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Studies on the molecular mechanisms of LuxR-type quorum sensing receptors in gram-negative bacteria

By

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Chapter 1:

An introduction for the public: how do bacteria communicate?

Contributions: This chapter was written by Irene M. Stoutland and edited by Helen E. Blackwell and Elizabeth Reynolds, with support from Professor Bassam Shakhashiri and Cayce Osborne at the Wisconsin Initiative for Science Literacy (WISL).

1.1 Introduction

Antonie van Leeuwenhoek (1632–1723) is known as the “father of microbiology” for his discovery of microscopic life. In 1676, he used a homemade microscope to observe and describe tiny living things he called “animacules” that we now know as bacteria. It is hard for me to imagine how outlandish such claims must have seemed to the scientific community at a time when “spontaneous generation” theory, which posited that life could arise spontaneously (such as maggots originating from meat), was a fierce debate. Though not formally trained as a scientist, van Leeuwenhoek developed some of the most sophisticated microscope technology of his time and used it to make startling biological discoveries.

As scientific understanding of the natural world has continued to accumulate over the several hundred years since van Leeuwenhoek’s discoveries, the barrier of entry to scientific research has grown. Whereas in the seventeenth century, the relatively easy-to-conceptualize ability to observe tiny things was at the cutting edge of microbiology, today’s molecular biologists spend years learning about the molecular structure of cells, DNA, and proteins; biochemical processes such as transcription, translation, cell division, metabolism, and reproduction; and complex techniques to track the invisible processes of DNA and RNA production, protein dynamics, intermolecular interactions, and chemical transformations in living systems—all just to catch up with the current state of the field. As a result, new discoveries are harder for people without extensive scientific training to understand and appreciate, and scientists risk doing their work in a vacuum, isolated from the input and interests of people who may be impacted by

scientific advancements and whose taxpayer dollars fund most academic research in the United States.

This chapter is my attempt to make the work I have accomplished over the last five years for my Ph.D. thesis accessible and interesting to anyone who is curious about what I do in the lab all day and why. While not nearly as groundbreaking or exciting as van Leeuwenhoek's discoveries, I hope the work I share provides a glimpse of how one graduate student can incrementally contribute to our understanding of the natural world.

The Wisconsin Initiative for Science Literacy (WISL) at UW–Madison provides a great service to the scientific community and the public at large in encouraging and sponsoring the creation of these chapters intended for a general audience. Many thanks to Professor Bassam Shakhashiri, Elizabeth Reynolds, and Cayce Osborne for creating this program and providing feedback on this chapter.

1.1.1 Bacteria: foes and friends

Bacteria live all around us. They coat the inside and outside of our bodies; they make their homes in plants, animals, soil, and air; they can even survive deep under the Earth's surface. In some situations, bacteria can be harmful: they can cause people to get sick, food to spoil, or crops to die. But bacteria have many beneficial roles as well. The microbes that live on and inside our bodies—our microbiome—play important roles in digesting our food, warding off pathogens, producing vitamins, affecting mood, and more. Bacteria are essential to producing popular fermented foods like sourdough, kombucha, yogurt, sauerkraut, and miso. Soil bacteria can enhance plant growth, disease resistance, and crop yields. Some bacteria can have beneficial or neutral roles

under certain conditions but cause illness or other negative effects under other conditions. Defining the factors that influence bacterial behavior could enable strategies to encourage the beneficial effects of bacteria while limiting the negative ones. For example, as rising antibiotic resistance continues to threaten humanity's ability to fight bacterial infections, exploring alternative strategies to influence bacterial behavior is increasingly urgent. Antibiotics kill bacteria, but any bacteria that develop a way to resist the antibiotic's effect can survive and prosper. Strategies to reduce the harmful effects of bacteria without killing them exert less selective pressure for the bacteria to develop resistance. During my time in graduate school, I have been part of a laboratory that works to understand how bacteria communicate and develop strategies to alter bacterial behavior by interfering with that communication.

1.1.2 How do bacteria communicate?

It may be tempting to think of bacteria as isolated organisms, since these single-celled creatures lack many of the senses, such as sight or hearing, that more complex organisms use to observe the world and communicate with one another. But despite their size and simplicity, bacteria can communicate both within and across species to coordinate complex group behaviors. The ability of bacterial cells to communicate with one another was first discovered in a species called *Vibrio fischeri*, which lives in the ocean and produces light, or bioluminescence, under certain conditions. *Vibrio fischeri* can colonize a specialized "light organ" inside the Hawaiian bobtail squid, and light produced by the bacteria helps the squid camouflage itself at night. The squid's light organ, full of glowing bacteria, acts like a flashlight that points down, masking the

shadow that the squid would otherwise cast on a moonlit night and helping it hide from predators and prey below.

Vibrio fischeri does not produce light all the time. In the lab, cultures of *Vibrio fischeri* remain dark during early stages of growth, then suddenly light up. How does *Vibrio fischeri* know when to start producing light? At early stages of growth in a bacterial culture, the bacteria are dilute, but as the bacteria multiply, the density of bacteria increases. Researchers discovered in the late 1960s that when bacteria in the early, dark stages of growth were exposed to growing media from a mature, bioluminescent culture, the early culture started to produce light. Eventually, researchers figured out that each bacterial cell produces a low level of chemical signal. As the population of bacteria grows, the concentration of the chemical signal also increases. Individual bacteria can detect when there is a high concentration of chemical signal, and that tells them that they are in a dense group and should initiate group behaviors—in the case of *Vibrio fischeri,* bioluminescence. In the experiment just described, the media from the mature cultures had a high concentration of chemical signal, which made the bacteria in the younger culture react as if they, too, were in a dense group. We now know that many species of bacteria use chemical signals to sense and respond to population density, a process called quorum sensing.

1.1.3 How does quorum sensing work?

Quorum sensing helps bacteria avoid wasting energy on behaviors that are only useful in certain situations, typically behaviors that are beneficial to a dense group of bacteria but not to a solitary bacterium. For example, a single bacterium cannot produce

enough light to make a difference in its environment, but a large group of bacteria can. In the decades since scientists first detected quorum sensing, researchers have discovered multiple types of quorum sensing systems, deduced the chemical structure of the signals involved, and identified the proteins involved in making and sensing the signals.

Bacteria have many specialized proteins with different jobs within the bacterial cell. Proteins with the ability to detect and respond to chemical signals are called receptors. Receptors play an important role in linking environmental signals to changes in gene expression, allowing a bacterium to adjust its lifestyle in response to its environment. The process by which a receptor detects a chemical signal used to be described as a key (the chemical signal) fitting into a lock (the receptor). We now know that both chemical signals and receptors often change shape to fit together more tightly, like a hand fitting into a glove. The chemical signal fits into a pocket in the receptor and changes the shape of the pocket and often the overall shape of the receptor, which changes how the receptor behaves. In many of the figures I show in this chapter, quorum sensing receptors are represented as simple shapes, but receptor structures are actually complex and have many moving parts (**Figure 1.1**). Researchers have identified the specialized receptors that detect the chemical signals that mediate quorum sensing, but we still do not fully understand how these receptors change shape when they interact with quorum sensing signals or how the receptor's shape controls its behavior. My work has focused on understanding how chemical signals change the shape and behavior of quorum sensing receptors.

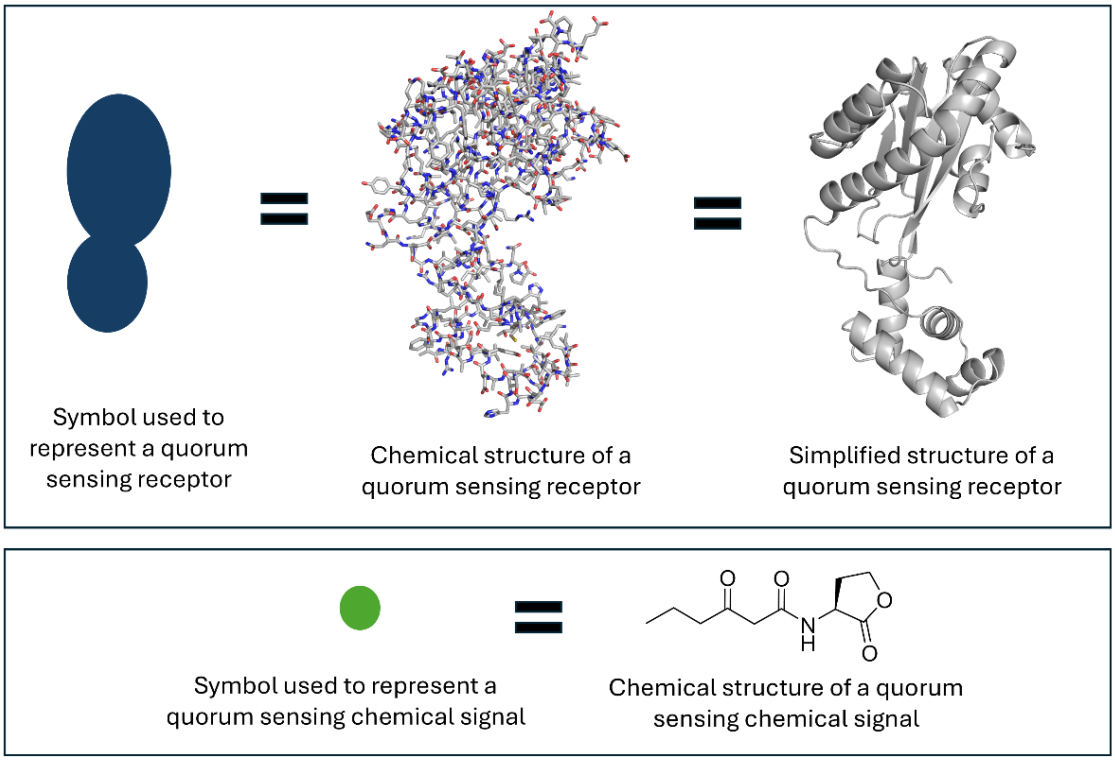

Figure 1.1. Different representations of quorum sensing receptors and signals. *Top left*, a symbol used to represent a quorum sensing receptor. *Top middle*, the chemical structure of a quorum sensing receptor, where each line segment represents a chemical bond and each vertex is an atom. *Top right*, a simplified version of the receptor's chemical structure, with ribbons and spirals used to represent specific structural configurations that are common in proteins. *Bottom left,* a symbol used to represent a chemical signal used for quorum sensing. *Bottom right,* chemical structure of a chemical signal used for quorum sensing, where each line segment represents a chemical bond, unlabeled vertices represent carbon atoms, and "O," "N," and "H" represent oxygen, nitrogen, and hydrogen atoms, respectively.

There are several types of quorum sensing systems, like different languages, which rely on distinct chemical signals and protein receptors. My work has focused on the LuxI/R quorum sensing system, which is present in many common species of bacteria with effects that can be beneficial (the squid flashlight, increased crop growth) or detrimental (animal and plant diseases, food spoilage). In the LuxI/R system, a protein called LuxI produces specific chemical signals that are released into the

environment. As the density of the bacterial population grows, so does the concentration of the chemical signal. Once the chemical signal concentration is high enough, the LuxR receptors inside each bacterium can interact with the chemical signal and detect that a quorum exists. When a chemical signal interacts with a receptor, it changes the receptor's ability to stick (a.k.a. bind) to DNA. When bound to DNA, the LuxR receptor recruits other proteins that make an RNA copy of the nearby DNA sequence (the gene), and that RNA is eventually converted into a protein, which can alter the behavior of the bacterial cell.

The DNA sequence of a gene determines what protein will be produced. DNA is made up of strings of A's, T's, G's, and C's, and each set of three DNA letters codes for one amino acid. Proteins are strings of amino acids. Some proteins make the bacteria bioluminescent by producing a small molecule that glows, while others break down sources of carbon so the bacteria can use them for energy; still others create chemicals that kill neighboring bacteria or assemble into structures that allow the bacterium to swim around. The LuxR receptor allows the bacteria to change which genes it expresses in response to chemical signals. The genes that a bacterium expresses determine which proteins are present, which changes what bacterium can do and how it behaves. (**Figure 1.2**).

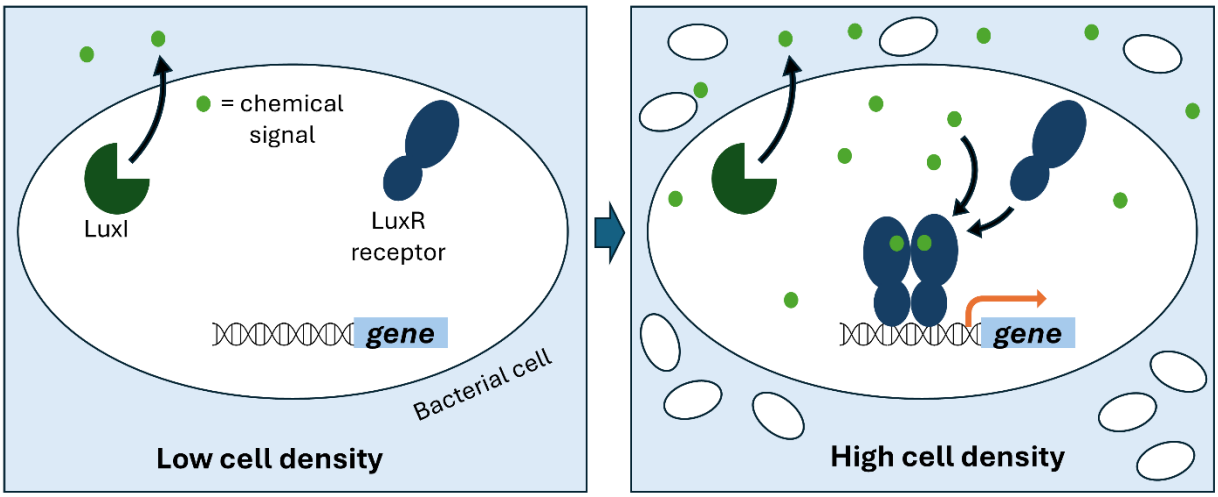

Figure 1.2. Bacteria use chemical signals to sense when they are in a group. Each bacterial cell produces a low level of chemical signal using a protein called LuxI. When a bacterial cell is alone, the concentration of chemical signal remains low. When there is a dense group of bacteria, a high concentration of signal can accumulate, which causes the LuxR receptor to activate specific genes that change bacterial behavior.

1.2 Project 1: Protein chimeras to study molecular mechanism

1.2.1 Why do we care about the molecular details of bacterial communication?

Diverse species of bacteria use quorum sensing to regulate a range of behaviors, such as the production of complex chemicals, the excretion of proteins that help digest nutrients in the environment, the formation of colonies that stick to surfaces, and the establishment of symbiotic relationships with other organisms. In some species, quorum sensing controls when bacteria engage in behaviors that help them infect a host, so interfering with bacteria's ability to produce or detect chemical signals that mediate quorum sensing could be a way to help fight infections.

Designing chemical signals that affect the activity of LuxR receptors is a way to better understand their function and can help us to control how bacteria behave. Over the last ~30 years, research groups around the world have designed such chemical signals to learn about how quorum sensing happens and to develop strategies to control

bacterial behavior. One of the barriers to developing chemical signals that have the desired effect on bacteria is that we do not fully understand the process by which the chemical signals affect LuxR receptors. If we knew how the shape of the receptor changed when it interacted with a chemical signal and what features of the chemical signal caused that change, it would be easier to design new chemical signals that had the desired effect on a receptor and therefore on bacterial behavior. My Ph.D. thesis aimed to address this gap in knowledge by asking: how does chemical signal change the shape and behavior of LuxR receptors?

1.2.2 What are the molecular details of bacterial communication?

Different LuxR receptors respond to chemical signals in various ways. Some receptors can bind to DNA only when they interact with an activating chemical signal. Other receptors only bind to DNA without chemical signal, and when the receptor interacts with chemical signal, it is no longer able to bind to DNA. Both receptor types help bacteria respond to chemical signals, but some do it by turning genes on and some do it by turning genes off. Remarkably, these receptor types show opposite responses to the exact same chemical signal. How does an identical signal cause two different receptors to change their behavior in opposite ways?

LuxR receptors have two parts, represented by two ovals in **Figures 1.1-1.4**. One of the parts binds to the chemical signal, and the other part binds to DNA. Is the ability of some LuxR receptors to bind to DNA only in the presence of chemical signal and others to bind to DNA only in the absence of chemical signal due to the characteristics of the signal-binding part of the protein, the DNA-binding part, or some

interaction between the two? To investigate this question, I merged receptors active in the presence of signal, called associative receptors, with receptors active in the absence of signal, called dissociative receptors. We call these mutant proteins “chimeras” because, like the mythological beast with the body of a lion, head of a goat, and serpent for a tail, these proteins combine pieces of receptors from different species.

1.2.3 What can protein chimeras teach us about quorum sensing?

Compared to proteins, DNA is relatively simple to generate in a lab and introduce into bacteria; we provide the DNA blueprint, and the bacteria do the hard work of building complex proteins. Bacteria are especially amenable to expressing artificially introduced genes because they take up small circles of DNA called plasmids from the environment. By introducing a plasmid with a specific DNA blueprint that codes for a LuxR receptor, we can make bacteria produce that LuxR receptor protein.

I made plasmid blueprints for protein chimeras using a technique called polymerase chain reaction, or PCR, that allows a strand of DNA to be copied many times (this same technique is used for some COVID-19 tests by allowing viral DNA to be copied millions of times so that it is easier to detect). I copied parts from two different plasmids and joined them together to make a plasmid expressing half of one LuxR receptor linked to half of a different LuxR receptor.

Since LuxR receptors naturally regulate gene expression, we can introduce another plasmid into the bacteria that allows the LuxR receptor to regulate production of a protein that produces light. When the LuxR receptor is activated by interacting with a chemical signal, the bacteria start to glow (**Figure 1.3**).

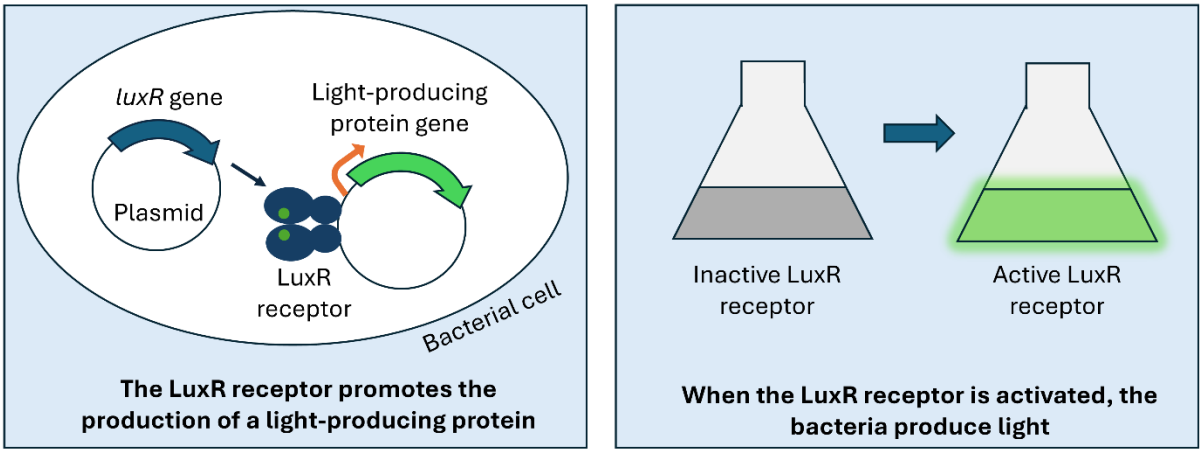

Figure 1.3. We measure LuxR receptor activity using bacteria that produce light when the LuxR receptor is active. A circle of DNA called a plasmid causes the bacteria to produce the LuxR receptor. When the LuxR receptor is active, it binds to another circle of DNA and causes a light-producing protein to be created.

Once I had bacteria expressing LuxR receptor chimeras, made by fusing half of an associative receptor (active in the presence of chemical signal) with half of a dissociative receptor (active in the absence of chemical signal), it was time to test their ability to bind to DNA and thus alter gene transcription. I grew bacteria expressing these chimeras in the presence and absence of chemical signal and measured the light produced under each condition. When I combined the signal-binding part of an associative receptor and the DNA-binding part of a dissociative receptor, the resulting chimera bound to DNA only in the presence of chemical signal, making the chimera associative. When I made the opposite combination (the signal-binding part of a dissociative receptor and the DNA-binding part of an associative receptor) the mutant bound to DNA in the absence of signal, making it dissociative. These results revealed that the part of the protein where the chemical signal binds determines whether the receptor binds to DNA in the presence or absence of chemical signal (**Figure 1.4**).

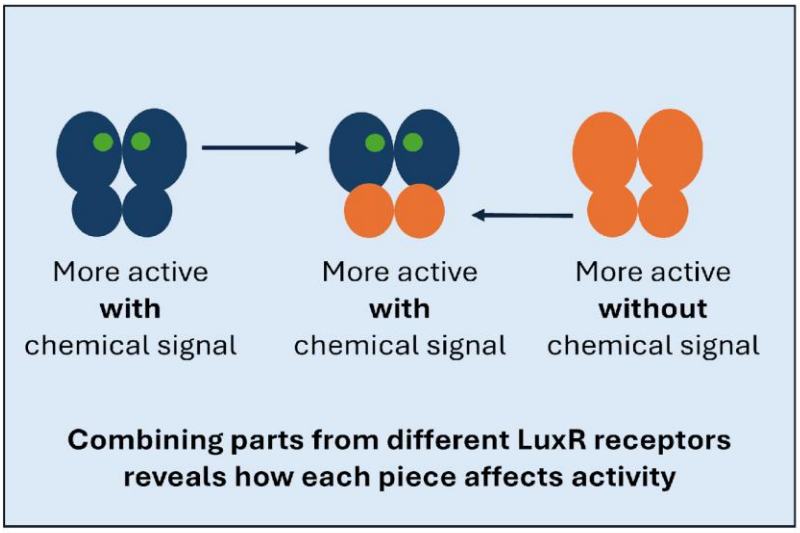

Figure 1.4. Mutant LuxR receptors help us understand the role of different parts of the protein. I combined a piece of a LuxR receptor that is more active with chemical signal and a piece of a receptor that is more active without chemical signal and found that the part of the protein where the chemical signal binds determines whether the receptor is more active with or without chemical signal.

This discovery helps us understand how specific parts of a receptor make some LuxR receptors behave differently than others. We still do not fully understand why some receptors bind to DNA only with chemical signal and others bind to DNA only without chemical signal, but we are a step closer to figuring out which parts of the protein make the difference.

1.3 Project 2: What does the protein look like?

1.3.1 How can we take a picture of a protein?

Another strategy I used to study LuxR receptors was to create a 3D model of the protein's chemical structure, which allowed me to visualize how it changed shape when it was active or inactive. Proteins are really tiny, too small to see with a microscope, so to image a protein, we need a technique called X-ray crystallography.

Even though (and maybe because) I knew little about what the protein crystallography process would entail, I dove right in. First, I used bacteria to make the protein I was interested in, a LuxR receptor called MrtR that comes from soil bacteria. Once I had isolated pure MrtR protein, I tried to grow crystals of MrtR. Some molecules form crystals under natural conditions—like grains of salt, quartz crystals, or rock candy. Proteins do not usually form crystals, but when a high concentration of protein is combined with certain chemicals, sometimes crystals form. To produce useful data, protein crystals need to be large enough, solid, and single; a crystal branched like a snowflake or a bunch of small crystals stuck together will not do. We cannot predict what precise conditions will make crystals form, and different proteins often require different conditions. Tiny variations in the types and concentrations of chemicals, temperature, pH, dust, and humidity can make the difference between success and failure in growing protein crystals. A (probably apocryphal) legend told in crystallography circles is about a scientist who had great success growing protein crystals, when suddenly the crystals stopped growing. The scientist tried to replicate exactly what he had done to grow the crystals and adjusted all the parameters he could think of that might make a difference. In the end, it turned out that around the time the crystals stopped growing, the scientist had shaved his beard. Tiny beard hairs had been getting into the protein solution and helping the crystals start growing!

Undaunted by the fickle nature of protein crystallography, I tried a bunch of different conditions in hopes of finding one that would grow MrtR crystals. Through many rounds of trial and error, tips from friends with experience in the area, internet searches, and hours spent pipetting miniscule volumes of liquid, I found conditions that

grew acceptable crystals (**Figure 1.5**). Then I set out for the crystallography center on campus, where an expert helped me freeze the crystals in a billowing vat of liquid nitrogen, pack them in a large metal canister, and ship them to a specialized facility called a synchrotron where data could be collected.

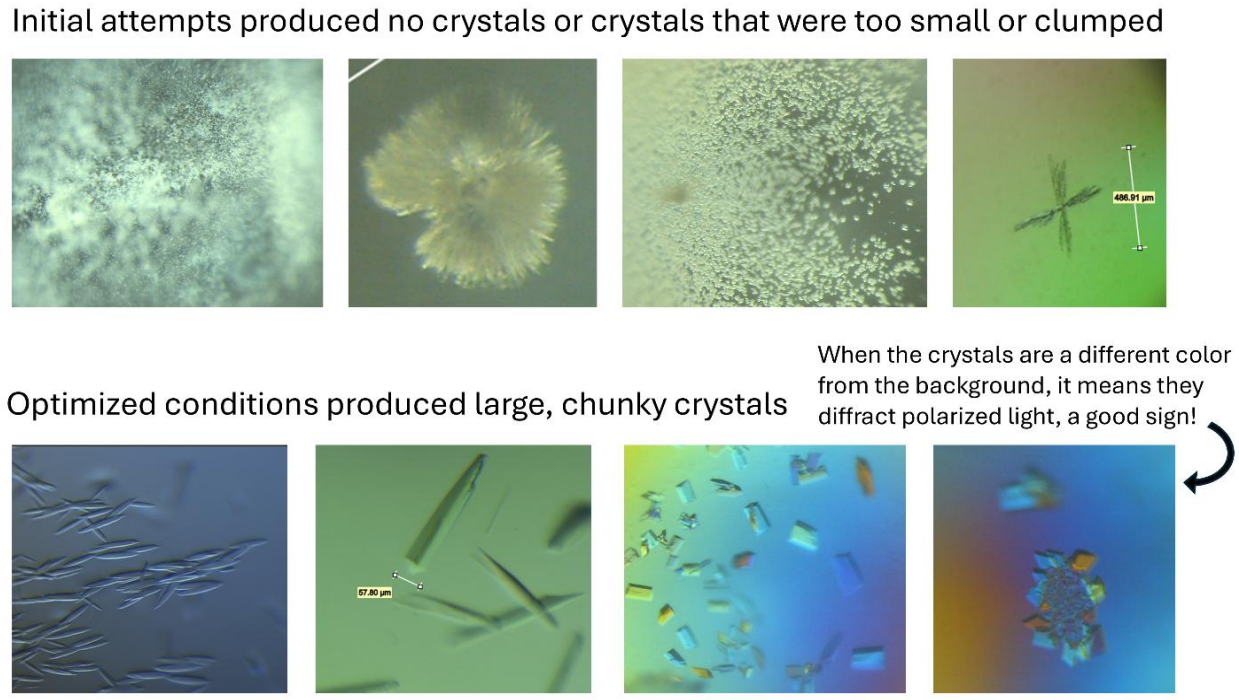

Figure 1.5. Images of some protein crystals I grew for this project. The crystals are made up of tens or hundreds of thousands of proteins but are still too small to see with the naked eye. These pictures are taken through a microscope, and the colors come from polarized light being diffracted by the plastic container the crystals are in and the crystals themselves.

When the crystals arrived at the synchrotron, they would be shot with X-rays to learn about the protein's molecular structure. A synchrotron accelerates electrons to nearly the speed of light as they travel around a ring about half a mile in circumference. The fast-moving particles emit high-energy X-rays that are focused into beams billions

of times brighter than X-rays generated from conventional sources, such as those used for medical imaging. When the X-rays hit the crystal, the atoms that make up a protein scatter the rays and produce a diffraction pattern, like how a prism diffracts sunlight into a rainbow. A computer program can work backwards from the diffraction pattern produced by the protein crystal to create a rough map of the protein structure—a map that represents the density of electrons within the crystal and helps us infer the locations of the atoms that make up the protein.

To convert the diffraction pattern into an electron density map, the computer program needs a starting prediction of the protein structure. In the past, people have used structures of similar proteins or incorporated special elements into their proteins to get around the need for a starting model. More recently, a machine learning program called AlphaFold has been developed that can predict protein structures, an advancement recognized by the 2024 Nobel Prize in Chemistry. I used the structure of MrtR predicted by AlphaFold as a starting model, which allowed the computer program to generate an electron density map without prior experimental information about the protein.

After generating the map, the next step was to adjust the starting protein model based on the map and information about ideal geometry for chemical bonds. I needed to adjust the position of each atom; add hundreds of water molecules to the model; build missing portions of the protein; and decide whether questionable blobs represented protein, water molecules, other small molecules, or background noise. In some cases, a single atom appeared to be in two places at once! I was running into one of the limitations of protein crystal structures—proteins are constantly moving around in

solution, but I was trying to create a static image. Crystals are made up of thousands of repeating units, like a piece of graph paper where each square represents a protein, but in three dimensions instead of two. If a group of atoms spends time in multiple positions, some of the proteins in the crystal might be trapped in a different position than others, and the electron density map will present the average. It can be easy to look at a model of a protein and think of it as an objective truth—what could be more reliable than an actual picture of a protein? But working with these models made me appreciate how much ambiguity can exist in the data. Some features of the model are crystal clear while others are difficult to decipher. Crystal structures are still immensely useful in understanding how proteins work, but looking at the data (the map) directly can reveal nuances that are not captured in the final model (**Figure 1.6**).

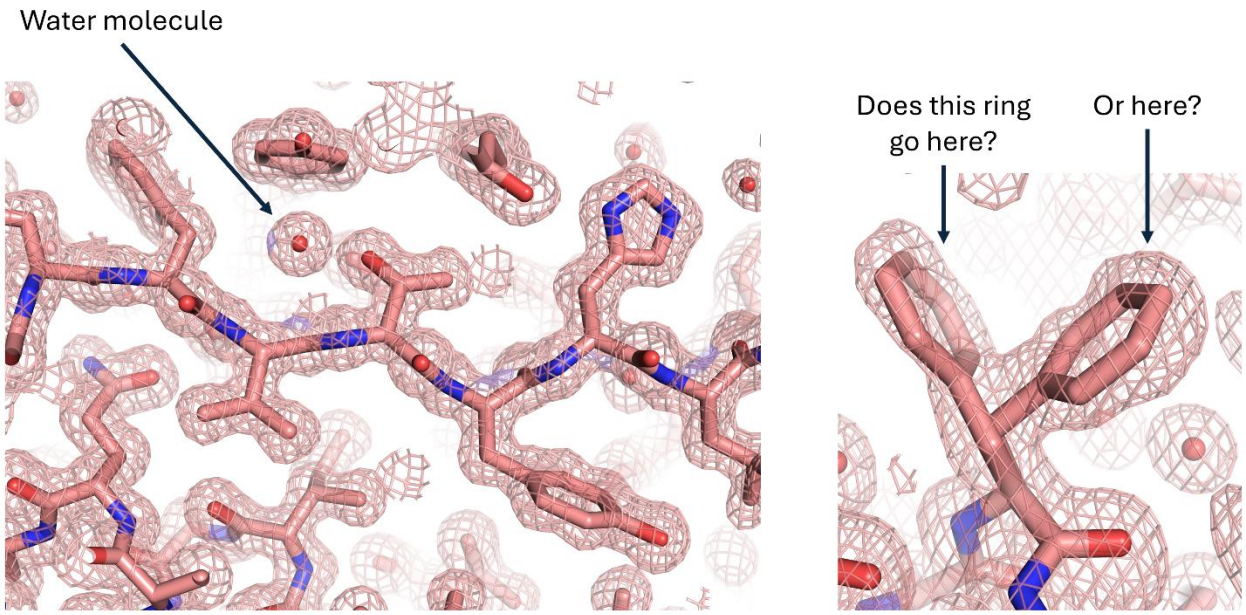

Figure 1.6. A protein model with electron density. To build the model, we fit the protein (sticks) into the density (mesh). Each stick segment represents a chemical bond, and each vertex is an atom. Carbon atoms are shown in pink, oxygen atoms in red, and nitrogen atoms in blue. Sometimes part of the structure adopts multiple positions (right).

The models of MrtR showed us precisely how the chemical signal fits into a pocket in the receptor and how the receptor's shape changes when it interacts with different chemical signals. These results help us understand the details of why different chemical signals change MrtR behavior in different ways. With this knowledge, we can predict how other chemical signals might affect the receptor and how changing the structure of the receptor itself might change its behavior.

1.3.2 If AlphaFold can generate a picture of a protein using machine learning, why do we need to go to all the trouble of collecting and processing experimental data?

AlphaFold represents a dramatic improvement in protein prediction technology, but it still struggles to predict how a protein's structure will change in different conditions, such as in the presence of different chemical signals. With our experimental data, we can compare the shape of the protein in active and inactive conformations. As protein prediction technology improves, it may be better able to predict how proteins change conformation in different conditions. These models are trained with experimental data, so additional experimental data will help improve the predictive capabilities of programs like AlphaFold. In the case of MrtR, the experimental structure of the protein bound to an activating chemical signal closely matched the AlphaFold prediction, but the experimental structure of MrtR bound to an inhibiting chemical signal did not (**Figure 1.7**). In addition, the predicted model was surprisingly different from experimental structures of similar proteins. Without experimental data to support the unusual structure, it would have been difficult to be confident in its validity.

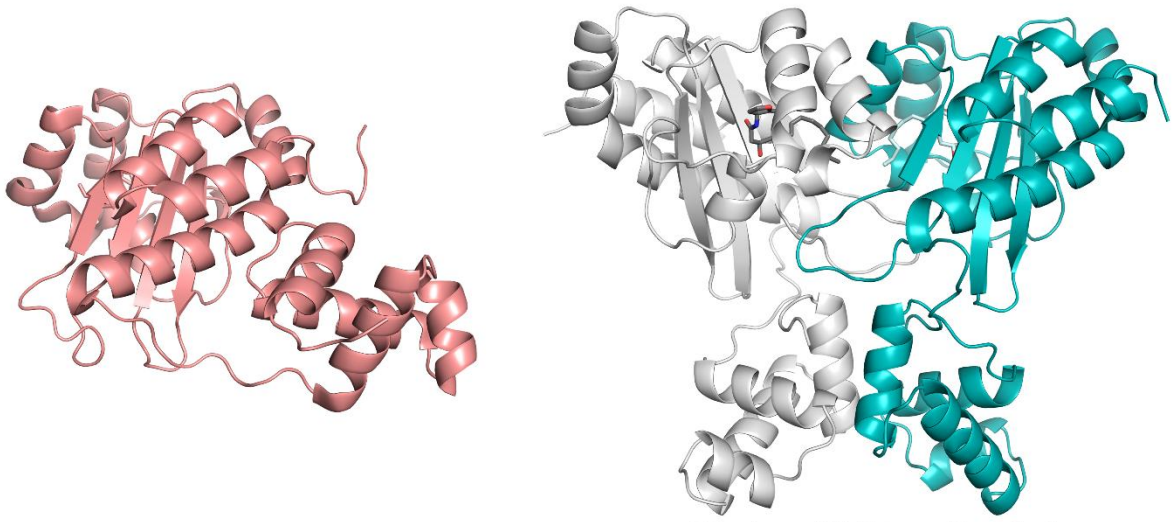

Figure 1.7. Model of MrtR with inhibitor or activator. The protein is shown as a cartoon to make it easier to see the overall shape. The inactive version (left) adopts a different shape than the active version (right).

1.4 Wrapping up and looking forward

Although there is still much to learn about how LuxR receptors work, not to mention thousands of predicted LuxR receptors that have yet to be characterized, my Ph.D. thesis work helps fill in a tiny piece of the puzzle. Someday, the molecular details I have uncovered about how these receptors work might help researchers better understand how bacteria interact with each other, how to design new chemicals to control bacterial behavior, or how to design new receptors to use in biotechnology applications.

Meanwhile, I am moving on to a different lab where I hope to master a new technique to study how proteins work. The basic sequence of a protein is encoded in an organism's DNA, but there are hundreds of tags and other alterations that can be added

to a protein to change its function. These modifications can be difficult to detect because some are short-lived or low in abundance, and a single protein could have many different combinations of modifications. By very precisely measuring the mass of a protein, then fragmenting it and measuring the masses of the pieces, it is possible to identify how and where a protein has been modified. I am excited to learn how to perform this technique and use it to study proteins with important jobs in human cells.