The Role of the Nitrogen Assimilation Control protein (NAC) in the Response of *Klebsiella pneumoniae* to Nitrogen Limitation

by

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Abstract

The nitrogen assimilation control protein of *Klebsiella pneumoniae* provides a link between the specialized nitrogen regulatory system that requires RNA polymerase bearing σ^{54} and the major form of the cellular RNA polymerase bearing σ^{70}. NAC both activates and represses transcription. In order to activate transcription NAC requires a 15bp core consensus site of ATA-N_9-TAT. This consensus site is able to function when centered at -64, -59, -47, and -44 at the characterized NAC sites. Data present in this thesis demonstrates that the mechanism of NAC transcriptional activation at these sites is flexible and that a site natively centered at -64 can function at -59 or -47 suggesting that NAC might activate transcription in a similar manner from all sites. This suggests that the NAC binding site is functionally flexible and that NAC control of a promoter might be easy to gain.

The functional flexibility of NAC may allow NAC to control many promoters. Some of the data presented here demonstrates that under nitrogen limitation NAC is bound to 98 unique regions of the chromosome in *K.pneumoniae*. Most of these regions contain genes involved in nitrogen metabolism but some contain genes that are involved in carbon and energy acquisition or cellular growth rate control. This suggests an expanded and flexible role of NAC in the response of *K.pneumoniae* to nitrogen limitation. NAC might play a role in regulating other aspects of cellular physiology in addition to nitrogen metabolism in response to nitrogen limitation.

In addition to the flexibility of NAC in responding to nitrogen limitation, other stress responses help fine-tune the response to nitrogen limitation. Data presented in this thesis demonstrate that the stringent response concurrently regulates at least one promoter (*codBp*) that is regulated by NAC in response to nitrogen limitation. In this case the regulation by the two systems is in the opposite direction, NAC activating the promoter and the stringent response repressing the promoter. The ability to fine-tune the response of metabolic genes suggests that the nitrogen stress response in *K.pneumoniae* is flexible in the genes it controls, how they are controlled, and the degree to which they are regulated.

Chapter I

Introduction

Nitrogen Regulation

Enteric bacteria such as *Escherichia coli* and *Klebsiella pneumoniae* can use various compounds as their sole source of nitrogen when ammonia, the preferred source of nitrogen, is unavailable. A system to use ammonia preferentially is present in both of these bacteria (2). *K. pneumoniae* is considered a nitrogen generalist and is capable of using a variety of amino acids, amino sugars, nucleotides, and inorganic compounds as the sole source of cellular nitrogen (140). The ability to use a large number of nitrogen sources allows the bacteria to survive periods of low concentration or absence of ammonia.

Under conditions where ammonium concentration outside the cell is high (> 1 mM), passive diffusion is strong enough to provide the cells with enough nitrogen for cellular growth (70). Under conditions where ammonium concentration outside the cell is lower, a system to transport ammonium into the cell is needed. Ammonium is transported into the cells under these conditions via the AmtB protein (59). The function of the AmtB protein of *E.coli* has been extensively studied (55, 57, 69, 75, 150). The AmtB protein spans the inner membrane and connects the periplasmic space to the cytoplasmic space (69, 150). The AmtB protein forms a pore as a homotrimer and

transports ammonium directly (55). It is thought that AmtB binds to ammonium on its periplasmic side at a site referred to as S1 (55, 69, 150). The nature of the pore seems to suggest that it would be incapable of transporting an ion due to hydrophobicity (69, 150). Most data seems to suggest that the ammonium ion bound to the periplasmic site is deprotonated and then transported through the pore as ammonia (34, 55). Some groups suggest that the proton is also transported across the pore separately (34, 81). Data distinguishing these two models are not available. The activity of the AmtB pore is modulated by association with GlnK (57). GlnK regulates the AmtB pore (Figure 1.1) by plugging the pore when extracellular ammonium concentration is high and by unplugging the pore when the extracellular ammonium concentration is in the low μM level (57). To maintain low ammonia concentration inside the cell, glutamine synthetase (GS) acts as an intracellular sink for ammonia, quickly converting ammonia transported into the cell into glutamine, thus keeping the intracellular ammonia concentration low (56). Since GS is the sink for NH_3, the concentration of glutamine reflects the availability of ammonia. This complex regulation allows the cell to sense the extracellular ammonium concentration and respond by activating the AmtB pore when ammonium concentrations are near the K_m of AmtB for ammonium (57).

The conversion of ammonia to glutamine and glutamate is highly regulated in enterobacteria. The enzyme glutamine synthetase (GS) acts as a dodecamer (37) and produces glutamine from ammonia and glutamate in an ATP dependant manner (146). There are two pathways for the production of glutamate. Glutamate dehydrogenase (GDH) produces glutamate directly from α-ketoglutarate and ammonia (122). GDH has a high K_m for ammonia (ca. 1 mM) and thus only plays a role in the production of

glutamate under conditions of high intracellular ammonia (122). The other enzyme responsible for the production of glutamate is glutamate synthase (GOGAT), which uses α-ketoglutarate and glutamine to produce two molecules of glutamate (139). *K. pneumoniae* and *E. coli* use the GS/GOGAT system for a majority of the production of glutamine and glutamate (9).

In enterobacteria, the nitrogen status of the cell is sensed via the level of glutamine, which is directly related to the concentration of ammonium outside of the cell (52). As the cells become nitrogen limited, the concentration of glutamine in the cell can drop more than 10 fold (from 3mM to 0.3mM). Uridylyltransferase/uridylyl-removing enzyme (UTase/UR), the product of the *glnD* gene, senses glutamine via a direct and concentration dependent interaction (62-64). Under conditions of nitrogen excess, GlnD, is bound by glutamine and acts as a UR catalyzing the removal of uridylyl modifications from P_{II} family proteins such as GlnK and GlnB (1, 36, 37, 62, 91, 119). Unmodified GlnK is able to interact with and plug the AmtB pore (Fig 1.1), preventing the pore from transporting ammonia into the cell (27, 56, 57). Unmodified GlnB is also able to interact with NtrB, the sensor kinase of the NtrBC two-component system, and stimulate its phosphatase activity (Fig 1.1), maintaining NtrC in an unphosphorylated form and unable to activate transcription (4, 62-64). Unmodified GlnB also interacts with adenylyl transferase (ATase), the product of the *glnE* gene, allowing the adenylylation of GS monomers inactivating the enzyme since glutamine levels in the cell are high and no synthesis of glutamine is necessary (1, 4, 5, 8, 54, 60, 61, 64, 74, 91, 119, 128, 143). Thus when glutamine (i.e. ammonia) is abundant AmtB is plugged, *glnA* transcription is off and GS is inactivated. Under conditions of nitrogen limitation, sensed by GlnD as

low glutamine concentration, however, GlnD is not bound to glutamine and acts as an UTase, catalyzing the uridylylation of GlnK and GlnB (62). GlnK-UMP_3 is unable to interact with the AmtB pore thus AmtB transports any available ammonia into the cell for glutamine synthesis (27, 56-58). GlnB-UMP_3 interacts with Adenylyltransferase (ATase), catalyzing the removal of deactivating adenylyl modifications from GS monomers activating the enzyme and allowing glutamine synthesis via GS (8, 31, 64, 74, 91). GlnB-UMP_3 is also unable to stimulate the phosphatase activity of NtrB leading to NtrB kinase activity and the accumulation of the transcriptional activator NtrC~P (3, 33, 49, 68, 93, 106). Thus when glutamine (i.e. ammonia) is scarce, AmtB is unplugged, *glnA* transcription is on, GS is active, and NtrC is phosphorylated.

NtrC~P is a transcriptional activator of promoters transcribed by RNA polymerase bearing the alternative sigma factor, σ^{54} (47, 51). Promoters controlled by RNA polymerase bearing σ^{54} require NtrC~P bound to an upstream or downstream enhancer element in order for transcriptional activation to occur (51, 113, 123). While this allows for tight control of these promoters and makes transcription dependent on the uridylylation state of GlnB, which in turn monitors the concentration of glutamine, it places specific constraints on the promoter that may not be compatible with other transcriptional regulatory systems (95). The extent of NtrC~P regulation has been characterized in *E. coli* via microarray analysis (152). The nitrogen sensory and regulatory cascade results in the regulation of genes transcribed by RNA polymerase bearing the specialized nitrogen stress sigma factor, σ^{54}. Some of the genes controlled by the nitrogen stress response, however, are controlled by promoters transcribed by RNA

polymerase bearing σ^{70} and require an additional factor, the nitrogen assimilation control protein, NAC, for transcriptional regulation during the nitrogen stress response.

Identification of the Nitrogen Assimilation Control protein (NAC)

The discovery and characterization of the nitrogen assimilation control protein (NAC) of *K. pneumoniae* began with a peculiar observation. Many of the genes that utilize non-glucose molecules as sources of carbon are repressed in the presence of glucose (even when the molecule is present) and this repression is only relieved once the glucose has been exhausted (87, 88). This effect is now referred to as catabolite repression (29, 38, 85). Histidine can be utilized as a source of carbon or nitrogen (86, 102, 103). Utilization of histidine as a carbon source is controlled by catabolite repression and, in the presence of glucose, histidase is not produced even when histidine is present (87, 88). Neidhardt *et al* (1957) noticed that cells grown in the presence of glucose were able to utilize histidine as a sole source of cellular nitrogen but only in the absence of ammonia (102, 103). This was the first reported example of an enzyme overcoming the effects of catabolite repression in the presence of glucose. It became apparent, however, that the "unique" derepession of histidase in the presence of glucose was not actually unique. Prival *et al* (1971) reported that proline oxidase of *K. pneumoniae* was also derepressed under nitrogen limiting conditions in glucose media but not in the presence of ammonia (114). The deprepression of histidase and proline oxidase was selective and those catabolite-repressed enzymes (e.g. β-galactosidase) that degrade molecules that do not contain nitrogen were not able to overcome catabolite repression under conditions of nitrogen limitation (114). Additionally, this relief of

repression was independent of cAMP and therefore, was not direct relief of catabolite repression in response to a poor nitrogen source (114). In fact, cells grown in the presence of glucose and with a poor nitrogen source such as histidine or proline demonstrate an increased catabolite repression when compared to cells grown in glucose and ammonia (114). Interestingly, this relief of catabolite repression was not present in the closely related bacterium *Salmonella enterica* serovar Typhimurium, which was unable to use histidine or proline as a sole source of nitrogen in the presence of glucose, suggesting that this system was regulatory in nature and that *S. enterica* lacked this regulation (114). Taken together, these studies provided the first hints of the NAC protein's role in the regulation of genes involved in the metabolism of nitrogen under conditions of nitrogen limitation.

The *nac* gene was identified in 1983 (7). A strain containing the *gltB200* and *glnL45* alleles is a glutamate auxotroph because it lacks the activity of GOGAT and is constitutive for the Ntr system, causing repression of the other enzyme that forms glutamate, GDH (17, 89). This mutant was reverted to glutamate independence (7). This strain is capable of utilizing histidine as its sole nitrogen source but is unable to grow on high or low concentrations of ammonia (16, 17). Four classes of glutamate independent revertants were isolated. The first class could grow on high ammonia as nitrogen source but not on low ammonia concentration or histidine; these mutants were $NtrC^-$ and could neither repress GDH activity nor derepress histidase or GS (7). No growth on low ammonia indicates that they were unable to activate GS in response to low ammonia and these strains are glutamine bradytrophs even in high ammonia (7). The second class of revertants could grow on high ammonia and use histidine as a sole source of nitrogen.

The revertants mapped to the *gdhA* region and were mutants that could no longer be repressed efficiently (6). The third class of mutants appeared to be like the wild type strain and could grow on high and low concentrations of ammonia and use histidine as a sole source of nitrogen; the identity of the mutation responsible for this revertant phenotype is unknown (7). The last and largest class of revertants was able to grow on high and low ammonia but failed to utilize histidine as a sole source of nitrogen (7). These mutants were not in NtrC because NtrC was still able to activate GS in response to low nitrogen (7). However, these mutants not only failed to repress GDH formation under nitrogen limiting conditions but also failed to derepress histidase and proline oxidase (7). This phenotype remained when both the *glnL45* and *gltB200* alleles were replaced with WT alleles and did not map to any locus known to regulate the response to low nitrogen (7). The locus was named *nac* for nitrogen assimilation control and the changes in the formation of three enzymes involved in nitrogen metabolism suggested that this locus played a regulatory role in the nitrogen stress response (7).

Early studies of NAC were bolstered when the *hut* operons were mapped and sequenced (14, 15, 105). The genes the encode the first two enzymes of the histidine utilization pathway, *hutUH*, are transcribed in what appeared to be a polycistronic mRNA transcribed by a promoter controlled by RNA polymerase bearing σ^{70} (105). This was different than other genes involved in the nitrogen stress response that were controlled by RNA polymerase bearing σ^{54}, the sigma factor specific to the nitrogen stress response (105). This suggested that the *nac* locus might be affecting the nitrogen stress response to genes that are not controlled by RNA polymerase bearing σ^{54}. The *nac* locus was cloned in 1990 and it was determined that one gene in the region was responsible for the

phenotypes seen in the glutamate independent revertants of *glnL45 gltB200* strains that were unable to utilize histidine as a sole source of nitrogen (11). This gene, *nac*, is approximately 1kb in length and is able to complement the *nac-1* mutant by restoring both the repression of GDH and the derepression of histidase and proline oxidase under nitrogen limiting conditions (11). Interestingly, the *nac* clone is able to derepress histidase in *S. enterica* under nitrogen limitation in the presence of glucose (11). It had been previously established that *S. enterica* lacked the ability to derepress histidase in response to nitrogen limiting conditions (12). These data suggest that NAC is both necessary and sufficient to provide regulation to histidase, proline oxidase, and GDH under nitrogen limiting conditions and that the formation of histidase in *S. enterica* can be derepressed by the *K.pneumoniae nac* gene. Expression of NAC under conditions of nitrogen excess demonstrated that NAC was both necessary and sufficient to activate the formation of histidase and urease and repress GDH (127). The role of NAC in the response to nitrogen limitation was further investigated by examining the formation of enzymes known to be regulated by the nitrogen stress response in WT and Nac^- strains (83). In addition to histidase, proline oxidase, and GDH, NAC activats the formation of urease under nitrogen limiting conditions but does not regulate the formation of GS, asparaginase, tryptophan permease, or NifL (83). This suggested that NAC regulates a subset of the enzymes involved in the nitrogen stress response.

The mechanism by which NAC regulates a subset of the nitrogen stress response had not yet been established. The clone of *nac* was sequenced in 1993, and the translation of the open reading frame (ORF) identified NAC as a member of the LysR family of transcriptional regulators (126). The LysR family proteins are DNA binding

transcriptional regulators that respond to cellular stress, unique metabolites, and toxic compounds (44). As a member of the LysR family (most of which require a physiological co-effector to activate the protein), it was expected that NAC would have a co-effector (84, 124). For most LysR family members the co-effector interacts directly with the protein and changes the nature of the tetramer interactions such that the tetramer changes the pattern of binding sites it is able to recognize and bind (107, 151). The co-effectors of LysR proteins are often small molecules but may be a modification, such as the formation of a disulfide bond (e.g. OxyR), or another protein (e.g. GcvA, a LTTR, complexes with GcvR), and the co-effectors seem to all have physiological relevance to the conditions requiring a regulatory change for the specific LysR family member (66, 67, 107, 151). To better understand how NAC functions as a transcriptional regulator, an understanding of how LysR family proteins function is necessary.

The LysR Family of transcriptional regulators

To respond to stressful situations cells must increase the levels of proteins that will aid in the recovery and adaptation to the stress and decrease the level of proteins that function to increase or prolong the stress. One of the best ways for bacterial cells to alter their protein profile is by adjusting the level of transcription from the genes encoding these proteins. A relatively simple RNA polymerase composed of five subunits performs bacterial transcription. Four of these subunits (β, β', α, and ω) are present in the core of RNA polymerase as $\beta\beta'\alpha_2\omega$ (18). Unlike the four subunits composing the core RNA polymerase, the fifth subunit, σ, is variable and gives the RNA polymerase binding site specificity (30). Enterobacteria have relatively few variants of σ (147). One σ subunit is

responsible for the transcription of most of the genes involved in a majority of the cell's functions; in enterobacteria this is σ^{70} (19). The other sigma factors are often responsible for responding to specific cellular stresses such as heat shock (σ^{32}), membrane stress (σ^{E}), and nutrient limitation (σ^{S} and σ^{54}) and play a relatively minor role outside of these special conditions (43, 117, 118, 145).

Besides changing the site to which RNA polymerase binds by altering the sigma factor present in the holoenzyme, transcription can be regulated by additional protein factors referred to as transcription factors whose function is to change the amount of transcription performed by RNA polymerase at a given promoter rather than change the set of sites recognized by RNA polymerase. In general most transcription factors are composed of at least two domains, a DNA binding domain (DBD) and a regulatory domain (RD). Bacterial transcriptional regulators are classified by the sequence and position of their DBD (108). Currently, there are sixteen major classes of bacterial transcription factors (108). Unlike sigma factors, transcription factors are numerous in the bacterial genomes (108). A recent (May 2009) search of the 1016 completed bacterial genome sequences of the Integr8 database revealed 127,128 members of the 16 major classes (CRP-FNR, IclR, CSD, DeoR, AsnC, ArsR, MerR, LacI, NtrC/Fis, MarR, LuxR, GntR, OmpR, AraC, TetR, and LysR) of bacterial transcriptional regulators (115). This suggests that on average each sequenced bacterial genome has 125 different transcriptional factors. The largest family of bacterial transcription factors is the LysR family with 23,627 members in the completed bacterial genomes (Figure 1.2). The LysR family is large, and the proteins regulate diverse stress responses within the cells (84).

Henikoff *et al* identified the LysR family in 1988 as a group of nine proteins that shared a high level of sequence similarity (44). The family was named after the first characterized member of the family, LysR, a transcriptional activator of the *lysA* gene (135). LysR is the product of the gene divergently transcribed from *lysA* (135) and while many of the characterized LysR family members are divergently transcribed from a gene they regulate it is becoming clear that LysR family members are global regulators and many regulate genes that are distant from their own (45, 152).

Members of the LysR family respond to diverse stresses on the cell such as nutrient limitation (e.g. NAC, ArgP, CbbR, and CysB), harmful environments (e.g. OxyR, AmpR, CynR, and CbnR), changes in cellular lifestyle (e.g. NodDIII, ToxR, and RovM), or the presence of unique metabolites (e.g. NocR, IlvR, and MdcR) and help the cell adjust their transcription profile to the stressful conditions (7, 26, 42, 46, 48, 76, 90, 96, 101, 107, 116, 137, 142, 144). Despite their broad range of functions and their presence in a diverse range of bacterial species, LysR family regulators are similar in both structure and mechanism of regulation (84). LysR type transcriptional regulators can be broken into two regions by functional domain (Figure 1.3). The C terminal region (pfam ID: LysR_substrate, ca. amino acids 110-C term) contains the tetramerization domain and co-effector binding domain (53, 72, 78). The N terminal and C terminal regions are separated by a short (ca. 10 amino acid), flexible linker region allowing the tetramer to assume multiple conformations (124). The N terminal region (pfam ID: HTH_1, ca. N term-100 amino acids) contains the DNA binding domain and the dimerization domain (32, 66, 67, 132, 144, 151). Interestingly, out of 19,414 proteins in the pfam database with HTH_1, 18,125 also have LysR_substrate suggesting that these

two functional domains have coevolved in bacteria (131). The N terminus of LysR family proteins is highly conserved and easily recognizable among members of the LysR family (84, 124). The alignment of a sample of well-characterized LysR family members demonstrates the N terminal similarity (Figure 1.4). The LysR DBD is a member of the winged helix-turn-helix (wHTH) family of DNA binding domains (84). The DNA binding domain (DBD) of LysR family members recognize a dyad of 13bp that can be generalized as T-N_{11}-A (84, 124). Most LysR family members interact with DNA as a tetramer which is actually a dimer of dimers and change the binding sites they are able to recognize by binding a physiological co-effector molecule (84, 124). The recognition of a physiological co-effector molecule allows the LysR family members to respond quickly to changes in environmental conditions.

The predicted domain architecture of the LysR family is confirmed by structural studies. LysR family members have been difficult to crystallize due to the insolubility of the HTH DNA binding domain (97). Although, the structures of eleven of the LysR family members have been solved (24, 32, 71, 97, 130, 134, 138, 141, 149). Of the eleven solved structures, only two full-length crystal structures have been solved (71, 97). For the remaining nine only the structure of the C-terminal domain has been solved (24, 28, 32, 129, 130, 134, 138, 141, 149). A representative example of the full-length structure, CbnR, is shown in Figure 1.5A (10). As expected, CbnR crystallized as a tetramer, and the structure of CbnR demonstrates that the tetramer is composed of two equivalent dimers (Figure 1.5B). Interestingly, while the dimers are equivalent, each dimer is composed of two monomers in different conformations (97). One monomer is in a compact conformation (Figure 1.5C) with an acute angle formed between the N

terminal and the C terminal domains; the other monomer is in the extended conformation (Figure 1.5C) with an obtuse angle between the N and C terminal domains (97).

The CbnR tetramer has an ellipsoid shape of the dimensions, 130 x 78 x 60 Angstroms (97). As expected from structural predictions each monomer of CbnR is composed of two domains: the DBD (A.A. 1-87) and the SBD (A.A. 91-296). As the only well studied full-length structure CbnR provides the best template to study the interactions that allow monomers of LysR family members to form dimers. In CbnR, the interactions between two monomers to form a dimer occurs in the N terminal region near the linker connecting the N terminal and C terminal domains between two anti-parallel α-helixes (97). The authors proposed that the lack of heterodimers of LysR family members is most likely due to unique monomer:monomer interactions formed by each family member, which is consistent with a lack of amino acid similarity among the LysR family members in the region (97).

The C terminal domains of LysR family members, while not highly similar on a sequence level have very similar structures (130). The structural similarity of the crystallized LysR family members suggests that structural data may be extrapolated to other LysR family members where large quantities of biochemical and genetic data exist but no crystal structure is available. The tetramer form of LysR family members seems to require specific interactions from each monomer and heterotetramers of LysR family members do not form for the same reason that heterodimers of the family members do not form (24, 97, 130). These interactions allow the LTTR proteins to form an elliptical structured tetramer.

Most LysR family members change the type of DNA binding sites they recognize by rearranging their tetramer interactions in response to a co-effector binding at their C terminal domain (20, 32, 39, 45, 48, 65, 72, 73, 78, 130, 133). Binding co-effector causes a change in tetramer interactions leading to changes in the arrangement and spacing of the DBD of each dimer within the tetramer, without changing the structure of the DBD in each dimer, thus allowing the protein to recognize a different set of binding sites and changing the regulatory profile of the protein (32). In general, in the absence of co-effector the dimer subunits of tetrameric LysR family members interact with more distantly spaced sites (Figure 1.6) creating a longer footprint (72, 73, 78). The binding sites for LysR proteins are often numbered numerically for each helical turn. Using this numbering system, in the absence of co-effector, most LysR family members bind to sites 1,2,4,5 with a helical turn separating the binding site of each dimer (1,2-helical turn-4,5) creating a bend in the DNA (72, 73, 78). In the presence of co-effector, most tetrameric LysR family members interact with sites that can be number 1,2,3,4 and lack the helical turn separating the binding sites of each dimer creating a shorter footprint often with a lower angle DNA bend (72, 73, 78). Some promoters contain 1,2,3,4,5 arranged sites and thus can bind both types of tetramers; other promoters contain only one set of sites and will only interact with a tetramer is in the appropriate conformation (74, 75, 80). Structural studies have provided insight into how LysR family members interact with their co-inducing molecule. These regions involved in co-effector binding are near regions involved in tetramer formation suggesting that the binding of co-effector might occlude regions that could interact in the absence of co-effector (24, 32, 97, 130).

Regardless of the conformation of the tetramer, LysR family members interact with DNA through a highly conserved HTH domain. Mutants in the DBD prevent LysR family members from interacting with their binding sites on the DNA. By structural analysis the wHTH is composed of thee α-helices and one β-strand (97). The wHTH is held together by hydrophobic core interactions between the α-helices and β-strand. Mutations disrupting this core (e.g. CysB L44R, GcvA V32A, and OxyR L32F) are particularly detrimental to the DNA binding ability of LysR family members (66, 72, 78). The crystal structure of CbnR shows that two uncharged and two charged residues of the recognition helix are exposed to the solvent and are likely candidates for interacting with the DNA (97). Not surprisingly, mautations in other LysR proteins around this region (e.g. CysB E11K, NahR R21W, OxyR R4C, and GcvA S38P) are detrimental to protein:DNA interactions (66, 72, 78, 125). The high level of conservation among the N terminus of the LysR family of transcriptional regulators coupled with the detrimental effect of mutations in this region suggest that LysR family members might interact with DNA via a conserved mechanism.

It is expected that mutants that are unable to bind DNA would be unable to activate or repress transcription from the promoters they regulate. Another class of LysR mutant fails to repress transcription but still bind a subset of their binding sites. Mutants in this class have been characterized as negative control mutants that prevent the tetramer from interacting with the distantly spaced sites (e.g. NahR T130M, and NAC L111K, L125R) on the DNA because the changes perturb tetramer formation (53, 121, 125).

Mutations of LysR family members that allow the protein to assume only one conformation of tetramer have also been found. These mutations affect the interaction

between LysR family members and their co-effector molecules and can have two phenotypes. The first phenotype (insensitivity to co-effector), prevents the LysR family member from interacting or responding to co-effector (e.g. CysB M160I, T196I, A244V, A247E) and therefore causes the LTTR to become stuck in the long form and only capable of binding the more distantly spaced binding sites (78). To date, these mutations have been found only in CysB to date but it would not be surprising to see similar mutations in other LysR family members since they depend on co-effector to change tetramer conformation to the form that binds the short-footprint. The opposite effect can be seen in mutants that act as if they are constitutively bound to co-effector (e.g. NahR M116I, CatM R156H, OxyR T100I, XapR V104E, CysB A227D, OccR F113L, GltC T99A, and Cbl T102W) and are unable to assume the conformation required to form tetramer that recognizes 1,2,4,5 sites (23, 50, 65, 72, 78, 104, 110, 133). Mutants that are stuck in one form of the tetramer lie within regions defined by the crystal structures as forming the tetramer interface, providing good correlation of biochemical and structural evidence that these regions are critical to the formation of the different forms of tetramer.

LysR family members activate transcription of genes involved in responding to their stress response. Mutational analysis of LysR family members has revealed changes that block activation of transcription while maintaining the ability to form protein:DNA interactions. Three LysR proteins, NAC, GcvA, and CysB have positive control mutations in the N terminal wHTH DBD (NAC H26D, GcvA F31A, CysB Y27G, T28A, and S29A) that do not disrupt DNA binding but produce a protein-DNA complex that is unable to activate transcription (66, 78, 120). It is possible that these mutations interfere with a protein:RNA polymerase interaction and may define an activating region of the

LysR family. Interactions between the C terminus of the α subunit of RNA polymerase and LysR family members have been suggested by experiments using both scanning mutagenesis of the C-terminal domain of the α subunit of RNA polymerase (α-CTD) and two-hybrid studies between the α-CTD and LysR family members (67, 73, 79, 92, 109). The proposed interaction region between LysR family members and the α-CTD of RNA polymerase seems to involve the region surrounding amino acid 273 of the α-CTD referred to as the 273 determinant, and first defined as the contact site on RNA polymerase for the transcriptional activator Fis (13, 79, 94). This might suggest a conserved mechanism of transcriptional activation by LysR family members that possibly involves contacting the α-CTD of RNA polymerase at the 273 determinant.

In summary, the LysR family is the largest known family of bacterial transcriptional regulators in the sequenced bacterial genomes. The LysR family of proteins regulates a diverse set of responses to cellular stress and metabolism cues, and most seem to recognize these cues by interacting with a physiological co-effector. The interaction with the physiological co-effector changes the tetramer interactions of the LysR family members and changes the DNA site recognized by the protein, thus changing the regulatory profile of the protein. The crystal structures available provide evidence that the extensive biochemical analysis of the LysR family has defined the appropriate interaction domains.

NAC as a transcriptional regulator

The role of NAC in regulating the nitrogen metabolism of *K.pneumoniae* at the transcriptional level led to a search for the presence of NAC regulation and the *nac* gene

in other closely related bacteria that utilize the NtrBC two-component system to regulate their nitrogen metabolism. The closely related species *Klebsiella oxytoca* has NAC and NAC regulates transcription of the *putP* promoter (136). To determine the presence of the *nac* gene in other enterobacteria, southern blotting was performed with a probe corresponding to the *nac* gene of *K. pneumoniae* against *S. enterica* and *E. coli* genomic DNA for the presence of a *nac* gene. Under conditions of low stringency (to allow hybridization in the presence of probe mismatches), a signal was seen for *E. coli* genomic DNA but not *S. enterica* genomic DNA, suggesting that a *nac* gene is present in *E.coli* but not *S. enterica* (98). The lack of an observable *nac* gene in *S. enterica* was not surprising since *S. enterica* is phenotypically the same as a *nac-1* mutant (fails to derepress histidase or proline oxidase in the presence of glucose under nitrogen limiting conditions) suggesting that NAC is not present or not functional in *S. enterica* (12). Interestingly, *K. pneumoniae* NAC brought the *hutU* promoter (*hutUp*) from *S. enterica* under control of the nitrogen regulation allowing derepression in the presence of glucose under nitrogen limiting conditions but failed to regulate *putPp* in *S. enterica* (11). This data suggests that not only is NAC not present or not functional in *S. enterica* but also that some of the operons that may have been under control of NAC in the last common ancestor of *K. pneumoniae* and *S. enterica* might have lost the *cis* DNA elements required for NAC control in some cases (12). NAC plays a regulatory role in *E. coli* affecting the ability of *E. coli* to grow on some nitrogen sources (cytosine, arginine, and serine). Additionally, a twofold repression of GDH formation is lost in the *nac* mutant strain of *E. coli*, suggesting that while this isn't the strong regulation seen in *K. pneumoniae,* NAC is still playing a role in regulating transcription in response to nitrogen limitation (98). An

alignment of NAC protein sequence from *K. pneumoniae* and *E. coli* demonstrates that the two proteins are only 80% identical, a low value for conserved genes between *E. coli* and *K. pneumoniae* (98). The N-terminal domains are 90% identical between the two organisms but the C-terminal domains are only 73% identical between the two organisms. The lack of strong conservation in the C-terminal region suggests a lack of selective pressure to maintain this region (98). In both *K. pneumoniae* and *E. coli*, the *nac* gene is flanked by two asparaginyl-tRNA genes, *asnU* and *asnV*, that are 86 bp in length and 100 percent identical (Figure 1.7). In *K. pneumoniae* the *erfK* gene is downstream from *nac* and *cbl*. In *E. coli*, however, the *erfK* gene is upstream from the *asnV* gene (Figure 1.7). In the genome of *S. enterica* there is no match to the NAC open reading frame from either *K. pneumoniae* or *E. coli*. Interestingly, if the genomic region around *erfK* in *S. enterica* is examined (Figure 1.7), it appears that a recombination event between the two *asn* genes might have occurred causing the loss of the *nac* and *cbl* genes (98). Thus, *K. pneumoniae*, unlike the other enterobacteria, seems to have maintained a major role for NAC in the response to nitrogen stress. This makes *K. pneumoniae* an ideal organism for the study of NAC.

In the absence of co-effector, the LTTR forms the long form of the tetramer and binds to sites 1,2 and 4,5 forming the long footprint on the DNA that often shows substantial hypersensitivity in the region between sites 2 and 4 (72, 73). In the presence of an effector, the interactions between two dimers are modified, tetramers assume the short form, recognizing sites 1,2 and 3,4 resulting in the shorter footprint on the DNA (72, 73). Thus, LysR family members may recognize specific patterns of binding sites in

the presence or absence of co-effector; switching between these two conformations and the patterns of sites that they recognize requires the co-effector.

It seemed likely that NAC would have a physiological co-effector associated with nitrogen metabolism that allowed repression of *gdhAp* and activation of *hutUp*, *ureDp*, and *putPp*. Transposon mutants were isolated using TN5-*tac1*, which contains a *tac* promoter facing in the outward direction (25, 127). These mutants removed the control of NAC from the nitrogen stress response and made the expression of the *nac* gene dependent on the lactose analog and inducer of the *tac* promoter, IPTG (127). Under fully induced conditions, the NAC produced from the *tac* promoter was fully capable of activating the transcription of the *ureD* and *hutU* promoters as well as repressing transcription of the *gdhA* promoter, suggesting that nitrogen-limiting conditions are not needed to activate the NAC protein and, that once translated, the NAC protein is functional regardless of the nitrogen status of the cell (127). Thus, NAC performs all of the known functions without the need for the physiological conditions associated with its stress response, suggesting that NAC is unlike most LysR family members. It appears that NAC is not directly regulated by a physiological co-effector but instead, is indirectly regulated by the presence of glutamine and its influence on the levels of NtrC~P which activates transcription of σ^{54} dependent promoters. If NAC is produced by gratuitous induction it is capable of fully activating and repressing promoters under its control even under conditions of nitrogen excess.

The *tac-nac* fusion was used to determine the site selectivity for NAC. Site selectivity seems to be inherent in NAC affinity for a particular binding site. A titration of NAC levels by IPTG demonstrated that the formation of active urease is 100 percent

derepressed and the activity of GDH is 100 percent repressed before the activity of histidase is derepressed at all suggesting that NAC has the highest affinity for *ureDp* and the lowest affinity for *hutUp* (127). This may allow NAC to use energetically cheaper (i.e. fewer ATP molecules required for synthesis) sources of ammonia such as urea before catabolizing more expensive sources of ammonia such as histidine (127).

A screen was performed to isolate mutants of NAC that failed to repress the *gdhA* promoter (where NAC function requires a tetramer) but maintained activation of transcription at the *ureD* promoter (53). Using this screen the mutant NAC^{L111K} was isolated (53). The WT NAC protein elutes from a gel filtration column at a molecular weight consistent with a tetramer, but NAC^{L111K} elutes from the gel filtration column at a molecular weight consistent with a dimer (53). This confirms the hypothesis that the two dimers bound to the distant sites of the *gdhA* promoter tetramerize and that this tetramer interaction is important for strong repression of the promoter (41, 53). Interestingly, this mutant is still able to activate *ureDp* and *hutUp* as well as repress the *nac* promoter suggesting that tetramerization might not be essential to the physiological function of NAC at these sites (121). Another, non-tetramerizing mutation was designed by comparing NAC to the sequence of OxyR where the crystal structure suggests that I110 and L124 of one dimer interacts with F219 (without co-effector) or A232 (with co-effector) to form a tetramer (24). The equivalent amino acids to I110 and L124 of OxyR in NAC from an alignment of the two proteins are L111 and L125 (121). Since a mutation in L111 had been isolated as a non-tetramerizing mutation it suggested that L125 of NAC might also be required to form a tetramer (121). Indeed, NAC^{L125R} expressed in *K. pneumoniae* cells failed to provide strong repression of *gdhAp* but

activated transcription of *hutU* and *ureD* (121). This suggests that the amino acids L111 and L125 are required for NAC to form any tetramer interaction.

Once the basic requirements for tetramer formation were identified, there existed two models for how NAC could recognize different patterns of tetramer site without a co-effector: (i) NAC might be flexible and able to bind both patterns of sites with the same tetramer interactions or (ii) NAC, like other LysR family members, makes two distinct types of tetramer interactions between which it can exchange freely without the use of a co-effector. LysR family members are known to change the interactions between dimers in response to co-effector leading to a form of tetramer that binds a shorter footprint. NAC is capable of switching between the two forms of tetramer without the presence of a co-effector (Figure 1.8). The *nac* promoter DNA, to which NAC binds as the long form of the tetramer, is capable of competing NAC tetramer away from the *cod* promoter, to which NAC binds as the short form of the tetramer (Figure 1.8). This data suggests that, unlike other LysR family members, NAC is produced in a form that recognizes both the short and long form of the binding site and can interchange between these two forms of the tetramer freely. Data from OxyR showed that different amino acids were responsible for the interactions between two dimers in the presence and absence of co-effector (24). It was also noted that amino acids between the region 217-233 of characterized LysR family members was highly conserved (Figure 1.4). To determine the role played by the residues in this region in the formation of both the long and short forms of NAC tetramer, mutagenesis of this region in NAC was performed (120). Mutations in NAC that failed to assume the form of tetramer required to interact with DNA as one form of the tetramer but maintained the ability to assume the other form of tetramer were isolated (120).

Replacement of I222, T223, or A230 with an arginine prevented NAC from forming a tetramer at long-footprint sites such as the *nac* promoter but unlike NAC^{L111K} or NAC^{L125R} these mutations do not prevent a tetramer of NAC binding to the short tetramer footprint such as the *codB* promoter (120). For a NAC tetramer to interact with promoters containing the long-footprint site, it seems that L111 and L125 of one dimer might interact with I222/T223/A230 of another dimer (Figure 1.9). On the other hand, G217 mutations of NAC prevented formation of NAC tetramer at short-footprint sites but not at long-footprint sites (120). Thus, for a NAC tetramer to interact with promoters containing the short-footprint site, it seems that L111 and L125 of one dimer might interact with G217 of another dimer (Figure 1.9). These data led to a model of NAC tetramer formation that involves the interaction of L111 and L125 of one dimer with either I222/T223/A230 or G217 of the other dimer to form the conformations required to bind sites containing long or short-footprints respectively (Figure 1.9). Interestingly, if double mutations of NAC are created that disrupt both types of tetramer interactions ($NAC^{G217R, I222R}$ and $NAC^{G217R, A230R}$), NAC is unable to form a tetramer at either site (Figure 1.9), and only interacts with the DNA as a dimer (121). This means that NAC, like other LysR family members, binds at least two types of tetramer footprints and that the interactions required to form each type of tetramer are different. Unlike other LysR family members, NAC is capable of switching between these two tetramer conformations without a physiological co-effector.

Tetramerization is required for NAC to strongly repress transcription of the *gdhA* promoter but it seems that both forms of the tetramer are capable of repressing *gdhA* transcription, suggesting that the mechanism of NAC repression of *gdhAp* is different

than the interactions between NAC and the *nac* or *cod* promoters. NAC binds to two sites at *gdhA*, an upstream site from -102 to -74 and a downstream site from +42 to +71 (41). At first glance, these would appear to be two dimers of NAC bound to the DNA at different positions leading to the repression of *gdhAp* transcription. Strong NAC repression of *gdhAp*, however, is only seen when NAC is able to tetramerize, and mutant forms of NAC which are unable to form tetramers (NAC^{L111K} and NAC^{L125R}) do not strongly repress the *gdhA* promoter (121). Additionally, mutants of *gdhAp* lacking the upstream and downstream site do not have strong NAC mediated repression likely because the NAC is unable to form the tetramer due to the lack of a second binding site (41). This suggests that a third type of tetramer:DNA interaction is made by NAC, one that might have the formation of the NAC tetramer causing a loop to form in the DNA that prevents transcription and strongly represses the *gdhA* promoter.

At the *ureD*, *putP*, and *hutU* promoters NAC interacts with the DNA as a dimer as suggested by the smaller 26 bp footprints (21, 22, 40). At this class of site the negative control mutations, NAC^{L111K} and NAC^{L125R}, did not affect the mobility of the bound fragment, suggesting that NAC:NAC tetramer interactions does not play a role at these sites and that NAC occupies these sites as a dimer this is also true for wild type NAC, even when tetramer interactions are possible (53, 121).

The dimer as a functional unit of LysR family members is not well characterized. Other than NAC, there are only two other reported examples (TrpI and MetR) where LysR proteins are active as dimers (20, 80). TrpI is a dimer in the absence of its inducer, indoleglycerol phosphate (InGP), and only occupies one of the sites in the region it protects (20). In the presence on InGP TrpI forms a tetramer and occupies an additional

site and is able to activate transcription of the *trpBAp* (20). On the other hand, MetR, under low concentration, associates with the *glyA* promoter as a dimer and weakly represses transcription (80). Functional dimers seem to be rare among the LysR proteins. The conformation of the dimer as a functional unit is unknown, since all current crystallographic data is of the tetrameric form of the proteins. We suspect the conformation of the C-terminal regions of the monomer within a single dimer might be different than the conformation seen in the tetramer crystal structures. It is known that the linker between the N and C terminal domains is exposed and particularly sensitive to cleavage by protease (in fact C-terminal fragments for some of the crystal structures of LysR family members were generated using this sensitivity) and this cleavage separates the two functional domains of the protein (24, 32, 71, 97, 130, 133, 134, 138, 141, 149). If the C-terminus is required for the function of the dimer and it might be possible that the dimer folds in a manner that might protect the linker region from protease. The C terminus of NAC, however, seems dispensable for dimer binding since truncations of NAC that contain only 100 or 86 N terminal amino acids and have lost over two thirds of the protein length are fully functional at dimeric sites (99, 121). These truncated proteins are unable to provide strong repression of *gdhAp*, which is not surprising since they lack the regions of NAC required for forming a tetramer (99, 121). These data suggest that, at sites where it interacts with DNA as a dimer, NAC does not require the C-terminal domain.

NAC binding sites can be broken into two groups of sites by the core consensus found within the footprint. NAC has two possible roles at a promoter, (i) transcriptional activation and (ii) transcriptional repression (Figure 1.10). Similar to other members of

the LysR family NAC has a consensus binding sequence among all of its sites that matches the 13 nucleotide dyad of T-N_{11}-A (40, 84, 124). Each of these types of sites contains five triplets of nucleotides and these triplets are numbered one through five from promoter-distal to promoter-proximal. NAC sites can be partitioned into two groups of sites, those present at promoters repressed by NAC (*nac* and *gdhA*) which contain a consensus of ATA-N_9-GAT (or the compliment ATC-N_9-TAT) and those present at activation sites (*ureD*, *codB*, *putP*, *hutU*, and *dadA*) which contain the sequence ATA-N_9-TAT (21, 22, 40, 84, 100, 124). This suggests that the function of a NAC binding site can be determined based on the sequence of the core consensus (Figure 1.10). Additional data shows that the NAC binding site from the *gdhA* promoter (NBS^{gdhA}) fails to bring a *lac* promoter under control of NAC when positioned at -64, the position of NBS^{hutU} in its native promoter and a position at which NBS^{hutU} is able to bring a *lac* promoter under control of NAC (112).

To determine the regions that are required to make the *gdhA* promoter NAC binding site into an activation-binding site, a mutagenesis screen was performed (112). Only in cases where the fifth triplet (GAT) changed to TAT and the fourth triplet changed to TnG was strong transcriptional activation observed (112). These changes make the *gdhA* promoter NAC repression site essentially into the *hutU* promoter NAC activation site. These data suggest that not only are there different sequence determinants present in repression and activation binding sites, but that repression sites lack a property present in the activation binding sites that allow NAC to activate transcription when bound to these sites.

The DNA determinants required for transcriptional activation by NAC have been best characterized at the *hutU* promoter. The core 28 bp binding-site that is protected from DNaseI digestion by NAC at the *hutU* promoter is able to bring transcription of a *lac* promoter under control of NAC (111). The 28 bp binding-site will bring a *lac* promoter under control of the nitrogen stress response via NAC when centered at -64 from the start of transcription, the native spacing found at the *hutU* promoter (111). The ability of the NAC binding site alone to bring a promoter under control of NAC suggests that all information required for NAC control is present in this short sequence. Additionally the NAC binding site from *hutUp* (NBS^{hutU}) maintained NAC mediated transcriptional control of a *lac* promoter when centered at -54, -52, and -42 but failed to maintain control at -69, -59, -49, and -47 (111), suggesting that NAC is flexible in its ability to assume control of transcription and can do so from different positions as long as the face of the helix on which NAC sits is maintained. It was surprising therefore, that NBS^{hutU} failed to maintain NAC control of the *lac* promoter when positioned at -59 and -47 since they are the native positions for the NBS at the *codB* and *ureD* promoters, respectively, and NAC activates these promoters from these spacings (77, 100). These data suggested that there may be intrinsic differences between the sites for activation depending on the position at which they are centered.

The molecular mechanism of NAC mediated activation from the *hutUp* NAC binding site has been characterized. Mutations of the conserved ATA and TAT disrupt NAC binding to the promoter (112). Since NAC fails to associate with promoters containing mutations in the conserved triplets, it also fails to activate transcription from promoters containing changes to these regions (112). Additionally mutations to the A in

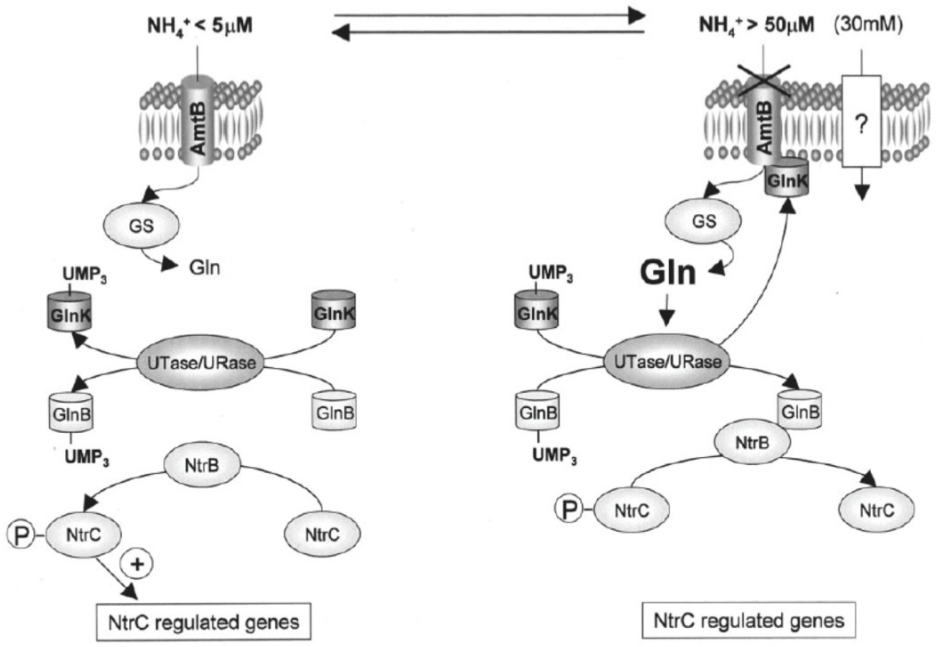
the second triplet also causes NAC to fail to recognize the site *in vitro* and an *in vivo* loss of NAC mediated activation occurs (112). This suggested that the distal half-site containing the first and second triplets was important for strong NAC binding to the *hutUp* NAC binding site. Replacement of the second triplet of the *hutUp* NAC binding site with the sequence of the fourth triplet caused an increase in NAC affinity for the site but a loss of *in vivo* transcriptional activation by NAC (112). The opposite replacement, where the sequence of the fourth triplet was replaced by the sequence of the second triplet, had low affinity for NAC *in vitro* but strong *in vivo* NAC mediated transcriptional activation proportional to its *in vitro* affinity for NAC (112). These data suggested that the two halves of the NAC binding site from the *hutU* promoter played distinct roles with the promoter distal half-site (first and second triplets) playing an important role in forming a stable NAC:DNA interaction whereas the promoter proximal half-site (fourth and fifth triplet) plays an important role in NAC mediated transcriptional activation (Figure 1.11). The half-sites must be in the proper orientation for strong NAC mediated activation of the *hutU* promoter *in vivo,* and a construct where the proximal and distal half-sites were used to replace each other had strong NAC affinity *in vitro* but failed to show strong NAC activation *in vivo* (112). These data suggest that the interaction between NAC and the *hutU* promoter is asymmetric.

The asymmetry of the *hutU* promoter NAC binding-site suggests that the response of NAC to the proximal half-site is causing the activation of transcription at this promoter. A mutation of NAC, NAC^{H26W}, fails to activate transcription of *hutUp* but was still able to repress transcription of *gdhAp*, was created by analogy to other LysR proteins where positive control mutants had been isolated by genetic screens (66, 73, 120). This

suggests that NAC^{H26W} is either unable to respond to the proximal half-site or unable to communicate the response to RNA polymerase. In the crystal structures of CbnR, the equivalent amino acid, H26, is in the HTH region and would lie close to the DNA (97). It has been demonstrated that some LTTR proteins contact the C-terminal domain of the α subunit of RNA polymerase, and the region around H26 might be a candidate area in looking for a NAC:RNA polymerase interaction (67, 79, 109, 132). Interestingly, NAC^{H26W} maintains NAC activation of the *ureD* promoter, suggesting that the mechanism of NAC mediated activation at the *hutU* promoter might not be universal (120). This is not surprising since NBS^{hutU} failed to bring the transcription of a *lac* promoter under NAC control at the positions of -59 and -47, positions where NAC is able of activating transcription at the *codB* and *ureD* promoters (77, 100, 111). This suggests that there may be different activating sites that are responsible for activating from different faces of the helix and that these sites may not be interchangeable. The mechanism of transcriptional activation by NAC at the *ureD* promoter and the *codB* promoter from opposite faces of the helix is unknown. The failure of NAC to activate transcription from these spacings with the *hutU* promoter NAC binding site suggests it is possible that a different mechanism might be responsible for the transcriptional activation of *ureDp* and *codBp* by NAC.

NAC plays a large role in the nitrogen stress response in *K. pneumoniae* but appears to be lost from *S. enterica* and in the process of being lost in *E. coli*. The extent to which NAC is involved in the nitrogen stress response of *K. pneumoniae*, however, is unknown. In *E. coli* it is known that NAC regulates nine promoters in response to nitrogen limitation (*codBA, ompF, oppABCDF, ydcSTUV, yedL, gabDTPC, nupC,*

dppABCDF, and *fklB-cycA*) via analysis of transcripts from WT and Nac$^-$ cells (152). The proteins produced by these operons all play a role in nitrogen metabolism. It is known that NAC in *K. pneumoniae* regulates operons that are not present (*hut*) or not regulated (*put*) by NAC in *E. coli* (21, 40). The sequencing of three *K. pneumoniae* strains (MGH78578, 342, and NTUH-K2044) will make the process of expanding the extent of the NAC regulon in *K. pneumoniae* nitrogen stress response easier (35, 82, 148). *K. pneumoniae* is capable of utilizing many compounds as a sole source of nitrogen. NAC might play a role in bringing the genes, some of which may be designed for other functions (*hut* and carbon metabolism), required to metabolize these compounds under control of the nitrogen stress regulon.



From Jarvelle *et al* 2004 (ref 57)

Figure 1.1: The nitrogen stress response. Adapted from Jarvelle *et al* 2004 (57). Under nitrogen limiting conditions low levels of glutamine are sensed by GlnD, which is an active UTase under these conditions. GlnD uridylylates both glnK and GlnB preventing them from interacting with AmtB and NtrB respectively. GlnK-UMP is unable to interact with AmtB, thus allowing the pore to transport what ammonia is available outside the cell in where it is converted by GS to glutamine. GlnB-UMP is unable to interact with NtrB. This allows NtrB to phosphorylate NtrC, which in turn is a transcriptional regulator for genes of the nitrogen stress response. Under conditions of nitrogen excess high levels of glutamine are sensed by GlnD. GlnD is an active uridylyl-removing enzyme when bound to glutamine. GlnB and GlnK are both deurydlylated and able to interact with NtrB and AmtB respectively. GlnB stimulates the phosphatase activity of NtrB maintaining NtrC in the unphosphoylated, inactive state. GlnK interacts with AmtB blocking the pore and preventing the import of ammonia into the cell via the pore.

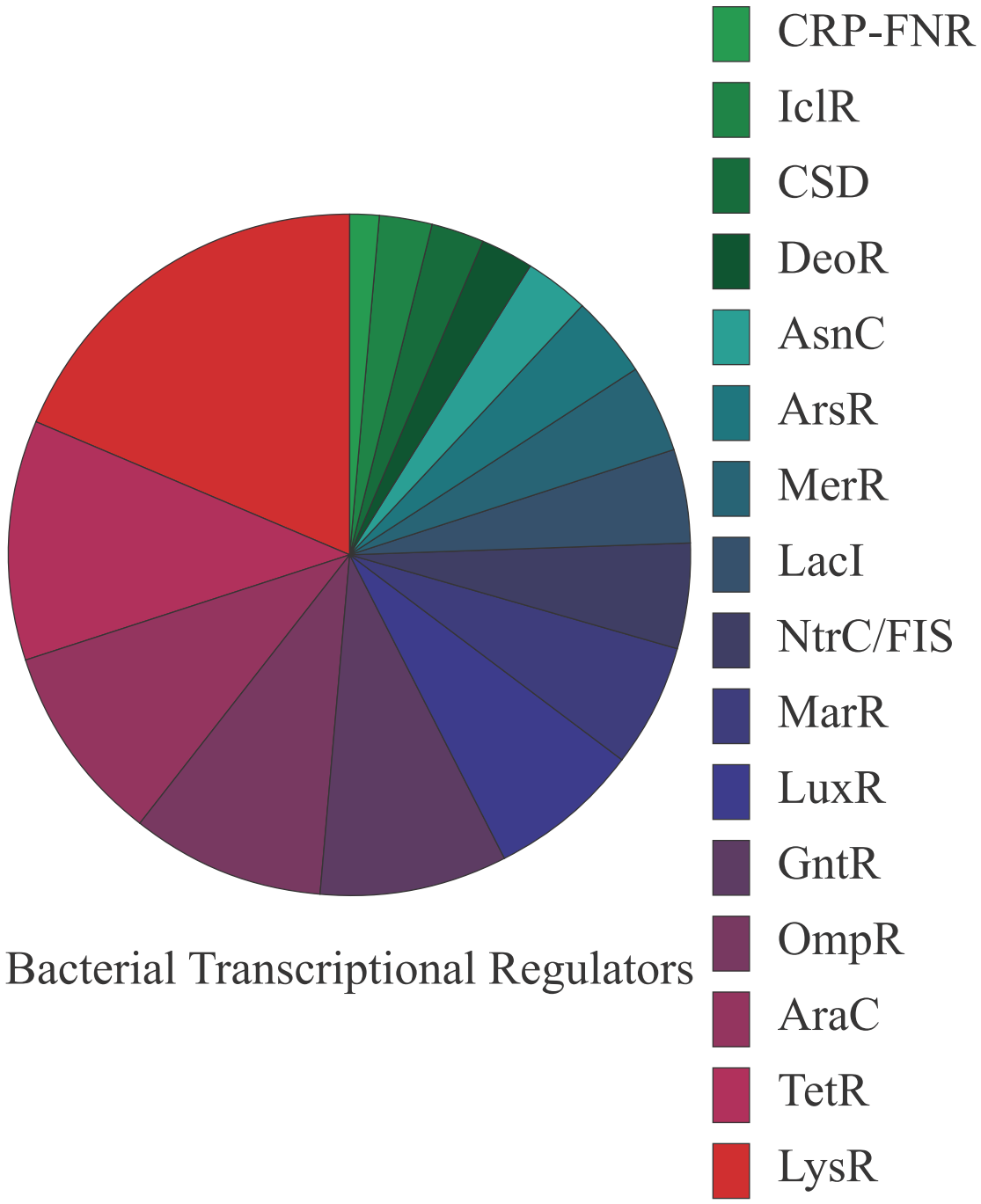


Figure 1.2: Bacterial transcriptional regulators. The sixteen largest families of bacterial transcriptional regulators from a search of the Integr8 database (15 May 2009) completed bacterial genomes were plotted as a pie chart. The families are displayed from least populated to most populated. The LysR family is displayed in red.

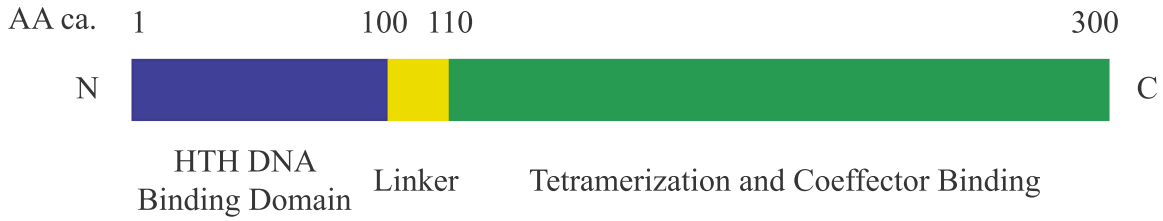


Figure 1.3: LysR proteins have two functional domains. The N terminal domain involved in DNA binding and dimerization is shown in blue. The C terminal tetramerization and coeffector-binding domain is shown in green. A short unstructured 10 amino acid linker region shown in yellow connects the two domains.

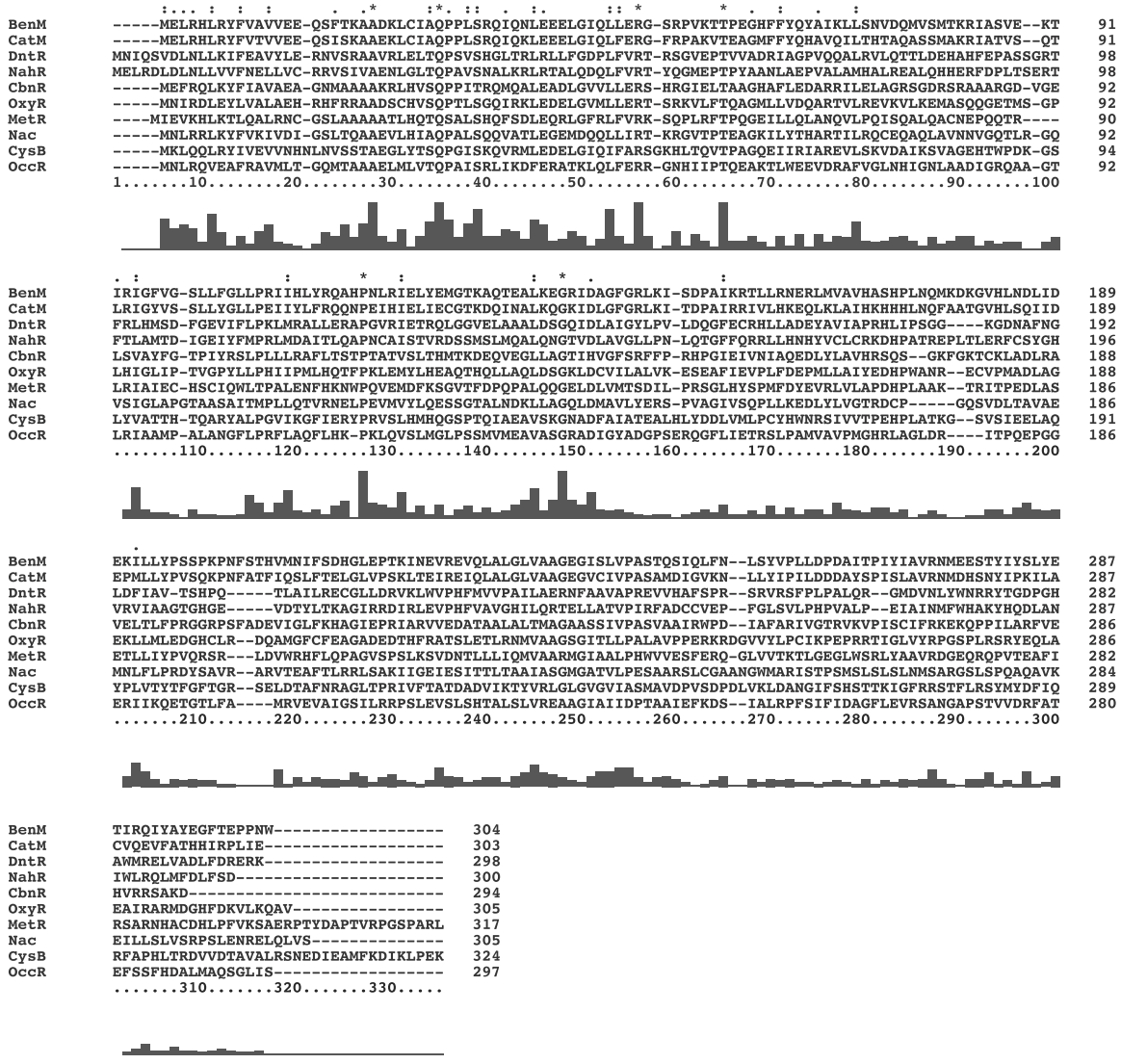


Figure 1.4: Alignment of well-characterized LysR family members. A clustalW alignment of well characterized LysR family members. Bar chart below the alignment shows the level of conservation among the LysR family members used in the alignment. Positions of exceptional conservation are shown above the alignment with * meaning complete conservation of a single residue, a : indicates strong group conservation, a . indicates weak group conservation.

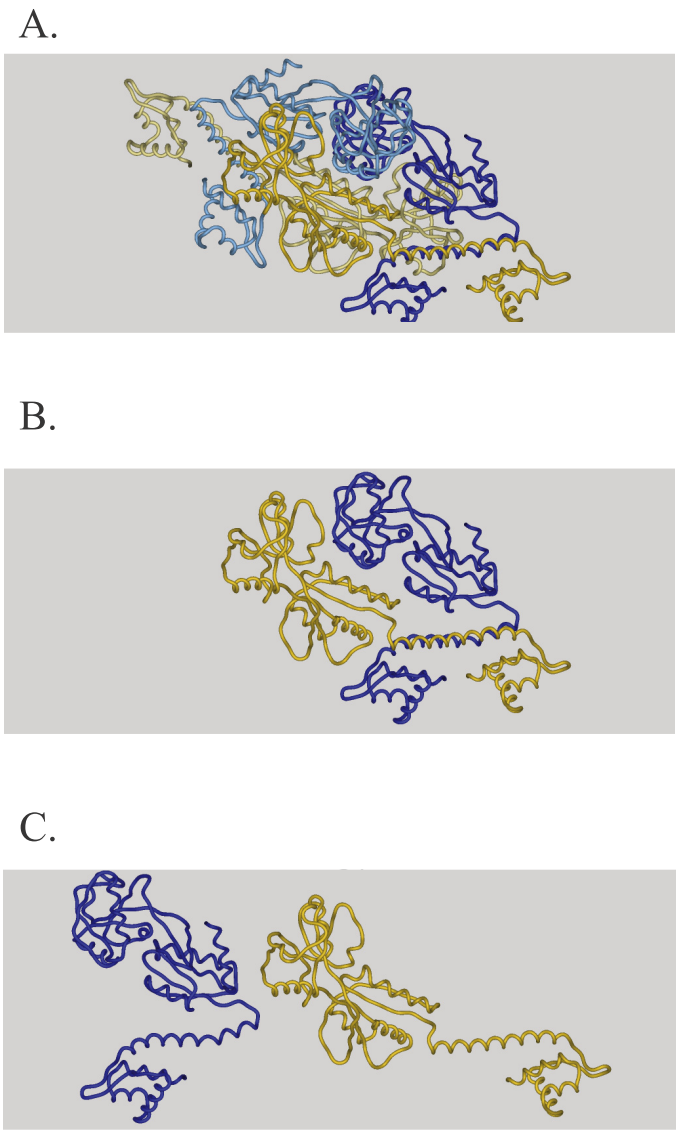


Figure 1.5: Two monomer conformations of LysR family members. (A) Projection of the tetramer of pdb file 1lZ1, the CbnR crystal structure. Extended monomers are shown in yellow and light yellow. Compact monomers are shown in blue and light blue. (B) One dimer of CbnR from the projection in A. The compact monomer is blue and the extended monomer is yellow. The long dimerization domain is seen as the horizontal alpha helix. (C) The extended and compact monomers from panel B separated from each other. The extended monomer is yellow and the compact monomer is blue. Notice the angle formed between the N terminal and C terminal domains in each type of monomer.

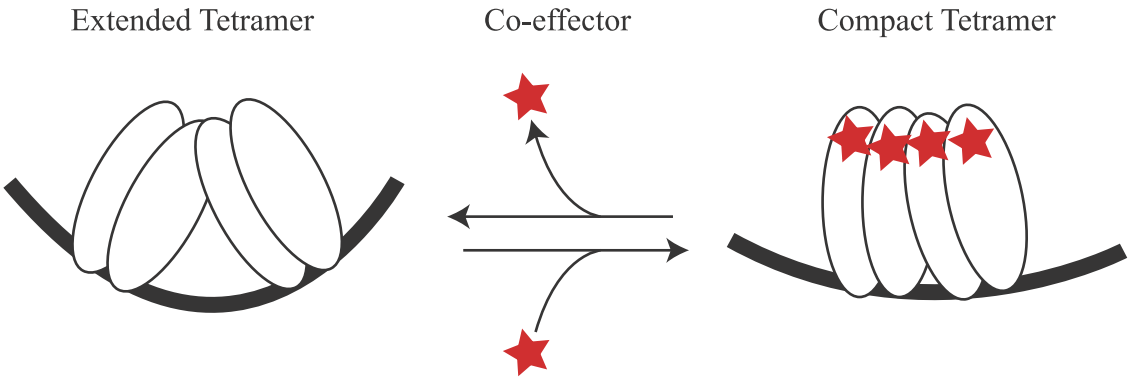


Figure 1.6: Model of LysR family member tetramer conformations. In the absence of co-effector (red star) most LysR family members form an extended tetramer and associate with distantly spaced DNA binding sites. Binding DNA in this manner causes a high angle DNA bend that often leads to DNaseI hypersensitivity of the region between the two sites. In the presence of co-effector many LysR family members form a compact tetramer that recognizes and binds more closely spaced sites. The interaction of co-effector associated LysR family members are characterized by a low angle bend and decreased hypersensitivity of the region between the two sites.

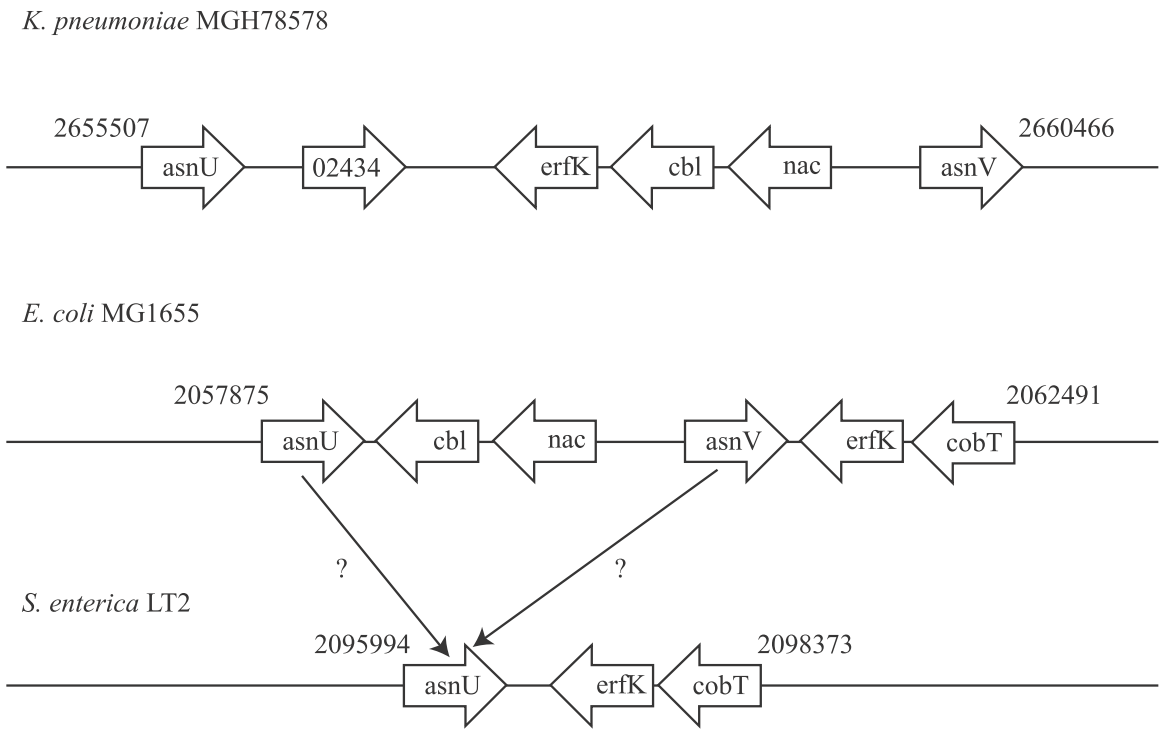


Figure 1.7: Map of the *nac* locus. Map of the region of the genome containing *nac* in *K. pneumoniae* MGH 78578 and *E. coli* MG1655. In both cases the *nac* gene is flanked by 100 % identical 86 bp tRNAs *asnU* and *asnV*. These regions do vary as *erfK* is upstream of *nac* in *E. coli* but downstream of *nac* in *K. pneumoniae*. The map of a similar region in *S. enterica* is also shown. A proposed recombination (arrows) event between the two identical tRNA genes in *E. coli* could have led to the genomic structure of this region in *S. enterica*.

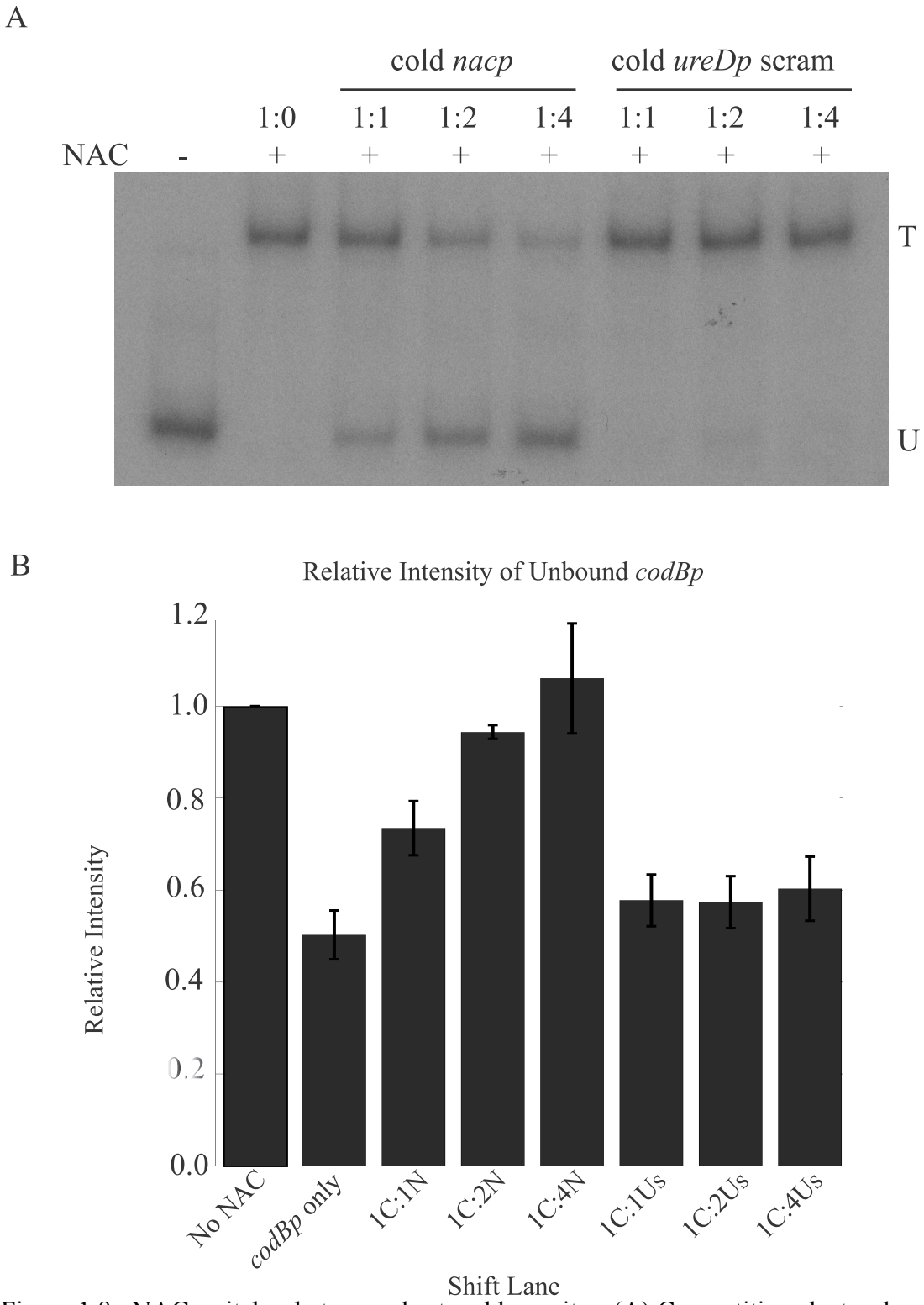
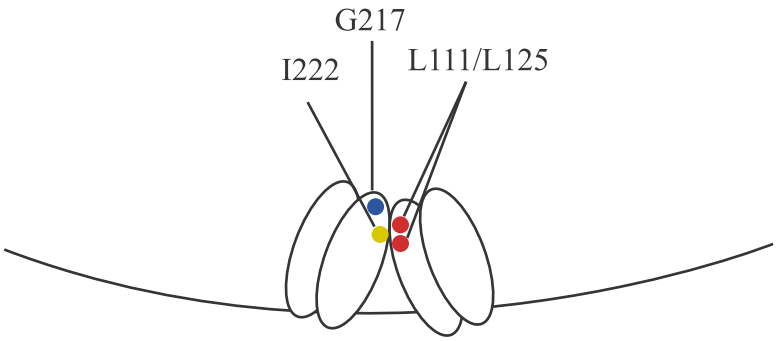
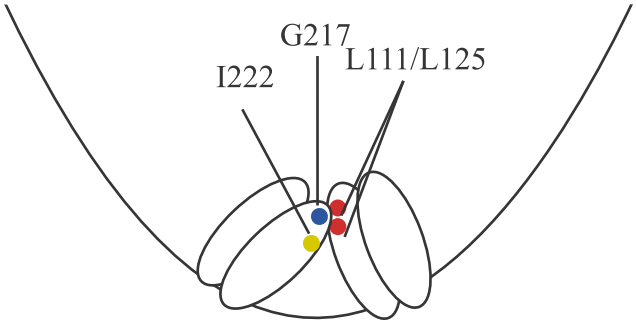


Figure 1.8: NAC switches between short and long sites. (A) Competitive electrophoretic mobility shift assay with labeled *codBp* competed with unlabelled *nac* promoter or scrambled *ureD* promoter. Ratio of labeled *cod* promoter : competitor is shown above each lane. (B) Bar chart of relative density of the unshifted *codB* promoter fragment from 3 independent experiments error bars indicate standard error.



Compact Tetramer



Extended Tetramer

Adapted from C. Rosario (120)

Figure 1.9: Tetramer interactions for NAC recognition of short and long sites. At sites such as that found in the *cod* promoter NAC forms a compact tetramer. To form a compact tetramer the L111 and L125 (red circles) of one dimer interacts with I222, T223 and A230 (yellow circle) of the other dimer. To interact with long sites such as the site found in the *nac* promoter L111 and L125 of one dimer (red circles) interact with G217 of the other dimer (blue circle).

A **Activation Sites**

hutU	ATA	ACA	AAA	TTG	TAT
putP	ATA	AGC	GTT	TCG	TAT
ureD	ATA	GGG	TAT	TTG	TAT
dadA	ATA	AAT	AAA	GCG	TAT
codB	ATA	TAA	AAA	ATA	TAT
cons.	ATA	...	...	...	TAT

B **Repression Sites**

gdh_u	ATA	ACT	AAA	AGC	GAT
gdh_d	ATA	AGG	GGA	ACT	GAT
nac_s	ATA	AGG	GAT	AGT	GAT
nac_w	ATA	CCA	CAA	TCT	GCT
cons.	ATA	...	...	...	G T

Figure 1.10: Known NAC binding sites. (A) An alignment of the five triplets from the promoters activated by NAC. The consensus is shown below the alignment. (B) Alignment of the five triplets of the NAC binding site from the 4 binding sites from promoters repressed by NAC. Beneath the alignment the consensus sequence is shown.

Figure 1.11: Model of NAC activation of the *hutU* promoter. NAC binds to the site centered at -64 with respect to the start of transcription. The distal half-site (open box) is required for strong NAC binding and the proximal half-site (checked box) is required for activation. The response of NAC to the proximal half site is represented by a change in the monomer of NAC interacting with this site.

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Chapter II

An Expanded Role for the Nitrogen Assimilation Control protein (NAC) in the Response of *Klebsiella pneumoniae* to Nitrogen Stress

Abstract

Klebsiella pnuemoniae is able to utilize many nitrogen sources, and the utilization of some of these nitrogen sources is dependent on the Nitrogen Assimilation Control (NAC) protein. Seven NAC regulated promoters have been characterized in *K. pnuemoniae*, and nine NAC regulated promoters have been found by microarray analysis in *Escherichia coli*. So far all characterized NAC regulated promoters have been directly related to nitrogen metabolism. We have used a genome wide analysis of NAC binding under nitrogen limitation to identify the regions of the chromosome associated with NAC in *K. pneumoniae*. We found NAC associated with 99 unique regions of the chromosome under nitrogen limitation. *In vitro*, 84 of the 99 regions associate strongly enough with purified NAC to produce a shifted band by electrophoretic mobility shift assay. Primer extension analysis of the mRNA from genes associated with seventeen of the fragments demonstrated that at least one gene associated with each fragment was NAC regulated under nitrogen limitation. The large size of the NAC regulon in *K. pneumoniae* indicates that NAC plays a larger role in the nitrogen stress response than it does in *E. coli*. Although a majority of the genes with identifiable functions that associated with NAC under nitrogen limitation are involved in nitrogen metabolism, smaller subsets are

associated with carbon and energy acquisition (18 genes), and growth rate control (10 genes). This suggests an expanded role for NAC regulation during the nitrogen stress response, where NAC not only regulates genes involved in nitrogen metabolism but also regulates genes involved in balancing carbon and nitrogen pools and growth rate.

Introduction

Klebsiella pneumoniae is considered a nitrogen generalist as it can use many compounds as a sole source of nitrogen including most amino acids, most nucleosides and bases, many amino sugars, many other organic compounds, and even some inorganic compounds (39). In most cases the utilization of alternative nitrogen sources is regulated by the availability of the preferred nitrogen source, ammonia (25). A global regulatory two-component system, NtrBC, responds to low concentrations of ammonia and is responsible for most of the nitrogen regulation seen in *K. pneumoniae* (25).

The Ntr system regulates some of the nitrogen response directly (25). Direct regulation by the Ntr system places specific constraints on the promoter of the regulated gene in that it requires a promoter that is transcribed by RNA polymerase bearing the alternative sigma factor σ^{54} and a distant enhancer element (25). One gene that is directly regulated by the Ntr system in response to nitrogen limitation is the Nitrogen Assimilation Control protein, NAC (10, 22).

NAC binds to and regulates transcription from promoters controlled by RNA polymerase bearing σ^{70}, thus providing a critical link between the specialized Ntr system

and genes controlled by promoters transcribed by the major cellular RNA polymerase (4, 13). Unlike the Ntr protein, NAC binds close to the start of transcription at the promoters it regulates (6, 11, 14, 17, 19, 29, 33). In contrast to the complex regulatory features required for a promoter to be controlled by the Ntr system, NAC regulation requires as little as a 15bp binding site that can be inserted within the promoter (32). This suggests that it is easier to bring genes into the nitrogen regulon through the use of NAC rather than direct control by the Ntr system.

Seven NAC regulated operons (*hutUH*, *ureDABCEFG*, *dadAX*, *nac*, *gdhA*, *putA*, and *codBXA*) have been characterized in *K. pneumoniae* to date (6, 11, 13, 14, 17, 19, 29). In *E. coli* the global extent of the NAC regulon has been examined by microarray analysis (43). Nine operons (*codBA, ompF, oppABCDF, ydcSTUV, yedL, gabDTPC, nupC, dppABCDF,* and *fklB-cycA*) showed differential expression between Nac^+ and Nac^- *E. coli* cells under nitrogen limitation (43). Like the known NAC regulated genes in *K. pneumoniae*, these operons are involved in nitrogen metabolism. Interestingly, several of the operons known to be NAC regulated in *K. pneumoniae* are either not present or not NAC regulated in *E. coli*. This raises the question, what is the size and breadth of the NAC regulon in *K. pneumoniae*? No microarray of *K. pneumoniae* is available to look at changes in transcription between Nac^+ and Nac^- strains under Nitrogen limitation. To examine the size and breadth of the NAC regulon in *K. pneumoniae* without microarray analysis we used Chromatin Immunoprecipitation (ChIP) to separate DNA bound to NAC under nitrogen limitation from the unbound portions of the chromosome.

Materials and Methods

Strains, media, and growth

The isogenic strains used in this study referred to as Nac^+ [KC6119 (*str-6 Δrbs753* ΩCB1618, Φ(*ureDp1'-'lacZ*))] (19) and Nac^- [KC6447 (*str-6 Δrbs753* ΩCB1618, Φ(*ureDp1'-'lacZ*) *nac-2*)] are derived from W70 (23). The *nac-2* is a replacement of the 19 bp upstream of the NAC start codon through the stop codon with a *frt* flanked kan^R cassette (7). Growth under nitrogen limitation was in W4 minimal medium supplemented with 0.4% (w/v) glucose and 0.2% (w/v) monosodium glutamate (22). *K. pneumoniae* cells were grown at 30°C. All *E. coli* strains were grown at 37°C in L broth and on plates of L broth solidified with 1.5% Bacto agar (28). To select for plasmids and chromosomal markers the media were supplemented with 100μg/ml ampicillin or 50μg/ml kanamycin.

ProteinA-Antibody beads and immunoprecipitation

Polyclonal anti-NAC antibody was generated in rabbits (Cocalico Biologicals Inc.) as previously described (35). In order to increase the specificity of the serum for NAC, the serum from rabbit 2F was incubated with acetone-precipitated protein from the Nac^- strain (15). Purified NAC antibody was directly crosslinked to protein-A sepharose beads (GE lifesciences) as described (15). To test for purity immunoreactive protein was precipitated from Nac^+ or Nac^- cell-free lysate prepared from cells grown under nitrogen limiting conditions. Cells were grown to mid log phase (50 Klett units). Cells were harvested and washed with one culture volume of cold 1% KCl. Cells were resuspended in 1 mL cold ChIP lysis buffer (50mM HEPES/KOH pH 7.5, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate, and 1mM PMSF) and lysed via 5 cycles of sonication at power level 5 for 15 seconds with 2 minutes on ice between cycles

with a Fisher microprobe sonicator (15). To precipitate NAC protein, 40µl of α-NAC bead slurry was added to each sample. Samples were rotated at 4°C for 1 hour. The beads were pelleted, a cell lysate sample was removed, and the beads were washed 5 times in 0.5ml of ChIP lysis buffer. The beads were resuspended in 30µl of 2x Lammeli loading buffer (18) and boiled for 5 minutes. Proteins from the cell-free lysate (10µl) and the immunoprecipitation (10µl) were separated by discontinuous SDS-PAGE on a 15% Tris-glycine gel. The proteins were transferred from the gel to 0.2 micron nitrocellulose in 1x Dunn's modified carbonate buffer (10mM $NaHCO_3$, 3mM Na_2CO_3 pH 9.9) in 20% (v/v) methanol at 50 volts for two hours at 4°C (8). The membrane was incubated in blocking buffer (TBS-T [25 mM Tris-HCl, 3mM KCl, 140 mM NaCl, 0.05% (v/v) Tween-20 pH7.4], 1% milk [w/v], 1% BSA[w/v]) for 1 hour (26). The membrane was washed twice in TBS-T. The membrane was treated with primary antibody (purified α-NAC), diluted 1:2500 in blocking buffer, for 1 hour. The membrane was washed twice in TBS-T. The membrane was treated with secondary antibody (Sigma Goat anti-rabbit IgG-HRP), diluted 1:10000 in blocking buffer, for 1 hour. The membrane was washed twice in TBS-T. The membrane was treated with dura west substrate (ThermFisher Scientific Inc.) and exposed to film.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was essentially performed as previously described (31, 36). Briefly, both the Nac^+ and Nac^- strains of *K. pneumoniae* were propagated under nitrogen limiting conditions at 30°C. The cells were grown to mid log phase (50 Klett units). Formaldehyde was added to each culture to a final concentration of 333 mM to crosslink protein to DNA. After 15 minutes of incubation the crosslinking

reaction was quenched with the addition of glycine to 125mM. Cells were pelleted and washed once with one culture volume of cold 1% KCl. Cells were resuspended in 0.5mL cold ChIP lysis buffer. Cells were lysed and DNA was fragmented by 5 cycles of sonication for 15 seconds at an intensity of 5 with a Fisher microprobe sonicator. This intensity of sonication lysed the cells and sheared the DNA to an average fragment size of 500bp (Data not shown). To immunoprecipitate NAC-DNA complexes 40µl of α-NAC bead slurry was added to each sample. The immunprecipitations were rotated at 4°C for 1 hour. The beads were pelleted and 100µl of supernatant was removed (input DNA) to provide confirmation that all PCR targeted sites were recovered in the total DNA sample. Beads were resuspended in 1 ml of buffer, rotated at room temperature for 5min, and pelleted (1000 x g, 1 min, 4°C); twice with 1mL ChIP lysis buffer, once with high salt ChIP lysis buffer (500mM NaCl), once with ChIP wash buffer (10mM Tris-HCl pH 8.0, 250mM LiCl, 0.5% NP-40, 0.5% Sodium deoxycholate, and 1mM EDTA), and once with TE (10mM Tris 1mM EDTA pH8.0). NAC-DNA complexes were removed from the beads by incubation in 100 µl ChIP elution buffer (50mM Tris-HCl pH 8.0, 10mM EDTA, and 1% SDS) at 65°C for 15 minutes. The crosslinks were reversed at 65°C overnight. Both input and immunoprecipitated DNA samples were treated with proteinase K (0.5µg/mL) for 2 hours at 37°C to decrease the protein concentration in the final samples. DNA was purified via Qiagen Qiaquick spin kits and eluted in 50µl TE pH 8.0.

Multiplex PCR

As a specificity control, the total and immunoprecipitated DNA samples were analyzed by multiplex PCR targeting the *ureD* promoter region, a region 1kb downstream

of this region, and a region 5kb downstream of this region (primers are available on request). PCR was performed using Invitrogen supermix with the primers at concentrations to provide equal levels of amplification at the same number of cycles. Samples were separated on a 5% TBE-PAGE gel at 10V/cm for 1 hour, stained with ethidium bromide, and visualized with a CCD UV imager (Alpha-InnoTec GmbH)

Cloning of immunoprecipitated DNA fragments

Immunoprecipitated DNA from the Nac^+ strain was blunted using the Klenow fragment of *E. coli* DNA polymerase I (26). This DNA was then ligated with pUC19, which had been digested with SmaI and dephosphorylated. Ligations were incubated overnight at 14°C. TOP-10 (F- *mcrA* Δ[*mrr-hsdRMS-mcrBC*] Φ80 *lacZΔM15* Δ*lacX74 recA1 araD139*Δ[*ara-leu*]*7697 galU rpsL endA1 nupG*) competent *E. coli* cells were transformed with the ligations and plated on L-plates supplemented with ampicillin. Plates were incubated for 14 hours at 37°C. Individual colonies from the transformation plates were purified by single colony isolation on the same media. Plasmid was recovered from each colony and sequenced using M13-20 and M13-RSP primers (16) at the University of Michigan DNA Sequencing core facility.

Purification of NAC

Purification of untagged NAC was performed as previously described (13). Purity was monitored by SDS-PAGE followed by coomassie blue staining using the method of Fairbanks (9). The NAC used in these experiments was estimated to be greater than 90% pure. Protein concentration was determined by the method of Lowry using bovine serum albumin (BSA) as a standard (20). Purified NAC was stored at -20°C in 50% glycerol.

Electrophoretic Mobility Shift Assay (EMSA)

The fragments from individual clones were amplified using HiFi supermix (Invitrogen corp.), plasmid DNA as template, and M13-20 and M13-RSP primers specific to pUC19 (27). PCR amplified fragments were purified by the WizardSV PCR cleanup kit (Promega) and eluted in ddH2O. DNA concentration was determined using the A_{260} of the purified DNA. Gel mobility shifts were performed as described (35). Briefly, purified NAC was diluted in buffer 6 (50% Glycerol, 125mM NaCl, 50mM NaH_2PO_4[pH 7.0], 1.25mM $MgCl_2$, 0.5mM 2-mercaptoethanol, 1mg/ml BSA). Buffer 6 or 0.48 pmol of NAC in buffer 6 were mixed with 0.24 pmol of DNA in a volume of 10µl. After 20 minutes at room temperature, 1 µl of 10x loading buffer (25% Ficoll, 100mM Tris-HCl, 10mM EDTA, 0.05% (w/v) cresol red, and 0.05% (w/v) Orange G, pH7.4) was added to each reaction. 10µl of this mixture was loaded onto a gel (5% polyacrylamide buffered with 0.5x TBE) that had been pre-run for 30 min at 10V/cm. Samples were separated at 10 V/cm for 1 hr, stained with ethidium bromide, and visualized with an UV imager (Alpha-InnoTec GmbH).

Primer Extension Analysis

Primer extension was performed essentially as described (42). Briefly, total cellular RNA was purified from Nac^+ and Nac^- strains cultured under nitrogen limitation. Primers of 20-22 nucleotides in length were designed to anneal to appropriate sequences within the first 100bp of the coding sequence of mRNA from the *ureD*, *argP*, *yceO*, *ybjP*, *folA*, *ldcA*, *ygdQ*, *yeiG*, *oppA*, *mltE*, *holE*, *yobA*, *gyrA*, *mreB*, *pduW*, *tuf*, *rpmI*, *secY*, and *glgX* genes (primers sequences available upon request). ^{32}P-labelled primers were annealed with 5µg of total cellular RNA and first strand synthesis was performed using

superscript II reverse transcriptase (Invitrogen) as described (42). Primer extension products were mixed 1:1 with 2x formamide loading buffer (60% v/v Formamide, 4mM Tris HCl pH 7.0, 10mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol), Heated to 95°C for 5 minutes, and 5µl of each product was loaded on a pre-run 8% modified TBE urea PAGE sequencing gel. Gels were run for 1.5-3 hr at 1500 volts, transferred to a filter paper support, dried, and exposed to film at -80°C for 96-300 hours prior to film development.

DNaseI footprinting

Footprinting was performed as described (13). Briefly, 5 µg of purified, single EcoRI digested plasmid DNA was end labeled using Klenow fragment (26). Labeled fragments were counter digested with SalI generating a long fragment containing the labeled insert and a short 12bp labeled fragment. Excess nucleotide and enzyme were removed using Qiagen nucleotide removal kit. Purified fragments were mixed with 0.5µg, 1.58 µg, or 5.0 µg of purified NAC in buffer 6 or buffer 6 alone. Reactions were incubated at room temperature for 45 min. 5 µl of DNaseI core buffer or 5 µl of DNaseI core buffer containing $2.8x10^{-3}$ units of DNaseI (Hoffmann-La Roche Ltd.) was added to each reaction. Reactions were incubated for 1 min at 37°C. Stop and precipitate mix (5µg/ml yeast tRNA, 375mM ammonium acetate, 95% (v/v) ethanol) was added to each reaction and reactions were immediately placed in a dry ice/ethanol bath for at least 15 minutes. Precipitated DNA was pelleted, washed, dried, resuspended in 12 µl of Formamide loading buffer, heated to 90°C for 2 min, and 5 µl of each sample was loaded onto a pre run 8 % modified TBE urea PAGE sequencing gel. To localize footprints, an A+G chemical sequencing ladder was run with each set of samples (3). The resulting

products were separated for 3 hr at 1500 volts constant. The gel was immobilized on filter paper, dried and exposed to film at -80°C for 24 hours.

Database searching

Sequences from the ChIP clones were assembled and submitted as nucleotide BLAST searches to the NCBI non-redundant nucleotide database (34). Regions of identity were analyzed against the sequenced *K. pneumoniae* genomes (12, 21). In cases where the region of highest identity did not occur in *K. pneumoniae* the organism of highest identity is listed in table 1.

Results

NAC Coimmunoprecipitated with known NAC binding sites.

Cell-free lysates prepared from Nac^+ and Nac^- cells grown under nitrogen limiting conditions were incubated with anti-NAC beads (see Materials and Methods). As expected, the α-NAC beads immunoprecipitated NAC protein from Nac^+ cells but were unable to precipitate NAC protein from Nac^- cells (Figure 2.1), confirming that the *nac-2* allele results in cells devoid of the NAC protein. Importantly, NAC was the only detectable species pulled down by this antibody, confirming that the α-NAC beads were highly specific for NAC.

The same stock of anti-NAC beads was used to immunoprecipitate NAC-DNA complexes from a strain carrying the *ureDp1* promoter (known to bind NAC) fused to a promoterless *lacZ* and inserted into the *rbs* locus (13). To test the specificity of the ChIP, the immunoprecipitated and the input DNA samples were probed by multiplex PCR for a known NAC binding site and nearby regions not thought to interact with NAC (Figure

2.2). Figure 2.2A shows the location of the probes used in the multiplex PCR reaction. The promoter fragment (P) is 198bp in length and contains the *ureDp* NAC binding site; the 1kb downstream fragment (1kb ds) is 119bp in length, is located within the *lacZ* ORF, and does not contain any apparent match to the NAC binding site consensus; the 5kb downstream fragment (5kb ds) is 300bp in length, lies in the *yieO* open reading frame downstream from the *ureDp-lacZ* fusion in the *rbs* landing pad (the fragment corresponds to nucleotides 4556246-4556545 in the MGH78578 complete genome sequence), and does not contain any apparent match to the NAC binding site consensus. The multiplex PCR showed that the immunoprecipitated DNA was enriched for the promoter region containing a NAC binding site but was not enriched for downstream regions that did not contain NAC binding sites (Figure 2.2B).

The *K. pneumoniae* NAC Regulon is Large

DNA that coimmunoprecipitated with NAC from two independent ChIP experiments was cloned into pUC19. In total, 299 clones were recovered. The DNA sequence of the cloned material in each of these 299 plasmids was determined and analyzed. These plasmids contained 111 unique DNA fragments. The sequence of these fragments was submitted against the non-redundant nucleotide residue collection at NCBI as BLAST searches to identify the DNA fragment (34). The 111 unique fragments represented 99 different regions of the *K. pneumoniae* chromosome. A list of all 99 unique regions from the library can be found in Table 2.1.

NAC Interacts with library fragments *in vitro*

As an independent confirmation of NAC interaction, an *in vitro* electrophoretic mobility shift assay (EMSA) was performed on each fragment from the library.

Fragment DNA was amplified from the library using the pUC19 specific primers M13-20 and M13-RSP (27). For comparison and as a positive control the well-characterized NAC binding site from *hutUp* was included on each gel (13, 32, 33). At the concentration of NAC used approximately one half of the *hutU* promoter fragment was shifted in the EMSA. A representative gel showing examples of the range of mobility shifts for the library fragments are shown in Figure 2.2. About 49 of the 111 unique fragments (including the fragment carrying the *dppA* promoter and that carrying the KPN_02730 region) showed a mobility shift at least as strong as that seen for the *hutUp* fragment (Figure 2.3). Another 46 fragments (including the *oppA* and KPN_01903 fragments) showed less shifted material than *hutUp* but still significantly higher than control non-specific DNA (Figure 2.3). For 17 of the unique fragments (including KPN_04049), no shifted material was detected in this assay (Figure 2.3). Therefore, 95 of the 111 unique fragments bound purified NAC strongly and stably enough to be detected in an electrophoretic mobility shift assay.

To examine the ability of NAC to directly interact with the promoter regions of the four genes from *E. coli* that show NAC regulation under nitrogen limitation but were not isolated in our screen these regions were amplified via PCR and their ability to interact with purified *K. pneumoniae* NAC was examined. In vitro the *ompF*, *nupC*, and *yedL* promoter regions from *E. coli* all interacted strongly with NAC (data not shown). The *cycA* promoter did not interact with NAC *in vitro* (data not shown). These results will be addressed in the discussion.

The NAC Regulon is Broad

Many of the cloned genes could be identified by their similarity to well characterized genes from *E. coli* or other *K. pneumoniae* strains (Table 2.1). Of the 48 genes for which a function could be assigned, 25 (52%) appear to be involved in nitrogen metabolism. Seventeen of the other genes are homologs of genes for which a putative general function can be ascribed (e.g. *yhfM* or *KPN_01903*). A majority of those (11/17) are also thought to function in nitrogen metabolism. In all, about 55% (36/65) of the genes for which a function is known or hypothesized seem to play a role in nitrogen metabolism. Of the remaining 45%, the majority (18/33) are predicted to function in carbon and energy acquisition (e.g. *glgX* or *malZ*). This suggests that the NAC regulon (and by extension the Ntr regulon) may be broader than previously thought.

NAC regulates genes from ChIP library

To correlate NAC binding with NAC regulation, primer extension was performed on total cellular RNA isolated from Nac^+ and Nac^- strains grown under conditions of nitrogen limitation. Nineteen genes identified in the ChIP library (*ureD*, *argP*, *yceO*, *ybjP*, *folA*, *ldcA*, *ygdQ*, *yeiG*, *oppA*, *mltE*, *holE*, *yobA*, *gyrA*, *mreB*, *pduW*, *tuf*, *rpmI*, *secY*, and *glgX*) were chosen for primer extension analysis. Eight of the nineteen genes (*ureD*, *yceO*, *ybjP*, *folA*, *ldcA*, *oppA*, *gyrA*, and *rpmI*) showed a positive regulation by NAC while six showed negative regulation (*argP*, *ygdQ*, *holE*, *yobA*, *secY*, and *glgX*) (Figure 2.4). NAC regulation of four genes (*yeiG*, *pduW*, *mreB* ,and *tuf*) could not be determined because no primer extension product was observed. One gene (*mltE)* showed no NAC mediated regulation under conditions of nitrogen limitation. This gene is divergently transcribed from *ldcA* and was isolated on the same fragment as *ldcA*, a gene that did

show NAC dependent regulation by primer extension. Thus primer extension data suggest that most if not all of the fragments contain a NAC-regulated gene.

Identification of NAC binding sites

NAC has been shown to bind to DNA with two distinctly different footprints (35). At some promoters NAC binds to DNA as a dimer (at *hutUp, ureDp, dadAp*, and *putAp*) and protects a small region (ca. 25-28bp) from DNaseI (6, 13, 17). This region is centered on a 15 bp core-binding site whose key components have been summarized as ATA-N_9-TAT (13, 32). At other promoters NAC binds to the DNA as a tetramer (at *nacp* and *codBp*) and protects a larger region (ca. 56-74 bp) containing at least one full NAC binding site and one partial NAC binding site (29, 35).

In vitro DNaseI footprints were performed on the ChIP library fragments corresponding to the promoter regions of *dppA* and *oppA* in the presence and absence of NAC. The NAC footprint on the *dppA* fragment protected a region of approximately 27 bp (Figure 2.5A). A footprint of this length is consistent with a dimer of NAC bound to this DNA fragment (13, 35). The protected region of the *dppA* fragment contained one 15 bp sequence that resembled the consensus NAC binding site, varying by only one nucleotide (Figure 2.5B). In addition, the *dppA* site contains an oligo T stretch within the 15 bp core site (Figure 2.5B), a characteristic found in several other sites where NAC binds as a dimer (13, 33). This site also shows a hint of hypersensitivity at one edge that has been seen previously at NAC dimer binding sites (13, 33).

NAC protected a 66 bp region in the *oppA* promoter (Figure 2.5A). Central to the protected region of *oppA* are three NAC induced hypersensitive sites (Figure 2.5A). The protected region on this fragment contains two regions that resemble a consensus NAC

binding site (ATA-N_9-TAT), the first, ATG-N_9-TAT, is also seen in the *nac* promoter (35). This site contains an oligo A stretch a common feature among NAC binding sites. The second region contained a site similar to that found at *dppA* (Figure 2.5B), ATT-N_9-TAT with an internal oligo T stretch within the core 15bp-binding site.

Discussion

To explore the extent of the NAC regulon we utilized ChIP to identify sites on the chromosome that NAC bound *in vivo* under conditions of nitrogen limitation and we were surprised to find that the NAC regulon may not be exclusively limited to operons involved in nitrogen metabolism. Our screen isolated 99 unique regions of the *K. pneumoniae* chromosome. Of the 99 isolates, 88 were isolated only once, suggesting that the screen may not be saturated. Interestingly, some sites that were isolated multiple independent times (e.g. *astA* and *mdtC*) failed to interact with NAC *in vitro* suggesting that other components present *in vivo* might be required for NAC interaction with these promoters. As expected, a majority of the fragments isolated by ChIP were in the promoter regions of genes that are involved in the metabolism of potential nitrogen sources. Some of these fragments contained known NAC binding sites (*ureD*, *dadA*, *gdhA*, and *codB*) that have been previously characterized (13, 14, 17, 19, 29, 32, 33). However, not all characterized NAC binding sites were isolated (*nac*, *hutUH*, and *putP*), also suggesting that the screen may not be saturated.

Microarray data for the NAC regulon in *E. coli* and our ChIP data for NAC binding sites in *K. pneumoniae* present very different pictures of the NAC regulon. Zimmer *et al* identified nine NAC regulated operons (*codBA*, *ompF*, *oppABCDF*,

ydcSTUV, *yedL*, *nupC*, *gabDTPC*, *dppABCDF*, and *fklB-cycA*) in their microarray studies of *E. coli* (43). In *K. pneumoniae* cells that were nitrogen limited, NAC bound to regions near homologs of five of the nine operons found by Zimmer *et al* (*KPN_00577* (*gabD*), *KPN_02036* (*codBA*), *oppA*, *ydcS*, and *dppA*). There are several possibilities as to why four of the *E. coli* operons that demonstrate NAC regulation were not found in our screen. The *yedL* gene of *E. coli* which NAC regulation is not present in any of the sequenced *K. pneumoniae* strains and thus might not be present in our strain either (12, 21, 41). The *flkB-cycA* promoter from *E. coli* did not interact with purified NAC *in vitro* and thus we suspect that the regulation of this gene is indirect. The remaining two promoters of *E. coli*, *ompF* and *nupC*, both interact with purified NAC *in vitro*. The regulation of *ompF* and *nupC* might be different between *K. pneumoniae* and *E. coli*. The regions upstream of *ompF* in *E. coli* show little sequence similarity to the regions upstream of the putative *ompF* homolog in the sequenced *K. pneumoniae* strains suggesting that NAC may not bind to this region in *K. pneumoniae*. One of the regions bound by NAC in *K. pneumoniae* is the region upstream of the *rstB* gene, a member of the RstB/RstA two-component system (30). In *E. coli* it is known that the RstB/RstA two-component system regulates the expression of *ompF*. It is possible that in *K. pneumoniae* that regulation of *ompF* expression by NAC is indirect with NAC regulating the expression of RstB. All three sequenced *Klebsiella* species contain two homologs of the *nupC* gene of *E. coli* suggesting that *nupC* regulation might be different between *E. coli* and *Klebsiella*.

From our data it seems that the NAC regulon of *K. pneumoniae* is substantially larger than that of *E. coli*. There are two possible explanations for the apparent difference

in the size of the NAC regulon between these two species. The first explanation is that the NAC regulon in *K. pneumoniae* actually is substantially larger. Some of the genes regulated by NAC in *K. pneumoniae* are not present in *E. coli* K12, an example is the *ureDABCEFG* operon, which is known to be NAC regulated in *K. pneumoniae* but is not present in *E. coli* K12. In fact, 43 of the 99 unique isolates from *K. pneumoniae* have no homolog in the sequenced K12 strains of *E. coli*. The second possible explanation is that microarray data might not show NAC regulation of genes that require both NAC and a physiological induction. An example would be the *gcvT* promoter. In *E. coli* it has been established that the *gcvT* promoter is repressed by the regulator GcvA in the absence of the physiological inducer, glycine (40). Such genes, requiring induction, might have mRNA levels that might not respond to NAC under repressing conditions.

Primer extension data shows that NAC regulation correlates well with NAC binding during nitrogen limitation. The only promoter that did not show NAC regulation, *mltE*, was isolated on the same fragment as *ldcA*, a gene that demonstrates NAC regulation under nitrogen limitation. The large size of the NAC regulon in *K. pneumoniae* suggests that the ability to use many compounds as a sole source of nitrogen may have been achieved via an expanded NAC regulon.

It had been suggested in *E. coli* that *dppAp* and *oppAp* might be controlled by RNA polymerase associated with the alternative sigma factor σ^{54}, suggesting direct regulation by the Ntr system, but these promoters seem to be regulated by NAC in *E. coli* and associated with NAC in *K. pneumoniae* under nitrogen limitation (43). An *in silico* search of the promoter regions of the *K. pneumoniae* MGH78578 *oppA* and *dppA* revealed no matches to the σ^{54} consensus site (37), supporting the conclusion that these

promoters are regulated by NAC, which would suggest that RNA polymerase associated with σ^{70} controls these promoters. A search of these promoters from *K. pneumoniae* and *E. coli* demonstrate that they contain a close match to the σ^{70} consensus sequence that is conserved between the two species (1, 2). The predicted start of transcription from our primer extension analysis matches well with this predicted promoter. Additionally, our primer extension analysis of *oppA* demonstrates NAC dependant regulation under nitrogen limitation supporting the regulation of *oppA* by NAC seen in *E. coli*. The region protected by NAC lies approximately 100-150 nucleotides upstream of the predicted promoter.

As expected, the large collection of sites to which NAC binds *in vivo* under nitrogen limitation contains many operons involved in nitrogen metabolism. Under nitrogen limitation, NAC also associated with 18 operons involved in carbon and energy acquisition suggesting that NAC may be regulating these operons. These results were unexpected since the *E. coli* NAC regulon seems to regulate only genes involved in nitrogen metabolism (43). In enteric bacteria, carbon and nitrogen metabolism are intimately linked. Nitrogen limitation is perceived as carbon excess (24) and carbon limitation is perceived as nitrogen excess (5). Thus it would not be unreasonable to find NAC playing a role in balancing carbon and nitrogen metabolism. Interestingly, a small number of the operons associated with NAC *in vivo* are involved in cellular processes associated with controlling growth rate such as transcription (*rstB*, *slrR*, KPN_*00054*, *KPN_02732*, *KPN_03248*, *KPN_03329*, and *KPN_03826*), translation (*tuf*, *rpmI*, and *ykgO*), DNA replication (*holE, gyrA*), and cell division (*mreB*). Under growth rate limiting conditions cells must modify a range of cellular processes to allow a slower

growth rate in response to a lack of macromolecular building-blocks (38), and perhaps NAC provides a fine-tuning to this regulatory necessity.

In summary, the NAC regulon of *K. pneumoniae* is both larger and broader than expected. The nitrogen stress regulon may include not only genes to provide nitrogen, but also genes to balance carbon and energy metabolism and growth rate in response to limitation of nitrogenous building blocks. This suggests an expanded role of NAC regulation in *K. pneumoniae* during nitrogen limitation where NAC regulates a large number of nitrogen metabolism genes but also plays a role in tuning cellular process to the stressful conditions of rate limiting growth.

Name[a]	EMSA[b]	Presumed Function
Previously Characterized		
dadA	+	D Amino Acid Dehydrogenase
ureD(2)[c]	+	Urease
gdhA (4)	+	Glutamate Dehydrogenase
codB [KPN_02036][d]	-	Cytosine deaminase
Present in *E.coli*		
abgR	+	Putative Transcriptional Regulator LysR type
argA	+	N-acetylglutamate Synthase
argP (2)	+	DNA Binding Transcriptional Activator
argR		Argining Repressor
aroP	+	Phenylalanine/tyrosine/tryptophan APC transporter
aspA	+	Aspartate ammonia-lyase
astA (2)	-	Arginine succinyltransferase
betA [KPN_00584]	+	Choline dehydrogenase
cysE	+	Serine acetyl transferase
pagP [crcA]	+	Palmitoyl transferase for lipidA
dapB	+	dihydrodipicolinate reductase
dppA	+	Dipeptide ABC transporter
dos	-	Heme Regulated phosphodiesterase (MG1655)[e]
folA	+	Dihydrofolate reductase
gcvT (2)	+	Aminomethyltransferase
glgX	+	Glycogen phosphorylase-limit dextrin α-1,6-glucohydrolase
glpB	-	Anaerobic glycerol-3-phosphate dehydrogenase (MG1655)
gyrA	+	DNA gyrase subunit A
gshA	+	Glutamate-cysteine ligase
kduD	+	2-deoxy-D-gluconate 3-dehydrogenase
leuO	+	Leucine Transcriptional Activator
ldcA	+	L, D-carboxypeptidase
lipA	+	Lipoate Synthase
lpxD	+	UDP-3-O-[3-hydroxymyristoyl]glucosamine N-acetyltransferase
malZ	-	Maltrodextrin Glucosidase
mdtC [yegO] (3)	-	Multidrug efflux pump
mreB	+	Longitudinal peptidoglycan synthesis
mtfA [yeeI]	+	mlc titration factor
oppA	+	Oligopeptide ABC transporter
pduW	+	Propionate Kinase
rpmI	+	Ribosomal protein L35
rstB	+	Sensor Kinase of rstBA system
rtn	+	EAL domain containing lipoprotein
secY [prlA]	+	Preprotein translocase secY

slt	+	Lytic murine transglycosylase, soluble
srlR	+	Sorbital utilization regulator
tfaD	+	DLP12 prophage tail fiber assemnbly protein (MG1655)
tuf	+	Elongation Factor Tu
yabI	+	Conserved inner membrane protein
ybhK	+	Putative phosphatase sulfatase
ybjP	+	Predicted lipoprotein
yceO	+	Hypothetical protein involved in biofilm formation
ydcS	+	Putative Spermadine/Putrescene transporter
yebO	+	Predicted protein of unknown function
yebW	+	Predicted protein of unknown function
yeiG (2)	+	S-lactoylglutathione hydrolase/ S-formylglutathione hydrolase
ygdQ	+	Predicted inner membrane tranport protein
ygbL	-	Putative aldolase
ygeG	+	Predicted chaparone (E.coli MG1655)
yhfM(2)	+	Putative Amino acid/amine transporter (fructoselysine)
yjcD	+	Predicted Permiase
ykgO	+	Predicted ribosomal protein (MG1655)
yobA/holE	+	yobA: conserved protein holE: DNA polymerase III θ subunit
yohC/yohD	+	yohC: transport protein yohD: integral membrane protein
ECUMN_2308	+	Conserved hypothetical protein (E.coli UMN026)
ECUMN_2314	+	Hypothetical protein (E.coli UMN026)

Present in Klebsiellae

phnV	+	Spermidine transport system E
KPN_00054	+	Two component system sensor kinase
KPN_00115	+	Sodium galactoside symporter family permease
KPN_00525	-	Putative aldol/keto reductase of the tas family
KPN_00576/577	+	576: put. Oxidoreductase 577: 4-aminobutyrate amino acid transferase
KPN_00598	+	Putative ABC transporter
KPN_00637	-	Putative Kinase
KPN_00644	+	Phosphoglycolate phosphatase
KPN_01054	+	Putative arginase/agmatinase/formimionoglutamate hydrolase
KPN_01121	+	4-hydroxy-L-threonine phosphate dehydrogenase
KPN_01319	-	7 alpha hydroxysteroid dehydrogenase
KPN_01711	+	Hypothetical protein
KPN_01755	+	Putative transporter
KPN_01781	+	Glutamine Amidotransferase
KPN_01789/01790	+	1789: put. transcriptional regulator 1790: put. Allantoin permiase
KPN_01903	+	Luciferase like monoxygenase
KPN_02168	+	Putative outermembrane lipoprotein
KPN_02171	+	Putative LysR Regulator

KPN_02570	+	Putative phosphinothricin N-acetyltransferase
KPN_02730 (2)	+	Hypothetical protein involved in capsule biosynthesis
KPN_02732	+	Putative CynR cyanate utilization regulator
KPN_03071	-	Maltoporin precursor
KPN_03248	+	Repressor of gntKU and gntT involved in gluconate utilization
KPN_03328/3329	+	3328: conserved protein yqfB 3329: putative regulator
KPN_03826	+	Putative Regulatory protein
KPN_03937	-	Putative Electron Transport protein Fe-S cluster
KPN_04048	-	Hypothetical Protein
KPN_04411	+	Putative cytochrome oxidase
KPN_04634	+	Putative cytoplasmic protein
KPN_04639	+	Putative selanocysteine synthase
KPN_04732	+	Oxidoreductase similar to some short chain dehydrogenases
KPN_pkpn3p05913	+	Hypothetical protein of unknown function
wbbO	+	Galactosyl transferase (K.pneumoniae NTUH-K2044)
wcaN	+	Predicted subunit with galU (K.pneumonaie NTUH-KP13)
KPK_4440	+	Radical SAM domain protein (K.pneumoniae 342)
Present in *K.penumoniae* W70 and other Organisims		
smal_2561/2562	-	smal_2561 agmatine deaminase or smal_2562 LTTR (S.maltophilia R551-3)
Unique (Not in NCBI)[f]		
Unique A	+	Unknown
Unique B	+	Unknown
Unique C	+	Unknown

[a]If no ORF was located on the fragment the name of the nearest ORF is listed.

[b]Presence of a shifted band in the presence of NAC during an Electrophoretic Mobility Shift Assay (EMSA).

[c]Number in parenthesis indicates number of independent isolates when greater than 1

[d]Gene name from *K.pneumoniae* MGH78578 when different than *E.coli* K12 is listed in brackets.

[e]In cases where MGH78578 is not the organism with highest identity, the organism of highest identity is listed.

[f]Sequences have no matches of significant similarity in the non-redundant NCBI sequence database.

Table 2.1: A list of genes in chromsomal regions bound by NAC in conditions of nitrogen limitation in *K. pneumoniae* W70

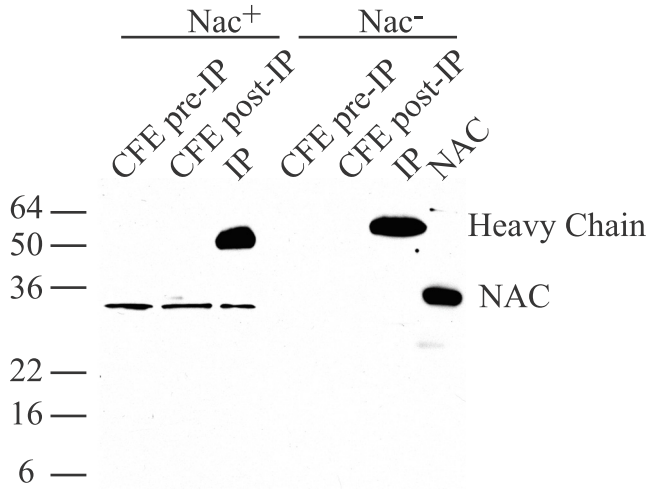

Figure 2.1: Specificity of precipitation by anti-NAC beads. Nac^+ and Nac^- cells were grown under nitrogen limitation. Samples of cell-free extract (CFE) were taken before (pre-IP) and after (post-IP) incubation with anti-NAC beads and separated by SDS-PAGE. A western blot was prepared and developed as described in the materials and methods with rabbit anti-NAC as the primary antibody and goat anti-rabbit IgG conjugated to HRP as secondary antibody. The intense cross-reacting bands in the IP samples are IgG heavy chain reacting with the secondary antibody

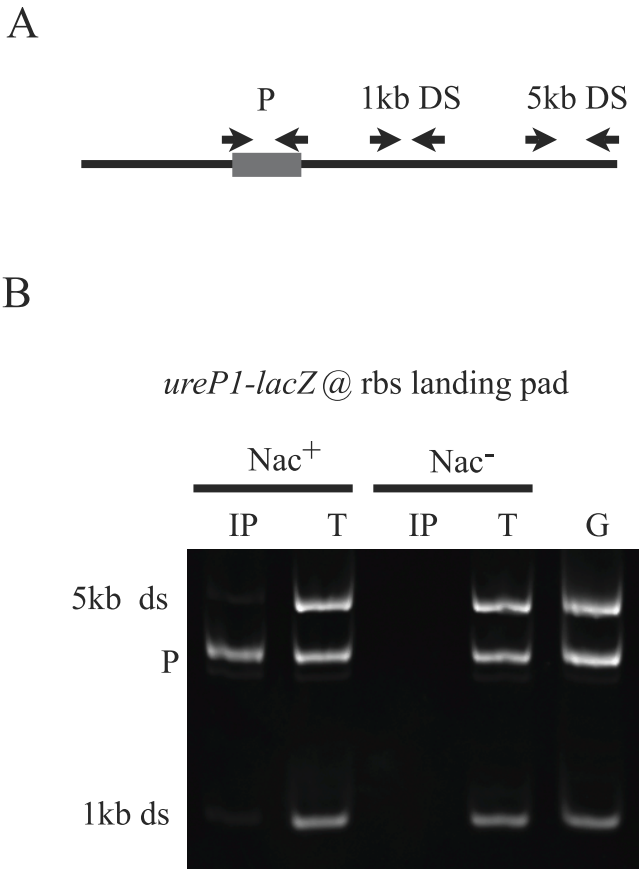

Figure 2.2: Selectivity of NAC:DNA co-immunoprecipitation. (A) A schematic diagram of the regions targeted by the multiplex PCR of ChIP samples. The gray box indicates the promoter region containing the NAC binding site. Arrows indicate primers used to amplify target fragments within the promoter (P), 1kb downstream (1kb DS), or 5kb downstream (5kb DS) of the NAC binding site. (B) Results of multiplex PCR on immunoprecipitated DNA [IP] or total input DNA [T] from a chromatin immunoprecipitation experiment on nitrogen limited Nac^+ and Nac^- strains. As a control, untreated genomic DNA from a Nac^+ strain was also subject to multiplex PCR and run on the same gel [G]. Multiplex PCR samples were separated on 5% TBE-PAGE gels and stained with ethidium bromide.

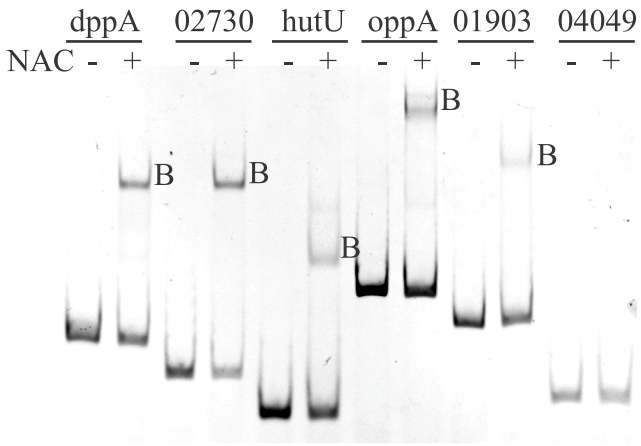

Figure 2.3: Electrophoretic mobility shift assay of ChIP clones. Fragments were selected to show the representative range of NAC binding seen for different fragments. The *hutUH* promoter fragment was used as a control on this and all gels. Reactions contained 0.24pmol of DNA and either buffer 6 (-) or 0.48pmol of NAC(+). Reactions were separated on 5% TBE-PAGE and stained with ethidium bromide. Fragment sizes were: dppA (179bp), KPN_02730 (159bp), hutU (107bp), oppA (277bp), KPN_01903 (232bp), and KPN_04049 (130bp). Bands corresponding to NAC-DNA complexes are labeled with a B.

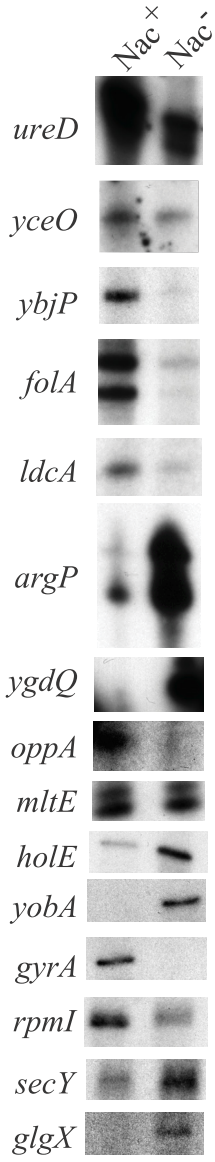

Figure 2.4: Primer extension analysis of mRNA from Nac^+ and Nac^- strains grown under conditions of nitrogen limitation. Targets were chosen from those found in the ChIP screen (Table 2.1).

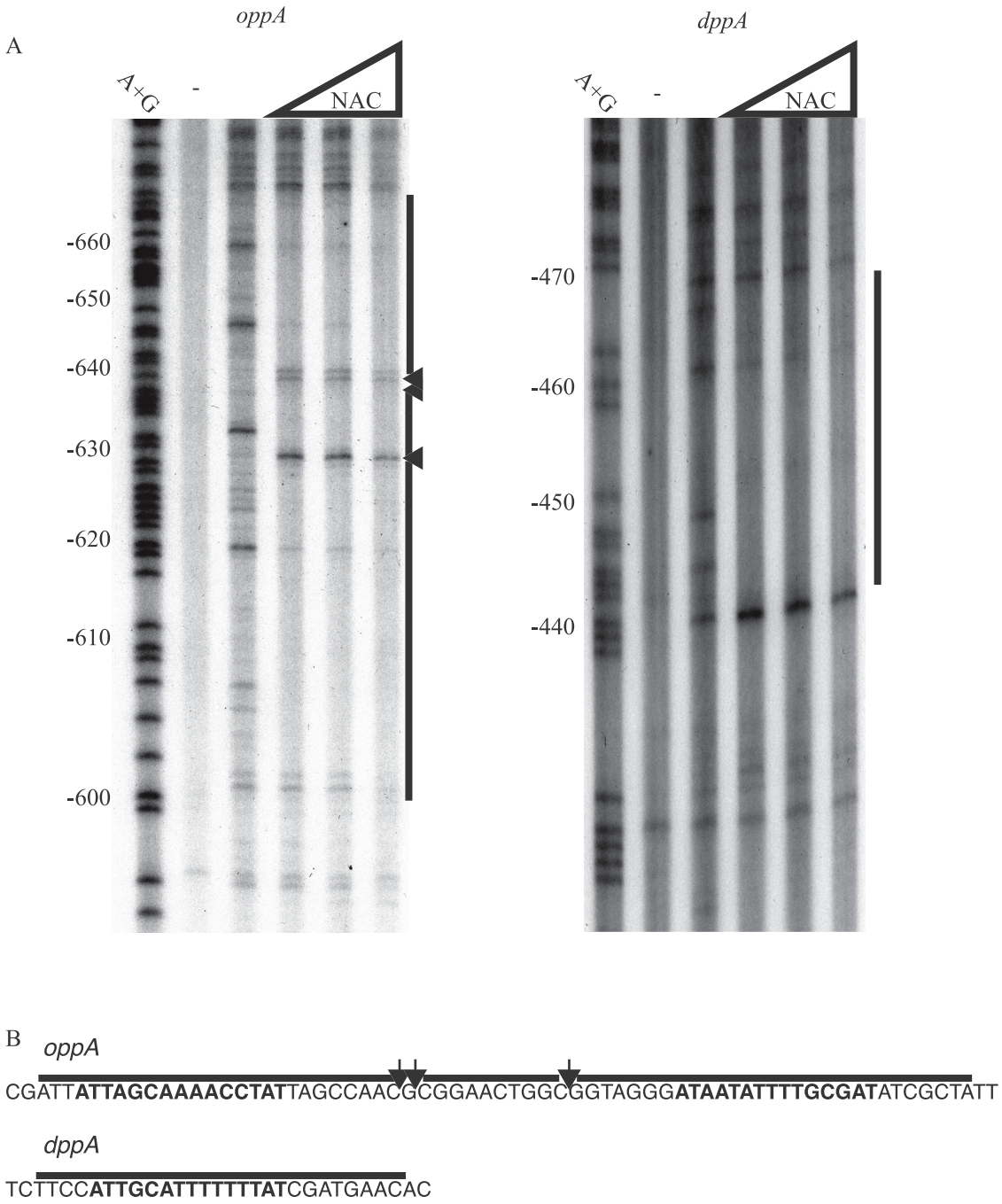

Figure 2.5: Template strand footprints of NAC bound to *oppA* or *dppA* promoter fragments. (A) End labeled DNA fragments were mixed with buffer only or increasing concentrations of NAC prior to treatment with DNaseI. A chemical A+G ladder of each fragment was also generated. Samples were separated on an 8 % modified TBE urea gel, dried, and exposed to film. A solid line next to the gel shows protected regions. Arrowheads indicate hypersensitive sites. (B) The DNA sequence of footprinted regions of *oppA* and *dppA* fragments. Lines above the sequence indicate regions of the template strand protected from DNaseI in the presence of NAC. Arrowheads indicate hypersensitive sites. Bold text indicates close matches to the NAC consensus site.

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Chapter III

Properties of the NAC-Binding Site within the *ureD* Promoter of *Klebsiella pneumoniae*

Abstract

The Nitrogen Assimilation Control protein (NAC) is a LysR-type transcriptional regulator that activates transcription when bound to a DNA site centered at a variety of distances from the start of transcription. The NAC-binding site from the *hutU* promoter (NBS^{hutU}) is centered at -64 relative to the start of transcription, but can activate the *lacZ* promoter from sites at -64, -54, -52, and -42, but not from sites at -47 or -59. The NBSs from the *ureD* promoter (*ureDp*) and *codB* promoter (*codBp*), however, are centered at -47 and -59, respectively, and NAC is fully functional at these promoters. To understand this apparent contradiction, we compared the activity of NBS^{hutU} and NBS^{ureD} within the context of *ureD*p as well as within *codBp*. NBS^{hutU} was functional at -47 within *ureDp* and at -59 within *codBp*. NBS^{hutU} is asymmetric with its promoter-proximal half site required for allowing NAC to activate transcription and its promoter-distal half-site required for tight binding of NAC. NBS^{ureD} is also asymmetric with its promoter proximal half site required for allowing NAC to activate transcription, however, both of the half sites in NBS^{ureD} allow tight binding of NAC. The asymmetry is even more pronounced for NBS^{ureD} than for NBS^{hutU}; inverting NBS^{ureD} abolished more than 99% of NAC's ability to activate *ureDp*. The key to the asymmetry lies in the fourth triplet of a

15 bp consensus binding sequence, especially the G at position -42 or the T at -44. Changing G-42 to T or T-44 to G drastically reduced the ability of NAC to activate transcription (to 6% and 15% of wild type, respectively). This suggests that the positional specificity of a NBS is dependent on the promoter in question and is more flexible than previously thought, allowing considerable latitude both in distance and face of the DNA helix for the NBS relative to RNA polymerase.

Introduction

Many transcriptional regulators in bacteria activate transcription from multiple classes of binding sites. One of the best-characterized examples of regulators with multiple classes of activation binding sites is the CRP/FNR class of bacterial transcriptional regulators (10). The CRP protein has three classes of binding site, each with a unique spacing with respect to the start of transcription (1, 8). Each of the classes of the CRP binding site has unique contacts with RNA polymerase representing different faces of the CRP dimer, and these contacts are important for the ability of CRP to activate transcription from the specific class of site (10). The elucidation of the mechanism at each class of binding site has led to greater understanding of how the CRP/FNR class of regulators activates transcription. Another regulator with binding sites at different positions is the Nitrogen Assimilation Control protein (NAC) from *Klebsiella pneumoniae*.

NAC mediated transcriptional activation has been characterized at five promoters (*hutU*, *dadA*, *putP*, *codB*, and *ureD*). Each of the promoters contains a dimer binding site that is centered at a different spacing with respect to the start of transcription; e.g., *putP* (-65), *hutU* (-64), *codB* (-59), *ureD* (-47), and *dadA* (-44). This pattern suggests that there

may be multiple classes of NAC binding sites (2, 7, 9, 11, 17). Interestingly, unlike the different classes of CRP binding sites, which vary by spacing, the NAC binding sites vary by both spacing and face of the DNA helix (e.g. *hutU* and *codB* are one half a turn of the helix apart placing NAC on opposite faces of the helix) suggesting that the sites could either be classified based on relative distance or by the face of the helix with respect to the start of transcription.

NAC mediated transcriptional activation of the *hutU* promoter is the best characterized (19). NAC protects a region of 26 bp from DNaseI digestion while bound to the promoter as a dimer (7). Included in the 26 bp protected region of NAC activated promoters is a 15 bp site, which is essential for binding and activation, and which has a consensus sequence among the NAC activated promoters of ATA-N_9-TAT (19). All the necessary information for NAC transcriptional activation from the *hutU* promoter NAC binding site (NBS^{hutU}) is contained within the 26 bp protected site, which is able to bring the *lac* promoter under control of the nitrogen regulon in a NAC dependent manner (18). The 26 bp protected NAC binding site of the *hutU* promoter is able to bring a *lac* promoter under control of NAC not only at the native spacing of -64 but also when centered at -54, -52, and -42 but not when centered at -74, -69, -59, -49, or -47, which suggested that NBS^{hutU} might require the appropriate face of the helix rather than a unique distance to allow NAC mediated transcriptional activation (18). Interestingly, the *ureD* and *codB* promoters have NAC binding sites centered at positions that were forbidden for NBS^{hutU} at a *lac* promoter (-47 and -59, respectively), suggesting that they might represent a separate class of NBS.

NBS^{hutU} is asymmetric with the proximal half site providing strong transcriptional activation by NAC and the distal half site providing strong NAC-DNA interaction (19). Perturbation of either the proximal or distal half site leads to decreased NAC mediated activation of the *hutU* promoter either by damaging the ability of bound NAC to activate or by interfering with NAC-DNA interactions, respectively (19). NBS^{hutU} requires the proximal and distal half sites in the appropriate orientation for strong NAC binding and activation in a directional manner. The locations of the NBSs at *hutUp* (-64) and *ureDp* (-47) are such that the promoter proximal edge of a NAC dimer bound at NBS^{hutU} and the promoter distal edge of a NAC dimer bound at NBS^{ureD} might be in the same general position relative to RNA polymerase. This suggested that NBS^{ureD} might be functionally inverted relative to NBS^{hutU}. Little was known about the requirements for NAC transcriptional activation at *ureDp*. The inability of NBS^{hutU} to function in the context of the *lac* promoter at the -47 and -59 position raised the question: Is the NBS from the *ureD* promoter functionally different from the *hutU* NBS?

Materials and Methods

Strains and Bacterial Growth

K. pneumoniae strains used in this study were derived from strain W70 (14). Strains used in this study are shown in table 3.1. Cells cultured under nitrogen limiting conditions were grown in W4 salts containing 0.4% (w/v) glucose and 0.2% (w/v) monosodium glutamate (13). For general use as rich medium for cloning and propagation of cells L Broth was used (16). For selection of alleles and plasmids antibiotics were used at the following concentrations: ampicillin (100μg/ml), kanamycin

sulfate (50μg/ml), streptomycin sulfate (50μg/ml), and tetracycline hydrochloride (25μg/ml). Solid media were supplemented with 1.5% Bacto Agar.

Genetic Techniques

DNA manipulation was carried out as described by Maniatis (15). Mutant promoters were amplified via PCR using wild-type promoter templates and primers containing the desired mutations. In this study the 15 bp essential NAC binding site plus 6 bp upstream and downstream were used as the NAC binding site. The sequences of the sites in the study are NBS^{ureD} GATGACATAAGCGTTTCGTATGACCGG, NBS^{codB} CTCATTCATATAAAAAATATATTTCCCC, and NBS^{hutU} CGCAATATAACAAAATTGTATCATTTC. The *ureDp* fusions consist of 120 bp of the promoter region plus the first 64 bp of *ureD* fused to the *lacZ* open reading frame (11). The *codBp* fusions contain 127 bp of the promoter region plus the first 76 bp of *codB* fused to the *lacZ* open reading frame (17). The DNA sequences of primers used in this study are available on request. PCR products were cloned into the EcoRI and BamHI sites of pRS415 (21). The DNA sequence of each cloned fragment was determined to ensure that no unwanted mutations had been introduced. In order to integrate the fusions into the chromosome of *K. pneumoniae*, the fusions were subcloned into a *pir*-dependent vector, pCB1583, based on pKAS46 (6, 22). Fusions were integrated into the *rbs* landing pad as previously described (6). Transduction of the *nac-2* allele, an in-frame deletion of the NAC open reading frame with the *aph-1* cassette replacing the deleted material (3), was performed utilizing $P1_{vir}$ grown on KC5447 as previously described (5).

NAC Purification

K. pneumoniae NAC was purified from *E. coli* cells as described (7). Purity was monitored via SDS-PAGE and staining by the method of Fairbanks (4). Purified protein was quantified by the method of Lowry relative to a BSA standard (12). Protein was diluted 1:1 in glycerol and stored at -20^{o}C.

Electrophoretic Mobility Shift Assay

Gel mobility shifts were performed with purified NAC as described (7). Briefly, 6µl (0.08 pmol) of purified PCR-amplified fragments of *ureDp*, *codBp*, or mutant promoter regions were mixed with 4µl of buffer 6 (50% Glycerol, 125mM NaCl, 50mM NaH_2PO_4 [pH 7.0], 1.25mM $MgCl_2$, 0.5mM 2-mercaptoethanol, 1mg/ml BSA) containing 0, 0.07, 0.14, or 0.28 pmol of purified NAC. Reactions were incubated at room temperature for 20 min. 1µl of 10X loading buffer (25% Ficoll, 100mM Tris-HCl, 10mM EDTA, 0.05% (w/v) cresol red, and 0.05% (w/v) orange G, pH7.4) was added to each reaction. 10µl of each reaction was loaded on a pre-run gel (5 % polyacrylamide buffered with 0.5x TBE). Bound and unbound species were separated by electrophoresis at 10V/cm for 60 min. Gels were stained with ethidium bromide (40µg/ml) and then destained with water. The mobilities of the dimer-bound and tetramer-bound promoter fragments were determined by comparison to known shifts of *ureDp* (dimer) and *codBp* (tetramer) as shown previously (17, 20).

β-galactosidase Assay

10 ml cultures were grown in 125 ml sidearm flasks at 30^{o}C and 250 rpm to mid-log phase (50 Klett units, ca. 1.2 x 10^8 cfu/ml). Cells were washed once with one culture volume of cold 1% KCl. Cells were resuspended in 1/10 culture volume of cold 1% KCl. Assays were performed on whole cells permeabilized by detergent as previously

described (13). Three different volumes of 10X cells were assayed for each culture and each strain was cultured at least three independent times. Values for specific activity are reported as nanomoles of product formed per minute per milligram total protein (as determined by the method of Lowry) at 30^{o}C (12).

Results

NBS^{hutU} functions in the *ureD* and *codB* promoters

Since NBS^{hutU} was unable to function from the spacing of -47 or -59 at the *lac* promoter we wanted to test whether there were two classes of NAC binding sites. Both the *ureD* and *codB* promoters demonstrate strong NAC mediated activation even though NBS^{ureD} is centered at -47 and NBS^{codB} is centered at -59, forbidden positions for NBS^{hutU} in the context of the *lac* promoter. NBS^{hutU} (15 bp core + 6 bp upstream and downstream) was used to replace the native NBS within the context of the *ureD* or *codB* promoters. The core binding sites share identity only at the first and last triplet of the 15 bp consensus (Figure 3.1A). Since NBS^{hutU} was unable to function at *lacZp* from -47 and -59 we might expect NBS^{hutU} to be non-functional from -47 at *ureDp* and from -59 at *codBp*.

NAC formed a stable interaction, as assayed by electrophoretic mobility shift assay (EMSA), with *ureDp* containing NBS^{hutU} and *codBp* containing *NBS*^{hutU} (Figure 3.1B). NAC interacted with NBS^{hutU} as a dimer within the context of *ureDp* and as a tetramer within the context of *codBp* determined as described in the materials and methods. This is not surprising since NAC associates with the native *ureDp* as a dimer and the native *codBp* as a tetramer (20). Since NAC was able to associate with NBS^{hutU}

in both *ureDp* and *codBp*, the ability of NAC to activate transcription from these constructs was tested.

Transcriptional fusions joining the *ureD* and *codB* promoters and these same promoters containing NBS^{hutU} to the *lacZ* open reading frame were integrated in single copy into the *K. pneumoniae* chromosome in Nac^+ and Nac^- strains. The strains were grown under nitrogen limiting conditions and assayed for β-galactosidase activity (Figure 3.1C). Transcription of all four promoters was dependent on NAC. The NAC mediated activation seen from the *ureD* promoter containing NBS^{hutU} was similar to the 8-fold activation of the native *hutUp* by NAC (19). NBS^{hutU} was also able to activate transcription when positioned at -59 in the context of the *codB* promoter. In this case, the maximal activation was similar to the maximal activation seen for the wild type *codB* promoter rather than the wild type *hutUp*. Clearly, the positions of -47 and -59 are not forbidden for NBS^{hutU}, at least in the context of the NAC activated promoters, *ureDp* and *codBp*. The basal level of activity from both the *ureD* and *codB* promoters containing NBS^{hutU} was higher than that of the wild-type *ureD* or *codB* promoters, and this will be addressed in the discussion.

NBS^{ureD} is asymmetric

NBS^{hutU} is asymmetric, and its asymmetry is important for NAC activation of the *hutU* promoter (19). Within the context of *hutUp*, the promoter-distal half-site of NBS^{hutU} is important for strong binding of NAC and the promoter-proximal half-site is important for activation of transcription by NAC (19). We reasoned the NBS^{ureD}, located closer to the promoter, might display different requirements. The role of each half-site of NBS^{ureD} was examined by creating mutant constructs with either two distal or two

proximal half-sites (Figure 3.2A). The distal half-site (shown in the open arrow) was used to replace the proximal half site (shown in the cross-hatched arrow) to create the construct referred to as distal-distal (D-D). The opposite replacement was made to create the proximal-proximal construct (P-P). NAC bound to promoters containing the D-D and P-P NBS mutants in a manner similar to the wild type *ureD* promoter (Figure 3.2B). This suggested that unlike NBS^{hutU}, both half sites of NBS^{ureD} were sufficient for strong NAC binding.

Since both the D-D and P-P bound NAC well, we next tested their ability to function in NAC-mediated activation of *ureDp*. Fusions of *ureD* promoters containing the P-P or D-D mutant NBSs to *lacZ* were integrated in single copy into the *K. pneumoniae* chromosome in Nac$^+$ and Nac$^-$ strain backgrounds. NAC was unable to activate transcription from the promoter containing the D-D mutant NAC binding site (Figure 3. 2C), even though NAC was capable of binding strongly to this site (Figure 3.2B). In contrast, the NAC mediated activation of the promoter containing the P-P mutant was even stronger than the wild type *ureD* promoter (Figure 3.2C). These data suggested that at least one proximal half site is required for NAC transcriptional activation from NBS^{ureD}.

NBS^{ureD} is directional

We next asked if the orientation of the proximal half site was important. To test this we constructed *ureD* promoters containing an inverted NBS^{ureD} or a scrambled NBS^{ureD} (Figure 3.3A). As expected, both the inverted NBS and the wild-type NBS bound NAC well, and the scrambled NBS failed to bind NAC (Figure 3.3B). Single copy promoter-*lacZ* fusions with either the inverted or scrambled NAC binding site were

integrated into the *K. pneumoniae* chromosome. Not surprisingly, the scrambled site to which NAC did not bind *in vitro* demonstrated little or no NAC-mediated transcriptional activation (Figure 3.3C). The inverted site, to which NAC bound well *in vitro*, also showed very little NAC-mediated activation, with only 5% as much as the wild-type NBS (Figure 3.3C). This suggested that NBS^{ureD} requires the proximal half-site in the appropriate orientation for NAC to activate transcription.

The Fourth trinucleotide is critical for NAC activation

The D-D mutant that failed in activation despite strong binding contained only two changes from the wild-type NBS^{ureD}. The two changes occurred in the fourth triplet of the five triplet NAC consensus binding-site, and the mutations correspond to nucleotides -44 and -42 with respect to the start of transcription (Figure 3.4A). Since the D-D construct had lost NAC mediated transcriptional control of the *ureD* promoter, the role of each of these changes, T-44G and G-42T, was assessed.

EMSA was performed with *ureD* promoters containing the D-D, T-44G, and G-42T NBS^{ureD} mutations. NAC stably interacted with both single mutations about as well as it did with the wild type site (Figure 3.4B). Single copy promoter-*lacZ* fusions of the mutant promoters integrated into the *K. pneumoniae* chromosome were assayed in Nac^+ and Nac^- backgrounds (Figure 3.4C). The promoter that contained the G-42T mutation had NAC-mediated activation that was only 6% of the wild-type *ureDp* (Figure 3.4C). The promoter that contained the T-44G mutation was slightly better, with 15% of the NAC-mediated activation of wild type *ureDp* (Figure 3.4C). These data suggest that nucleotides T-44 and G-42 work synergistically to elicit NAC-mediated activation of the *ureD* promoter.

Two nucleotides in the fourth triplet of the core NBS^{ureD} were critical for NAC-mediated activation of the promoter but were non-essential for NAC binding to the promoter. This raised the question, does the other nucleotide within the fourth triplet, C-43, which remained unchanged in both the P-P and D-D constructs, play a role in NAC-mediated transcriptional activation of the *ureD* promoter. The role of C-43 in NAC mediated transcriptional activation of the *ureD* promoter was examined by changing C-43 to the other three nucleotides creating the constructs C-43A, C-43T, and C-43G (Figure 3.5A). The ability of these three constructs to interact with NAC *in vitro* and *in vivo* was examined to determine the role of C-43 in both NAC binding and NAC mediated transcriptional activation under nitrogen limiting conditions.

NAC bound all three of the C-43 substitution mutations *in vitro* (Figure 3.5B). Single copy integrants of the mutant promoters fused to *lacZ* were assayed in the Nac$^+$ and Nac$^-$ backgrounds (Figure 3.5C). All three of the promoters containing NAC binding sites with C-43 substitution mutations showed less activation in response to NAC than the wild-type *ureD* promoter. The C-43A and C-43T mutations had slightly less NAC mediated activation, 65 % and 61 % of the wild type *ureDp* activity, respectively (Figure 3.5C). The promoter containing the C-43G mutant had activation that was 30 % of the wild type *ureD* promoter.

Discussion

Previous data had suggested the NAC-mediated activation of transcription required that NAC be located on the same face of the DNA helix as RNA polymerase but that the distance from the RNA polymerase was somewhat flexible (18). The NBS^{hutU}

brought the *lac* promoter under control of NAC when placed at -64, -54, and -42, all positions on the same face of the helix. Transcriptional activation by NAC at the closer distances was weaker than that seen from -64, suggesting that NBSs located close to the promoter are less effective (18). The NBS^{hutU} failed to bring the *lac* promoter under control of the nitrogen regulon when located at -69, -59, and -47, all positions on the opposite face of the helix (18). Interestingly, NBS^{ureD} is located at the "weaker" spacing and on the "forbidden" face of the helix and demonstrates strong NAC dependent transcriptional activation (11). The data presented here show that NBS^{hutU} was able to activate the *ureD* promoter even at -47, a position where it failed to activate the *lac* promoter. In addition, NBS^{hutU} activated the *codB* promoter even when at -59, a position from which it failed to activate at the *lac* promoter. Thus, there appears to be considerable flexibility in how NAC activates transcription. We noted that many constructs with altered NBSs had elevated levels of basal activity when compared to the wild type promoters. The replacement of NBS^{ureD} with NBS^{hutU} changed the nucleotide composition as close to the promoter as -34, and these changes increased the A/T richness of the -35 region. Likewise, replacement of NBS^{codB} with NBS^{hutU} increased the A/T richness of the -60 region of the *codB* promoter. These changes appear to have improved the strength of the promoters and increased their basal level of expression. Nevertheless, NAC was still able to activate expression from these promoters.

NBS^{hutU} contains two functionally important regions; the promoter-distal half-site (D) that is important for NAC binding affinity and the promoter-proximal half-site (P) that is important for NAC transcriptional activation of the *hutU* promoter (19). For NAC to yield strong activation, the P-site must be located in the proper orientation (facing the

promoter), suggesting that for NBS positioned at -64 the activation of transcription by NAC occurs in a directional manner (19). The different locations of NBS^{hutU} and NBS^{ureD} at their native promoters might suggest that if the protein extends to the edge of its footprint, the monomer of the NAC dimer associated with the P-site of NBS^{hutU} might be at a position similar to that of the monomer associated with the D-site of NBS^{ureD}. Thus we might have expected that the D-site of NBS^{ureD} might be the active region, in contrast to the case with NBS^{hutU}. Surprisingly, it was the P-site of NBS^{ureD} was important for NAC mediated transcriptional activation from this site rather than the D-site as hypothesized. The DNA sequence of the fourth triplet of NBS^{ureD} (the only triplet mutated in the D-D construct) suggested that T-44 and G-42 might play a synergistic role in NAC-mediated transcriptional activation of *ureDp*. The activation by NAC from NBS^{ureD} at the position of -47 is also directional, requiring not only that a P-site be present but also that it be in the appropriate orientation.

The observation that it is the proximal half-site of the NBS that is required both at the -64 and -47 location raises several questions about NAC mediated transcriptional activation; (i) What is the nature of the change in NAC in response to the proximal half-site? (ii) Does NAC, like other LysR family members, contact RNA polymerase, and if so, are the RNA polymerase-NAC interactions the same at the different positions of NBS, or does NAC contact different subunits of polymerase when bound to NAC binding sites positioned at -47 and -64?

Strain	Genotype	Reference
K.pneumoniae		
KC2653	Δ*bla-2 hutC515 str-6*	6
KC5447	*hutC515 nac-2*	This Work
KC6119	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1618, Φ(*ureDp'-'lacZ*)	This Work
KC6373	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1747, Φ(NBS^{hutU}-*ureDp'-'lacZ*)	This Work
KC6374	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1748, Φ(NBS^{codB}-*ureDp'-'lacZ*)	This Work
KC6385	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1754, Φ(*codBp'-'lacZ*)	This Work
KC6403	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1765, Φ(*ureDp(inv)'-'lacZ*)	This Work
KC6404	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1766, Φ(*ureDp(sca)'-'lacZ*)	This Work
KC6424	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1747, Φ(NBS^{hutU}-*ureDp'-'lacZ*) *nac-2*	This Work
KC6425	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1748, Φ(NBS^{codB}-*ureDp'-'lacZ*) *nac-2*	This Work
KC6426	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1765, Φ(*ureDp(inv)'-'lacZ*) *nac-2*	This Work
KC6427	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1766, Φ(*ureDp(sca)'-'lacZ*) *nac-2*	This Work
KC6447	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1618, Φ(*ureDp'-'lacZ*) *nac-2*	This Work
KC6448	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1754, Φ(*codBp'-'lacZ*) *nac-2*	This Work
KC6635	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1849, Φ(*ureDp(D-D)'-'lacZ*)	This Work
KC6636	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1850, Φ(*ureDp(P-P)'-'lacZ*)	This Work
KC6638	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1849, Φ(*ureDp(D-D)'-'lacZ*) *nac-2*	This Work
KC6639	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1850, Φ(*ureDp(P-P)'-'lacZ*) *nac-2*	This Work
KC6804	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1887, Φ(*ureDp(ideal)'-'lacZ*)	This Work
KC6805	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1896, Φ(*ureDp(T-44G)'-'lacZ*)	This Work
KC6813	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1894, Φ(*ureDp(G-42T)'-'lacZ*)	This Work
KC6842	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1887, Φ(*ureDp(ideal)'-'lacZ*) *nac-2*	This Work
KC6844	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1896, Φ(*ureDp(T-44G)'-'lacZ*) *nac-2*	This Work

KC6845	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1894, Φ(*ureDp(G-42T)'-'lacZ*) *nac-2*	This Work
KC6953	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1944, Φ(*ureDp(C-43T)'-'lacZ*)	This Work
KC6963	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1917, Φ(*ureDp(C-43A)'-'lacZ*)	This Work
KC6980	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1944, Φ(*ureDp(C-43T)'-'lacZ*) *nac-2*	This Work
KC6981	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB1917, Φ(*ureDp(C-43A)'-'lacZ*) *nac-2*	This Work
KC7109	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB2073, Φ(*ureDp(C-43G)'-'lacZ*)	This Work
KC7110	Δ*bla-2 hutC515 str-6 Δrbs753* ΩCB2073, Φ(*ureDp(C-43G)'-'lacZ*) *nac-2*	This Work
E.coli		
DH5α	*fluA2* Δ(*argF-lacZ*)*U169 phoA glnV44* Φ80 Δ(*lacZ*)*M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	23

Table 3.1: Strains used in this study.

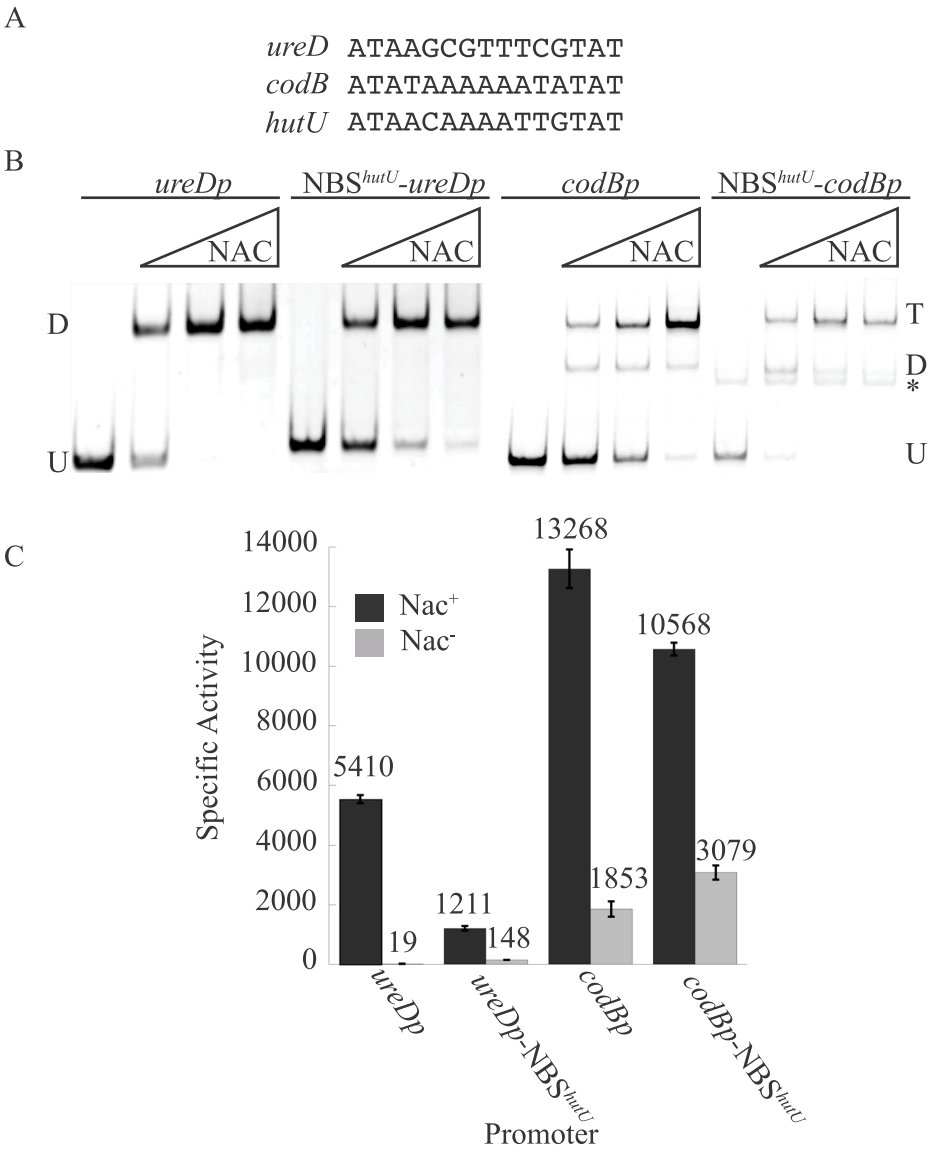

Figure 3.1: Functional equivalence of the NAC binding sites. (A) Alignment of the core, 15 nucleotide, NAC binding sites from the *ureD*, *codB*, and *hutU* promoters. (B) Electrophoretic Mobility Shift Assay (EMSA) of the *ureDp*, NBS^{hutU}-*ureDp*, *codBp*, or NBS^{hutU}-*codBp* fragments mixed with buffer 6 and increasing concentrations of NAC. U indicates the mobility of the unbound DNA, D indicates the band corresponding to a dimer of NAC associated with the DNA, T indicates the band corresponding to a tetramer of NAC associated with the DNA, and * indicates a PCR artifact in the NBS^{hutU}-*codBp* mobility shifts that is not NAC reactive. (C) β-galactosidase activity of *ureDp*, NBS^{hutU}-*ureDp*, *codBp*, or NBS^{hutU}-*codBp* as lacZ fusions integrated in Nac$^+$ (dark gray) or Nac$^-$ (light gray) backgrounds grown under nitrogen limiting conditions. Bars indicate standard error.

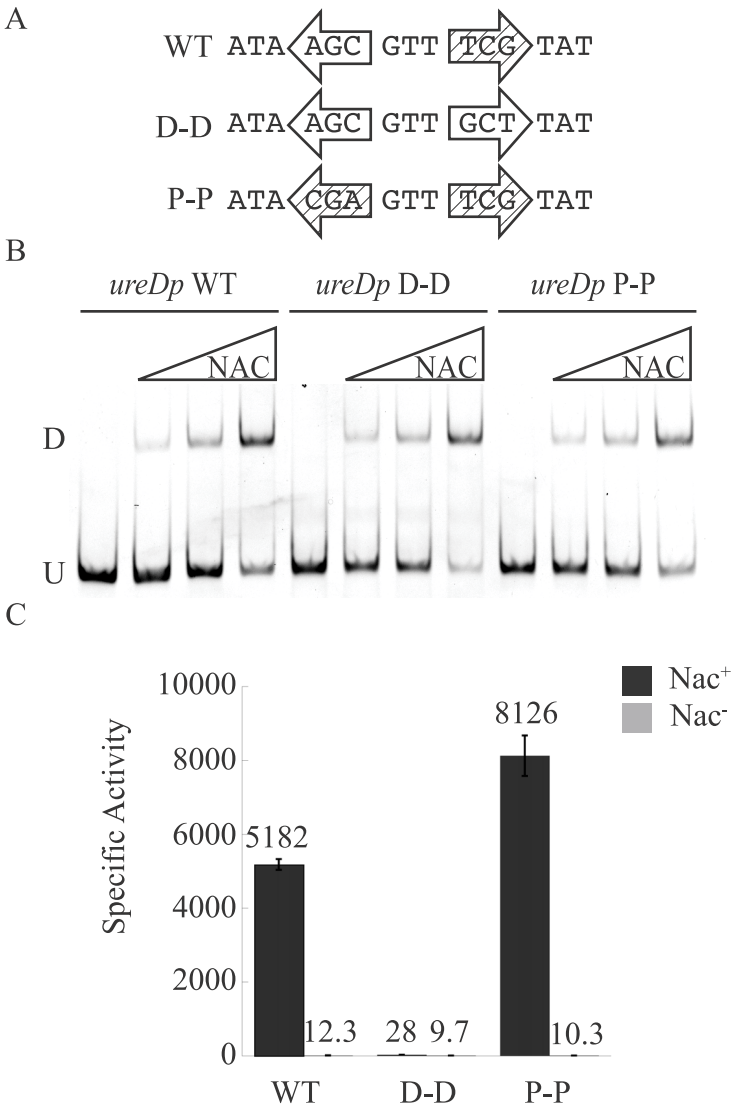

Figure 3.2: Asymmetry of the *ureD* promoter NAC binding site. (A) Alignment of the core, 15 nucleotide, NAC binding site of the wild-type *ureD* NAC binding site, Proximal-Proximal (P-P), and Distal-Distal (D-D) mutants. The proximal half site is shown as a crosshatched arrow and the distal half site is an open arrow. (B) EMSA of the *ureDp*, D-D, and P-P fragments mixed with buffer 6 and increasing concentrations of NAC. U indicates the mobility of the unbound DNA and D indicates the band corresponding to a dimer of NAC associated with the DNA. (C) β-galactosidase activity of *ureDp*, D-D, and P-P mutants as lacZ fusions integrated in Nac^+ (dark gray) or Nac^- (light gray) backgrounds grown under nitrogen limiting conditions. Bars indicate standard error.

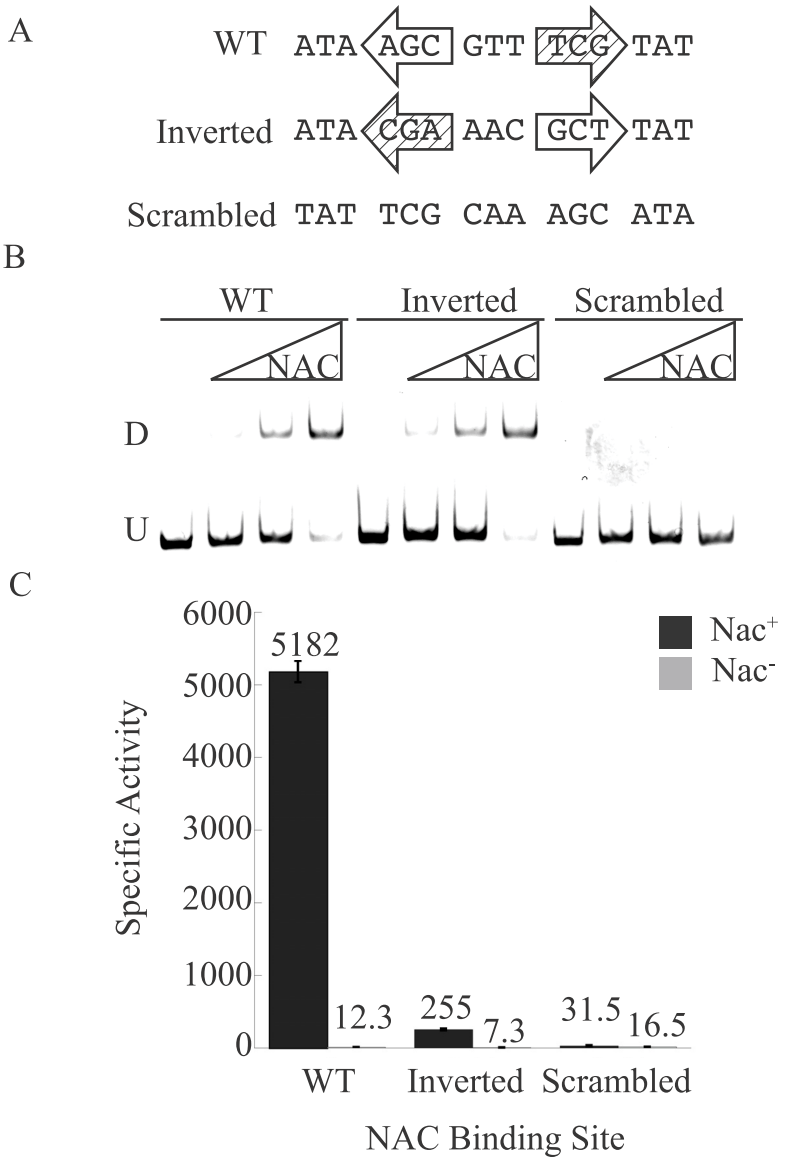

Figure 3.3: Directionality of the *ureD* promoter NAC binding site. (A) Alignment of wild-type, inverted, and scrambled mutant containing *ureD* promoter NAC binding sites. The distal half site is shown as an open arrow and the proximal half site is a cross-hatched arrow. (B) EMSA of wild-type, inverted, and scrambled *ureDp* fragments. U indicates the mobility of the unbound DNA and D indicates the band corresponding to a dimer of NAC associated with the DNA. (C) β-galactosidase activity of wild-type, inverted, and scrambled containing *ureDp* as lacZ fusions integrated in Nac^+ (dark gray) or Nac^- (light gray) backgrounds grown under nitrogen limiting conditions. Bars indicate standard error.

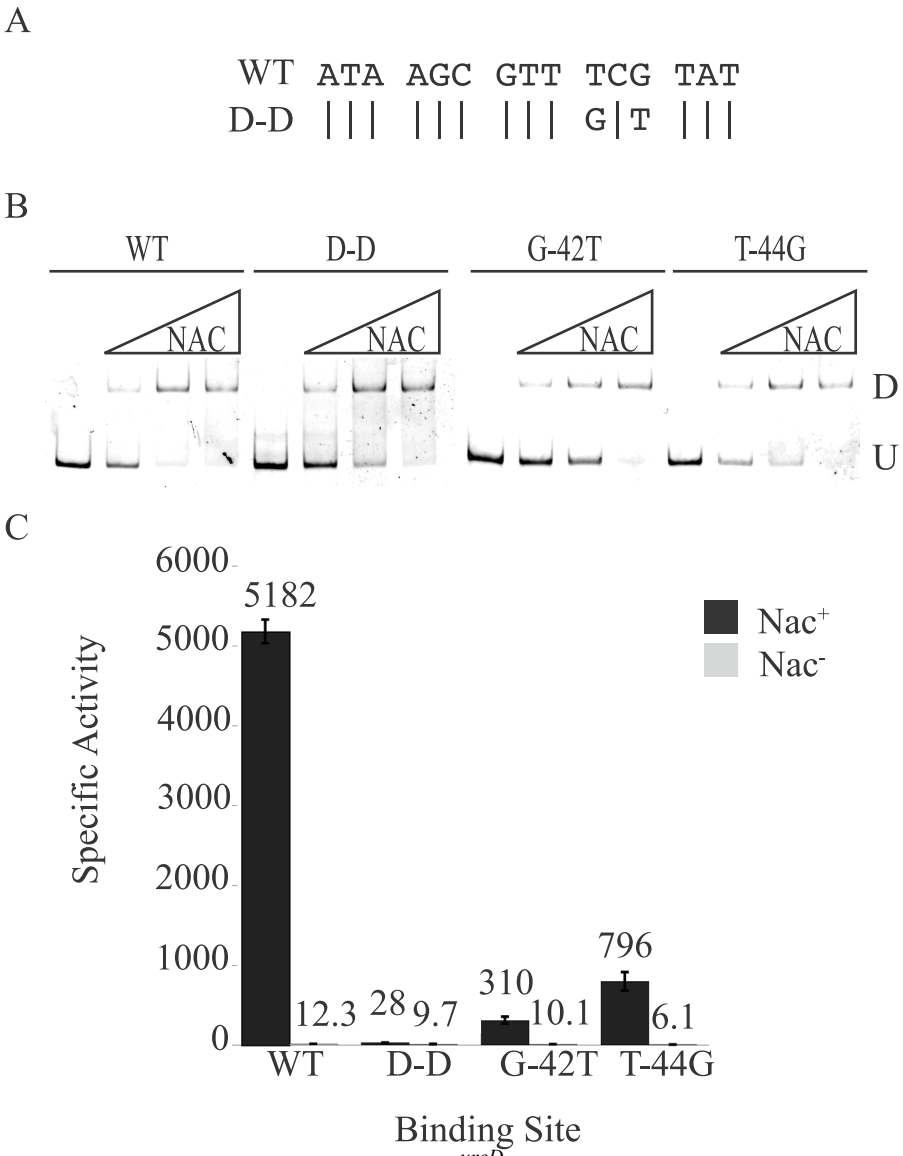

Figure 3.4: Role of T(-44) and G(-42) at NBS^{ureD}. (A) Alignment of wild-type and D-D *ureDp* NAC binding sites. (B) EMSA of wild-type, D-D, G-42T, and T-44G containing *ureD* promoter fragments. U indicates the mobility of the unbound DNA and D indicates the band corresponding to a dimer of NAC associated with the DNA. (C) β-galactosidase activity of wild-type, D-D, G-42T, and T-44G containing *ureDp* as lacZ fusions integrated in Nac$^+$ (dark gray) or Nac$^-$ (light gray) backgrounds grown under nitrogen limiting conditions. Bars indicate standard error.

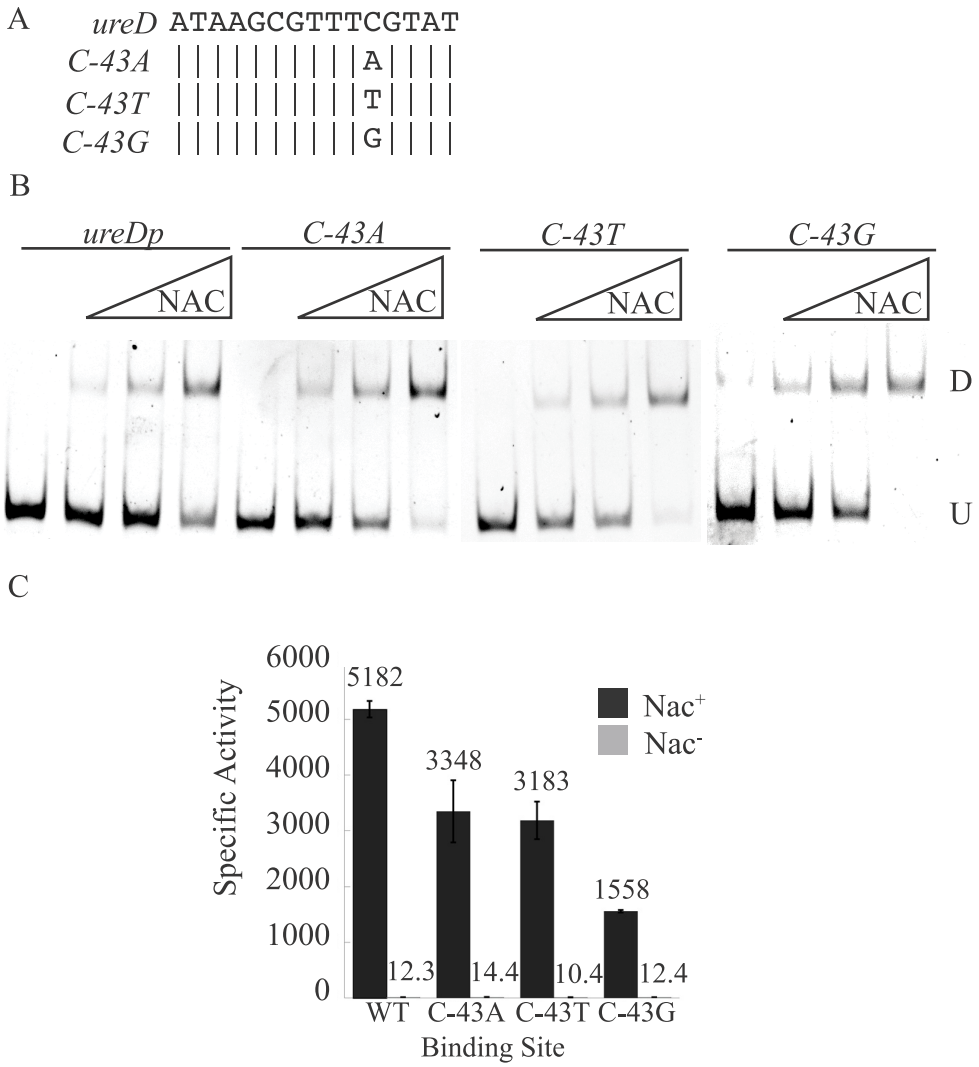

Figure 3.5: Role of C(-43) at NBS^{ureD} . (A) Alignment of wild-type, C-43A, C-43T, and C-43G *ureDp* NAC binding sites. (B) EMSA of wild-type, C-43A, C-43T, and C-43G containing *ureD* promoter fragments. U indicates the mobility of the unbound DNA and D indicates the band corresponding to a dimer of NAC associated with the DNA. (C) β-galactosidase activity of wild-type, C-43A, C-43T, and C-43G containing *ureDp* as lacZ fusions integrated in Nac$^+$ (dark gray) or Nac$^-$ (light gray) backgrounds grown under nitrogen limiting conditions. Bars indicate standard error.

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Chapter IV

Complex Regulation of the *codB* promoter of *Escherichia coli* by the Nitrogen Assimilation Control protein and the non-DNA Binding Transcriptional Regulator DksA

Abstract

The *codB* promoter from *E. coli* is regulated by at least three different responses, the nitrogen stress response (via NAC), nucleotide pool imbalance (via PurR), and the stringent response (via ppGpp). In a wild-type strain the expression level of the *codB* promoter does not change between conditions of nitrogen excess and severe nitrogen limitation. However, NAC is required for *codBp* expression under conditions of nitrogen limitation to counteract a growth-rate dependent repression under these conditions. The growth-rate repression of the *codB* promoter is relieved by deletion of the non-DNA binding transcription factor DksA , which is known to play a role in the stringent response. Interestingly, NAC is no longer able to activate transcription of the *codB* promoter in the absence of DksA. These data suggest that NAC and DksA are regulating the transcription of the *codB* promoter concurrently under conditions of nitrogen limitation. Thus, it is possible that these two forms of regulation might allow the cell to maintain the level of *codB* promoter expression as the cells become nitrogen limited.

Introduction

Enterobacteria have a highly controlled response to the stress of nitrogen limitation. Nitrogen limitation is sensed as an intracellular deficit of glutamine (18). In response to low levels of glutamine the transcriptional activator NtrC~P accumulates (17). NtrC~P regulates transcription of genes involved in the response to nitrogen limitation and requires the genes be transcribed by RNA polymerase bearing the alternative sigma factor σ^{54} (17, 30). Most of the genes activated by NtrC~P in response to nitrogen limitation act directly to increase the concentration of ammonia and therefore glutamine inside the cell (38). Many of these genes are regulated directly by NtrC~P (38). However, some genes are regulated indirectly via the nitrogen assimilation control protein, NAC, a member of the LysR family of transcriptional regulators (7, 23). NAC acts to link the specialized system involving NtrC~P and RNA polymerase bearing σ^{54} to genes transcribed by RNA polymerase bearing σ^{70}, the major cellular form of the holoenzyme (16). NAC regulates transcription of metabolic genes that influence the amount of ammonia available to the cell, activating the expression of genes that increase the amount of free ammonia and repressing the expression of genes whose function requires high concentrations of ammonia (16, 20, 21, 23, 27, 28, 33). The Ntr system and NAC allow the cell to recover from nitrogen stress by allowing the cell to assimilate ammonia from alternative sources such as amino sugars, nucleotides, and amino acids.

The utililization of amino acids might put the Ntr response and NAC at odds with another regulatory system that is induced during nitrogen limitation, the stringent response (19). Since the nitrogen available to the cell is sensed via the concentration of glutamine, an amino acid, nitrogen limitation can be defined as amino acid limitation

(18). Amino acid limitation leads to the accumulation of non-aminoacylated tRNAs, activating the stringent response and leading to the accumulation of guanosine-tetraphosphate (ppGpp) inside the cell (10). The stringent response regulates transcription during amino acid starvation by shortening the half-life of the RNA polymerase open complex (5, 6). The ribosomal RNA promoters normally have a very short half-life for the open complex and thus are repressed by additional shortening of open complex half-life (6). In contrast, the promoters for the genes involved in the biosynthesis of amino acids have a relatively long open complex half-life and thus are not repressed by the stringent response (6). Transcription of amino acid biosynthesis genes increases during the stringent response due to increased availability of RNA polymerase that has been freed from the rRNA promoters by the shortened half-life of the open complex (6). This causes a shift in cellular physiology toward the production of amino acids to alleviate amino acid starvation.

There are two enzymes involved in the synthesis of ppGpp in *E. coli* (3, 32, 37). RelA and SpoT are both capable of synthesizing ppGpp during amino acid starvation, though RelA performs the majority of this synthesis during amino acid starvation (8, 32). Some of the transcriptional changes in response to ppGpp require the non-DNA binding transcriptional regulator DksA (9). DksA interacts directly with ppGpp and may act to change the stability of the RNA polymerase open complex by binding in the secondary channel of RNA polymerase (29). Once the cell has recovered from amino acid starvation the stringent response is terminated by SpoT dependent hydrolysis of ppGpp (32).

The regulation of the *codB* promoter is complex. The *codB* promoter is regulated by the availability of ammonia in a NAC dependent manner (28). Under nitrogen limiting conditions, when the ammonia concentration is low, NAC activates transcription from the *codB* promoter (28). The purine repressor, PurR, also regulates transcription of the *codB* promoter in response to the concentration of purines in the cell (2). Under high purine concentration, transcription of the *codB* promoter is repressed (2). Cells that were nitrogen limited concurrent with purine excess demonstrated that the regulation of *codBp* by NAC and PurR appeared to be independent and cumulative, with each mechanism of regulation contributing to the overall transcription of the promoter (2). Levels of the *codB* transcript are decreased in a WT strain under isoleucine starvation and this decrease is relieved in a ppGpp^0 (Δ*relA* Δ*spoT*) strain, suggesting that ppGpp is either directly or indirectly causing the levels of *codB* transcript to change under amino acid starvation (36). This additional level of regulation is uncharacterized with respect to the regulation of the *codB* promoter under nitrogen limitation.

Materials and Methods

Strains and Plasmids

All *K. pneumoniae* strains were derived from W70 (24). All *E. coli* strains were derived from YMC9 with the exception of TE2680 (4, 12). The fusion of the *codB* promoter from *E. coli* was created in pRS415 and contains 127 bp of promoter plus the first 76 bp of *codB* fused to the *lacZ* open reading frame (34). The *codBp-lacZ* fusion was integrated into the *K. pneumoniae* chromsome using a landing pad system as described (15). For construction of the *E. coli* strain containing a single copy integrant of

the *codBp-lacZ* fusion the fusion was first subcloned into pRS551 (34). The *codBp-lacZ* transcriptional fusion was integrated in single copy on the *E. coli* chromosome replacing the *trp* operon as described (12). The fusion was moved into YMC9 using $P1_{vir}$ mediated transduction of the linked Km^R allele (12). The *dksA::cm* and *nac::cm* deletions were generated by deleting the open reading frame of these genes and replacing the deleted segment with *cat* from pKD3 as previously described (11). The *nac::cm* cassette was removed by recombination of the flanking *frt* sites using flp recombinase (11). The *dksA::cm* allele was transferred to the *nac::frt* strain using $P1_{vir}$ mediated transduction selecting for the associated Cm^R marker (26). The *nac-2* allele, a deletion of the *nac* ORF and 19 bp upstream of the translational start site was transduced into the *K. pneumoniae* strain containing the *codB-lacZ* fusion using $P1_{vir}$ grown on KC5249 (14)

Growth and Media

For assays, strains were grown in W4 minimal salts (23). The media were supplemented with 0.4% (w/v) glucose and 0.2% or 0.04% (w/v) nitrogen source (ammonia sulfate, monosodium glutamate, glutamine, or casitone). Minimal media used for the growth of *E. coli* strains was also supplemented with 1μg/ml thiamine and 20μg/ml L-tryptophan (13). *E. coli* strains were grown at 37°C and *K. pneumoniae* strains were grown at 30°C. All cultures were grown to 50 Klett units (ca. 1×10^8 cells/ml of culture). For maintenance and genetic manipulation of strains L Broth was used (26). To select for plasmid and chromosomal markers media were supplemented with the following antibiotics when necessary: ampicillin (100μg/ml), kanamycin sulfate (50μg/ml), or chloramphenicol (25μg/ml). When solid medium was necessary bacto agar was added to a final concentration of 1.5% (w/v).

β-galactosidase Assays

β-galactosidase assays were performed essentially as described (23). Briefly, cells from 10 ml cultures were washed once in cold 1% (w/v) KCl and then resuspended as 10X cells in 1 ml cold 1% (w/v) KCl. The 10X cells were permeabilized with CTAB for β-galactosidase assays (23). Results are reported as units of β-galactosidase per milligram total protein as determined by the method of Lowry (22). Each strain was cultured at least three independent times and each 10X cell stock was assayed at three different concentrations.

Results

NAC regulates *codBp*$_{E.\ coli}$ in *K. pneumoniae*

As part of another study (31), we examined the ability of *K. pneumoniae* to regulate the *codB* promoter from *E. coli* (*codBp*$_{Eco}$). A *codBp*$_{Eco}$-*lacZ* fusion was integrated into the *K. pneumoniae* chromosome in single copy via a landing-pad technique (15). The ability of *K. pneumoniae* to regulate *codBp*$_{eco}$ had been demonstrated by gratuitously overproducing NAC$_{Kpn}$ in *E. coli* (28). In a *K. pneumoniae* strain where NAC was produced normally from its own promoter, *codBp*$_{eco}$ was activated in response to nitrogen limitation in a NAC dependent manner in *K. pneumoniae* (Figure 4.1). The *codB* promoter from *E. coli* showed a seven-fold NAC dependent activation between the wild type strain and a *nac* mutant under conditions of nitrogen limitation (Figure 4.1). Interestingly, *codBp*$_{eco}$ showed no change in the level of transcription in a wild type strain under conditions of nitrogen excess (GNgln) and severe nitrogen limitation (Gglt) suggesting that some other factor was repressing the transcription of the promoter under

conditions of nitrogen limitation (Figure 4.1). In a wild type strain transcription of *codbp*$_{eco}$ was highest under moderate nitrogen limitation, suggesting that NAC was activating transcription and the repression was not yet fully active (Figure 4.1). In the Nac$^-$ strain, transcription of *codBp*$_{eco}$ was highest under the conditions of nitrogen excess (GNgln) where the cells were growing at the fastest rate (Figure 4.1). Under conditions of moderate nitrogen limitation (Ggln.04), *codBp*$_{eco}$ had a transcriptional activation that was approximately 2.5 fold decreased from the conditions of nitrogen excess (Figure 4.1). Under the most limiting growth conditions (Gglt), which corresponded to the slowest growth-rate, the transcription of *codbp*$_{eco}$ was the lowest, with a 2.8 fold decrease in activity from the moderately limiting conditions and a 6.5 fold decrease in activity from the conditions of nitrogen excess (Figure 4.1), suggesting that the repression might be dependent on growth-rate.

codBp expression is repressed by slow growth-rate in _E. coli_

To see if *codBp* was repressed by slow growth-rate in *E. coli*, a *codBp-lacZ* fusion was integrated into the chromosome in single copy using a landing pad method (12). Nac$^+$ and Nac$^-$ strains containing the fusion were grown under conditions of nitrogen excess (GNgln) and nitrogen limitation (Gglt) to determine the effect of slow growth-rate induced by severe nitrogen limitation on *codBp* transcription in *E. coli*. Similar to the Nac$^+$ *K. pneumoniae* strain, the Nac$^+$ strain of *E. coli* demonstrated no apparent regulation of *codBp* by nitrogen limitation (Figure 4.2). However, the Nac$^-$ strain demonstrated a repression of *codBp* transcription under conditions of nitrogen limitation (Figure 4.2). This suggested that NAC might be overcoming a slow growth-rate repression of *codBp* transcription that occurred in conditions of nitrogen limitation.

Recently, Traxler *et al* (2008) demonstrated that the *codB* transcript of *E. coli* is repressed in wild type strains under isoleucine starvation and that this repression was dependent on ppGpp (36). We wondered if the slow growth-rate repression of *codBp* under conditions of nitrogen limitation was related to the stringent response.

DksA is required for slow growth-rate dependent repression of *codBp*

To determine if DksA played a role in regulating the transcription of *codBp* in response to slow growth-rate induced by nitrogen limitation wild type, Nac^-, $DksA^-$, and Nac^- $DksA^-$ strains were grown under conditions of nitrogen excess and nitrogen limitation. Due to poor growth of $DksA^-$ strains on minimal media, nitrogen limitation was achieved by adding various concentrations of casitone to the minimal media. The wild type strain showed no change in *codB* promoter transcription between conditions of nitrogen excess and nitrogen limitation (Figure 4.3). Transcription from *codBp* was repressed in the Nac^- mutant under conditions of nitrogen limitation (Figure 4.3). This is in agreement with the results using defined minimal media (Figure 4.2). The $DksA^-$ strains showed approximately 3 fold higher activity of the *codB* promoter under conditions of nitrogen excess or nitrogen limitation (Figure 4.3). In contrast to the $DksA^+$ strain, the $DksA^-$ strain showed little or no NAC-mediated activation under conditions of nitrogen limitation.

Discussion

The *cis* regulatory elements for both NAC activation and slow growth-rate control of $codbp_{eco}$ are recognized by their effectors in *K. pneumoniae*. When integrated into the *K. pneumoniae* chromosome in single copy, $codBp_{eco}$–*lacZ* demonstrated a NAC-dependent activation in nitrogen limiting conditions. This agreed with the previous data,

which showed that plasmid-encoded NAC from *K. pneumoniae* could regulate $codBp_{eco}$ in *E. coli* cells (28). However, relying only on the wild type strain one could conclude that *K. pneumoniae* NAC was unable to activate transcription of $codBp_{eco}$-*lacZ* in *K. pneumoniae* cells, suggesting a NAC independent repression of $codBp_{eco}$ under nitrogen limiting conditions. The repression of *codBp* under nitrogen limiting conditions also occurs in *E. coli*, suggesting that this is not an effect of placing an *E. coli* promoter in *K. pneumoniae*.

Previous data suggest that transcription of the *codB* promoter of *E. coli* is repressed in wild type cells starved for isoleucine (36). In cells that cannot produce ppGpp (Δ*relA* Δ*spoT*), the repression of the *codB* promoter in response to isoleucine starvation is not seen (36). We wondered if the slow growth-rate repression of the *codB* promoter seen under nitrogen limiting conditions was dependent on ppGpp. Unfortunately, *relA spoT* strains failed to grow under our conditions of nitrogen limitation or excess most likely due to the multiple auxotrophies of this mutant (37). Much of the transcriptional regulation of ppGpp requires the non-DNA binding transcriptional regulator DksA and deletion of DksA seems to uncouple much of the ppGpp effect on transcription (1). By growing the $DksA^-$ strains in conditions of limiting casitone the role of DksA, and possibly ppGpp, in the slow growth-rate regulation of the *codB* promoter could be tested. The $DksA^-$ mutant demonstrated a relief of repression of the *codB* promoter under conditions of nitrogen excess and nitrogen limitation suggesting that the slow growth-rate mediated repression of the *codB* promoter might require the presence of DksA.

While the DksA$^-$ cells demonstrated a relief of slow growth-rate repression, a loss of NAC-mediated transcriptional activation under nitrogen limiting conditions was also seen in DksA$^-$ strains . There are four models that might explain why NAC only activates transcription of *codBp* in the presence of DksA. (i) Perhaps, in the absence of DksA, the *codBp* is already maximally active. If this were the case, NAC would not be able to further activate transcription of *codBp*. (ii) NAC might only activate transcription of *codBp* when RNA polymerase is bound to the promoter in complex with DksA-ppGpp. This model might disagree with previous *in vitro* data demonstrating that NAC was capable of activating transcription from the *codB* promoter using purified RNA polymerase (28). It is possible, that the high concentrations of NAC *in vitro* might have overcome or masked the requirement of DksA for transcriptional activation of the *codB* promoter by NAC. (iii) NAC might function at the same step of transcription inhibited by DksA at *codBp*. In this model, once the repression of *codBp* by DksA was relieved there might not be a need for NAC activation because the inhibition of this step would no longer exist. (iv) NAC mediated-activation might be blocked by a product produced by the DksA$^-$ strain. ppGpp and DksA regulate the expression of many different genes during the stringent response and it would not be surprising to find that DksA$^-$ cells might contain different elements than a wild type strain under conditions that induce the stringent response (36). Our data do not allow us to distinguish among these models.

Ntr-independent nitrogen regulation has been noted previously at the *ureD* promoter (*ureDp2*) under conditions of nitrogen limitation (21). This response might also be due to the stringent response regulating the transcription of this promoter. It would make sense that the stringent response would activate the urease gene cluster as

this group of genes allows the production of ammonia from a non-amino acid source, urea, allowing the cell to create more amino acids.

Name	Genotype	Ref
E.coli		
YMC9	*endA1 thi-1 hsdR17 supE44 ΔlacU169*	
BW20767	*RP4-2 tet:Mu-1 Kan::Tn7 integrant leu63::IS10 recA1 CreC510 hsdR17 endA1 zbf-5 uddA (ΔMuI):pir*$^{+}$ *thi*	25
DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	35
TE2680	F- λ- *IN(rrnD-rrnE)1 Δ(lac)X74 rpsL galK4 recD1903::TN10D-tet TrpDC700::putPA1303::[Kan*^{S}*-Cam*^{R}*-lac]*	12
EB6862	F- λ- *IN(rrnD-rrnE)1 Δ(lac)X74 rpsL galK4 recD1903::TN10D-tet TrpDC700::putPA1303::Φ1874/xhoI[Kan*^{R}*-codBAp-lac]*	This Work
EB7112	*endA1 thi-1 hsdR17 SupE44 ΔlacU169 TrpDC700::putPA1303::Φ1874/xhoI[Kan*^{R}*-codBAp-lac]*	This Work
EB7118	*endA1 thi-1 hsdR17 SupE44 ΔlacU169 TrpDC700::putPA1303::Φ1874/xhoI[Kan*^{R}*-codBAp-lac] Δnac::frt*	This Work
EB7125	*endA1 thi-1 hsdR17 SupE44 ΔlacU169 TrpDC700::putPA1303::Φ1874/xhoI[Kan*^{R}*-codBAp-lac] ΔdksA::Cam*^{R}	This Work
EB7126	*endA1 thi-1 hsdR17 SupE44 ΔlacU169 TrpDC700::putPA1303::Φ1874/xhoI[Kan*^{R}*-codBAp-lac] Δnac::frt ΔdksA::Cam*^{R}	This Work
K.pneumoniae		
KC2553	*Δbla-2 hutC515 str-6*	15
KC5447	*Δbla-2 hutC515 nac-2*	This Work
KC6385	*Δbla-2 hutC515 str-6 Δrbs753* ΩCB1754 Φ(*codBp'-'lacZ*)	This Work
KC6448	*Δbla-2 hutC515 str-6 Δrbs753* ΩCB1754 Φ(*codBp'-'lacZ*) *nac-2*	

Table 4.1: Strains used in this study.

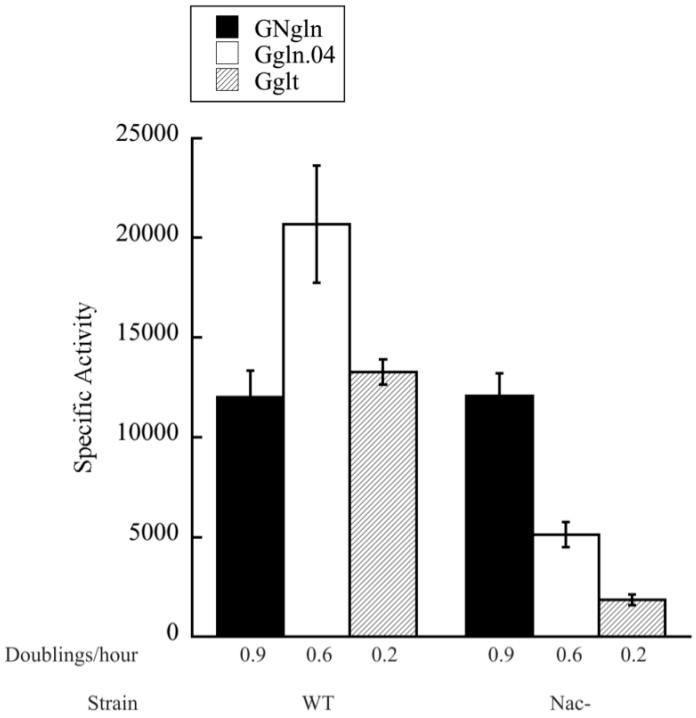

Figure 4.1: *codBp*$_{E.coli}$ is NAC regulated in *K.pneumoniae*. WT or Nac$^-$ cells containing a chromosomal *codBp*$_{E.coli}$-*lacZ* fusion were grown in W4 medium containing 0.2% (w/v) glucose and the following nitrogen sources: 0.2% (w/v) ammonia and 0.2% (w/v) glutamine (solid), 0.04% (w/v) glutamine (open), or 0.2% (w/v) monosodium glutamate (cross-hatched) to mid-log phase. Detergent permeabilized cells were assayed for β-galactosidase activity. Doublings/hour for each strain grown in each medium is shown below the appropriate bar. Error bars show the standard error for three separate experiments.

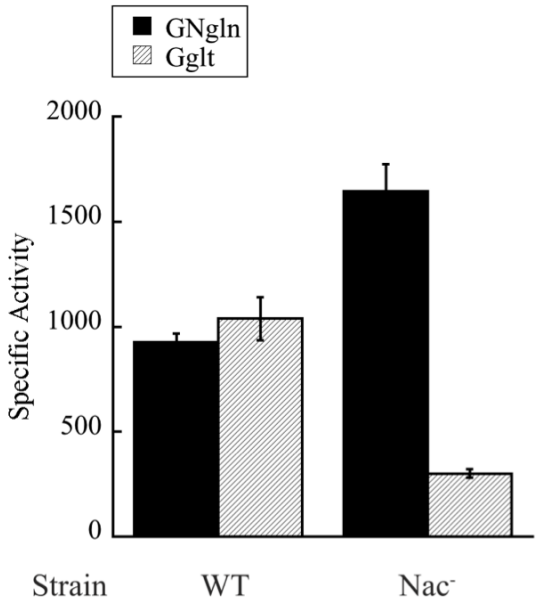

Figure 4.2: *codBp* is growth rate regulated in *E. coli*. WT or Nac$^-$ cells containing a chromosomal *codBp-lacZ* fusion were grown in W4 medium containing 0.2% (w/v) glucose, 1μg/ml thiamine, 20μg/ml tryptophan, and the following nitrogen sources: 0.2% (w/v) ammonia and 0.2% (w/v) glutamine (solid) or 0.2% (w/v) monosodium glutamate (cross-hatched) to mid-log phase. Detergent permeablized cells were assayed for β-galactosidase activity. Error bars indicate the standard error.

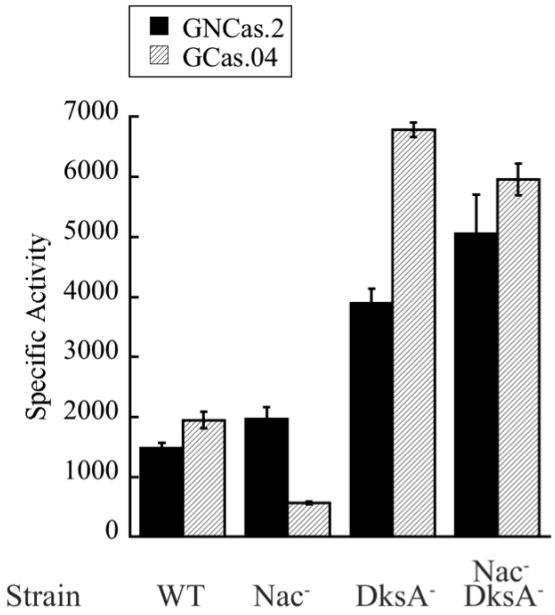

Figure 4.3: DksA is required for growth rate control of *codBp*. Wild-type, Nac$^-$, DksA$^-$, or Nac$^-$ DksA$^-$ strains containing a chromosomal *codBp-lacZ* fusion were grown in W4 medium containing 0.2% (w/v) glucose1µg/ml thiamine, 20µg/ml tryptophan, and the following nitrogen souces: 0.2% (w/v) ammonia and 0.2% (w/v) casitone (solid), or 0.04% casitone (cross-hatched) to mid-log phase. Detergent permeabilzed cells were assayed for β-galactosidase activity. Error bars indicate the standard error.

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Chapter V

General Discussion

Perturbations in cellular physiology occur during a change in bacterial lifestyle such as infecting a host, switching from planktonic to biofilm growth, progressing through the bacterial cell cycle, and changing environmental conditions. All of these changes in lifestyle alter the metabolic requirements of the cell (1, 3, 6, 16). The cell induces stress response operons to respond and overcome the insult and return cellular physiology to its normal state. Understanding how these stress-response regulons bring about changes to cellular physiology both related and unrelated to their specific response will provide clues to how the cell responds to changes in lifestyle (11). Often perturbations in cellular physiology involve nutrient limitation and thus require the modification of cellular metabolism.

Cellular metabolism is tightly controlled by the availability of macronutrients. Of all the nutrients available to the cell, carbon and nitrogen seem to be the only two that cannot be limited simultaneously (2, 14). This suggests that the balance of the carbon and nitrogen pools available to the cell might tightly regulate cellular metabolism. Under conditions of carbon limitation the cell is able to activate genes that utilize alternative carbon sources in order to produce energy and assimilate carbon (9). Interestingly, this system also regulates some of the ammonia assimilation machinery (15, 17). The transcriptional regulator involved in the carbon stress response, CAP-cAMP, activates the

formation of glutamine synthase and glutamate dehydrogenase under conditions of carbon limitation and also represses the formation of glutamate synthetase and glutaminase (15). The role of catabolite repression in altering the levels of four enzymes involved in ammonia fixation suggests that the availability of carbon plays a role in regulating the metabolism of nitrogen. Moreover, acetyl phosphate, a key metabolite in the glycolytic pathway of aerobic cells, plays a critical role in initiating the Ntr response to nitrogen limitation (5, 7, 10). The role of the nitrogen stress-response in regulating genes involved in carbon and energy acquisition has been established as part of this thesis. One example of the nitrogen stress response regulating genes involved in carbon and energy acquisition is the negative regulation of *glgX* transcription by the nitrogen assimilation control protein (NAC). The negative regulation of this promoter by NAC might decrease the formation of the first enzyme in glycogen debranching, a pathway that eventually leads to glucose (4). This suggests that while a majority of the nitrogen or carbon stress response deal with regulating genes directly involved in the metabolism and physiology of nitrogen and carbon, respectively, these responses play a role in adjusting the cellular metabolism of each other. The ability to regulate metabolism of other macronutrients might aid in the recovery from either carbon or nitrogen limitation thus restoring metabolic balance to the cell more quickly.

Another mechanism that unites different aspects of cellular physiology is the interplay between the nitrogen stress response and the stringent response. Under conditions of nitrogen limitation the nitrogen stress response activates genes involved in releasing free ammonia from compounds such as nucleotides and amino acids while repressing genes that are involved in the assimilation of ammonia. On the other hand,

under conditions of nitrogen limitation the stringent response results in an activation of genes that assimilate ammonia into amino acids. It would seem that these two metabolic stress responses might be at odds with each other during nitrogen limitation. However, the two stress responses may act in concert during nitrogen limitation with the nitrogen stress response yielding ammonia from overabundant nitrogenous compounds and the stringent response activating transcription of genes encoding for enzymes that assimilate the newly freed ammonia into amino acids that are limiting. Some of the genes that demonstrate regulation by the Ntr system or NAC in response to nitrogen limitation demonstrate additional regulation under these conditions that is independent of both Ntr and NAC (8, 18). In this thesis the interplay between the stringent response and the nitrogen stress response has been demonstrated at the *codB* promoter. The stringent response negatively regulates the transcription of *codB* promoter and NAC positively regulates the transcription of the *codB* promoter in response to nitrogen limitation. The NAC activation of the *codB* promoter seems to be needed only in the presence of a stringent response. In the absence of DksA, NAC was unable to activate transcription from the *codB* promoter. The interplay between the stringent response and the nitrogen stress response is not fully characterized but it might be expected that the two stress responses may act in concert to rebalance the cellular pools of nitrogenous compounds.

NAC activates transcription from different spacings and different faces of the helix with respect to RNA polymerase (8, 12, 13). Previous data suggested that NAC binding sites were limited and might only allow NAC to activate transcription when positioned on the face of the helix found in the native promoter (13). This suggested that the ability of NAC to activate transcription was fairly flexible; as long as NAC bound

DNA on the appropriate face of the helix it was competent to activate transcription (13). Work presented in this thesis demonstrates that the same NAC binding site was capable of activating transcription from the opposite face of the helix. This suggests that the constraint on activation from specific faces may be a property of the specific promoter rather than of NAC or the NAC binding site. This suggests that the mechanism by which NAC activates transcription might be more flexible than originally thought. NAC seems to be capable of activating transcription from a large variety of positions, and while not every promoter will be activated by each spacing and position, many promoters could potentially be controlled by NAC at some spacing and position. Perhaps the flexibility inherent in NAC-mediated transcriptional activation explains the ability of NAC to regulate transcription at many different promoters. This flexibility in activation may lend clues to how the NAC regulon in *K. pneumoniae* evolved to contain not only so many genes but also genes not directly involved in nitrogen metabolism.

The large NAC regulon of *K. pneumoniae* is a stark contrast to the more limited role NAC plays in the response to nitrogen limitation in the related enterobacterium, *E. coli* (19). *K. pneumoniae* is a soil bacterium and in that environment it is conceivable that it spends most of the time growing on sources of carbon other than glucose and sources of nitrogen other than ammonia and thus a broader response to the stress of nutrient limitation might help *K. pneumoniae* survive long term in these conditions. *K. pneumoniae* has approximately 800 more genes than *E. coli* and one might expect that, as a soil bacterium, many of these additional genes would be responsible for transporting and metabolizing the various carbon and nitrogen sources present in the soil.

The requirements for NAC control at a promoter might be generalized as containing the degenerate NAC binding site and a promoter recognized and bound by RNA polymerase bearing σ^{70}. The ability of NAC to control transcription from many positions might explain the ability of NAC to bring many different genes under control of the nitrogen regulon of *K. pneumoniae*. The genes regulated by NAC during nitrogen limitation contribute to the ability of *K. pneumoniae* to use a diverse set of compounds as the sole source of nitrogen and increase the ability of *K. pneumoniae* to survive long-term in the absence of the preferred nitrogen source, ammonia. The ability of a cell to respond to stress is key to survival not only during nutrient poor growth but also in response to changes in the lifestyle of the organism. A better understanding of cellular response to metabolic stress will provide insight into the role physiology plays in all cellular functions.

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