is also able to bind to DNA, causing deamination and base mispairing.

NO, a primary source of RNS, is generated either by phagocytes within host cells or by bacterial chemical reactions that generate NO as the by-product of nitrate and nitrite

reduction to ammonia (Lundberg *et al., 2*004). The review by Fang (2004) indicated that NO, produced by an animal's macrophage via the enzymatic activity of nitric oxide synthase 2 (NOS2), has a role in killing pathogenic bacteria.

During anaerobic growth in the presence of nitrate, energy is conserved via coupled reactions of nitrate reduction and the creation of a proton gradient that leads to ATP synthesis by proton motive force. Dissimilatory nitrate reduction is a process utilized to generate NO_2^-, which is further reduced to ammonia (NH_4^+). It was assumed that NO is endogenously produced as the by-product of nitrite metabolism generated by nitrite reductase NirB and NrfA, and nitrate reductase NarG is possibly responsible for NO production (Corker and Poole, 2003; Gilberthorpe and Poole 2008; Metheringham and Cole, 1997; Ralt *et al*., 1988; Weiss, 2006).

In 2008, Gilberthorpe and Poole indicated that the mutant cells that lack NarG are unable to produce NO. Vine *et al*. (2011) reported the method using beta-galactosidase assay to measure NO accumulation in *E. coli* based on NsrR, the transcription factor that is sensitive to NO. Contrary to the previous finding by Corker and Poole (2003) and Weiss (2006) who identified that the majority of NO is produced as the by-product of nitrite metabolism generated by nitrite reductase NrfA and NirB, Vine *et al*. (2011) suggested that the majority of NO in cytoplasm was generated as a side product during nitrite reduction by membrane associated nitrate reductase NarG, whereas NirB and NrfA nitrite reductases are not the major sources of NO from nitrite (Vine *et al.,* 2011). This discovery gave answers to the controversial question of how endogenous NO is generated in *E. coli.*

The nitrate reductase NarG was also reported to be the major source of S-nitrosylation activity in *E. coli* during anaerobic respiration (Seth *et al.,* 2012). The level of accumulated S-

nitrosylated cysteines in *ΔnarG* strain was reduced ~80% compared to the wild-type strain. It was reported that S-nitrosylation of proteins that occurred in anaerobic conditions in the presence of nitrate is controlled by OxyR, the transcription factor that is previously thought to be activated only under aerobic condition via oxidation (Seth, *et al., 2*012; Foster *et al.,* 2009; Bosworth *et al.,* 2009). A high amount of S-nitrosylated proteins was detected in a *ΔoxyR* strain. Therefore, it was suggested that OxyR is involved in protection against nitrosative stress, possibly by protein denitrosylation to limit amounts of S-nitrosylation.

Various genes, such as *hcp*, *yeaR*, *hmpA* and *ytfE*, were identified to be OxyR-dependent (Seth *et al., 2*012) and activated in response to nitrosative stress (Filenko *et al., 2*007; Hyduke *et al.,* 2007; Overton e*t al.,* 2006; Pullan *et al.,* 2007). It was also reported that a high induction level of *norV* and *norW,* which encode NO reductase proteins, was detected in the absence of OxyR (Seth *et al., 2*012). However, the previous discovery by Filenko *et al* (2007), based on microarray analysis and bioinformatic studies, revealed that all of *hcp, hmpA* and *ytfE* are strongly regulated by NsrR, the NO-sensing transcription regulator. It was also reported that NsrR is involved in regulation of the nitrate reduction pathway (Bodenmiller *et al.,* 2006; Filenko *et al., 2*007).

NsrR is a protein in Rrf2 family that contains the helix-turn helix motif for DNA binding and an iron-sulphur (4Fe-4S or 2Fe-2S) cluster that functions as a sensor of NO (Tucker *et al.,* 2008; Tucker *et al.,* 2010). The Fe-S cluster of NsrR directly senses NO, resulting in nitrosylation and inhibition of DNA binding. In *E. coli,* NsrR plays an important role in the resistance against nitrosative stress (Tucker *et al.,* 2010). Karlinsey *et al* (2012) used qRT-PCR and microarray analysis to confirm that *hcp-hcr, ygbA, ytfE* and *hmp* are NsrR regulated genes.

Over the course of evolution, *E. coli* has acquired several systems that enable them to detect and detoxify NO to prevent cellular damage caused by RNS. Hcp, a hybrid cluster protein that contains iron-sulphur cluster motif, was previously reported to be a hydroxylamine reductase; however, a high Km for hydroxylamine was detected (3-fold greater than the concentration that inhibits growth). The transcriptomic studies showed that the *hcp* expression is induced when cells encounter nitrosative stress, suggesting that it has a role in protecting against nitrosative damage more likely than detoxifying hydroxylamine (Cabello *et al., 2*004; Overeijnder *et al.,* 2009; Vine and Cole, *2*011). YgbA has a motif found in ferredoxin oxidoreductase, suggesting that it also contains iron-sulphur cluster (Finn *et al., 2*010). YtfE, a di-iron protein for an assembly of the iron-sulphur binding motif, has a role in restoring damaged iron-sulfur centers caused by nitrosative stress (Justino *et al.,* 2006; Justino *et al.,* 2007; Overton et al., 2008; Vine *et al.,* 2010). Hmp detoxifies NO particularly in aerobic environments by converting NO to NO_3^- (Hernandez-Urzua *et al*., 200; Stevanin *et al., 2*002; Svensson *et al., 2*010). The Km value for the NO dioxygenase activity of Hmp in *E. coli* is ~2400 $\mu M^{-1}S^{-1}$. The Vmax (maximal turnover) for NO measured in cells grown in aerobic and anaerobic conditions are 670 s^{-1} and 0.02 s^{-1} respectively (Gardner, *2*005; Gardner and Gardner, 2002). In 2002, Gardner and Gardner revealed the experiments using Hmp-deficient and overexpressing mutants, showing that Hmp afforded negligible NO detoxification (NO is reduced to N_2O) under anaerobic conditions.

E. coli uses alternative anaerobic detoxifying enzymes, including flavorubredoxin (NorV) and cytochrome-c nitrite reductase (NrfA), which convert NO to non-toxic metabolites N_2O or NH_3 (Gardner *et al.,* 2002; Gomes *et al.,* 2002; Hausladen *et al., 1*998; Mill *et al.,* 2005; Poock *et al.,* 2002; Wang *et al.,* 2010; Wonderen *et al.,* 2008). In 2011, Vine and Cole (2011) indicated that 4 proteins in *E. coli*, including NirB, NrfA, NorVW and Hmp,

play roles in NO reduction. There are alternative mechanisms that directly repair damage caused by RNS, e.g. base excision repair machinery deals with damaged and incorrect bases (Grossman and Kovalsky, 2001), the O_6-alkylguanine DNA alkyltransferase (Ogt) acts as a DNA-repair protein in response to a presence of nitrate, a precursor of nitric oxide (Constantinidou *et al.,* 2006; Potter *et al.,* 1987).

The *narG, napF, nrfA* and *nirB* genes, encoding nitrate and nitrite reductases, are regulated by Fnr (the regulator of nitrate and fumarate reduction), which contains a 4Fe-4S cluster for DNA binding function. Whether or not Fnr acts as a NO sensor was unclear. Members of the Fnr regulon, i.e. *nrf,* should be responsive to the presence of NO if Fnr acts as a NO sensor. However, no significant result from microarray studies was obtained to confirm that NO controlled the *nrf* expression. This challenged the idea that Fnr might be one target of the damage caused by NO, but not the NO sensor (Justino *et al., 2*005; Spiro 2006; Spiro 2007; Vine and Cole, 2011).

1.4 The adaptive response

It is essential for bacteria to have DNA integrity in order to exist despite a change of environmental conditions. In general, reactions contributing to DNA damage are methylation, oxidation, deamination and depurination (Ames, 1989; Totter, 1980). The presence of RNS is an important factor that increases mutation rates due to DNA damage caused by DNA structural alteration and chemical changes in DNA base pairing (Dizdaroglu, 1993; Epe, 1993). NO and its derived compounds are capable of inducing nitrosation, nitration and deamination in DNA (Oshima and Bartsch, 1994; Routledge *et al.,* 1994; Weiss, 2006). Failure to repair DNA base damage results in an inaccurate replication and accumulation of

heritable mutations that affects cell function, as well as genome instability (Friedberg *et al.*, 2006).

Methylation of the DNA bases occurs via nucleophillic substitution when the electron from the DNA bases replaces the departing anion of methylating agents, which serves as strong electrophiles (Garcia-Santos *et al.*, 2001). Several nitroso-compounds have been identified as potential sources of methylating agents. Nitrosation of peptides occurs at the terminal primary amine or at any N-atoms, yielding diazopeptides and N-nitrosopeptides respectively. Degradation of diazo-compounds generates diazomethane that acts as a methylating agent and cause DNA damage (Garcia-Santos *et al.*, 2001). Shuker and Margison (1997) suggested that azasarine, an alkylating adduct that arises from nitosated glycine and its derivatives, causes methylation at O_6-G in DNA (Shuker and Margison , 1997; Zurlo e*t al.*, 1982).

Nitrosation of amines, amino acids and peptides is a source of mutagenic metabolites that function as methylating agents, modifying DNA at different sites and causing mutagenic lesions. *E. coli* has evolved to acquire adaptive responses that directly protect and repair DNA against methylation damage of nucleotides caused by NO and its derived products (Kunisaki and Hayashi, 1979; Sedgwick, 1997). Several enzymes, such as DNA methyltransferases, DNA glycosylases and DNA dioxygenase, are involved in mechanisms to repair DNA methylation damages.

DNA-methyltransferases, Ada and Ogt, can directly remove the methyl group on the exocyclic oxygens of the modified base to its cysteine residue (Harrison *et al.*, 1999; Mackay *et al.*, 1994; Traverna and Sedgwick, 1996; Sedgwick 1997; Sedgwick, 2004). The *ada* gene is identified as one of the best characterised components of the adaptive response. Ada is the

enzyme that contains an alkyltransferase (ATase) activity. Its C-terminal domain is able to repair methylated guanine nucleotides, while the N-terminal domain is responsible for repairing methylated phosphotriesters (Shevall and Walker, 1991). The methylated guanine pairs up with thymine instead of cytosine during the replication process, hence, causing inaccurate DNA replication and subsequent mutation. Furthermore, double/single-strand DNA breaks are a consequence of the mismatch base pairs (Lawley and Phillips, 1996). Ada also acts as a major transcription factor in the adaptive response by up-regulating various genes related to DNA repair due to alkylating damage such as *alkA, alkB* and *ada* itself (Teo *et al.,* 1986; Volkert, 1988; Yu *et al.,* 2006).

Potter *et al.* (1987) tried to isolate the *ada* gene but discovered a smaller one encoding a distinct protein called Ogt that is similar to the C-terminal domain of Ada. The *ogt* gene encodes O6-alkylguanine DNA alkyltransferase, an enzyme that responds to nitrate, a precursor of NO. It repairs alkylated DNA by transferring an alkyl group from alkylguanine residue in the damaged DNA to itself (Constantinidou *et al.,* 2006; Potter *et al.,* 1987).

The second helix of the DNA binding motif of either Ada-C terminal domain or Ogt contains a region called "arginine finger" that promotes the flipping of O_6-meG from the DNA base stack. The base is then captured by a crosslink with the Cys residue, and demethylated inside the substrate-binding pocket of the proteins (Daniels and Tainer, 2000; Duguid *et al.,* 2003; Wibley *et al.,* 2000).

DNA glycosylase AlkA, an alternative protein that repairs DNA methylation damage, repairs the damaged base via hydrolysis of the glycosyl bond. An additional endonuclease is required to remove the DNA backbone at the baseless region, and then the gap is repaired by

activities of DNA polymerase and DNA ligase (Memisoglu and Samson, 2000). DNA dioxygenase AlkB also catalyses base reversion, using oxidative demethylation mechanism.

1.5 Regulation of stress response

Bacteria have evolved stress response systems to control cellular stability and maintain equilibrium in order to survive under fluctuating environments. Cells sense different surrounding stresses and respond via a complex regulatory network using the appropriate gene expression and protein functions. The stress situations can vary such as temperature stress, osmotic stress, metal ion stress, oxygen stress, etc (Chowdhury *et al.,* 1996).

The transcriptional regulation of genes involved in stress responses protects bacteria from DNA/protein damage and allows them to tolerate stress. Different sigma factors are activated when *E. coli* face different stresses in the environment. These sigma factors direct RNA polymerase to the specific promoters in order to initiate transcription of the corresponding genes required for specialized stress management. Some repressors are inactivated under specific stress conditions, resulting in derepression of stress-responsive promoters.

When the environment switches from aerobic to anaerobic, gene expression in *E. coli* is regulated by an ArcAB and Fnr-dependent regulatory network. Fnr regulates transcription of multiple respiratory genes such as *frd* and *nar*, which encode proteins for respiration using fumarate and nitrate as alternative electron acceptors in the absence of oxygen (Jones and Gunsalas, 1987; Lambden and Guest, 1976; Stewart, 1982). ArcAB is also responsible in

switching from aerobic to anaerobic conditions by repressing the transcription of several genes involved in the aerobic mechanisms.

RNS are generated as by-products of nitrate/nitrite reduction and acts as potent mutagenic nitrosating agents. In order to survive under nitrosative stress, a number of systems in *E. coli* have been evolved to detoxify nitric oxide, as well as to repair its damage. The expression of *norV, norW* and *hmpA* was found to be upregulated under nitrosative stress. All three genes have been shown to detoxify nitric oxide (Constantinidou *et al.,* 2006; Flatley *et al.,* 2005; Justino *et al.,* 2005). Nitrite, a precursor of nitric oxide, also induces transcription of several genes involved in protection against nitrosative stress such as *hmpA, norV, ytfE* and *yeaR-yeaG* (Constantinidou *et al.,* 2006; Filenko *et al*., 2007; Poole *et al.,* 1996; Bower *et al., 2*009).

Repression of the *hmpA* expression is mediated by Fnr (fumarate and nitrate reductase regulatory protein) and NsrR (the regulator of nitrosative stress) (Bodenmiller and Spiro, 2006; Filenko *et al.,* 2007). Nitrosative stress negates their functions, resulting in de-repression of *hmpA* transcription (Crack *et al.,* 2008; Cruz-Ramos *et al.,* 2002). Also, there is evidence showing that the global regulator, ArcA, also plays a role in nitrosative stress tolerance (Lu *et al., 2*002).

DNA damage caused by nitrosative stress can be repaired by a number of SOS proteins in which the synthesis is induced by DNA damage and by a presence of nitric oxide (Constantinidou *et al.,* 2006; Filenko *et al*., 2007; Justino, 2005). The adaptive response by SOS proteins are involved in repairing DNA damage by various mechanisms such as excision repair, recombination and error prone repair. *E. coli* also possess proteins that belong to the transcription-repair coupling factor (TCRF) family, which act as a DNA-dependent ATPase

and releases stalled RNA polymerase from the sites of DNA lesion; hence, resulting in the recruitment of other DNA repair factors (Selby and Sancar, 1990; Selby and Sancar, 1993).

E. coli can regulate genes in response to unrelated stimuli if those stimuli normally occur before the product of the genes are required (Mitchell *et al.,* 2009). Nitric oxide causes some amino acids to become DNA methylating agents (Kunisaki and Hayashi, 1979; Taverna and Sedqwick, 1996). NarL, in response to nitrate, activates transcription of the *ogt* gene, which encodes the protein that repairs DNA damage caused by methylation. Therefore, *E. coli* provides a prophylactic mechanism as an adaptive response against the future endogenous RNS that will be produced during nitrate/nitrite metabolism (Squire *et al.*, 2009; Weiss, 2006).

1.6 An overview of bacterial RNA polymerase and promoter recognition

In *E. coli,* the multisubunit DNA-dependent RNA polymerase is the enzyme responsible for all transcriptional processes. The core RNA polymerase, with a molecular mass of 400 kDa, is comprised of 2α, β, β' and ω subunits as illustrated in Figure 1.2 (Zhang *et al*., 1999). These subunits are homologous in sequences, functions and structures, with the core RNA polymerase subunits found in other bacteria as well (Ebright, 2000). The β and β' subunits form a "crab-claw" structure that contains the active site with Mg^{2+} located at the cleft (Cramer *et al.,* 2001; Hirata *et al.,* 2008; Zhang *et al.,* 1999). The internal channel is occupied by a DNA template for the synthesis of the RNA-DNA hybrid transcript using NTPs that enter the active site via the secondary channel while the transcript is extruded through the RNA exit channel (Naryshkin *et al., 2*000; Vassylyey *et al.,* 2007).

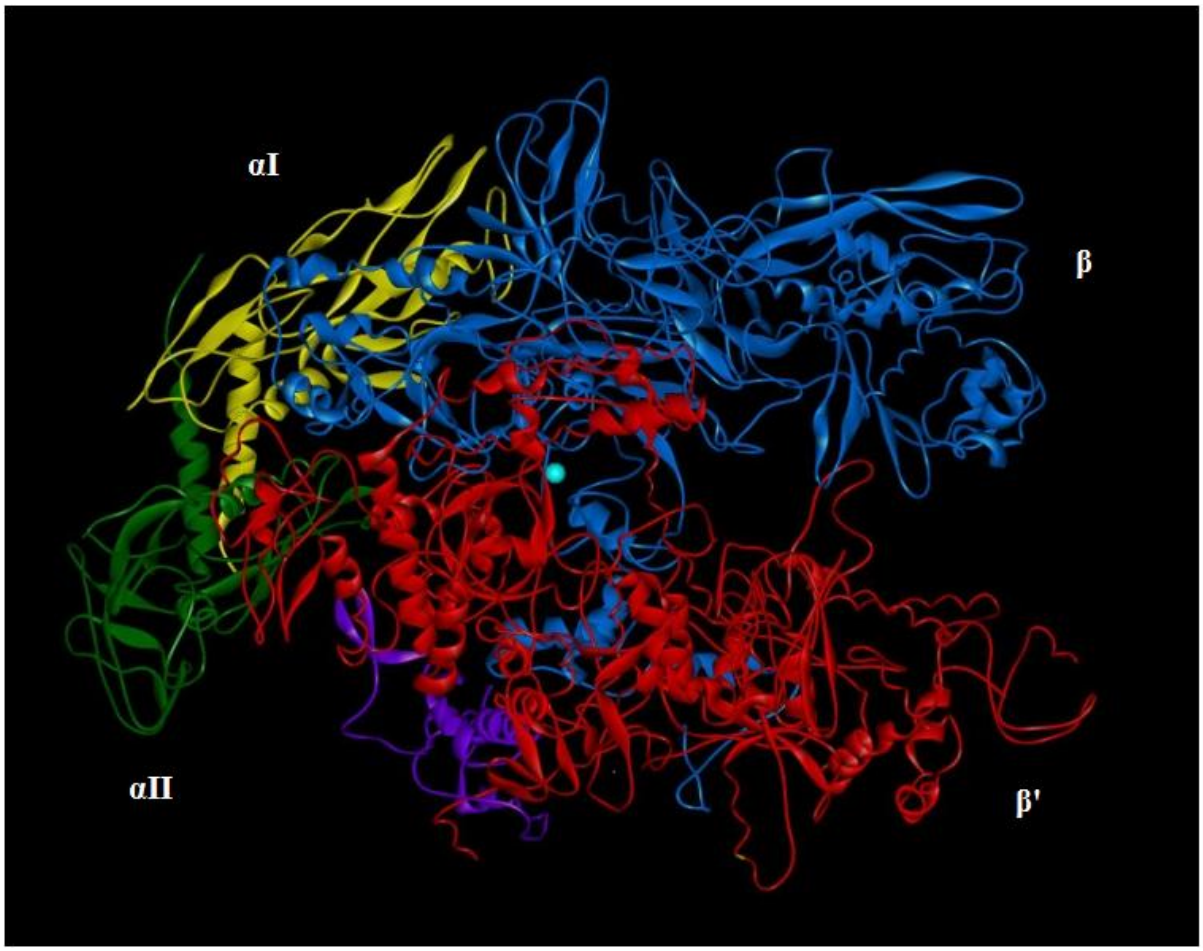


Figure 1.2 Structural model of the bacterial core RNA polymerase

Figure shows the core RNA polymerase structure of *Thermus aquaticus.* The αI, αII, β and β' are shown in yellow, green, blue and red respectively with Mg^{2+} shown as a blue sphere positioned at the centre of the active site (Zhang *et al.*, 1999).

RNA is synthesized until termination signals are reached. Rho (ρ) protein is a termination factor that attaches to a recognition site on mRNA and moves along the mRNA to access the terminator site at the RNA-DNA hybrid (Das, 1993). Transcription is terminated when the ρ protein interacts with RNA polymerase, causing the release of RNA polymerase from DNA (Ciampi, 2006). There is an alternative termination process that does not require the ρ factor. The secondary structure of the synthesized RNA itself creates a hindrance that interrupts RNA polymerase activity. This causes RNA polymerase to dissociate from DNA and terminate transcription (Artsimovitch and Landick, 2000).

The two RNA polymerase α subunits are identical. Each subunit consists of an amino-terminal domain (αNTD) and a carboxy-terminal domain (αCTD), which is connected by a flexible linker (Blatter *et al.,* 1994; Ebright and Busby, 1995). The αNTD dimer interacts with β and β' to assemble the core subunits, whereas the αCTDs function as DNA binding domains (Blatter *et al.,* 1994; Gaal *et al.,* 1996; Murakami *et al.,* 1996; Zhang *et al.,* 1999). The ω subunit acts as a chaperone to assist RNA polymerase stabilization (Darst *et al.*, 1989; Minakhin *et al.,* 2001; Mukherjee and Chatterji, 1997).

To initiate transcription, the core-RNA polymerase must associate with a σ subunit to form the holo-RNA polymerase that recognizes specific DNA elements at the promoter region (Burgess *et al.*, 1969; Ishihama *et al.*, 1973). The σ^{70} subunit is the house-keeping sigma factor encoded by the *rpoD* gene. Ishihama (1973) revealed that σ^{70} regulates most of the transcriptional units in *E. coli* (~1,000 transcription units). There are also 6 other alternative σ factors in *E. coli,* each of which is responsive to the presence of different stimuli and recognizes different DNA sequences at the cognate promoter regions (Gross *et al.*, 1998; Ishihama, 1999).

Holo-RNA polymerase recognizes 3 specific elements including the -10 element, the -35 element and the UP element. DNA sequences at positions -10 and -35 relative to the transcript start site are recognized by domain 2.4 and domain 4 of σ^{70} (Murakami *et al.*, 2002a; Campbell *et al.*, 2002). The consensus sequence for a -10 element recognized by σ^{70} is TATAAT. Rosenberg and Court (1979) revealed that the first 2 bases and the last base of the -10 element are conserved throughout most of the promoters. The consensus sequence of the -35 element recognized by σ^{70} is TTGACA. The first 3 bases of the -35 element are highly conserved (Rosenberg and Court, 1979). The UP element is ~20 bp with an AT-rich region located upstream the -35 element and it is recognized by the αCTD of RNA polymerase (Gourse *et al.*, 2000). The consensus binding sequence of the proximal UP element is AAAAAARNR (R=A or G), while the consensus sequence for the distal one is AWWWWWTTTTTT (W= A or T) (Estrem *et al.*, 1999).

Other elements that recognize σ factors are the extended -10 element and the discriminator. Domain 3 of σ^{70} recognizes the extended -10 element, which is the TG base pairs located at position -13 and -14 upstream of the -10 element (Barne *et al.*, 1997; *et al.*, Murakami *et al.*, 2002). It is an important element in some promoters that contain low affinity -10 and -35 elements (Mitchell *et al.*, 2003). The discriminator is the region recognized by domain 1.2 of σ^{70}. It is located between the transcript start site and the -10 element. The interaction at this region is thought to stabilize open complex formation (Haugen *et al.*, 2006; Haugen *et al.*, 2008). The diagram illustrated in Figure 1.3 shows binding of RNA polymerase to specific recognition sites of DNA at a bacterial promoter.

There is a limited supply of RNA polymerase in cells. This results in competition among different promoters for RNA polymerase binding. Recognition elements that resemble the consensus serve as good binding sites for RNA polymerase subunits, however, there is no

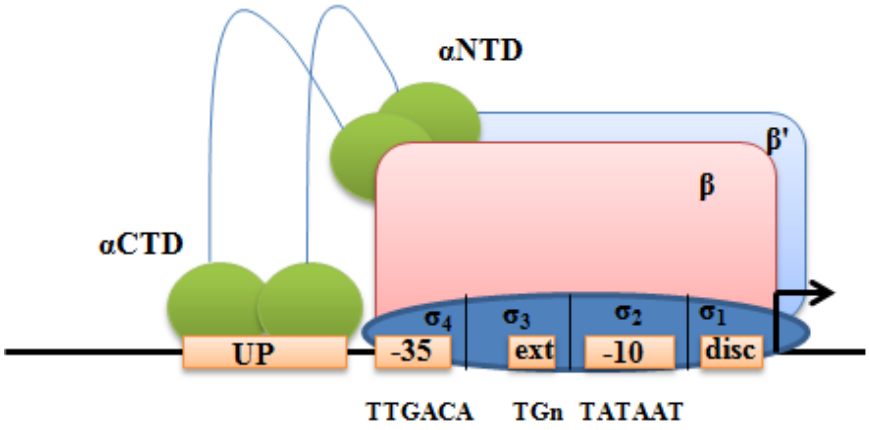


Figure 1.3 Binding of RNA polymerase to specific recognition elements at the promoter

Figure is adapted from Browning and Busby (2004). The figure shows the holo-RNA polymerase binding to the promoter at different specific DNA elements. Interactions are made between αCTD and UP element; region 4 of σ^{70} and -35 element; region 3 of σ^{70} and the extended -10 element (ext); region 2 of σ^{70} and -10 element; region 1 of σ^{70} and the discriminator element (disc).

such natural promoter that contains perfect recognition sites. Therefore, transcription factors are required in order to increase RNA polymerase binding efficiency and rates of gene transcription.

1.7 Transcript initiation

The holo-RNA polymerase binds to double stranded DNA at specific recognition elements (-10, extended -10, -35 elements) of the promoter, resulting in a closed complex formation. Then, DNA undergoes conformational changes that unwind the DNA helix structure in order to initiate transcription. After DNA is destabilized, a transcriptional bubble is formed at the DNA unwinding region involving the -10 element and the transcript start site (Murakami *et al.*, 2002; Murakami and Darst, 2003). The DNA template strand accesses the active site and repositions region 1 of sigma, allowing the downstream DNA to enter the active site (Makler *et al.,* 2002; Murakami *et al.,* 2002b). DNA is further unwound from positions -11 to +3. Then, NTPs enter the active site and the phosphodiester bonds are formed (Murakami *et al.*, 2002a; Murakami and Darst, 2003). The synthesized transcript is sent directly to the RNA exit channel. This action destabilizes the interaction between the promoter -35 element and sigma region 4, resulting in the dissociation of the sigma factor from the core RNA polymerase (Murakami *et al.,* 2002b; Nickels e*t al.,* 2005; Vassylyev *et al.*, 2002). Transcript elongation continues after promoter clearance. The diagram in Figure 1.4 shows the assembly of a transcription initiation complex during the different steps of transcription.

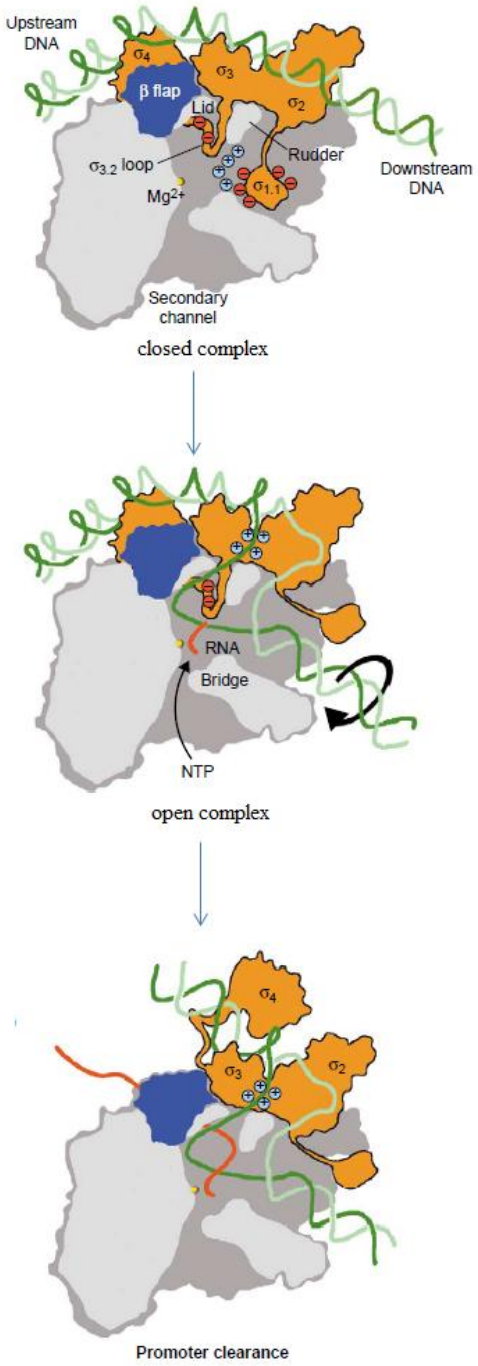


Figure 1.4 Transcription initiation by RNA polymerase

Figure shows transcription initiation steps in a cross sectional view. The sigma subunit is in orange. The holo-RNA polymerase β flap is in blue. The rest of RNA polymerase is in grey. DNA template and non-template strands are dark green and light green respectively. Synthesized RNA is in red. Diagrams show the transition from a closed complex to an open complex until promoter clearance stage (Figure is taken from Murakami and Darst, 2003).

1.8 Regulation of transcription initiation

The *E. coli* genome contains ~2,000 transcription units. Regulation of transcription initiation allows only needed RNA transcripts to be synthesized as there are limited supplies of RNA polymerase. The prioritized genes are transcribed by free RNA polymerase in response to environmental stimuli to ensure that gene expression occurs at the right time. There are several factors that control RNA polymerase distribution across the chromosome including DNA sequences of the promoters, small ligands, chromosomal folds and competitions among σ factors for RNA polymerases and transcription factors (Browning and Busby, 2004).

Transcription factors are DNA-binding proteins that bind to specific sequences at target promoters to up- or down-regulate transcription initiation. Activities of the different transcription factors are regulated by various mechanisms; the expression level of transcription factors, ligand binding, covalent modification and factor sequestration. In *E. coli*, there are ~300 different transcription factors functioning as activators or repressors (Perez-Reuda and Collado-Vides, 2000).

Many transcription factors regulate just one promoter but there are some transcription factors designated as global regulators (Browning and Busby, 2004). Figure 1.5 illustrates the *E. coli* transcriptional regulatory network. Seven global transcription factors including CRP, FNR, Fis, IHF, H-NS, Lrp and ArcA regulate the expression of the large numbers of transcription units (~50%) (Martinez-Antonio and Collado-Vides, 2003). This work will focus on a transcription factor, called NarL, and its function in transcription regulation. More details about NarL are discussed in Section 1.7. Many transcription factors that act as activators make direct contacts with RNA polymerase at the target promoter site. Interactions often

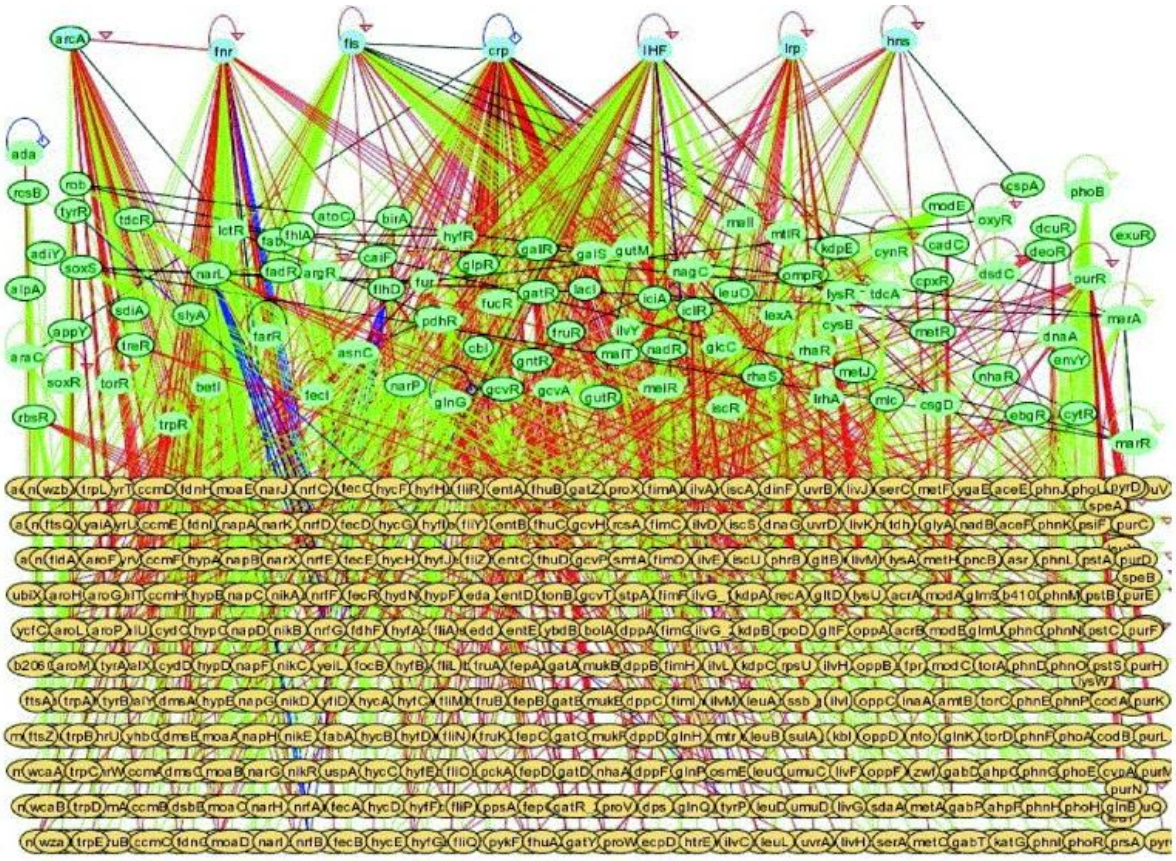


Figure 1.5 The transcriptional regulatory networks of *E. coli*

Yellow ovals represent different genes. Transcription factors are shown as green ovals, while global transcription factors are shown as blue ovals. Green and red lines indicate activation and repression. Dark blue lines represent dual regulation (Figure is taken from Martinez-Antonio and Collado-Vides, 2003).

occur between transcription factors and the C-terminal domain of the α subunit and the σ subunit of RNA polymerase in order to recruit RNA polymerase and facilitate promoter isomerization (Hochschild and Dove, 1998). Some activators bind to DNA and carry out their function by altering DNA conformation, resulting in more efficient binding of RNA polymerase to the promoter region.

CRP, FNR, ArcA and LrP are global regulators that control metabolism in cells. Other global regulators (Fis, H-NS, IHF) are classified as nucleoid associated proteins (NAPs). NAPs are small proteins (<20 kDa) that regulate gene expression via DNA bending (Ali Azam *et al.,* 1999; Marr *et al.,* 2008). The formation of nucleoid structures also affects the RNA polymerase distribution between different promoters and hence, in turn, influences transcription programs. This is particularly relevant in many genes that are repressed by Fis during a rapid growth of *E. coli* as RNA polymerase enzymes are required for the synthesis of more essential proteins and cell replication when the availability of RNA polymerase is limited (Ishihama, 2000). However, binding of Fis at some promoters recruits RNA polymerase and stimulates the transcription initiation process.

Another factor that regulates transcription initiation is the σ factor. RNA polymerase holo-enzyme is formed by the association of σ factor with the core RNA polymerase (Burgess e*t al.,* 1969). The σ factor recognizes specific promoter elements; hence it directs RNA polymerase to the right position and facilitates DNA melting (Gross *et al.,* 1998). Competition among different σ factors for association with limited amount of RNA polymerase to form holo-RNA polymerase enzymes is a major controlling factor that determines which genes will be encoded (Gruber and Gross, 2003).

In *E. coli,* there are 6 alternative σ factors that regulate different sets of genes in response to varying environmental conditions or particular growth phases. (Gruber and Gross, 2003; Ishihama, 2000; Maeda *et al.,* 2000). However, transcription regulation by σ factors can be interfered by anti-σ factors that occlude the binding surface of σ factors or directly break down σ factors. Mechanisms involving the release σ factors from its associated anti-σ factors can be achieved by covalent modification, proteolysis and anti-anti-σ factors (Campbell *et al., 2*008).

1.9 Transcription repression

Simple transcription repression takes place when repressors bind to the target promoters and interrupt the transcription initiation process. There are 3 repression mechanisms including steric hindrance, DNA bending and interaction between a repressor and an activator (anti-activation) (Browning and Busby, 2004). Steric hindrance is a mechanism in which a transcription factor binds near core promoter elements or DNA sites for activators, thus preventing RNA polymerase and activators from being able to access the DNA (Izu *et al.,* 2002). An example of the transcription factor that represses transcription via the steric hindrance mechanism is CRP. It directly binds to DNA between positions -20 and +11 and obscures the access of RNA polymerase to the *cya* promoter (Aiba, 1985).

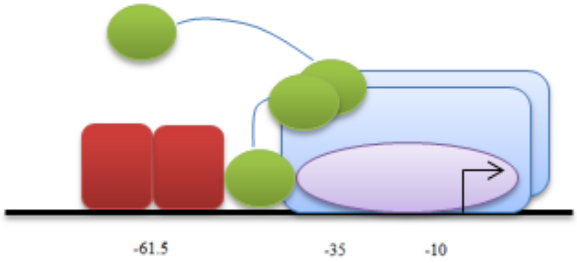
The second common repression mechanism is via DNA bending. Binding of repressors can induce DNA distortion at the promoter region and inhibit transcription initiation. An example of the promoter that is repressed by DNA bending is the *nirB* promoter. Binding of Fis and IHF induces DNA bending and DNA structure contortion (Browning e*t al., 2*000). The third repression mechanism is achieved by anti-activation.

Transcription factors that act as anti-activators may disrupt an interaction between a nearby activator and RNA polymerase. An example of a transcription factor that functions as an anti-activator is CytR. In several promoters, the binding of CytR to the promoter inhibits transcription initiation as it prevents an interaction between CRP and αCTD (Valentin-Hansen, 1996).

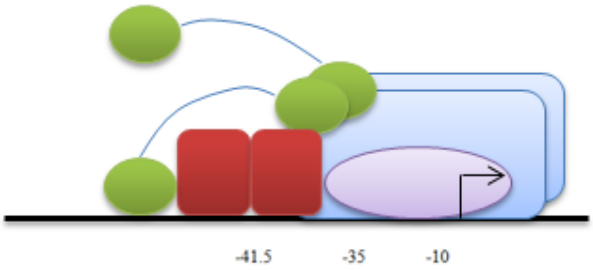
1.10 Transcription activation

Transcription factors can induce 3 simple mechanisms of transcription activation (Browning and Busby, 2004). The first mechanism is transcription activation by promoter remodelling. An example of this pathway is the activation of the σ^{70} dependent promoter by MerR, a transcription factor that manipulates a distance between -10 and -35 elements allowing optimal RNA polymerase recognition (Summer, 1992; Brown *et al.,* 2003). The second mechanism is the binding of transcription factors to an enhancer in order to create a DNA loop that supports an interaction between regulators and RNA polymerase. Also, the interaction of RNA polymerase-factors can induce structural changes in RNA polymerase during an open complex formation (Rappas *et al.,* 2007). The third mechanism is an RNA polymerase-factor interaction that recruits RNA polymerase to the promoter region. The mechanism is divided into 3 groups (class I, II, III) based according to the locations in which the transcription factors bind to DNA and how they come into contact with RNA polymerase. Figure 1.6 illustrates the 3 distinct mechanisms of simple transcription activation.

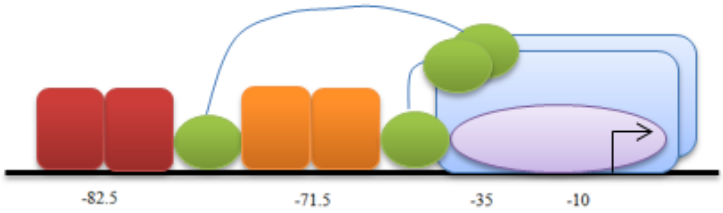
Class I mechanism



Class II mechanism



Class III mechanism (I+I)



Class III mechanism (I+II)

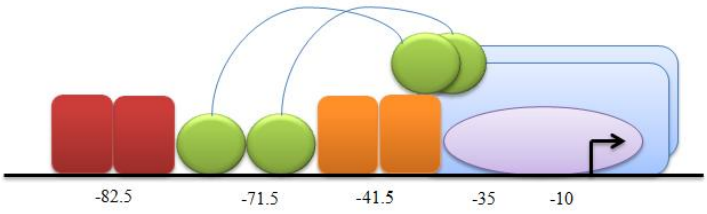


Figure 1.6 Simple mechanisms of promoter activation

Figure illustrates 3 simple mechanisms of promoter activation. The locations of RNA polymerase subunits at the promoter are indicated. Transcription factors are denoted by rectangles. Green circles denote α subunits of RNA polymerase. Purple ellipses denote σ^{70}, which is combined with the core RNA polymerase.

Class I promoters are regulated by a single activator that binds to a target sequence upstream the -35 element and makes a contact with the downstream αCTD of RNA polymerase. The linker between the αCTD and the αNTD is flexible; hence, it allows the αCTD to bind to several locations. However, the DNA site for an activator must be on the same DNA helical face as the binding site of RNA polymerase (Gaston *et al.,* 1990; Zhou *et al.,* 1994). The best understood transcription factor is cyclic-AMP receptor protein (CRP). At class I dependent promoter, such as the *lac* operon, CRP binds to position -61.5 relative to the transcript start site and interacts with αCTD to recruit RNA polymerase (Zhou *et al.,* 1994).

Class II promoters are regulated by a single activator that binds to the site overlapping or upstream the -35 element but downstream the DNA site for αCTD. In this promoter configuration, the activator may interact with multiple subunits of RNA polymerase; α (CTD orNTD), σ and β. CRP activates the *gal* promoter by binding to DNA at position -41.5, which overlaps with the -35 element. An upstream CRP monomer interacts with αCTD that binds to the DNA region upstream of the CRP binding site. The downstream CRP monomer forms contacts with the αNTD and the σ region 4 (Gaston *et al.,* 1990; Li *et al.,* 1998).

Class III promoters are regulated by two or more transcription factors. The activators can either be the same or different transcription factors. It is thought as a combination of activation mechanisms involving class I and class I, or class I and class II. In this promoter configuration, multiple contacts with RNA polymerase are made (Busby and Savery, 2007; Joung *et al.,* 1993; Joung *et al.,* 1994). An example is the *ansB* promoter, which has CRP located at position -91.5 and FNR located at position -41.5 (Scott *et al.,* 1995).

All class I, II, III activators bind to DNA on the same face of DNA helix as RNA polymerase. However, a strange observation in transcription activation has been found in

Bordetella pertussis. Some virulence genes are regulated by BvgA response regulator with unusual orientation of RNA polymerase and transcription factors (Decker *et al.,* 2012). The architecture of P_{fhaB} is different from the class I and II promoters mentioned before. The molecular structural model of the transcription initiation complex showed that σ region 4 is kept at -35 element in the same orientation. The αCTD is located on the same DNA region where a BvgA dimer binds to DNA but on the different DNA helical face (Boucher *et al., 2*003). Another example of an unusual promoter architecture is found at the *fim* promoter, which contains σ region 4, αCTD and a BvgA dimer that bind at the -35 element but on different DNA helical faces (Bouncher *et al.,* 2003; Decker, 2011).

A vast majority of genes are regulated by more than one transcription factor, resulting in complexity due to the integration of effects from multiple stimuli. Apart from the mentioned mechanisms (integration of independent contacts with RNA polymerase), different regulatory outcomes can be regulated by co-dependence of activation due to co-operative binding of activators (Barnard *et al., 2*004). Different transcription factors regulate the promoter by binding to multiple target binding sites in response to different signals to produce an appropriate output at the right time. For example, the *lac* operon is up-regulated by CRP in an absence of glucose but down-regulated by LacI in an absence of lactose. This allows the cells to benefit from the synthesized products as glucose is a better energy source than lactose (Lewis, 2005).

1.11 Two component systems

Transcription factors couple intracellular and extracellular stimuli to transcription. Signaling molecules inside the cells vary e.g. ligands, ions, O_2, etc. Extracellular signaling

requires signals moving from outside to inside the cells. The process is called signal transduction in which environmental stimuli interact with cellular receptors and create cellular actions by altering intracellular molecules. A two-component system is a typical pathway that enables signal transduction in prokaryotes to respond to the changes of environmental conditions. The system is composed of a sensor kinase and its cognate response regulator.

The sensor kinase is a transmembrane protein that senses environmental stimuli outside the cell. Autophosphorylation occurs at the histidine residue located at the cytoplasmic domain. Subsequently, the signal is transferred to the cognate response regulator and triggers phosphorylation at the conserved aspartate residue in the receiver domain. Phosphorylation induces conformational change of the response regulator, causing it to become competent for binding to the DNA recognition site at the promoter region in order to activate transcription (Hoch, 2000). The schematic event of the two-component system is illustrated in Figure 1.7. An example of the basic two-component system is a BvgAS regulatory system in *B. pertussis.* In response to relevant environmental stimuli, the expressions of more than 100 virulence genes are regulated by the sensor kinase BvgS and the cognate response regulator BvgA (Bootsma *et al.,* 2002; Lacey, 1960; Scarlato *et al., 1*991).

Basic chemical reactions of phosphoryl transfer in a two-component system are as follows:

Autophosphorylation : HK-His + ATP ↔ HK-His~P + ADP

Transfer of a phosphoryl group: HK-His~P + RR-Asp ↔HK-His+RR-Asp~P

Dephosphorylation : RR-Asp~P+H_2O ↔ RR-Asp +Pi

***HK = histidine kinase, RR = Response regulator

The numbers of two-component proteins vary among different bacterial species. None has been found in *Mycoplasma genitalium,* while 70 have been discovered in *B. subtilis* (Mizuno,

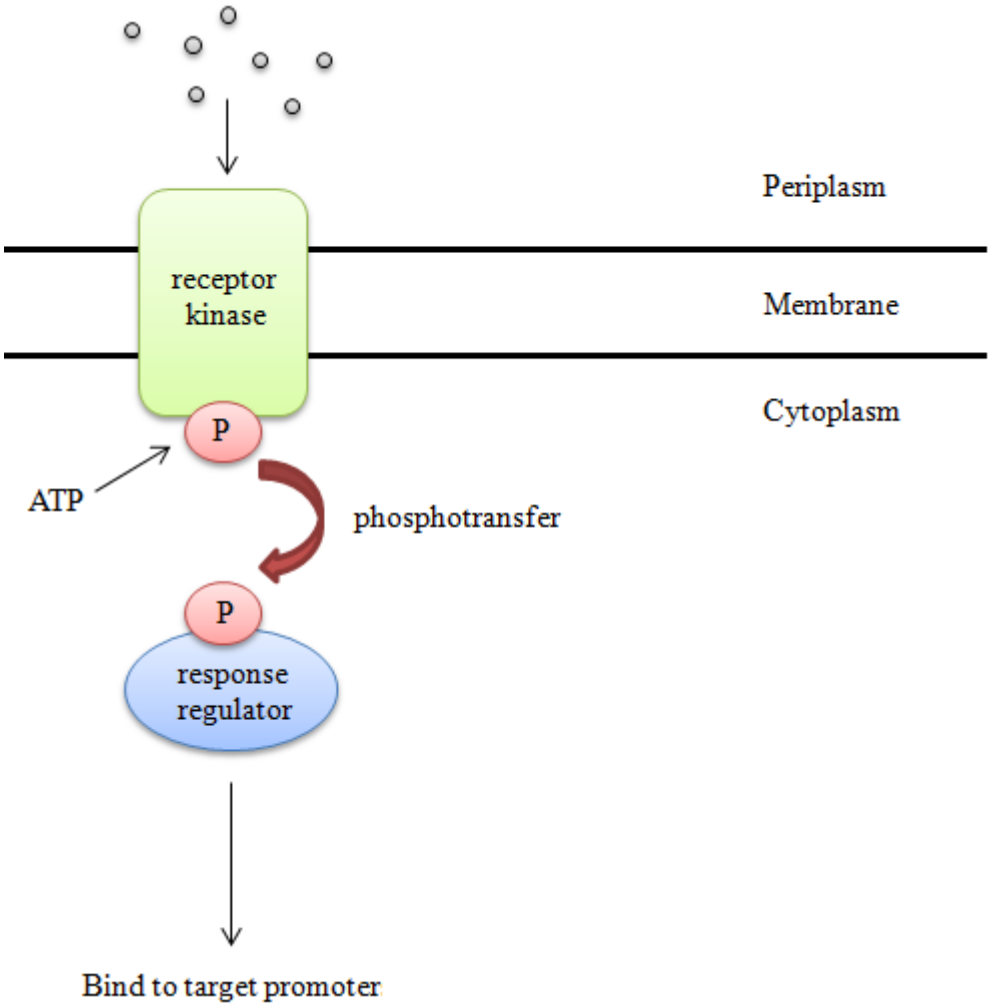


Figure 1.7 Schematic events in two-component system

Signals from outside the cell trigger conformational changes in the transmembrane sensor kinase via phosphorylation using ATP. The phosphate group is then transferred to the cognate response regulator. The phosphorylated response regulator becomes competent for binding to the target promoter and regulates transcription.

1997; Fabret *et al.*, 1999). *E. coli* contains more than 30 two-component systems that regulate gene expression, flagella direction, enzyme activities, etc (Mizuno, 1997).

There are variations in the phosphorylation scheme of the two-component system in which the same response regulator is phosphorylated by several sensor kinases or by a single sensor kinase that controls multiple response regulators. One example is found in regulation of nitrate responsive genes (Li *et al.*, 1995). Complex signal transduction processes involve multiple pathways of phosphoryl transfer reactions, e.g. a mechanism of sporulation in *B. subtilis* is regulated via His-Asp-His-Asp phosphorelays (Appleby *et al.*, 1996; Burbulys, 1991; Parkinson, 1993). The phosphorelay cascade consists of multiple intermediate proteins linked by a number of phosphotransfer actions.

1.12 Nitrate responsive two-component systems (NarXL and NarQP)

NarXL and NarQP are Nar two-component regulatory systems that regulate many nitrate and nitrite responsive genes (Stewart, 1993). The transmembrane NarX and NarQ function as sensor kinase proteins. The cytoplasmic NarL and NarP proteins are response regulators that function as DNA-binding components. A diagram showing the signal transduction pathway of the NarXL two-component system is illustrated in Figure 1.8. NarX and NarQ sensor kinase proteins are homologous proteins comprising 2 domains; the periplasmic domain and the cytoplasmic domain.

The periplasmic domain contains a conserved P-box of 17 amino acids, which detects the presence of nitrate and nitrite (Cavicchioli *et al.*, 1996). The signal triggers an autokinase activity in the cytoplasmic domain at residue His_{399} of NarX (His_{370} for NarQ) (Cavicchioli *et al.*, 1995). The phosphoryl group is transferred through the cytoplasm to a conserved aspartate

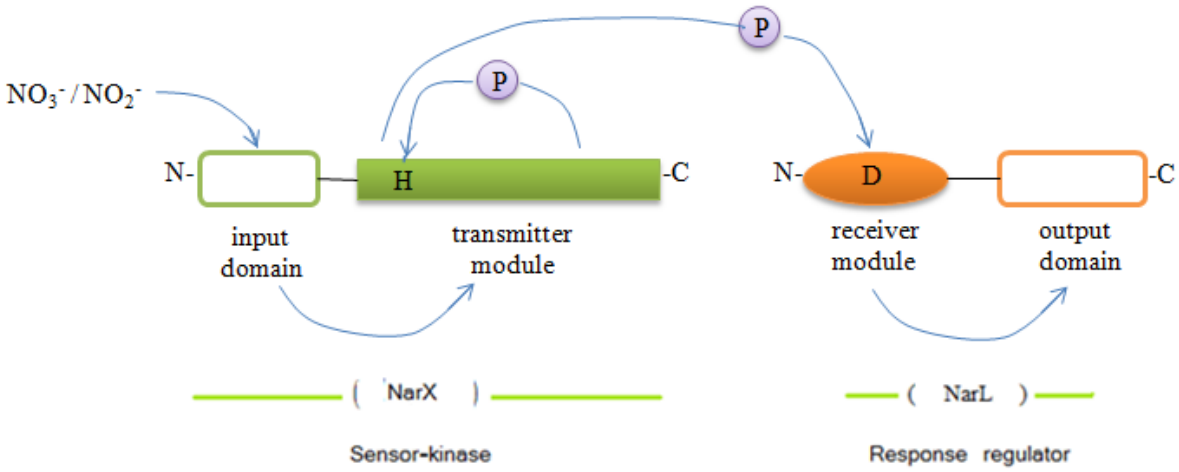


Figure 1.8 The signal transduction in the NarXL two-component system

Figure shows the phosphotransfer pathway in a two-component system, which is composed of sensor kinase NarX and NarL response regulator. NO_3^-/NO_2^- triggers an autophosphorylation reaction at a conserved histidine residue in the cytoplasmic transmitter module of NarX. A phosphoryl group is transferred to an aspartate residue of NarL, causing a conformational change that separates its two domains. Then, the C-terminal domain of Narl is capable of DNA binding at the target promoter region.

residue on the cognate response regulator NarL and NarP (Egan and Stewart, 1991). Cross regulation between NarXL and NarQP two-component systems have been studied. Nitrate-induced phospho-NarX phosphorylates both NarL and NarP, however, in the presence of supplemented nitrite, only NarP is phosphorylated, while NarL is dephosphorylated (Huynh and Stewart, 2011; Lee *et al.*, 1999; Noriega *et al.*, 2011; Williams and Stewart, 1997).

NarL and NarP response regulators consist of the N-terminal domain (receiver domain) and the C-terminal domain (DNA binding domain) linked by a flexible tether. The N-terminal domain is composed of 5 stranded parallel β-sheets surrounded by α2, α3, α4 at one side and α1 and α5 at the other side of the sheet. The C-terminal domain is composed of 4 α helices including support helix 7, scaffold helix 8, DNA-recognition helix 9 and DNA-dimerization helix 10. Helices 8 and 9 form a helix-turn-helix motif, which enables DNA binding (Kahn and Ditta, 1991; Galperin, 2006).

In a non-phosphorylated form, the C-terminal DNA binding domains of NarL and NarP response regulators are ineffective as it is obscured by the N-terminal domain (Baikalov *et al.,* 1996). The N-terminal domain is phosphorylated at the conserved aspartate residue, causing weak interactions at the interdomain interface (Egan and Stewart, 1991). This allows NarL to undergo a conformational change that separates its two domains by ~30Å and exposes some inaccessible residues required for dimerization and DNA binding (Baikalov, 1996; Zhang *et al.,* 2002; Zhang *et al.,* 2003).

Dimerization of two NarL monomers is formed by Van der Waals interaction between their α10 helices. A NarL dimer orients the N-terminal region of α9 recognition helix into the major groove of the DNA where Lys188, Val189 and Lys192 of NarL make contacts with the

major groove's floor (Mariz *et al.,* 2002). It has been identified that the consensus heptameric sequence of NarL is TACYYMT (Y=C/T, M= A/C) (Tyson *et al.,* 1994). Both NarL and NarP bind to a site arranged in an inverted repeat as TACYYMTnnAKRRGTA (K=G/T, R=A/G) (Darwin *et al.,* 1997; Dickerson *et al.,* 2002). The crystal structure of NarL-DNA complex is illustrated in Figure 1.9.

1.13 Regulation of RNS detoxification

E. coli has evolved systems that sense and cope with cytoplasmic RNS toxicity. Mostly, levels of NO and its precursors regulate the expression of various genes involved in NO detoxification and DNA damage repair (Poole *et al.,* 1996; Tucker *et al.,* 2005). In *E. coli*, flavorubredoxin NorV and flavoprotein NorW are involved in NO reduction to nitrous oxide under anaerobic conditions (D' Autreaux e*t al.,* 2005). The expression of *norVW* is regulated by σ^{54} and NorR, an NO responsive response regulator (Justino *et al.,* 2005a). The work of Gardner *et al.* (2003) indicated that the expression of *norVW* is absolutely abolished when a mutation is introduced to *norR.*

In addition to NorR, NsrR is another transcription factor that responds to the presence of NO and nitrosative stress (Bodenmiller and Spiro, 2006; D' Autreaux *et al., 2*005). NsrR contains an NO-sensitive iron-sulphur centre. Nitrosylation of this cluster results in the loss of DNA binding efficiency of NsrR, thus it cannot function as a repressor of the *nsrR* regulon (Bodenmiller and Spiro, 2006; Tucker *et al., 2*008). The recognition sequences of NsrR have been found in many promoters such as *hmpA, ytfE* and *ygbA,* which are believed to be involved in dealing with nitrosative stress (Justino et *al., 2*005; Rodinovo *et al.,* 2005b)

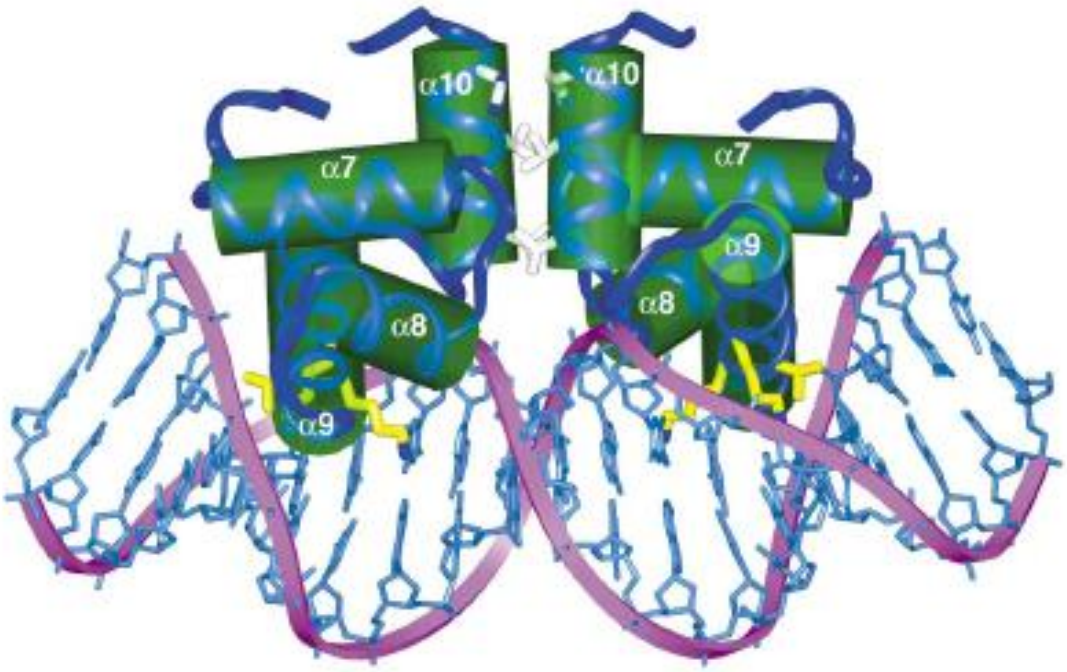


Figure 1.9 NarL-DNA complex

Figure shows a crystal structure of NarL-DNA complex. The NarL dimer interface is formed between helices 10 of two DNA binding domains. The recognition helices 9 are inserted into DNA major groove floors (Dickerson *et al.*, 2002).

1.14 Regulation of the *ogt* promoter

From the work of Constantinidou e*t al.* (2006), the transcriptomic studies indicated that the *E. coli ogt* promoter is up-regulated by NarL in response to the presence of nitrate irrespective of Fnr. The completed *ogt* promoter architecture was demonstrated by Squire *et al.* (2009). It contains 2 DNA sites for NarL, each of which is organized as a 7-2-7 inverted repeat (Lin *et al.,* 2007; Squire *et al., 2*009). The NarL I site is centered at position -78.5, while the NarL II site is centered at position -45.5 relative to the transcript start site. DNase I footprint with the *ogt* promoter fragments containing one of the two NarL sites mutagenised showed that NarL is able to bind to each site independently.

As NarL and NarP are homologous response regulators, the DNA sequence in the *ogt* promoter recognized by NarL should also be recognized by NarP. However, NarP does not bind to the *ogt* promoter *in vitro* despite the presence of high NarP concentrations. NarP alone plays a small role on the *ogt* promoter activity *in vivo* (Squire *et al.,* 2009; Tyson *et al.,* 1994).

Transcription of the *ogt g*ene is also regulated by Fis, a DNA-binding protein that helps to compact the bacterial chromosome (nucleoid). It can bind to DNA nonspecifically and functions as a nucleoid associated protein that organizes and maintains the structures of nucleic acids. This DNA compaction effects distribution of RNA polymerase across regulatory regions. Fis is also involved in transcription regulation of several genes related to nitrate/nitrite respiration (McLeod and Johnson, 2001). Fis can bend DNA from less than 50° to greater than 90° due to a number of basepairs coming into contact with Fis at the Fis binding site (Pan *et al.,* 1996).

Although Fis can bind to the DNA sites that contain weakly-related sequences, it preferentially binds to the DNA site of GNtYAaWWWtTRaNC (Y=C/T, W=A/T, R=G/A)

(Hengen *et al.,* 1997). Fis regulates the *ogt* promoter by binding to a single Fis site located at position -82, which overlaps with the NarL I site, (Squire *et al.,* 2009). Therefore, it represses the *ogt* promoter by competing with NarL for the overlapping binding site. Cellular Fis abundance is high during an early exponential growth (~50,000 molecules) but low in the post-exponential phase and a stationary phase (Ball *el al.,* 1992; Mcleod and Johnson, 2001; Ali Azam., 1999).

1.15 Thesis outline

Transcription of the *ogt* gene in *E. coli* is induced by nitrate via the two-component transduction system NarXL. The *ogt* gene encodes O6-alkylguanine DNA alkyltransferase, which is responsible for repairing DNA damage caused by RNS that are generated as by-products during nitrate respiration. This thesis studies the different distinct architectures of the *ogt* promoter in order to better understand the mechanisms of activation by NarL and how the transcription of the *ogt* promoter can be regulated.

Chapter 3 focuses on transcription activation by NarL at different *ogt* promoter derivatives in order to study the factors that influence the functions of NarL. The optimal locations of the DNA site for NarL are also identified. NarL is composed of an N-terminal and a C-terminal domain. Phosphorylation triggers domain separation and allows the C-terminal domain of NarL to bind to DNA and activate the promoter. To understand the functions of each domain more clearly, Chapter 4 focuses on the regulation of the *ogt* promoter by the full-length NarL and the C-terminal domain of NarL, liberated from the N-terminal domain. In addition, results from half-site experiments are also used to identify the NarL monomer that is involved in transcription activation.

Chapter 5 focuses on the data from alanine scan libraries of α subunit of RNA polymerase obtained from the work of David Chismon, the University of Birmingham (2010). Together with the data from NarL positive control mutant experiments and epistasis studies, this work aims to identify potential interacting residues between NarL and αCTD. Results from DNase I footprinting and FeBABE [Iron (S)-1-(p-bromoacetamidobenzyl)ethylene-diaminetetraacetate] footprinting experiments are used to create the structural model of transcript initiation complex at the *ogt* promoter. The previous work from Squire *et al.* (2009) suggested that the *ogt* promoter is activated by NarL via class III mechanism. Results from the current work will reveal that the regulation of the *ogt* promoter by NarL does not represent a class III but an unrecognized mechanism of activation.

Chapter 2: Materials and Methods

2.1 Materials

Most chemicals used in experiments were purchased from Fisher Scientific, Sigma-Aldrich and Oxoid unless otherwise stated. Radionucleotides were obtained from MP Biomedicals. Synthetic oligodeoxynucleotides were supplied by Alta Biosciences laboratory, the University of Birmingham. DNA ladder, Phusion polymerase, restriction endonucleases, calf intestinal alkaline phosphatase and T4 DNA ligase were purchased from New England Biolabs. dNTP mix was purchased from Bioline. 6X Orange loading dye was supplied by Fermentas. Sybrsafe was purchased from Invitrogen. DNase I was purchased from Roche. All chemicals and enzymes were used and stored according to instructions from the manufacturers. Purified NarL protein and the FeBABE-tagged RNA polymerase were kindly donated by Dr. Douglas Browning and Dr. David Lee, the University of Birmingham.

2.1.1 Media

Media were prepared using distilled water then the solutions were autoclaved for 15 minutes at 120°C, 1 atmosphere. LB (Luria Broth) was purchased from Sigma-Aldrich. It contains 10g/l tryptone, 5 g/l yeast extract and 5 g/l NaCl. 1X LB was made by dissolving 20 g LB powder in 1 litre of distilled water. 1 litre of MS (Minimal Salts medium) contains 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/l $MgCl_2.7H_2O$, 10 g/l $MnCl.4H_2O$, 4 g/l $FeCl_2.6H_2O$, 1 g/l $CaCl_2.6H_2O$, 20 ml/l HCl), 1 ml sodium selenate (1mM), 1 ml ammonium molybdate (1 mM), 0.1 g casein digest, 0.05 g yeast digest, 0.32 g sodium fumarate, 8 ml 50% glycerol. SOC media was purchased from Sigma-Aldrich. SOC contains 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 8.6 mM NaCl, 3.5 mM KCl, 20mM $MgSO_4$, 20 mM glucose. LB agar was

purchased from Oxoid. It was prepared by dissolving 28 g LB agar powder in 1 litre of distilled water. MacConkey lactose agar was purchased from Difco**.** It was prepared by dissolving 50 g powder in 1 litre of distilled water.

2.1.2. Buffers and reagents

Solutions were autoclaved for 15 minutes at 120°C, 1 atmosphere or sterilized using 0.2 µm filters. Buffers and reagents used are described in Table 2.1

2.1.3. Antibiotics

Stock solutions were prepared by dissolving antibiotics in distilled sterile water and stored at -20°C. It was added to media for selecting strains carrying plasmids with antibiotic markers. The 100 mg/ml stock solution of Ampicillin was made to 100 µg/ml final concentrations after it was added into media. The 10 mg/ml stock solution of Kanamycin was made to 50 µg/ml final concentrations. The 20 mg/ml stock solution of Tetracycline was made to 20 µg/ml final concentrations.

2.1.4. Bacterial strains

Bacterial strains used in experiments are listed in Table 2.2. Strains were streaked on agar plates containing antibiotics. Plates were incubated at 37°C overnight and stored at 4 °C. An overnight culture was prepared from a single colony picked by a wooden toothpick and inoculated into 5 ml 2X LB media containing relevant antibiotics. The culture was incubated

Table 2.1 Buffers and reagents used in the studies

Name	Composition
General Buffer	
Tris-EDTA (TE) buffer	10 mM Tris-HCl, 1 mM EDTA
Preparing competent cell	
TFB 1	100 mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$, 15% glycerol
TFB2	10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15% glycerol
Gel electrophoresis	
Agarose solution	0.8-1.5% agarose powder was dissolved in 0.5X TBE buffer and boiled for 20-25 seconds.
5X TBE buffer stock	0.445 M Tris-HCl, 0.445 M boric acid, 0.01 M EDTA. The stock was diluted to 0.5X TBE for using in agarose gel electrophoresis
7.5% Acrylamide solution	125 ml ProtoGel acrylamide (from 30% w/v stock solution purchased from National Diagnostics), 100 ml 5X TBE buffer, 20 ml glycerol per 500 ml dH_2O.
Beta-galactosidase assay	
Sodium carbonate (Na_2CO_3)	1 M contains 105.99 g Na_2CO_3/1 litre dH_2O
Z buffer	0.75 g KCl, 0.245 g $MgSO_4.7H_2O$, 8.53 g Na_2HPO_4, 4.87 g $Na_2H_2PO_4.2H_2O$ per 1 litre dH_2O. 2.70 ml β-mercaptoethanol was added immediately before used
1% sodium deoxycholate	1% w/v of deoxycholic acid sodium salt

Table 2.1 (continued)

Name	Composition
Electrophoretic mobility shift assays (EMSA)	
Sephadex G-50 solution:	5g Sephadex was suspended in 50 ml TE buffer and stored at 4°C.
10X HEPES-glutamate buffer	200 mM HEPES, 50 mM $MgCl_2$, 500 mM potassium glutamate, 10 mM dithiothreitol. Diluted in HEPES dilution buffer (10% v/v HEPES-glutamate buffer, 10% v/v BSA, 80% v/v dH_2O) prior to use.
10X binding buffer	1 M potassium glutamate, 100 mM K_2HPO_4, 100 mM KH_2PO_4, 10 mM EDTA, 500 µM DTT.
Fixing solution	10% v/v acetic acid, 10% v/v methanol
DNase I footprinting	
10X HEPES/glutamate buffer	200 mM HEPES, 50 mM $MgCl_2$, 500 mM potassium glutamate, 10 mM dithiothreitol. Diluted in HEPES dilution buffer (10% v/v HEPES glutamate buffer, 10% v/v BSA in dH_2O) prior to use.
10X binding buffer	1 M potassium glutamate, 100 mM K_2HPO_4, 100 mM KH_2PO_4, 10 mM EDTA, 500 µM DTT.
DNase I stop solution	0.3 M sodium acetate, 10 mM EDTA
Gel loading buffer:	95% v/v deionized formamide, 20 mM EDTA, 0.05% xylene cyanol FF, 0.05% bromophenol blue

Table 2.2 Bacterial strains (*E. coli* K-12)

Strains	**Genotype**	**Source**
JCB 387	Δ*nir*, Δ*lac*	Page *et al*., 1990
JCB 3875	JCB387 Δ*narP252*::Tn*10*d(Cm)	Page *et al*., 1990
JCB 3883	JCB387 Δ*narL*	Tyson *et al*., 1994
JCB 3884	JCB387 Δ*narL*Δ*narP252*:: Tn*10*d(Cm)	Tyson *et al*., 1994
JCB3871	JCB387 Δ*fis985*::StrR^{R}	Wu *et al.*, *2*004

shaking at 200 rpm, 37°C overnight. For long term storage, strains were maintained in 2XLB media containing 15% glycerol at -80°C.

2.2. Methods

2.2.1. Gel electrophoresis

Agarose gel electrophoresis

0.5X TBE buffer containing 0.8-1.5% agarose was boiled in microwave oven until totally dissolved. 0.8% agarose solution is used in order to image large fragments e.g. plasmids. Concentration of 1-1.5% agarose is used in order to image smaller fragments such as PCR products. 10,000X Sybrsafe (Invitrogen) was added to make the final concentration of 1X before agarose was poured to the casting plate. DNA solutions were mixed with 1/5th volume of 6X Orange DNA loading dye. Gels containing DNA samples and DNA ladder (100 bp ladder or 2-log ladder) were run at 100V in 0.5X TBE buffer for ~30-35 minutes or when DNA fragments separated adequately for observation using a UV trans-illuminator (Clare Chemical).

Polyacrylamide gel electrophoresis

Polyacrylamide gels were used for imaging very small DNA fragments (<0.5 kb). Gels contained 7.5% (w/v) acrylamide, 4% glycerol, 0.01 volume of 10% (w/v) ammonium persulphate, 0.001 volume of TEMED in 1X TBE buffer. Samples of DNA were mixed with 6X Orange DNA loading dye to a final concentration of 1X. Gels containing DNA samples and DNA ladder (100 bp ladder or 2-log ladder) were run at 36mA in 1X TBE buffer ~40

minutes (for 100 x 70 x 1.5 mm gel). Before imaging, gels were stained in 1X TBE containing Sybrsafe.

Sequencing polyacrylamide gel electrophoresis

In DNase I footprint assays, a large acrylamide sequencing gel (40cm x 30cm x 0.4cm) was used and run at 60 W in 1X TBE. The 6% acrylamide gel (SequaGel) was prepared then pre-run for 1 hour. DNA samples and loading buffer were mixed together and incubated for 2 min at 90 °C prior loading. The gel was run for 1.5-2 hours. Fixing solution was used to fix DNA then the gel was transferred to filter paper and let dried at 80 °C for up to 30 minutes. After being exposed overnight to a phosphor screen, the gel was scanned and analyzed using a BioRad Molecular Imager FX supplied with QuantityOne software.

2.2.2. DNA extraction

DNA purification by Qiaquick kit

The QIAgen QiaQuick PCR purification kit provides spin-columns containing silica membrane. In an appropriate pH condition and high salt, DNA binds to the membrane while proteins and free nucleotides pass through columns. To extract DNA from an agarose gel, the regions of gel containing DNA fragments were isolated using a scalpel. Then, the QIAgen Qiaquick Gel Extraction kit was used following instructions from the manufacturer to purify DNA. Finally, DNA was eluted to 50 ml EB buffer.

Electroelution

Regions of polyacrylamide gel containing DNA fragments of interest were isolated using a scalpel. Gels were transferred into a dialysis tube (clipped at one end) of 5-10 cm containing 200 µl of 0.1 TBE buffer. The dialysis tube was sealed at another end and run for 30 minutes at 26 mA. Then, the buffer in the tube was transferred to a new microcentrifuge tube for further DNA purification by phenol-chloroform extraction and ethanol precipitation.

Phenol-chloroform extraction and ethanol precipitation

The DNA solution was mixed vigorously with the same volume of phenol-chloroform then centrifuged at 13,000 rpm for 1 minute. The sample was separated to two layers. Contaminated proteins were separated from the DNA samples and appeared in a lower organic layer. 200 µl of an upper aqueous layer containing DNA was carefully transferred into a new microcentrifuge tube. Then, concentrated DNA was extracted from the DNA solution by ethanol precipitation.

The DNA solution was mixed with 10% (v/v) sodium acetate, 1% (v/v) 1M $MgCl_2$, 2 volume of 100% ice-cold ethanol and incubated for 15 minutes at -80 °C. The sample was centrifuged for 15 minutes at 14,000 rpm (4°C) then supernatant was removed. The DNA pellet was re-suspended with 70% ice-cold ethanol and re-centrifuged for 10 minutes at 14,000 rpm. The supernatant was discarded. The DNA pellet was dried for 10 minutes under vacuum to get rid of ethanol. Finally, the DNA pellet was dissolved into 50 µl sterile distilled water.

Plasmid DNA isolation

Plasmids used in this study are described in Table 2.3, which lists their useful functions. The structures of each plasmid are shown in Figures 2.1-2.10. To extract plasmid DNA in a small scale (miniprep), a single colony was picked and inoculated into 5ml of 2X LB containing relevant antibiotics. The culture was incubated overnight at 200 rpm in 37°C. Cell pellet was collected by centrifugation at 4,000 rpm for 1 minute. The QIAprep Mini-prep Spin Kit was used following providing protocol based on alkaline lysis.A large scale of pure plasmid DNA was extracted using QIAprep Spin Maxiprep kit. 250 ml of 2X LB supplemented with relevant antibiotics was inoculated with 1 ml of an overnight culture of cells carrying plasmids of interest. The culture was grown shaking at 37 °C overnight. Cells were collected by centrifugation for 30 minutes at 4,000 rpm, 4°C. Plasmid DNA was extracted using the maxiprep kit following instructions from the manufacturer.

Table 2.3 plasmids used in this study

Plasmid	Description
pRW50	Low copy-number plasmid containing tetracycline resistance marker, used for cloning *EcoRI-HindIII* promoter fragment to construct *lacZ* fusions for beta-galactosidase assays. The plasmid contains the *lac* operon carrying *lacZ, lacY, lacA* genes (Lodge *et al*, 1992).
pSR	High copy-number plasmid containing an ampicillin resistance marker used for general cloning of *EcoRI-HindIII* fragment (Kolb *et al*, 1995)
pJW15Δ100	High copy-number plasmid containing the *melR* promoter and coding region (first 100 codons). Used for cloning *EcoRI-HindIII* fragment (ampicillin resistance). Codon 1 and 2 of *melR* carrying the NsiI site (Kahramanoglou *et al.*,2006).
pLG339	Low copy-number plasmid for cloning. Contains kanamycin and tetracycline resistance markers (Stocker *et al.*, 1982)
pDLC1	pJW15Δ100 derivative containing *NsiI-HindIII* fragment carrying NarL-CTD (Chismon, 2010)
pDLC3	pLG339 derivative containing *BamHI-EcoRI* fragment carrying NarL-CTD (Chismon, 2010)

Table 2.3 (continued)

Plasmid	Description
pDLC5	pLG339 derivative containing *BamHI-EcoRI* fragment carrying *narXL* operon and regulatory region(Chismon, 2010)
pDLC8	pJW15Δ100 derivative containing *NsiI-HindIII* fragment carrying NarP-CTD (Chismon, 2010)
pDLC15	pJW15Δ100 derivative containing *NsiI-HindIII* fragment carrying *NarP* gene and regulatory region (Chismon, 2010)
pGEMTeasy	Cloning vector for PCR products. Provided by Promega.

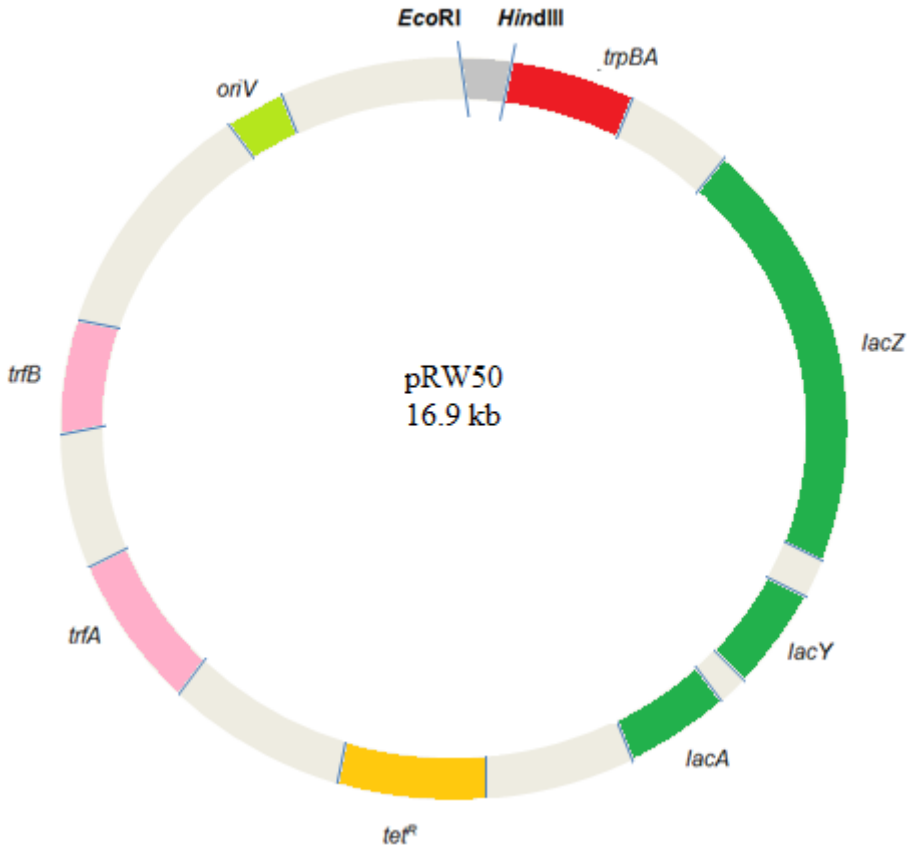


Figure 2.1 Map of pRW50

Figure shows a map of *lac* expression vector pRW50 used for measuring activities of transcription fusions (by cloning *EcoRI-HindIII* promoter fragment upstream of the *lacZYA* operon). The origin of replication is *oriV*. Replication genes (*trfB* and *trfA*) and tetracycline resistance marker tet^R are also shown.

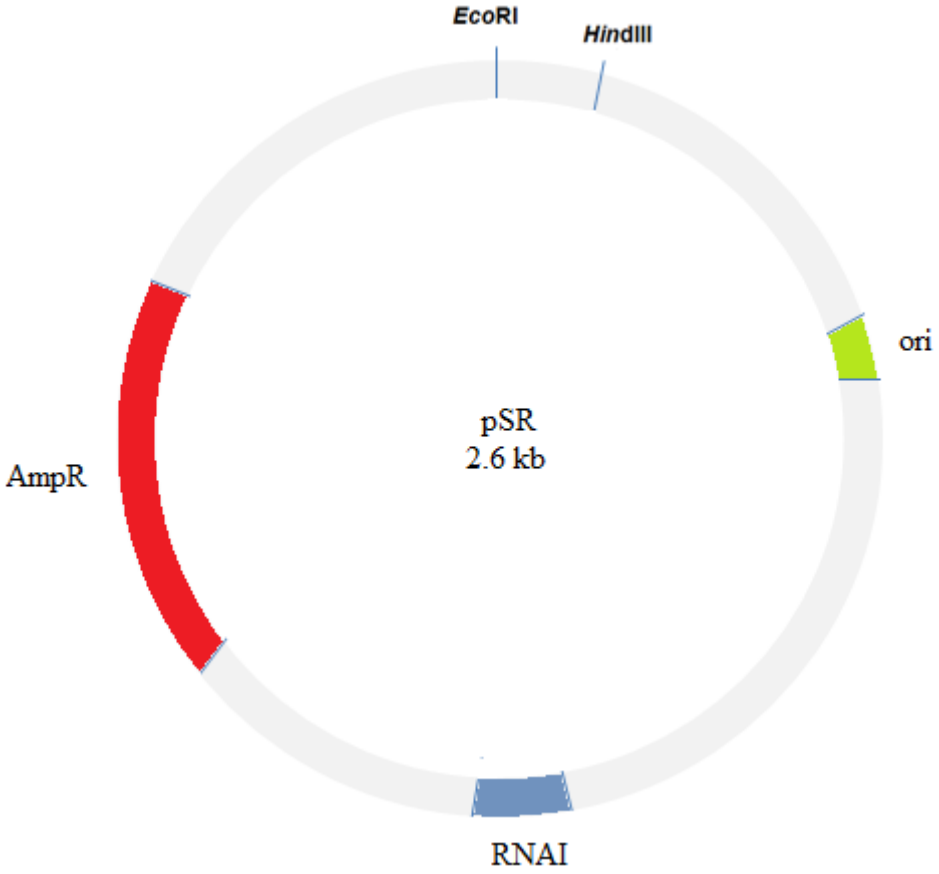


Figure 2.2 Map of pSR

Figure shows a map of high copy cloning vector pSR containing restriction sites for cloning *EcoRI-HindIII* fragment. Also shown are Ampicillin marker *(*AmpR) and the origin of replication *(ori). RNAI* gene is essential for replication control.

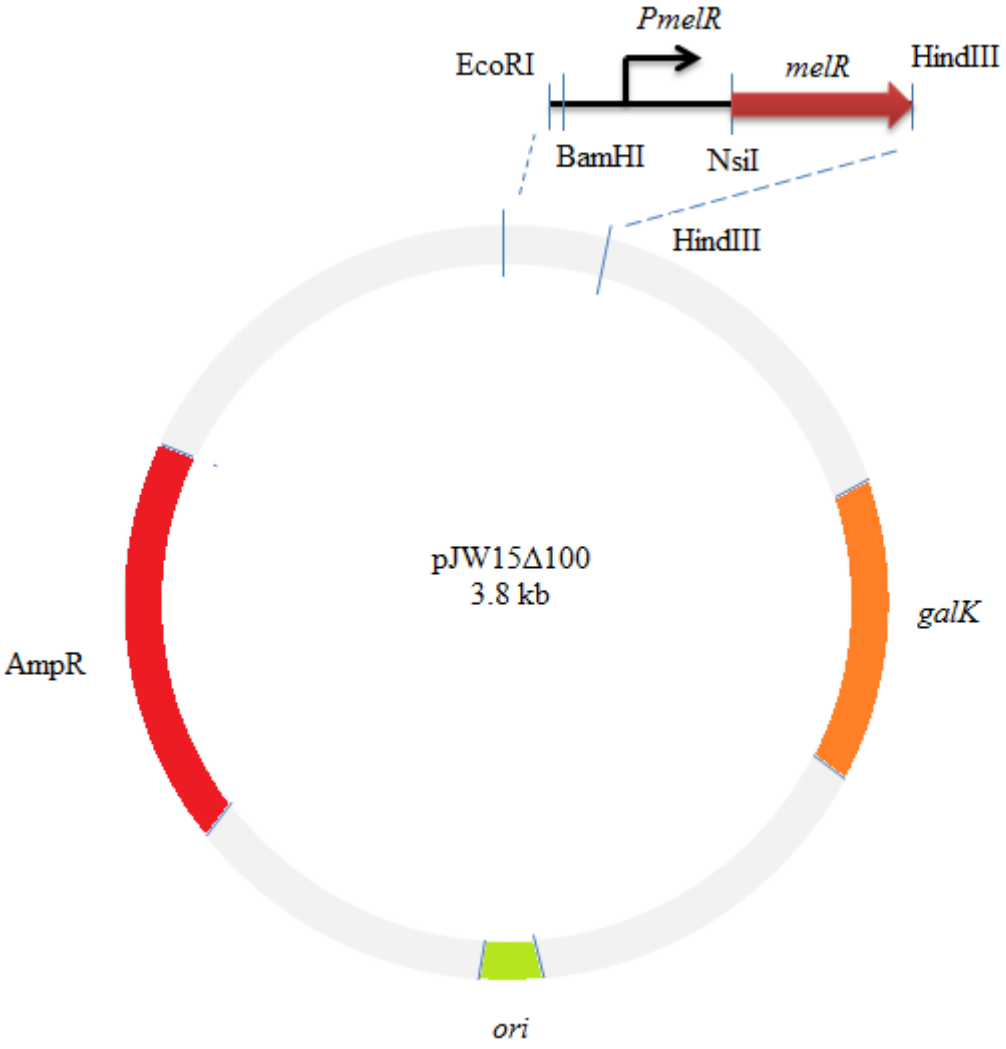


Figure 2.3 Map of pJW15Δ100

Figure shows a map of pJW15Δ100 plasmid. The plasmid contains *melR* with codons 1-99 deleted. Also shown is the *galK* gene, ampicillin resistance marker (AmpR) and the origin of replication (*ori*)

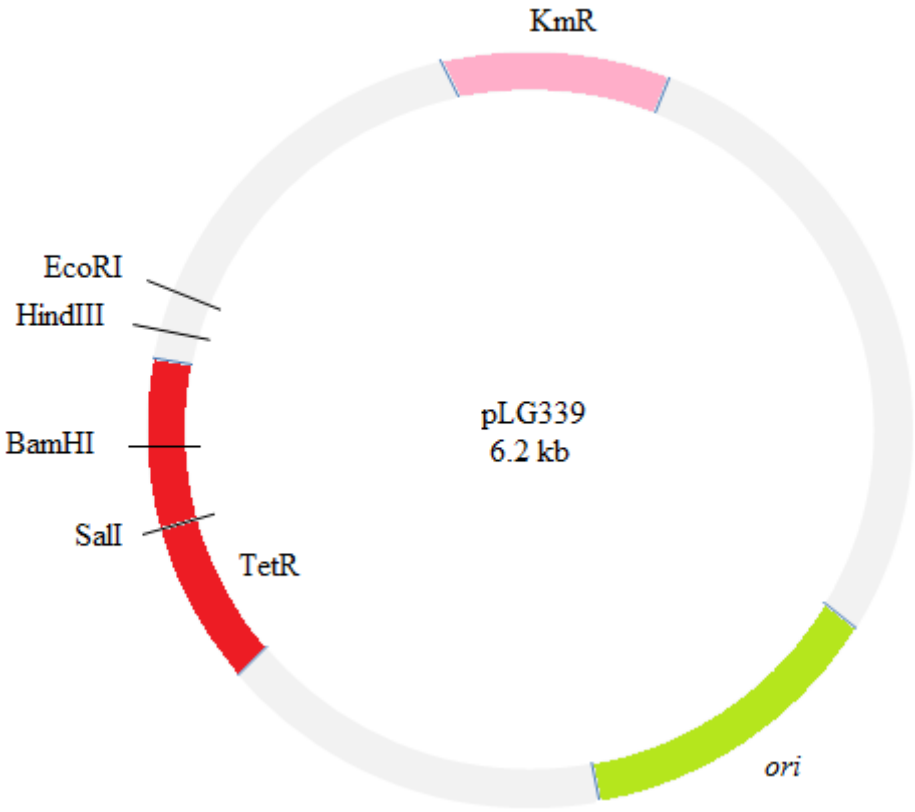


Figure 2.4 Map of pLG339

Figure shows a map of pLG339, a low copy-number plasmid. Also shown are kanamycin resistance marker (KmR), tetracycline resistance marker (TetR) and the origin of replication (*ori*)

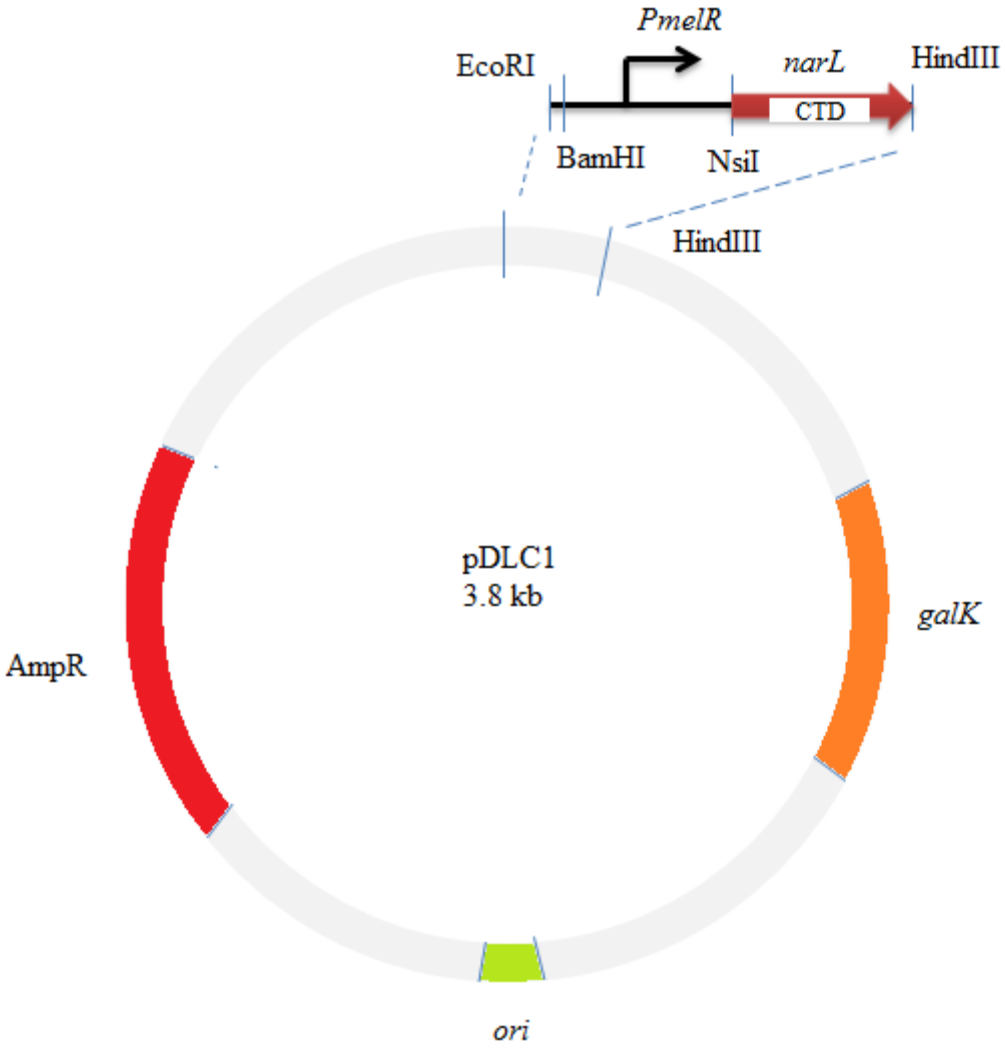


Figure 2.5 Map of pDLC1

Figure shows a map of pDLC1, a derivative plasmid of pJW15Δ100 that carries a DNA fragment encoding NarL-CTD at the *melR* coding region. Thus, the *melR* promoter drives NarL-CTD expression.

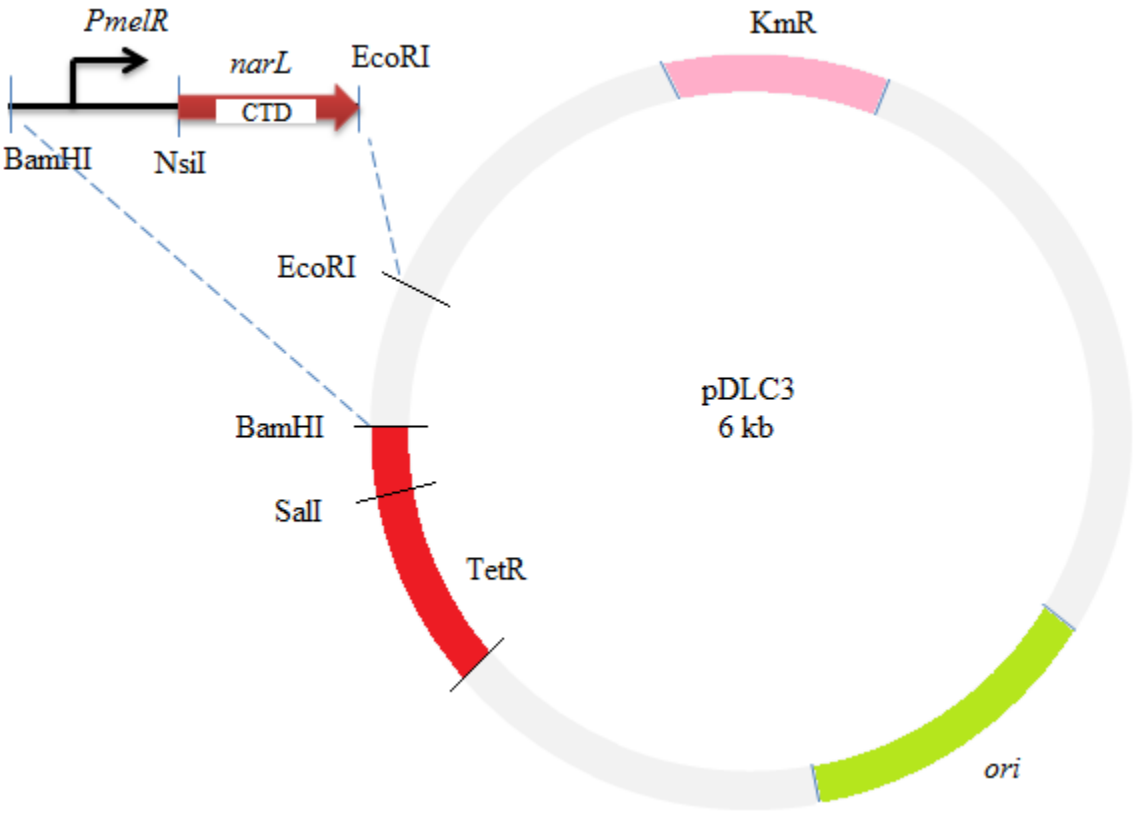


Figure 2.6 Map of pDLC3

Figure shows a map of pDLC3, a derivative of pLG339 plasmid. The region between *BamHI* and *EcoRI* sites at the TetR gene carries a DNA fragment encoding NarL-CTD driven by the *melR* promoter.

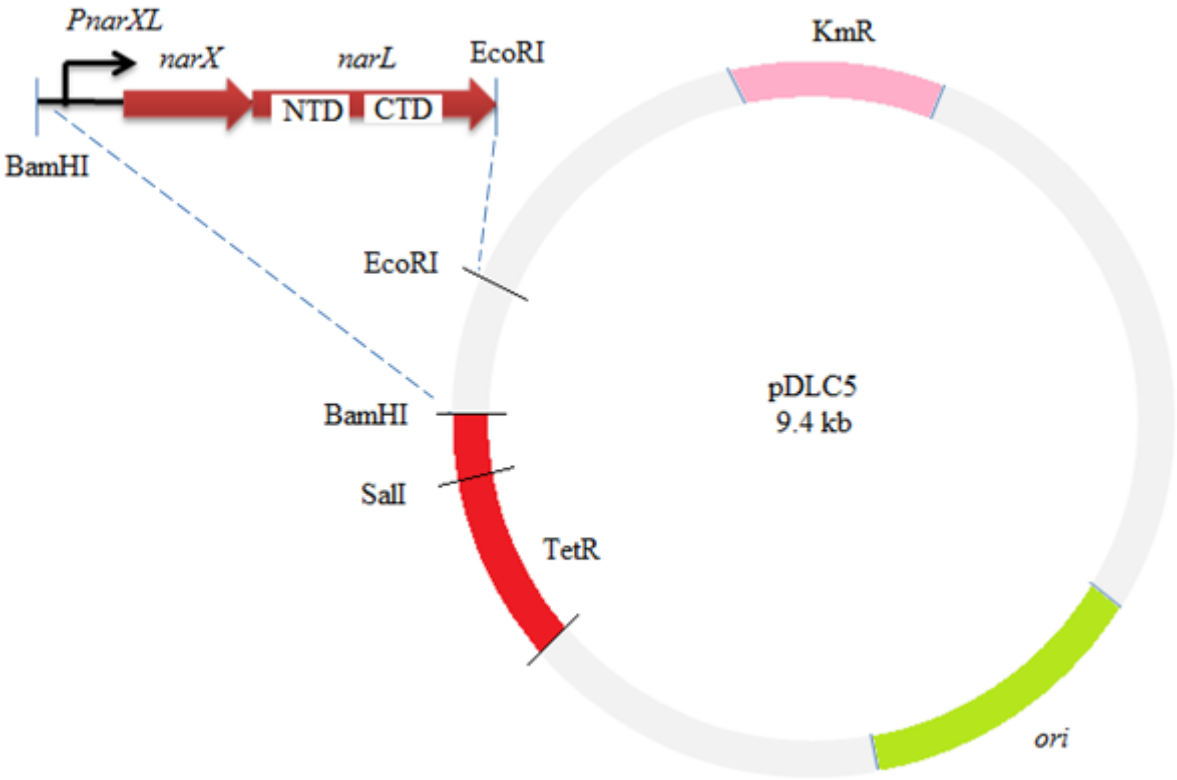


Figure 2.7 Map of pDLC5

Figure shows a map of pDLC5, a derivative of pLG339 plasmid. A region of TetR gene between *BamHI* and *EcoRI* sites carries a DNA fragment encoding *narXL* operon.

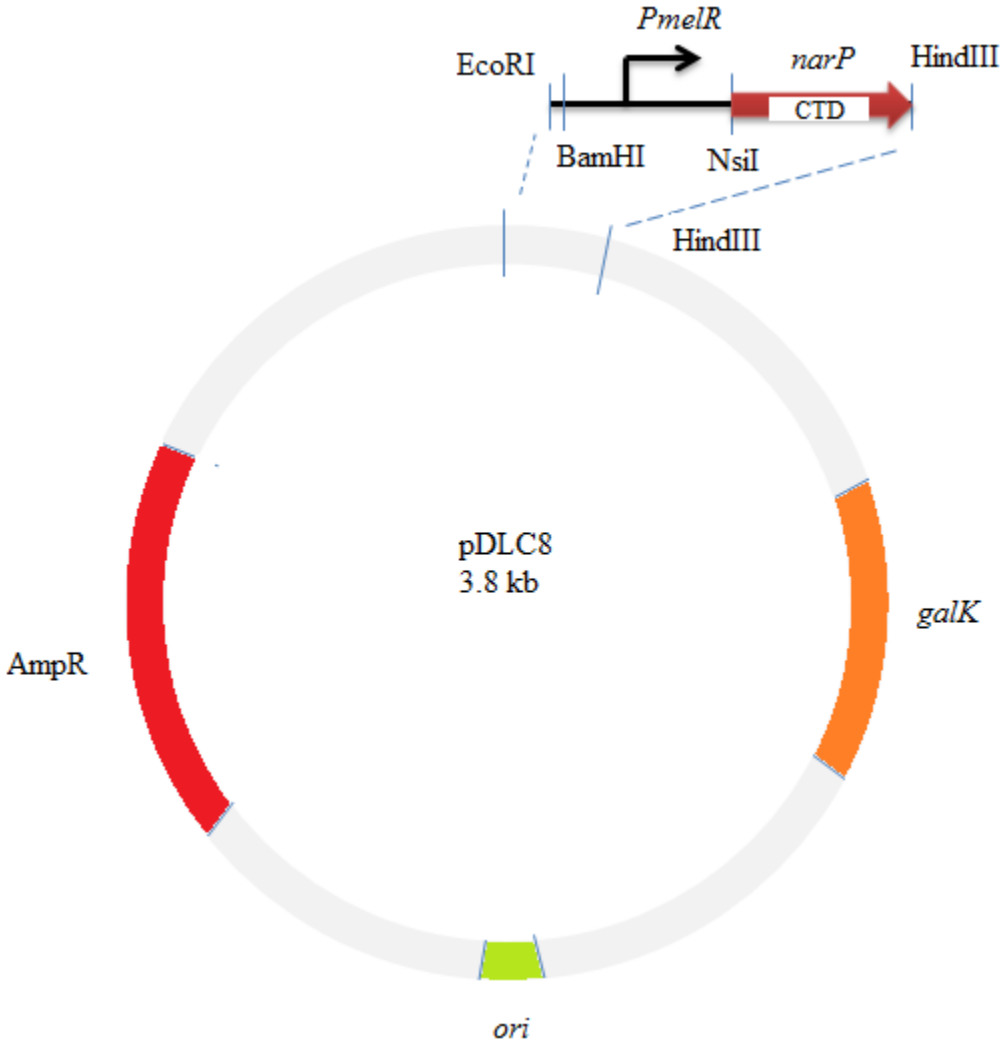


Figure 2.8 Map of pDLC8

Figure shows a map of pDLC8, a derivative plasmid of pDLC1. The region between *NsiI* and *HindIII* sites carries a DNA fragment encoding NarP-CTD driven by the *melR* promoter.

Figure 2.9 Map of pDLC15

Figure 2.9 shows a map of pDLC15, a derivative of pJW15Δ100 plasmid that carries a DNA fragment encoding the *narP* gene and its promoter.

2.2.3 DNA restriction

5% (v/v) restriction enzymes (NEB or Fermentas) were used in DNA restriction. To digest plasmid DNA for cloning preparation, the reaction was incubated at 37°C for at least 3 hours. Incubation time for PCR product digestion was ~30-60 minutes. After digestion, DNA was purified using PCR purification kit (QIAquick). For the second digest, another enzyme with an appropriate buffer was used. Another way is to use FastDigest enzymes with the same buffer for a double digest. Finally, DNA was collected after DNA fragments were separated by electrophoresis.

2.2.4 Dephosphorylation of vector DNA

To prepare vector DNA for cloning, 6 cell cultures of JCB387 containing the desired plasmids were incubated overnight in 5 ml of 2X LB medium. Cells were harvested by centrifugation at 4,000 rpm, then plasmids were extracted using a QIAprep mini-prep spin kit. Plasmid DNA was eluted in EB buffer to a final volume of 300 µl. Restriction enzymes were used to digest plasmids following by adding 5 µl of calf intestinal phosphatase; CIP (NEB) to the mixture for at least 30 minutes to remove 5′ phosphates and prevent plasmid re-ligation. CIP is active in NEBuffer 3 (100mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$). It also works well in NEBuffer 2 and 4, which are used for digestion with *EcoRI* and *BamHI*. QIAquick PCR purification kit was used for purification.

2.2.5 DNA ligation

The desired DNA fragment was inserted into the restriction sites of a plasmid vector using T4 DNA ligase (NEB) as a catalyst to form phosphodiester bonds between 2 nucleotides. The 20 µl reaction contains 2 µl of dephosphorylated vector, 1 µl of T4 DNA ligase, 2 µl of 10X T4 ligase buffer and 10 to 15 µl of inserted DNA fragments in sterile distilled water. The mixture was incubated at room temperature for at least 30 minutes then 1 to 5 µl of the reaction was taken for transformation.

2.2.6 Transformation

Preparation of competent cells

A culture in 5 ml 2X LB media was incubated shaking overnight at 37°C. The next day, 100 ml of 2X LB in a conical flask was inoculated with 1 ml of the overnight culture. The flask was incubated shaking until an optical density at 650 nm (OD 650) reached ~0.4-0.5. The flask was incubated in ice for another 10 minutes then the content was centrifuged at 4,000 rpm, 4°C for 9 minutes. The supernatant was discarded. 30 ml of TFB1 buffer was added to suspend cell pellet. The tube was placed in ice for at least 90 minutes. Then cells were centrifuged again at 4,000 rpm, 4°C for 9 minutes. The supernatant was discarded then cell pellet was re-suspended in 4 ml of TFB2 buffer. Aliquots were prepared (300 µl for each aliquot) and immediately frozen at -80°C.

Transformation

The frozen competent cells were defrosted by placing on ice for 10 minutes. 40 µl of thawed competent cells were transferred to a new 0.5 ml micro-centrifuge tube and mixed

with 1 μl of plasmid DNA (5-10 μl in case of ligation mixture). The tube was incubated in ice for 30 minutes. Cells were heat-shocked by incubating in water bath at 42°C for 42 seconds then immediately put back in ice for 5 minutes. 250 μl of SOC broth (Sigma) was added. Cells were incubated shaking at 37°C for 30 minutes. Finally, 100 μl of the transformation mixture was plated onto the agar plate containing appropriate antibiotics. Plates were incubated at 37°C overnight.

2.2.7 Polymerase Chain Reaction (PCR)

PCR is a method using DNA polymerase (Phusion) and oligonucleotide primers (all primers used were synthesized by Alta Bioscience, the University of Birmingham, UK) to amplify DNA template. The Phusion™ Hotstart polymerase contains 5′-3′ DNA polymerase activity and 3′-5′ exonuclease activity. The primer lists used in this study are shown in Table 2.4. The 50 μl of a standard PCR reaction contains 10 μl of 5X HF buffer, 0.1 μM of each primers, 1μl of the template DNA, 0.25 mM of each dNTP, 0.5 μl of Phusion polymerase.

Table 2.4 primers used in this study

name	Description	Sequence (5′-3′)
pRWup	Forward primer, anneals upstream *EcoRI* site of pRW50	CCCTGCGGTGCCCCTCAAG
pRWdown	Reverse primer, anneals downstream *HindIII* site of pRW50	GCAGGTCGTTGAACTGAGCCTGAAATTCAGGATCTT
pSRup	Forward primer, anneals upstream *EcoRI* site of pSR	ACCTGACGTCTAAGAAACC
pSRdown	Reverse primer, anneals downstream *HindIII* site of pSR	ATCCAGATGGAGTTCTGAGG
Ogt41 inst5	Used in inverse pcr to construct ogt104183 promoter fragment.	GGGAGATCTATCCACTTAGCTTTTTGGTGCTA
Ogt41 inst10	Used in inverse pcr to construct ogt104188 promoter fragment.	GGGAGATCTCTGATATCCACTTAGCTTTTTGGTGCTA
Nar I inst DN	Used with either Ogt41 inst5 or Ogt41 inst10 to construct ogt104183 and ogt104188 promoter fragment	CCCAGATCTGGGCAAGACTACCCATTAATA
Ogt41 Nar I MT	Used with D10527 to construct ogt104152 promoter fragment	ACGCGAAACTGGGTACCTCTTTAGAGGTAGTCTTGCCCTA
Ogt52 Nar II MT	Used with D10527 to construct ogt105241 promoter fragment	TGCCCTATCCACTTACTTATTAATGGGTATGGCTGCTGAT
Ogt 10 MT	Used with D10527 to mutate base 2 of -10 element of the promoter fragment	TTGCTGGCGTGGTCTCTTGTCGGTCTGCCG
Ogt4168up	Forward primer to produce megaprimer for construction of ogt104168 promoter fragment, used with D10527	ACTTATTAATGGGTAATCCACTTAGCTTTT
Ogt4168 DN	Reverse primer to produce megaprimer for construction of ogt104168 promoter fragment, used with D10520	AAAAGCTAAGTCGATTACCCATTAATAAGT
Ogt4158up	Forward primer to produce megaprimer for construction of ogt104158 promoter fragment, used with D10527.	ACTTATTAATGGGTACTTTTTGGTGCTATG
Ogt4158 DN	Reverse primer to produce megaprimer for construction of ogt104158 promoter fragment, used with D10520	CATAGCACCAAAAAGTACCCATTAATAAGT

Table 2.4 (Continued)

name	Description	Sequence (5′-3′)
Ogt4145up	Forward primer to produce megaprimer for construction of ogt104145 promoter fragment, used with D10527.	ACTTATTAATGGGTATGGCTGCTGATGTTG
Ogt4145 DN	Reverse primer to produce megaprimer for construction of ogt104145 promoter fragment, used with D10520	CAACATCAGCAGCCATACCCATTAATAAGT
Ogt41-1up 2dn	Forward primer to produce megaprimer for construction of ogt1041_1+2- promoter fragment, used with D10527	ACGCGAAACTGGGTACTTATTATTCGCTAGTCTTGCCCTA
Ogt41-1dn 2up	Forward primer to produce megaprimer for construction of ogt1041_1-2+ promoter fragment, used with D10527	ACGCGAAACTGGGTAGTTACTAATGGGTAGTCTTGCCCTA
Ogt52-3up 4dn	Forward primer to produce megaprimer for construction of ogt1052_3+4- promoter fragment, used with D10527	TGCCCTATCCACTTACCTCTTTGGTGCTATGGCTGCTGAT
Ogt52-3dn 4up	Forward primer to produce megaprimer for construction of ogt1052_3-4+ promoter fragment, used with D10527	TGCCCTATCCACTTAGCTTTTTAGAGGTATGGCTGCTGAT
Ogt417 MT	Used with D10527 to construct ogt10417 promoter fragment	TCTGAATTCTAATGGGTAGTCTTGCCCTAT
Nar208A ST	Forward primer to introduce alanine substitution to position 208 of NarL	CGCGTGGAAGCAGCGGCATGGGTGCATCAGGAG
Nar178A ST	Forward primer to introduce alanine substitution to position 178 of NarL	AAGATGATTGCCGCCCGCCTGGATATCACC
Nar179A ST	Forward primer to introduce alanine substitution to position 179 of NarL	ATGATTGCCCGCGCCCTGGATATCACCGAA
Nar181A ST	Forward primer to introduce alanine substitution to position 181 of NarL	ATTGCCCGCCGCCTGGCCATCACCGAAAGC
Nar178E ST	Forward primer to substitute position 178 of NarL by glutamate	AAGATGATTGCCGAGCGCCTGGATATCACC
pRE273R FW	Used in inverse PCR with pRE273R RW to construct pREII-E273R	AACTGCCTTAAAGCACGAGCTATCCACTATATCGGTGATCTG
pRE273R RW	Used in inverse PCR with pRE273R FW to construct pREII-E273R	ATAGTGGATAGCTCGTGCTTTAAGGCAGTTAGCAGAGCGGAC

Table 2.4 (Continued)

name	Description	Sequence (5′-3′)
Ogt52A1 up	Used with D10527 to produce megaprimer to construct ogt1052A1 promoter fragment	GTATGGCTGCTGTGTTGCTGGCGT
Ogt52A2 dn	Used with D10527 to produce megaprimer to construct ogt1052A2 promoter fragment	GTATGTCTGCTGTGTTGCTGGCGT

Sterile distilled water is added to the PCR sample to make up 50 µl volume. There are 3 stages within 1 cycle of PCR. Denaturation is the first stage that DNA strands are separated by high temperature. The second stage, called the annealing stage, occurs when temperature is cooled down to the optimum point that two primers can bind to the template DNA. Finally, temperature is increased again during the extension stage, which DNA are replicated by DNA polymerase. The cycle of 3 stages is repeated ~30 rounds to amplify the DNA fragments. In this study, the temperature was set at 98 °C, 1.30 minutes for an initial denaturation. 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, 15seconds per kb for extension. The final extension stage was set at 72°C for 5 minutes then PCR products were cooled down to 4 °C.

Biomix Red™ (Bioline) was used for colony PCR to check whether cells contain plasmids carrying particular DNA fragments. After an initial denaturation at 95°C for 10 minutes, cycles were set as 95°C, 30 seconds for denaturation; 42-55°C, 1 minute for annealing and 71°C, 15 seconds for extension. The final extension step was set at 71°C for 7 minutes. A single colony was picked and suspended in a PCR sample containing 25 µl Biomix Red and 1 µl of each oligonucleotide primer made up to a 50 µl final volume.

2.2.8 Construction of promoter fragments

EcoRI-HindIII promoter fragments used in this study are listed in Table 2.5**.** Sequences of promoter fragments are shown in Figure 2.10-2.13.

Base insertion between NarL sites of the ogt promoter

The DNA sequence of the ogt100 promoter was confirmed by the Functional Genomics and Proteomics Laboratory, the University of Birmingham. To construct the *ogt* promoter fragment that carries inserted bases between two NarL sites, the ogt1041 promoter fragment was amplified from the pRW50 carrying the ogt100 promoter. PCR products were purified then digested with *EcoRI* and *HindIII* and run on an agarose gel. The *EcoRI-HindIII* promoter fragment (~300 bp) was extracted from the gel and cloned into the pSR vector. Plasmids were introduced into JCB387 (Both NarL and NarP are present in cells) then transformants were plated on nutrient agar containing ampicillin.

Inverse PCR was used to insert 5 and 10 bp between the NarL I and NarL II binding sites to construct the ogt104183 and ogt104188 promoter fragments respectively. A forward primer (Ogt41 inst5, Ogt41 inst10 for 5 and 10 bp insertion respectively) and a reverse primer (Nar I inst DN) were designed to add additional 5/10 bp to the 5' end as well as to carry the *BglII* restriction site. Diagrams of the method are illustrated in Figure 2.14. Products from inverse PCR were cut with *BglII* then ligated to produce circular plasmids. The recombinant plasmids were cut by *EcoRI* and *HindIII*, purified and ligated into the prepared pRW50 vector.

Table 2.5 *EcoRI-HindIII* promoter fragments used

Name	Description	Source
ogt100	The *ogt* promoter fragment of *E. coli* (carries sequences from -270 to +50)	Squire *et al.*, 2009
ogt102	Derivative of the ogt100 promoter fragment (carries P84G and P63C mutations)	M. Xu
ogt104	Derivative of the ogt100 promoter fragment (carries P51G and P40C)	M. Xu
ogt1041	Derivative of the ogt100 promoter fragment (carries P80T, P77A, P75G, P73G, P51G, P40C)	D. Browning
ogt1052	Derivative of the ogt100 promoter fragment (carries P84G, P63C, P51C, P48C, P44A, P42A)	D. Browning
ogt100A-12C	Derivative of the ogt100 promoter fragment (carries P12C)	This work
ogt1041A-12C	Derivative of the ogt1041 promoter fragment (carries P12C)	This work
ogt1052A-12C	Derivative of the ogt1052 promoter fragment (carries P12C)	This work
ogt104152	The ogt1041 promoter fragment with sequence of the NarL I site replaced by the NarL II site from the ogt1052 promoter	This work
ogt105241	The ogt1052 promoter fragment with sequence of the NarL II site replaced by the NarL I site from the ogt1041 promoter	This work
ogt104188	The ogt1041 promoter fragment with 10 bp inserted between position -63, -64	This work

Table 2.5 (continued)

Name	Description	Source
ogt104183	The ogt1041 promoter fragment with 5 bp inserted between position -63, -64	This work
ogt104173	The ogt1041 promoter fragment with position -70 to -66 deleted	This work
ogt104168	The ogt1041 promoter fragment with position -70 to-61 deleted	This work
ogt104168A-12C	Derivative of the ogt104168 promoter fragment (carries P12C)	This work
ogt104163	The ogt1041 promoter fragment with position -70 to -56 deleted	This work
ogt104158	The ogt1041 promoter fragment with position -70 to -51 deleted	This work
ogt104153	The ogt1041 promoter fragment with position -70 to -46 deleted	This work
ogt104145	The ogt1041 promoter fragment with position -70 to -38 deleted	This work
ogt1041_1+2-	Derivative of the ogt1041 promoter fragment carrying P73C, P75C, P77T	This work
ogt1041_1-2+	Derivative of the ogt1041 promoter fragment carrying P80C, P84G	This work
ogt10417	Derivative of the ogt1041 promoter fragment with position -270 to -80 deleted	This work

Table 2.5 (continued)

Name	Description	Source
ogt1052_3+4-	Derivative of the ogt1052 promoter fragment carrying P40C, P42T, P44G	This work
ogt1052_3-4+	Derivative of the ogt1052 promoter fragment carrying P48T, P51C	This work
yeaR100	The *yeaR* promoter fragment of *E. coli* (carries sequences from -294 to +96)	D. Squire

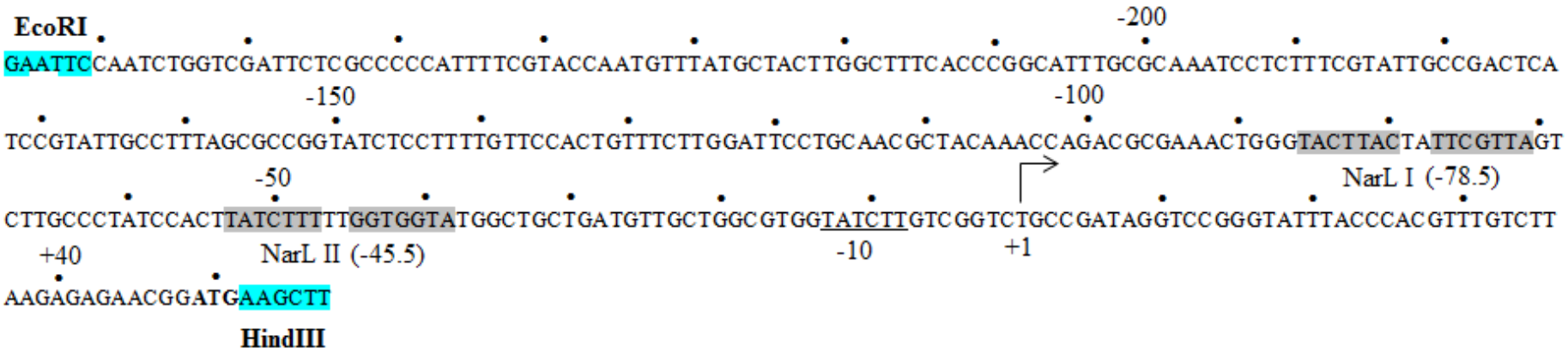


Figure 2.10 The *ogt* promoter fragment

The DNA sequence of the ogt100 promoter fragment. *EcoRI* and *HindIII* restriction sites are labeled and shaded in blue colour. The transcript start site is labeled +1 with a bent arrow and -10 element is underlined (Potter *et al.*, 1987). The translation start codon is shown in bold letters. The NarL binding sites are labeled and shaded in grey colour (Squire *et al.*, 2010).

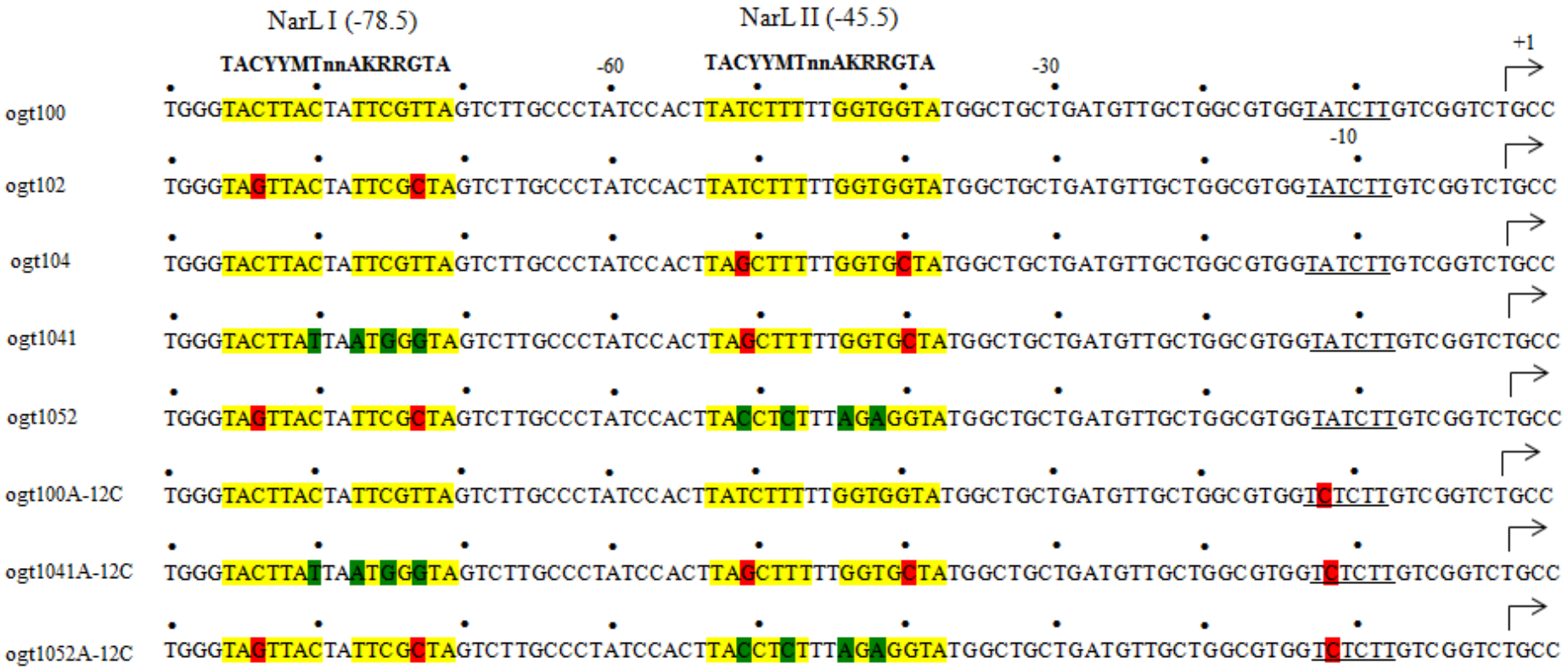


Figure 2.11 Partial sequences of the *ogt* promoter fragments used

Figure shows partial sequences of the *ogt* promoter fragments used in this study. The sequences outside the partial sequences are as same as in the ogt100 fragment. The consensus for both NarL binding sites is identified as **TACYYMTnnAKRRGTA**. Green shading indicates that the bases are mutated to improve affinities of the DNA sites for NarL. Red shading indicates that the bases are mutated to worsen affinities of the DNA sites for NarL. The -10 elements are underlined and the transcript start sites are indicated by bent arrows.

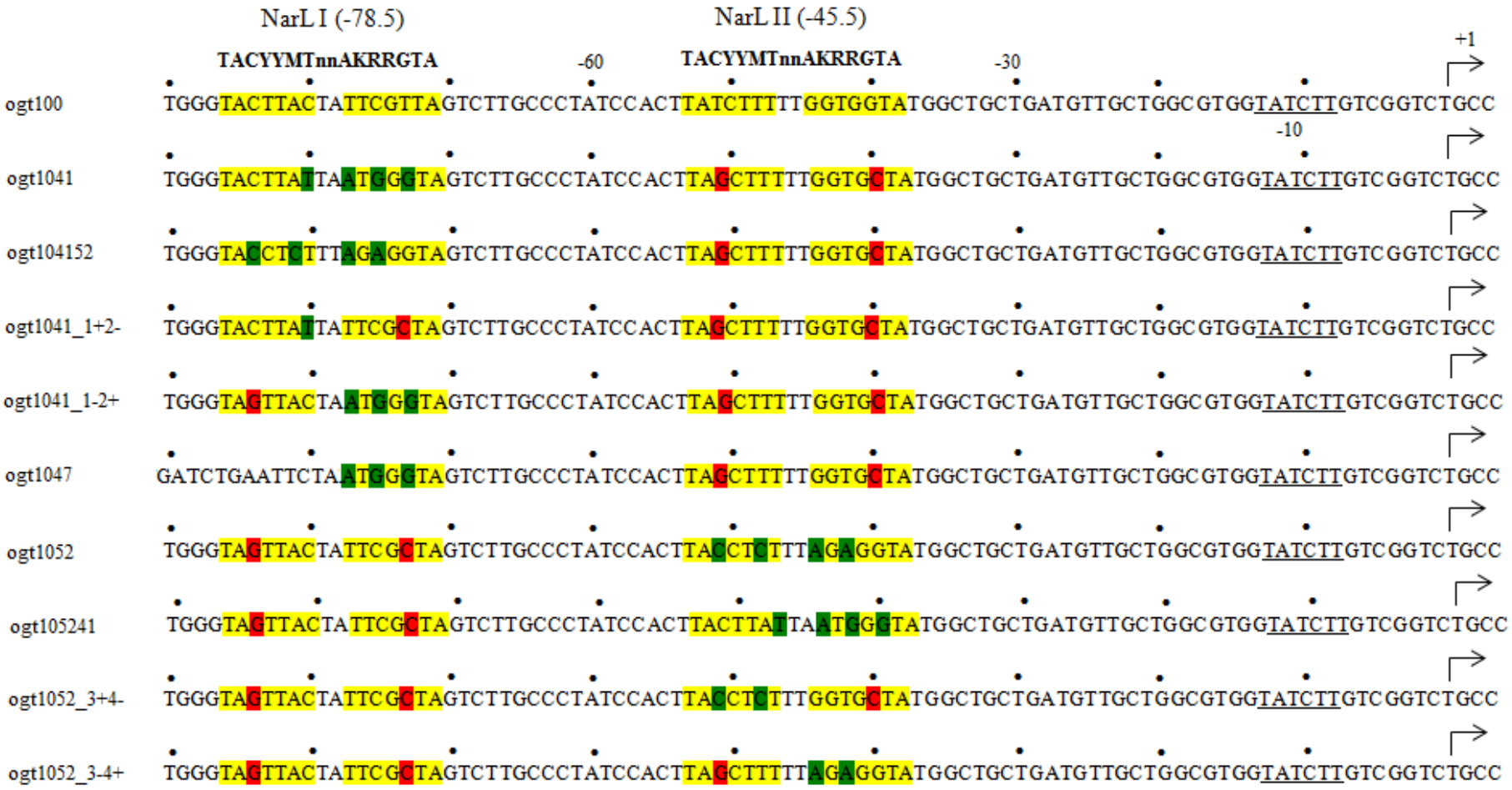


Figure 2.12 Partial sequences of the *ogt* promoter fragments used (2)

Figure shows partial sequences of the *ogt* promoter fragments used in this study. The sequences outside the partial sequences are as same as in the ogt100 fragment. The consensus for both NarL binding sites is identified as **TACYYMTnnAKRRGTA**. Green shading indicates that the bases are mutated to improve affinities of the DNA sites for NarL. Red shading indicates that the bases are mutated to worsen affinities of the DNA sites for NarL. The -10 elements are underlined and the transcript start sites are indicated by bent arrows.

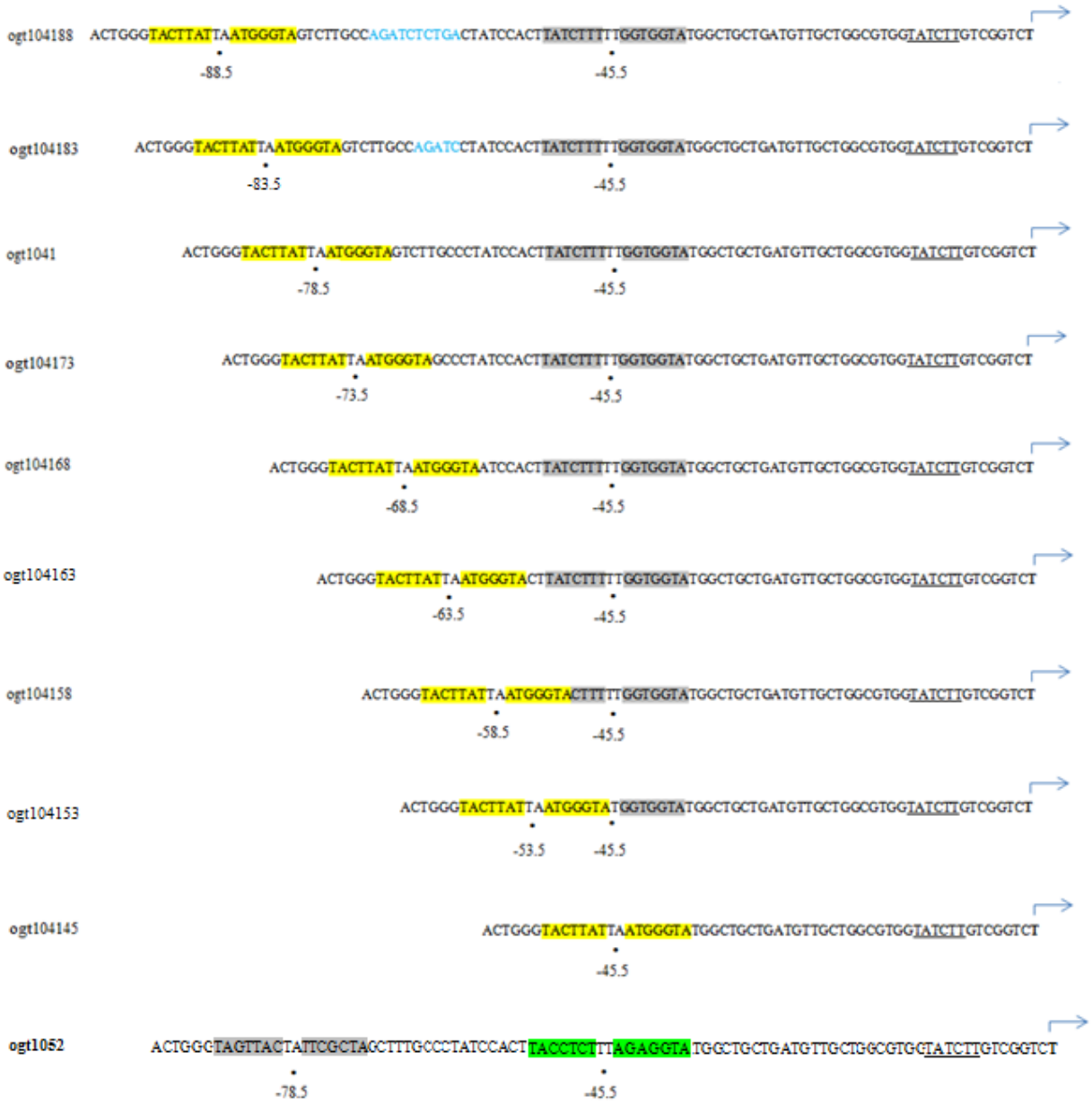


Figure 2.13 Partial sequences of the *ogt* promoter fragments carrying the NarL I site at different locations.

DNA sequence of the NarL I site is shaded in yellow. Sequences in blue colour denote inserted bases. The NarL I site was moved to position -88.5, -83.5, -73.5, -68.5, -63.5, -58.5, -53.5 and -45.5 relative to the transcript start site.

Site-directed mutagenesis by megaprimer PCR

Megaprimer PCR was used to introduce a point mutation at the specific base. Diagrams of the method are illustrated in Figure 2.15. The pRW50 plasmid carrying the desired promoter fragment was used as a template. A mutagenic primer (listed in Table 2.3) carrying point mutations at specific bases was used as a forward primer together with pRWdown in PCR to synthesize a megaprimer. The purified megaprimer was used as a reverse primer together with pRWup in another round of PCR with the same template. This step produced a full-length promoter fragment with mutations at the desired positions. PCR products were purified and cut with *EcoRI* and *HindIII* then cloned into the pRW50 vector.

Base deletion by overlapping PCR

In this study, ogt104173, ogt104168, ogt104163, ogt104158, ogt104153 and ogt104145 promoter fragments were produced by overlapping PCR to delete base-pairs between NarL sites and move the NarL I binding site to position -73.5, -68.5, -63.5, -58.5, -53.5 and -45.5 respectively. Diagrams of the method are illustrated in Figure 2.16. Two PCR products were generated separately. Both contain sequence identity at one end, allowing them to anneal to each other in the next PCR. Then, DNA extension was performed using DNA polymerase to synthesize full-length promoter fragments with deletion of specific bases. PCR products were purified and cut with *EcoRI* and *HindIII* then cloned into the pRW50 vector.

2.2.9 DNA sequencing

Sequencing was achieved by Functional Genomics and Proteomics Laboratory, the University of Birmingham. For plasmid sequencing, 3 μl of plasmid was mixed with 3.2 μl

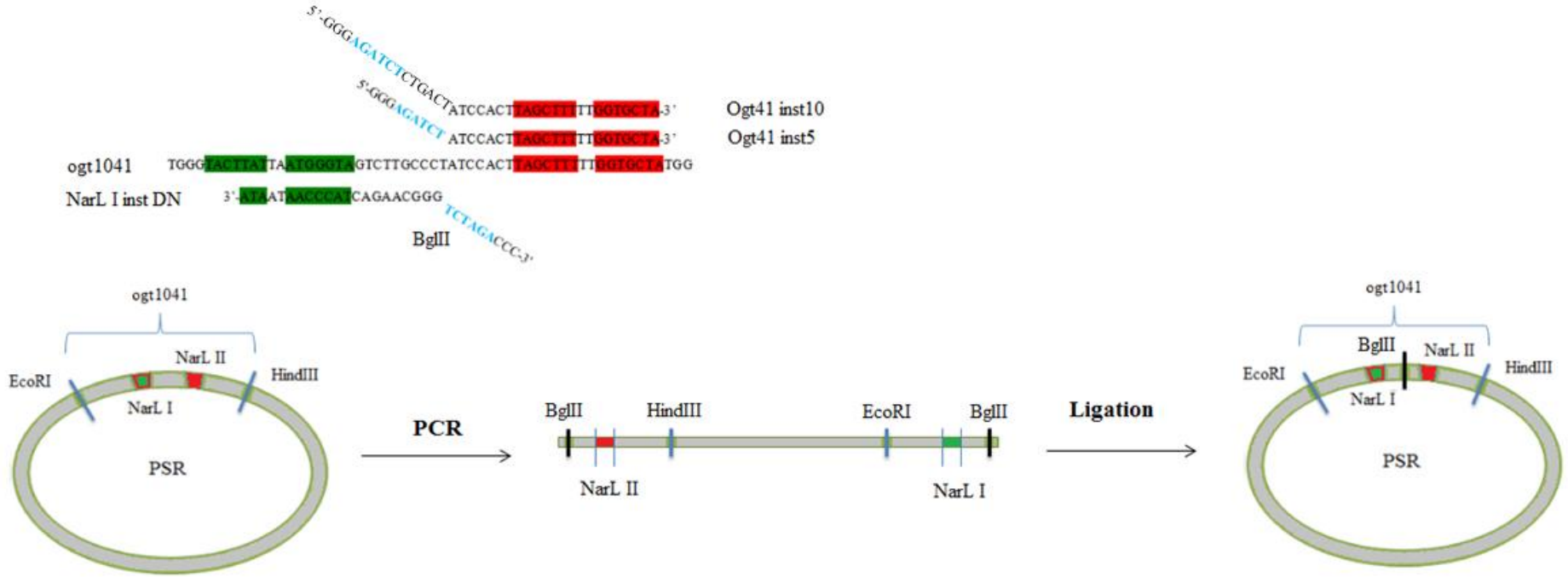


Figure 2.14 Insertion of 5/10 bp to the ogt1041 promoter

Forward primers Ogt41 inst5 and Ogt41 inst10 (each carries the *BglII* restriction site) were used with a reverse primer NarL I inst DN in inverse PCR in order to insert 5 and 10 bp between the NarL sites of the ogt1041 promoter. the PCR products contain two *BglII* site at the 5' and 3' end. The fragment was cut by *BglII* and re-ligated to produce a circular plasmid containing additional 5 and 10 bp between two NarL sites

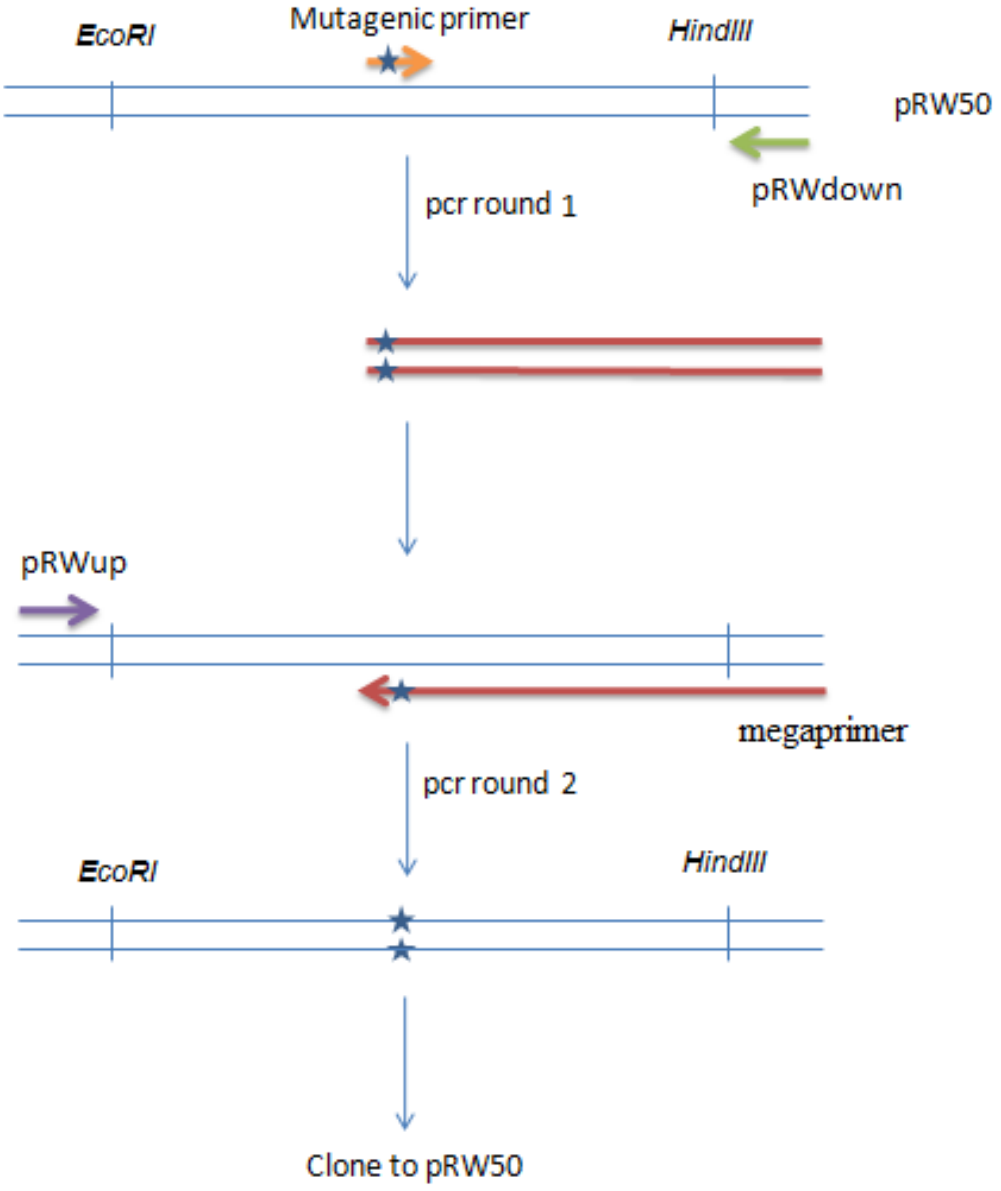


Figure 2.15 Site-direct mutagenesis by megaprimer PCR

Megaprimer PCR technique was used to introduce point mutations to specific bases in the *EcoRI-HindIII* fragment. The template was pRW50 carrying the promoter fragment of interest. A mutagenic primer was used as a forward primer together with pRWdown in the first PCR to generate PCR products containing mutation at the desired position. The PCR product (megaprimer) was used as a reverse primer together with pRWup primer to amplify the original template.

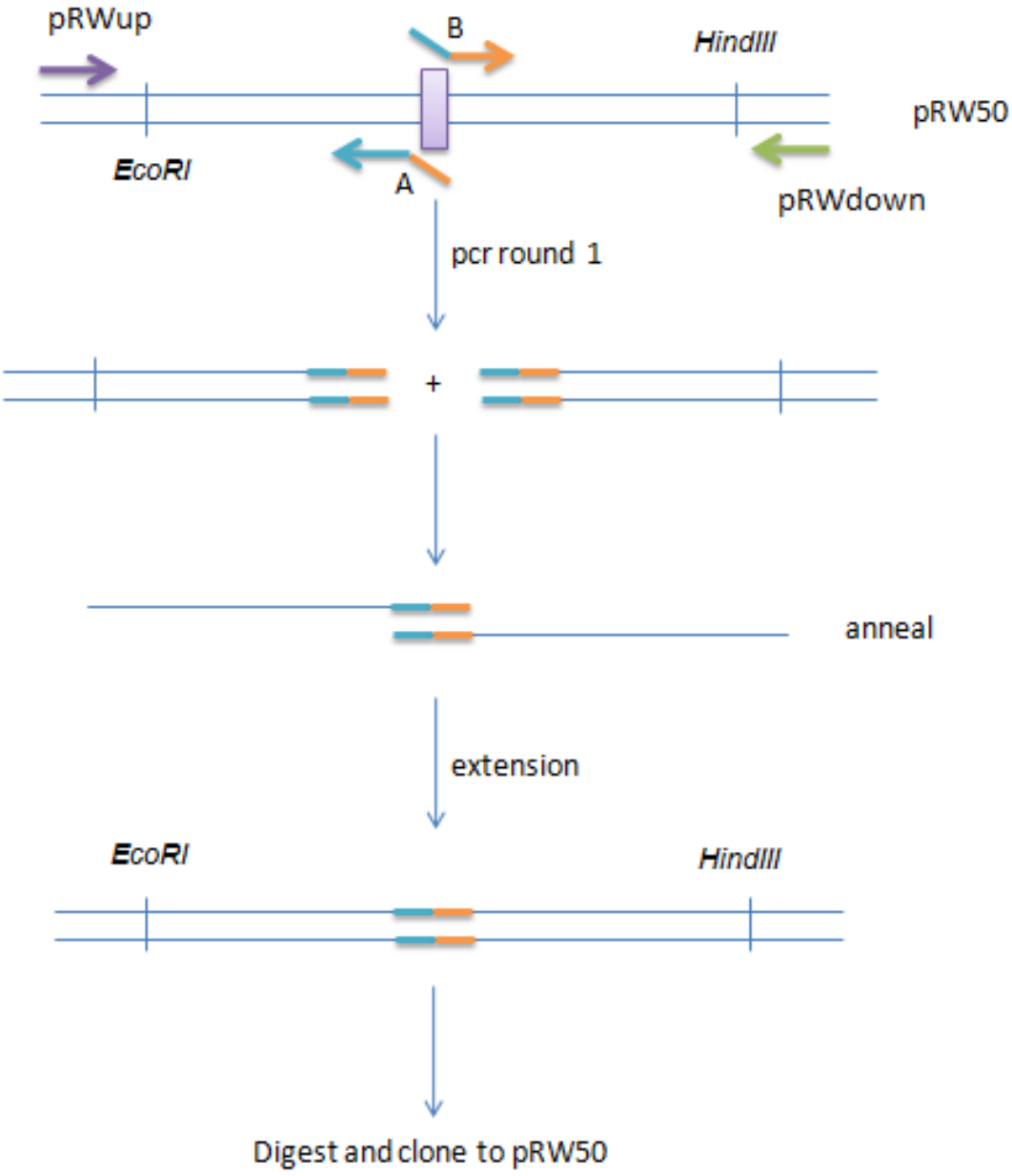


Figure 2.16 Base deletions by overlapping PCR

The DNA region to be deleted was shown as a purple box. Two PCRs were performed separately in the first PCR. Primers at the overlapped region have DNA sequences complementary to each other. PCR products from the first PCR were used as primers in the second PCR; hence, they anneal to each other. Replication was performed by DNA polymerase to generate a full-length DNA fragment.

of 10 μM sequencing primers and 3.8 μl sterile distilled water. For PCR sequencing, 6.8 μl of purified PCR product was mixed with 3.2 μl of 10 μM sequencing primers.

2.2.10. Beta-galactosidase assays (Miller, 1972)

To observe an activity of the desired promoter *in vivo,* beta-galactosidase activities is measured in cells carrying the desired promoter::*lacZ* fusions. A single distinct colony containing the promoter::*lacZ* fusion was inoculated in 2X LB and grown at 37°C overnight. To assay anaerobic activities, 500 μl of overnight cultures was inoculated into growth tubes containing 10 ml of 1X minimal salts medium and grown at 37°C without shaking until an optical density (OD) of 650 nm reached ~0.5-0.6.

To assay anaerobic activities in a presence of nitrate, 400 μl of overnight cultures was inoculated into 10 ml minimal salts medium supplemented with 20 mM sodium nitrate until OD_{650} reached ~0.5-0.6. Then, the growth tubes were placed on ice. 2 ml from each tube was transferred to new tubes and lysed by mixing with 30 μl of 1% sodium deoxycholate and 30 μl of toluene. Tubes were shaken at 100 rpm for 20 minutes to evaporate toluene. Assay tubes containing 2 ml of Z buffer were pre-warmed at 37 °C before adding 100 μl of lysate to each tube. The reaction was started by adding 0.5 ml of 13 mM ONPG solution (at 37 °C). To stop the reaction, 1M sodium carbonate solution was added at the time yellow colour developed. The OD_{420} was measured. The following formula was used to calculate beta-galactosidase activity.

Beta-galactosidase activity = (A x C x OD_{420} x 1000) / (4.5 x T x V x OD_{650})

A = final assay volume (3.6 ml)

C = coefficient of converting from OD_{650} to mg dried weight of protein = 2.5

OD_{420} = optical density at 420 nm

1000/4.5 = conversion factor of OD_{420} to nmol of o-nitrophenol

T = reaction time (min)

V = volume of lysate used in assay (ml)

OD_{650} = optical density at 650 nm

In this study, value of beta-galactosidase activities were the average of three replicates

2.2.11. Electro mobility shift assays (EMSAs)

The *EcoRI-HindIII* promoter fragments were cloned into pGEM-T easy vectors and introduced into JCB387. Plasmids were isolated by miniprep then cut with *EcoRI* and *HindIII* enzymes. DNA fragments were dephosphorylated by treating with CIP then run on 7.5% polyacrylamide gel and purified by electroelution. To prepare radio-labelled DNA fragment, 1 µl T4 polynucleotide kinase (NEB) was used to label the 5' end of promoter fragments (15-17 µl) with [γ^{32}P]-ATP in a 20 µl final volume. After 30 minutes of incubation at 37°C, the sample was passed through a Sephadex G-50 column containing suspension of 400 µl 50% Sephadex G-50 and centrifuged at 3500 rpm to remove unincorporated [γ^{32}P]-ATP. The

column containing labeling fragments was placed in a new tube and further centrifuged at 3500 rpm.

NarL proteins (donated by Dr. Douglas Browning, the University of Birmingham) were incubated with acetyl phosphate for 30 minutes for pre-phosphorylation. Different concentrations of phosphorylated NarL proteins were incubated at 37 ℃ for 30 minutes with 0.2 ng of P32-labelled promoter fragments, 1 µl binding buffer, 1 µl glycerol (50% v/v), 5µg BSA and 250 ng herring sperm DNA. Samples of DNA-protein complexes were loaded on 6% of polyacrylamide gel (contains 2% glycerol) and run in 0.5X TBE buffer at 160 V for ~2 hours. After electrophoresis, the gel was fixed by soaking in a fixing solution (10% acetic acid+10% methanol) for 15 minutes. The gel was vacuum-dried and exposed to a Fuji phosphor screen. Molecular imager FX with OuantityOne was used to analyze radio-labelled DNA fragments.

2.2.12. DNase I footprinting

DNA fragments were cloned into pGEM-T easy vectors and introduced into JCB387. 100 µl maxiprep of plasmids was cut by 8 µl *HindIII* and water was added to make a 120 µl total volume. Fragments were treated with CIP to dephosphorylated the 5' end. After phenol-chloroform treatment and ethanol precipitation, DNA was cut by 8 µl *AatI* in a 80 µl total volume. DNA fragments were run on 7.5% polyacrylamide gel, purified by electroelution following by phenol-chloroform extraction and ethanol precipitation. The *AatI-HindIII* fragments were re-suspened in 50 µl TE buffer. Then, T4 polynucleotide kinase was used to label γ-^{32}P on the fragments at the *HindIII* end.

A Maxam-Gilbert (GA) ladder was used to calibrate DNA footprinting reaction. The GA sequence ladder was prepared following the method from Maxam and Gilbert (1980). A total volume of 12 µl containing 3-4 µl of radiolabelled *AatI-HindIII* fragments were incubated at room temperature with 50 µl formic acid for 90 seconds. 200 µl of 0.3 M sodium acetate and 700 µl ice-cold 100% ethanol was added to stop the reaction. After DNA precipitation, the DNA pellet was re-suspended and incubated with 100 µl of 1 M piperidine for 30 minutes at 90°C. DNA was extracted by ethanol precipitation then the pellet was re-suspended in 20 µl loading buffer. The GA ladder was cleaved at G and A, allowing determination of positions where proteins bind to DNA.

DNase I footprinting was used to map positions of NarL and RNA polymerase on the *ogt* promoter fragments. 0.3 µl of 500 nM acetyl phosphate was used to phosphorylate 3 µl NarL (provided by Dr. Douglas Browning, the University of Birmingham) in 2.7 µl HEPES-glutamate buffer. The reaction was diluted in HEPES dilution buffer to different required concentrations and mixed with radiolabelled *AatI-HindIII* fragments. DNase I footprinting can be used to determine positions of holo-RNA polymerase as well. A total volume of 20 µl DNase I footprinting reaction contains NarL/RNA polymerase (different concentration as required), 1 µl labeled *AatI-HindIII* fragments, 2 µl HEPES-glutamate buffer, 1 µl BSA and sterile distilled water. Each reaction was incubated with DNase I solution (Roche Applied Science). After 30-90 seconds, DNase I stop solution was added to stop the reaction following by phenol-chloroform extraction and ethanol precipitation. Samples were re-suspended in loading buffer and run on 6% acrylamide sequencing gel.

2.2.13. FeBABE footprinting

In this work, a location of αCTD at the *ogt* promoter was determined by the FeBABE footprinting experiment. RNA polymerase holoenzyme was labelled with FeBABE at either position 273 or 302 of α subunits (donated by Dr. David Lee, the University of Birmingham). 200 nM of FeBABE-tagged RNA polymerase was incubated at 37 ℃ for 20 minutes with radiolabelled *AatI-HindIII ogt* promoter fragments and 3.2 µM pre-phosphorylated NarL in 25 µl final volume of HEPES-glutamate buffer containing 0.5 g/ml BSA. Then, sodium ascorbate and hydrogen peroxide was added to a final concentration of 5mM and 0.06% respectively to start DNA cleavage. FeBABE stop solution was added to the reaction after 10 minutes of incubation. Then DNA fragments were purified and re-suspended in loading buffer for denaturing gel. DNA cleavage patterns were analyzed by electrophoresis on 6% acrylamide sequencing gel.

Chapter 3: Regulation of the *E. coli ogt* promoter

3.1 Introduction

Transcription of genes regulated in a nitrate responsive manner is mediated by the paralogous response regulators, NarL and NarP (Stewart and Rabin, 1995). NarL and NarP act as activators or repressors at several individual promoters and their binding site configurations in the promoter regions differ from one promoter to another (Constantinidou *et al.*, 2006; Stewart and Rabin, 1995; Barnard *et al.*, 2004).

Most bacteria have systems to protect themselves against threats from RNS generated during nitrate and nitrite respiration (Berks *et al.*, 1995). In *E. coli,* the O^6-alkylguanine-DNA alkyltransferase (Ogt) is one of the DNA repair enzymes that has evolved to cope with DNA methylation damage caused by RNS. Expression of the *ogt* gene is induced by NarL in response to the presence of nitrate (Constantinidou *et al.*, 2006). The *ogt* promoter contains two DNA sites for NarL centered at position -78.5 (the NarL I site) and position -45.5 (the NarL II site) relative to the transcript start site. The partial base sequence of the *ogt* promoter fragment is illustrated in Figure 3.1. Transcription activation of the *ogt* promoter requires binding of NarL to both DNA sites. This work aims to investigate the roles of NarL and NarP response regulators in relation to the organization of the *ogt p*romoter.

3.2 Effects of the consensus binding site for NarL on the *ogt* promoter activity

Derivatives of the wild-type *ogt* promoter (ogt100) were constructed by introducing point mutations into the DNA sites for NarL to increase or decrease the binding affinity of NarL. The base sequences of the NarL binding sites in each *ogt* promoter derivative are

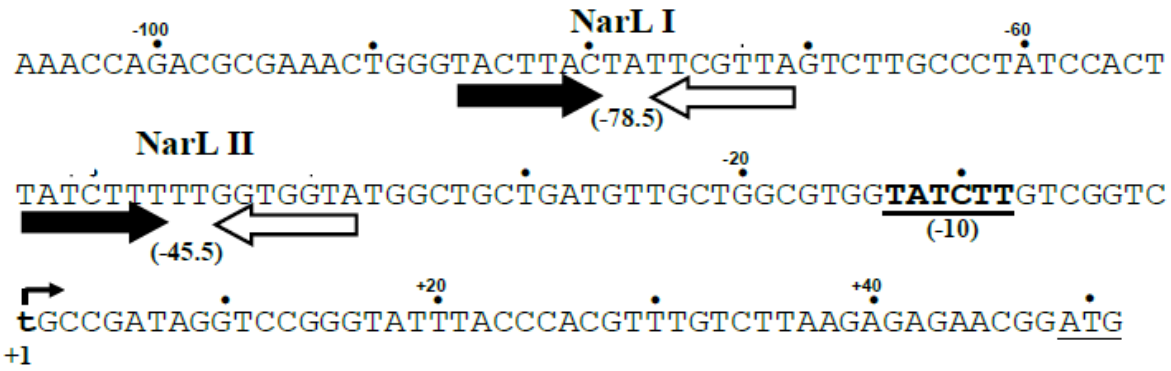


Figure 3.1 Partial base sequence of the *ogt* promoter fragment

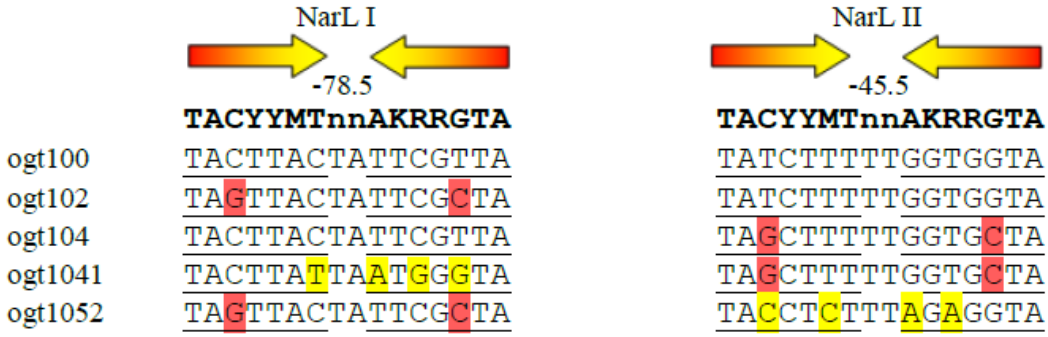
The figure shows a partial base sequence of the ogt100 promoter fragment from position -106 to +51 relative to the transcript start site (+1). Two DNA sites for NarL are identified by inverted arrows and the centre of each site is designated relatively to the transcript start site. The -10 element is underlined and boldface. The transcript start site is indicated by a bent arrow on the lower-case letter. The translation start codon (ATG) is underlined (Figure is taken from Squire *et al.*, 2009).

illustrated in Figure 3.2 panel A. DNA fragments containing the *ogt* promoter region from positions -270 to +51 were cloned into a *lac* expression vector (pRW50). Then, recombinants were introduced into JCB387 (both NarL and NarP are present in the cells). Cells were grown in minimal salts medium anaerobically with or without 20 mM nitrate to measure beta-galactosidase activities.

Results illustrated in Figure 3.2 panel B show that both DNA sites for NarL are required to activate the ogt100 promoter (both NarL sites are wild-type). Expression of the ogt100 promoter::*lacZ* fusion is low without nitrate induction. In the presence of nitrate, the activity of the ogt100 promoter increases ~3-fold. The ogt102 promoter contains the NarL I site mutated to disrupt NarL binding while the NarL II site remains functionally intact as the wild type. In contrast, the ogt104 promoter contains the wild-type NarL I site and the disrupted NarL II site. NarL fails to activate both the ogt102 and the ogt104 promoters. This suggests that both NarL binding sites are crucial as NarL fails to activate the *ogt* promoter that contains only one DNA site for NarL.

This work also examined the activities of the *ogt* promoter derivatives that contain a single NarL site that was modified to better resemble the consensus sequence. The ogt1041 and the ogt1052 promoters contain a single near-consensus NarL binding site at position -78.5 and -45.5 respectively. Results illustrated in Figure 3.2 panel B show that the activity of the ogt1041 promoter induced by nitrate is comparable to the activity of the ogt100 promoter. This indicates that NarL can activate the *ogt* promoter that contains only one NarL binding site at position -78.5 if the site is modified to match the consensus. When the consensus NarL site is located at position -45.5, a high level of expression of the ogt1052 pomoter::*lacZ* fusion was found despite the lack of nitrate supplementation. In the presence of nitrate, the ogt1052 promoter activity is induced ~2-fold greater.

A.



B

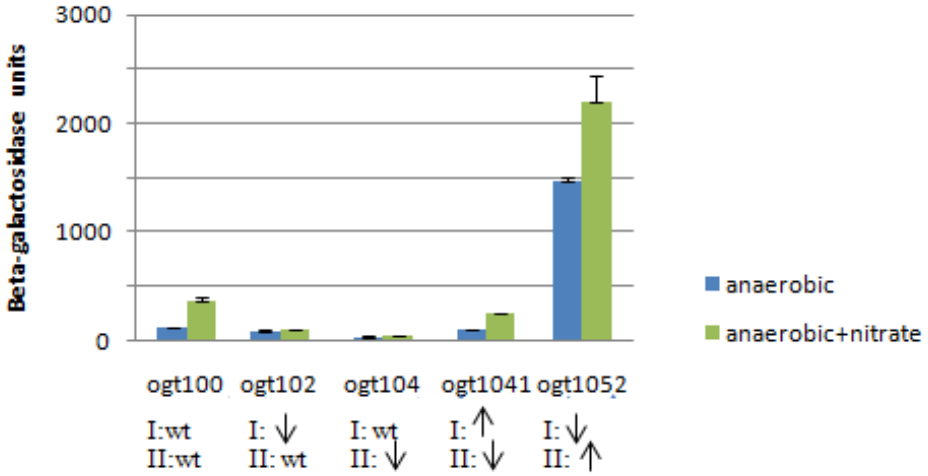


Figure 3.2 Changes in the DNA sites for NarL of the *ogt* promoter

Panel A shows base sequences of two DNA sites for NarL in the ogt100, ogt102, ogt104, ogt1041 and ogt1052 promoter fragments. The positions relative to the transcript start site are indicated above the NarL sites. The 7-2-7 consensus binding sequence is shown in boldface. The red highlights indicate base changes that disrupt NarL binding. The yellow highlights indicate base changes that make the sites better resemble the consensus.

Panel B shows beta-galactosidase activities measured in JCB387 (L^+P^+) carrying the ogt100, ogt104, ogt1041, ogt102 and ogt1052 promoter::*lacZ* fusions. The status of each NarL binding site is given below the fragment name. WT indicates that the site is wild-type. Modifications are described by arrows. Improved sites, which resemble the consensus NarL binding site, are indicated by an upward arrow. Downward arrows indicate that the sites are disrupted. Cells were grown in minimal salts medium anaerobically with or without 20 mM nitrate. Beta-galactosidase activities were measured during an exponential growth phase (OD_{650} = 0.5-0.6).

Taken together, the *ogt* promoter activity is influenced by sequences of the DNA sites for NarL. Both NarL binding sites are required in order to activate the wild-type *ogt* promoter. NarL can activate the *ogt* promoter that contains a single DNA site for NarL, only if the site is modified to closely resemble the consensus binding site. Furthermore, the promoter organizations have an effect on levels of NarL induction. The highest promoter activity was achieved using the near-consensus NarL site located at position -45.5 relative to the transcript start site.

The base sequences of the *ogt* promoter derivatives were altered; hence, it is possible that transcription starts at different positions in comparison to that of the wild-type promoter. To investigate whether the ogt1041 and the ogt1052 promoters are not alternative promoters, the promoter derivatives were disrupted at the second base of the -10 element (TATCTT to TCTCTT). Results illustrated in Figure 3.3 show that the promoter activities decrease in cells containing either the ogt1041-12C or the ogt1052-12C promoter::*lacZ* fusions (the promoters carry P12C). This indicates that the same -10 hexamer used in the wild-type *ogt* promoter is required, thus, the activities obtained experimentally for the ogt1041 and the ogt1052 promoter derivatives were not the result of transcription at alternative promoters.

3.3 Nitrate induction of the *ogt* promoter derivatives

From Section 3.2, NarL can activate the *ogt* promoter that contains a single DNA site for NarL if the site better resembles the consensus sequence. The activity of the ogt1041 promoter, which has a single NarL site located at position -78.5, is comparable to that of the ogt100 promoter that carries two wild-type DNA sites for NarL at positions -78.5 and

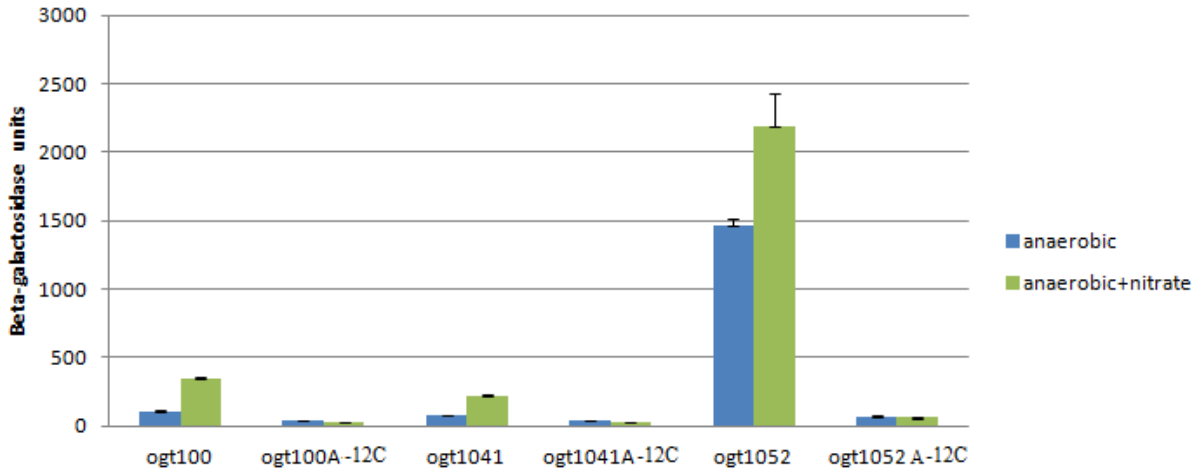


Figure 3.3 Mutation at base 2 of -10 element decreases the *ogt* promoter activities

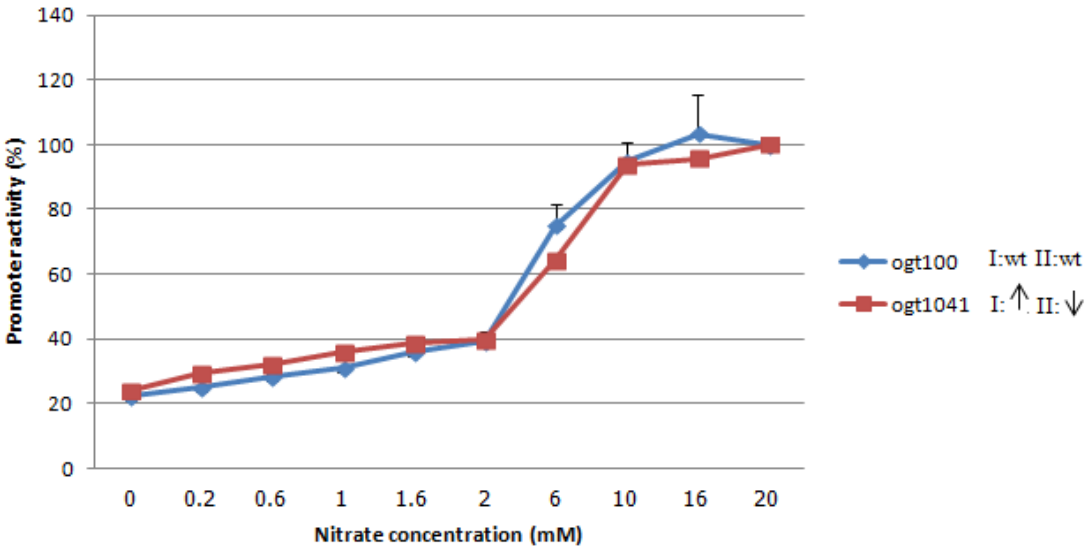
The -10 element of the ogt100 (I:wt II:wt), ogt1041 (I ↑ II ↓) and ogt1052 (I ↓ II ↑) promoters was disrupted (base A at position -12 was mutated to C). Beta-galactosidase activities measured in JCB387 (L^+P^+) containing the ogt100, ogt1041 and ogt1052 promoter::*lacZ* fusions were compared to activities in cells containing the ogt100-12C, ogt1041-12C and ogt1052-12C promoter::*lacZ* fusions (contain P12C). Cells were grown in minimal salts medium at 37°C anaerobically with or without 20 mM nitrate.

-45.5. This work used beta-galactosidase assays to measure the activities of the ogt100 promoter and the ogt1041 promoter in response to different nitrate concentrations. It is possible that a lower amount of phosphorylated NarL is sufficient to induce transcription initiation at the ogt1041 promoter as NarL efficiently binds to the high-affinity target site in the promoter region for a longer period.

Results illustrated in Figure 3.4 panel A show the beta-galactosidase activities measured in cells carrying the ogt100 and ogt1041 promoter::*lacZ* fusions as percentages of the promoter activity measured in cells grown anaerobically in minimal salts medium with 20 mM nitrate. There is a basal level of NarL induction when cells are grown in media with <2.0 mM nitrate. The promoter activity is increased by ~20% at a concentration of 2.0 mM. Increasing nitrate concentration results in higher expression levels of both the ogt100 and ogt1041 promoter::*lacZ* fusions. Promoter activities reach the plateau phase at ~10 mM. Data show that the activities of both the ogt100 and ogt1041 promoters are comparable at all concentrations of nitrate.

Results illustrated in Figure 3.4 panel B show that a lower concentration of nitrate is sufficient to activate the ogt1052 promoter. The promoter activity is high despite the absence of nitrate. Increasing nitrate concentration results in higher activities of the ogt1052 promoter. The plateau phase is reached at a concentration of ~10 mM.

A.



B.

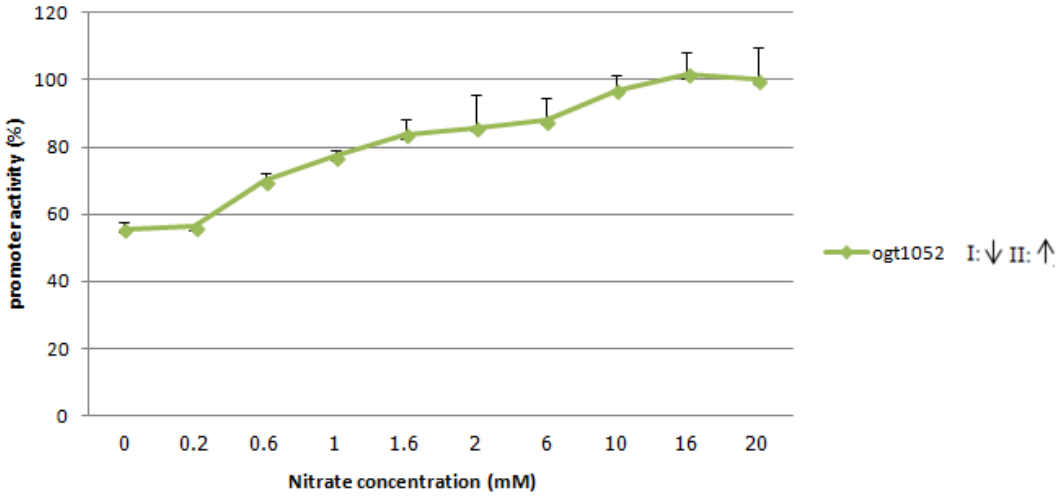


Figure 3.4 Activities of the *ogt* promoter at different nitrate concentrations

JCB387 Cells were grown in minimal salts medium supplemented with different concentrations of sodium nitrate (0-20 mM).

Panel A shows the ogt100 and ogt1041 promoter activities.

Panel B shows the ogt1052 promoter.

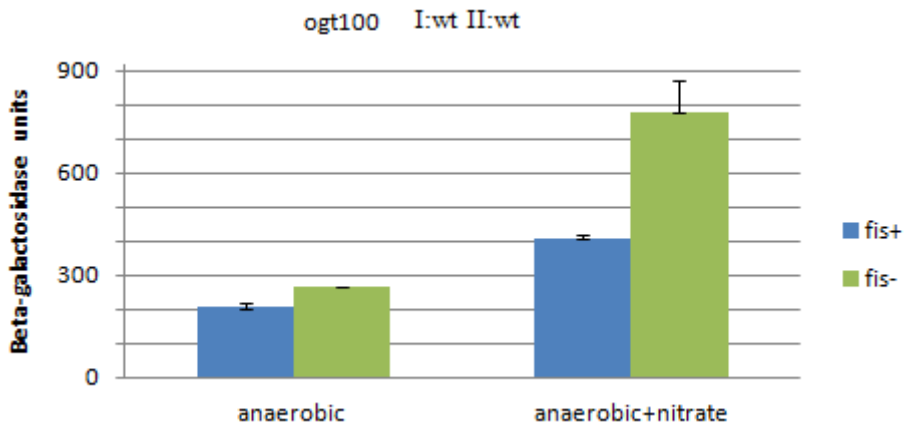
Promoter activities are shown as percentages of the activity obtained from cells carrying the *ogt* promoter::*lacZ* fusion, grown anaerobically in minimal salts medium with 20 mM nitrate.

3.4 Effects of Fis on the *ogt* promoter activity.

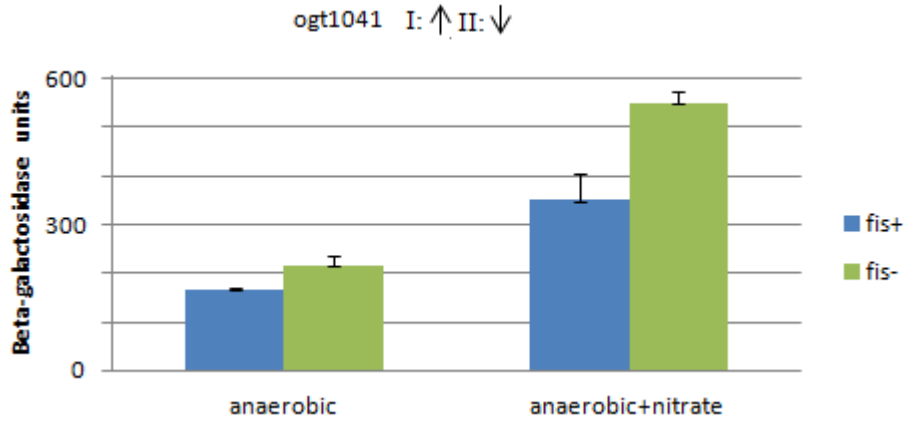
Fis is a DNA-binding protein that plays several roles in the maintenance and organization of the nucleoid structures (Cho *et al.*, 2008; Schneider *et al.*, 2001). It also functions as a regulator of transcription. Fis represses the *ogt* promoter activity by binding to the DNA target site that overlaps with the NarL I site (Squire *et al.*, 2009). The aim of this study is to investigate Fis effects on activities of the ogt100, ogt1041 and ogt1052 promoters. Since the ogt100 and ogt1041 promoters require the binding of NarL to the NarL I site, the promoter activities measured in cells that lack Fis (JCB3871) should increase. Fis does not affect NarL binding at the NarL II site; hence, the ogt1052 promoter activity measured in JCB3871 should be similar as that of JCB387.

Effects of Fis were investigated by measuring beta-galactosidase activities in JCB3871 (Fis^-) and JCB387 (Fis^+) cells carrying the ogt100, ogt1041 and ogt1052 promoter::*lacZ* fusions. Results illustrated in Figure 3.5 show that the activities of the ogt100, ogt1041 and ogt1052 promoters are higher in cells that lack Fis. This suggests that the presence of Fis has an effect on all the *ogt* promoter derivatives. Activities of the ogt100 promoter and the ogt1041 promoter are comparable in JCB387, however the activities of those measured in JCB3871 increase by ~2 and ~1.5-fold respectively. In the absence of Fis, the ogt100 promoter provides a better promoter organization for NarL induction than the ogt1041 promoter.

A.



B.



C.

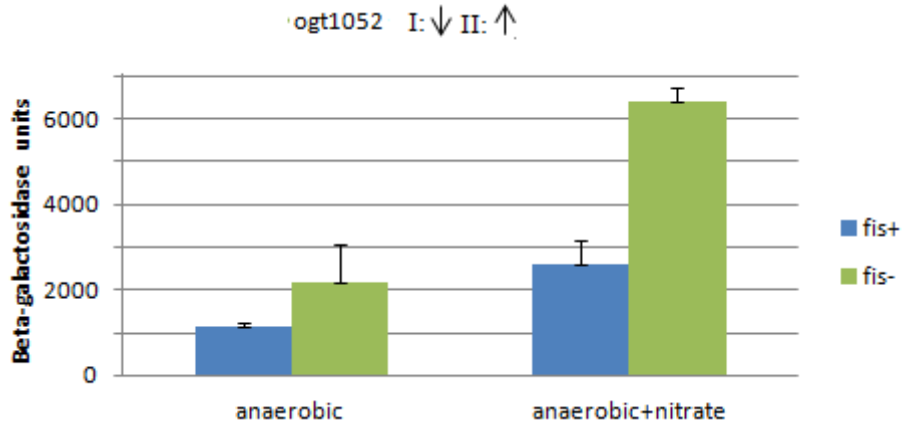


Figure 3.5 Effects of Fis on the *ogt* promoter activities

The figures show beta-galactosidase activities measured in JCB387 (Fis+) and JCB3871 (Fis$^-$) carrying the ogt100 (panel A), ogt1041 (panel B) and ogt1052 (panel C) promoter::*lacZ* fusions. Cells were grown at 37°C in minimal salts medium anaerobically with or without 20 mM sodium nitrate.

3.5 NarL and NarP dependent regulation of the *ogt* promoter

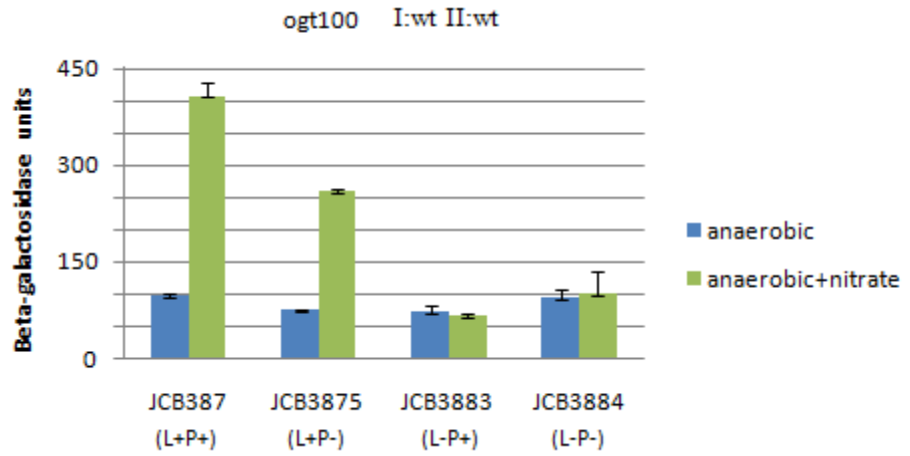
Results from Section 3.2 show that NarL can activate the ogt100 promoter, as well as its derivatives including the ogt1041promoter and the ogt1052 promoter. Since NarL and NarP are homologous regulators, it is possible that NarP also binds to the DNA sites for NarL and regulates the *ogt* promoter as well. In this work, beta-galactosidase activities were measured in various cell strains including JCB387 (both NarL and NarP are present in cells: L^+P^+), JCB3875 (cells contain only NarL: L^+P^-), JCB3883 (cells contain only NarP: L^-P^+) and JCB3884 (none of NarL and NarP are present in cells: L^-P^-) to investigate the roles of NarL and NarP in the regulation of different *ogt* promoter derivatives.

Results illustrated in Figure 3.6 panel A show beta-galactosidase activities measured in JCB387 (L^+P^+), JCB3875 (L^+P^-), JCB3883 (L^-P^+) and JCB3884 (L^-P^-) carrying the ogt100 promoter::*lacZ* fusion. Nitrate induction was found when NarL is present in cells (JCB387, JCB3875). The ogt100 promoter activity in JCB387 is ~1.6-fold higher compared to that of the JCB3875 cells. There is no nitrate induction in JCB3883 and JCB3884. This indicates that NarP alone cannot activate the ogt100 promoter.

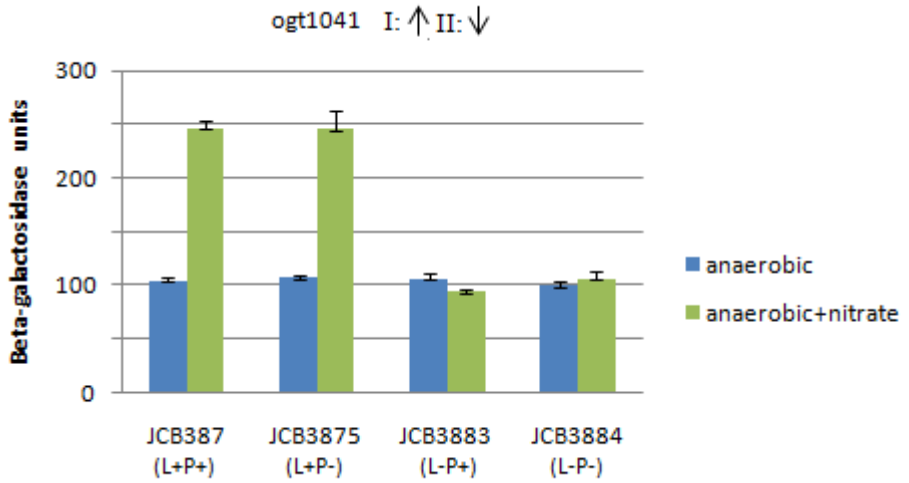
Results illustrated in Figure 3.6 panel B show NarL/NarP dependent activation of the ogt1041 promoter, which contains a single near-consensus NarL I site while the NarL II site is modified to disrupt NarL binding. The ogt1041 promoter activity measured in cells containing both NarL and NarP (JCB387) was identical to that in cells containing only NarL (JCB3875). In JCB3883 (L^-P^+), NarP alone is unable to activate the promoter. This indicates that NarP cannot activate the *ogt* promoter carrying a single NarL site located at position -78.5.

Results illustrated in Figure 3.6 panel C show that the expression of the ogt1052 promoter::*lacZ* fusion is induced by either NarL or NarP. The ogt1052 promoter contains a

A.



B



C

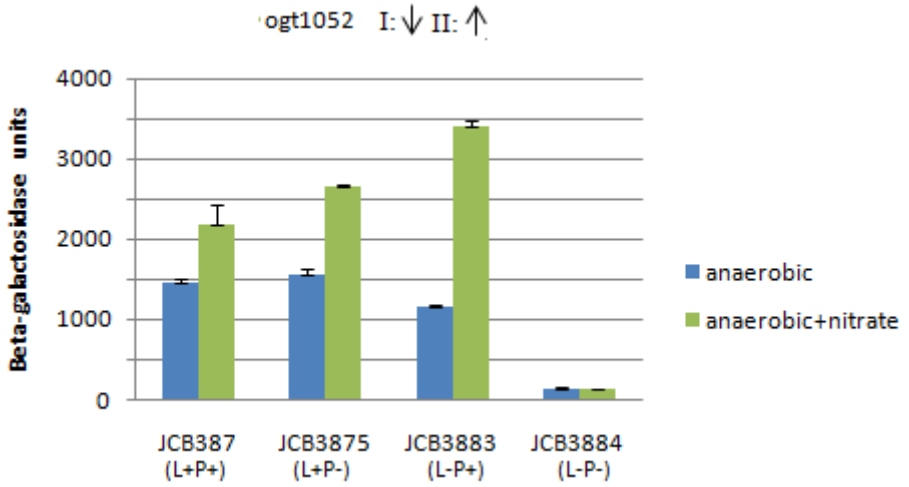


Figure 3.6 NarL and NarP dependent activation of the *ogt* promoter derivatives

Beta-galactosidase activities were measured in JCB387 (L+P), JCB3875 (L+P), JCB3883 (L^-P^+) and JCB3884 (L-P) carrying the ogt100 (panel A), ogt1041 (panel B) and ogt1052 (panel C) promoter::*lacZ* fusions. Cells were grown in minimal salts medium anaerobically with or without 20 mM sodium nitrate.

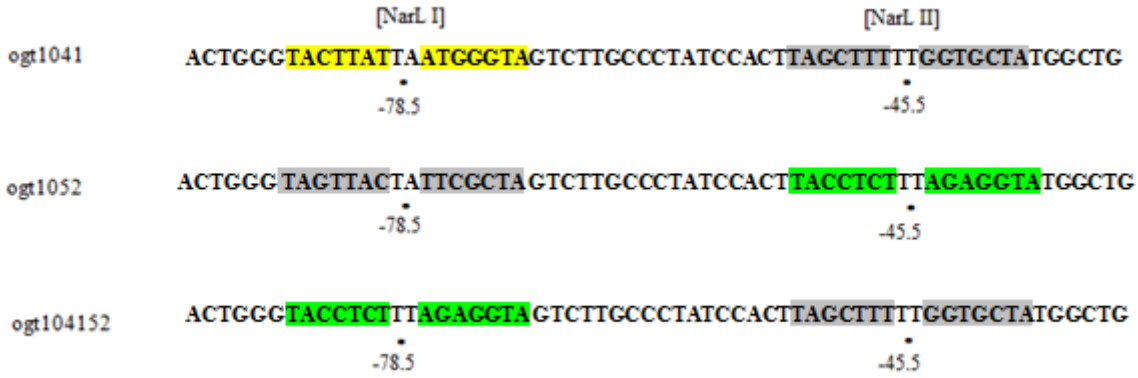
disrupted NarL I site, while the NarL II site is modified to better resemble the consensus sequence. The ogt1052 promoter activity is high even in the absence of nitrate. With nitrate, the promoter activity is induced even further. The induction was also found in cells that contain only NarP (JCB3883). This indicates that NarP is able to activate the *ogt* promoter carrying a single NarL site located at position -45.5.

3.6 Sequence and location of the DNA site for NarL: which plays a bigger role?

Both the ogt1041 promoter (I↑II↓) and the ogt1052 promoter (I↓II↑) contain a single near-consensus DNA site for NarL (at positions -78.5 and -45.5 respectively). DNA sequence of the NarL site in the ogt1041 promoter is different from the sequence of the NarL site in the ogt1052 promoter. This study is aimed to investigate whether the location or the DNA sequence of the NarL binding site plays a more significant role in the transcription activation of the *ogt* promoter. NarL and NarP dependent activation were examined when the sequence of the NarL site was fixed and its location was altered, comparing to when the location of the NarL site is fixed while its DNA sequence is altered.

Figure 3.7 panel A illustrates a partial base sequence of the ogt104152 promoter fragment, which contains a single NarL site located at position -78.5 and the site is modified to match the near-consensus NarL II site of the ogt1052 promoter. Therefore, both the ogt1041 and the ogt104152 promoters contain a single near-consensus NarL site at the same location (position -78.5) but the sequences of the sites are different. The pRW50 containing either the ogt1041 or the ogt104152 promoter::*lacZ f*usions was introduced into JCB387 (L^+P^+), JCB3875 (L^+P^-), JCB3883 (L^-P^+) and JCB3884 (L^-P^-). Cells were grown in minimal salts medium anaerobically with or without 20 mM nitrate. Beta-galactosidase activities were

A.



B.

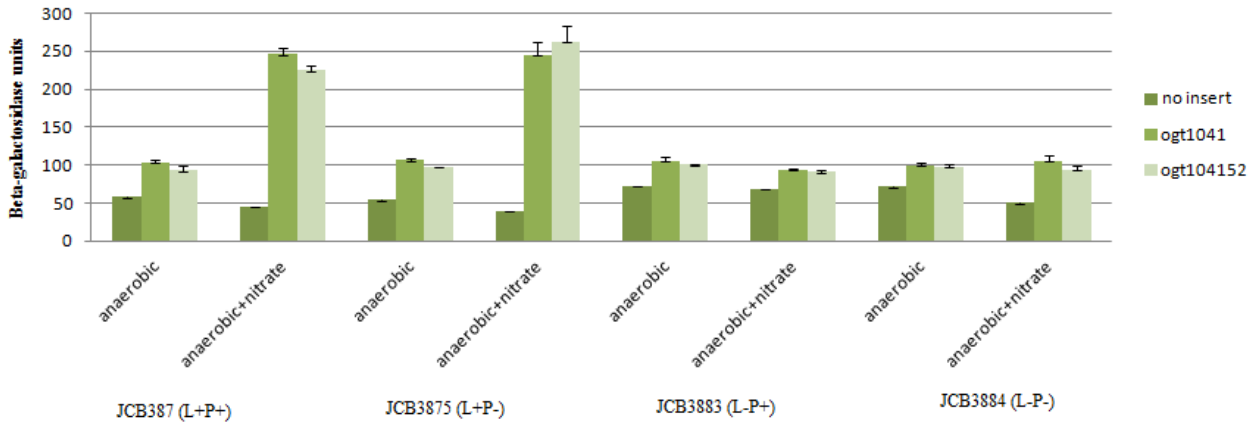


Figure 3.7 Altering the near-consensus sequence of the NarL I site

Panel A shows a partial base sequence of the ogt104152 promoter fragment (I↑II↓), which is constructed by replacing the NarL I site of the ogt1041 promoter (I↑II↓) with the NarL II site of the ogt1052 promoter (I↓II↑). The near-consensus sequence of the DNA site for NarL is coloured yellow for the ogt1041 promoter and green for the ogt1052 promoter. The disrupted NarL binding site is coloured grey.

Panel B shows beta-galactosidase activities measured in JCB387 (L^+P^+), JCB3875 (L^+P^-), JCB3883 (L^-P^+) and JCB3884 (L^-P^-) that contain pRW50 carrying either the ogt1041 promoter or the ogt104152 promoter. Cells were grown in minimal salts medium anaerobically with or without 20 mM nitrate.

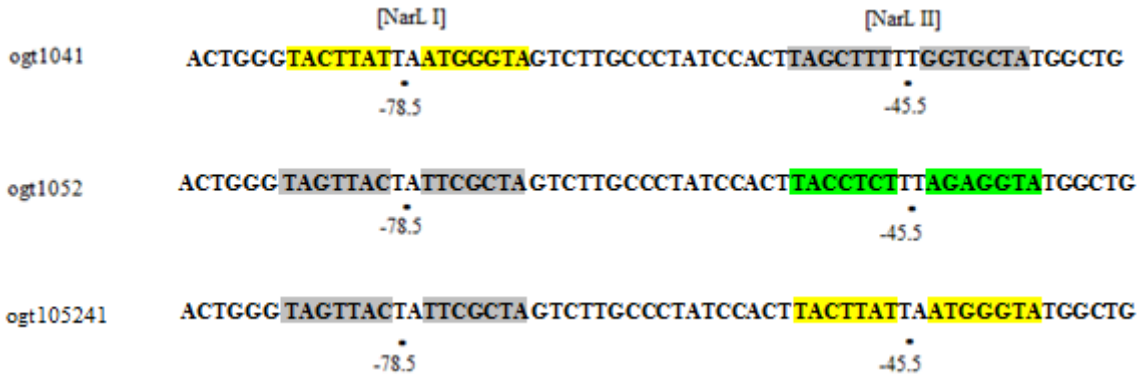
measured during the mid-exponential growth phase.

Results illustrated in Figure 3.7 panel B show that expressions of the ogt1041 promoter::*lacZ* fusion and the ogt104152 promoter::*lacZ* fusion occur only in cells that contain NarL. In cells JCB387 (L^+P^+) and JB3875 (L^+P^-), an induction of the ogt1041 promoter is comparable to that of the ogt104152 promoter. No induction is found in JCB3883 (L^-P^-), suggesting that NarP cannot activate both the ogt1041 and the ogt104152 promoters. Likewise, no induction is found in JCB3884 (L^-P^-). This indicates that the promoter activities obtained are induced only by NarL. To conclude, the ogt1041 and ogt104152 promoter activities are similar although the sequences of the NarL site are different as both are induced by NarL but not NarP.

Figure 3.8 panel A shows the partial base sequences of the ogt1041, the ogt1052 and the ogt105241 promoter fragments. The ogt105241 promoter (I↓II↑) has the DNA site for NarL centered at position -45.5 and the site resembles the NarL I site of the ogt1041 promoter (I↑II↓). Both the ogt1052 promoter and the ogt105241 promoter have the near-consensus NarL site located at the same position (-45.5) but the sequences of the sites are different. The pRW50 plasmids carrying either the ogt1052 promoter or the ogt105241 promoter were introduced into JCB387 (L^+P^+), JCB3875 (L^+P^-), JCB3883 (L^-P^+) and JCB3884 (L^-P^-) to measure β-galactosidase activities.

The effect of different base sequences of the NarL II site are illustrated in Figure 3.8 panel B. In the absence of nitrate, the expression of the ogt1052 promoter::*lacZ* fusion is ~2-fold higher than that of the ogt105241 promote::*lacZ* fusion. With the supplement of nitrate, the promoter activities are induced further in cells that contain either NarL or NarP. In

A.



B.

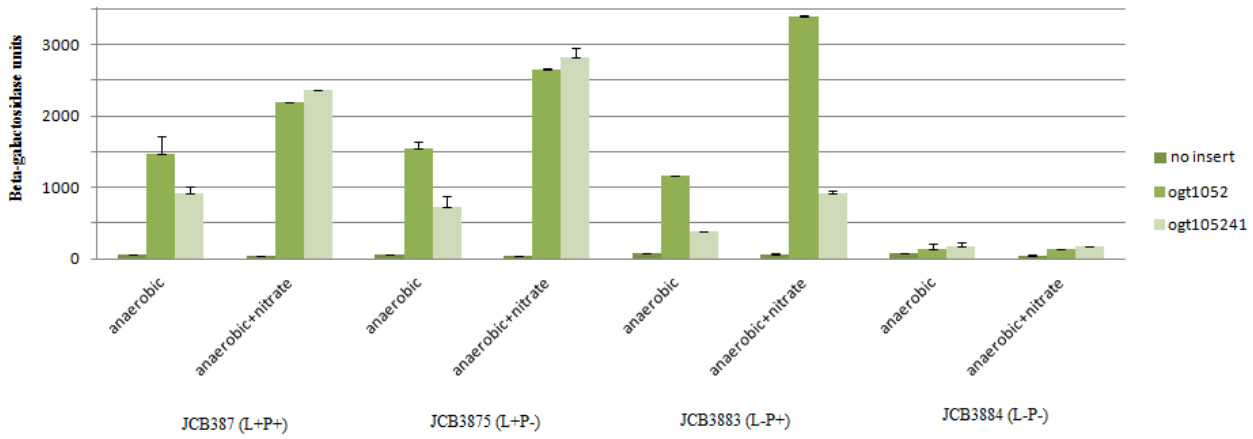


Figure 3.8 Altering the near-consensus sequence of the NarL II site

Panel A shows partial base sequences of the ogt105241 promoter fragment (I↓II↑), which is constructed by replacing the NarL II site of the ogt1052 promoter (I↓II↑) with the NarL I site of the ogt1041 promoter (I↑II↓). The near-consensus NarL site is coloured in yellow for the ogt1041 promoter and in green for the ogt1052 promoter. The disrupted NarL binding site is coloured in grey.

Panel B shows beta-galactosidase activities measured in JCB387 (L^+P^+), JCB3875 (L^+P^-), JCB3883 (L^-P^+) and JCB3884 (L^-P^-) that contain pRW50 carrying either the ogt1052 or the ogt105241 promoters. Cells were grown in minimal salts medium anaerobically with or without 20 mM nitrate.

JCB387 (L^+P^+) and JCB3875 (L^+P^-), the activities of the ogt1052 promoter and the ogt105241 promoter are comparable. In JCB3883 (L^-P^+), the activity measured in cells carrying the ogt1052 promoter are ~3.5-fold higher than in cells carrying the ogt105241 promoter. This suggests that altering the DNA sequence of the NarL site has an effect on the *ogt* promoter if the site is located at position -45.5. There is no expression of both the ogt1052 and the ogt105241 promoter::*lacZ* fusions in JCB3884 (L^-P^-), indicating that all the observed activities are induced by NarL or NarP.

To conclude, the location of the NarL site has an effect on NarL and NarP induction of the *ogt* promoter. NarL is able to activate the *ogt* promoter containing a single NarL site at either position -78.5 or position -45.5. NarP can activate the *ogt* promoter containing the NarL site at position -45.5 but it fails to activate if this site is moved to position -78.5. Taken together, NarL induction is dependent more on the location of the DNA site for NarL than its sequence. NarL dependent activities of the *ogt* promoter derivatives are the same despite altering the sequence of the NarL site. NarP induction depends not only on its location but also the sequence of the NarL site. Altering sequences of the site at position -45.5 results in different promoter activities induced by NarP.

3.7 Effects of locations of the NarL site on the *ogt* promoter

Results from Section 3.2 indicate that NarL can activate the *ogt* promoter that contains only one DNA site for NarL. The ogt1041 promoter and the ogt1052 promoter have a single NarL site centered at position -78.5 and -45.5 relative to the transcript start site respectively. NarL induction is high when the DNA site for NarL is located at position -45.5 (~9-fold higher than that of the ogt1041promoter). The aim of this work is to investigate the effects of

location of the NarL site on the *ogt* promoter activity. Inverse PCR was used for DNA insertion downstream the NarL site of the ogt1041 promoter. Insertion of 10 bp moves the NarL site further upstream but the site is still located on the same face of the DNA helix. In contrast, adding 5 bp moves the NarL site to the opposite helical face. The *ogt* promoter derivatives ogt104188 and ogt104183 contain the NarL site centered at position -88.5 and -83.5 respectively.

Overlapping PCR was used to delete 5, 10, 15, 20, 25 and 33 bp downstream the NarL site of the ogt1041 promoter to move the NarL site from position -78.5 to position -73.5, -68.5, -63.5, -58.5, -53.5 and -45.5 respectively. The *ogt* promoter derivatives are named ogt104173, ogt104168, ogt104163, ogt104158, ogt104153 and ogt104145. Partial base sequences of the promoter fragments used in this study are illustrated in Figure 3.9. The promoter fragments were cloned into pRW50 and introduced into JCB387. Beta-galactosidase activities were measured in cells grown in minimal salts medium anaerobically with or without 20 mM nitrate.

Results illustrated in Figure 3.10 show that NarL fails to activate the *ogt* promoter containing the NarL site at position -88.5 or -83.5, indicating that they are not the optimal locations for NarL to regulate the *ogt* promoter. Expression of the ogt promoter derivative::*lacZ* fusions is increased when the NarL site was moved 10 bp downstream to position -68.5 (ogt104168). Nitrate induces the ogt104168 promoter activity to ~5-fold greater than that of the ogt1041 promoter. It is possible that binding of NarL to DNA at position -68.5 better recruits RNA polymerase due to an optimal interaction between NarL and αCTD.

Minimal activation levels were observed when the NarL site was centered at position -73.5, -63.5, -58.5 and -53.3, suggesting that these locations are not the appropriate

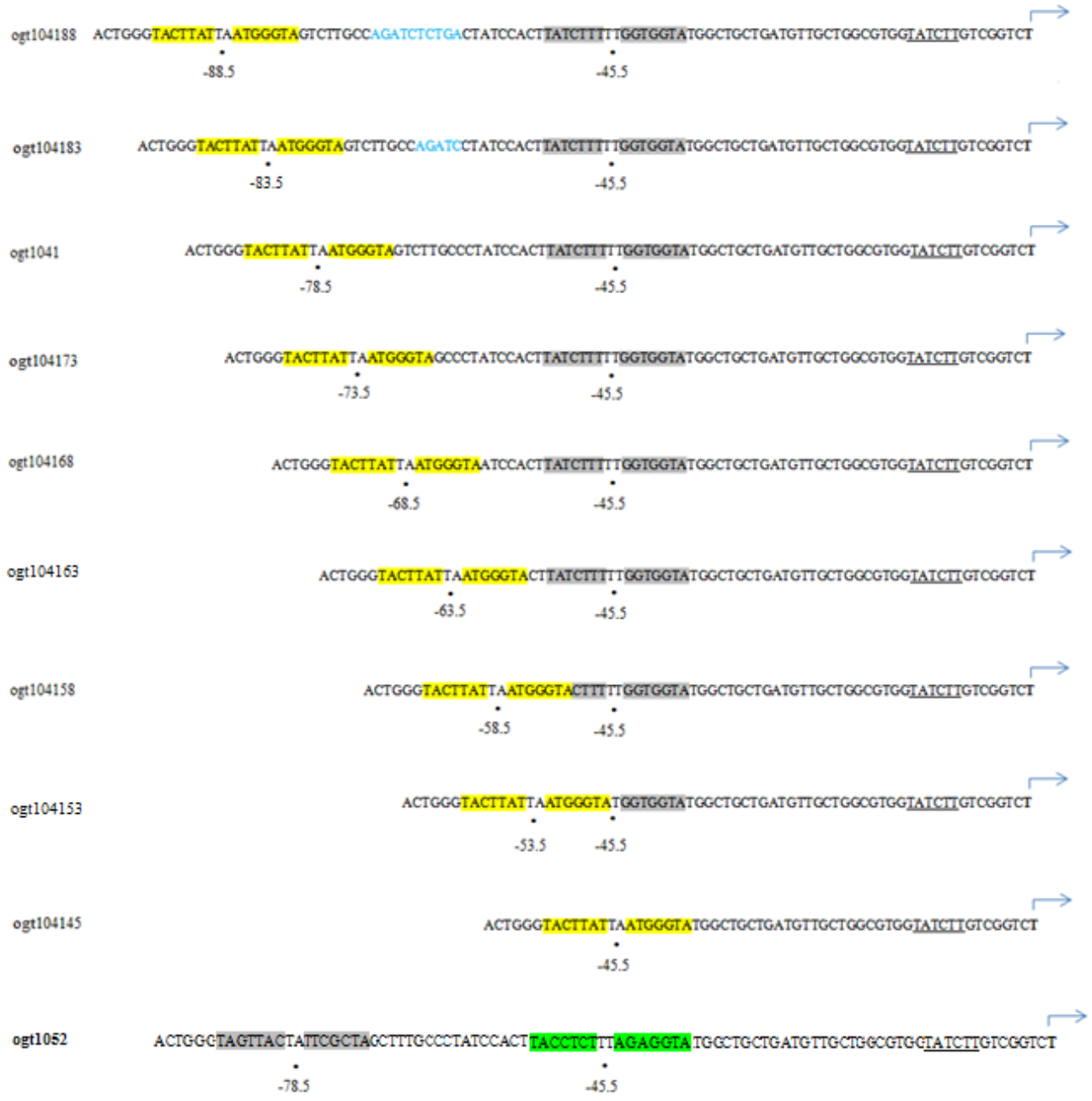


Figure 3.9 Partial base sequences of the *ogt* promoter fragments carrying the NarL site at different locations.

The DNA sequence of the near-consensus NarL I site is coloured yellow. Sequences in blue colour denote inserted bases downstream the NarL site. The *ogt* promoter derivatives were constructed by moving the NarL site to position -88.5, -83.5, -73.5, -68.5, -63.5, -58.5, -53.5 and -45.5 relative to the transcript start site.

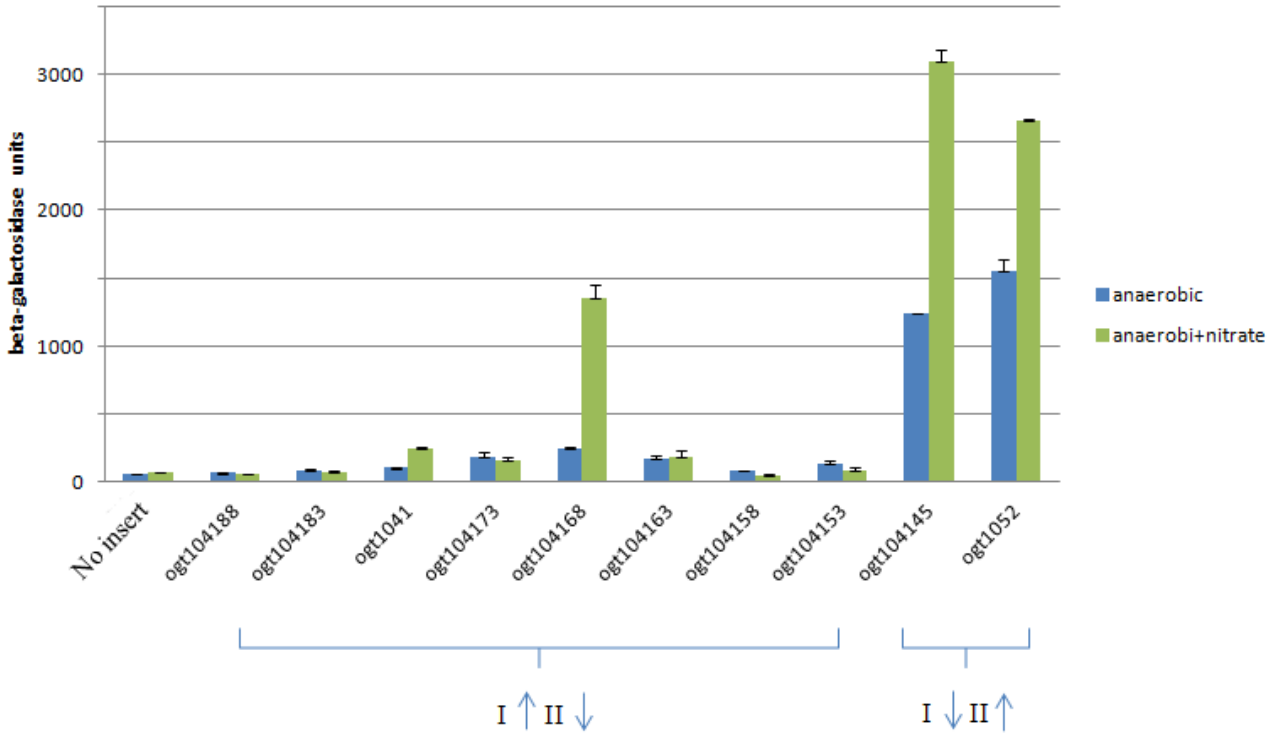


Figure 3.10 Altering locations of the NarL site

Beta-galactosidase activities were measured in JCB387 (L^+P^+) that contain pRW50 carrying the *ogt* promoter derivatives with the NarL site located at different positions. Cells were grown in minimal salts medium in anaerobically with or without 20 mM nitrate. Beta-galactosidase activities were measured after reaching the mid exponential growth phase.

positions for NarL on the DNA helix. In the ogt104145 promoter, the NarL site is located at position -45.5, which is adjacent to the -35 element. In the presence of nitrate, the ogt104145 promoter activity is ~12-fold greater than that of the ogt1041 promoter and comparable to the activity of the ogt1052 promoter, which also has a single NarL site located at position -45.5 but the sequence of the site is different.

3.8 Location of -10 element in the ogt104168 promoter

The previous results indicate that activity of the ogt1041 promoter is lower than that of the ogt104168 (~5.5-fold) and the ogt1052 promoter (~10-fold). In Section 3.2, it was confirmed that the same promoter as that of the wild-type *ogt* promoter also functions for the ogt1041 and the ogt1052 promoters as well. This work is aimed to examine whether the same -10 element as in the wild-type *ogt* promoter is required for the ogt104168 promoter.

The ogt104168 promoter was mutated at base two of -10 element (TATCTT to TCTCTT) to disrupt transcription of the *ogt* gene. The DNA fragment, named ogt104168A-12C, was cloned into pRW50 and introduced into JCB387 (L^+P^+). Cells were grown anaerobically with or without 20 mM nitrate to measure beta-galactosidase activities. Results illustrated in Figure 3.11 show that there is no induction in cells containing the ogt104168A-12C promoter::*lacZ* fusion. This indicates that transcription at the ogt104168 promoter requires the same -10 element as that used in the wild-type *ogt* promoter, and the observed ogt104168 promoter activity does not result from NarL induction of an alternative promoter.

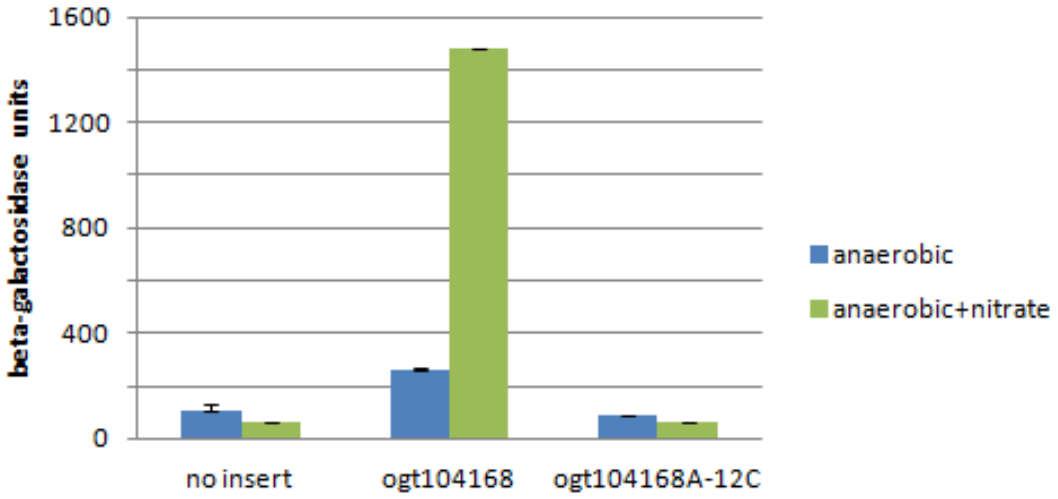


Figure 3.11

Beta-galactosidase activities were measured in JCB387 (L^+P^+). that contain pRW50 carrying the ogt104168 promoter or the ogt104168A-12C promoter (base 2 of -10 element was mutated from A to C to disrupt transcription). Cells were grown in minimal salts medium anaerobically with or without 20 mM nitrate. Beta-galactosidase activities were measured after reaching the mid exponential growth phase.

3.9 Discussion

Architecture of the ogt promoter

Synthesis of O6-alkylguanine-DNA-alkyltransferase (Ogt), a protein that is produced prior to the production of RNS to act as a DNA repairing enzyme, is induced by nitrate. Nitrate is a precursor of NO, thus, it provides a prophylactic mechanism against future DNA-methylation damage caused by NO and its derived compounds. NarX senses nitrate availability in environments and transfers the signal to the cognate NarL response regulator. Transferring a phosphate group to NarL triggers a conformational change and allows the C-terminal domain of NarL to bind to the target DNA binding sites and in turn, activates the *ogt* promoter.

The *ogt* promoter consists of two NarL sites arranged as a 7-2-7 inverted repeat at position -78.5 (the NarL I site) and position -45.5 (the NarL II site) relative to the transcript start site. This study confirms that transcription initiation at the *ogt* promoter requires both DNA sites for NarL. The mutation introduced to inactivate one of the NarL sites results in reduced NarL induction. Considering the locations of the two NarL sites in the *ogt* promoter region, it suggests that NarL may activate the wild-type *ogt* promoter via a class III mechanism. Further investigation is required to confirm the location of NarL on the DNA helix in order to classify the *ogt* promoter architecture and to better understand the activation mechanism of NarL at the *ogt* promoter. More will be discussed in Chapter 5.

NarL is a versatile response regulator as it can activate not only the ogt100 promoter but also both the ogt1041 and the ogt1052 promoters that contain a single DNA site for NarL at position -78.5 and position -45.5 respectively. The NarL-dependent activity of the ogt1052 promoter is higher than that of the ogt100 and the ogt1041 promoters. It is possible that the

location of the DNA site for NarL in the ogt1052 promoter (position -45.5) serves as an optimal location for NarL to recruit RNA polymerase and accelerate an isomerization. Further investigation to determine an interaction between NarL and RNA polymerase will be discussed in Chapter 5.

Activation levels of the *ogt* promoter depend on nitrate availability. In the presence of low nitrate concentrations, NarL still efficiently binds to the high-affinity NarL site (Darwin *et al.*, 1997; Wang and Gunsalus, 2000). Therefore, a low nitrate concentration is sufficient to activate the ogt1052 promoter, which contains the near-consensus NarL site centered at position -45.5. NarL is also able to bind to a low-affinity binding site in the presence of high nitrate concentration (Darwin *et al.*, 1007; Wang and Gunsalus, 2000). This coincides with the data that activation of the ogt100 promoter, which carries two wild-type NarL sites, requires higher nitrate concentrations. The ogt1041 promoter has one NarL site modified to better resemble the consensus. Therefore, it is expected that NarL induction of the ogt1041 promoter can occur at a lower nitrate concentration in comparison to the ogt100 promoter. However, this study shows that the activation levels of both the ogt100 and the ogt1041 promoters are similar for all nitrate concentrations. Induction of the ogt1041 promoter still requires a high level of nitrate to be present. This may be due to an effect from another repressor (discussed in the next section) that also binds to the same DNA site for NarL. More phosphorylated NarL is needed to compete for the target site; hence, it requires supplementation with a high nitrate concentration.

Fis effect on nitrate induction

During a rapid growth phase, transcription of many genes in *E. coli* is limited due to a fixed amount of RNA polymerase (Ishihama, 2000). The work from Squire *et al.* (2009) indicated that Fis, a nucleoid associated protein, restricts nitrate induction of the *ogt* promoter in vivo and in vitro. Fis binds to the *ogt* promoter region at around position -82. The Fis binding site overlaps with the NarL I site as illustrated in Figure 3.11. There is competing between Fis and NarL to control transcription of the *ogt* gene. Binding of Fis causes steric hindrance and interrupts access of NarL (Grainger *et al.,* 2008; Browning *et al.,* 2005).

Fis interrupts the binding of NarL at position -78.5, which is a crucial target site in order to activate the ogt100 and the ogt1041 promoter. In JCB3871 cells (Fis^-), the ogt100 promoter activity is ~2-fold higher than in JCB387 cells (Fis+). There should be a smaller effect from Fis repression on the ogt1041 promoter as the promoter has the NarL site modified to increase the affinity for NarL. Data show that the ogt1041 promoter activity measured in Fis^- cells is higher than in Fis^+ cells (<2-fold). This suggests that Fis still disrupts the binding of NarL to the near consensus NarL I site but once NarL binds to the target site, it binds strongly and causes transcription activation to occur although the promoter contains only one DNA site for NarL.

It was thought that Fis does not have an effect on the expression of the ogt1052 promoter::*lacZ* funcion as the NarL site at position -45.5 is not the target site of Fis. The results show that the ogt1052 promoter activity in Fis^- cells is ~ 2.5-fold higher than in Fis^+ cells, indicating that there is still an effect from Fis. At the ogt1052 promoter, Fis does not block the NarL binding but its role in contributing to chromosome compaction may repress open

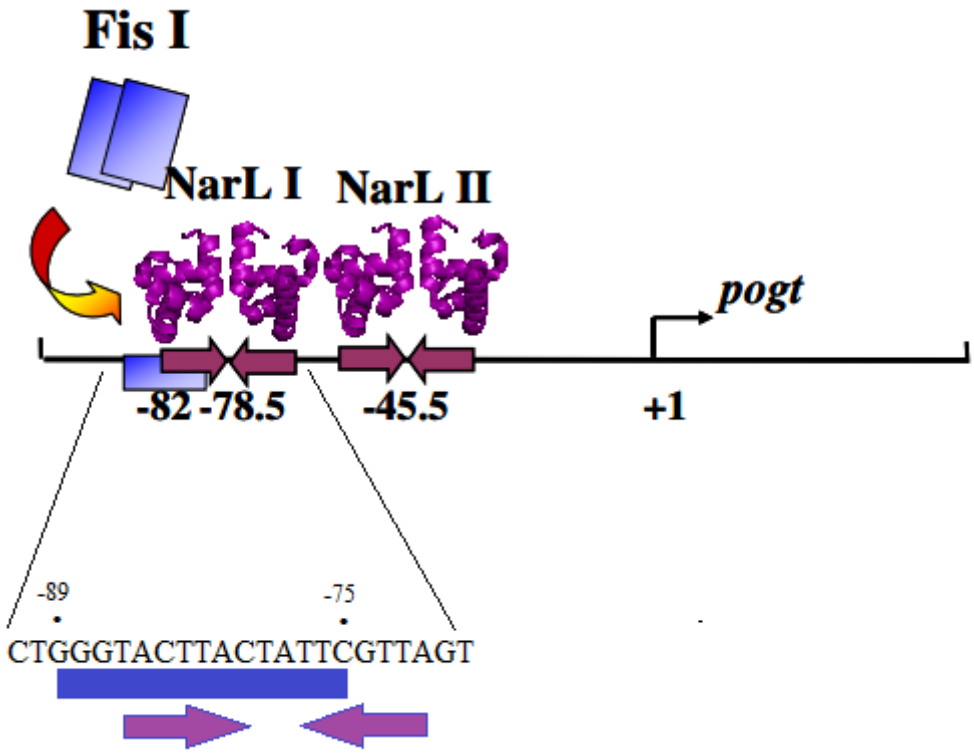


Figure 3.11 DNA sites for Fis and NarL at the *ogt* promoter

The *ogt* promoter has two NarL sites organized as 7-2-7 inverted repeat. The NarL sites (purple arrow) are centered at position -78.5 and -45.5 relative to the transcript start site (+1). The blue box denotes the DNA site for Fis.

complex formation at the *ogt* promoter via DNA folding or bending (McLeod and Johnson, 2001).

NarL/NarP-dependent regulation of the ogt promoter

There is a great loss of the ogt100 promoter activity when NarP is not present in cells. Therefore, not only does NarL play a role in the regulation of the *ogt* promoter, but also NarP is required for the maximal activation. However, NarP alone has limited abilities to up-regulate the *ogt* promoter, as there is no induction in cells that contain only NarP. The ogt1052 promoter is activated by either NarL or NarP. The promoter activity is higher when there is only NarP in cells, leading to the idea that NarP is more competent in activating the ogt1052 promoter but the binding of NarP is antagonized by NarL. At the ogt1041 promoter, transcription initiation is induced only by NarL. Although NarL and NarP are homologues and their amino acid sequences share high similarities, they are still different in terms of their structures. It is possible that the DNA sites for NarL are sub-optimal for NarP binding.

NarP activates the *ogt* promoter by binding to DNA at position -45.5 only. It is unable to activate the promoter by binding to DNA at position -78.5 although the site is modified to resemble the NarL site at position -45.5. Darwin *et al.* (1997) reported that NarP only binds to the 7-2-7 binding site while NarL is also able to bind to the target site arranged in alternative configurations. The NarL I site (position -78.5) overlaps with the Fis binding site. Therefore, NarP binding is interrupted as the target site arranged as a 7-2-7 configuration is interfered with Fis.

The location of the DNA site for NarL plays a crucial role in activation of the *ogt* promoter. Altering DNA sequences of the NarL site at either position -78.5 or position -45.5 does not cause a great change in the level of NarL induction, as long as the site resembles the

consensus (TACYYMnnAKRRGTA). However it has an effect on NarP induction if the DNA sequence of the site located at position -45.5 is altered. The ogt10521 promoter has the NarL II site replaced by the NarL I site, which serves as a sub-optimal binding site for NarP. This results in a very low NarP induction level in comparison to the ogt1052 promoter.

Altering the location of the DNA site for NarL

Apart from the affinities of the DNA site for NarL, the activities of the *ogt* promoters are also dependent on the locations of the NarL site in the promoter region. Different *ogt* promoter derivatives contain the near-consensus NarL site located at different locations on the DNA helix. NarL can regulate the *ogt* promoter only when it binds to an optimal position. A high induction occurs when the promoter contains the DNA site for NarL at a favorable location where NarL, RNA polymerase and DNA form a transcript initiation complex. Kaplan and Friedman (2012) indicated that the recruitment of transcription factors by the transcriptional machinery takes time (Garcia *et al., 2*012). The promoter strength depends on how long a transcription factor stays bound. Binding of NarL to the target site at the optimal location for long period results in high induction of the *ogt* gene. There is a possibility that an interaction between NarL, DNA and RNA polymerase at the right position leads to stabilization of the DNA-factor complex (Polach and Widom, 1995; Voss *et al.*, 2011).

Chapter 4: NarL and NarP dependent regulation of the *ogt* promoter

4.1 Introduction

The *E. coli* NarL response regulator consists of an N-terminal domain and a C-terminal domain. The N-terminal receiver domain carries a phosphorylation site while the C-terminal domain forms a typical helix-turn-helix motif and functions in DNA binding (Gelparin, 2006; Aravind *et al.*, 2005; Henikoff *et al.*, 1990). The two domains are linked together by a flexible tether of 13 residues.

The NarX and NarQ sensors sense the presence of nitrate and nitrite in the environments and phosphorylate either NarL or the homologous protein NarP (44% identity and 64% similarity). The amino acid sequences of both NarL and NarP are illustrated in Figure 4.1. Phosphorylation of the NarL receiver domain at the conserved residue Asp59 triggers a conformational change and allows the DNA binding domain to recognize and regulate the target promoter (Egan and Stewart, 1991; Zhang *et al.*, 2003). The consensus recognition sequence for NarL and NarP is TACYYMT (Y=C/T, M=A/C). The work of Darwin *et al.* (1997) indicated that both NarL and NarP bind as a dimer to heptameric sites arranged as an inverted repeat with two base-pair spacing (7-2-7).

The crystal structure of the non-phosphorylated NarL protein has been solved. The dimerization helix of the NarL C-terminal domain (NarL-CTD) is packed against the domain linker, and the recognition helix is occluded by the N-terminal domain. Phosphorylation of the N-terminal domain triggers domain rearrangement and allows NarL dimerization to occur (Baikalov *et al.*, 1996; Eldridge *et al.*, 2002). A NarL dimer interacts with DNA via residues Lys188, Val189 and Lys192, which are all located in the recognition helix (α9) of the C-terminal domain (Maris *el al.*, 2002; Maris *et al.*, 2005).

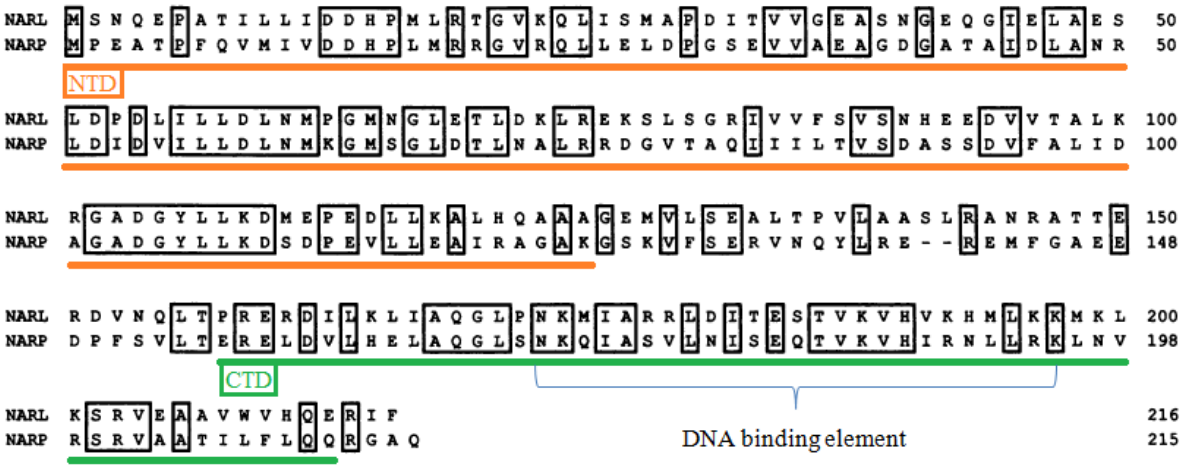


Figure 4.1 Amino acid sequences of NarL and NarP

Identical amino acid residues of NarL and NarP are shown in black boxes. The amino acid sequences of the N-terminal domain and the C-terminal domain are red-underlined and green-underlined respectively. The DNA binding elements are also identified (the figure is adapted from Baikalov *et al.*, 1996; Rabin and Stewart, 1993).

4.2 Activation of the *ogt* promoter by NarL-CTD and NarP-CTD

Maris *et al.* (2002) indicated that NarL-CTD, liberated from the receiver domain, can bind freely to the target binding site. This work aimed to examine whether NarL-CTD and NarP-CTD can activate the *ogt* promoter. The DNA fragments encoding NarXL and NarL-CTD were cloned into pLG339, while the DNA fragments encoding NarP and NarP-CTD were cloned into pJW15. Subsequently, recombinant plasmids were introduced into JCB3884 (lacks NarL and NarP) that contained pRW50 carrying the ogt100 promoter (I:wt II:wt). Cells were grown anaerobically with or without 20 mM nitrate, and beta-galactosidase activities were measured.

The results illustrated in Figure 4.2 show that there is no expression of the ogt100 promoter::*lacZ* fusion in cells carrying empty pLG339, as well as in cells that carry pLG339 encoding NarL-CTD. Hence, NarL-CTD alone is unable to activate the ogt100 promoter. Nitrate fails to induce expression of the ogt100 promoter::*lacZ* fusion in cells that contain pJW15 encoding either NarP or NarP-CTD, as the promoter activities are identical to those in cells carrying plasmids with no insert. Results suggest that NarL-CTD, NarP and NarP-CTD fail to activate the ogt100 promoter. Hence, only the full-length NarL is competent in activating the transcription at the wild-type ogt100 promoter.

The same method was used to measure beta-galactosidase activities in JCB3884 (L^- P^-) that contains pRW50 carrying the ogt1041 promoter (I ↑ II ↓). Results illustrated in Figure 4.3 show the expression of the ogt1041 promoter::*lacZ* fusion in cells that contain empty pLG339, pLG339 encoding NarXL and NarL-CTD, empty pJW15, pJW15 encoding NarP and NarP-CTD. Nitrate induction occurs only in cells that contain plasmids encoding NarXL.

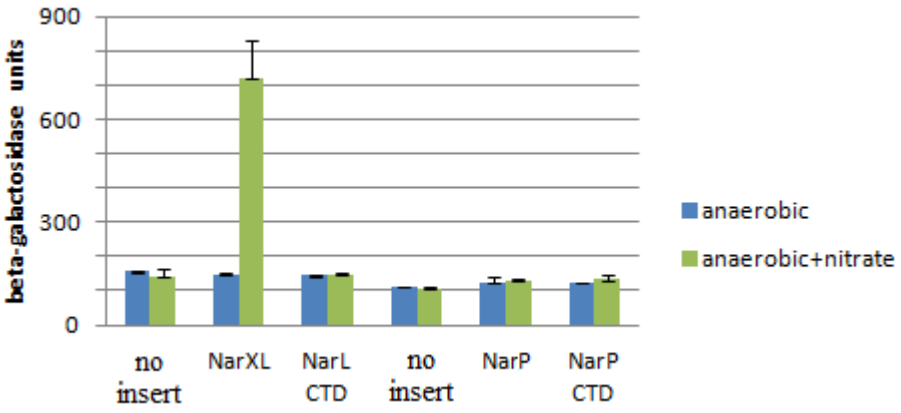


Figure 4.2 Expression of the ogt100 (I:wt II:wt) promoter::*lacZ* fusion in cells that carry plasmids encoding full length or CTD of NarL and NarP

Figure shows expression of the ogt100::*lacZ* fusion measured in cells strain JCB3884 (L^-P^-) that carry plasmids encoding NarXL, NarL-CTD, NarP and NarP-CTD, compared to in cells carrying plasmids with no insert. Cells were grown anaerobically in minimal salts medium with or without 20 mM nitrate.

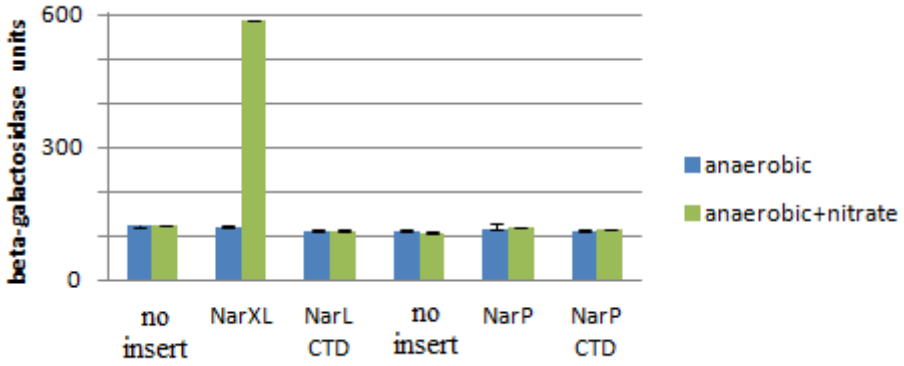


Figure 4.3 Expression of the ogt1041 (I ↑ II ↓) promoter::*lacZ* fusion in cells that carry plasmids encoding full length or CTD of NarL and NarP

Figure shows expression of the ogt1041 promoter::*lacZ* fusion measured in cells strain JCB3884 (L^-P^-) that carry plasmids encoding NarXL, NarL-CTD, NarP and NarP-CTD, compared to in cells carrying plasmids with no insert. Cells were grown anaerobically in minimal salts medium with or without 20 mM nitrate.

Expression of the ogt1041promoter::*lacZ* fusion in cells that contain pLG339 encoding NarL-CTD is identical to that in cells containing empty pLG339. Hence, NarL-CTD is unable to activate the ogt1041 promoter. In addition, NarP and NarP-CTD fail to induce the expression of the ogt1041 promoter::*lacZ* fusion. Taken together, only the full-length NarL is able to activate the ogt1041 promoter.

The same method was used to measure beta-galactosidase activities in JCB3884 (L^-P^-) that contains pRW50 carrying the ogt104168 promoter. The ogt104168 promoter contains a single NarL site centered at position -68.5 relative to the transcript start site. Results from Chapter 3 indicated that the activation of the ogt104168 promoter was induced in JCB387 (L^+P^+). This work aimed to investigate the activation of the ogt104168 promoter by NarL, NarP, NarL-CTD and NarP-CTD (liberated from the N-terminal receiver domain). Results illustrated in Figure 4.4 show the expression of the ogt104168 promoter::*lacZ* fusion in cells that contain empty pLG339, pLG339 encoding NarXL and NarL-CTD, empty pJW15, pJW15 encoding NarP and NarP-CTD. Only the full-length NarL is competent to activate the ogt104168 promoter.

Finally, beta-galactosidase activities were measured in strain JCB3884 (L^-P^-) that contain pRW50 carrying the ogt1052 promoter (I ↓ II ↑). Results illustrated in Figure 4.5 show expression of the ogt1052 promoter::*lacZ* fusion in cells that contain empty pLG339, pLG339 encoding NarXL and NarL-CTD, empty pJW15, pJW15 encoding NarP and NarP-CTD. High promoter activities are found in cells that contain pLG339 encoding either NarXL or NarL-CTD even in an absence of nitrate. This indicates that full-length NarL and NarL-CTD are able to activate the ogt1052 promoter. Expression of the ogt1052 promoter::l*acZ* fusion is also high in cells that contain pJW15 encoding NarP and NarP-CTD.

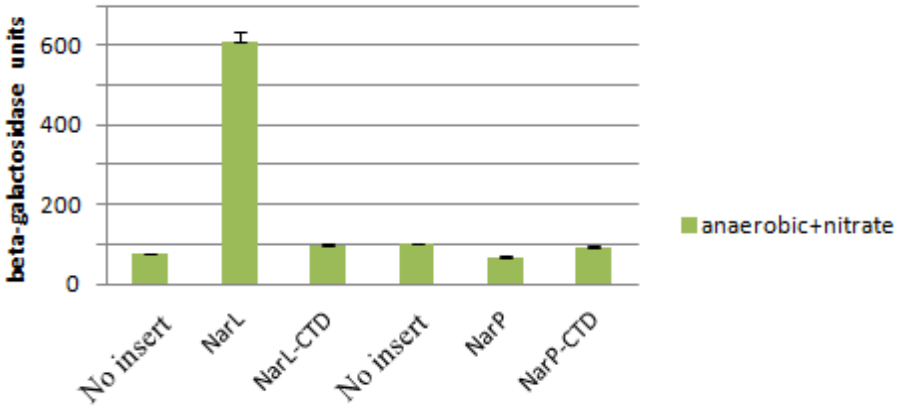


Figure 4.4 Expression of the ogt104168 promoter::*lacZ* fusion (the NarL site is centered at position -68.5) in cells that carry full-length or CTD of NarL and NarP

Expression of the ogt104168 promoter::*lacZ* fusion is measured in JCB3884 (L^-P^-) containing empty plasmids comparing to in cells that carry plasmids encoding NarL, NarL-CTD, NarP and NarP-CTD. Cells were grown anaerobically in minimal salts medium 20 mM nitrate to measure beta-galactosidase activities.

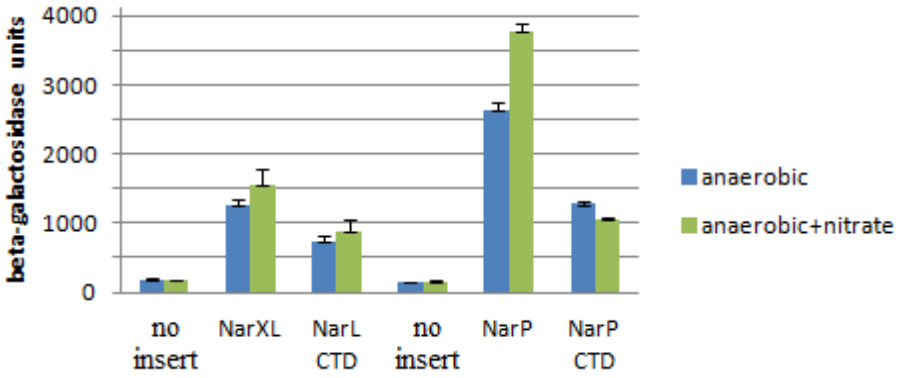


Figure 4.5 Expression of the ogt1052 (I ↓ II ↑) promoter::*lacZ* fusion in cells carrying full-length or CTD of NarL and NarP

Expression of the ogt1041 promoter::*lacZ* fusion is measured in JCB3884 (L^-P^-) carrying empty plasmids comparing to in cells that carry plasmids encoding NarXL, NarL-CTD, NarP and NarP-CTD. Cells were grown anaerobically in minimal salts medium with or without 20 mM nitrate.

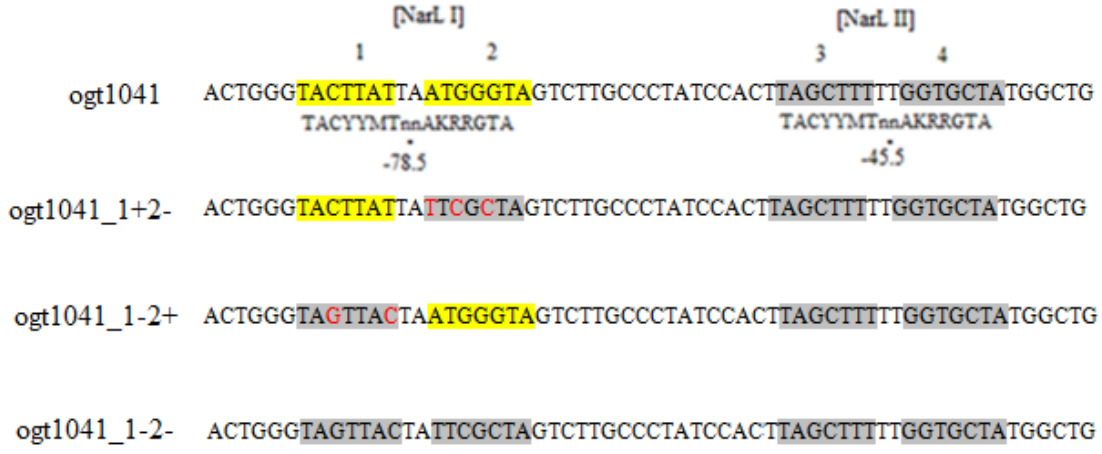
NarP can induce the promoter activity further in the presence of nitrate. Hence, both NarP and NarP-CTD can activate the ogt1052 promoter. Taken together, NarL is able to activate all the ogt100, ogt1041, ogt104168 and ogt1052 promoters, while NarL-CTD, NarP and NarP-CTD can activate the ogt1052 promoter but fail to activate the ogt100, ogt1041 and ogt104168 promoters.

4.3 Half-site studies

The *ogt* promoter consists of 2 DNA sites for NarL, each of which is arranged as an invert of two heptameric sites with 2 base spacing (7-2-7). Altogether, NarL occupies 4 half sites; half site 1 = the upstream half of NarL I site, half site 2 = the downstream half of NarL I site, half site 3 = the upstream half of NarL II site, half site 4 = the downstream half of NarL II site. This work aimed to examine whether site-specific mutation at each half site has any effects on NarL-dependent activation, and to determine whether or not NarL is able to activate the *ogt* promoter containing a single half-site. This will lead to a better understanding of NarL functions and how NarL interacts with αCTD.

Each heptameric site was modified to be similar or dissimilar (denoted by +/- respectively) to the consensus binding site of NarL. Figure 4.6 panel A shows partial base sequences of the ogt1041_1+2-, the ogt1041_1-2+ and the ogt1041_1-2- promoter fragments in comparison to the ogt1041 fragment. These DNA fragments were cloned into pRW50 vector and introduced into JCB387 (L^+P^-) for beta-galactosidase assays. The results illustrated in Figure 4.6 panel B show that nitrate fails to induce expression of the ogt1041_1+2- promote::*lacZ* fusion. In contrast, the ogt1041_1-2+ promoter activity is comparable to that of the ogt1041 promoter. This indicates that NarL, which binds to the downstream half of the

A.



B.

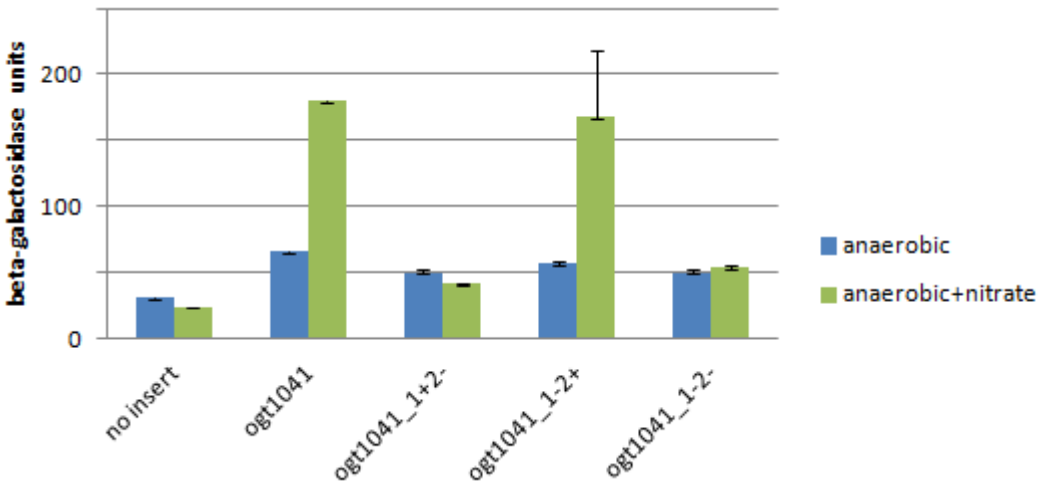


Figure 4.6 Site specific mutations at each heptameric site of the NarL I site in the ogt1041 promoter (I ↑ II ↓)

Panel A shows partial base sequences of the ogt1041, ogt1041_1+2- and ogt1041_1-2+ promoter fragments. DNA sites for NarL are highlighted in yellow with numbers above the sequences. Number 1 and 2 denote the upstream and the downstream half of the NarL I site while 3 and 4 denote the upstream and the downstream half of the NarL II site. Site specific mutations were introduced to each half site 1, 2 of the ogt1041 promoter. The mutated bases are written in red. The consensus sequences are given underneath the DNA sites for NarL. Symbol + and - indicate that the sequences is similar and dissimilar to the consensus. Disrupted NarL binding sites are coloured grey.

Panel B shows expression of the ogt1041 promoter::*lacZ* fusion in comparison to the ogt1041_1+2- promoter::*lacZ* fusion and the ogt1041_1-2+ promoter::*lacZ* fusion. Cells were grown in minimal salts medium anaerobically with or without 20 mM nitrate. Beta-galactosidase activities were measured after reaching the mid exponential phase.

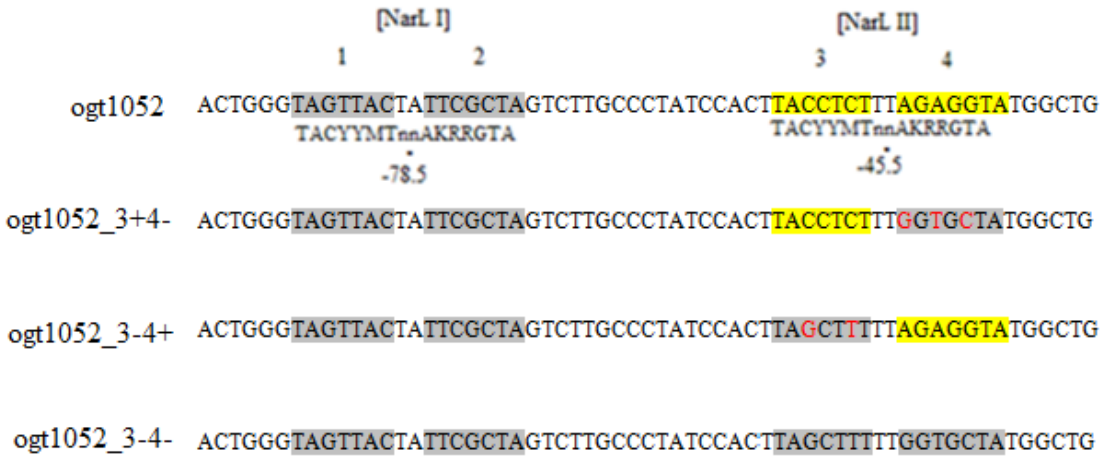
NarL I site, plays a role during the activation of the ogt1041 promoter. No induction was found when both half sites are disrupted.

Figure 4.7 panel A shows the partial base sequences of the ogt1052_3+4-, the ogt1052_3-4+ and the ogt1052_3-4- promoter fragments in comparison to the ogt1052 promoter fragment. Results illustrated in Figure 4.7 panel B show expression of the ogt1052 promoter::*lacZ* fusion comparing with the ogt1052_3+4- promoter::*lacZ* fusion and the ogt1052_3-4+ promoter::*lacZ* fusion. High nitrate induction still occurs in cells that contain pRW50 carrying the ogt1052_3+4- promoter, which has only half site 3 resembling the consensus. However, the activity level is ~2/3 lower than the ogt1052 promoter activity. There is minimal induction in cells that contain pRW50 carrying the ogt1052_3-4+ promoter, which has only half site 4 resembling the consensus. This suggests that only NarL, which binds to the upstream half of the NarL II site, plays a role during the activation of the ogt1052 promoter.

4.4 Binding of NarL to the NarL I site that carries one poor half site

Results from Section 4.3 showed that NarL is able to activate the ogt1041_1-2+ promoter but not the ogt1041_1+2- promoter. This suggests that NarL, which makes a contact with the αCTD, is located on the downstream half of the NarL I site. This work aimed to investigate whether NarL binds to the ogt1041_1-2+ promoter region as a monomer to the high-affinity half site or whether it still binds as a dimer despite the fact that one half site is mutated to disrupt NarL binding.

A.



B.

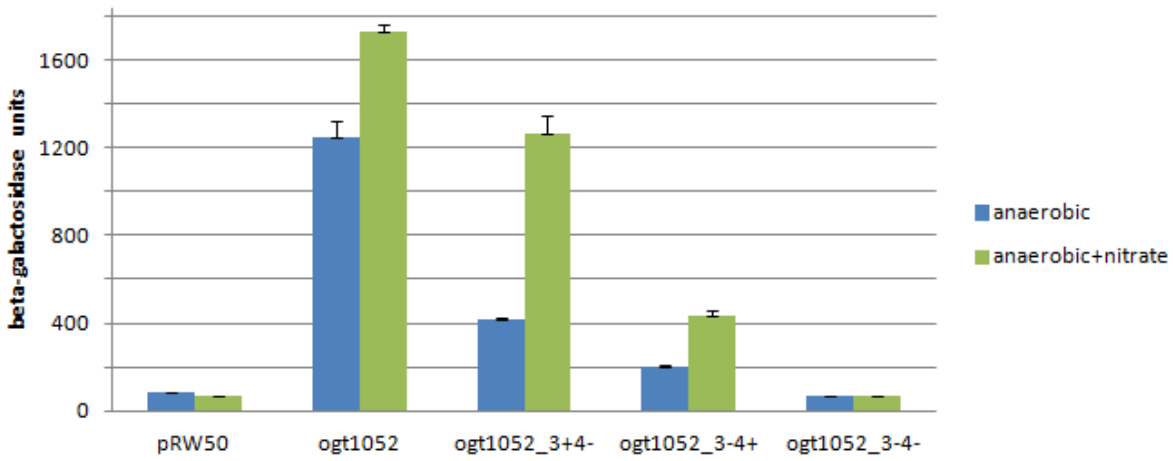


Figure 4.7 Site specific mutation at each heptameric site of the NarL II site of the ogt1052 promoter (I ↓ II ↑)

Panel A shows partial base sequences of the ogt1052, ogt1052_3+4-, ogt1052_3-4+ and ogt1052_3-4- promoter fragments. DNA sites for NarL are highlighted in yellow with numbers above the sequences. Number 1 and 2 denote the upstream and the downstream half of the NarL I site while 3 and 4 denote the upstream and the downstream half of the NarL II site. Site specific mutation was introduced to each half site 3 and 4 of the ogt1052 promoter. The mutated bases are written in red. The consensus binding sequences are given underneath the DNA sites for NarL. Symbol + and - indicate that the sequences is similar and dissimilar to the consensus. Disrupted NarL binding sites are coloured in grey.

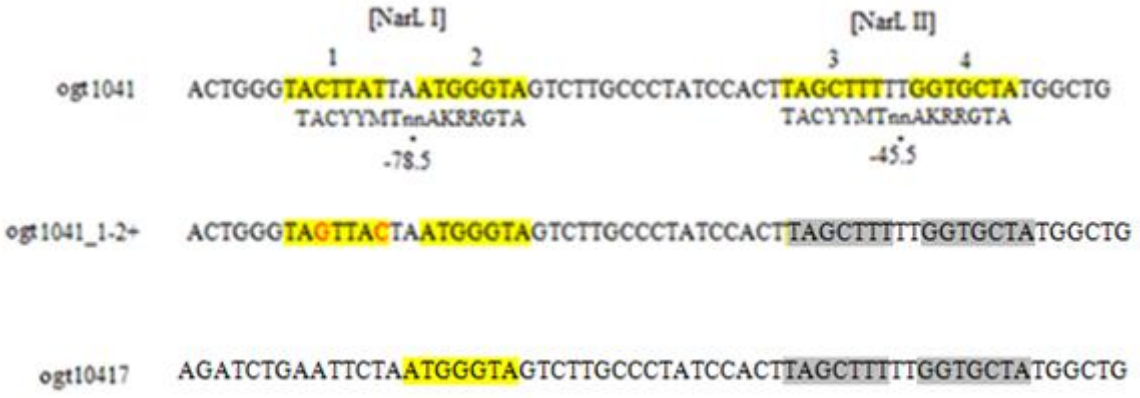
Panel B shows expression of the ogt1052 promoter::*lacZ* fusion in comparison to the ogt1052_3+4- promoter::*lacZ* fusion and the ogt1052_3-4+ promoter::*lacZ* fusion. Cells were grown in minimal salts medium anaerobically with or without 20 mM nitrate. Beta-galactosidase activities were measured after reaching the mid exponential phase.

Figure 4.8 panel A shows the partial base sequences of the promoter fragments of the ogt1041 (both half sites 1 and 2 have high binding affinities), the ogt1041_1-2+ (contains a low-affinity half site 1 and a high-affinity half site 2) and the ogt10417 (half site 1 is deleted while half site 2 has a high affinity for NarL). Results illustrated in Figure 4.8 panel B show that the expression levels of the ogt1041 and the ogt1041_1-2+ promoter::*lacZ* fusions are identical, which is ~ 2-fold greater than that of the ogt10417 promoter. This suggests that NarL still binds as a dimer to the NarL I site despite the upstream half site having a poor affinity for NarL. Compared to the ogt1041 promoter, activity of the ogt10417 promoter decreases by ~50% due to deletion of the whole upstream half site.

4.5 Binding of NarL to the NarL II site that carries one poor half site

Binding of NarL to the NarL II binding site is investigated *in vitro* by the EMSA technique with phosphorylated NarL and ^{32}P radiolabelled fragments of the ogt1052_3+4+, the ogt1052_3+4-, the ogt1052_3-4+ and the ogt1052_3-4- promoters. Partial base sequences of the promoter fragments are shown in Figure 4.9 panel A. The results illustrated in Figure 4.9 panel B show that the shift is detected when the concentration of NarL is high enough. When the ogt1052_3+4+ promoter fragment is tested, a single shift appears due to the binding of a NarL dimer to the NarL II site, which contains two high-affinity half sites. Also, binding of NarL to the ogt1052_3+4- promoter fragment results in a single shift at the position corresponding to the NarL-bound ogt1052_3+4+ promoter fragment. This suggests that NarL still binds as a dimer to the NarL site that contains one poor downstream half site.

A.



B.

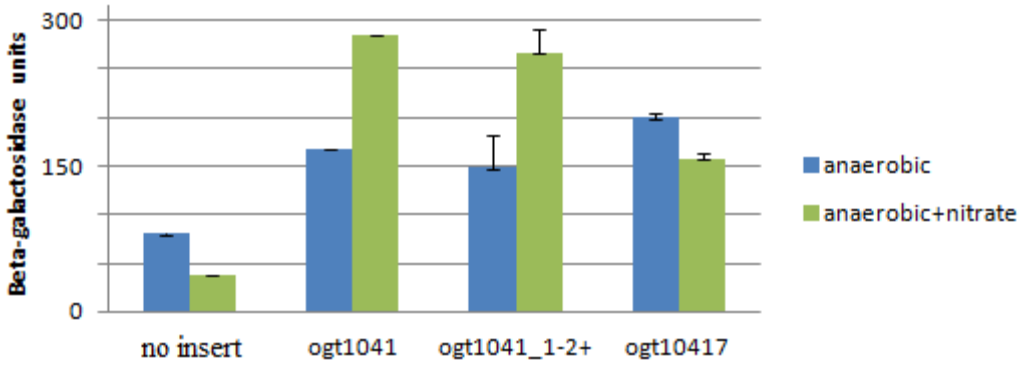


Figure 4.8 Effects of half site on binding of NarL to the NarL I site

Panel A shows partial base sequences of the ogt1041 and the ogt1041_1-2+ promoter fragments in comparison to the ogt10417 promoter fragment. DNA sites for NarL are highlighted in yellow with numbers above the sequences. Number 1 and 2 denote the upstream and the downstream half of the NarL I site while 3 and 4 denote the upstream and the downstream half of the NarL II site. Site specific mutation was introduced to each half site 1 and 2 of the ogt1041 promoter. The mutated bases are written in red. The consensus binding sequences are given underneath the DNA sites for NarL. Symbol + and - indicate that the sequences are similar and dissimilar to the consensus respectively. Disrupted NarL binding sites are coloured grey.

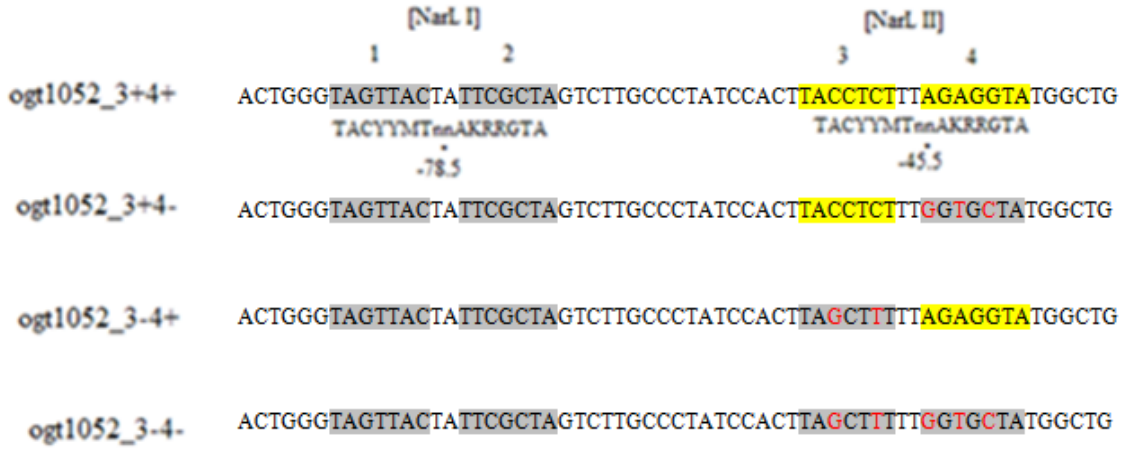
Panel B shows expression of the ogt1041 promoter::*lacZ* fusion comparing to the ogt1041_1-2+ promoter::*lacZ* fusion and the ogt10417 promoter::*lacZ* fusion. Cells were grown in minimal salts medium anaerobically with or without 20 mM nitrate. Beta-galactosidase activities were measured after reaching the mid exponential phase.

Figure 4.9 Effects of half site on binding of NarL to the NarL II site.

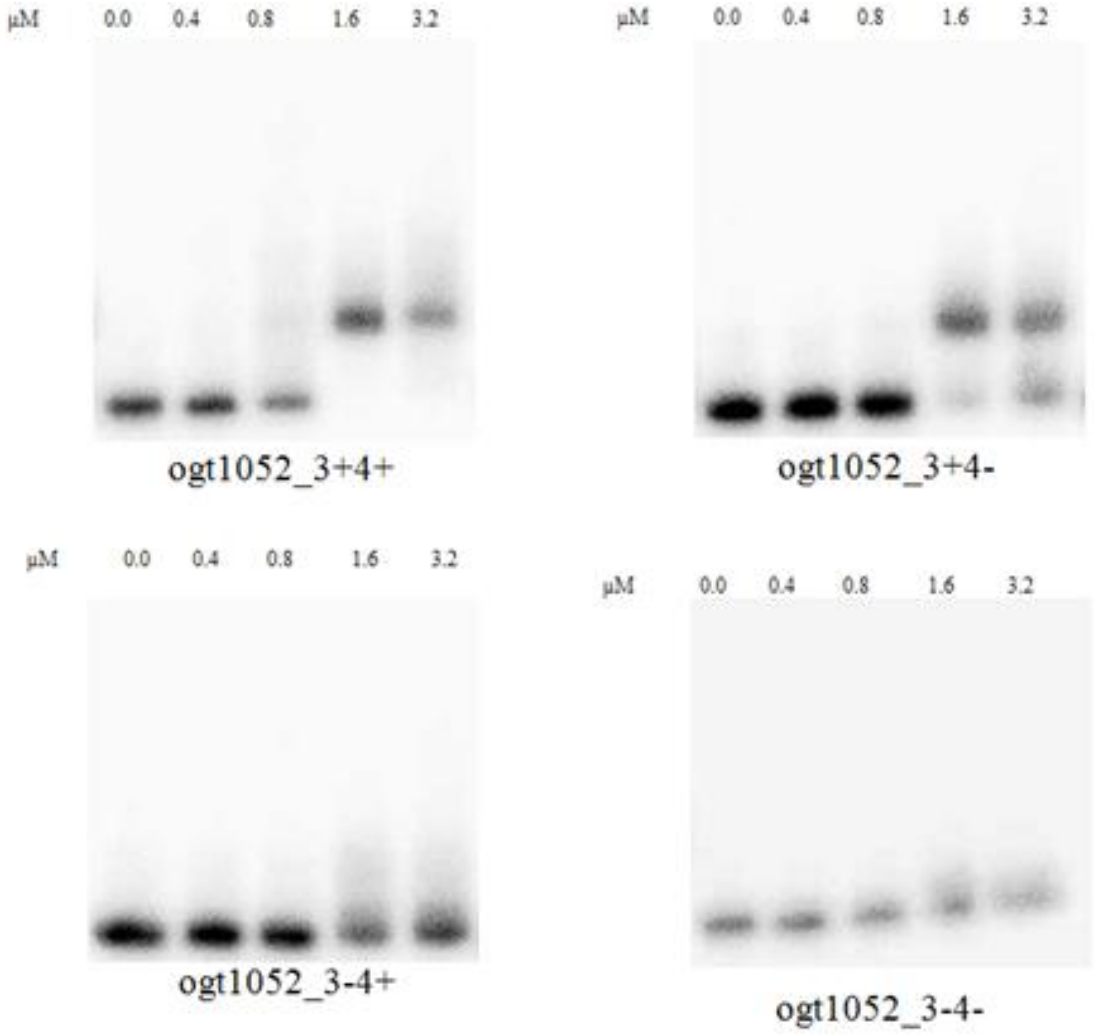
Panel A shows partial base sequences of the ogt1052_3+4+, ogt1052_3+4-, ogt1052_3-4+ and ogt1052_3-4- promoter fragments. DNA sites for NarL are highlighted in yellow with numbers above the sequences to denote half site names. Number 1 and 2 denote the upstream and the downstream half of the NarL I site while 3 and 4 denote the upstream and the downstream half of the NarL II site. Site specific mutation was introduced to each half site 3 and 4 of the ogt1052 promoter. The mutated bases are written in red. The consensus binding sequences are given underneath the NarL sites. Symbol + and - denote that the sites are similar and dissimilar to the consensus respectively. The disrupted sites are coloured grey.

Panel B shows results of EMSA experiments with NarL and ^{32}P end-labelled fragments of the ogt1052_3+4+, the ogt1052_3+4-, the ogt1052_3-4+ and the ogt1052_3-4- promoters. NarL concentrations used are; lane 1=no protein, lane 2= 0.4 µM, lane 3=0.8 µM, lane 4=1.6 µM, lane 5=3.2 µM

A.


[NarL I]
[NarL II]
1
2
3
4
ogt1052_3+4+
ACTGGGTAGTTACTATTCGCTAGTCTTGCCCTATCCACTTACCTCTTTAGAGGTATGGCTG
TACYYMTnnAKRRGTA
TACYYMTnnAKRRGTA
-78.5
-45.5
ogt1052_3+4-
ACTGGGTAGTTACTATTCGCTAGTCTTGCCCTATCCACTTACCTCTTTGGTGCTATGGCTG
ogt1052_3-4+
ACTGGGTAGTTACTATTCGCTAGTCTTGCCCTATCCACTTAGCTTTTTAGAGGTATGGCTG
ogt1052_3-4-
ACTGGGTAGTTACTATTCGCTAGTCTTGCCCTATCCACTTAGCTTTTTGGTGCTATGGCTG

B.


μM
0.0
0.4
0.8
1.6
3.2
ogt1052_3+4+
μM
0.0
0.4
0.8
1.6
3.2
ogt1052_3+4-
μM
0.0
0.4
0.8
1.6
3.2
ogt1052_3-4+
μM
0.0
0.4
0.8
1.6
3.2
ogt1052_3-4-

Results show that NarL does not bind to the ogt1052_3-4+ promoter fragment. This suggests that half site 3 has a greater role in the initial NarL binding than half site 4. No shift was found when tested with the ogt1052_3-4- promoter, indicating that NarL does not bind to the NarL II site, which has both of its half sites disrupted.

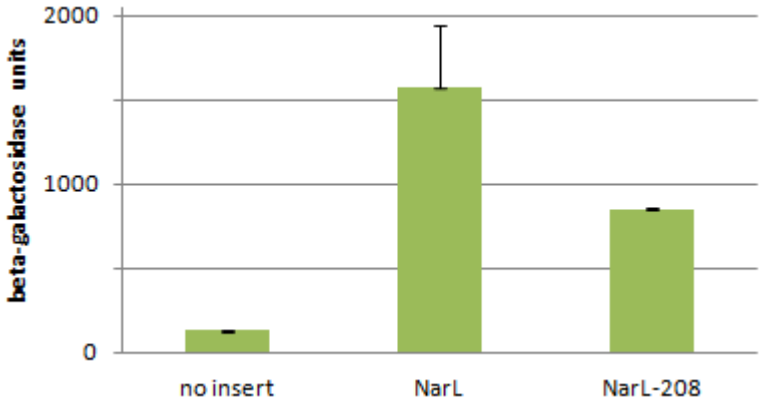
4.6 Activation of the *ogt* promoter by NarL dimers

Results from the EMSA experiment (Section 4.4) suggested that NarL still binds as a dimer to the ogt1052_3+4- promoter although the NarL site has the poor downstream half site. The work of Maris *et al.* (2002) indicated that the DNA target site was recognized by the NarL dimer. The crystal structure of a NarL-CTD dimer in a complex with DNA has been solved by Baikalov *et al.* (1998). Dimerization occurs between α10 helices of two NarL monomers, and a NarL dimer positions its dimerization interface perpendicular to the DNA minor groove. This work aimed to determine whether NarL dimerization is required for the binding of NarL to the NarL II site in order to activate the ogt1052 promoter derivatives.

A dimer of NarL is formed by an interaction between residues V208 of each monomer (Maris *et al.*, 2002). Alanine substitution was introduced to V208 of the full-length NarL and NarL-CTD, then the fragments encoding NarL and NarL-CTD with V208A were cloned into pJW15. Recombinants were introduced into JCB3884 (L^-P^-) that contain pRW50 carrying the ogt1052 promoter or the ogt1052_3+4- promoter. Beta-galactosidase activities were measured in cells grown anaerobically with 20 mM nitrate.

Results in Figure 4.10 show that expression of the ogt1052 promoter::*lacZ* fusion in JCB3884 (L^-P^-) containing NarL with V208A decreases ~2-fold from the expression

A.



B.

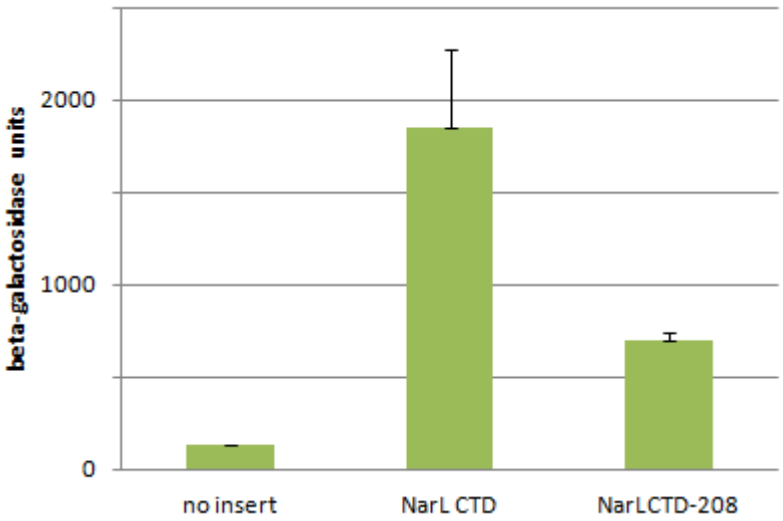


Figure 4.10 Effects of alanine substitution at V208 of NarL on the ogt1052 promoter

Panel A shows beta-galactosidase activities measured in JCB3884 (L^-P^-) carrying the ogt1052 promoter::*lacZ* fusion and pJW15 encoding NarL protein with/without an alanine substitution at V208.

Panel B shows beta-galactosidase activity measured in JCB3884 (L^-P^-) carrying the ogt1052 promoter::*lacZ* fusion and pJW15 encoding NarL-CTD with/without an alanine substitution at V208.

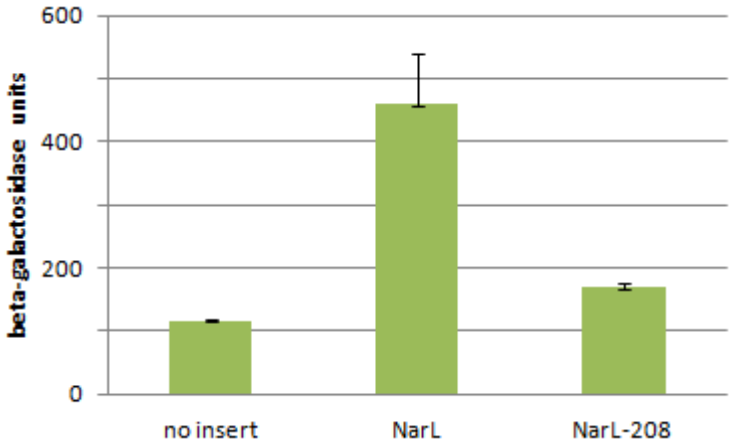
Cells were grown in minimal salts medium anaerobically with 20 mM nitrate. Beta-galactosidase activities were measured after reaching the mid exponential growth phase.

level induced by the wild-type NarL. Results illustrated in Figure 4.11 show the expression of the ogt1052_3+4- promoter::*lacZ* fusion in JCB3884 (L^-P^-) containing NarL-CTD with or without an alanine substitution at V208. The promoter activity induced by the wild-type NarL-CTD is >2-fold greater than when it is induced by NarL-CTD carrying the V208A substitution. To conclude, disruption of the NarL dimerization interface by an alanine substitution at residue 208 results in decreasing abilities of NarL and NarL-CTD to activate the ogt1052 promoter and the ogt1052_3+4- promoter. This indicates that NarL binds to the ogt1052_3+4- promoter region as a dimer even though the DNA target site contains only one functioning heptameric site.

4.7 NarL and NarP dependent activation of the *ogt* promoter that contains one half site for NarL

From Section 4.3, NarL successfully activated the ogt1052_3+4- promoter (contains the upstream half of the NarL II site) but failed to activate the ogt 1052_3-4+ promoter (contains the downstream half of the NarL II site). Together with data from the EMSA experiment, it suggests that the upstream half site of the NarL II binding site plays a more crucial role in facilitating the binding of a NarL dimer. This work aimed to examine the effects of losing a half site on NarL and NarP functions. The pRW50 carrying the ogt1052_3+4- promoter and the ogt1052_3-4+ promoter were introduced into JCB387 (L+P+), JCB3875 (L^+P^-), JCB3883 (L^-P^+) and JCB3884 (L^-P^-). Cells were grown anaerobically with or without 20 mM nitrate. Beta-galactosidase activities were then measured after reaching the exponential growth phase.

A.



B.

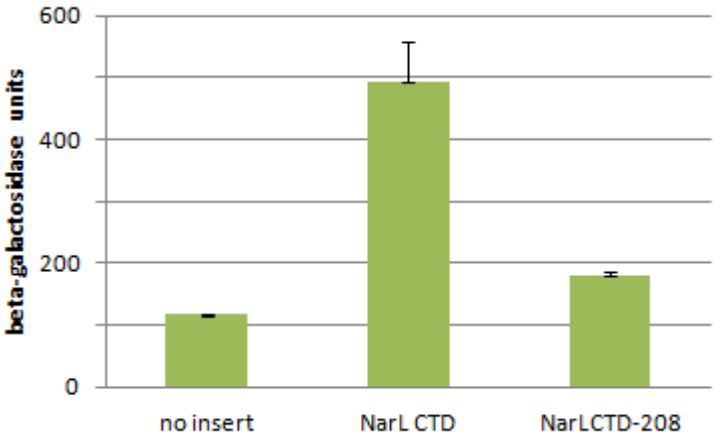


Figure 4.11 Effects of alanine substitution at V208 of NarL on the ogt1052_3+4- promoter

Panel A shows beta-galactosidase activities measured in JCB3884 (L^-P^-) carrying the ogt1052_3+4- promoter::*lacZ* fusion and pJW15 encoding NarL protein with/without an alanine substitution at V208.

Panel B shows beta-galactosidase activities measured in JCB3884 (L^-P^-) carrying the ogt1052_3+4- promoter::*lacZ* fusion and pJW15 encoding NarL-CTD with/without an alanine substitution at V208.

Cells were grown in minimal salts medium anaerobically with 20 mM nitrate. Beta-galactosidase activities were measured after reaching the mid exponential growth phase.

The pRW50 carrying the ogt1052_3+4- promoter were introduced into JCB387, JCB3875, JCB3883 and JCB3884 for beta-galactosidase assays. The results illustrated in Figure 4.12 show that there must be NarL in cells in order to activate the ogt1052_3+4- promoter, while NarP alone fails to induce the activation. Beta-galactosidase activities measured in JCB387 (L^+P^+) and JCB3875 (L^+P^-) are ~6 times and ~5 times higher than in JCB3883 (L^-P^+). No induction is found in JCB3884 (L^-P^-); hence, the obtained promoter activities in alternative strains are the result of regulation by NarL and NarP.

In addition, pRW50 plasmids carrying the ogt1052_3-4+ promoter were used to transform JCB387, JCB3875, JCB3883 and JCB3884 for beta-galactosidase assays. Results show that no induction is found in JCB3875 (L^+P^-), JCB3883 (L^-P^+) and JCB3884 (L^-P^-). Minimal expression (~2-fold greater than that measured in JCB3884) occurs in JCB387 (L^+P^+). This suggests that neither NarL nor NarP can activate the ogt1052_3-4+ promoter.

4.8 Regulation by full-length NarL and NarL-CTD when the NarL II site contains only one half site

The previous results indicated that NarL-CTD was able to activate the *ogt* promoter that carries a single NarL site centered at position -45.5. To activate the ogt1052_3+4- promoter, NarL still binds as a dimer to the NarL site that lacks the downstream half site. This work aimed to investigate NarL and NarL-CTD dependent activation of the ogt1052 promoter derivatives that contain a single half site for NarL. The pLG339 encoding NarXL and NarL-CTD were introduced into JCB3884 (L^-P^-) carrying the ogt1052_3+4- promoter::*lacZ* fusion or the ogt1052_3-4+ promoter::*lacZ* fusion for beta-galactosidase assays.

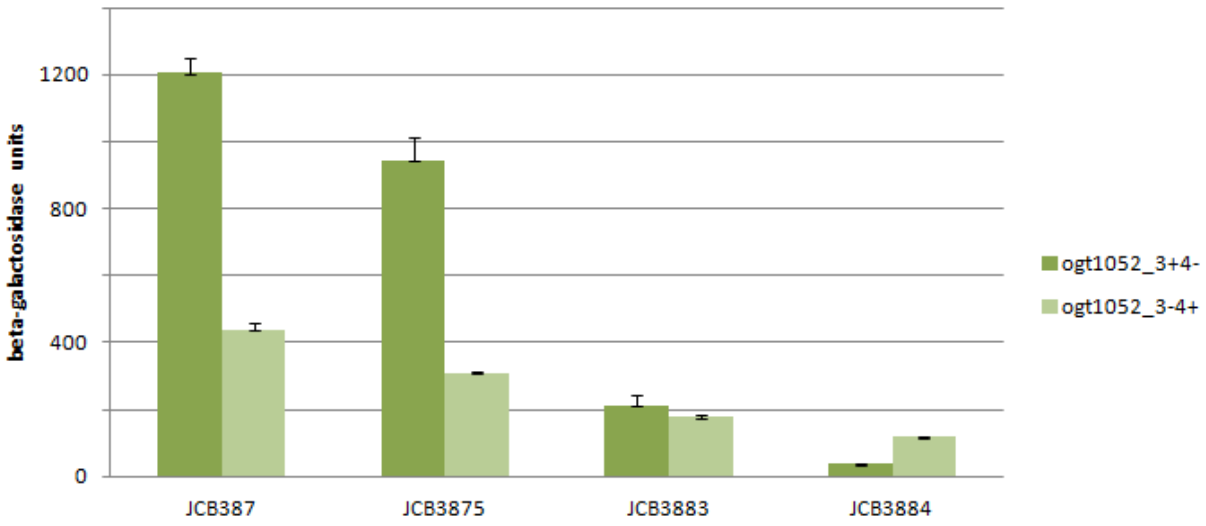


Figure 4.12 NarL and NarP dependent activation of the *ogt* promoter containing a single half site at the NarL II site

Figure shows beta-galactosidase activities measured in JCB387 (L+P+), JCB3875 ($L^{+}P^{-}$), JCB3883 ($L^{-}P^{+}$) and JCB3884 ($L^{-}P^{-}$) that contain pRW50 carrying either the ogt1052_3+4- promoter or the ogt1052_3-4+ promoter. Cells were grown in minimal salts medium anaerobically with 20 mM nitrate. Beta-galactosidase activities were measured after reaching the mid exponential growth phase.

Results illustrated in Figure 4.13 panel A show that a high expression of the ogt1052_3+4-promoter::*lacZ* fusion is induced by NarL. NarL-CTD fails to activate the ogt1052_3+4-promoter. Results illustrated in Figure 4.13 panel B show that the ogt1052_3-4+ promoter activity, induced by NarL, is ~50% less than that of the ogt1052_3+4- promoter. No induction is found in cells carrying NarL-CTD. Taken together, unlike the full-length NarL, NarL-CTD liberated from the N-terminal receiver domain cannot activate the *ogt* promoter that carries a single heptameric site for NarL. This work suggests that lacking the N-terminal domain affects the ability of NarL-CTD to regulate the *ogt* promoter that carries a low-affinity binding site for NarL dimer.

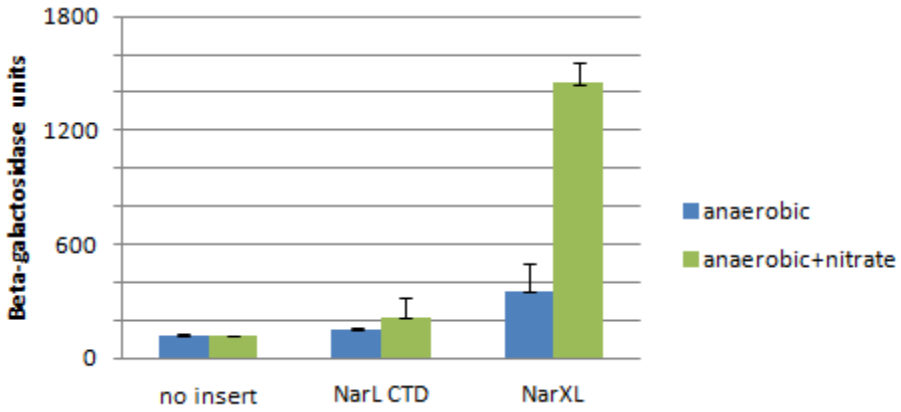
4.9 Discussion

Roles of the N-terminal domain (NTD) and the C-terminal domain (CTD) of NarL/NarP

NarL and NarP response regulators are homologous proteins with a high degree of correspondence between two sequences (44% identity), indicating that both have similarity in function. This study shows that NarL, in comparison to NarP, can activate a wider range of *ogt* promoter derivatives. This might be due to some distinct structural differences between the two proteins. The CTD and full-length versionsof both NarL and NarP can activate the ogt1052 promoter, which contains the DNA site for NarL centered at position -45.5. NarL can activate the ogt100 promoter and the ogt1041 promoter but NarP cannot.

Results show that the NTD of NarL is required for the activation of the ogt100 and ogt1041 promoters. This agrees with the work from Lin *et al.* (2010) who suggested that the

A.



B.

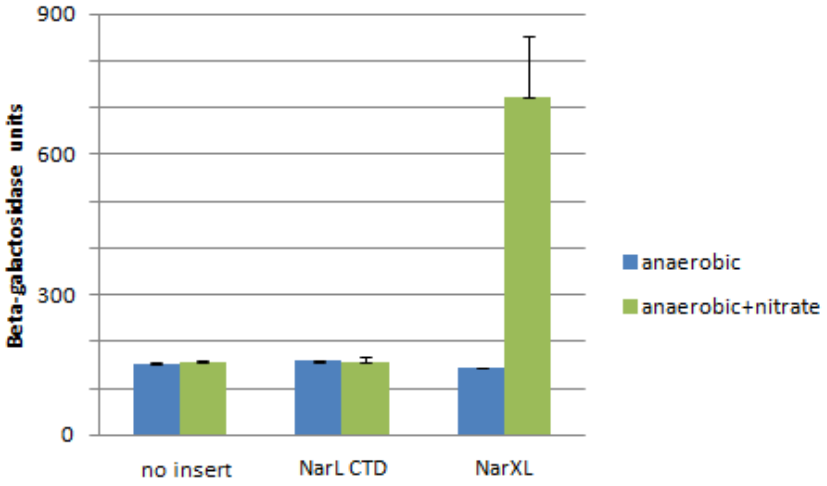


Figure 4.13 Regulation of the *ogt* promoter by full-length NarL and NarL-CTD when the NarL II site contains a single half site

Panel A shows beta-galactosidase activities measured in JCB3884 (L^-P^-) containing pRW50 carrying the ogt1052_3+4- promoter and pLG339 encoding either NarL-CTD or NarXL.

Panel B shows beta-galactosidase activities measured in JCB3884 (L^-P^-) containing pRW50 carrying the ogt1052_3-4+ promoter and pLG339 encoding either NarL-CTD or NarXL.

Cells were grown in minimal salts medium anaerobically with or without 20 mM nitrate.

NTD of NarL contains necessary determinants for promoter activation via class I and class III mechanisms. Nevertheless, further studies are needed to determine the specific region of the NarL-NTD that interacts with RNA polymerase. Interestingly, activation of the ogt1052 promoter does not require the NTD of both NarL and NarP, however the promoter activity is less than when it is induced by the full length proteins.

NarL can activate all of the ogt100, ogt1041 and ogt1052 promoters. NarP, as well as NarL-CTD and NarP-CTD, are competent in only activating the ogt1052 promoter. This might result from different transcription mechanisms due to different relative placements of NarL/NarP and αCTD at the promoters. From the unpublished work of David Chismon (2011), the alanine scan library of αCTD was used to determine the critical residues for NarL-dependent promoter activation. It was found that there was more deviation from the wild-type expression level when measured in cells carrying the ogt100 and the ogt1041 promoter::*lacZ* fusions, compared to in cells carrying the ogt1052 promoter::*lacZ* fusion.

Half-site studies

The DNA site for NarL is composed of an inverted repeat of heptameric sites with 2 base spacing (Lin *et al.*, 2007; Squire *et al.*, 2009). From the half-site experiment, the downstream half of the NarL site centered at position -78.5 (the NarL I site) was crucial for the expression of the ogt1041 promoter::*lacZ* fusion. This suggests that NarL, which binds to the downstream half of the NarL I site, plays a role in the *ogt* promoter activation and interacts with the αCTD.

The ogt1052 promoter has a single NarL binding site centered at position -45.5 (the NarL II site) adjacent to the -35 element. High nitrate induction still takes place although the downstream half site is modified to have a low affinity for NarL. This suggests that NarL,

which is located at the upstream half of the NarL II site, plays a role during the activation of the *ogt* promoter.

From the results from half-site experiments, it is thought that the wild-type *ogt* promoter has one αCTD that makes a contact with NarL located at the downstream half of the NarL I site, while another αCTD interacts with NarL located at the upstream half of the NarL II site. The site specific mutagenesis of NarL and alanine scan libraries of the α subunit of RNA polymerase have been studied to determine the residues of interaction between αCTD and NarL. This will be discussed in further detail in Chapter 5.

NarL binds to the ogt promoter region as a dimer

Phosphorylation of NarL response regulator allows two NarL monomers to dimerize and bind as a dimer to the specific target binding site in the *ogt* promoter region. The ogt1041_1-2+ promoter has the NarL I site of which the downstream half site has a high affinity for NarL, while the upstream half is modified to less likely resemble the consensus region (2 mismatches). Expression of the ogt1041_1-2+ promoter::*lacZ* fusion is comparable to that of the ogt1041 promoter::*lacZ* fusion. The ogt10417 promoter was constructed by introducing differences to the consensus region, specifically in the upstream half of the NarL I site (5 mismatches), which resulted in a decrease in the promoter activity by nearly 50%. This indicates that NarL prefers to bind to DNA as a dimer, and the target site arrangement as a 7-2-7 inverted repeat serves as the optimal binding configuration of the DNA site for NarL. This agrees with the work from Maris *et al.* (2005) who revealed that the 7-2-7 configuration provides an affinity 3-to 5-fold higher than others.

Results from the EMSA experiment with NarL and radiolabelled DNA fragments of the *ogt* promoter derivatives (ogt1052_3+4+, ogt1052_3+4-, ogt1052_3-4+ and ogt1052_3-4)

indicate that NarL still binds as a dimer to the *ogt* promoter carrying only a single upstream half of the NarL II site but no evidence of NarL binding is found when the promoter carries a single downstream half site. It is possible that each base functions differently to facilitate an initial binding of NarL due to its position on the DNA helix; hence, introducing mutations to different bases may result in an unequal loss of NarL binding affinity. Some mutated bases might influence interactions with the side chains of NarL that are inserted into the major groove or disrupt water-mediated DNA recognition. This explains why introducing mutations to some bases at the upstream half of the NarL II site results in a decrease of the promoter activity.

Although NarL recognition involves the extensive NarL-DNA contact, only 3 protein side chains (Lys-188, Val-189, Lys-192) of NarL-CTD come into direct contact with the DNA major groove floor. Some certain bases are located at the positions of non-contacted regions, allowing flexibility that mutation of these base pairs may not affect NarL recognition (Maris *et al.*, 2005). This may explain why there is still a high promoter activity when some bases at the downstream half of the NarL II site are mutated.

A hydrophobic 800 Å solvent-excluded dimerization interface is formed by residues 204, 208 and 211 between helices 10 of two NarL proteins. The ogt1052_3+4- promoter activity is reduced in cells carrying NarL and NarL-CTD with an alanine substitution at residue V208. This indicates that there is a higher tendency for two NarL monomers to dimerize even though the DNA site for NarL contains only the upstream half site. The NarL-DNA recognition involves a gradual bending mechanism, which is achieved by NarL dimerization. Also, the structure of the DNA region that interacts with a NarL dimer is transitioned from B to A form, resulting in a specific recognition of the target site by NarL (Mais *et al.*, 2002).

Regulation of the ogt promoter carrying a single half of the NarL II site

The DNA sequences and configurations of the heptameric sites for NarL affect the DNA structural recognition such as DNA groove width and DNA backbone flexibility. Hence, introducing mutations to different half sites causes different effects on NarL recognition and NarL function. A greater NarL induction is found when the NarL II site contains the upstream half, compared to when the site contains only the downstream half. As previously discussed that NarL tends to bind as a dimer despite the fact that the binding site contains one half site, it suggests that the upstream half of the NarL II site contributes more in NarL dimer recognition than the other half.

In addition, the 1052_3+4- promoter activity, induced by NarL-CTD, is lower in comparison to when it is induced by NarL. NarL-CTD contains all the essential determinants for DNA binding; however, lacking the NTD still reduces the level of induction. It is possible that some residues of the NTD have a role in transcription activation (Lin *et al., 2*010).

NarP is unable to activate the *ogt* promoter containing a single half of the NarL II site. It has been reported that the phosphorylated NarP is restricted to binding to the target site arranged as heptameric pairs only. It apparently interacts weakly to the DNA sites of alternative binding configurations (Darwin *et al.,* 1996 & Darwin *et al.,* 1997).

Chapter 5: Mechanisms of activation by NarL at the *ogt* promoter

5.1 Introduction

Results from Chapter 3 indicate that NarL activates the wild-type *ogt* promoter by binding to 2 DNA sites for NarL located at position -78.5 (NarL I site) and -45.5 (NarL II site) relative to the transcript start site of the *ogt* promoter. This agrees with the work of Squire *et al.* (2009) that transcription of the *ogt* gene requires NarL binding at both NarL sites. Data show that NarL also activates the *ogt* promoter containing a single DNA site for NarL if the site better resembles the consensus sequence.

NarL binds to the ogt1041 promoter at a site located at position -78.5. This suggests that NarL possibly activates the ogt1041 promoter via a class I mechanism. NarL binds to the ogt1052 promoter at a site located at position -45.5 adjacent to the -35 element. In a class II promoter, an activator binds to the DNA region upstream or overlapping the -35 element and also in most cases contacts domain 4 of σ (Browning and Busby, 2004; Dove *et al.,* 2003). Hence, the ogt1052 promoter may act as a class II promoter. Both class I and class II activators make a direct protein-protein interaction with αCTD that is located on the same face of DNA helix. This work is aimed to define the residues of interaction between NarL and αCTD and to identify their orientations on the DNA helix at the *ogt* promoter.

From the Ph.D work of David Chismon (2010), a single alanine substitution has been introduced to residues 255-329 of the α subunit of RNA polymerase to determine important residues for activation of the *ogt* promoter. The pHTfI and pREII encoding α derivatives were introduced into JCB3875 (L^+P^-) that contained pRW50 carrying the wild-type ogt100 promoter (I:wt II:wt). Cells were grown anaerobically with or without 20 mM nitrate to measure expression of the ogt100 promoter::*lacZ* fusion. Figure 5.1 to 5.3 show data from the Chismon PhD thesis and from the basis of work described in this chapter.

Results illustrated in Figure 5.1 show that NarL-dependent activation of the ogt100 promoter is impaired when residues 258, 266, 273, 287, 288, 290, 293, 307 of α are substituted by alanine. Alanine substitution has the most effect when it is introduced to residue 273.

Results illustrated in Figure 5.2 show beta-galactosidase activities measured in cells that contain pRW50 carrying the ogt1041 promoter (I ↑ II ↓) and pHTf or pREII encoding α derivatives. Alanine substitutions of αCTD at residues 259, 261, 271, 273, 280, 288, 290, 293, 296, 302 and 328 impair the promoter activation. Again, alanine substitution has the most effect when it is introduced to residue 273

Results illustrated in Figure 5.3 show beta-galactosidase activities measured in cells that contain pRW50 carrying the ogt1052 promoter (I ↓ II ↑) and pHTfI or pREII encoding α derivatives. Alanine substitutions of αCTD at residues 259, 261, 268, 273, 287, 288, 293, 295, 296, 299, 302, 310, 312, 313 and 320 impair the activation. Alanine substitution has the most effect when it is introduced to residue 273

Residues involved in activation of the *ogt* promoter were mapped on the αCTD structural model (Jeon *et al.,* 1995). Most of these residues are located on the surface of the αCTD. Residue 273, which is required for activation of all the ogt100, ogt1041 and the ogt1052 promoters, is a surface-exposed residue. Hence, this agrees to the idea that the NarL directly contacts with αCTD to facilitate binding of RNA polymerase at the *ogt* promoter.

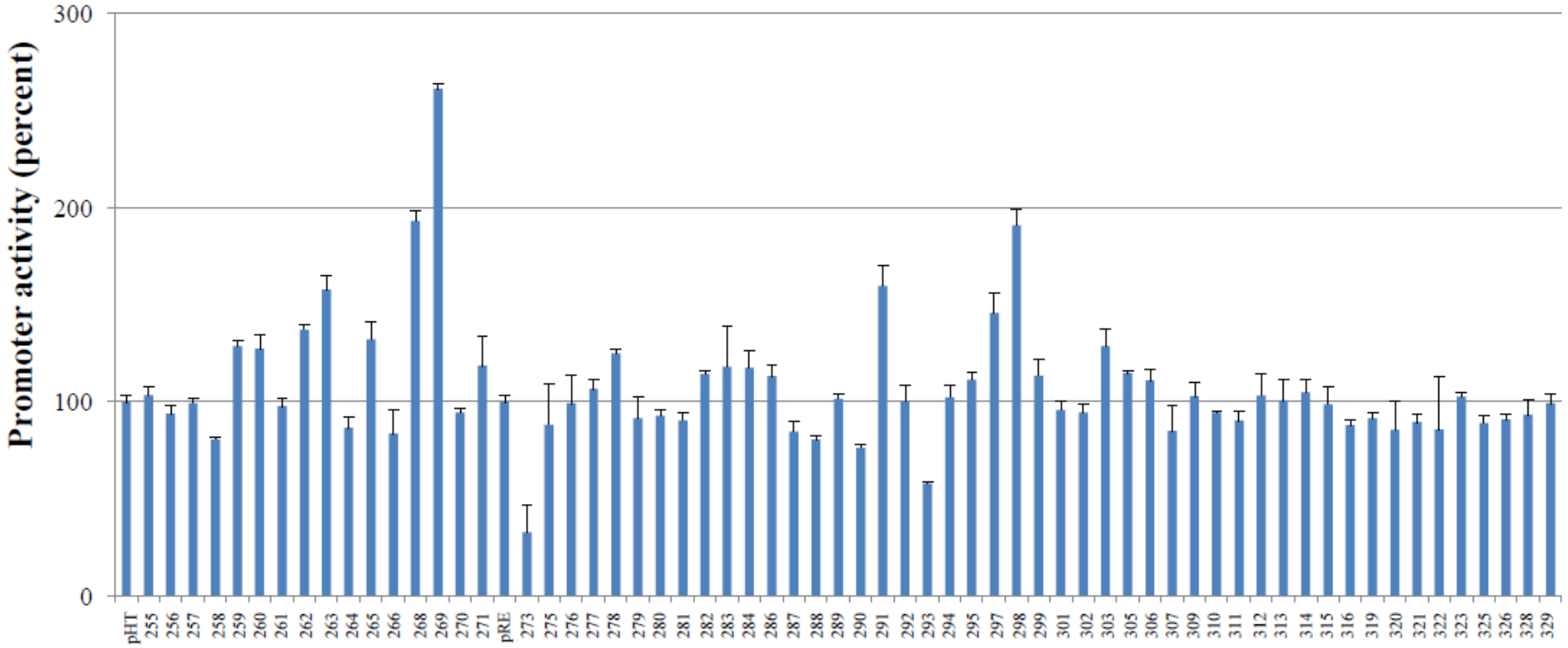


Figure 5.1 Important residues of αCTD for activation of the ogt100 promoter

Beta-galactosidase activities were measured in JCB3875 (L^+P^-) that contained pRW50 carrying the ogt100 promoter together with pHTf1 or pREII encoding α derivative with a single alanine substitution (residue 255-329). The promoter activities are presented as percentages of the activity measured in cells that carry plasmids encoding the wild-type α (648 Miller units) (taken from Ph.D. thesis of David Chismon, 2010)

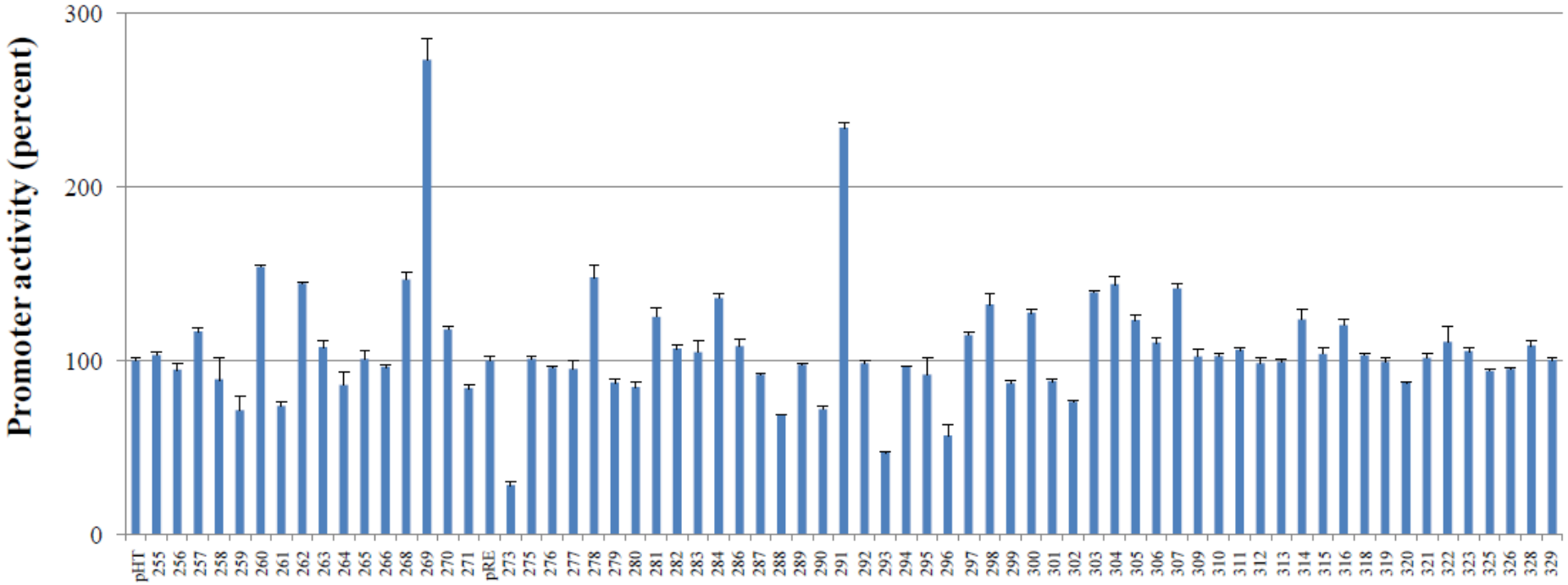


Figure 5.2 Important residues of αCTD for activation of the ogt1041 promoter

Beta-galactoisidase activities were measured in JCB3875 (L^+P^-) that contained pRW50 carrying the ogt1041 promoter together with pHTf1 or pREII encoding α derivative with a single alanine substitution (residues 255-329). The promoter activities are presented as percentages of the activity measured in cells that carry plasmids encoding the wild-type α (405 Miller units) (taken from Ph.D. thesis of David Chismon, 2010).

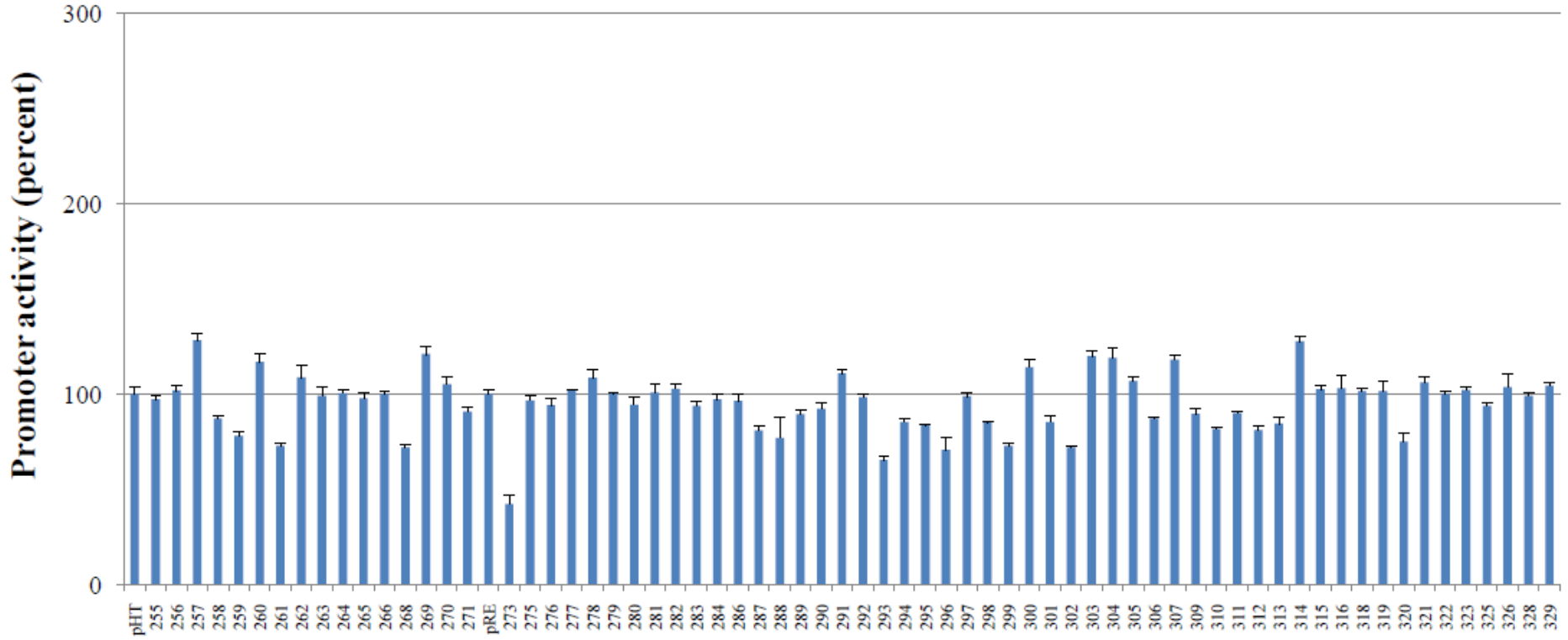


Figure 5.3 Important residues of αCTD for activation of the ogt1052 promoter

Beta-galactosidase activities were measured in JCB3875 (L^+P^-) that contained pRW50 carrying the ogt1052 promoter together with pHTf1 or pREII encoding α derivative with a single alanine substitution (residues 255-329). The promoter activities are presented as percentages of the activity measured in cells that carry plasmids encoding the wild-type α (2,120 Miller units) (taken from Ph.D. thesis of David Chismon, 2010).

5.2 Studies of NarL positive control mutants

NarL is composed of 2 domains, the N-terminal domain (NTD) and the GerE-family C-terminal domain (CTD). The CTD of NarL is a DNA binding domain that contains a tetrahelical helix-turn-helix motif. Phosphorylation of NarL by either NarX or NarQ in response to nitrate and nitrite triggers conformational change and domain separation. This allows the CTD to bind to the DNA target region and regulate gene expression (Galperin, 2006). This work is aimed to identify amino acid residues of NarL that are involved in positive control (PC) mutations. Mutations at these residues have no effect on DNA binding but the mutants lose their abilities to function as transcription activators. Alanine substitutions of NarL at residues corresponding to PC substitutions for TraR, an alternative GerE-family regulatory protein, have been examined by Lin and Stewart (2010) with the *narG*, *fdnG*, *napF* and *yeaR* operon control regions (Lin and Stewart, 2010; Qin *et al.*, 2009; White and Winans, 2005). Experiments focusing on some of these residues were designed to determine the effects of these PC NarL substitutions on transcription at the *ogt p*romoter.

The pRW50 plasmids containing the ogt1041 promoter (I↑ II ↓) were introduced into cells strain JCB3884 (L^-P^-) that carried pLG339 encoding NarXL with a single alanine substitution at residues R178, R179 and D181. Cells were grown anaerobically with or without 20 mM nitrate for beta-galactosidase assays. Results illustrated in Figure 5.4 show that activities of the ogt1041 promoter, induced by NarL carrying alanine substitutions at residues 178, 179 or 181, are decreased ~65%, ~35%, ~35% respectively in comparison to the promoter activity induced by the wild-type NarL. A great loss of induction is found in cells that carry NarL with an alanine substitution at residue 178.

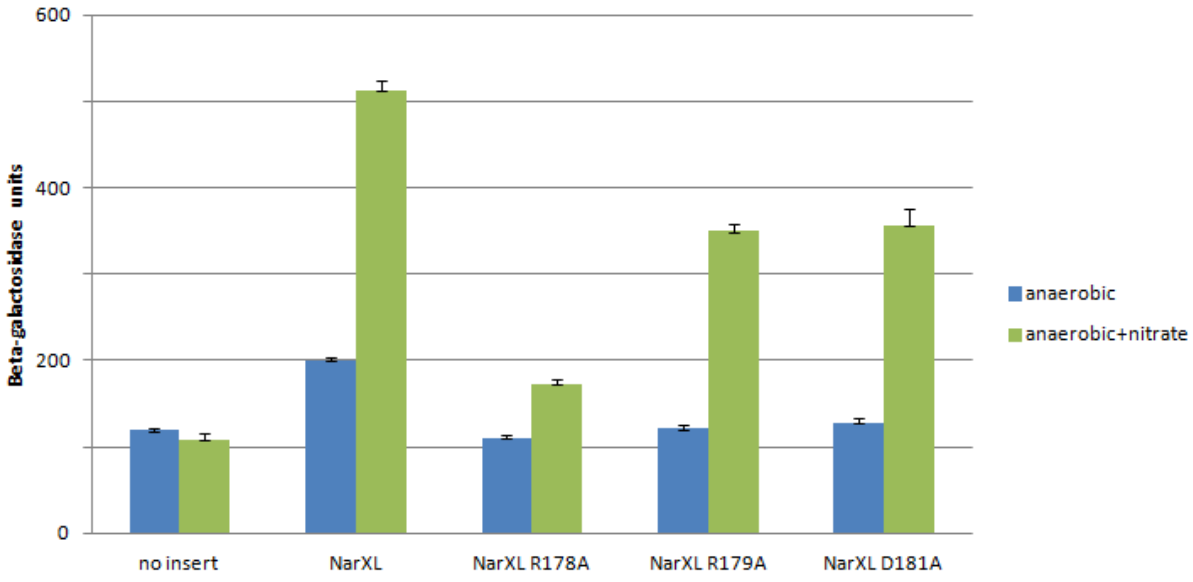


Figure 5.4 Effects of alanine substitutions at different residues of NarL on the ogt1041 promoter (I↑II↓)

Beta-galactosidase activities were measured in JCB3884 (L^-P^-) that contained pRW50 carrying the ogt1041 promoter and pLG339 encoding NarXL with a single alanine substitution at residues 178, 179 and 181. Cells were grown in minimal salts medium anaerobically with or without 20 mM nitrate.

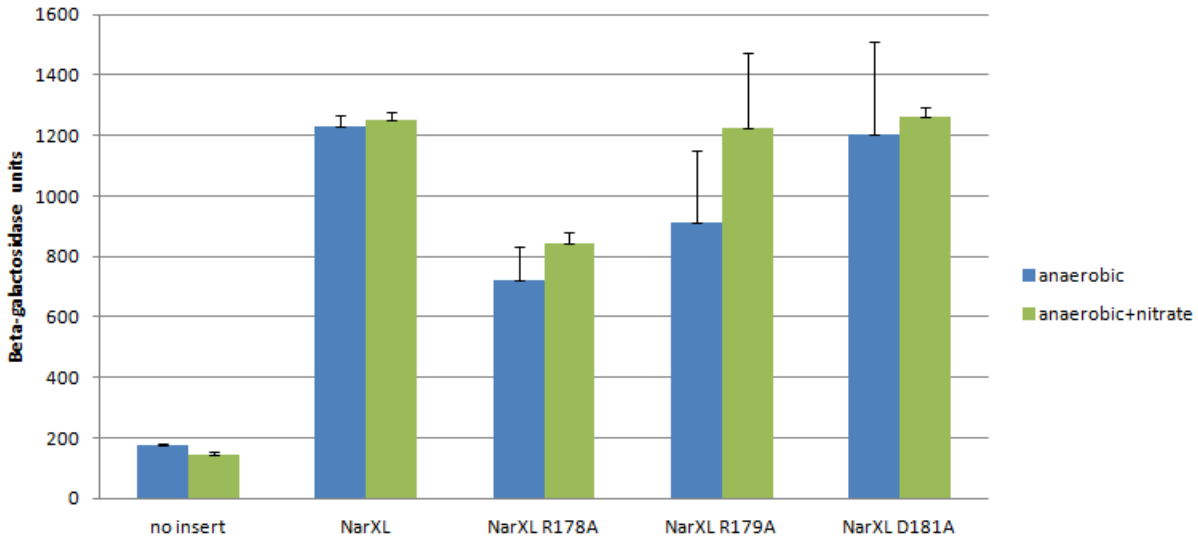


Figure 5.5 Effects of alanine substitutions at different residues of NarL on the ogt1052 promoter (I↓II↑)

Beta-galactosidase activities were measured in JCB3884 (L^-P^-) that contained pRW50 carrying the ogt1052 promoter and pLG339 encoding NarXL with a single alanine substitution at residues 178, 179 or 181. Cells were grown in minimal salts medium anaerobically with or without 20 mM nitrate.

The experiment was repeated to examine activity of the ogt1052 promoter (I ↓ II ↑) in cells that contain pLG339 encoding NarXL with a single alanine substitution at residues R178, R179 and D181. Results illustrated in Figure 5.5 show that the ogt1052 promoter activity is decreased when an alanine substitution is introduced to residue 178.

Alanine substitution at the surface-exposed R178 residue of NarL has the strongest effect on both the ogt1041 promoter and the ogt1052 promoters. There is a possibility that it is an important residue that interacts with αCTD of RNA polymerase (Lin and Stewart, 2010). Further experiments were designed to examine if NarL with alanine substitution at R178 still has a near-normal binding efficiency.

The *ynfE* gene encodes a protein paralogous to the dimethyl sulfoxide reductase (Lubitz and Weiner, 2003). Recent studies have revealed that the *ynfE* expression is repressed in anaerobic conditions with a presence of nitrate. Phosphorylated NarL binds to the target DNA site that overlaps the -10 element of the *ynfE* promoter (Browning *et al.*, 2002; Constantinidou *et al.*, 2006; Kang *et al.*, 2005; Xu *et al.*, 2009). Results illustrated in Figure 5.6 show that NarL with an alanine substitution at R178 strongly represses the *ynfE* expression. The *ynfE* promoter activity in cells containing NarL with R178A is identical to that in cells containing wild-type NarL. This indicates that residue 178 of NarL is involved in transcription activation but does not function in DNA binding.

Results from Chapter 4 show that the CTD of NarL, alone without the NTD, is able to induce the expression of the ogt1052 promoter::*lacZ* fusion. An alanine substitution was introduced to residue 178 of NarL-CTD. Beta-galactosidase activities were measured in cells carrying the ogt1052 promoter::*lacZ* fusion and pJW15 encoding either wild-type NarL-CTD or NarL-CTD with R178A. Results illustrated in Figure 5.7 show that the ogt1052 promoter

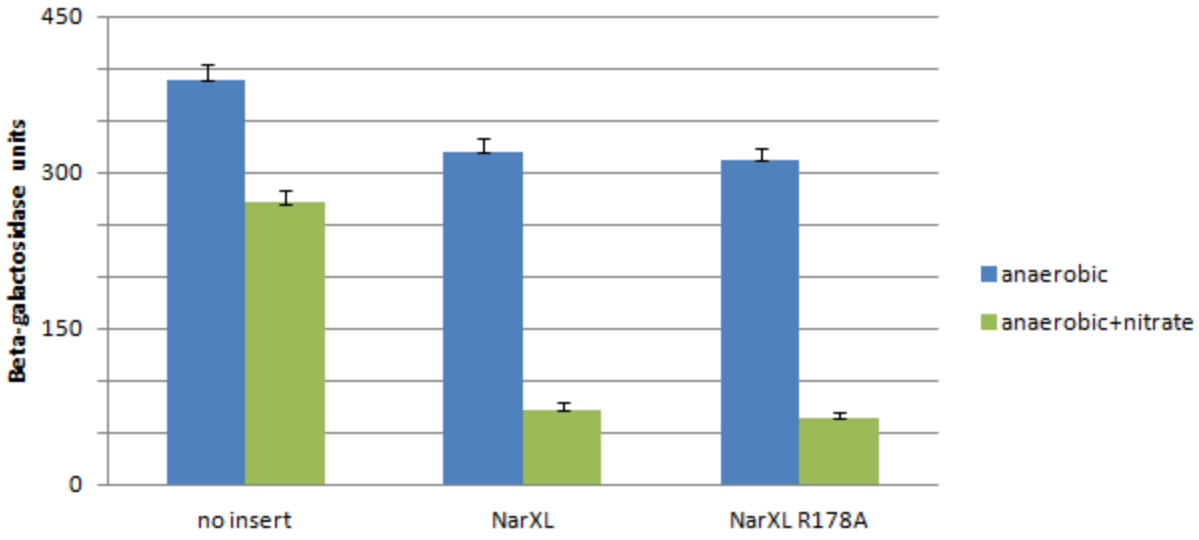


Figure 5.6 Activities of the *ynfE* promoter repressed by NarL

Beta-galactosidase activities were measured in JCB3884 (L^-P^-) carrying the *ynfE* promoter::*lacZ* fusion and pLG339 encoding NarXL with or without an alanine substitution at residue 178. Cells were grown in minimal salts medium anaerobically with or without 20 mM nitrate.

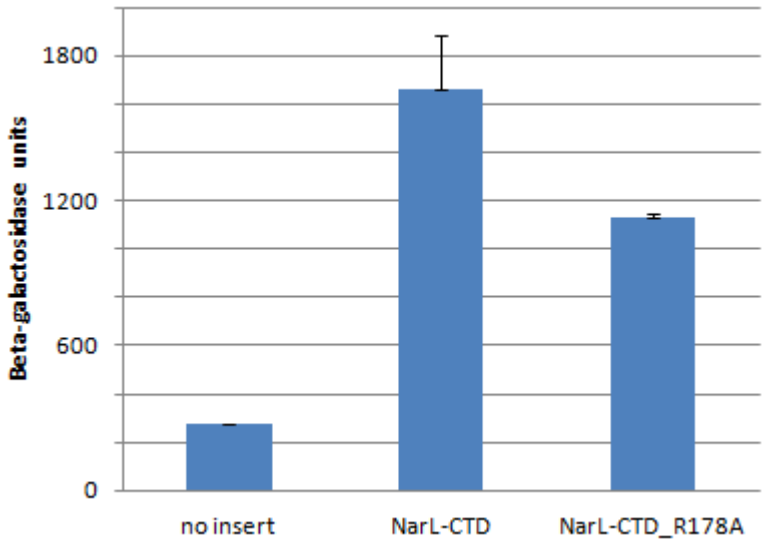


Figure 5.7 Effect of alanine substitution at residue 178 of NarL-CTD on the ogt1052 promoter activity (I↓II↑)

Beta-galactosidase activities were measured in JCB3884 (L^-P^-) carrying the ogt1052 promoter::*lacZ* fusion and pJW15 encoding NarL-CTD with or without an alanine substitution at residue 178. Cells were grown in minimal salts medium anaerobically.

activity, induced by NarL-CTD with R178A, is ~65% of that induced by the wild-type NarL-CTD. Results illustrated in Figure 5.8 show that the expression of the *ynfE* gene, repressed by NarL-CTD with R178A, is comparable to when it is repressed by the wild-type NarL-CTD. Hence, this indicates that residue 178 does not function in DNA binding but is involved in transcription activation of the ogt1052 promoter.

5.3 Epistasis experiment

From an alanine scanning mutagenesis of αCTD (Section 5.1), substitution at residue 273 has the largest effect on activities of all of the ogt1041 (I ↑ II ↓), the ogt1052 (I ↓ II ↑) and the ogt100 (I:wt II:wt) promoters. The NarL positive control mutant experiment (Section 5.2) indicates that alanine substitution at residue 178 of NarL affects activities of both the ogt1041 promoter and the ogt1052 promoters. Hence, there is a possibility that residue 273 of α subunit makes a contact with residue 178 of NarL regulator in order to recruit RNA polymerase to the *ogt* promoter.

Epistasis experiments were designed to examine a NarL-αCTD interaction at the ogt1052 promoter. The pLG339 plasmids encoding wild-type NarXL, NarXL-R178A (alanine substitution is introduced to R178 of NarL) or NarXL-R179A (alanine substitution is introduced to R179 of NarL) were introduced into JCB3884 (L^-P^-) that contained the ogt1052 promoter::*lacZ* fusion and pREII (encodes wild-type α) or pREII-273 (encodes α derivative with an alanine substitution at residue 273). Cells were grown in minimal salts medium anaerobically with or without 20 mM nitrate. Beta-galactosidase activities were measured after reaching the mid-exponential growth phase.

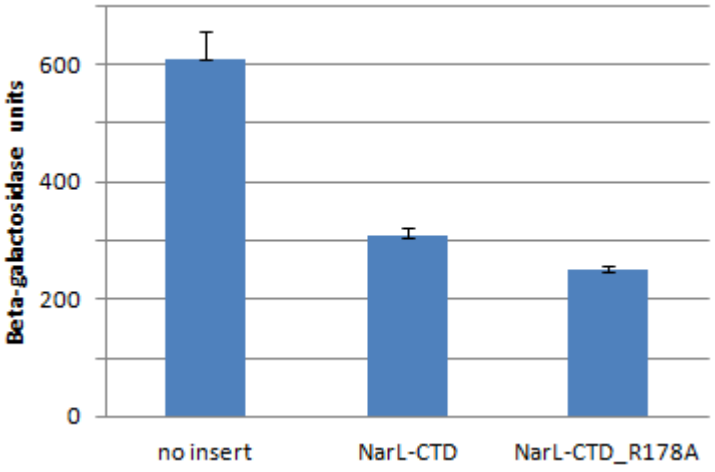


Figure 5.8 Activity of the *ynfE* promoter repressed by NarL-CTD

Beta-galactosidase activities were measured in JCB3884 (L^-P^-) carrying the *ynfE* promoter::*lacZ* fusion and pDW15 encoding NarL-CTD with or without an alanine substitution at residue 178. Cells were grown in minimal salts medium anaerobically.

Results illustrated in Figure 5.9 show that the beta-galactosidase activity is decreased (~60% of the wild-type value) in cells containing either pREII-273 or NarL-R178A, compared to the wild-type activity. The activity measured in cells containing NarL-R178A together with pREII-273 is similar to that measured in cells containing NarL-R178A together with pREII, and in cells containing NarXL together with pREII-273. This suggests that residue 178 of NarL makes a direct contact with residue 273 of the αCTD. Therefore, residue 178 of narL is epistatic to residue 273 of α subunit of RNA polymerase. Alanine substitution at residue 179 of NarL has no effect on the ogt1052 promoter activity, thus residue 179 of NarL is not required for transcription activation.

Strain JCB3884 (L^-P^-) carrying the ogt1052 promoter::*lacZ* fusion and pLG339 encoding wild-type NarXL, NarXL-R178A or NarXL-R179A was introduced with either pHTfI (encodes wild-type α) or pHTfI-261 (encodes α derivative with an alanine substitution at residue 261). Cells were grown in minimal salts medium anaerobically with 20 mM nitrate for beta-galactosidase assays. Residues 261 and 273 are located on the opposite face of the αCTD. Results illustrated in Figure 5.10 show that there is no effect of an alanine substitution at residue 261 of α subunit both in cells carrying wild-type NarL and in cells carrying NarL-R179A. The expression levels, in cells carrying pHTfI-261 and in cells carrying pHTfI, are identical. An alanine substitution at residue 178 of NarL results in decreasing the promoter activities (~60% of the wild-type value) both in cells containing pHTfI and in cells containing pHTfI-261. These suggest that residue 261 does not interact with NarL, and that residue 178 of NarL, but not residue 179, is involved in transcription activation.

The same experiment was repeated with the ogt1052_3+4- promoter that contains a single upstream half of the NarL site located at position -45.5. Results from Chapter 4 indicated that NarL still binds as a dimer to the ogt1052_3+4- promoter although the target

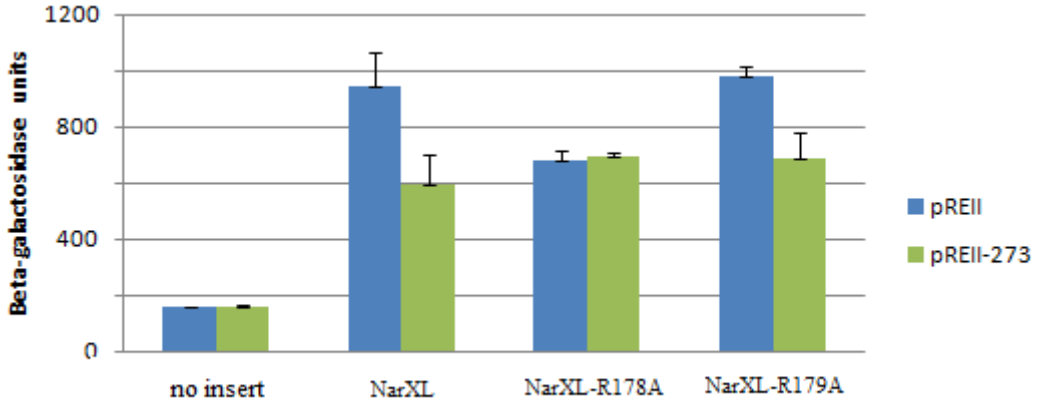


Figure 5.9 Activities of the ogt1052 promoter in cells containing wild-type/derivatives of NarL and pREII

Beta-galactosidase activities were measured in JCB3884 (L^-P^-) containing the ogt1052 promoter::*lacZ* fusion and pLG339 encoding wild-type NarXL, NarXL-R178A or NarXL-R179A together with pREII (encodes wild-type α) or pREII-273 (encodes α derivative with an alanine substitution at residue 273). Cells were grown in minimal salts medium anaerobically with 20mM nitrate.

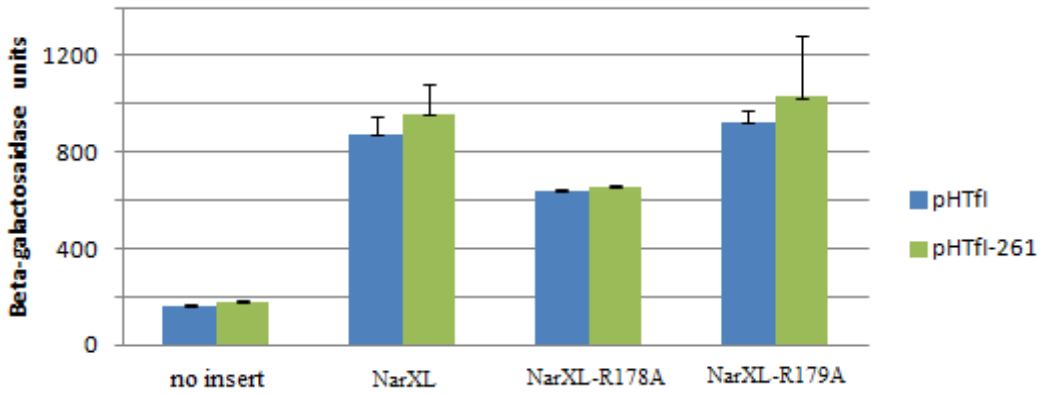


Figure 5.10 Activities of the ogt1052 promoter in cells containing wild-type/derivatives of NarL and pHTfI

Beta-galactosidase activities were measured in JCB3884 (L^-P^-) containing the ogt1052 promoter::*lacZ* fusion and pLG339 encoding wild-type NarXL, NarXL-R178A or NarXL-R179A together with pHTfI (encodes wild-type α) or pHTfI-261 (encodes α derivative with an alanine substitution at residue 261). Cells were grown in minimal salts medium anaerobically with 20mM nitrate.

site has a low binding affinity for a NarL dimer. The pLG339 encoding wild-type NarXL, NarXL-R178A and NarXL-R179A were introduced into JCB3884 (L^-P^-) containing the ogt1052_3+4- promoter::*lacZ* fusion. Then, cells were transformed with either pREII or pREII-273 and grown in minimal salts medium anaerobically with 20 mM nitrate for beta-galactosidase assays.

Results illustrated in Figure 5.11 show that the ogt1052_3+4- promoter activity is decreased (~50% of the wild-type activity) in cells that contain either pREII-273 or NarL-R178A. The promoter activity measured in cells containing NarL-R178A together with pREII is similar to that measured in cells containing NarL together with pREII-273, as well as in cells carrying NarL-178A and pREII-273. This suggests that residue 178 of NarL interacts with residue 273 of α subunit of RNA polymerase. An alanine substitution at residue 179 of NarL results in ~50% decrease of the promoter activity, suggesting that residue 179 of NarL is also involved in the ogt1052_3+4- promoter activity.

Strain JCB3884 that carry the ogt1052_3+4- promoter::*lacZ* fusion and pLG339 encoding wild-type NarXL, NarXL-R178A or NarX-R179A were transformed with either pHTfI or pHTfI-261 for beta-galactosidase assays. Results illustrated in Figure 5.12 show that there is no effect of an alanine substitution at residue 261 of the α subunit both in cells carrying wild-type NarL and in cells carrying NarL-R179A. The expression levels, in cells carrying pHTfI in comparison to in cells carrying pHTfI-261, are identical.

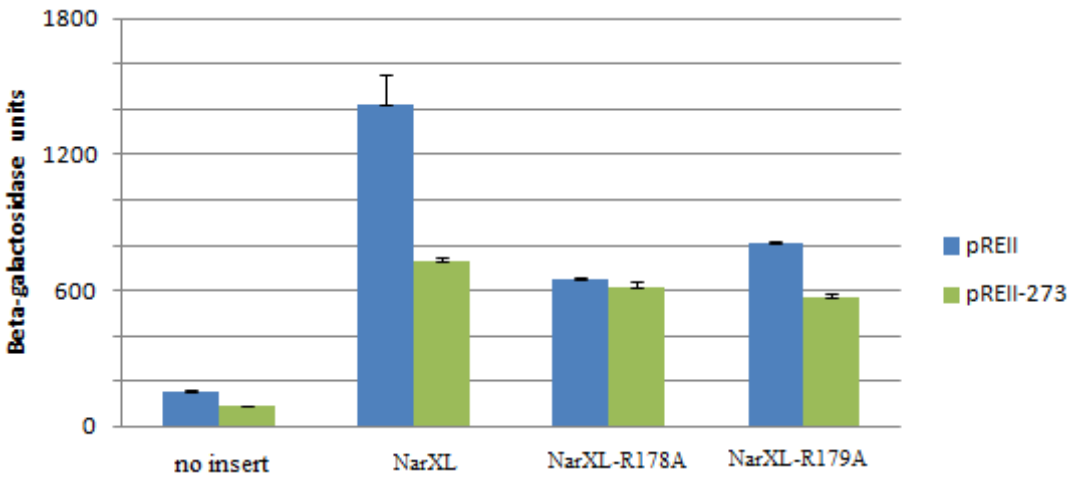


Figure 5.11 Activities of the ogt1052_3+4- promoter in cells containing wild-type/derivatives of NarL and pREII

Beta-galactosidase activities were measured in JCB3884 (L^-P^-) containing the ogt1052_3+4-promoter::*lacZ* fusion and pLG339 encoding wild-type NarXL, NarXL-R178A or NarXL-R179A together with pREII (encodes wild-type α) or pREII-273 (encodes α derivative with an alanine substitution at residue 273). Cells were grown in minimal salts medium anaerobically with 20mM nitrate.

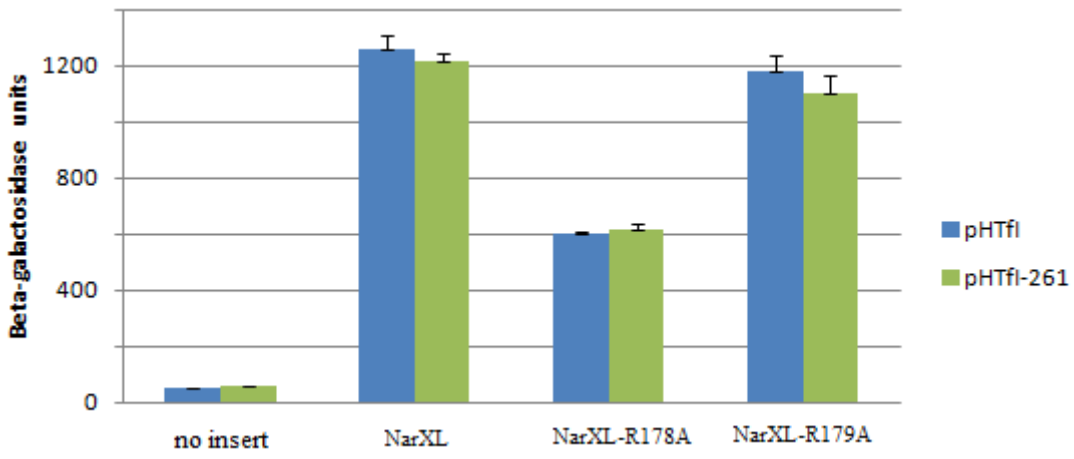


Figure 5.12 Activities of the ogt1052_3+4- promoter in cells containing wild-type/derivatives of NarL and pHTfI

Beta-galactosidase activities were measured in of JCB3884 (L^-P^-) containing the ogt1052_3+4- promoter::*lacZ* fusion and pLG339 encoding wild-type NarXL, NarXL-R178A or NarXL-R179A together with pHTfI (encodes wild-type α) or pHTfI-261 (encodes α derivative with an alanine substitution at residue 261). Cells were grown in minimal salts medium anaerobically with 20mM nitrate.

5.4 Charge inversion

Results from the epistasis experiments (Section 5.3) suggested that residue 178 of NarL may interact with residue 273 of the α subunit of RNA polymerase. Residue 178 of NarL is arginine (R) while residue 273 of α subunit is glutamate (E). These amino acids contain opposite charged groups. The positively charged side chain of R178 of NarL may be attracted to the negatively charged side chain of E273 of α subunit through a protein-protein ionic interaction. Hence, introducing an alanine substitution to either R178 or E273 might disrupt an attractive force that pulls two proteins to each other, resulting in decreasing the promoter activity. This work is aimed to investigate whether the ionic interaction between residue 178 of NarL and residue 273 of α subunit is required, using charge inversion experiments.

The pLG339 plasmids encoding NarXL-R178E (residue 178 of NarL was substituted by glutamate) was introduced into JCB3884 (L^-P^-) cells containing the ogt1052 promoter::*lacZ* fusion. Cells were grown anaerobically with 20 mM nitrate for beta-galactosidase assays. A negatively charged glutamate substitution was introduced to residue 178 of NarL to create a pushing force between two interacting residues that carry like-negatively charged side chains. Due to losing an attractive force between NarL and αCTD, it was expected that substitution by glutamate at residue 178 of NarL has a greater effect on the ogt1052 promoter activity than substitution by alanine.

Results illustrated in Figure 5.13 show that substitution of residue 178 of NarL by glutamate results in a greater effect on the ogt1052 promoter activity. The ogt1052 promoter activity, in cells carrying NarL-R178E, is only ~40% of the wild type level. To confirm that NarL interacts with αCTD via an attractive force between two opposite charged

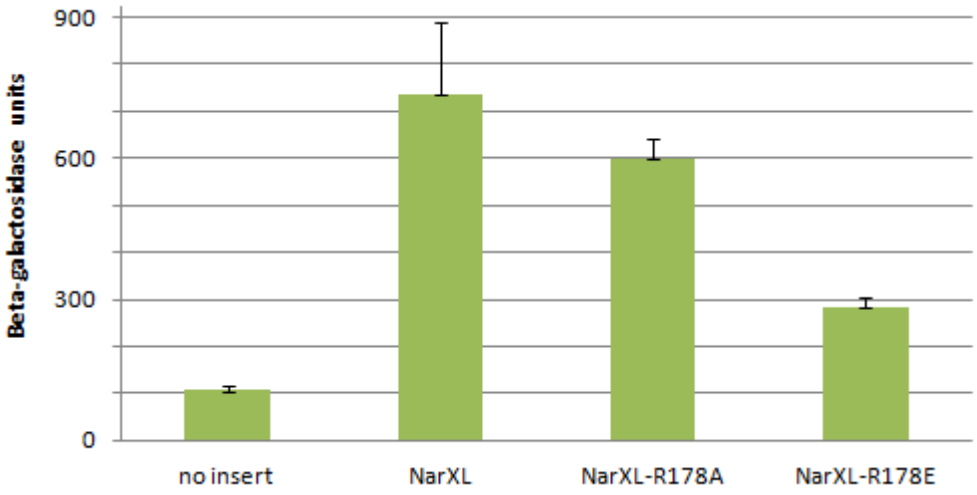


Figure 5.13 Activities of the ogt1052 promoter in cells carrying NarL with alanine and glutamate substitution at residue 178

Beta-galactosidase activities were measured in JCB3884 (L^-P^-) carrying the ogt1052 promoter::*lacZ* fusion and pLG339 encoding wild-type NarL or NarL derivatives with alanine or glutamate substitution at residue 178. Cells were grown in minimal salts medium anaerobically with 20 mM nitrate.

residues, charge inversions were introduced to both interacting residues. It was expected that charge inversion of both residues had no effect on the ogt1052 promoter activity as the attractive force between the opposite charges still remained.

Results illustrated in Figure 5.14 show that expression of the ogt1052 promote::*lacZ* fusion is decreased (~20% and ~35% of the wild-type level) in cells carrying either NarL-R178E or pREII-E273R (encodes α derivative with an arginine substitution at residue 273) respectively. It is expected that the ogt1052 promoter activity measured in cells containing both NarL-R178E and pREII-E273R is comparable to the activity measured in cells containing wild-type NarL and pREII (encodes wild-type α). The data show that NarL-R178E fails to induce the ogt1052 promoter activity. Hence, the charge inversion experiment does not agree with the hypothesis that a NarL-αCTD interaction is formed by an attractive force between two opposite charged residues. However, other factors might interrupt the ionic interaction when charge inversion was introduced to each of NarL and αCTD.

5.5 Mutation at -35 element of the *ogt* promoter

To start transcription, the holo-RNA polymerase binds to 4 specific DNA elements for promoter recognition including the -10 element, the extended -10 element, the -35 element and the UP element. The -10 and -35 elements are located around 10 and 35 base-pairs upstream the transcript start site, and recognized by domain 2 and 4 of σ^{70} (Campbell *et al.*,

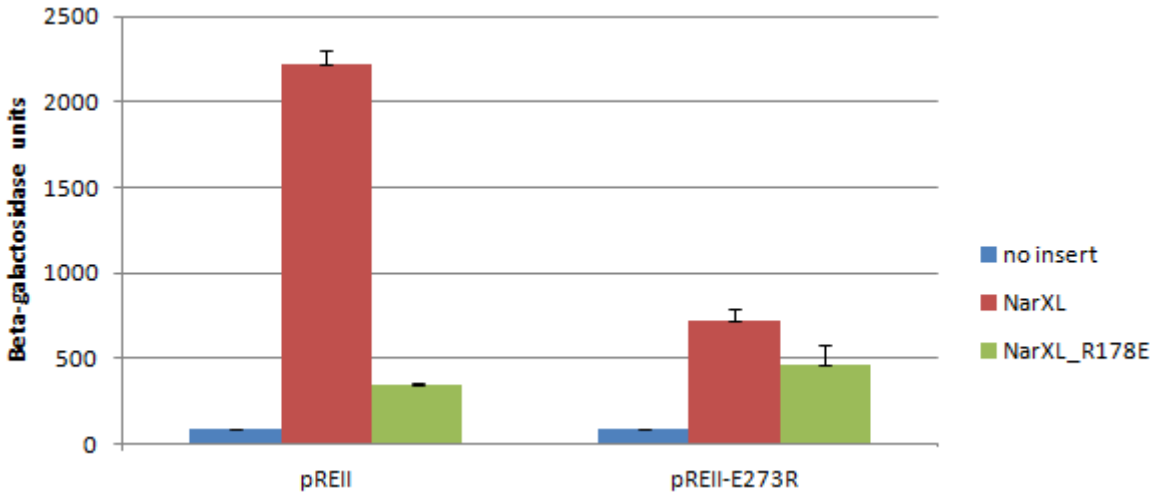


Figure 5.14 Activities of the ogt1052 promoter when ionic charges of interacting residues between NarL and α subunit are inverted

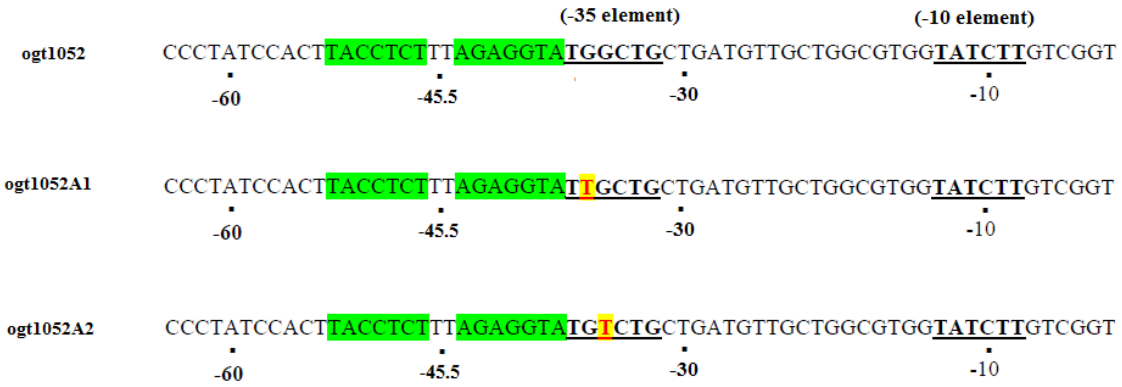
Beta-galactosidase activities were measured in JCB3884 (L^-P^-) containing the ogt1052 promoter::*lacZ* fusion and pLG339 encoding wild-type NarXL or NarXL-R178E (positively charged arginine at position 178 of NarL is inverted to negatively charged glutamate) together with pREII (encodes wild-type α) or pREII-E273R (negatively charged glutamate at position 273 of α is inverted to positively charged arginine). Cells were grown in minimal salts medium anaerobically with 20mM nitrate.

2002). The consensus sequences have been determined to be TATAAT and TTGACA for -10 and -35 element respectively. Results from Chapter 3 indicated that mutation at base 2 of the -10 element disrupts transcription of the *ogt* gene. Each of the ogt1041 promoter and the ogt1052 promoter is the same promoter as the wild-type *ogt* promoter as they use the same -10 element. The potential -35 element of the *ogt* promoter is located adjacent downstream the NarL II site, 18 bp upstream -10 element. This work is aimed to investigate an effect of a specific base change in the -35 element to identify its location in the *ogt* promoter region and to examine whether it is required for transcription activation.

This work focuses on the ogt1052 promoter, which the earlier studies in Chapter 3 showed that the promoter activity is high even in an absence of nitrate. Mutations were introduced to different bases in the -35 element of the ogt1052 promoter. Figure 5.15 panel A illustrates partial base sequences of the ogt1052A1 and the ogt1052A2 promoter fragments in comparison to the ogt1052 promoter. The ogt1052A1 promoter contains the near-consensus -35 element due to mutation at base 2 of the hexamer (G→T). The ogt1052A2 promoter has the -35 element less similar to the consensus due to a mutation at base 3 of the hexamer (G→T).

The pRW50 plasmids carrying the ogt1052A1 and the ogt1052A2 promoters were introduced into JCB387 (L+P+). Cells were grown in minimal salts medium anaerobically with or without 20 mM nitrate for beta-galactosidase assays. Results illustrated in Figure 5.15 panel B show that high induction was found in cells containing either the ogt1052 promoter::*lacZ* fusion or the ogt1052A1 promoter::*lacZ* fusion. The promoter activities, especially the ogt1052A1 promoter, are high even in the absence of nitrate. In contrast, there is a minimal induction in cells carrying the ogt1052A2 promoter (~30% and ~25% of the ogt1052 and the ogt1052A1 promoter activities, respectively).

A.



B.

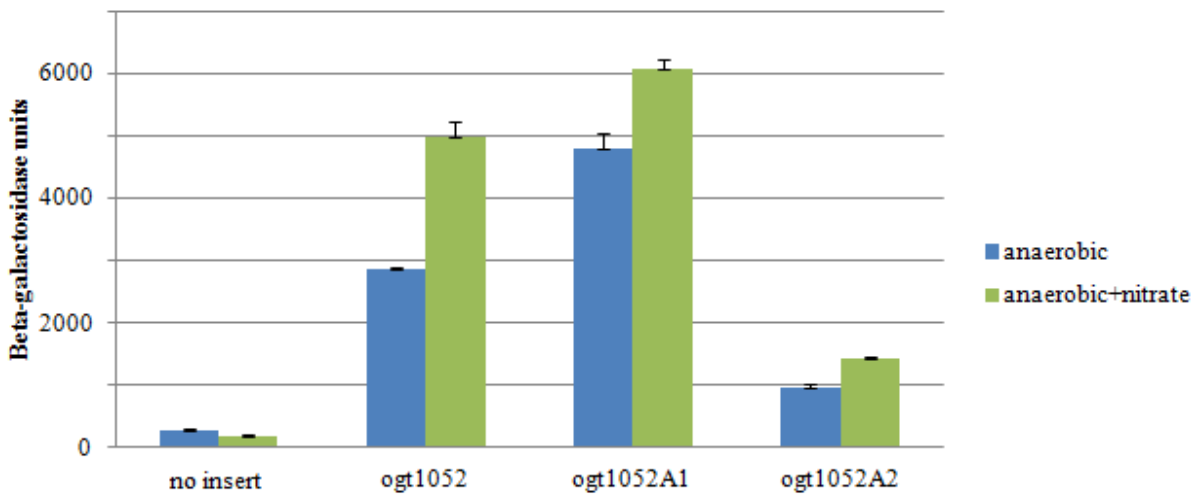


Figure 5.15 Activities of the *ogt* promoter containing mutation at -35 element (in JCB387)

Panel A shows partial base sequences of the ogt1052, ogt1052A1 and ogt1052A2 promoter fragments. The DNA sites for NarL are highlighted in green. The -35 element and -10 element are identified and underlined. The mutated bases are coloured red.

Panel B shows beta-galactosidase activities measured in JCB387 (L^+P^+) containing pRW50 carrying the ogt1052, ogt1052A1 or ogt1052A2 promoters. Cells were grown in minimal salts medium anaerobically with or without 20 mM nitrate. Beta-galactosidase activities were measured when cells reached the exponential phase.

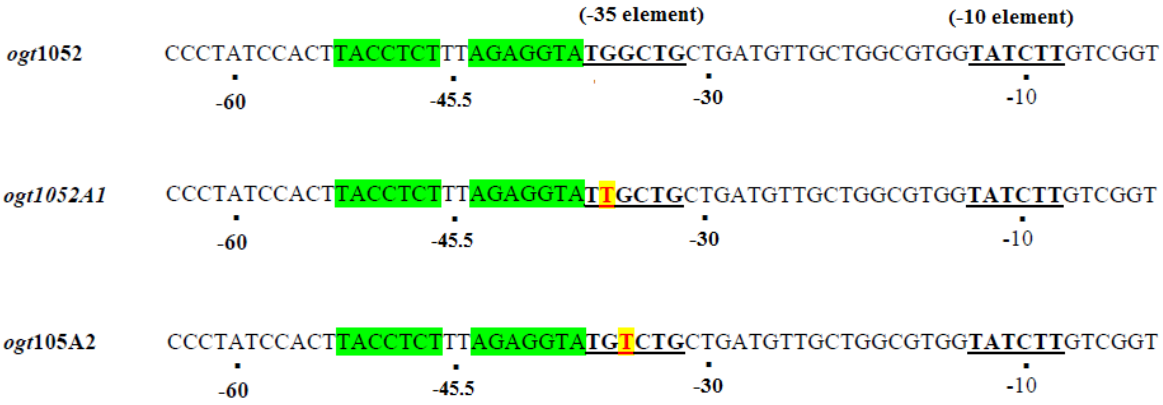
The experiment was repeated to measure beta-galactosidase activities in cells strain JCB3884 (L^-P^-) that contain pRW50 carrying the ogt1052, the ogt1052A1 or the ogt1052A2 promoters. Results illustrated in Figure 5.16 panel B show that no induction is found on both the ogt1052 promoter and the ogt1052A2 promoter. The ogt1052A1 promoter activity is ~6-fold higher, indicating that transcription still takes place. Strain JCB3884 lacks both NarL and NarP; hence, all observed promoter activities result from how well σ factor recognizes the DNA sequence of the hexamer that functions as the -35 element for promoter recognition.

5.6 Locations of NarL and αCTD at the *ogt* promoter

Data from experiments in Section 5.1-5.3 suggested that residue 178 of NarL interacts with residue 273 of αCTD. In addition, the results from Section 5.5 indicated that the -35 element, which is the DNA site for sigma region 4, is required for transcription of the *ogt* gene. To identify the locations of NarL and αCTD of RNA polymerase on the DNA helix, this work focuses on the ogt1052A1 promoter that contains a single near-consensus NarL site located at position -45.5, and also contains the improved -35 element, resulting in a high binding affinity for NarL and domain 4 of sigma factor.

The location of the NarL site in the ogt1052A1 promoter region was identified in vitro by DNase I footprinting. The ogt1052A1 fragment was end-labelled with ^{32}P-ATP and incubated with different concentrations of phosphorylated NarL then treated with DNase I to digest DNA at unprotected sites. Figure 5.17 shows the result of DNase I footprinting calibrated by the G+A sequencing reaction of radio-labelled ogt1052A1 fragment. A protected region, corresponding to the NarL binding site at position -45.5, is found when

A.



B.

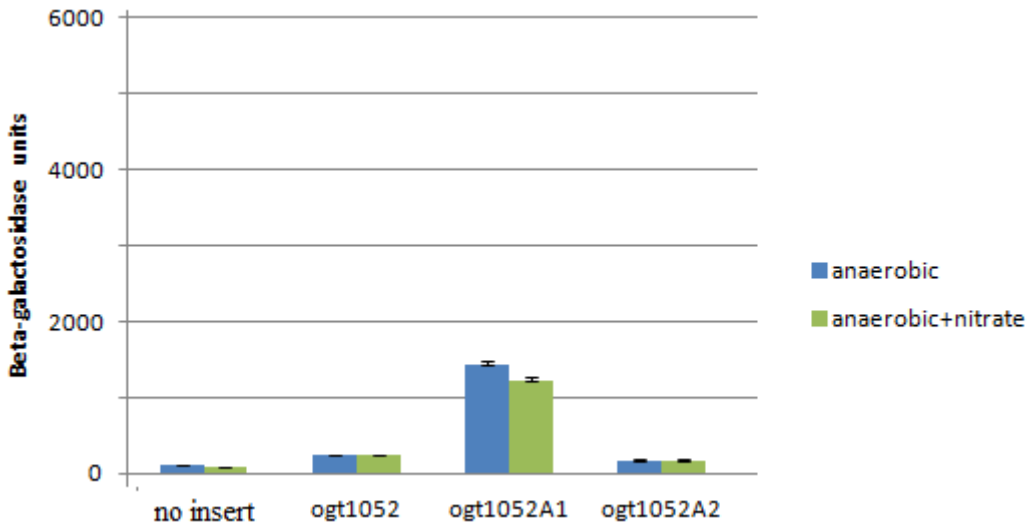


Figure 5.16 Activities of the *ogt* promoter containing mutations at the -35 element (in JCB3884)

Panel A shows partial base sequences of the ogt1052, ogt1052A1 and ogt1052A2 promoter fragments. DNA sites for NarL are highlighted in green. The -35 element and -10 element are identified and underlined. The mutated bases are coloured red.

Panel B shows beta-galactosidase activities measured in JCB3884 (L^-P^-) containing pRW50 carrying the ogt1052, ogt1052A1 or ogt1052A2 promoters. Cells were grown in minimal salts medium anaerobically with or without 20 mM nitrate. Beta-galactosidase activities were measured when cells were in the exponential phase.

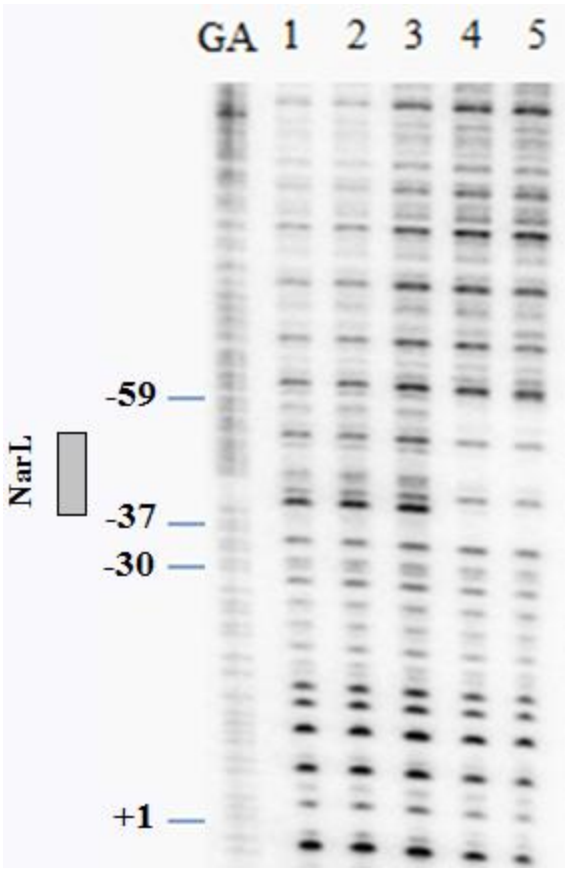


Figure 5.17 The DNA site for NarL at the ogt1052A1 promoter

The binding site of NarL at the ogt1052A1 promoter was identified by the DNase I footprinting experiment. Different concentrations of pre-phosphorylated NarL were incubated with the end-labelled ogt1052A1 promoter fragments then treated with DNase I. The digested fragments were calibrated using a G+A sequencing reaction. Lane 1: no protein, lane 2: 0.4 µM, lane 3: 0.8 µM, lane 4: 1.6 µM, lane 5: 3.2 µM. Location of the DNA site for NarL is indicated by a grey box.

incubated with 1.6 μM NarL.

Figure 5.18 shows the result of a DNase I footprint experiment of holo-RNA polymerase and phospho-NarL binding to the radio-labelled ogt1052A1 promoter fragment. In an absence of protein (lane 1), no protection region is found. The binding locations of NarL and RNA polymerase are shown in lane 2 and lane 3 respectively. Lanes 4-6 show protection regions when the ogt1052A1 fragments were incubated with 1.6 μM NarL and increasing concentrations of RNA polymerase. The results confirm that RNA polymerase binds to the ogt1052A1 promoter, with the αCTD located at the same DNA site as for NarL.

Locations of the αCTD binding sites were identified using a chemical nuclease. In this experiment, iron [S]-1-[p-bromoacetamidobenzyl] (FeBABE) was used as a DNA cleavage reagent. RNA polymerase was modified by conjugating FeBABE to residue 273 or 302. The locations of these residues are shown in a structural model of αCTD-DNA complex illustrated in Figure 5.19 panel A. The αCTD interacts with DNA through determinant 265 (coloured pink). Determinant 261 (coloured yellow) points downstream to sigma region 4 while determinant 287 (coloured green) points upstream. Residues 273 and 302 are coloured blue and red respectively, positioning on opposite sides of the αCTD (Lee *et al.,* 2003).

The ogt1052A1 promoter fragment was used in the FeBABE footprinting experiment in order to identify the location of αCTD that binds to the *ogt* promoter, independently and dependently of NarL. FeBABE, conjugated with α subunits of holo-RNA polymerase, was triggered to cleave DNA during an open promoter complex formation. A pattern of DNA cleavage is used to identify the location where αCTD binds to DNA.

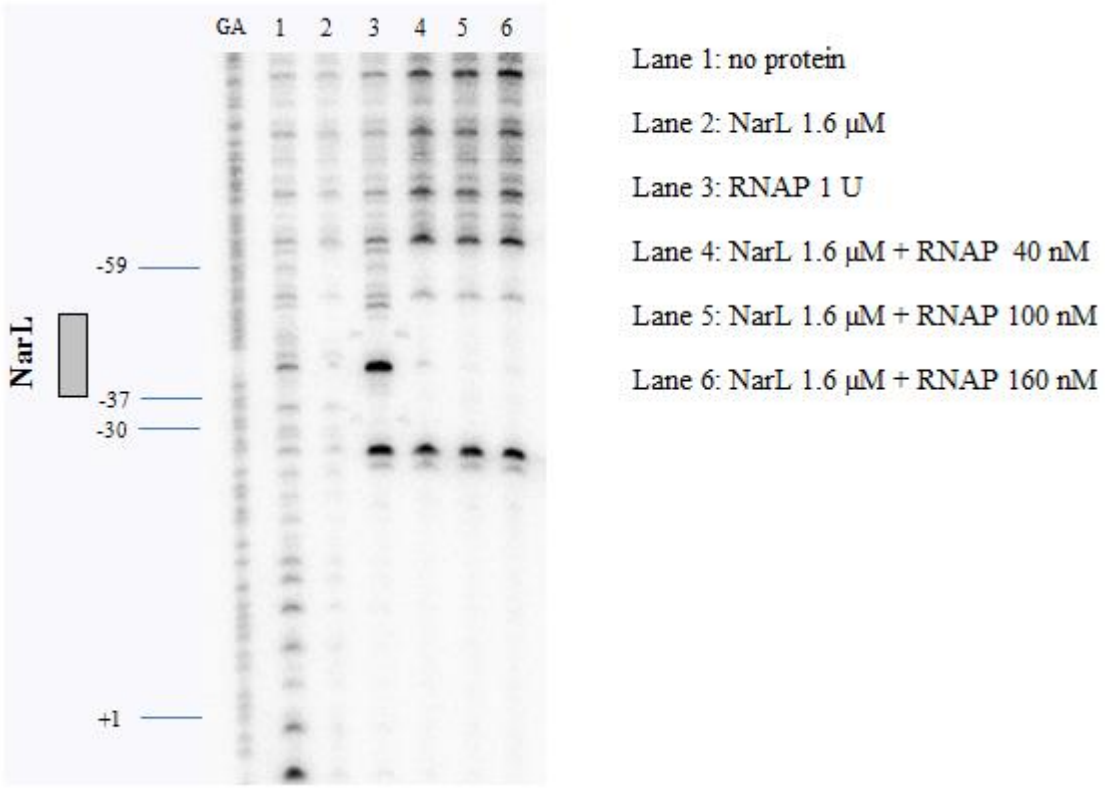
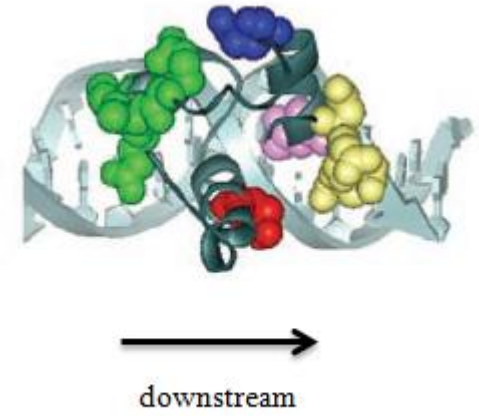


Figure 5.18 Locations of NarL and RNA polymerase at the ogt1052A1 promoter

The binding sites of NarL and RNA polymerase at the ogt1052A1 promoter are identified in the DNase I footprinting experiment. 1.6 µM pre-phosphorylated NarL and different concentrations of holo-RNA polymerase were incubated with the end-labelled ogt1052A1 fragments and treated with DNase I. The digested fragments were calibrated using a G+A sequencing reaction. Description of protein concentrations for each lane is shown alongside the figure.

A.



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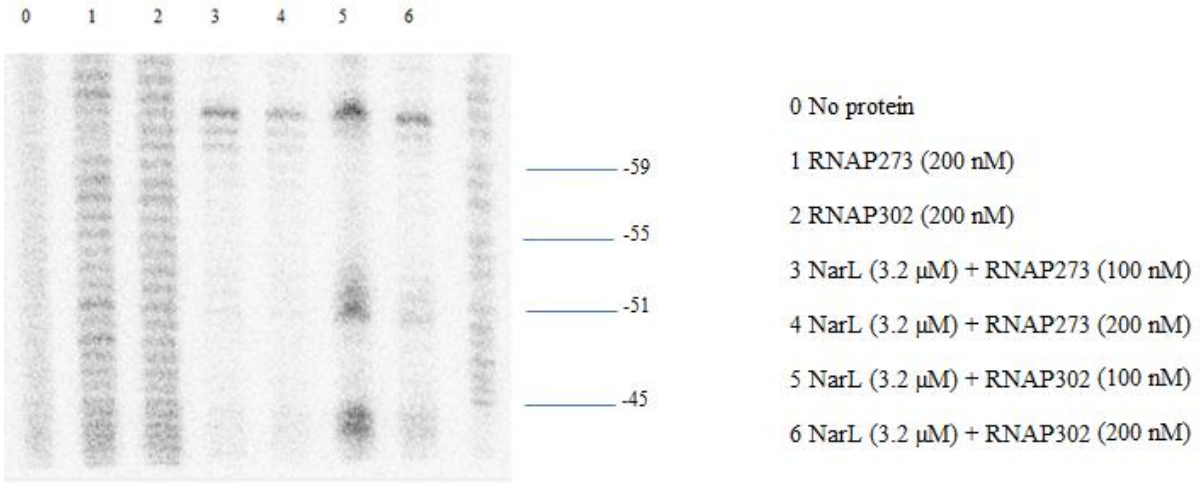


Figure 5.19 Location of FeBABE-tagged αCTD at the ogt1052A1 promoter

Panel A shows a model of αCTD-DNA complex. An arrow points to the downstream direction of DNA. Residues of determinant 261, 265, 273, 287 and 302 are coloured in yellow, pink, blue, green and red respectively.

Panel B shows the DNA cleavage patterns resulting from FeBABE that is attached on α subunits of RNA polymerase holo-enzyme. A description of each lane is shown alongside the figure. The G+A Maxam-Gilbert sequence ladder was used to calibrate cleaved positions.

Results illustrated in Figure 5.19 panel B show phosphorimager scans of DNA cleavage patterns due to binding of FeBABE-tagged RNA polymerase to the ogt1052A1 promoter. Lane 0: the control experiment (no protein). Lane 1 and 2 contain 200 nM of RNA polymerase carrying FeBABE at position 273 and 302 respectively. In the absence of NarL to fix the location of the αCTD, the clusters of DNA cleavage are not clearly detected. Lane 3 and 4 contain NarL and RNA polymerase with FeBABE tagged at position 273. None of the DNA cleavage is found. The previous results suggested that the αCTD interacts with NarL via residue 273. Conjugation of FeBABE to this residue may disrupt the protein-protein contact that recruits αCTD to DNA. Also, binding of NarL at the ogt1052 promoter may suppress DNA cleavage by FeBABE tagged to residue 273 of α subunit.

Lane 5 and 6 contain NarL and RNA polymerase with FeBABE tagged at position 302. The figure shows clusters separated by ~10 bp, indicating that two αCTDs position on the same face of DNA helix. The strongest DNA cleavage is found around position -42. This suggests that αCTD binds immediately upstream the -35 element, and the DNA site for αCTD overlaps with the NarL site centered at position -45.5. Hence, αCTD and NarL are located on the same DNA region but different faces of DNA helix. Also, this is only a possible orientation that allows residue 273 of αCTD to interact with residue 178 of NarL. This interaction can occur if they are located on the opposite faces of DNA helix from each other. A structural model of the *ogt* transcript initiation complex will be discussed further in the next section.

Discussion

Important residues of αCTD for NarL-dependent activation of the ogt promoter

The ogt100 promoter contains two crucial DNA sites for NarL located at position -78.5 and -45.5 relative to the transcript start site. This promoter organization possibly allows NarL form multiple contacts with RNA polymerase. Results represented in this chapter indicated that NarL is able to activate the *ogt* promoter containing a single NarL binding site if the site better resembles the consensus sequence. NarL activates the ogt1041 promoter by binding to the NarL site centered at position -78.5 where NarL can interact with the downstream αCTD and recruit RNA polymerase to the right position. The ogt1052 promoter is activated by NarL that binds to the NarL site centered at position -45.5 adjacent upstream the -35 element.

David Chismon (the University of Birmingham, 2010) studied NarL-dependent activation of the *ogt* promoter derivatives in cells carrying RNA polymerase with a single alanine substitution introduced to the αCTD. It was reported that the majority of important αCTD residues for activation of the ogt1041 promoter and the ogt1052 promoter are broadly similar. Some surface exposed residues of αCTD are possibly involved in protein-protein contacts. They form a cluster outside the αCTD; hence, NarL and αCTD may interact with each other via multiple distinct contacts such as those found in CRP-dependent promoters (Savery e*t al.*, 1998; Savery e*t al.*, 2002). Another possibility is that NarL makes a single extensive contact with the αCTD. Alanine substitution at residue 273 of αCTD highly impairs NarL induction of all the ogt100, ogt1041 and ogt1052 promoters. Furthermore, some of the mutated residues that improve transcription activation fall into the DNA binding region. This suggests that disruption of the αCTD-DNA interaction may help NarL to interact better and recruit αCTD to its optimal DNA site (Gourse and Gaal, 2000).

Data from αCTD alanine scan libraries and NarL positive control mutant experiments were used to identify potential residues of interaction between NarL and αCTD. To classify the mechanism of activation by NarL at the *ogt* promoter, DNase I footprinting and FeBABE footprinting experiments were used to confirm locations of NarL and αCTD on the DNA helical face. This will lead to more understanding of the *ogt* promoter architecture and to construct the protein-DNA structural model of the *ogt* promoter.

NarL-αCTD interaction

Based on the structure of NarL (Maris *et al.,* 2002), the NarL-CTD dimer in a complex with DNA at the *nirB* operon control region has been revealed by Lin and Stewart (2010) as illustrated in Figure 5.20. The NarL-TraR alignment was used for implication of residues of NarL chosen to be substituted by alanine in order to identify their functions. An activation region is formed by a group of residues located on one face of NarL. Alanine substitutions at these residues result in decreased transcriptional activation, but the mutants still maintain DNA binding efficiency (Rhodius et al., 1997).

Site-specific mutagenesis was used to identify surface exposed residues of NarL that interact with the αCTD of RNA polymerase. It was found that mutants R178A, R179A and D181A are denoted as positive control for the ogt1041 promoter. For the ogt1052 promoter, only R178A was denoted as a positive control mutant. Residues 178, 179 and 181 are located around the scaffold helix at positions where they can interact with αCTD.

Interactions between transcription factors and RNA polymerase are altered by how well the factor binds to the operator sequence. Therefore, a binding affinity for an operator

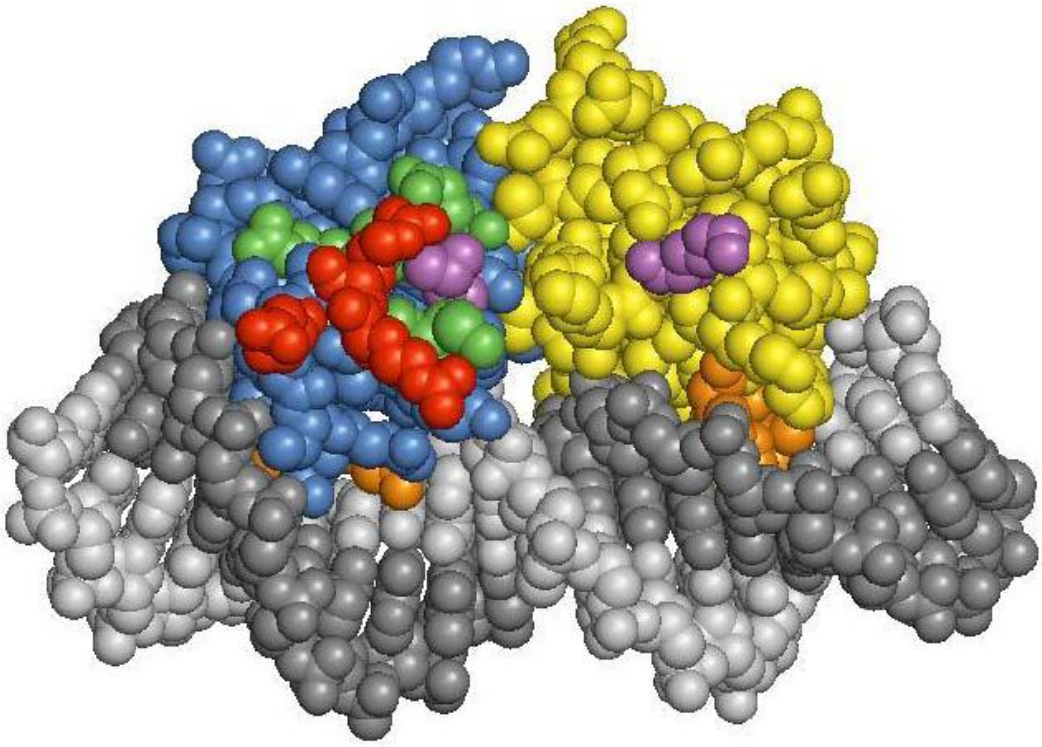


Figure 5.20 Important residues of NarL-CTD for transcription activation

The model shows a complex of NarL-CTD attached to DNA at the *nirB* operon control region. NarL protomers are coloured blue and gold. DNA strands are coloured light and dark grey. Red-highlighted residues represent PC phenotype for mutants (Arg-178, Arg-179, Asp-181). Residues Asp-162, Leu-166, Met-175 are coloured green for functional. Residues Leu-171 and Lys-199 are coloured magenta for deficient. Residues that function in DNA binding are coloured orange (Lys-188, Val-189, Lys-192) (figure is taken from Lin and Stewart, 2010).

also has an effect on promoter escape and gene expression (Garcia *et al.*, 2012). This agrees with results showing that an alanine substitution of NarL highly affects the *ogt* promoter activity when the promoter contains low-affinity site for NarL. The ogt1052_3+4- promoter has the weaker-affinity binding site for a NarL dimer, compared to the ogt1052 promoter. Hence, the ogt1052_3+4- promoter activity, induced by NarL–R178A, is lower.

Epistasis experiment

Alanine substitution at residue 273 of the RNA polymerase α subunit impairs NarL induction of all the ogt100, ogt1041 and ogt1052 promoters. Results from the NarL positive control mutant experiments indicated that the *ogt* promoter activities are low when an alanine substitution is introduced at residue 178. Confirmed by the epistasis experiments, the phenotype (expression of the ogt1052 promoter::*lacZ* fusion) due to a single alanine substitution at either residue 178 of NarL or residue 273 of αCTD is similar to that resulting from a pair of substitutions. Hence, residue R178 of NarL interacts with residue E273 of αCTD. The control experiment showed that mutants NarL-179 and αCTD-261 cause no effect on the phenotype, indicating that they are not involved in transcription activation.

A multiple sequence (from Molecular Modeling Database; MMDB) alignment of 65 proteins that carry GerE conserved domains, in complexes with DNA, was performed using MAFFT alignment program. There are 11 proteins including NarL that contain a positively charged, polar amino acid at the position corresponding to R178 of NarL. The charge inversion experiments argued that there is no induction in cells containing both NarL-R178E and pREII-E273R, thus a NarL-αCTD interaction does not result from an attraction force between two opposite charged residues. However, this may be due to effects of surrounding residues within the determinant. For example, the positively charged R178 is inverted to the

negatively charged glutamate, which is then attracted to the neighbor-positively charged R179. This action may interrupt an interaction between NarL and αCTD even though an attraction force between the two residues is still remained.

A significant phenotypic effect is found when the ogt1052_3+4- promoter is used in the epistasis experiment. It contains only a single upstream half of the NarL II site, resulting in less binding affinity for NarL. Interestingly, residue 179 of NarL also plays a role in transcription activation of the ogt1052_3+4- promoter. It is a neighbor-positively charged residue of R178. Mutant R179A may affect an orientation of residue 178, as well as its interaction with the αCTD. This also agrees to the earlier discussion that factor-RNA polymerase contacts are altered by how well the transcription factor binds to the operator sequence (Garcia *et al.*, 2012).

Orientation of NarL and αCTD at the ogt promoter

The DNase I footprinting experiment was used to confirm the location of the NarL site (position -45.5) and to confirm that RNA polymerase binds to the *ogt* promoter region. The promoter activity is decreased when the hexamer, located immediately downstream the NarL site, less resembles the consensus sequence of the -35 element. This suggests that the hexamer funtions as the -35 element, and is recognized by domain 4 of sigma subunit of RNA polymerase (Campbell *et al.*, 2002).

FeBABE, tagged on αCTD of RNA polymerase, was used as a DNA cleavage reagent to determine the location and orientation of αCTD in the transcript initiation complex at the ogt1052A1 promoter. The cleavage patterns suggest that αCTD binds to the DNA minor groove adjacent upstream -35 element; hence, it is located on the same DNA region as NarL. A diagram of transcript initiation complex at the ogt1052A1 promoter is illustrated in Figure

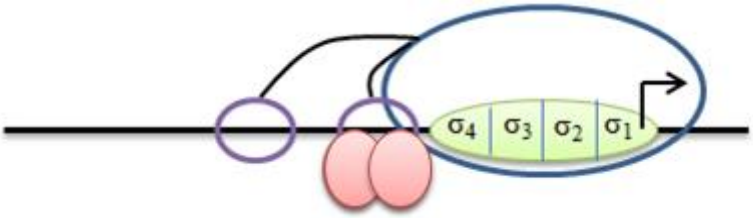
5.21 panel A. The αCTD is located on the different face of the DNA helix from NarL that is also located on the opposite DNA helical face from the sigma factor.

Results reported in this chapter are comparable with the work of Decker *et al.* (2011) who studied the *fim3* promoter, which is regulated by BvgA, a response regulator in NarL family. The binding site of BvgA is on the opposite face of DNA helix from sigma factor. The location of αCTD was identified using FeBABE-tagged RNA polymerase. The FeBABE footprinting experiment suggested that αCTD is located on a different DNA helical face from a BvgA dimer. Another comparable model is from the work of Kedzierska *et al.* (2003) who studied CII-dependent activation of the λ P_E promoter. CII is located on the opposite face of the DNA helix from sigma factor. One αCTD is located at position -41 on the opposite DNA helical face from CII. The binding sites of both BvgA and CII overlap -35 element of P_{fim3} and P_E, unlike the *ogt* promoter, which NarL is located upstream -35 element. However, the transcript initiation models at P_{fim3} and P_E suggest that it is possible to have a transcription factor and αCTD located on different faces of the DNA helix.

The αCTD binds to DNA through determinant 265, and interacts with activators through different surfaces at different bacterial promoters (Pan *et al.*, 1996; Benoff *et al.*, 2002; Cheng *et al.*, 2002; McLeod *et al.*, 2002). In the *ogt* promoter, residue 273 of the αCTD that binds to an UP element proximal subsite makes a direct contact with residue 178 of NarL located at position -45.5 on the opposite face of DNA helix. This action causes residue 261 (residues 257-259 and 261) of αCTD to point downstream toward the sigma subunit.

The work from Lee *et al.* (2003) has revealed architectures of the CRP-dependent promoters. In an absence of CRP, one copy of αCTD binds freely to DNA near position -41 while another is not fixed and binds to the upstream region (Naryshkin *et al.*, 200). The previous work from Chen *et al.* (2003) and Ross *et al.* (2003) suggested that determinant 261

A.



B.

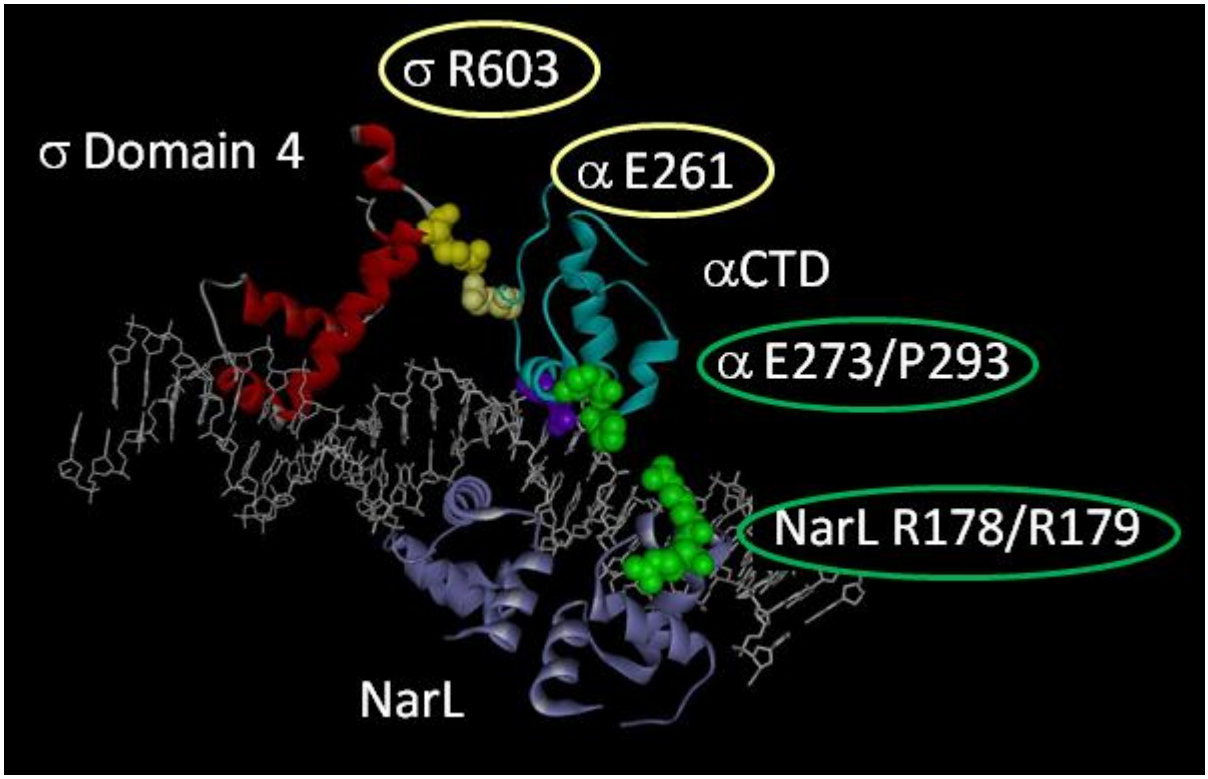


Figure 5.21 DNA-NarL-αCTD-σ_4 complexes at the *ogt* promoter.

Panel A shows locations of NarL and αCTD at the ogt1052 promoter. The αCTD, bound to UP element proximal subsite, is located on the same DNA region as a NarL dimer

Panel B shows a structural based model of a DNA-NarL-αCTD-σ_4 complex. DNA is shown in stick form. Sigma region 4 and αCTD in ribbon form are coloured red and blue respectively. The αCTD is located upstream the boundary of the -35 hexamer, thus it orients residue 273 to interact with residue 178 of NarL. A NarL dimer is centered at position -45.5 on the opposite face of DNA helix from αCTD.

of αCTD makes a contact with sigma domain 4 when αCTD is located at position -41. Current results indicate that the αCTD binds to DNA around position -42. Also, an alanine substitution at residue 261 of αCTD has no effect on the ogt1052 promoter activity, suggesting that it is not involve in transcription activation of the *ogt* promoter. It is possible that an interaction between NarL and αCTD at the ogt1052 promoter is sufficient to recruit RNA polymerase to the right position in spite of a substitution at residue 261.

Figure 5.21 panel B shows a structural based model of DNA-NarL-αCTD-σ_4 complex. DNA is shown in a stick form. A NarL dimer is centered at position -45.5 adjacent to the -35 element on the opposite face of DNA helix form sigma region 4. There is a contact between residue 178 of NarL and residue 273 of αCTD that binds to DNA immediately upstream -35 element, on the opposite face of DNA helix from NarL, with residue 261 pointing downstream toward sigma region 4 (Benoff *et al.,* 2002).

Chapter 6: Closing comments

The O6-alkylguanine DNA alkyltransferase (Ogt) is the DNA repair protein involved in a prophylactic stress response. The expression of *ogt* gene does not response directly to the RNS but it is induced by a presence of nitrate, a precursor of RNS. Therefore, it has time to produce Ogt before RNS are generated. This suggests that activation by NarL at the *ogt* promoter serves as prophylactic regulation to protect *E. coli* from the future DNA-methylation damage resulting from RNS.

The gene encoding Ogt is regulated by the NarXL and NarQP two component systems in response to a presence of nitrate. The work in Chapter 3 investigated effects of sequences and locations of the DNA site for NarL on the *ogt* expression. My work confirms that activation of the *ogt* promoter is induced by NarL in response to a presence of nitrate while Fis represses the *ogt* expression. The wild-type *ogt* promoter is not highly active as it contains the low-affinity DNA sites for NarL. Both NarL binding sites are crucial; however, NarL is still able to activate the *ogt p*romoter that contains a single DNA site for NarL if the site better resembles the consensus sequence.

Apart from affinity of the DNA site for NarL, the *ogt* promoter activity is also dependent on location of the NarL site in the promoter region. Recruitment of the transcriptional machinery by transcription factors takes time. Hence, the promoter strength depends on how long transcription factors stay bound to the DNA (Kaplan and Friedman, 2012). Interactions of NarL, DNA and RNA polymerase at the right position in the promoter region leads to stabilization of the DNA-factor complex. Binding of NarL to the target site at

the optimal location for a long period results in high induction of *ogt* expression (Polach and Widom, 1995; Voss *et al.*, 2011).

The DNA site for NarL was moved to different locations to identify the novel binding site for NarL. The *ogt* promoter activity is driven when the NarL site is located at some specific positions on the DNA helix, and the high level of activation occurs when the site is moved downstream closer to the location of RNA polymerase. NarP alone has a limited ability to up-regulate the *ogt* promoter. However, it is still able to activate the *ogt* promoter that carries a single near-consensus NarL site if the site is located immediately upstream the -35 element.

The current work also addresses effects of NarL binding site configurations. The C-terminal domain of NarL contains all of the essential determinants for DNA binding. The optimal NarL binding site configuration is arranged as two inverted heptameric sites with 2 base pair spacing (7-2-7). Binding of NarL as a dimer facilitates NarL-DNA recognition via a gradual bending mechanism. Hence, NarL has a strong tendency to bind as a dimer even though the DNA site for NarL includes one poor heptameric site. Binding as a monomer may not be sufficient for the promoter activation. Especially, NarP is restricted to bind only at the target site of 7-2-7 inverted repeat (Darwin *et al.*, 1996; Darwin *et al.*, 1997).

Each NarL and NarP consists of an N-terminal domain and a C-terminal domain linked by a flexible tether. The N-terminal domain carries a phosphorylation site for the regulator. The C-terminal domain forms a typical helix-turn-helix motif for DNA binding. The work in Chapter 4 investigated activation by NarL, NarL-CTD, NarP and NarP-CTD on the the *ogt* promoter derivatives. All the full length NarL, the full length NarP, NarL-CTD and NarP-CTD are competent for activation of the *ogt* promoter containing a single near

consensus NarL site located at position -45.5. However, only the full-length NarL is able to activate the wild-type *ogt p*romoter and the *ogt p*romoter containing a single near-consensus NarL site at position -78.5.

Mechanisms of activation by transcription factors can be classified into class I, II, III. In class I activation, a single activator binds upstream and makes a direct contact with the αCTD that is located on the same DNA helical face. At the *lac* promoter, which is the best characterized class I promoter, the DNA site for CRP is located at position -61.5. The CRP-αCTD interaction is accomplished by determinant 287 of αCTD and the activating region 1 (AR1) of CRP. AR1 is located on the surface of the downstream CRP subunit (Savery *et al., 2*002).

In class II activation, a single activator binds to the DNA region between the αCTD and -35 element. Both NarL and αCTD are located on the same face of the DNA helix. The best characterized class II-CRP dependent promoter is the *galP1* promoter that contains the CRP binding site located at position -41.5. Activation proceeds through 3 interactions between CRP and RNA polymerase. The upstream CRP monomer has AR1 contacting with determinant 287 of the αCTD. The downstream CRP monomer has AR2 and AR3 contacting with the αNTD and σ^{70} respectively (Busby and Ebright, 1999; Niu *et al.,* 1996; Savery *et al., 1*998; West *et al.,* 1993). A final class, a combination of either classes I+I or I+II, is classified as class III.

NarL regulators bind as dimers to two target sites at the *ogt* promoter and directly interact with αCTDs. One αCTD contacts with a NarL monomer located on the downstream half of the distal NarL site upstream the -35 elements. Another αCTD contacts with a NarL monomer located on the upstream half of the proximal NarL site adjacent upstream the -35

element. It is possible that NarL regulators activate the *ogt p*romoter via a class III mechanism as suggested by Squire *et al.* (2009). To test this hypothesis, further experiments were designed to determine if both NarL and αCTD are located on the same face of the DNA helix.

The work in Chapter 5 examined residues of interaction between NarL and αCTD. The previous PhD. work of David Chismon (2010), the University of Birmingham, used an alanine scan library of α subunit of RNA polymerase to determine important residues involved in activation of the *ogt* promoter. Together with data from the positive control mutant experiments and the epistasis experiments, my work suggests that residue 178 of NarL makes a direct contact with residue 273 of αCTD. However, this interaction cannot occur if both NarL and αCTD are located on the same face of the DNA helix.

Positions of NarL and αCTD were identified by DNase I footprint and FeBABE footprint experiments. It was confirmed that a NarL dimer binds to DNA immediately upstream -35 element, around the same region of the DNA site for αCTD but on the different face of DNA helix from αCTD. This unusual promoter configuration indicates that NarL, located at position -45.5, does not activate the *ogt p*romoter via a class II mechanism as expected. In a typical class II promoter, residue 287 of αCTD interacts with a downstream activator that is located on the same face of DNA helix as αCTD. For the *ogt* promoter with NarL located at position -45.5, the αCTD binds to its rightful place and contacts with a NarL dimer, which is positioned on the different DNA helical face, through residue 273.

Many studies of transcription factors have been designed to determine their functions on different gene promoters. The genome scale studies use microarray and genome sequencing techniques to examine the roles of an individual transcription factor, which genes it regulates, uuder what conditions and how it functions. NarL is a global transcription factor

that its structure and DNA binding mechanism has already been identified. There is still a gap in understanding of its mechanism of transcription activation. In the current work, site-specific mutagenesis and analysis of DNA-protein binding at the *ogt* promoter were used to address several questions of NarL functions and to characterize its mechanism of activation. Since there have been investigations of the NarL dependent promoters, further studies should examine whether the *ogt* promoter can be used as a model to define an unusual mechanism of regulation by NarL at other particular promoters.

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