# IclR-DNA Multimerization and Affinity

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# Abstract

Bacteria have a unique adaptation where they can bypass steps in the Tricarboxylic Acid (TCA) cycle to conserve carbon. The bypass is regulated by the Isocitrate Lyase Regulator (IclR) protein which works on the aceBAK operon. This bypass can be a target for antibacterial therapeutics that would not harm humans, as they do not possess the bypass. Previous researchers at Millersville prepared IclR with a C-terminal His-tag for purification, however, previous studies used IclR that did not have a tag. This study aimed to evaluate whether the His-tag has an effect on the IclR protein's ability to bind to DNA. The His-tag from purified IclR using enterokinase. Electromobility shift assay (EMSA) was then used to evaluate the binding of the IclR to aceBAK DNA with and without the His-tag at several concentrations and quantified. The His-tagged and tag-cleaved proteins had different banding patterns, especially at higher concentrations, indicating that a His-tag on the protein tends to stabilize multimerization. EMSA reactions were also performed using mutated protein where one residue, serine 147, was replaced with an alanine, as this serine is suspected to be important for multimerization. Reactions comparing His-tagged and cleaved S147A revealed a difference in binding activity as well as less overall multimerization when compared to wild type IclR.

# Background

Bacteria are found almost everywhere on Earth and are vital to both the environment and human life. However, not all bacteria are helpful as some species of bacteria will cause disease.[1] For example, the bacterium *Mycobacterium tuberculosis* will cause a patient to develop tuberculosis. Tuberculosis is one of the most powerful infectious diseases causing almost two million deaths annually.[2] Additionally, some strains of *Escherichia coli* can cause a plethora of issues including urinary tract infections, respiratory illness, and pneumonia.[3] Because bacteria are found in so many different environments, they face a variety of stressors. Many bacteria have developed mechanisms to aid in their survival in these stressful situations. Finding adaptations that are present in bacteria but not humans provides a promising route of study for new therapies, as this pathway could be shut down in the bacteria without harming the human. One survival mechanism found in both *M. tuberculosis* and *E. coli* is a glyoxylate shunt through the citric acid cycle (TCA) cycle.[2]

The TCA cycle is a series of reactions that power metabolism within cells (Figure 1). Intermediates in this cycle are used as starting materials for other essential metabolic pathways. In normal conditions, where all substrates are readily available, the TCA cycle can be expressed fully. However, in conditions where substrates, and specifically glucose, is scarcely available, some bacteria including *M. tuberculosis* and *E. coli* can utilize a glyoxylate shunt to skip steps of the TCA cycle (Figure 1). This bypass allows for conversion of isocitrate to malate by use of the enzymes isocitrate lyase and malate synthase. Isocitrate lyase converts isocitrate to succinate and glyoxylate while malate synthase catalysis the reaction of glyoxylate and acetyl-CoA to malate. The genes that code for these enzymes and the regulatory enzymes of the glyoxylate bypass are

found in the same operon, called aceBAK. These genes can be regulated by transcription factors upstream of the aceBAK operon.[4]

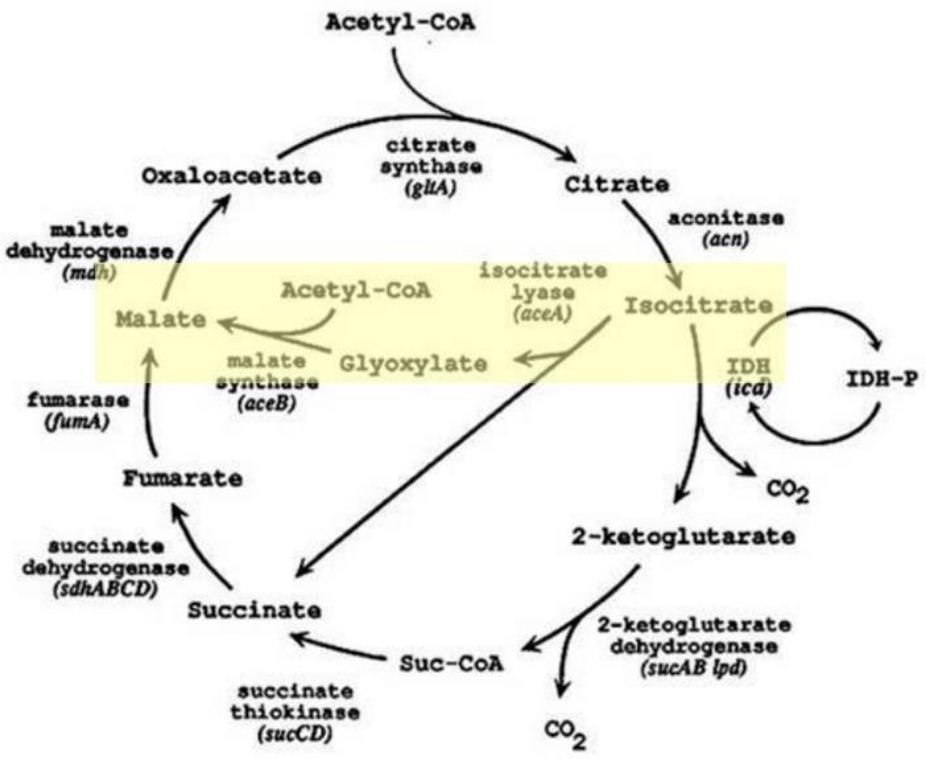



Figure 1. Full Citric Acid (TCA) cycle with glyoxylate bypass highlighted in yellow.[4] The enzymes utilized in the glyoxylate bypass are labeled with the operons that code for these enzymes. The operons together are referred to as the aceBAK operon.

In general, transcription factors are proteins that regulate the transcription of genes. The activities of these proteins determine how a cell functions and responds to environmental stressors. There are several different mechanisms by which transcription factors are able to regulate the transcription of genes. One method of defining these transcription factors is by the type of DNA binding domains they contain.

The transcription factor that regulates the glyoxylate bypass through the TCA cycle is the Isocitrate Lyase Regulator (IclR).[5] IclR has two binding domains; C-terminal and N-terminal. Its N-terminal binds to specific DNA motifs while its C-terminal has the ability to bind to effector molecules. IclR is known to have two antagonistic effector molecules; glyoxylate and pyruvate. In the presence of glyoxylate IclR is more likely to take on a dimer formation (Figure 2 A) which is the inactive state. This form allows for RNA polymerase to bind and initiate transcription of the aceBAK gene to commence. Alternatively, when pyruvate is bound to IclR it is more likely to be in the active tetrameric form of the protein (Figure 2 B). In the tetrameric form the protein will bind to the promotor, blocking RNA polymerase from binding so transcription of the gene will not occur (Figure 3).

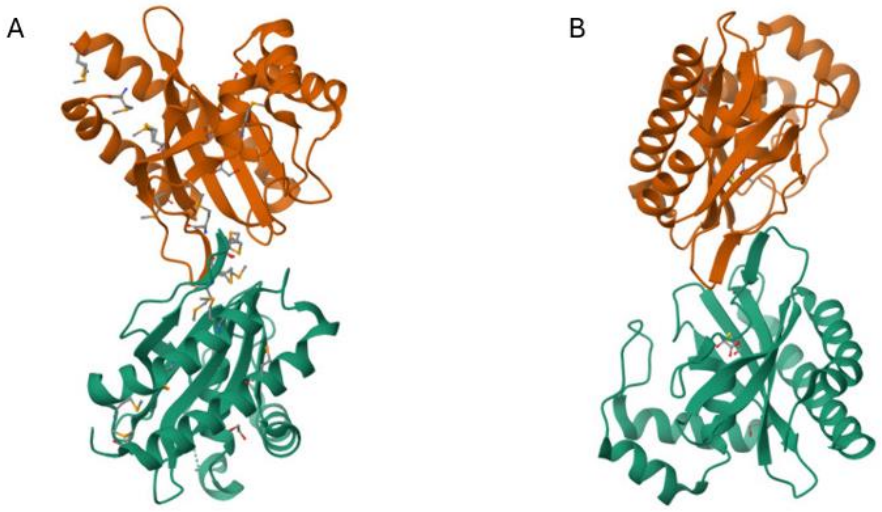



Figure 2. (A) Crystal structure of *E.coli* IclR C-terminal fragment with glyoxylate bound in the effector binding site (PDB 2O9A).[5] (B) Crystal structure of *E.coli* IclR C-terminal fragment with pyruvate bound in the effector binding site (PDB 2O99).[5]

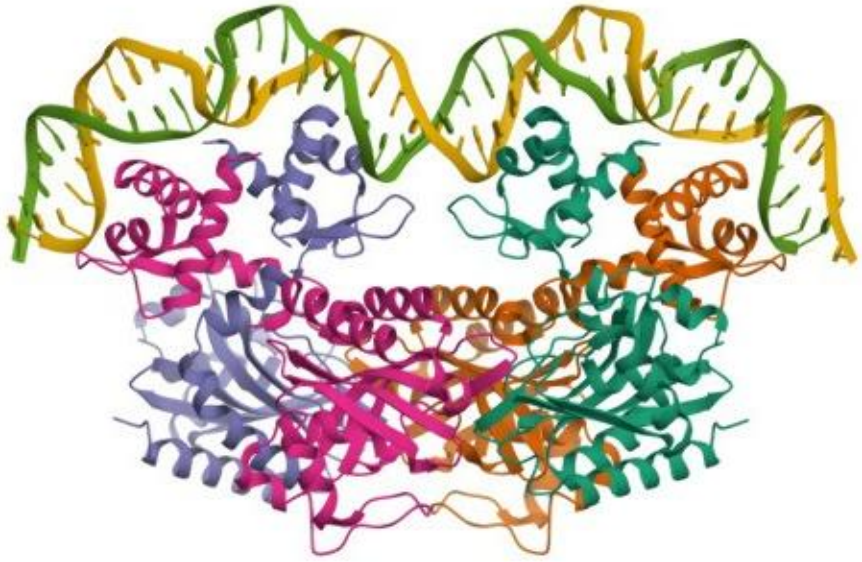


Figure 3. Crystal structure of IclR protein family member, TtgV in complex with its DNA operator. This protein is in a tetramer formation with each monomer being shaded a different color. IclR protein structure is highly conserved across different family members.[10]

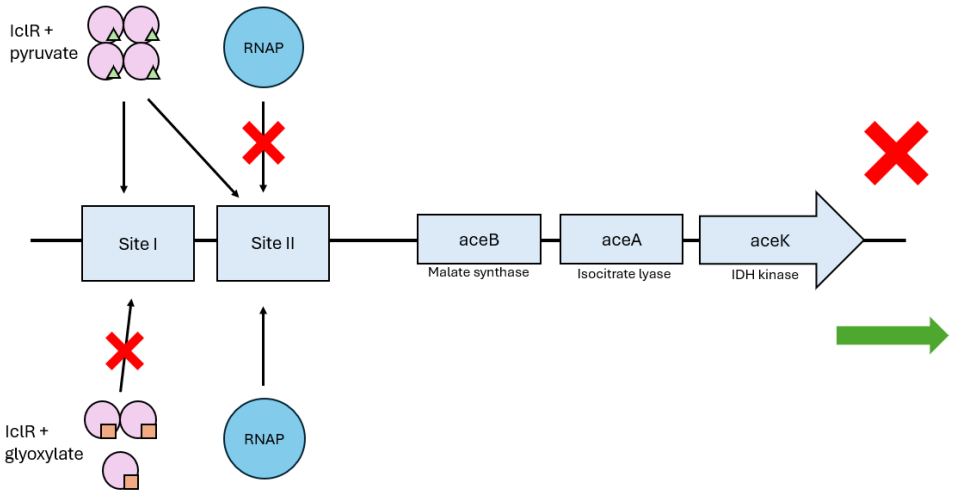



Figure 4. Simplified depiction of IclR-DNA binding and its effect on operon transcription. Pink circles represent the IclR protein monomers, green triangles represent pyruvate molecules, orange squares represent glyoxylate molecules. When glyoxylate binds to IclR a monomer or dimer is likely to form, allowing RNA polymerase to bind and transcription to occur. When pyruvate binds to IclR a tetramer is likely to form, blocking RNA polymerase from binding and preventing transcription.

Affinity tags are unique peptides attached to one terminus of a protein. These tags give the protein a specific characteristic that can be utilized when separating the protein out from many others. The first affinity tags were large proteins used almost exclusively for protein purification in *E. coli.* Today, there are many affinity tags to choose from which are optimized for specific modes of purification. In the case of this study, a polyhistidine tag was used. The polyhistidine tag on IclR was added to the C-terminus and contained six histidine residues which allowed for purification with a metal coordinated column. Most commonly used, and used in this experiment, is a Ni(II)-nitrilotriacetic acid (Ni-NTA) column, but several other metals can also be used. An enterokinase cleavage site was included between the His-tag and the rest of the IclR protein. This provides a convenient way to remove the His-tag if needed.

Another aspect of this experiment examined the effect of the His-tag on a mutated IclR protein. In previous experiments, residues Met-146, Leu-154, Leu-220, and Leu-143 were found to form a hydrophobic patch within the protein structure that interacts with pyruvate to stabilize a tetrameric form of IclR. Attempts to identify other residues that may affect IclR multimerization were pursued, including serine 147 which was suspected to be important for multimerization. A mutant was created via site-directed mutagenesis changing the serine to alanine. This mutated protein was treated the same as its wild type counterpart in this study to elucidate if the His-tag affects the binding affinity to DNA.

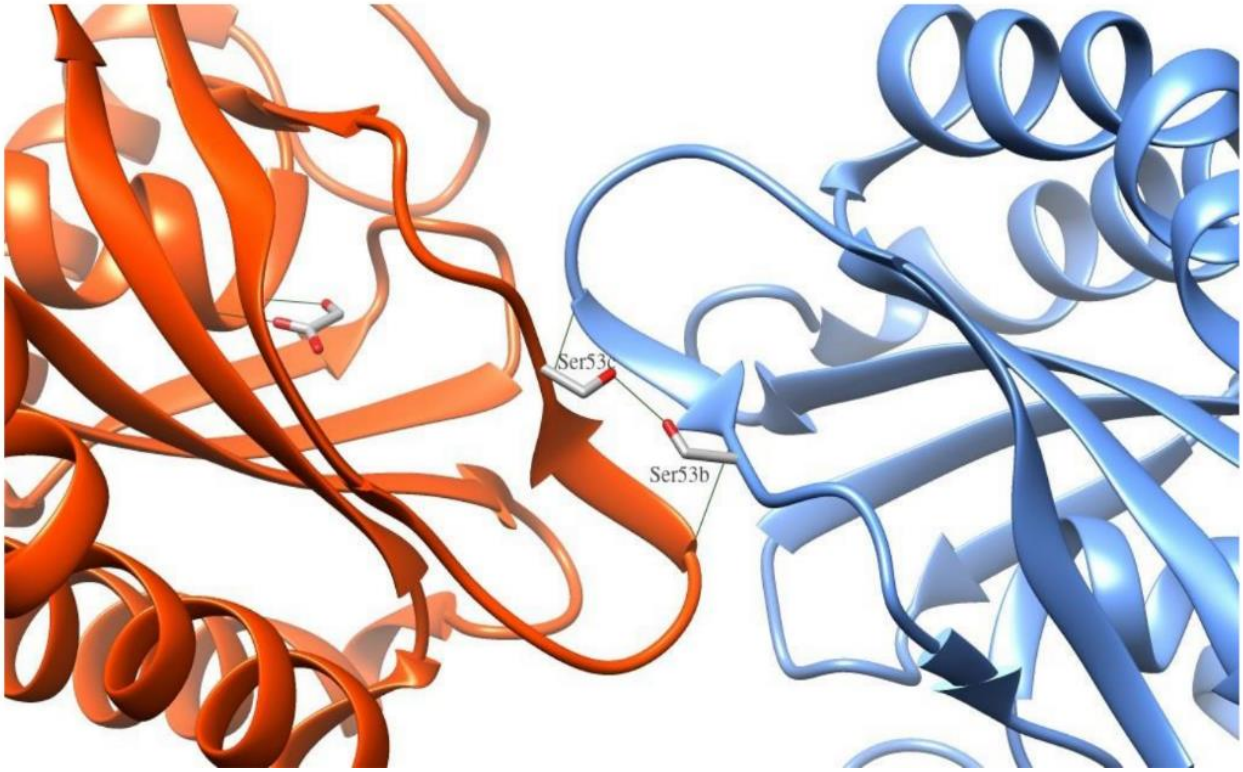



Figure 5. Hydrogen bonding between serine residues 53b and c in each monomer of dimeric *E. coli* IclR C-terminal fragments with glyoxylate bound in the effector binding site (PDB 2O99).[9]

# Materials and Methods

## DNA preparation

A PCR reaction was completed to amplify aceBAKp -120+17 using Taq polymerase (1.25 units/ 50µL), aceBAKp3 forward and reverse primers (0.5 µM), and concentrated aceBAK template DNA. 200 µL total was prepared in four 50 µL aliquots. This PCR program cycle consisted of 30 seconds at 95°C, followed by 29 cycles of 15 seconds at 95°C, 15 seconds at 55°C, and 45 seconds at 72°C, then held at 4°C.

The PCR product was confirmed on an agarose gel then cleaned using a QIAGEN QIAquick® Gel Extraction Kit. After cleaning, the concentration of the PCR product was determined using a Thermo Fischer NanoDrop 1000 Spectrophotometer. A sample of just the new DNA was run on a 5% TBE polyacrylamide gel (BioRad) under the same conditions as the later mentioned EMSA reactions, to ensure purity.

## Protein Purification and His-tag Cleavage

His- tagged IclR protein was purified for use at Millersville University using Qiagen QIAexpress Ni-NTA Fast Start columns. To cleave the His-tag from the purified IclR protein a procedure developed by Betel Erkalo was followed.[8] Enterokinase was used to cleave at an enterokinase site on the C-terminus of the IclR. The reaction mixture consisted of 25 µg protein, 4 µL 10x reaction buffer (0.2 M Tris-HCl, 0.5M NaCl, 20mM $CaCl_2$), 1 µL enterokinase light chain (NEB 16000 units/mL) and brought to a final volume of 20 µL with $dH_2O$. All components were mixed well and incubated at 25°C overnight, allowing cleavage to occur. The cleavage was confirmed using Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

## Electromobility Shift Assay (EMSA)

Binding was assessed using electrophoretic mobility shift assay (EMSA) with a 5% TBE polyacrylamide gel (BioRad). Each lane was loaded with a 20 µL reaction mixture consisting of 5 ng/µL aceBAK DNA, 2 mM pyruvate, 1X binding buffer (0.25 mM Tris-HCl, pH 7.5, 150 mM KCl, 10 mM $MgCl_2$, 0.01% TritonX-100, 1 mM DTT), and 25-350 nM IclR. One set of each protein concentration had the His-tag and the other set of each protein concentration that was treated for tag removal. Four controls were also added including a sample with no IclR protein, one with no aceBAK DNA, and two samples where the pyruvate is replaced with the same volume of 2 mM glyoxylate; one with His-tagged IclR and one with cleaved IclR. The gel was run at 100 V for 45 min at 4°C. After the gel was run, DNA was visualized using SYBR® Green stain (Invitrogen).

Table 1. Example reaction makeup for 75 – 350 nM IclR concentration.

| | 75nM WT IclR | 150nM WT IclR | 250nM WT IclR | 350nM WT IclR | 350nM WT IclR+G | NO DNA 250 WT IclR | NO IclR | 350 cleaved IclR+G | 250nM cleaved IclR | 250nM cleaved IclR | 150nM cleaved IclR | 75nM cleaved IclR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5 ng/µL DNA | 4µL | 4µL | 4µL | 4µL | 4µL | - | 4µL | 4µL | 4µL | 4µL | 4µL | 4µL |
| 1µM no HIs IclR | - | - | - | - | - | - | - | 7µL | 7µL | 5µL | 3µL | 1.5µL |
| 100nM no His IclR | - | - | - | - | - | - | - | - | - | - | - | - |
| 1µM WT IclR | 1.5µL | 3µL | 5µL | 7µL | 7µL | 5µL | - | - | - | - | - | - |
| 2mM pyruvate | 5µL | 5µL | 5µL | 5µL | - | 5µL | 5µL | - | 5µL | 5µL | 5µL | 5µL |
| 2mM glyoxylate | - | - | - | - | 5µL | - | - | 5µL | - | - | - | - |
| 1X Binding Buffer | 9.5µL | 8µL | 6µL | 4µL | 4µL | 10µL | 11µL | 4µL | 4µL | 6µL | 8µL | 9.5µL |
| Final volume | 20µL | 20µL | 20µL | 20µL | 20µL | 20µL | 20µL | 20µL | 20µL | 20µL | 20µL | 20µL |

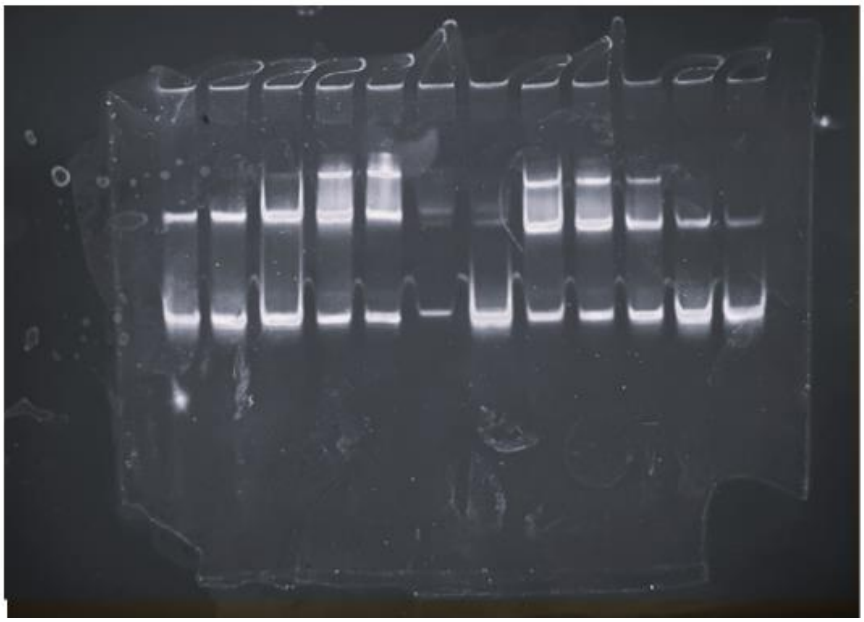
## Band Quantification

The software Image Studio Lite by LI-COR was used to quantify gel bands. The signal for each band present was corrected for background interference then compared to other bands in the same lane.

## Results and Discussion

To evaluate binding behavior several gels were run and their bands were quantified. In EMSA protein-nucleic acid complexes are separated based on size with smaller complexes moving faster, therefore moving further down the gel than larger complexes. Free nucleic acid would migrate the farthest. To begin, a range of protein concentrations was selected that would encapsulate the formation of both dimer and tetramer complexes. These concentrations were based on a previous study which evaluated how concentration affected multimerization. The starting range was 25 – 250 nM IclR. Reactions of both cleaved and tagged IclR were run on the same gel to provide a direct comparison.

| | WT IclR | | | | | | | | Cleaved IclR | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| DNA | + | + | + | + | + | - | + | + | + | + | + | + |
| IclR WT (nM) | 25 | 75 | 150 | 250 | 250 | 250 | 0 | 0 | 0 | 0 | 0 | 0 |
| IclR cleaved(nM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 250 | 250 | 150 | 75 | 25 |
| Glyoxylate | - | - | - | - | + | - | - | + | - | - | - | - |
| Pyruvate | + | + | + | + | - | + | + | - | + | + | + | + |



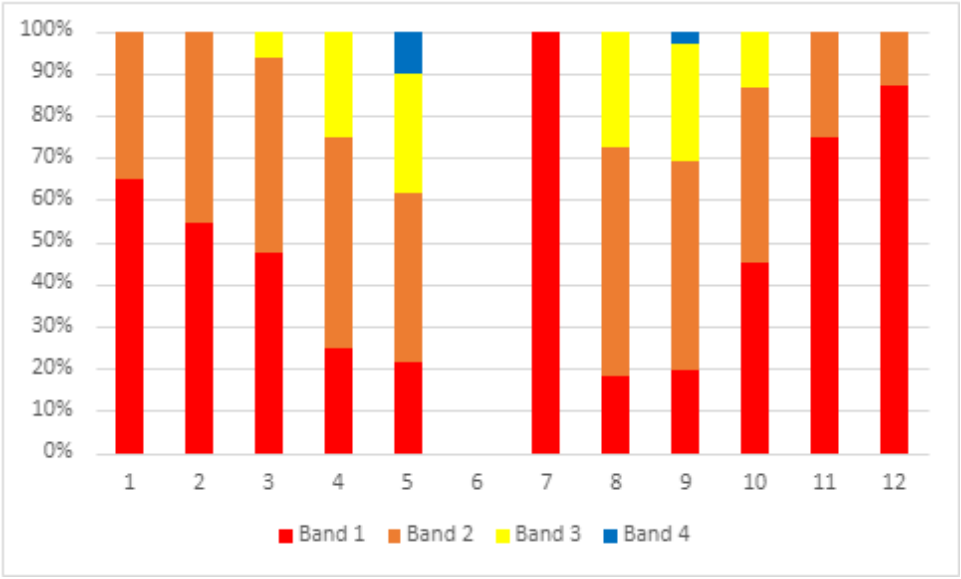



Figure 6. Gel 1 EMSA reaction comparing His-tagged and cleaved wild type IclR. Lanes labeled with reaction contents. EMSA performed using a 5% TBE polyacrylamide gel run at 100V in 4°C for 45 minutes and stained with SYBR® Green stain. Below is a plot of the quantification of bands compared by lane.

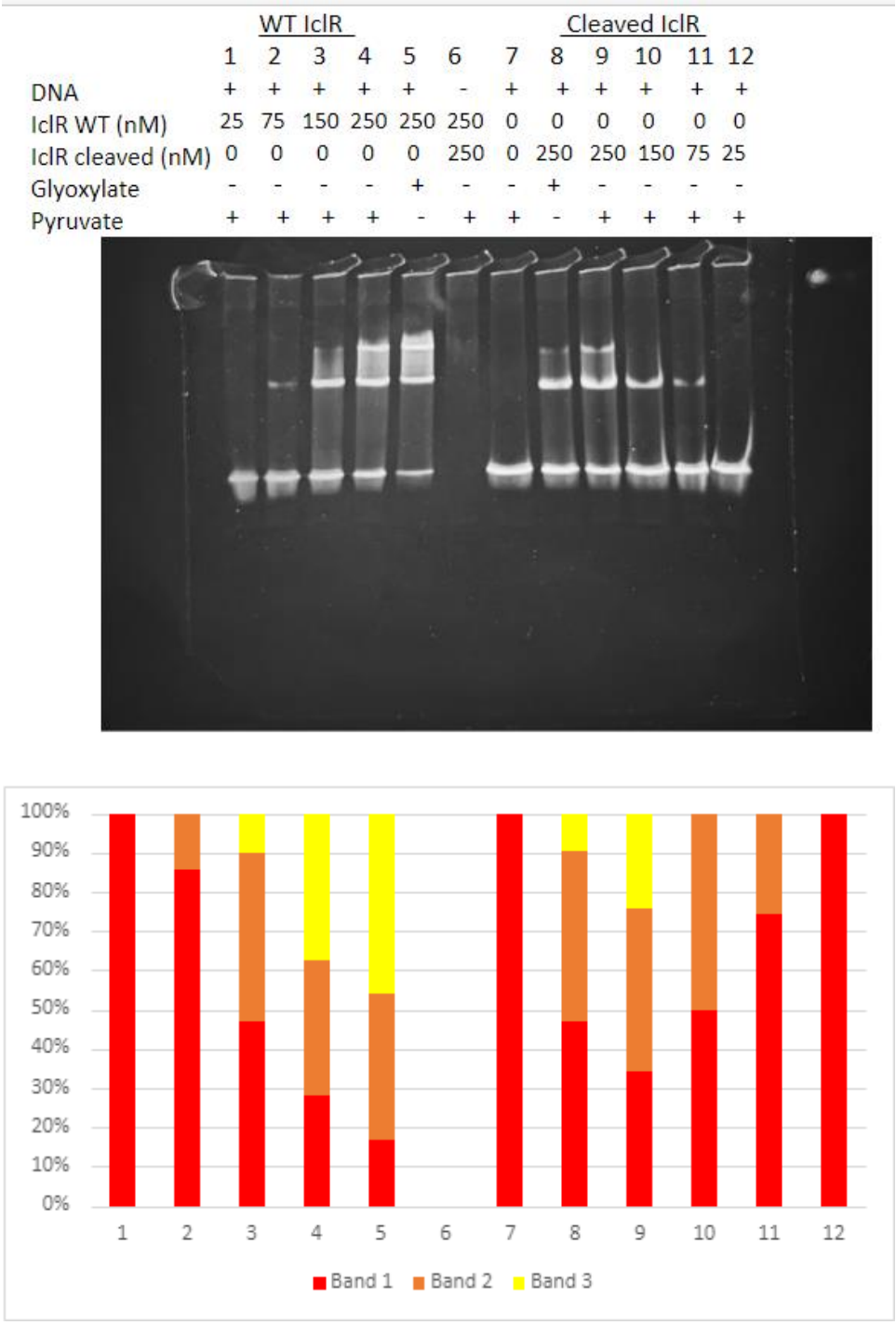



Figure 7. Gel 2 EMSA reaction comparing His-tagged and cleaved wild type IclR. Lanes labeled with reaction contents. EMSA performed using a 5% TBE polyacrylamide gel run at 100V in 4°C for 45 minutes and stained with SYBR® Green stain. Below is a plot of the quantification of bands compared by lane.

Each reaction on these gels contains aceBAK DNA, either pyruvate or glyoxylate, and either tagged or cleaved IclR protein. Additionally, there are two reactions (lanes 6 and 7) which have no IclR and no DNA respectively. The left half of each gel uses tagged IclR while the right half of the gel uses cleaved IclR. The edges of each gel (lanes 1 and 12) have the lowest protein concentration and the concentration increases as lanes move farther into the middle of the gel.

Looking at the first two gels, it can be seen that across both types of IclR (tagged and cleaved) higher concentrations of protein tend to more readily form multimeric complexes. This followed expectations set from previous experiments. However, slight differences can be noticed between the tagged and cleaved IclR. In gel 1, both types of proteins exhibited similar binding patterns as seen by the relative symmetry in both the gel image and the graph (Figure 8). A slight difference can be seen in the 250 nM protein concentration as the cleaved reaction had a small percentage of protein move up to form a fourth band (lane 9). Additionally, differences were observed in the glyoxylate reactions. Recall that glyoxylate is expected to cause IclR to take on an inactive dimer formation. However, in gel 1 the 250 nM tagged protein reaction with glyoxylate had some third and fourth band presence (lane 5). The 250 nM cleaved IclR with glyoxylate more so followed expectations and had a larger percentage of DNA in band 2, some in band 3, but none in band 4 (lane 8).

Looking at gel 2, there is less symmetry than in the previous gel (Figure 7). The tagged IclR reactions, as they move up in concentration, readily form higher multimeric structures as revealed by the drive up to form bands 2 and 3. The cleaved IclR on this gel began to form a second band at the same concentration (75 nM) as the tagged IclR (lanes 2 and 11). However, the tagged IclR began to move up to a third band at 150 nM while the cleaved IclR did not form a third band until the 250 nM concentration reaction. Directly comparing the 250 nM in each type

of IclR, the percentage of DNA in the third band is higher in the tagged (lane 4) than in the cleaved (lane 9). Also, similarly to gel 1, the glyoxylate reaction with the tagged IclR went against expectation and had a larger third band presence than did the same protein concentration with pyruvate (lanes 4 and 5). And again, like gel 1, the cleaved IclR behaves more along expectations and has a higher percentage of DNA in band 2 than in band 3 (lane 8).

After analysis of these initial gels, it was seen that there was very little multimerization when using the 25 nM concentration of protein. Because of this, it was decided that additional work would be done with a range of 75-350 nM protein. Two more gels using the new concentration were then run.

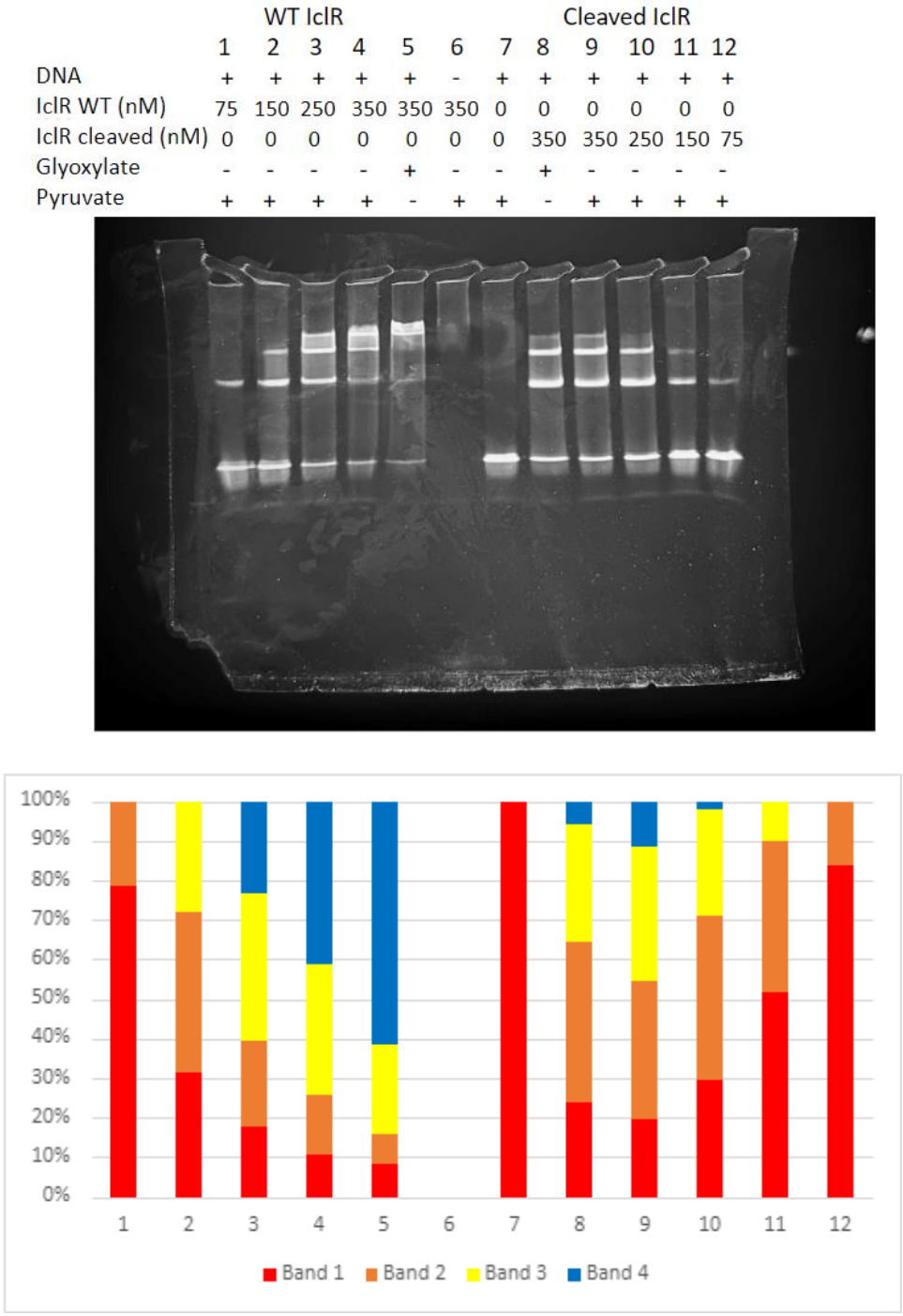



Figure 8. Gel 3 EMSA reaction comparing His-tagged and cleaved wild type IclR. Lanes labeled with reaction contents. EMSA performed using a 5% TBE polyacrylamide gel run at 100V in 4°C for 45 minutes and stained with SYBR® Green stain. Below is a plot of the quantification of bands compared by lane.

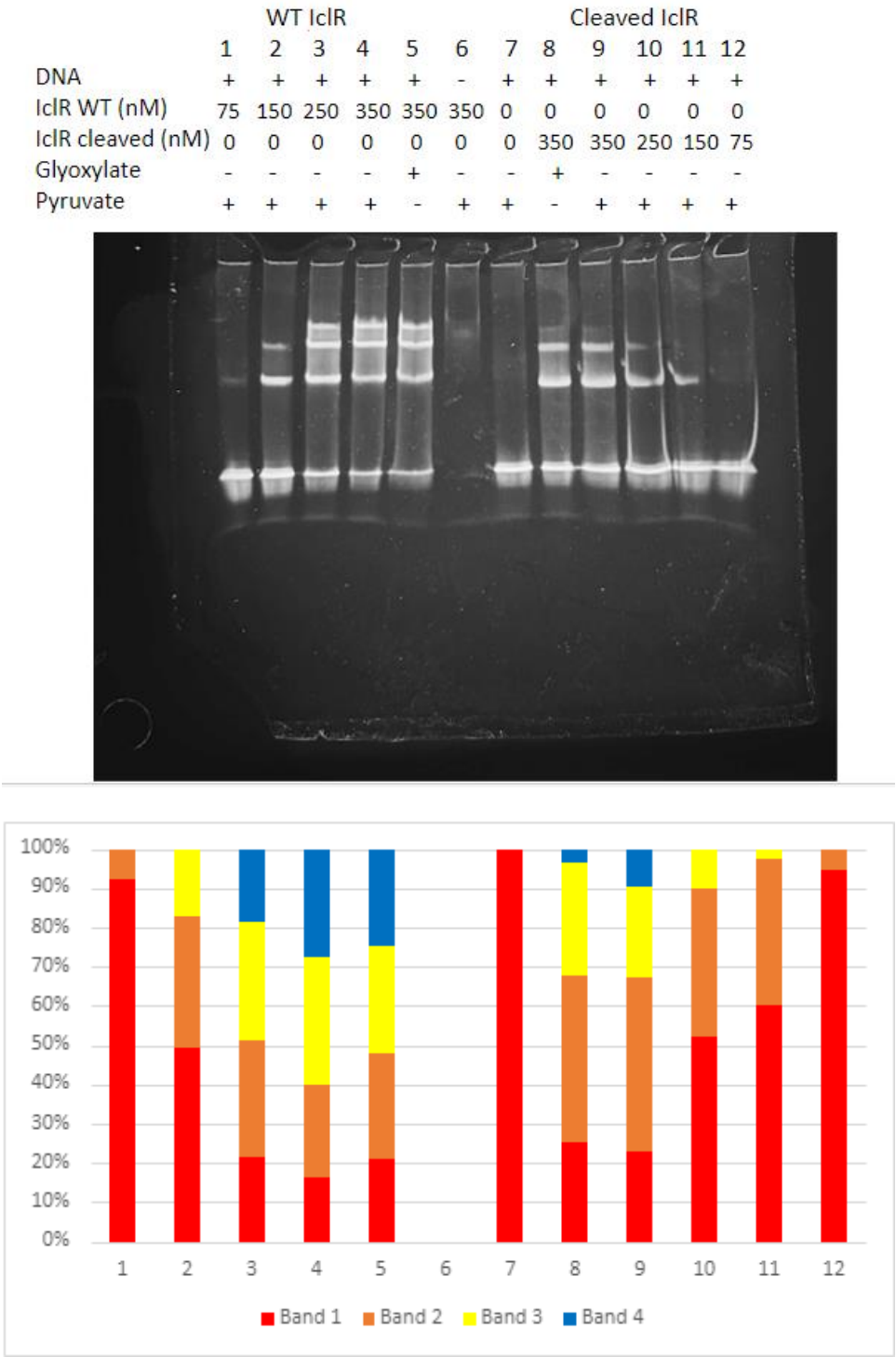



Figure 9. Gel 4 EMSA reaction comparing His-tagged and cleaved wild type IclR. Lanes labeled with reaction contents. EMSA performed using a 5% TBE polyacrylamide gel run at 100V in 4°C for 45 minutes and stained with SYBR® Green stain. Below is a plot of the quantification of bands compared by lane.

Gel 3 (Figure 8) shows a drastic difference between the tagged and cleaved IclR, especially at the higher concentrations. At 75 nM, binding seems to behave very similarly between each type of IclR (lanes 1 and 12). However, at 350 nM the DNA is about 40% in the third band while the cleaved IclR has only about 10% in the third band. The biggest difference in this gel is again seen in the glyoxylate reactions. The 350 nM tagged IclR with glyoxylate (lane 5) has the large majority of DNA driven up to the fourth band compared to only about 5% for the cleaved IclR (lane 8). This gel follows the trend set by the previous two where the tagged IclR with glyoxylate binds in a higher multimer despite being expected to bind as a dimer.

In gel 4 (Figure 9) once again, the two lowest concentrations of tagged and cleaved IclR had similar binding activities. At the 250 nM concentration, the tagged IclR reaction started moving up to form a fourth band (lane 3), but in the cleaved protein reaction the fourth band did not appear until the 350 nM concentration (lane 9). Comparing the 350 nM reactions, the tagged IclR had about 28% of DNA move up to the fourth band while the cleaved IclR had only 10% move up to the fourth band. The difference between the types of IclR at 350 nM was not as extreme as seen in gel 3 but did follow the same pattern. The gel 4 glyoxylate reactions also follow the trend seen in the previous gels as the tagged IclR glyoxylate reaction (lane 5) caused more drive into the fourth band than the cleaved IclR glyoxylate reaction (lane 8). However, a difference in this gel was that the tagged IclR at 350 nM with glyoxylate had slightly less fourth band presence than the tagged 350 nM concentration with pyruvate (lanes 8 and 9).

Table 2. Summary of band presence with increasing IclR concentration in tagged and cleaved IclR reacting with pyruvate

| Column | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Protein concentration (nM) and type | 25 WT | 75 WT | 150 WT | 250 WT | 350 WT | 350 Cleaved | 250 Cleaved | 150 Cleaved | 75 Cleaved | 25 Cleaved |
| First band presence (%) | 70.09 | 77.75 | 43.83 | 20.79 | 13.27 | 21.17 | 33.37 | 51.73 | 81.98 | 87.86 |
| Second band presence (%) | 29.91 | 22.25 | 40.85 | 30.97 | 19.59 | 39.70 | 42.06 | 41.73 | 18.02 | 12.14 |
| Third band presence (%) | - | - | 15.32 | 29.45 | 32.70 | 28.46 | 22.04 | 6.54 | - | - |
| Fourth band presence (%) | - | - | - | 18.80 | 34.44 | 10.67 | 2.52 | - | - | - |

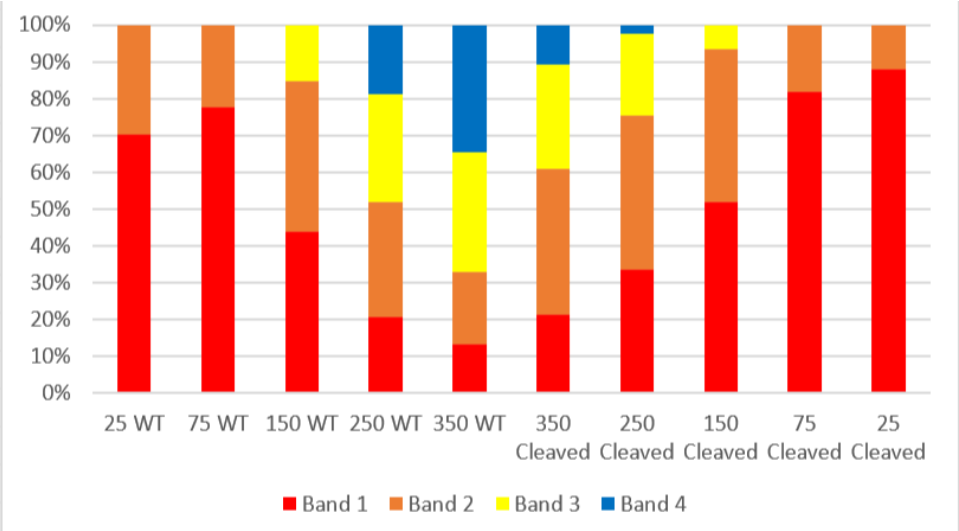



Figure 10. Plot comparing presence of bands in tagged and cleaved IclR in reactions with pyruvate.

Table 3. Summary of band presence of tagged and cleaved IclR at 250 nM in reactions with glyoxylate

| Column | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Protein concentration (nM) and type | 250 WT + pyruvate | 250 WT + Glyoxylate | 250 Cleaved + pyruvate | 250 Cleaved + Glyoxylate |
| First band presence (%) | 20.79 | 20.17 | 32.53 | 33.37 |
| Second band presence (%) | 30.97 | 40.21 | 48.86 | 42.06 |
| Third band presence (%) | 29.45 | 31.95 | 18.61 | 22.04 |
| Fourth band presence (%) | 18.80 | 7.67 | - | 2.52 |

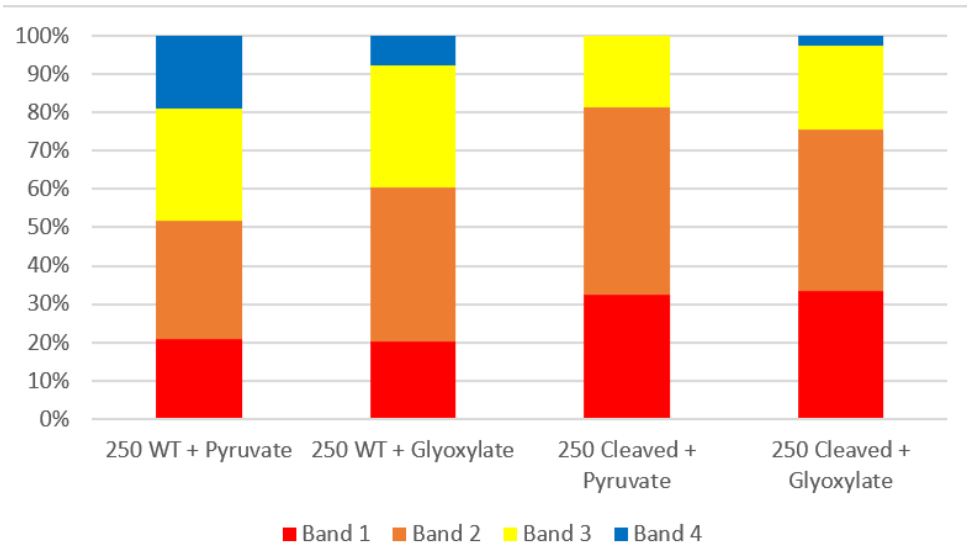



Figure 11. Plot comparing presence of bands in tagged and cleaved IclR at 250 nM in reactions with glyoxylate and pyruvate

Table 4. Summary of band presence of tagged and cleaved IclR at 350 nM in reactions with pyruvate and glyoxylate

| Column | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Protein concentration (nM) and type | 350 WT + pyruvate | 350 WT + Glyoxylate | 350 Cleaved +pyruvate | 350 Cleaved + Glyoxylate |
| First band presence (%) | 13.27 | 14.47 | 24.64 | 21.17 |
| Second band presence (%) | 19.59 | 17.46 | 41.24 | 39.69 |
| Third band presence (%) | 32.70 | 24.99 | 29.34 | 28.46 |
| Fourth band presence (%) | 34.44 | 43.08 | 4.78 | 10.67 |

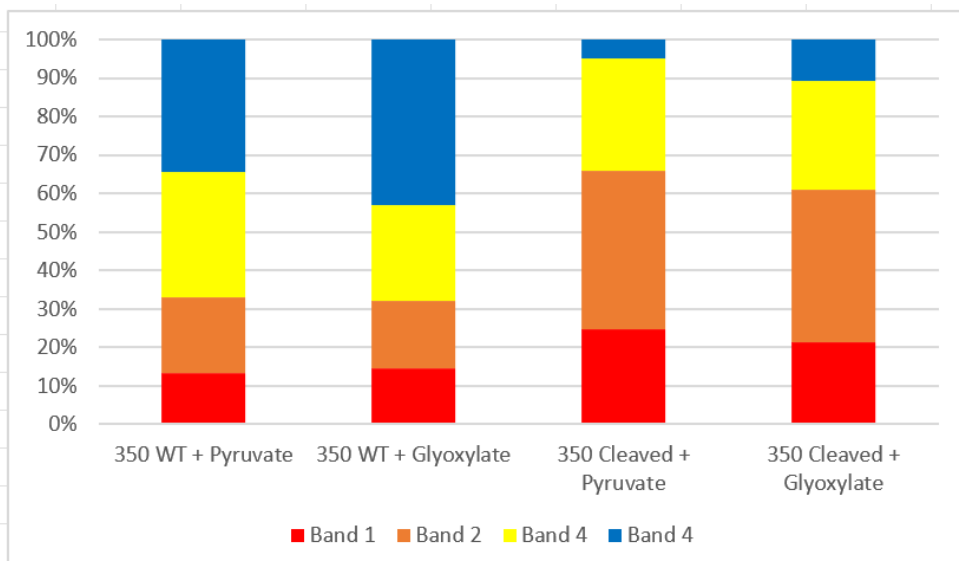



Figure 12. Plot comparing presence of bands in tagged and cleaved IclR at 250 nM in reactions with glyoxylate and pyruvate

Overall across all the gels, it appears that the tagged IclR multimerized at lower concentrations than the cleaved IclR. These differences in binding were seen more at higher concentrations. In reactions with glyoxylate, the tagged protein tended to drive binding up into higher multimer bands 3 and 4 while the cleaved protein tended to have less band presence in band 4 and more presence in band 2. This data raised a series of questions, with three main ones being: what level of multimerization are these proteins achieving, why is binding different with and without a His-tag, and why are the glyoxylate reactions behaving against expectations?

## Multimerization Labels

Throughout the results discussion of the gels the bands were being referred to simply as "band 1, 2, 3, and 4". Band 1 in all the gels is known to be the unbound aceBAK DNA. Originally, it was suspected that "band 2" was the DNA bound to a dimeric IclR and "band 3" was the DNA bound to the protein in a tetramer formation, however, when the concentration of protein was increased, the presence of a fourth band became more clear and this original line of thinking came into question. The alternative labeling of these bands has "Band 1" as unbound

DNA, “Band 2” as DNA and IclR monomer, “Band 3” as DNA and dimeric IclR, and “Band 4” as DNA and tetrameric IclR. Exactly identifying which label is accurate is unfortunately past the scope of the EMSA analysis performed in this study. In the future, analysis using blue native PAGE or HPLC gel filtration would be helpful in answering the band labeling question.

## His-tag Effect on Multimerization

Usually a His-tag is inconsequential to overall protein behavior because of their relatively small size and charge.[6] However, the results in these experiments show a different story. There are literature reports where a His-tag did affect protein binding behavior, creating discrepancies when reproducing experiments and creating new studies. The tag can cause interference by altering the protein’s ability to bind to ligands, cause aggregation, or change protein solubility. In cases where the His-tag did affect protein binding, it was found that the tag created conformational changes in the active site of the protein or acted as a weak competitive inhibitor.[7] Again, determination of what exactly is causing the His-tag to affect protein binding cannot be done using EMSA studies. If we’re hypothesizing that the His-tag is making changes in the protein’s structural arrangement, crystal structure analysis could reveal exactly what structural changes are taking place. In some cases, the actual His-tag is too flexible to appear in a 3D structure but if it is making structural changes these should be able to be seen in comparison to the cleaved protein crystal structure. It has been shown that intermolecular interactions between the methyl group on the pyruvate molecule and a hydrophobic patch formed by residues Met-146, Leu-154, Leu-220, and Leu-143 in the IclR stabilizes the tetramerization of IclR.[5] The His-tag maybe having some effect on this hydrophobic region increasing the binding affinity of the protein to the pyruvate, thus increasing the stability of the tetrameric state. However, in future experiments in future experiments it should be sufficient to just used cleaved IclR.

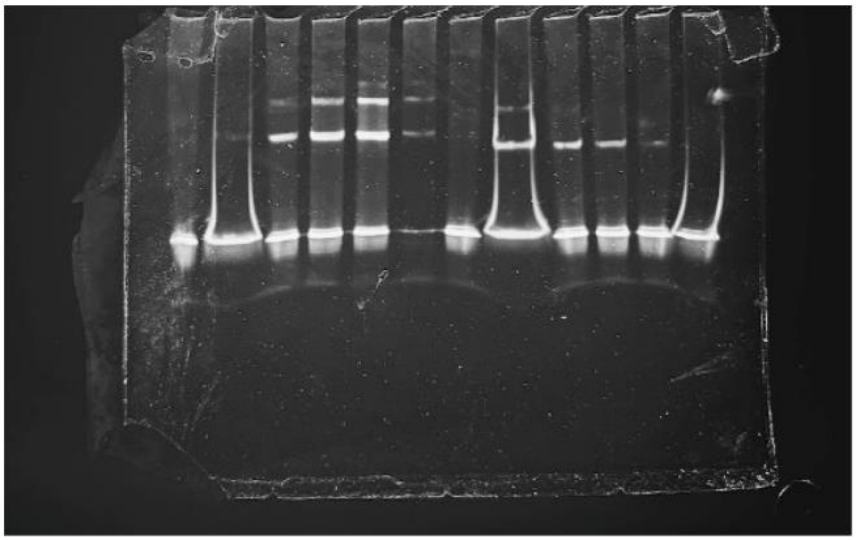
## Glyoxylate Reactions

It was seen that the tagged protein glyoxylate reactions often drove binding to higher multimeric complexes while cleaved proteins did not as readily form these higher multimeric complexes. Once again, these EMSA reactions show that there is a difference but does not allow for the exact reason to be determined. Attention should be turned to the 3D protein structure to identify this difference. Effector molecules bind to the C-terminal of the protein and interact with different specific residues depending on their structure. Through mutated protein analysis it has been found that the residues Leu-143 and Met-146 are important in glyoxylate binding and related to glyoxylate's dimer stability.[5] The His-tag could be having an effect on these residues and possibly be causing the glyoxylate to bind more like pyruvate and thus stabilizing the tetramer form over the dimer form.

## Mutant Studies

EMSA analysis was also performed using mutated S147A IclR. The concentration range for these reactions followed that of the latter half of the wild type analysis, 75-350 nM. All other reactions conditions were the same as the wild type analysis.

| | S147A IclR | | | | | | Cleaved S147A | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| DNA | + | + | + | + | + | - | + | + | + | + | + | + |
| IclR S147A (nM) | 75 | 150 | 250 | 350 | 350 | 350 | 0 | 0 | 0 | 0 | 0 | 0 |
| IclR cleaved (nM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 350 | 350 | 250 | 150 | 75 |
| Glyoxylate | - | - | - | - | + | - | - | + | - | - | - | - |
| Pyruvate | + | + | + | + | - | + | + | - | + | + | + | + |



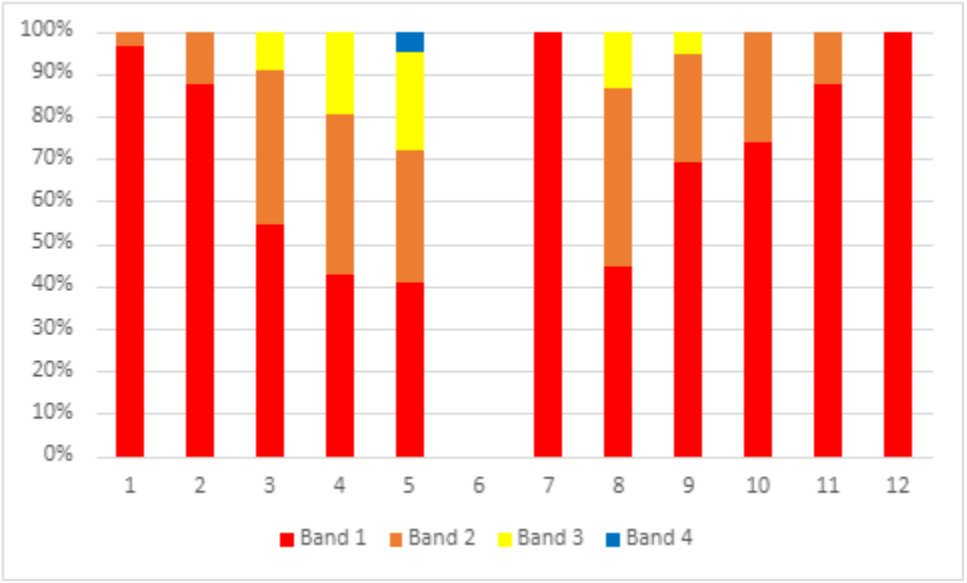



Figure 13. Gel 5 EMSA reaction comparing His-tagged and cleaved S147A IclR. Lanes labeled with reaction contents. EMSA performed using a 5% TBE polyacrylamide gel run at 100V in 4°C for 45 minutes and stained with SYBR® Green stain. Below is a plot of the quantification of bands compared by lane.

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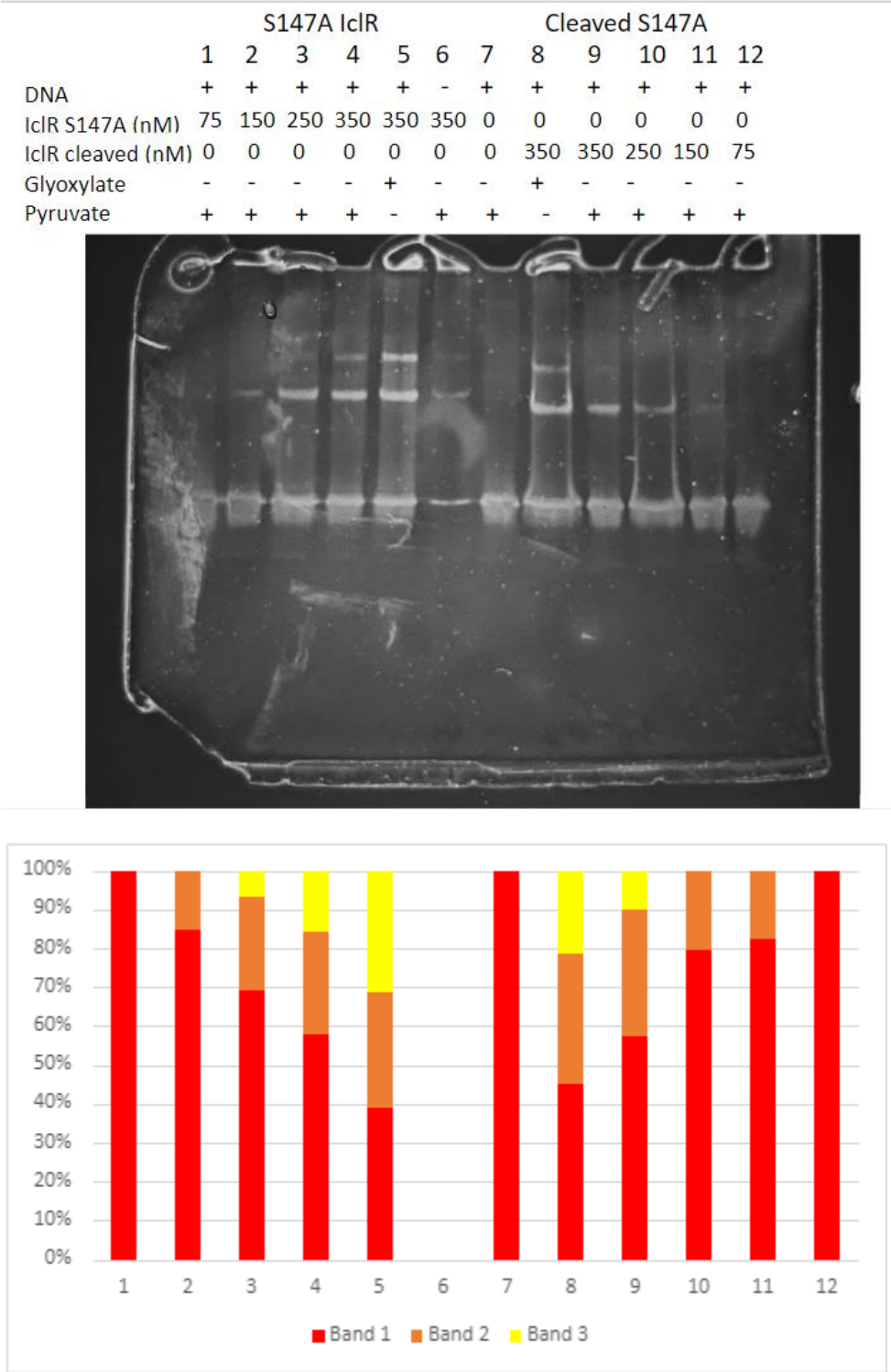


Figure 14. Gel 6 EMSA reaction comparing His-tagged and cleaved S147A IclR. Lanes labeled with reaction contents. EMSA performed using a 5% TBE polyacrylamide gel run at 100V in 4°C for 45 minutes and stained with SYBR® Green stain. Below is a plot of the quantification of bands compared by lane.

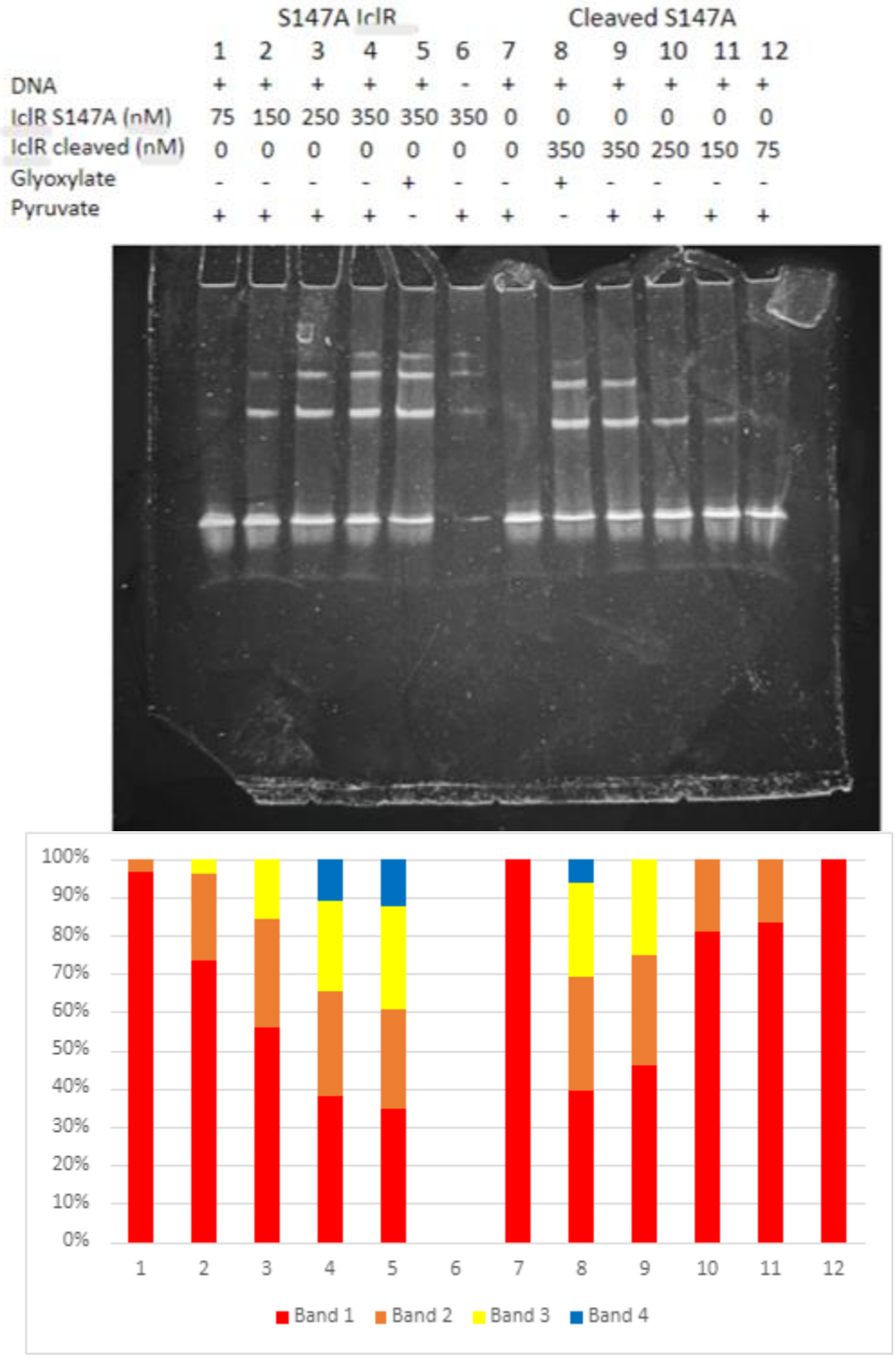


Figure 15. Gel 7 EMSA reaction comparing His-tagged and cleaved S147A IclR. Lanes labeled with reaction contents. EMSA performed using a 5% TBE polyacrylamide gel run at 100V in 4°C for 45 minutes and stained with SYBR® Green stain. Below is a plot of the quantification of bands compared by lane.

Table 5. Summary of band presence with increasing IclR concentration in tagged and cleaved S147A IclR reacting with pyruvate

| Column | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Protein concentration (nM) and type | 75 WT | 150 WT | 250 WT | 350 WT | 350 Cleaved | 250 Cleaved | 150 Cleaved | 75 Cleaved |
| First band presence (%) | 97.73 | 79.63 | 59.85 | 43.08 | 57.51 | 78.06 | 84.39 | 100 |
| Second band presence (%) | 2.27 | 16.36 | 29.65 | 28.29 | 28.96 | 21.94 | 15.61 | - |
| Third band presence (%) | - | 4.01 | 10.50 | 18.30 | 13.53 | - | - | - |
| Fourth band presence (%) | - | - | - | 10.33 | - | - | - | - |

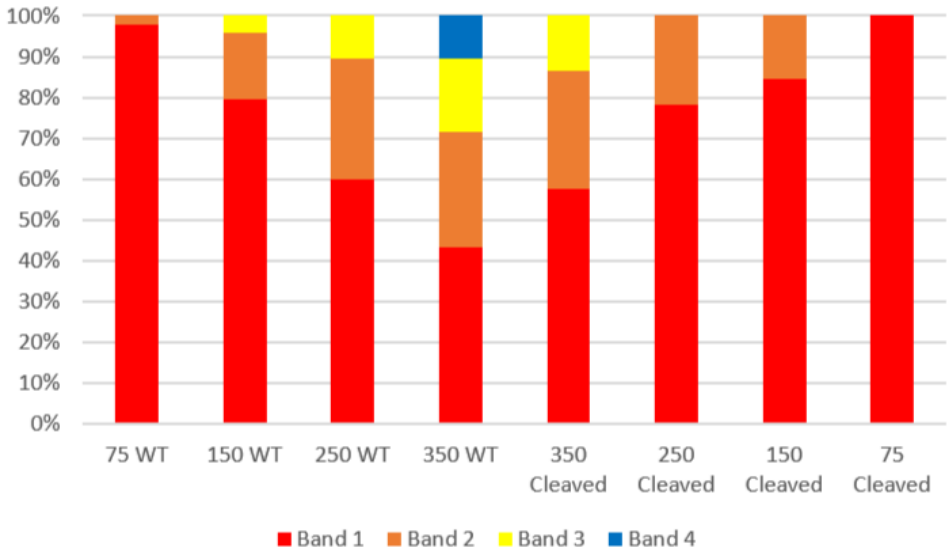


Figure 16. Plot comparing presence of bands in tagged and cleaved S147A IclR in reactions with pyruvate

The mutated S147A IclR reactions behaved similarly to the wild type IclR. Once again it was seen that the cleaved protein required higher concentrations to form higher multimeric states. Comparing the 250 nM concentration of tagged (lane 3) and cleaved IclR (lane 10), gels 5 through 7 showed the tagged IclR beginning to move up to form a third band however, in the cleaved protein the third band did not appear until 350 nM (lane 9). Overall, the mutated protein saw less drive up into multimeric states compared to the wild type IclR. This can be seen by the

large percentage of presence in Band 1 across the whole gel. In the mutated protein gels the Band 1 percentage mostly remains above 40% while in the wild type gels Band 1 presence is overall lower and went down to around 20% at the highest concentration.

Table 6. Summary of band presence of tagged and cleaved S147A IclR at 350 nM in reactions with pyruvate and glyoxylate

| Column | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Protein concentration (nM) and type | 350 S147A + Pyruvate | 350 S147A + Glyoxylate | 350 Cleaved S147A+Pyruvate | 350 Cleaved S147A+ Glyoxylate |
| First band presence (%) | 43.08 | 38.26 | 41.24 | 57.51 |
| Second band presence (%) | 28.29 | 28.85 | 33.61 | 28.96 |
| Third band presence (%) | 18.30 | 27.08 | 18.92 | 13.53 |
| Fourth band presence (%) | 10.33 | 5.82 | 6.24 | - |

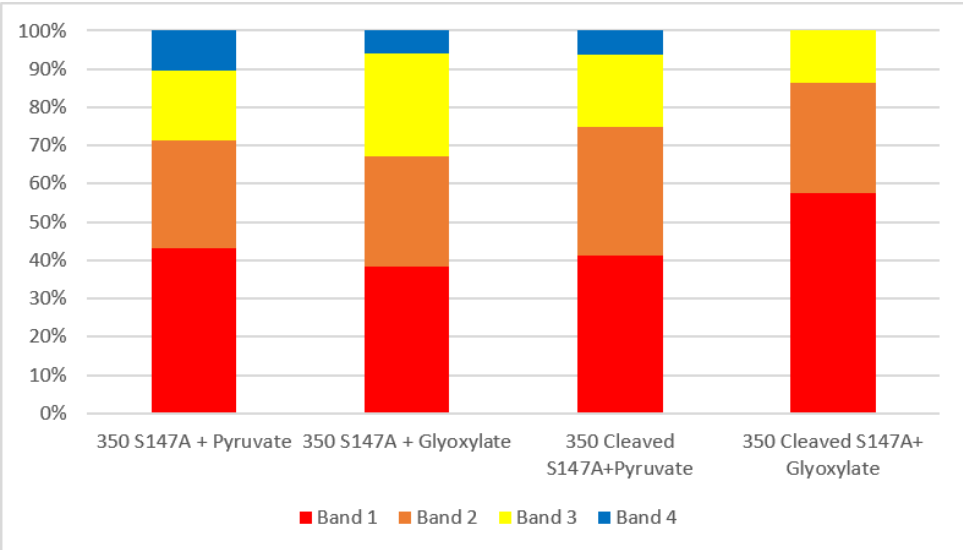


Figure 17. Plot comparing presence of bands in tagged and cleaved S147A IclR at 350 nM in reactions with glyoxylate and pyruvate

Several comparisons can be made in relation to the glyoxylate reactions with the S147A IclR. One comparison can be made between the S147A tagged and cleaved protein in a reaction with glyoxylate. The 350 nM tagged S147A with glyoxylate in gels 5, 6, and 7 showed a higher

percentages of DNA in band 3 and 4 while the 350 nM cleaved S147A with glyoxylate reactions had higher percentages of DNA in Bands 1 and 2. Gel 6 (Figure 14) also showed a higher percentage in Band 3 in the 350 nM tagged S147A with glyoxylate (lane 8) than in the same concentration with pyruvate (lane 9). Another comparison can be made between both forms of S147A IclR with glyoxylate and with pyruvate. Looking at the S147A IclR, there was more drive to higher multimerization units in the glyoxylate reaction compared to a pyruvate reaction at the same concentration. This aligned with what was found when working with tagged wild type IclR, which as mentioned, went against expectations as IclR bound to glyoxylate is expected to be less stable as a tetramer. One more comparison could be made between the cleaved S147A with glyoxylate and the cleaved wild type IclR with glyoxylate. The cleaved wild type IclR tended to have more drive to higher multimerization states when bound to pyruvate than with glyoxylate. However, the cleaved S147A behaved oppositely, having more drive to higher multimerization states in the reaction with glyoxylate rather than pyruvate.

The S147A mutant was less likely to produce bands indicative of multimerization and DNA binding is consistent with serine 147 being an important residue in DNA binding. The substitution of this residue with alanine seemed to decrease IclR's affinity to DNA in both tagged and cleaved reactions. In terms of the glyoxylate reactions, both the cleaved and tagged proteins had higher levels of multimerization than they did with pyruvate, so the tag does not seem to have an effect on multimerization. However, it seems that serine 147 is somehow involved in the destabilization of a tetrameric formation with glyoxylate.

## Conclusions and Future Work

This study aimed to evaluate if a polyhistidine tag on IclR protein alters its binding affinity to DNA and its ability to multimerize. To do this purified IclR protein underwent a cleavage reaction which removed the His-tag from the C-terminus of the protein. EMSA was then used to evaluate binding affinity and comparison of the tagged and cleaved IclR protein. Reactions included 20 ng aceBAK DNA, various concentrations of IclR protein, either tagged or cleaved, either pyruvate or glyoxylate, and binding buffer. DNA bands on the gels were stained and quantified. It was found that His-tagged IclR was able to multimerize at lower concentrations than cleaved IclR. Additionally, tagged IclR when bound to glyoxylate tended to form higher multimers than cleaved IclR with glyoxylate. These findings lead to the belief that the His-tag is having some stabilization affect on multimeric states of IclR.

EMSA reactions were also completed using mutated S147A IclR where serine residue 147 was replaced with alanine. S147A IclR with pyruvate behaved similarly to the wild type IclR with pyruvate. Tagged S147A again was able to multimerize at lower concentrations than cleaved S147A. Overall there was more unbound DNA with the S147A IclR than with the wild type IclR. Both tagged and cleaved S147A, when bound to glyoxylate, tended to be driven up into bands 3 and 4, signaling serine 147 might be important in destabilizing multimeric IclR.

In the future it would be helpful to perform gel filtration HPLC experiments to determine the exact size and various multimeric states of IclR. Additionally, exploration of alternate tags that may not affect DNA interaction and multimerization could be beneficial. Ultimately, future work should be done with IclR without a His-tag to mimic behavior of naturally occurring IclR protein.

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