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Graduate Studies

# IDENTIFICATION AND CHARACTERIZATION OF MUTATIONS IN *NMPR* THAT RESTORE TYPE-IV PILI DEPENDENT MOTILITY IN *MYXOCOCCUS XANTHUS*

A Manuscript Style Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Microbiology

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We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science – Clinical Microbiology.
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ABSTRACT

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*Myxococcus xanthus* is a Gram-negative bacterium that displays Type-IV pili dependent motility. These pili are transcriptionally regulated by the two-component system (TCS) PilSR. A strain of *M. xanthus* in which the response regulator (RR) *pilR* has been deleted is non-motile. However, after extended incubation, motility is restored due to mutations in a different TCS, NmpRSTU. Some of these identified mutations were in the RR *nmpR* and restored motility by causing NmpR to be in a constitutively active, or "ON" state. We sought to increase the number of known mutations in *nmpR* that would lead to this "ON" state and additionally characterize how these variants bind DNA compared to the wild type NmpR. A screen for *nmpR* mutants with restored motility was performed, and sequencing determined the site of mutation. Several mutations were identified in a linker domain of NmpR. To examine the impact of these mutations, $NmpR^{ON}$ variants were constructed, purified, and analyzed using electromobility shift assays (EMSAs) to characterize DNA binding. It was observed that the NmpR variants had larger shifts compared to wildtype NmpR, even when one of two NmpR-binding sites on the DNA was mutated. This suggests that the $NmpR^{ON}$ variants have increased oligomerization compared to the wildtype, which may explain how they are constitutively active. Since RRs are conserved across many bacterial species, this mutational analysis gives broad insight into how TCS can compensate for loss-of-function mutations.

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# INTRODUCTION

## The Complex Lifecycle of *Myxococcus xanthus*

*Myxococcus xanthus* is a Gram-negative, bacillus bacterium that is ubiquitous in soil environments. The survival of *M. xanthus* in this complex and dynamic environment relies on several multicellular, social behaviors such as motility, fruiting body development, and microbial predation. The predation strategy of *M. xanthus* is a cooperative group behavior and is thought to be the primary way *M. xanthus* acquires nutrients from its surroundings (1). Predation itself is a combination of behaviors that range from single-cell functions to population-level phenomena. For example, single cell *M. xanthus* lyse neighboring prey cells through direct contact (2), fusing directly with the target membranes and inducing lysis to ensure nutrients will not be utilized by surrounding organisms (3). Additionally, *M. xanthus* cells can lyse prey cells at a distance by releasing outer membrane vesicles (OMVs) that are loaded with hydrolases, phosphatases, and proteases that function in degradation of target cells (3). Subsequent released nutrients from lysed target cells are taken in by *M. xanthus* and metabolized. Finally, another important factor in nutrient acquisition is motility. *M. xanthus* uses a special type of social motility called rippling, in which cells move back and forth over an area multiple times in a coordinated fashion before moving on, forming a “ripple” or wave-like appearance that ensures nutrients are depleted (2).

When prey cells and nutrients are no longer available, *M. xanthus* begins to form aggregates, then a mound, and eventually a mature fruiting body containing spores (Fig. 1). The fruiting bodies formed by *M. xanthus* and other closely related genera/species take on a variety of morphologies and sizes (4). Within the fruiting body, different cell

types have particular roles. For example, peripheral rods are a special type of developmental cell that exist as undifferentiated cells in a monolayer on the outside of the fruiting body. It is proposed that non-reproductive peripheral rods allow the fruiting body to respond to sudden changes in nutrient availability (5). The rods maintain vegetative behavior even under unfavorable conditions, and it is suggested that when new food sources are available, they digest the nutrients and release soluble byproducts that trigger spore germination (6). Within the fruiting body, proper cells undergo sporulation and exist as myxospores that are resistant to harsh environmental conditions such as heat and desiccation. In conditions only slightly below the threshold of favorable, more rods than spores are present in the fruiting body. However, in extreme conditions, more spores are present than rods to increase the chance of long-term survival (5). The remaining cells that do not sporulate undergo autolysis (7). Autolysis is induced by the gene *mazF*, and its deletion results in a large drop in spore formation and eliminates developmental cell death (8). It is believed that autolysis releases compounds such as glucosamine and fatty acids that are necessary for the developing fruiting body and myxospores (9). When nutrients are available again, the peripheral rods send the signal to the myxospores to germinate, allowing *M. xanthus* to return to a vegetative state for renewed growth and nutrient acquisition.

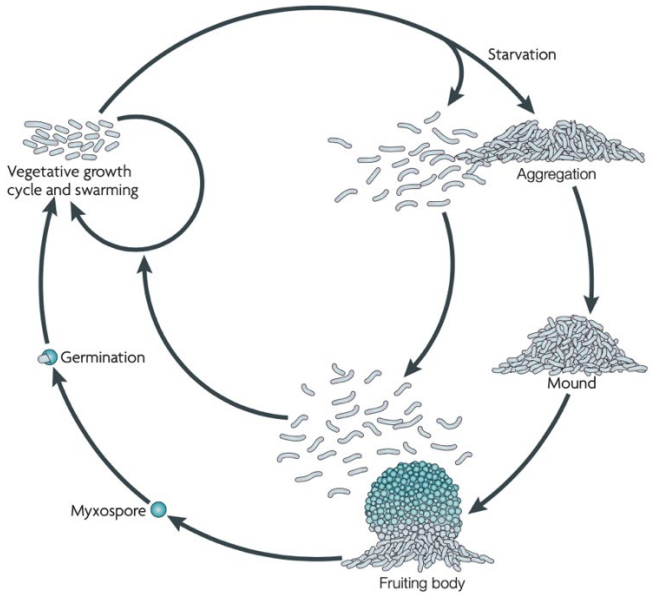


**Fig. 1. Life cycle of *M. xanthus*.** Under starvation conditions, cells aggregate and form a fruiting body, which is resistant to many adverse environmental conditions. When favorable conditions arise again, germination of the myxospores occurs and vegetative growth resumes. Imaged used with permission from (6).

## Social Motility

Motility is required for the complex *M. xanthus* cellular processes such as fruiting body formation, nutrient acquisition, and predation. *M. xanthus* uses two distinct motility mechanisms that have been termed adventurous (A) and social (S). A-motility is movement of individual cells over hard, dry surfaces whereas S-motility is multicellular movement across soft, wet surfaces (10, 11). S-motility is dependent on type-IV pili (T4P) and extracellular polysaccharides (EPS) (4, 11). T4P are defined by a conserved major pilin amino acid sequence found across many Gram-negative bacteria (12). T4P have a multitude of functions in bacteria including DNA uptake, surface sensing, protein secretion, and twitching motility (13). Polymerization and depolymerization of the pilin subunits is facilitated by interchangeable ATPases PilB and PilT, respectively (14). Due

to the repeated extension (polymerization) and retraction (depolymerization) of pili, this method of motility has a characteristic appearance that led to it being called “twitching” motility. Twitching motility is utilized by many other bacteria including the opportunistic pathogen *Pseudomonas aeruginosa* (15, 16).

The other necessary factor in *M. xanthus* T4P-dependent motility is EPS biogenesis. EPS functions to hold cells together through an extracellular matrix and promotes pili retraction (17). Mutants defective in EPS production are unable to undergo S-motility and as a result do not form proper fruiting bodies (18). Interestingly, these mutant strains have normal levels of T4P, demonstrating EPS is not necessary for T4P production, but rather likely functions in the pili extension and retraction (18). Furthermore, the EPS pathway works in parallel with the Frz pathway to regulate cellular reversal rates. As previously mentioned, cellular reversals are necessary for twitching motility, nutrient uptake, and for complex behaviors such as rippling. The actual amount of EPS is important and is tightly regulated. For instance, during high cell density, EPS concentration is also high and therefore promotes a lower cellular reversal frequency. Similarly, when EPS production was genetically deleted, cells reversed direction very frequently and were observed to be defective in S-motility (19).

## Two-Component Signaling Systems

To regulate its complex social behaviors, *M. xanthus* has extensive cellular signaling networks (1). In particular, *M. xanthus* encodes 272 two-component system (TCS) genes (20, 21). TCSs are a type of signal transduction system that allow prokaryotic cells to respond to environmental signals, typically through regulation of

gene expression. A prototypical TCS consists of a transmembrane sensor histidine kinase (SK) and a cytoplasmic response regulator (RR). The signaling cascade begins with the detection of an environmental signal by the SK. Collectively, numerous signals are sensed by bacteria via TCS. To name just a few: *P. aeruginosa* senses nitrogen and carbon concentration via NtrBC and CbrAB, respectively (22), *Bacillus subtilits* SK DesK detects changes in temperature (23), and *Klebsiella pneumoniae* CitA detects environmental citrate (24). After the environmental (or in some cases, cytoplasmic) signal is detected by the SK, the kinase domain autophosphorylates at a conserved histidine residue. Next, the activated SK phosphorylates the aspartic acid residue in the receiver domain of the cognate (interaction partner) RR via phosphotransfer from the SK to the RR (Fig. 2). This phosphorylation leads to a conformational change of the RR, which typically results in altered RR DNA binding properties and/or changes in the interactions with the RNA polymerase. The phosphorylated RR can bind upstream of the RNA polymerase binding site and enhance transcription, or it can bind downstream of the RNA polymerase binding site and inhibit or decrease transcription (25). In most cases, the phosphorylated RR will bind to a promoter via a helix-turn-helix (HTH) domain or similar DNA binding domain and promote transcription of a specific set of genes appropriate for the cellular response to the original signal sensed by the SK. Many cellular responses have been characterized include toxin production, biofilm formation, invasion/survival in the host, sporulation, and expression of virulence factors (26). For example, an RR in *K. pneumoniae* induces the expression of genes needed for citrate fermentation (24) and PhoPQ in *Salmonella* induces the expression of $Mg^{2+}$ transporter genes (27). Finally, most SKs also have phosphatase activity and dephosphorylate their

cognate RRs when the signal is no longer present (28), thus decreasing transcription of the regulated genes and restoring the transcriptional state of the cell in the absence of the signal.

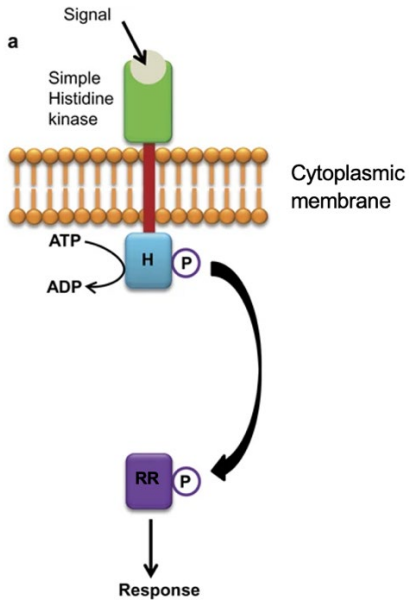


**Fig. 2. Activation and autophosphorylation of a prototypical TCS.** A signal is detected by the SK and results in autophosphorylation of the conserved histidine residue. The phosphate group is then transferred to a RR that can now regulate transcription of appropriate genes. Figure modified from (29).

## Response Regulator Families

Protein RR families are defined by their domains and the functional output of their response. All RRs function via phosphorylation of the conserved receiver domain, so additional domains and their biochemical interaction with the receiver domain define each family's structure and function. To briefly summarize these families: 1) the CheY family is found in organisms such as *Escherichia coli* and *Salmonella typhimurium* and consists solely of a single receiver domain that accepts a phosphoryl group. Phosphorylated CheY binds to the flagellar motor to promote clockwise rotation, and

therefore impacts tumbling behavior during chemotaxis (30, 31); 2) members of the OmpR family of RRs contain an N-terminal receiver domain connected via a flexible linker to a winged helix-turn-helix C-terminal domain that binds DNA (32). Phosphorylated OmpR in *S. typhyimurium* regulates porin genes, and when unphosphorylated, activates the acid stress response, essential for *S. typhimurium* virulence (33, 34). OmpR has the "classical" RR domain structure (receiver domain linked to a DNA-binding domain) and there are numerous examples across bacteria including OmpR-1 in *Rhodobacter sphaeroides* (35), RetPC57 in *Rhizobium etli* (36), and PrcR in *Lactobacillus casei* (37); 3) the NarL family of RRs contains the same linear domain architecture as the OmpR family, but the tertiary structures differ  NarL promotes nitrate respiration in *E. coli* under anaerobic conditions (38); and 4) the LytR family contains an N-terminal receiver domain and a unique LytTR DNA binding C-terminal domain and functions in autolysis in *S. aureus* (39). The LytTR regulatory systems (LRS) are a relatively newly characterized family, but when researchers modeled the conserved LRS of *Streptococcus mutans*, they found >4000 putative LRS operons that ranged across Gram-negative and Gram-positive organisms (40).

## NtrC-Like RRs

A fifth and distinct family of RRs is known as NtrC-like RRs, named after the first member of this family to be characterized, NtrC, which regulates the response to nitrogen in *E. coli* and *S. typhimurium* (41, 42). These proteins function like typical RRs but have a more complex domain architecture. NtrC-like RRs consist of the typical receiver and HTH domains, but also contain a well-conserved central ATPase domain.

The receiver domain and ATPase domain are separated by a Q linker, which predominantly takes on a coil or turn secondary structure (Fig. 3a,b) (43). Q linkers are typically 15-20 residues long and occur between domains in sensory and regulatory proteins in bacteria. They are low in arginine and rich in glutamine (Q), giving them their "Q linker" name (44). The sequences of Q linkers are not strongly conserved, and when amino acids were inserted into these linkers in NtrC, there was no impact on NtrC function (45). However, when the domains were expressed separately without the Q linker, the resulting protein did not function, indicating the linker tethering the domains is necessary for function (43).

Beyond the domain architecture of these RRs, another defining characteristic of NtrC-like RRs is their specific interaction with RNA polymerase holoenzymes containing the alternative sigma factor 54, $\sigma^{54}$. Unlike RNA polymerases containing the housekeeping sigma factor 70, $\sigma^{70}$, the $\sigma^{54}$ RNA polymerase holoenzyme recognizes and binds to promoter elements at the -12 and -24 position, but in a closed complex that cannot initiate transcription on its own (46). When phosphorylated, NtrC-like RRs interact with enhancer sites on DNA typically around 70-150 bp upstream of $\sigma^{54}$ binding sites (47). When NtrC-like RRs bind the enhancer, the DNA bends into a conformation that allows for interaction of the oligomer with $\sigma^{54}$ (Fig. 3c). This interaction results in an active ATPase domain that then hydrolyzes ATP and provides the energy required for the bound $\sigma^{54}$ RNA polymerase to form an open complex and initiate transcription (48). Overall, this specific and highly regulated NtrC-like RR interaction with $\sigma^{54}$ RNA polymerases allows for tighter control of gene expression (49).

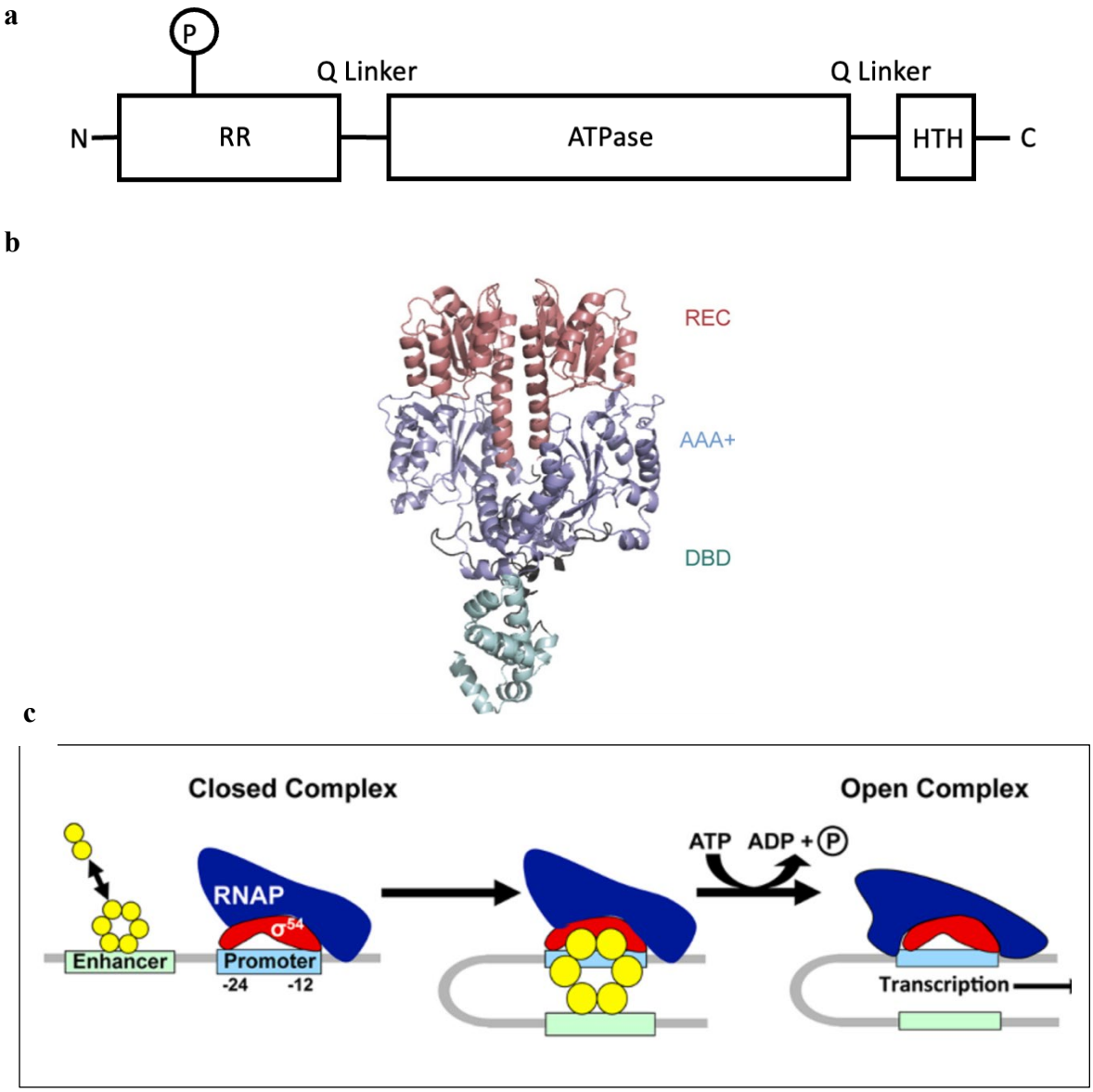


**Fig. 3. NtrC-like RR structure and function.** (a) The N-terminus of the NtrC-like RR consists of a receiver domain that gets phosphorylated by a cognate SK. The C-terminus consists of a HTH domain that binds DNA. Central to the protein structure is an ATPase domain. These domains are connected via flexible Q linkers. (b) The crystal structure of a dimer of NtrX from *Brucella abortus* (50), with each domain indicated by color. Rec indicates the receiver domain, AAA+ is the ATPase domain, and DBD (DNA binding domain) is the HTH domain. Image used with permission from (50). (c) The RNA polymerase holoenzyme complex on the left is stable and cannot become an open complex without the oligomerization of NtrC-like RRs (yellow circles, shown here as a hexamer). The formation of the oligomer drives the bending of DNA and the subsequent interaction with $\sigma^{54}$ stimulates ATP hydrolysis. Image modified from (51).

The NtrC-like RR family can be further delineated into two groups based on the positioning of the receiver domain within the oligomer and the impact of phosphorylation. First, in negatively regulated NtrC-like RRs, the receiver domain is structurally positioned to inhibit the ring assembly of the oligomer via a tight dimer interface (Fig. 4, 32). In this subfamily, the first Q linker between the receiver domain and ATPase domain has some secondary structure, typically an α helix, that participates in the dimer interaction (52). When phosphorylated, the receiver domain flips out of the way of the ATPase domain and formation of the ring (typically a heptamer) occurs (52). On the other hand, for the subfamily that has positive regulation, phosphorylation of the receiver domain activates this domain, allowing the α-helix 1 of the ATPase domain to directly interact with the adjacent α-helix 4 of the receiver domain, stabilizing the formation of the ring complex (typically a hexamer) (53). In this type of regulation the Q linker between the receiver domain and ATPase domain is less structured and is very flexible (52). In either case, once the ring is assembled, the ATPase domain hydrolyzes ATP and initiates transcription with the energy that is released.

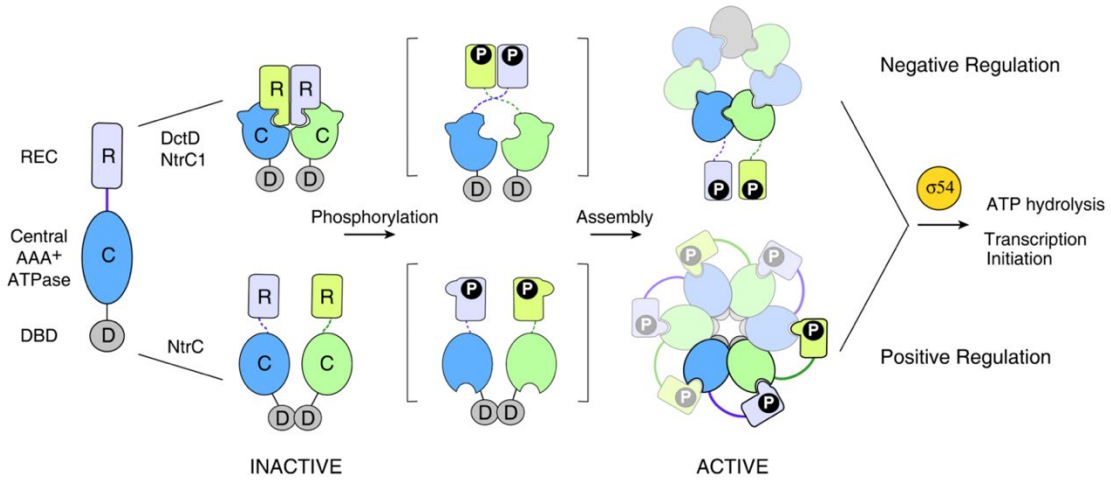


**Fig. 4. Positive vs. Negative Regulation of NtrC-like RRs.** Both positive and negatively regulated RRs have the same domain architecture but are regulated via two different mechanisms. In negatively regulated RRs, the ATPase can function without phosphorylation, but phosphorylation is needed to move the receiver domain out of the way to form the heptamer (top). In positively regulated RRs, phosphorylation of the receiver domain is necessary to form interactions between the ATPase and receiver domains to form the hexamer (bottom). Image used with permission from (32).

## TCS and Motility in *M. xanthus*

In *M. xanthus*, S-motility requires the TCS PilSR which is responsible for the gene regulation of T4P (4, 54). PilS functions to detect PilA and respond in different ways depending on the level of PilA. When the level of PilA is low, PilS phosphorylates its cognate NtrC-like RR PilR. PilR then binds the enhancer upstream of *pilA* and allows for initiation of transcription. The protein product, PilA, is the major pilin protein that comprises T4P. Under high levels of PilA, PilS will dephosphorylate PilR and *pilA* will be downregulated (55). Deletion of *pilR* in *M. xanthus* results in cells that are unable to transcribe *pilA* and are, therefore, unable to perform S-motility. Regulation of T4P by PilSR is conserved in *P. aeruginosa*, *Geobacter sulfurreducens*, and other bacteria. Additionally, mutational analysis has demonstrated roles for PilSR beyond regulation of

*pilA*. For example, in *P. aeruginosa* PilSR also regulates FleSR, which controls flagellum dependent swimming motility (16) and PilSR in *G. sulfurreducens* regulates genes involved in cell wall biogenesis and secretory pathways (56). Interestingly in *M. xanthus*, another TCS designated PilS2R2 is also necessary for motility. This system likely arose via a duplication event and regulates motility by a mechanism that is different than PilSR. Deletion of the RR *pilR2* does not impact PilA production, but it does affect T4P assembly, and therefore impacts S-motility (55). The Frz TCS is yet another TCS that assists in regulating S-motility in *M. xanthus*. As previously mentioned, the Frz pathway, specifically FrzS, contributes to cellular reversal rates. FrzS moves from one pole of an *M. xanthus* cell to the other and it is suggested that this allows FrzS to interact with channels that secrete EPS to confer EPS release (57). An additional TCS that has newly been revealed to play a role in S-motility of *M. xanthus* is NmpRSTU.

## NmpRSTU

While investigating the non-motile *M. xanthus ΔpilR* strain, it was observed that "flares" of motility spontaneously appeared (58). These flares were apparent after extended incubation of 1-2 weeks at 32°C. It was hypothesized that these flares with restored motility represented mutants that had acquired a mutation, allowing them to bypass the need for PilR. To identify the mutations, a portion of each flare was collected, DNA isolated, and the whole genome sequenced. This analysis revealed mutations in a previously uncharacterized TCS, NmpRSTU composed of NmpU, an SK; NmpS, a hybrid RR-SK; NmpT, a phospho-sink protein; and NmpR, an NtrC-like RR. NmpU is the first protein in the cascade of NmpRSTU that binds oxygen to determine if the system

is “ON” or “OFF”. In this case, the protoglobin domain of NmpU binds oxygen and is turned “ON” through autophosphorylation in the presence of oxygen. Unlike a prototypical SK, NmpU is soluble and senses the oxygen concentration within the cytoplasm. It is modeled that during high oxygen conditions NmpU is always “ON” and results in the phosphorylation of NmpS and NmpT. Importantly, phosphorylation of NmpS maintains NmpS in an “OFF” state. When NmpS is “OFF”, it cannot phosphorylate NmpR. However, under low oxygen conditions, NmpU is “OFF” and NmpS is unphosphorylated and “ON”, allowing it to phosphorylate NmpR. NmpR can then bind the *pilR* promoter and promote transcription of *pilR* (Fig. 5).

Seven of the nine mutant strains originally identified had a mutation in the SK *nmpU*, resulting in a frame shift or nonsense mutation. The only mutant within the RR *nmpR* was a missense mutation from valine to glutamic acid at position 87 (V87E). Mutational analysis suggests that the V87E mutation resulted in a gain-of-function and restored motility in the *ΔpilR* strain. When *nmpR* was deleted from an otherwise wild type strain, the cells remained motile. However, when *nmpRV87E* was deleted in the *ΔpilR* strain, motility was lost. This indicates that *nmpR* is not necessary for motility under wild type conditions; however, in the *ΔpilR* strain, the V87E mutation is responsible for the restored motility. To characterize how motility was restored, motility was assessed using a phosphomimetic variant (D54E) and a unphosphorylatable variant (D54A). It was found that the $NmpR^{D54E}$ variant restored motility to the Δ*pilR* strain whereas the $NmpR^{D54A}$ did not. With this information it was hypothesized that the $NmpR^{V87E}$ mutation resulted in a constitutively active NmpR either by making NmpR

more likely to be phosphorylated, by making it less likely to be dephosphorylated, and/or by mimicking the structure of a phosphorylated state.

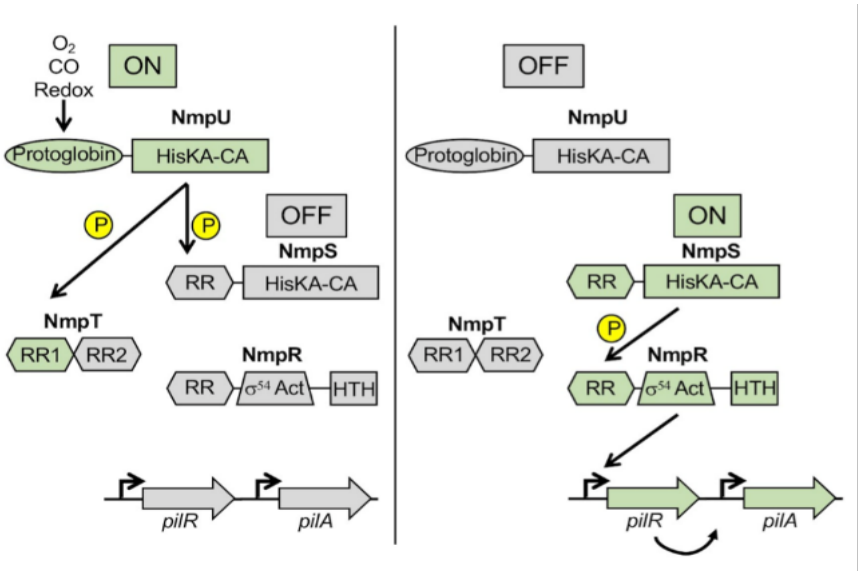


**Fig. 5. The signaling of the NmpRSTU TCS.** The signaling pathway is controlled by oxygen concentrations. In high oxygen conditions NmpU is always on and results in the phosphorylation of NmpS and NmpT, depicted on the left. Under low oxygen conditions, NmpU is off, so NmpS can phosphorylate NmpR which can promote transcription, depicted on the right. Figure used with permission from (58).

## Summary

TCS are important in regulating many cellular responses. *M. xanthus* is an ideal organism to study TCS due to their unusually high number of TCS encoded in their genome (20). SKs allow bacteria to detect environmental stimuli such as nitrogen concentration, carbon concentration, or temperature. Detection of these environmental stimuli promote changes in gene expression through phosphorylation that is essential for these cells to properly respond. For example, in *M. xanthus* PilA level is detected by PilS, part of the PilSR TCS. This allows PilR to regulate the level of PilA being expressed through a negative feedback loop. When *pilR* is deleted, *M. xanthus* cannot properly

regulate *pilA* and is no longer motile. However, over time *M. xanthus* routinely acquires mutations, especially in *nmpR*, that rescue motility.

NmpRSTU TCS is a system that functions to regulate genes under changing environmental oxygen concentrations. Constitutively "ON" NmpR variants bind upstream of *pilR* and therefore modulate transcription of *pilA*. Since NmpR is an NtrC-like RR, it is important to perform mutational analysis to determine how NmpR is becoming constitutively "ON" and, in this circumstance, rescuing *M. xanthus* motility. Like other NtrC-like RRs, it is speculated that under typical circumstances, NmpR oligomerizes to form a hexamer/heptamer, which can then bind to enhancer sites upstream of the RNA polymerase binding site. Binding of the oligomer and the ATPase activity of NtrC provide the energy necessary to bend the DNA into the open complex and initiate transcription in sigma 54-dependent genes. Through this project I investigated how the "ON" mutations influenced conformation change in the protein and how this change influenced DNA binding.

## EXPERIMENTAL OBJECTIVES

### Specific Aim 1A

*Mutations in* nmpR *result in gain-of-function of NmpR, particularly in amino acids that are highly conserved amongst NtrC-like RRs.*

Previous research has found that the non-motile *M. xanthus* Δ*pilR* regained motility after extended incubation (58). Although most of the mutations were found in *nmpU*, one was identified in *nmpR*. A second screen in a related background strain, identified an additional 12 mutations in *nmpR* (58). This second screen revealed mutations in the receiver and Q linker regions of *nmpR*. Therefore, I hypothesized that further mutations in *nmpR* would result in gain-of-function in NmpR, particularly in amino acids that are highly conserved amongst NtrC-like RRs. To test this, I repeated the suppressor mutation screen, isolating mutants with restored motility in the *M. xanthus* Δ*pilR* genetic background. This was done to see if new mutations continually arose that restored motility, or if there would reach a point of saturation in which there is a finite list of amino acid positions that when mutated can confer constitutive activity. I grew *M. xanthus* Δ*pilR* that contained an integrated plasmid conferring high expression of *nmpR* and allowed that strain to grow for an extended incubation period. I observed for flares, indicating that motility had been restored. Next chromosomal DNA isolation and PCR of *nmpR* was performed. Sequences of the amplified *nmpR* were analyzed against wild type *M. xanthus* DZ2 to identify the site of mutation in the restored motility strains.

## Specific Aim 1B

*Mutations found in conserved regions of NtrC-like RRs indicate biochemical or structural significance at the mutational sites.*

I further hypothesized that mutations found in this project may be similar to mutations previously found in other NtrC-like RRs. After mutants were harvested and sequenced, the sites of mutation were identified, and I performed an extensive literature review. Based on previously obtained mutants and preliminary research, similar mutants such as K104N (59) and D54E (60) have also been found in other studies in the literature. Since these sites of mutation were independently discovered in multiple studies, there is likely to be structural or biochemical importance to these specific amino acid sites.

## Specific Aim 2:

*$NmpR^{ON}$ variants demonstrate binding to DNA with increased oligomerization compared to wild-type NmpR.*

Oligomerization is crucial for NtrC-like RRs to initiate transcription. NtrC-like RRs (also known as bacterial enhancer binding proteins (bEBP)) have previously been shown to have altered oligomerization when mutated. For example, variants of bEBP PspF with an altered N64 and F85 positions have differing ATPase activity along with oligomeric states (61, 62). In addition, previous data in the Bretl lab showed increased oligomerization of the $NmpR^{V87E}$ variant compared to wild type (63).

Therefore, I hypothesized that $NmpR^{ON}$ variants will demonstrate binding to DNA with increased oligomerization compared to wild-type NmpR. To test this hypothesis, I performed EMSAs to determine if $NmpR^{ON}$ variants have altered DNA

binding. The focus of this aim was on mutations within the first Q linker region of NmpR because these mutations are typically in unstructured regions, yet several mutations were identified in this area that confer constitutive activity.

## MATERIALS AND METHODS

### Mutant Screening

*M. xanthus* strain JK4702 (DZ2 Δ*pilR nmpR*$^{high}$) was grown from a frozen stock at 32°C for 5 days on 1.5% agar Casitone Yeast Extract (CYE) (1% casitone, 0.5% yeast extract, 0.01M MOPS pH 7.6, and 0.1% $MgSO_4x7H_2O$) (Becton, Dickinson, and Company, Franklin Lakes, NJ). This strain has an integrated pET28a(+) plasmid that encodes for high expression of *nmpR* via the *hsp60* promoter. From the plates, 20 ml CYE broth was inoculated, and grown overnight at 32°C with shaking (220 rpm) at which time the $OD_{600}$ was measured. The bacteria were then subcultured to get the required optical density of 0.5-1.0 for experimentation (log-phase growth) following another overnight incubation. The next day, cells were harvested and concentrated by centrifugation, washed in 1 ml MMC (20 mM MOPS, pH 7.6, 4.0 mM $MgSO_4$, 2 mM $CaCl_2$), with a final resuspension to an OD equivalent of 2.2 in MMC. Ten microliters of this cell suspension was plated in the center of multiple 0.5% agar CYE plates, the standard percent agar for S-motility (11). Spots were allowed to dry onto the surface of the agar before placing the plates into a 32°C incubator. These plates were observed for restored motility over the course of two weeks (Fig. 6).

When a mutant flare of a distance of at least 1 cm from the non-motile colony was observed, a 1 cm x 1 cm square was cut from the plate using a flame-sterilized razor blade. The section was transferred to a new 0.5% agar CYE plate and incubated at 32°C for 5-7 days until the flare had moved from the center to exclude any non-motile cells that may have traveled along with the mutant motile cells on the original plate. Following this incubation period, the motile suppressor mutant strains were picked using the same

method and placed into 20 ml CYE broth to prepare the culture for chromosomal DNA isolation and long-term storage. Any sample that had not mutated (i.e. remained non-motile) after two weeks of incubation on the 0.5% agar CYE plates was passaged by the same method to a new 0.5% CYE agar plate at 32°C for an additional two weeks. Finally, any sample without motility flares was passaged once more and observed for motility. Thus, any particular sample was incubated for a maximum of 6 weeks. Any sample that had not mutated by the end of 6 weeks was noted and discarded. This entire procedure was repeated one additional time with an independent starter culture.

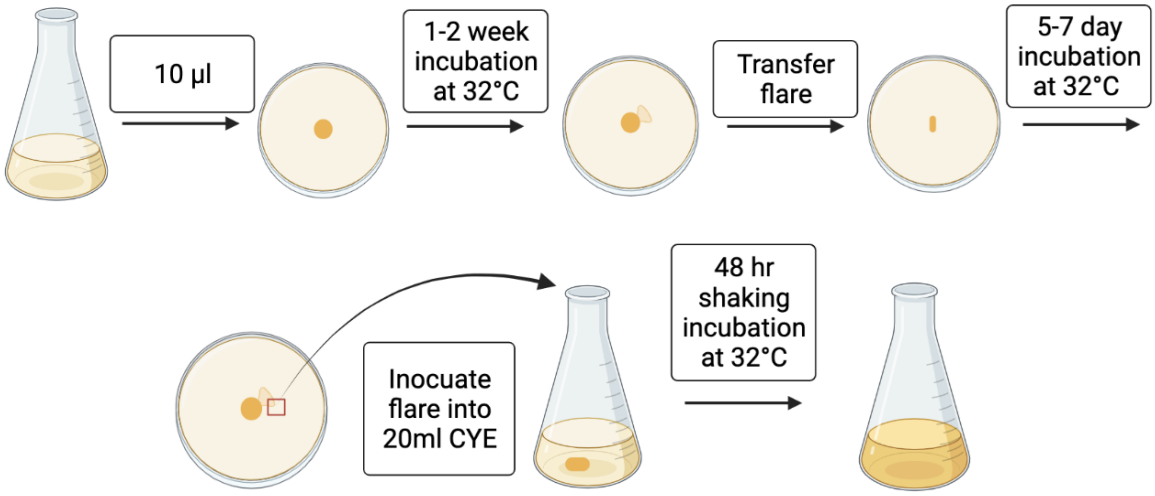


**Fig. 6. Mutant screening methods.** Mutants were isolated using a suppressor screen and a series of passages on CYE 0.5% motility agar. Image made with BioRender.

## Chromosomal DNA Isolation

Each mutant with restored motility was grown in 20 ml CYE broth overnight until it reached an $OD_{600}$ ~0.8. Three milliliters of each sample were centrifuged at 3000 x g for 10 min at room temperature. Then the supernatant was removed, and the cell pellet was resuspended in 4 ml SET buffer (75 mM NaCl, 25 mM EDTA, 10 mM Tris pH 7.5). To lyse the cells, 400 µl of 10% SDS was added, and the tube was inverted three times.

To reduce contamination of the DNA with cellular components, 1 µl of 20 mg/ml Proteinase K (New England Biolabs, Ipswich, MA) was added to the sample and then incubated for 1 hr at 55°C while occasionally inverting the tube. Then 10 µl of 20 mg/ml RNaseA (New England Biolabs, Ipswich, MA) was added and incubated at room temperature for 10 min. To precipitate the cellular components, 1/3 volume of 5 M NaCl was added to the solution with gentle rocking. One volume of chloroform was added, and the sample was rocked for 30 min. Next, samples were centrifuged at 3000 x g for 10 min at room temperature. The upper phase of the solution containing the DNA was transferred to a new tube, one volume of cold isopropanol was added, and the tube was rocked (Fig. 7). This was stored at -20°C overnight. The next day the samples were centrifuged at 3000 x g for 5 min at room temperature. The supernatant was decanted, and the DNA pellet was washed with 1 ml 70% ethanol. The solution was centrifuged at 13,000 rpm and all of the 70% ethanol was carefully removed. The DNA pellet was dissolved in ~200 µl of sterile water until resuspended and stored at 4°C.

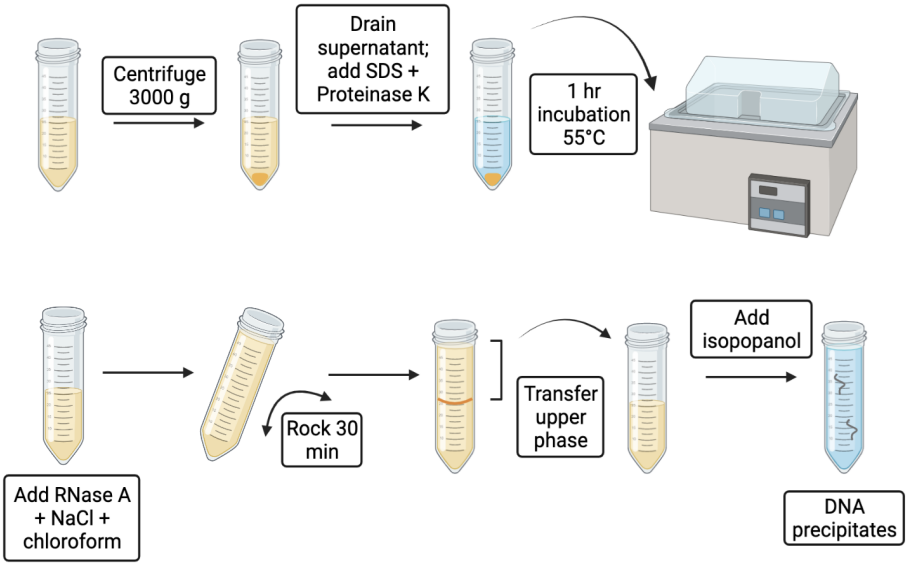


**Fig. 7. Chromosomal DNA isolation.** Mutant DNA was isolated using a chloroform extraction method. Image made with BioRender.

## PCR/Gel Electrophoresis

After chromosomal isolation was complete, DNA concentrations were determined by spectrophotometry (Nanodrop, Thermo Scientific, Waltham, MA). Samples were diluted to a concentration of 20 ng/µl for use as a PCR template. Fifty microliter PCR reactions consisted of: 1 µl of this template DNA, 25 µl Buffer K (Lucigen, Middleton, WI), 2.5 µl Hsp60Fwd primer (5' AATCTAGACTGGTGGTAGAGGATGGCCAG 3'), 2.5 µl 4240 full reverse primer (5' AAAAAGCTTCTACGACTCCGCGGCCAG 3'), 1 µl FailSafe polymerase (Lucigen, Middleton, WI), and 18 µl DI water, totaling 50 µl. Samples amplified with parameters of: denaturation 95°C for 30 sec, annealing 57°C for 30 sec, extension 72°C for 2.5 min. Samples were separated by electrophoresis for 30 min and 150V using a 1% agarose gel. DNA having the expected length of ~2000 bp (~1400 bp from *nmpR* and ~600 bp from the *hsp60* promoter) were considered positive (Appendix A).

## Preparation for Sanger Sequencing

A PCR cleanup kit (IBI Scientific, Dubuque, IA) was used to prepare the PCR products for sequencing per the manufacture's protocol with a final elution 15 µl elution buffer. DNA concentration of the PCR products was determined by Nanodrop and diluted to 40-50 ng/µl for sequencing. Products were sequenced with four primers to ensure full coverage of the *nmpR* sequence (Fig. 8): 4240 forward (5' AATCTAGAATGTCCCCACAGCGAATCCT 3'), 4240 rec reverse (5' AAAAAGCTTTCACTTCTCCAGCACCTTGCC 3'), ATPase forward (5' AAACATATGATCATCGGCGAATCGCC 3'), and 356 forward (5'

GCTGCTGTCCCAGGACTGGCCGGGC 3'). Five microliters of the appropriate primer was added to each tube along with 5 µl of the DNA template and sequenced at Eurofins Genomics (Louisville, KY). Sequences were analyzed with NCBI blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Clustal Omega (64) to identify mutation sites compared to *M. xanthus* DZ2 *nmpR* (*MXAN_4240*).

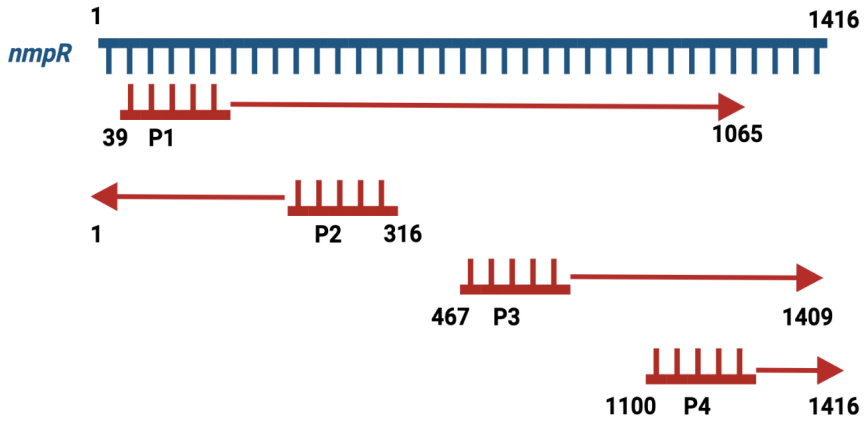


**Fig. 8. *nmpR* sequencing primers.** Four different primers were used for Sanger Sequencing to obtain coverage of the full sequence length of *nmpR*. Image made using BioRender.

## Plasmid Generation and Protein Purification

A previously constructed plasmid that contains *nmpR* in the expression vector pET-28a(+) (Novagen, Madison, WI) was used to perform site-specific mutagenesis for select mutants found within the Q linker (Table 1). To do this, NEBase changer was used (New England Biolabs, Ipswich, MA) per the manufacturers protocol. The resulting plasmid products were transformed into *E. coli* DH5 and the plasmids from the resulting transformants were purified using a miniprep plasmid kit (IBI Scientific, Dubuque, IA) and sequence confirmed. The confirmed plasmid was then transformed into *E. coli* BL21 (DE3) for protein production.

Transformants were grown on LB (lysogeny broth) agar with 50 μg/ml kanamycin at 37°C overnight. Then an isolated colony was inoculated into 5 ml of LB broth with 50 μg/ml kanamycin and grown at 37°C overnight shaking at 220 rpm. One milliliter of turbid broth was transferred into 250 ml terrific broth (12 g Bacto Tryptone, 24 g Yeast Extract, 4 ml glycerol, and 1 L DI water). A 40x super salts solution (501.6 g $K_2HPO_4$ and 92.5 g $KH_2PO_4$) was added to the terrific broth to a final 1x concentration and the culture incubated at 37°C until $OD_{600}$ ~0.5. Isopropyl -D-1-thiogalactopyranoside (IPTG) was added to the solution (1 mM final concentration) and the culture was further incubated overnight at 16°C shaking at 220 rpm. The culture was centrifuged at 6000 x g for 20 min at 4°C to pellet the cells. After the supernatant was removed, the pellet was transferred to a new tube and resuspended in wash buffer (50mM sodium phosphate pH 8.0, 300 mM NaCl, 1 mM imidazole). CellLytic powder (Sigma-Aldrich) was added to lyse the cells. This solution rocked for 1 hr and then centrifuged at 3000 x g at room temperature for 30 min. The lysate was added to a HIS-Select Cobalt Affinity Gel (Sigma-Aldrich) that was pre-equilibrated with distilled water and wash buffer (50 mM sodium phosphate pH 8.0, 1 mM imidazole, 300 mM NaCl). The column was washed twice with 10 ml of buffer containing 10 mM imidazole, then twice with 10 ml of buffer containing 20 mM imidazole, and finally the protein was eluted with 5 ml of buffer containing 250 mM imidazole. The eluent was dialyzed overnight at 4°C in dialysis buffer (125 mM NaCl, 1 M Tris pH 7.6, 1mM Dithiothreitol (DTT), 0.5 M EDTA, 1% Triton X-100, and 50% glycerol). The dialyzed protein was separated by SDS-PAGE to establish protein purity.

Table 1. List of primers for mutagenesis

| Primer | Sequence (5' to 3') |
|---|---|
| NmpR(R132W)F | CGAGGCGCTGTGGGACCAGGTGC |
| NmpR(R132W)R | GCCTCCTGGCGCAGGCTG |
| NmpR(D133N)F | GGCGCTGCGGAACCAGGTGCGCT |
| NmpR(D133N)R | TCGGCCTCCTGGCGCAGG |
| NmpR(Q134E)F | GCTGCGGGACGAGGTGCGCTCGC |
| NmpR(Q134E)R | GCCTCGGCCTCCTGGCGC |

**Electromobility Shift Assays (EMSA)**

PCR was used to generate DNA probes for the EMSAs. The *mxan_4236* promoter, the first gene in the putative operon containing *nmpR*, was used as the probe as it has been previously demonstrated NmpR binds at this location (58). Two microliters of 10 μM forward (5' AAATCTAGACTGGAGTCGTAGACGTGCACC 3') and reverse (5' AAAGGATCCCATGGCGGTACTCCTGGCA 3') primers for *mxan_4236* was added to a PCR tube along with 25 μl Buffer K, 20.5 μl DI water, 0.5 μl FailSafe polymerase, and 1 μl template *M. xanthus* DZ2 DNA for a total PCR reaction of 50 μl. The DNA was amplified with an annealing temperature of 57°C for 30 s and an extension time of 45 s. To confirm the PCR product, amplicons were separated on a 1.0 % agarose gel at 150 V for 30 min (Appendix B). The same PCR cleanup protocol as previously described was performed and then samples were diluted with DI water to 100 fmol/μl.

For the EMSAs, 8% glycine gels were prepared by adding 8 ml 30% acrylamide/bis-acrylamide solution (Bio-Rad), 3 ml 10x glycine buffer (2 ml 0.5M EDTA, 250 ml Tris pH 7.6, 144 g glycine, water to 1L), 20 μl TEMED

(tetramethylethylenediamine), 150 µl 10% APS (ammonium persulfate), to 19 ml DI water. Concentration of purified NmpR and $NmpR^{ON}$ was determined by a Bradford Assay (65) (Biorad, Hercules, CA) and dilutions of purified mutant protein were made so that the final amount of protein was 2, 4, 8, and 16 pmol/µl, confirmed via SDS-PAGE (Appendix C). To each 20 µl EMSA reaction, DI water, 1x EMSA buffer (20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.6, 10 mM $(NH_4)_2SO_4$, 1 mM EDTA, 1 mM DTT, 30 mM KCl, 0.20% Tween 20), 200 fmol *mxan_4236* DNA probe, and 1 µl of protein was added. Five microliters of bromophenol blue dye (25% TBE buffer (890 mM Tris base, 890 mM boric acid, and 20 mM EDTA pH 8.0), 34% glycerol, 32.5% DI water, and 0.2% wt/v bromophenol blue) was also added to each tube. Twenty microliters of each sample was loaded into glycine gels that had been equilibrated in running buffer for 45 min at 60 V. Following loading of the glycine gel the EMSAs were resolved at 60 V for 2 hr. Gels were stained in 1 µg/ml ethidium bromide for 15 min and then imaged on a Gel Doc EZ Imager (BioRad, Hercules, CA) (Appendix D).

# RESULTS

## Numerous Independent Mutations Result in a Constitutively Active NmpR

Following the motility suppressor screen, twenty-four independent mutant strains were isolated and sequenced. Of these, 19 unique mutations were identified. These mutations were all missense mutations found in the receiver domain, first Q-linker, or ATPase domain (Fig. 9). Mutations identified by independent investigators in the Bretl lab were compiled into one comprehensive figure (Fig. 10). The mutations that occurred with the highest frequency were found in the receiver and first Q linker domain. There were six identified mutations in the ATPase domain, but no mutations in the HTH domain. Since the receiver domain is the domain in which the protein gets switched "ON" via phosphorylation and subsequent conformation change, it is logical that the most mutations are within this domain. In addition, many mutations were seen in a short span of amino acids in the Q linker domain (AAs 121-145). For example, R136C was identified four independent times by three different researchers, indicating this residue within the Q linker could be especially important in keeping the protein in its "OFF" state. In addition, replicates of other mutations were also found in these same domains. For example, mutation K104N was seen 11 independent times in the mutant screens that were conducted by multiple researchers. This mutation is well known as a gain-of-function mutation in RRs (59, 60), and the hypothesized mechanism of rescue is explored further in the discussion portion. Other mutations such as M94V and H356Q were seen five independent times. Collectively, the frequency of these independently identified mutations strongly suggests these amino acids play a critical role in the structure and function of NmpR. The fact that no mutations in the HTH domain were found may

indicate that the HTH is necessary for RR function and may be less amenable to mutation. As discussed previously, the HTH domain functions in binding the protein to the DNA it regulates, so any mutation in this region may negatively impact the ability of the RR to bind and regulate gene expression.

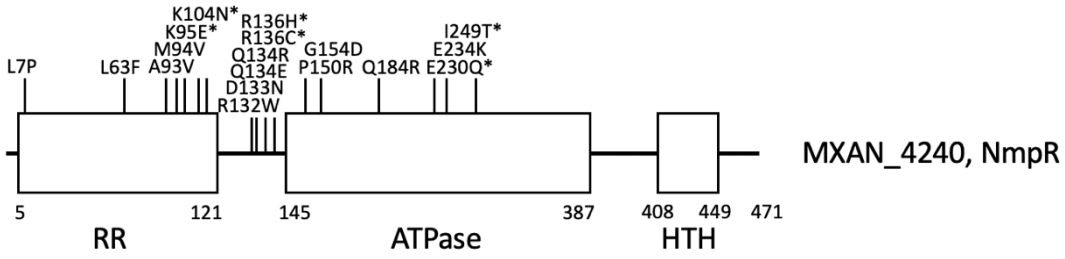


**Fig. 9. Gain-of-function mutations in *nmpR*.** Suppressor mutations from the *M. xanthus ΔpilR nmpR*$^{high}$ were isolated and subjected to Sanger sequencing. The mutational analysis revealed multiple mutations in the receiver, Q linker, and ATPase domains as depicted. Mutations with a * were identified twice independently.

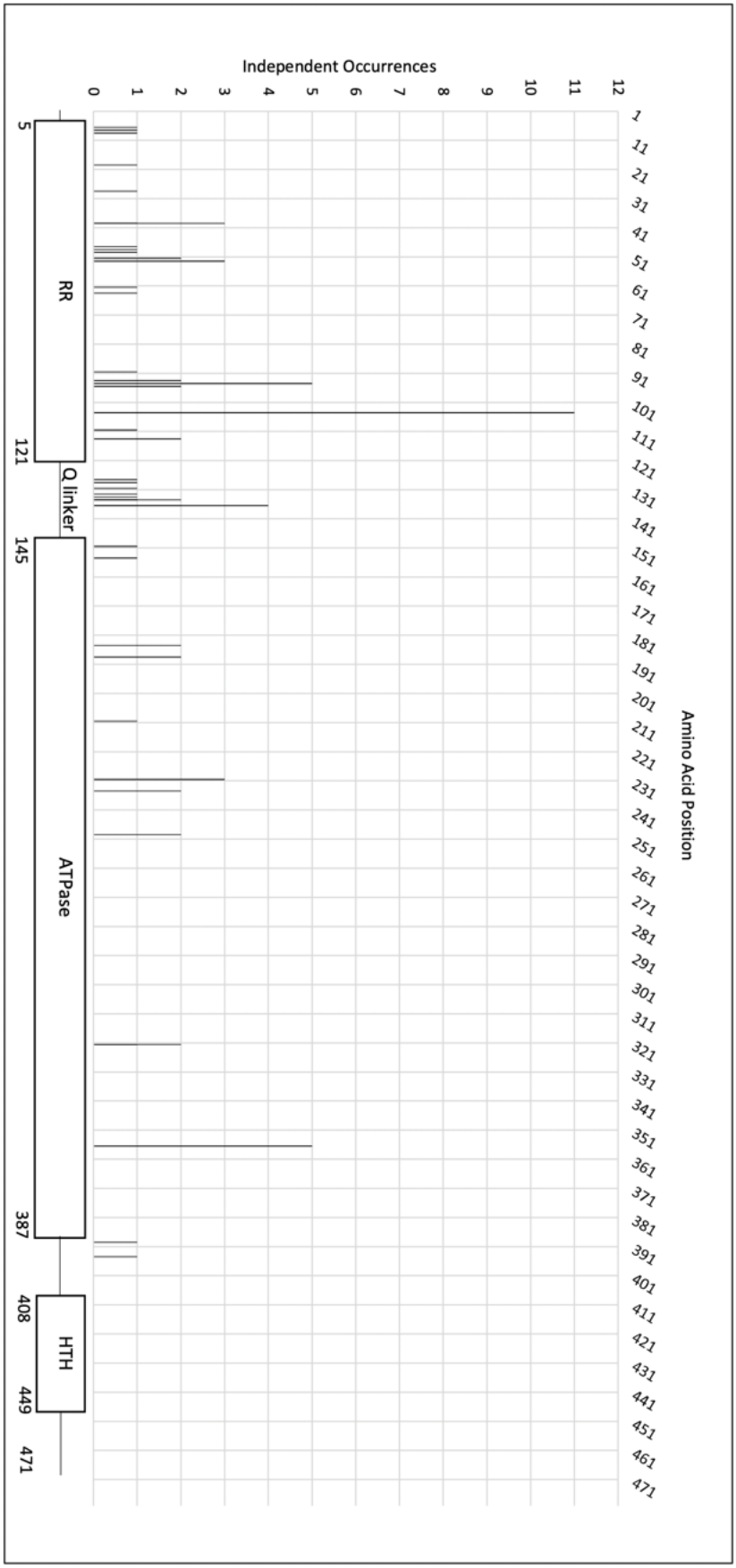
Independent Occurrences
0
1
2
3
4
5
6
7
8
9
10
11
12
Amino Acid Position
1
11
21
31
41
51
61
71
81
91
101
111
121
131
141
151
161
171
181
191
201
211
221
231
241
251
261
271
281
291
301
311
321
331
341
351
361
371
381
391
401
411
421
431
441
451
461
471
RR
5
121
Q linker
145
ATPase
387
408
HTH
449
471

**Fig. 10. Comprehensive map of *nmpR* mutations**. The mutant screen performed in this work was repeated by multiple individuals including Dan Bretl, Ph.D, Vanessa Giallombardo, and other undergraduate researchers in the Bretl lab. The x-axis indicates the amino acid position within *nmpR*, and the y-axis is the number of independent occurrences of these mutants. This data was compiled by Vanessa Giallombardo who also constructed this graph.

## NmpR Variants Have Increased Oligomerization Upon DNA Binding

To analyze the binding pattern of NmpR and NmpR$^{ON}$ variants to the native *mxan_4236* promoter, a series of EMSAs were performed. It is known that wild type NmpR binds upstream of *mxan_4236* with a pattern that indicates the formation of an oligomer in a protein concentration dependent manner (58, 66). Specifically, the wild type NmpR binds the DNA probe with a reproducible stepwise shift in binding that is protein concentration dependent, indicating a higher order complex has been formed (66). In this assay, the *pilA* promoter was used as a negative control because it is known that NmpR does not bind the *pilA* promoter (58). Further analysis by another graduate student in the Bretl lab indicated that there are two separate enhancer sites upstream of the promoter of *mxan_4236* on which NmpR binds (63). These sites are designated as "site 1" and "site 2", which are distal and proximal to the start codon, respectively (Fig. 11). When site 1 is mutated, NmpR can still bind to site 2 and form higher order structures, although not as well as with the wild type probe indicated by the unbound probe at the bottom of the wells and a reproducible "smear" of the shift (Fig. 12). When site 2 is mutated, NmpR is unable to bind to site 1. This indicates site 2 is necessary for wild-type NmpR binding to the *mxan_4236* promoter. In addition to wild type, EMSAs were performed on variants of NmpR including D54A (cannot be phosphorylated), D54E (mimics phosphorylation) and mutants within the receiver domain and Q linker domains. The D54A variant binds like wild type NmpR, which suggests the wild-type NmpR is in an unphosphorylated state in this assay. The D54E variant mimics phosphorylation and

oligomerizes well even with the smallest concentration of protein. The receiver and linker domain mutant EMSA patterns appear to follow the binding pattern like that of the D54E variant, indicating these mutants somehow mimic phosphorylation. Another observation with these "ON" variants is that they bind the DNA probe even when site 2 is mutated, unlike wildtype NmpR, showing they can oligomerize when only site 1 is available. Some of these variants even shift some DNA, albeit at a much lower amount when both sites 1 and 2 are mutated. The same type of pattern is seen in the *pilA* EMSAs (negative control), which is known NmpR does not bind. This possibly indicates some DNA-independent oligomerization that non-specifically shifts some DNA probe. Overall, NmpR mutants in both the receiver domain and Q linker domain shift both wild type and mutant *mxan_4236* better than wild type NmpR.

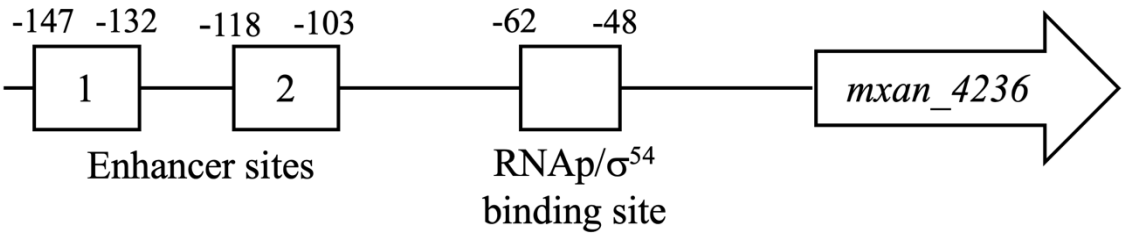


**Fig. 11. Map upstream of the *mxan_4236* promoter.** NmpR binds both enhancer sites 1 and 2 under typical conditions. The distance indicated are relative to the translational start site of *mxan_4236*.

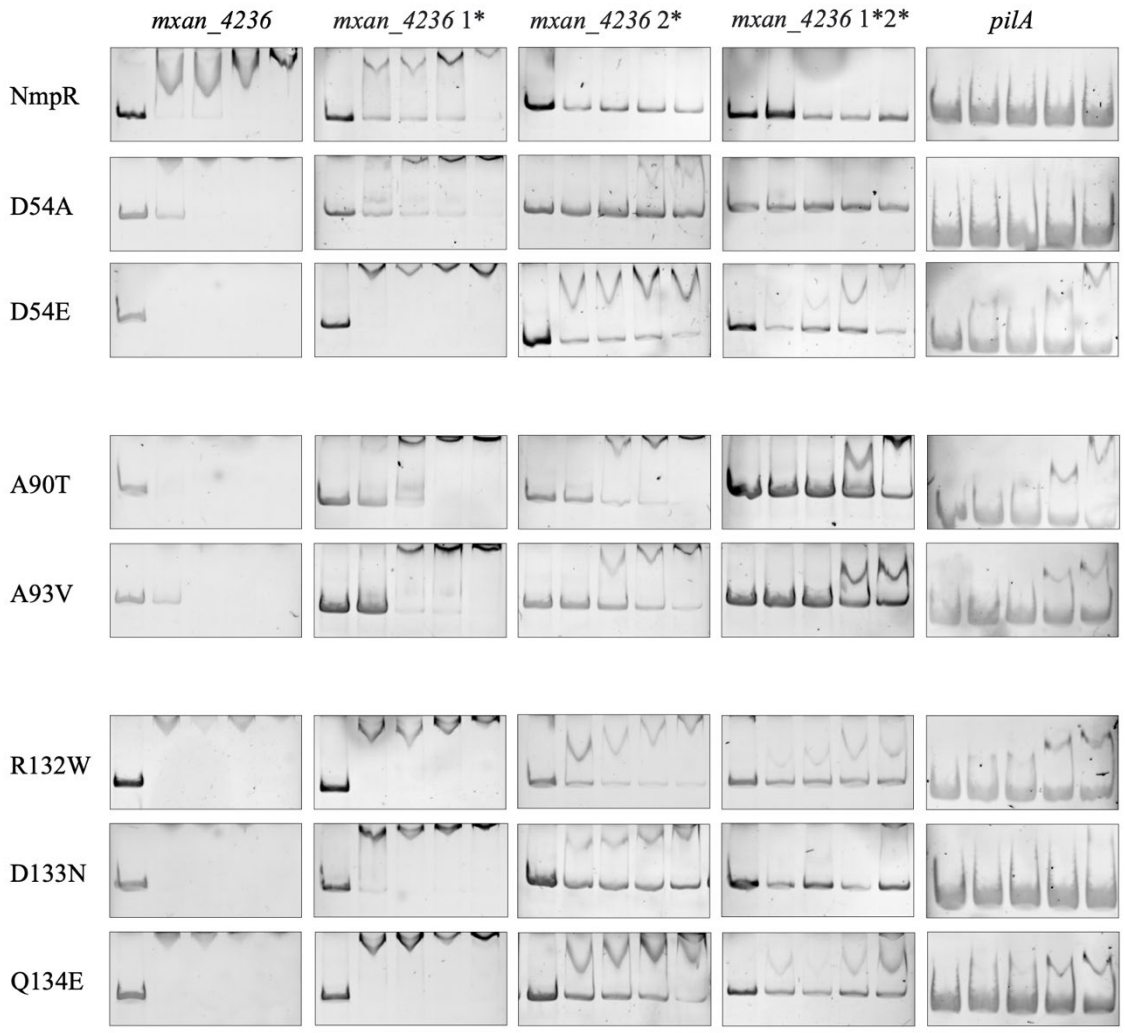


**Fig. 12. NmpR variant DNA binding pattern**. Electromobility shift assays (EMSAs) of NmpR variants to the *pilA* promoter (negative control) and the *mxan_4236* promoter were separated on 8% acrylamide gels for 2 hr at 60V. The protein concentrations in the wells are 0, 2, 4, 8, 16 pmol in lanes moving left to right. * denotes the corresponding mutated enhancer site.

## DISCUSSION

*M. xanthus* is a soil bacterium that relies on its multicellular behavior to survive in its changing environment. TCSs are utilized by *M. xanthus* to control its gene expression in response this highly dynamic environment. Specifically, RRs bind promoters and influence transcription of genes in response to the signals sensed by SKs. In the case of motility, *M. xanthus* uses a TCS called PilSR to promote transcription of *pilA* and therefore promote T4P production and social motility. When non-motile strains (*ΔpilR*) are left to incubate for long periods of time, mutant strains arise that acquire restored motility through a secondary TCS designated NmpRSTU. Specifically, NmpR, an NtrC-like RR, acquires gain-of-function mutations throughout the gene that confers the ability to bypass the otherwise necessary PilR. There are many mechanisms of how these mutations in NmpR may lead to this gain-of-function based on the contribution of each domain of NtrC-like RRs and the contribution of known mutations in other NtrC-like RRs.

### Receiver Domain

*Contributions of amino acids upstream of the phosphorylated aspartic acid residue*

Mutations within the first 20 AAs of the N-terminus of NtrC-like RRs are typically not in well conserved regions and do not have clear explanations indicating why these mutations confer constitutive activity. In the NtrC-like RR FlbD, which promotes flagellar gene transcription based on spatial and temporal cues in *Caulobacter crescentus*, amino acid substitutions at L9P, Q12P, L13R, and V17M (Table 2) were identified through the isolation of suppressor mutants of a non-motile strain (Δ*fliX,* a regulator of

temporal flagellar transcription) (67). All of these substitutions occurred at residues that are not well conserved except L13R, which is typically seen as an arginine in other RRs (67). Interestingly, most receiver domains across all RR families contain 2-3 aspartic acid residues near position 10 that have been proposed to assist in the phosphorylation reaction, possibly through $Mg^{2+}$ binding that FlbD lacks (30). In another NtrC-like RR DctD, point mutations V7M and L44P resulted in a constitutively active protein (68). DctD is found in *Sinorhizobium meliloti* and *Rhizobium leguminosarum* and controls expression of *dctA*, encoding a $C_4$-dicarboxylate transport protein. It is unknown how these mutations lead to constitutive activity, but it has been proposed that these variants may mimic a phosphorylated state or escape the negative autoregulation of the N-terminal domain.

Many mutations within this region of the receiver domain in NmpR conferred constitutive activity, including early mutations I6N, L7P, and V8A, all contained within the N-terminal β1 sheet. The very early positions I6 and L7 are not well conserved across a multiple sequence alignment, but V8 is conserved across four of the six NtrC-like RRs explored in this review (Table 2). It is not clear what specifically these residues are responsible for within the β1 sheet, but because their mutation leads to a rescue of function, they may play a role the change in conformation that happens upon phosphorylation.

Another group of mutations in NmpR occurred in the amino acids S47 through L52. Residues S47, P48, and A49 are within the α2-β3 connecting region, and L51 and L52 are in β3, both structures that undergo movement upon phosphorylation, leading to activation (Fig. 13, 69). Khalili and Wales predicted that upon phosphorylation, there is

movement of the α4 helix, followed by movement of the α2 helix, and finally a flip of the β3-α3 loop (69). Mutations of residues within α2-β3 could confer activation by altering the structure to mimic this movement proposed to occur after phosphorylation to induce protein activation.

Table 2. Constitutively Activating Mutations of NtrC-like RRs.

| NtrC AA[a] | Substitution | Protein | NtrC Domain | Species | Source |
|---|---|---|---|---|---|
| V6 | I6N | *mxan_4240* | REC | *M. xanthus* | (this study) |
| V6 | V7M | DctD | REC | *S. meliloti* | (68) |
| W7 | L7P | *mxan_4240* | REC | *M. xanthus* | (this study) |
| V8 | V8A | *mxan_4240* | REC | *M. xanthus* | (this study) |
| D12 | L9P | FlbD | REC | *C. crescentus* | (67) |
| I15 | Q12P | FlbD | REC | *C. crescentus* | (67) |
| R16 | L13R | FlbD | REC | *C. crescentus* | (67) |
| L19 | I19N | *mxan_4240* | REC | *M. xanthus* | (this study) |
| E20 | V17M | FlbD | REC | *C. crescentus* | (67) |
| L28 | Y28D | *mxan_4240* | REC | *M. xanthus* | (this study) |
| V39 | A39T<br>A39V | *mxan_4240* | REC | *M. xanthus* | (this study) |
| L43 | L44P | DctD | REC | *S. meliloti* | (68) |
| T47 | S47P | *mxan_4240* | REC | *M. xanthus* | (this study) |
| P48 | P48T | *mxan_4240* | REC | *M. xanthus* | (this study) |
| P48 | P41L | LuxO | REC | *V. fischeri* | (70) |
| D49 | A49P | *mxan_4240* | REC | *M. xanthus* | (this study) |
| L51 | V51M | *mxan_4240* | REC | *M. xanthus* | (this study) |
| L52 | L52R | *mxan_4240* | REC | *M. xanthus* | (this study) |
| D54 | D52A | Rrp2 | REC | *B. burgdorferi* | (71) |
| D54 | D61E | LuxO | REC | *V. harveyi* | (72) |
| D54 | D54E | NtrC | REC | *S. typhimurium* | (73) |
| D61 | D61V | *mxan_4240* | REC | *M. xanthus* | (this study) |
| L63 | L63F | *mxan_4240* | REC | *M. xanthus* | (this study) |
| P77 | P78S | DctD | REC | *S. meliloti* | (68) |
| I79 | I80N | DctD | REC | *S. meliloti* | (68) |
| T80 | T83I | DctD | REC | *S. meliloti* | (68) |
| D86 | D86N | NtrC | REC | *S. typhimurium* | (73, 74) |
| L87 | V87E | *mxan_4240* | REC | *M. xanthus* | (this study) |
| A89 | A89T | NtrC | REC | *S. typhimurium* | (73) |
| A90 | A90T | *mxan_4240* | REC | *M. xanthus* | (this study) |

| | | | | | |
|---|---|---|---|---|---|
| A93 | A93V | *mxan_4240* | REC | *M. xanthus* | (this study) |
| Y94 | M94V | *mxan_4240* | REC | M. xanthus | (this study) |
| Q95 | K95E<br>K95N | *mxan_4240* | REC | M. xanthus | (this study) |
| Q95 | K92 | NtrC1 | REC | A. aeolicus | (52) |
| A98 | A91D | LuxO | REC | *V. fischeri* | (70) |
| F99 | Y100D<br>Y100H | DctD | REC | *S. meliloti* | (68) |
| F99 | Y96F | NtrC1 | REC | *A. aeolicus* | (52) |
| D100 | D101G<br>D101K | DctD | REC | *S. meliloti* | (68) |
| Y101 | F94C | LuxO | REC | *V. fischeri* | (70) |
| Y101 | F94W<br>(F108W) | LuxO | REC | *V. harveyi* | (72) |
| Y101 | F108A<br>F108W | LuxO | REC | *V. cholerae* | (75) |
| L102 | I103T | DctD | REC | *S. meliloti* | (68) |
| K104 | K104Q | NtrC | REC | *E. coli* | (59) |
| K104 | K104R | NtrC | REC | *E. coli* | (60) |
| K104 | K104N | *mxan_4240* | REC | *M. xanthus* | (this study) |
| P105 | P98L<br>P98Q | LuxO | REC | *V. fischeri* | (70) |
| D107 | A107T | FlrC | REC | *V. cholerae* | (76) |
| D109 | E106S | NtrC1 | REC | *A. aeolicus* | (52) |
| E110 | E107S | NtrC1 | REC | *A. aeolicus* | (52) |
| A111 | L112P | DctD | REC | *S. meliloti* | (68) |
| A111 | L104Q<br>(L118Q) | LuxO | REC | *V. cholerae* | (77) |
| V112 | A107A<br>A107T | FlbD | REC | *C. crescentus* | (67) |
| A113 | V113A | *mxan_4240* | REC | *M. xanthus* | (this study) |
| A113 | V120E | LuxO | REC | *V. cholerae* | (75) |
| L114 | M114V | FlrC | REC | *V. cholerae* | (76) |
| L114 | T107R | LuxO | REC | *V. fischeri* | (70) |
| L114 | T114P | *mxan_4240* | REC | *M. xanthus* | (this study) |
| L114 | M114I | FlrC | REC | *V. cholerae* | (78) |
| V115 | V108G | LuxO | REC | *V. fischeri* | (70) |
| V115 | V115I | NtrC | REC | *E. coli* | (73) |
| R117 | K114E | NtrC1 | REC | *A. aeolicus* | (52) |
| I119 | T114N | FlbD | REC | *C. crescentus* | (67) |
| S120 | E117K | NtrC1 | R-C Linker | *A. aeolicus* | (52) |
| S120 | E121K | DctD | R-C Linker | *S. meliloti* | (68) |
| H121 | K122E<br>K122D | DctD | R-C Linker | *S. meliloti* | (68) |
| H121 | R114P | LuxO | R-C Linker | *V. fischeri* | (70) |

| | | | | | |
|---|---|---|---|---|---|
| H121 | H118E | NtrC1 | R-C Linker | *A. aeolicus* | (52) |
| H121 | D116G | FlbD | R-C Linker | *C. crescentus* | (67) |
| Q127 | E124K | NtrC1 | R-C Linker | *A. aeolicus* | (52) |
| Q127 | L132Q | DctD | R-C Linker | *S. meliloti* | (68) |
| Q127 | E127A | *mxan_4240* | R-C Linker | *M. xanthus* | (this study) |
| P128 | A128S | *mxan_4240* | R-C Linker | *M. xanthus* | (this study) |
| P128 | N125S | NtrC1 | R-C Linker | *A. aeolicus* | (52) |
| R129 | R129S | NtrC1 | R-C Linker | *A. aeolicus* | (52) |
| N130 | A130V | *mxan_4240* | R-C Linker | *M. xanthus* | (this study) |
| I131 | R136H<br>R136C<br>R136L | *mxan_4240* | R-C Linker | *M. xanthus* | (this study) |
| I131 | E131K | NtrC1 | R-C Linker | *A. aeolicus* | (52) |
| Q132 | R132W | *mxan_4240* | R-C Linker | *M. xanthus* | (this study) |
| Q132 | K132E | NtrC1 | R-C Linker | *A. aeolicus* | (52) |
| N/A[b] | D133N | *mxan_4240* | R-C Linker | *M. xanthus* | (this study) |
| N/A | Q134E | *mxan_4240* | R-C Linker | *M. xanthus* | (this study) |
| D139 | G145E | LuxO | R-C Linker | *V. cholerae* | (75) |
| I141 | V121G | FlbD | $\sigma^{54}$ Act | *C. crescentus* | (67) |
| A144 | D124N | FlbD | $\sigma^{54}$ Act | *C. crescentus* | (67) |
| P145 | P150R<br>P150L | *mxan_4240* | $\sigma^{54}$ Act | *M. xanthus* | (this study) |
| D149 | G154D | *mxan_4240* | $\sigma^{54}$ Act | *M. xanthus* | (this study) |
| S160 | S140F | FlbD | $\sigma^{54}$ Act | *C. crescentus* (now *C. vibrioides)* | (79) |
| S160 | S160F | NtrC | $\sigma^{54}$ Act | *S. typhimurium* | (74, 80, 81) |
| S160 | S160F<br>S160Y<br>S160W<br>S160C<br>S160L | NtrC | $\sigma^{54}$ Act | *S. typhimurium* | (82) |
| H179 | Q184R | *mxan_4240* | $\sigma^{54}$ Act | *M. xanthus* | (this study) |
| R183 | Q188R | *mxan_4240* | $\sigma^{54}$ Act | *M. xanthus* | (this study) |
| L204 | L185A | FlbD | $\sigma^{54}$ Act | *C. crescentus* | (67) |
| I205 | L210V | *mxan_4240* | $\sigma^{54}$ Act | *M. xanthus* | (this study) |
| R224 | C215Y | FlrC | $\sigma^{54}$ Act | *V. cholerae* | (76) |
| Q225 | E230Q | *mxan_4240* | $\sigma^{54}$ Act | *M. xanthus* | (this study) |
| E229 | E234K | *mxan_4240* | $\sigma^{54}$ Act | *M. xanthus* | (this study) |
| M244 | I249T | *mxan_4240* | $\sigma^{54}$ Act | *M. xanthus* | (this study) |
| V248 | V248A | NtrC | $\sigma^{54}$ Act | *S. typhimurium* | (74) |
| V248 | L228Q | FlbD | $\sigma^{54}$ Act | *C. crescentus* | (67) |
| V288 | V288I | NtrC | $\sigma^{54}$ Act | *S. typhimurium* | (74) |
| R315 | S321R<br>S321I | *mxan_4240* | $\sigma^{54}$ Act | *M. xanthus* | (this study) |
| D317 | D297E | FlbD | $\sigma^{54}$ Act | *C. crescentus* | (67) |

| | | | | | |
|---|---|---|---|---|---|
| R323 | H319R | LuxO | $\sigma^{54}$ Act | *V. fischeri* | (70) |
| Q327 | G333K | LuxO | $\sigma^{54}$ Act | *V. cholerae* | (75) |
| V328 | H324R | LuxO | $\sigma^{54}$ Act | *V. fischeri* | (70) |
| L339 | T335I | LuxO | $\sigma^{54}$ Act | *V. fischeri* | (70) |
| L351 | H356Q | *mxan_4240* | $\sigma^{54}$ Act | *M. xanthus* | (this study) |
| L351 | H331L<br>H331Q | FlbD | $\sigma^{54}$ Act | *C. crescentus* | (67) |
| P382 | R362 | FlbD | C-D Linker | *C. crescentus* | (67) |
| E384 | V389L | *mxan_4240* | C-D Linker | *M. xanthus* | (this study) |
| T389 | R394P | *mxan_4240* | C-D Linker | *M. xanthus* | (this study) |
| A391 | H396P | *mxan_4240* | C-D Linker | *M. xanthus* | (this study) |
| G455 | G455W | NtrC | HTH | *S. typhimurium* | (83) |
| N457 | N466Y<br>(N463) | NtrC | HTH | *S. meliloti* | (84) |
| R461 | R461Q | NtrC | HTH | *S. typhimurium* | (83) |
| N/A | V451 | FlbD | HTH | *C. crescentus* | (67) |

[a]Amino acid position
[b]N/A indicates residues that are not in NtrC

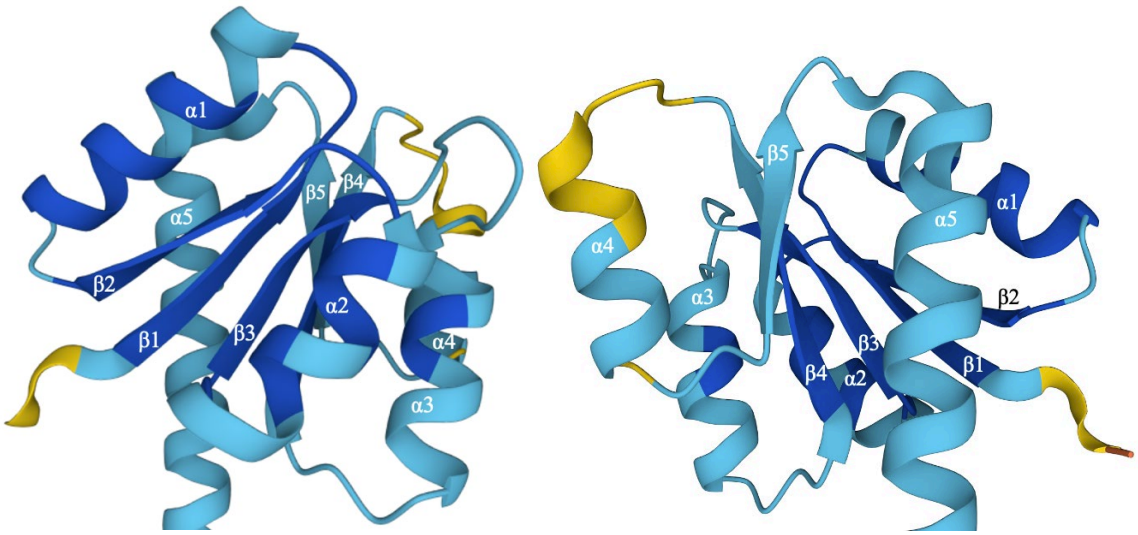


**Fig. 13. Predicted structure of NmpR.** The receiver domain of NmpR represented in two views with the β sheets and α helices indicated (AlphaFold ID: AF-Q1D4K8-F1).

*Site of phosphorylation mutants*

One of the gold standard methods to demonstrate a protein is a *bona fide* RR of any RR family is to mutate its proposed site of phosphorylation. When the predicted aspartic acid residue is mutated to an alanine, the protein is unable to be phosphorylated

and will lose its function (85). When this residue is mutated to a glutamic acid, the protein mimics phosphorylation at this residue and therefore has phospho-independent activity (85). There are countless examples of these site directed mutations in the literature, but a few examples in the family of NtrC include D61E of LuxO, D54E of NtrC, and D52E of Rrp2 (71–73).

*Contribution of amino acids after the phosphorylation site*

Some of the most frequently observed mutations in NtrC-like RRs occur in this region of the receiver domain (D54-I119). The most plausible explanation for how these mutations function in negatively regulated NtrC-like RRs is through mutations to affecting dimerization. In one study, researchers subjected the NtrC-like RR DctD to random mutagenesis and screened for proteins that were constitutively active. Out of 15 identified mutants, most of them occurred at this predicted dimer interface or directly adjacent to it including P78-L132 (68). Since DctD is negatively regulated, dimerization of residues in the receiver domain and the rigid helical linker domain (mainly in helix 5) inhibits oligomerization and is required to keep the protein in its "off" state (86). When phosphorylated, a change in conformation occurs in which the dimer stability is reduced and therefore the ATPase domain is active. Mutations to this dimer interface could alter the structure in such a way that activation of the centralized ATPase domain can at least partially escape repression by the receiver domain. Mutation K122E activated transcription the greatest, even greater than wildtype DctD. After researchers superimposed the crystal structures of wild type DctD and K122E, they saw that Lys 122 participates in an important interaction with Glu 121 and Asp 76, necessary for the proper

packing of helix 5, part of the dimer interface. The lack of these interactions could suggest how this mutation destabilizes the dimer and activates transcription even greater than wild type.

Another NtrC-like RR that functions through negative regulation is LuxO , which functions to promote biofilm formation and overall virulence in *V. cholerae* (75). Researchers have found mutations in LuxO near the dimer interface hypothesized by the crystal structure of DctD (68). LuxO mutations A91D - R114P were generated via random mutagenesis and map near this predicted dimer interface and could function like mutations in DctD, where the mutations induce an escape from repression of the ATPase domain (70). Several additional LuxO mutants in other *Vibrio* spp. near the predicted dimer interface likely act in the same manner (Table 2, (70).

In addition to LuxO and DctD, NmpR of *M. xanthus* is predicted to be negatively regulated due to its predicted secondary structure containing the long α helix in the Q linker (Fig. 13., AlphaFold ID: AF-Q1D4K8-F1, (52). Many mutations that induce constitutive transcription were found in the region after the phosphorylation site, including D61V and L63F. It has been found that residues between G59 and G62 (part of the β3-α3 loop) in particular are responsible for the twist motion of this loop that occurs after phosphorylation (87). After the twisting motion, there is a subsequential "flap" performed by M55-D61 that is a slower motion. Alterations in this movement, caused by mutants D61V or nearby L63F, could mimic this type of movement or structure that is formed post-phosphorylation. Another group of amino acid substitutions include V87E - K95N in NmpR and D86N, A89T in NtrC (73). These residues span the full α4 helix and additionally A90 and A93 are highly conserved and K95 is semi-conserved across NtrC-

like RRs. This particular helix is highly flexible in the unphosphorylated state and its change in conformation once phosphorylated is key to the function of the protein (88). Particularly in NtrC, which is positively regulated, residues 87 and 90 in helix 4 of are thought to play a crucial role in its interaction and subsequent activation of the ATPase domain because they are surface exposed (48). For example, a previously defined *nmpR* mutation resulting in a V87E amino acid substitution in the RR domain, is specifically within the α-helix 4 region of the RR (48). Additionally, the EMSA results from this study indicate increased oligomerization of mutants A90T and A93V compared to wild type (Fig. 14). Another previously found mutation K104N was also observed in this project (59). The lysine at this position is a strictly conserved amino acid across all RRs regardless of protein family (89, 90). At this position the lysine is absolutely required for the ability to dephosphorylate the RR with its hydrogen bond interaction with the phosphoryl group (90). Interestingly, mutations at this site that confer constitutive activity share the same charge (R or N) but seem to have the inability to be positioned correctly for the dephosphorylation. Thus, RR can be phosphorylated but are not dephosphorylated by their cognate SK.

Overall, the mutations found in this helix that confer constitutive activity are from a smaller amino acid to a larger or bulkier amino acid, which could play a role into the alteration of the position of the helix, assisting in mimicking phosphorylation.

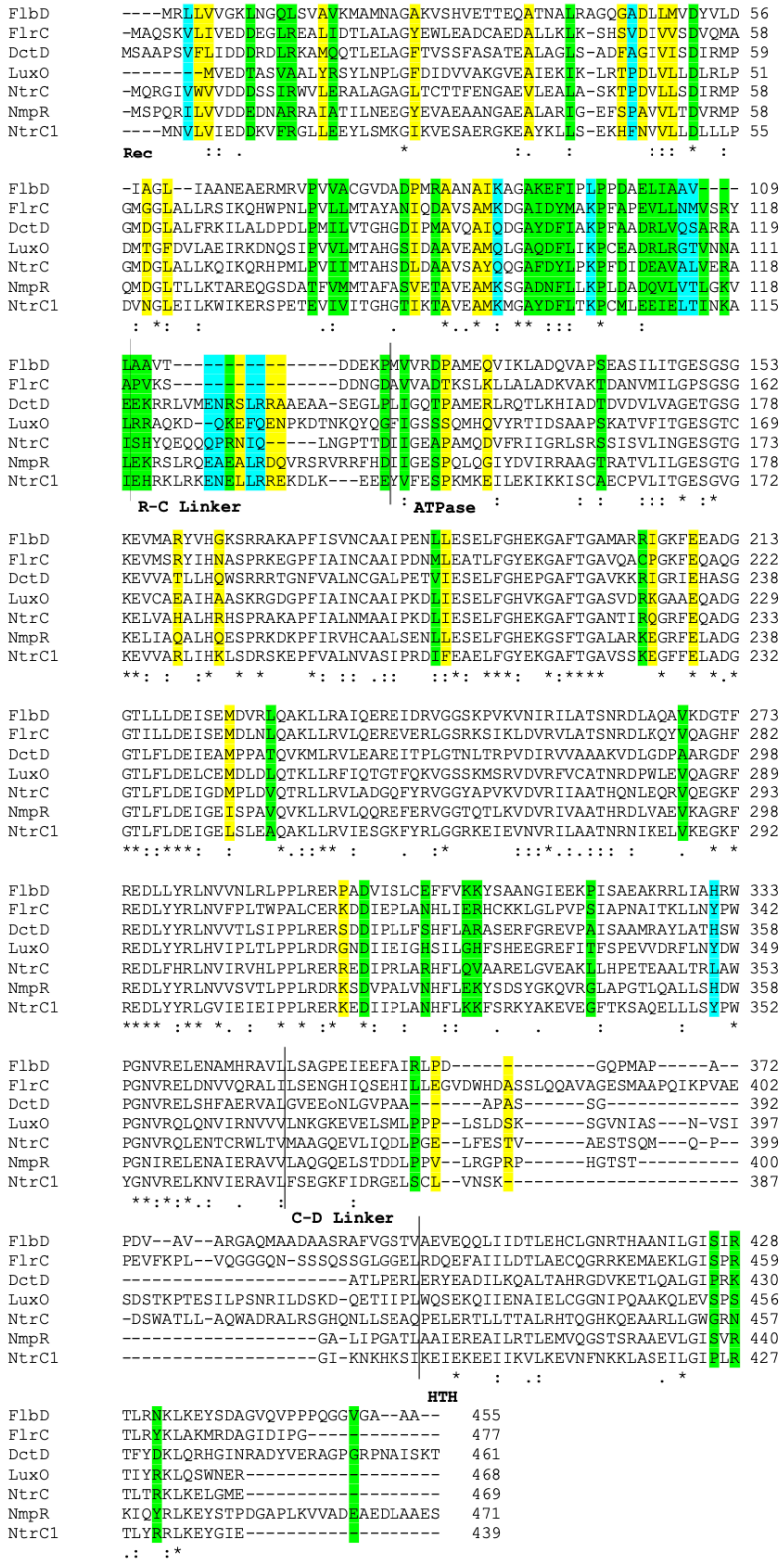


**Fig. 14. Multiple Sequence alignment of NtrC-like RRs.** The domains indicated are based upon the structure of NtrC and the alignment. Yellow indicates mutations found only in this study, green is mutations found only in the literature, and blue indicates mutants found both in this study and the literature.

## Q Linker

Interestingly, all mutations within the Q linker that conferred constitutive activity of NtrC-like RR occurred specifically in those that are negatively regulated, including LuxO, NmpR, DctD, and NtrC1. NtrC1 is found in *Aquifex aeolicus* and shares 60% sequence similarly with NtrC (91), but the specific cellular function of this protein is unknown. Researchers performed mutagenesis of the NtrC1 dimer interface predicted through the work on DctD (68) to identify constitutive mutants and found many mutants spanning the protein, including in the linker domain (52). Since these proteins are all negatively regulated, the α helix 5 extends from the receiver domain through this linker region, giving it secondary structure, unlike the unstructured linker region of positively regulated NtrC-like RRs. As described previously, the structured linker region of negatively regulated NtrC-like RRs is part of the dimer interface and is necessary to keep the protein in an off state (68). Mutations to this dimer, including the linker region, could allow the protein to escape inhibition of function by dimerization.

In our *nmpR* suppressor screen, a high number of mutants occurred independently in this small stretch of the protein, specifically between residues 132-136. Three of these mutants, R132W, D133N, and Q134E, were analyzed using an EMSA and similar binding patterns were seen between the three. When DNA probe with site 1 mutated was analyzed with these proteins, binding occurred even with very small quantities of protein. This is different than what was seen in the binding patterns for the few receiver domain mutants that were tested, which needed a slightly greater protein concentration to see similar affects as the Q linker mutants. With the linker mutants, it may be possible that

the change in size (R132W) or charge (D133N, Q134E) of the mutants in this region may bend or rearrange the linker into a locked "ON" position.

## ATPase Domain

The ATPase domain of an NtrC-like RR functions to hydrolyze ATP once the oligomer, typically a hexamer or heptamer, is formed. This process provides the necessary energy to the RNA polymerase to initiate transcription. Site 160 of NtrC is not well conserved across NtrC-like RRs but is typically seen as a serine or threonine residue (92). Many mutations at this site have been identified and fall into two categories of phospho-dependent or phospho-independent. Mutants S160F and S160Y are phospho-independent whereas S160W, C, and L are phospho-dependent (74, 82). S160F was identified through spontaneous suppressor strains (80) whereas the rest of the S160 mutations were identified using site directed mutagenesis (82). Authors of the site directed mutagenesis experiment suggest the phospho-dependent variants possibly are more receptive to cross talk, or phosphorylation via other HKs because when the cognate SK is present (NtrB) these mutants show greater activity. It has been suggested that specifically the phospho-independent mutant S160F favors the fully assembled ring structure, and may be another explanation for its ability to initiate transcription without phosphorylation (93). The exact function of S160 is not known so it is difficult to predict the direct effects of these substitutions on protein conformation change and function.

Another site of mutation found in multiple studies is V248 of NtrC (67, 74). This residue is directly adjacent to the highly conserved Q249 residue in α helix 9. Although the function of V248 is unknown, the fact that it is right next to a conserved residue may

indicate that the change in amino acid at this site could impact the position of Q249 and rescue transcription initiation. Another mutation downstream is V288I of NtrC (74). This mutant was shown to be phospho-independent as the protein was still functional when the N-terminal domain was deleted.

L351 is another site in which different researchers found constitutive mutations (this study, (67)). Although this residue is not conserved, it is seen typically as a H or Y in the NtrC-like RR alignment. These mutations (H356Q of NmpR and H331L or H331Q of FlbD) are located right before an α helix and all display a change in charge or polarity. These changes in charge and size likely impact the positioning of the α helix that L351 is directly adjacent to and could impact its function.

## Helix-Turn-Helix Domain

The HTH domain in an NtrC-like RR functions to anchor the protein to the enhancer sites upstream of the transcriptional start site. Although this domain is the smallest, it is arguably one of the most important as it determines the location of where the protein will bind the DNA and is therefore important for nucleotide specificity. In general, this domain lacks in mutations that confer constitutive mutants when compared to the other domains. Most mutations in this domain likely impair the ability of the protein to bind DNA, and therefore they can no longer regulate transcription. Two mutations that were relatively close in location were identified in this domain that promoted transcriptional activity, the first being N466Y identified in NtrC of *S. meliloti*. (84). Researchers were surprised to see that even with this mutation, this protein had high levels of activation of NtrC regulated genes such as *nifH*, *dctA*, and *fixA*. The researchers

hypothesized that due to the high concentration of NtrC in the experiment, it could be directly binding to $\sigma^{54}$ instead of the upstream promoter region, therefore bypassing the need for direct DNA binding for regulation. Similar observations were seen in other studies (94–96). Additionally, other researchers were able to show that the promoter of *glnA* could still be activated by NtrC even when all known binding sites on the DNA were deleted (97). Another less likely hypothesis proposed is that this substitution alters DNA binding specificity in a way such that it can bind activator sequences.

The second mutation found in this region is R461Q in NtrC of *S. typhimurium* through selecting for mutants that can grow without the addition of glutamine but fail to grow without arginine or alanine (83). This mutation, like the other, does not bind its typical promoters but will bind at very high concentrations of a strong enhancer. Even though binding was not as strong, stimulation of the ATPase activity appeared increase about 5.5-fold when the protein was phosphorylated and could activate transcription (83). However, without phosphorylation, this variant could not activate transcription unlike the other constitutive mutants discussed in this review.

## Concluding Thoughts

NtrC-like RRs are complex regulatory proteins that have repeatedly acquired single substitution mutations that restore function or turn the protein permanently "ON". These mutations are seen not only in the receiver domain, but throughout the protein domains. Literature review has suggested many hypotheses toward how these mutations are rescuing function in these proteins, but there are still many mutations with no clear explanation as their function has not yet been characterized. Many of the mutations found in the literature search also have been seen in other families of response regulators, such

as K104N in CheY (89, 90), suggesting there may be broad evolutionary pressures at these positions of RRs, generally.

Further investigation needs to be done on certain areas within NtrC-like RRs to understand how mutations affect the secondary and tertiary structure, and therefore function of the protein. Protein regions such as early in the receiver domain, later in the ATPase domain, and mutations in general in the HTH domain have yet to have clear explanations with solid evidence as to how mutations in these areas lead to a gain-of-function. Since these amino acids are not yet characterized in how they function in protein structure and activation, it is difficult to predict how mutations to these areas influence amino acid interactions and function.

The EMSAs generated in this study can give general insight into how mutants in the receiver domain and Q linker domain of negatively regulated NtrC-like RRs can rescue function. Mutants both in the receiver and Q linker domains were able to bind DNA probe better than wild type, even when mutated, indicating these mutations may mimic phosphorylation such that the protein is locked “ON” and can function regardless of its phosphorylation status.

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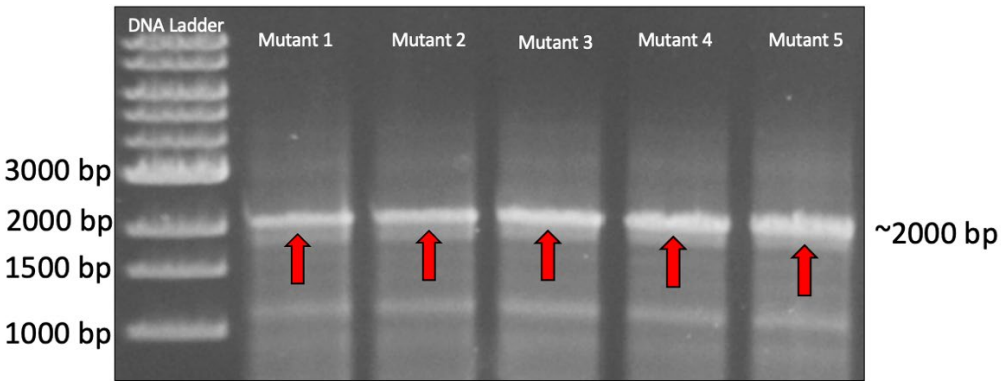
# APPENDIX A

PCR DETECTION OF *nmpR*

**Appendix A:** PCR Detection of *nmpR*

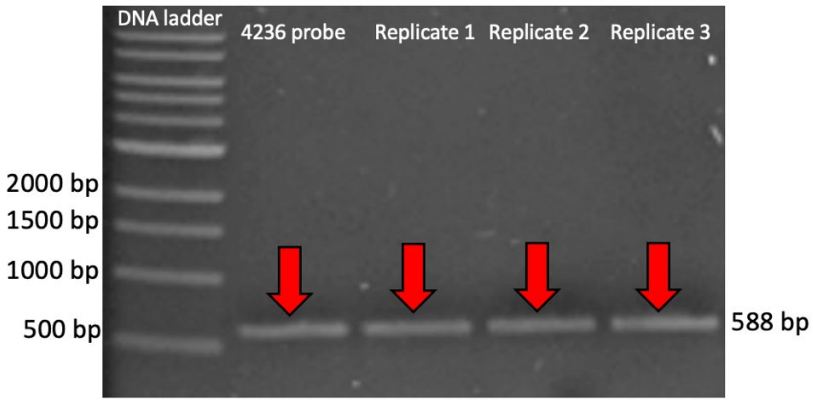



PCR of five *nmpR* mutants were confirmed by separation on a 1% agarose gel by comparison to 1 kb ladder. In each lane is apparent that there is an intense band right above at the 2000 bp marker. This confirms the presence of the *nmpR* gene in each of these mutants.

**APPENDIX B**

GENERATION OF *mxan_4236* PROMOTER PROBE

**Appendix B.** Generation of *mxan_4236* Promoter Probe

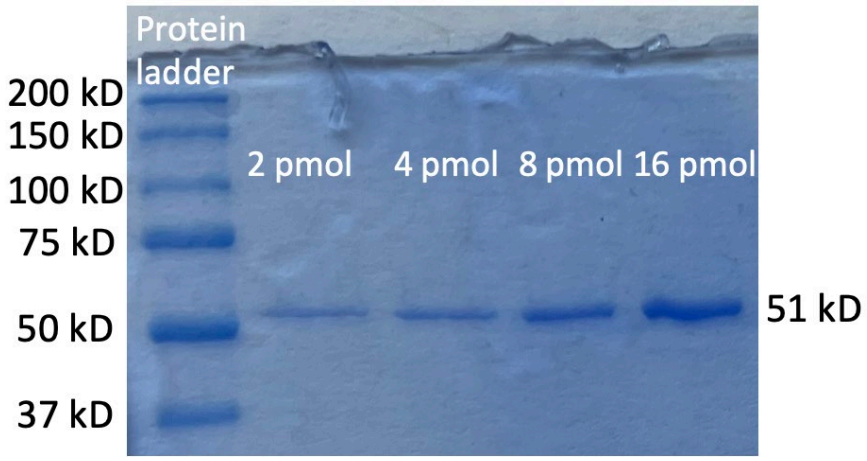



PCR for the 4236 probe was performed four times and separated on a 1% agarose gel beside a 1 kb DNA ladder. All four lanes have a single DNA product at the correct length of 588 bp, indicating the PCR worked and the probe can be used.

**APPENDIX C**

CONFIRMATION OF PROTEIN DILUTIONS VIA SDS-PAGE

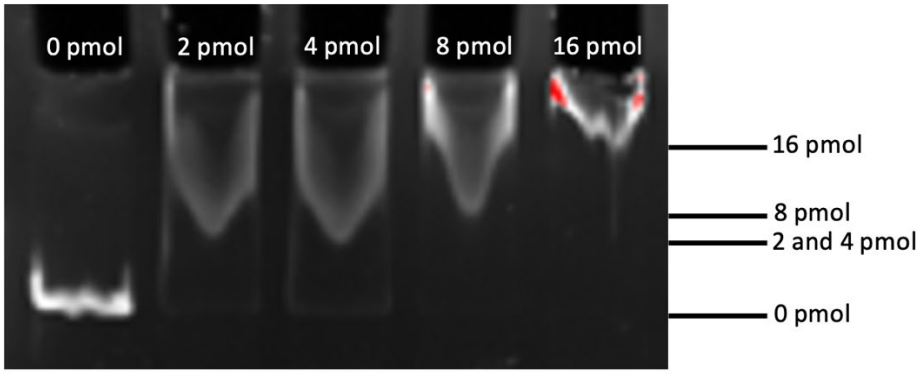
**Appendix C.** Confirmation of Protein Dilutions via SDS-PAGE




Wild type NmpR protein was diluted to the molar amount indicated at the top of each well. Samples were separated using a 12 % acrylamide resolving gel. All samples ran to the correct size of 51kD and there is increasing intensity of band size as protein concentration increases.

**APPENDIX D**
EXAMPLE OF NMPR EMSA RESULTS

**Appendix D.** Example of NmpR EMSA Results.




A test EMSA was performed using an 8% acrylamide gel at 60V for 2 hr. All lanes have 200 fmol *mxan_4236* and the amount of protein is shown in white text at the top of each well.