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UW-Madison Department of Chemistry
1101 University Avenue
Madison, WI 53706-1396
Contact: Prof. Bassam Z. Shakhashiri
bassam@chem.wisc.edu
www.scifun.org

May 2014

QUORUM SENSING INHIBITION IN *PSEUDOMONAS AERUGINOSA*: INVESTIGATIONS INTO MECHANISM AND RESISTANCE DEVELOPMENT

by

Joseph P. Gerdt

A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Chemistry)

at the

UNIVERSITY OF WISCONSIN-MADISON

2014

Date of final oral examination: 07/21/2014

The dissertation is approved by the following members of the Final Oral Committee:
Helen E. Blackwell, Professor, Chemistry
Samuel H. Gellman, Professor, Chemistry
Laura L. Kiessling, Professor, Chemistry and Biochemistry
Ronald T. Raines, Professor, Chemistry and Biochemistry
Douglas B. Weibel, Associate Professor, Chemistry and Biochemistry

CHAPTER 1:

BACTERIAL COMMUNICATION: WHAT IS IT? HOW DO THEY DO IT? AND WHAT HAVE WE LEARNED ABOUT IT?

1.1 Introduction

Bacteria are fascinating tiny organisms that have a tremendous impact on human lives and livelihood. These small organisms (or "microorganisms") are typically about 2–3 μm long. This length is almost a million times shorter than a human (on average 1.7 meters). Bacteria are often rod-shaped or spherical, but can also look like spirals and other shapes (see **Figure 1.1**). Just like humans, bacteria contain long chains (approximately 5 million base pairs) of deoxyribonucleic acid (DNA) that comprehensively encodes how a given bacterial cell should be constructed. This DNA instruction manual tells the bacterial cell how to make each of its thousands of proteins (large molecules that perform most tasks in a cell).

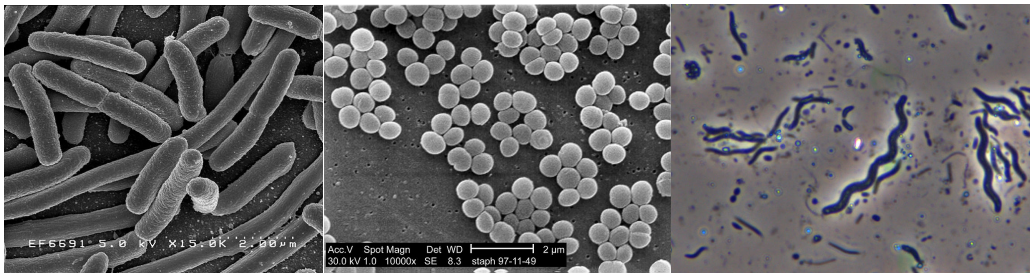

Figure 1.1. Microscopy images of bacteria.
(Left) *Escherichia coli* (i.e., *E. coli*) cells that are about 2.5 μm long. (Middle) *Staphylococcus aureus* cells that are about 0.5 μm in diameter. (Right) *Spirillum winogradskyi* cells that are about 3–4 μm long. All three images are public domain, accessed from Wikipedia.

Perhaps one of the most fascinating things about bacteria is how they can adapt to environmental changes. Imagine a bacterial cell as a self-sustaining factory of robots. It has a set of instructions that govern every aspect of how the factory should run (the DNA). And, it has thousands of different robots all with important functions (the proteins). Some robots maintain the physical structure of the factory. Some robots work to make sure all the other robots have fuel. Some robots make new robots as old ones break or as the factory determines that its output needs require a different collection of robots. Some robots get rid of waste or fix broken robots, and so

much more. Bacterial cells are similar—we need only replace the word "robot" with "protein" and the word "factory" with "cell" in the previous sentences to see that. Good factories must adapt to their situation. An ideal automobile factory would be able to make any vehicle possible. As demands change, perhaps the automobile factory should no longer make trucks, but instead make hybrid cars. In that case, an ideal factory would recognize this need, go to its instructions, and make robots that will break down the robots that are useful only for producing large trucks and use that material instead to make the robots needed to produce smaller hybrid cars. Many bacteria are incredibly adaptable, just like this ideal factory. As a few examples, they can adjust their set of proteins in order to feed on certain food sources when those are abundant, to secrete poisons (toxins) into the environment when they are around enemies, and to produce useful products to maintain a symbiotic relationship with a host organism.[1, 2]

Not only are bacteria fascinating in how they can respond to stimuli, but they are also incredibly important for human life (see **Table 1.1**). Most people are familiar with disease-causing bacterial pathogens, which are certainly significant to humans,[3] but bacteria intersect with our lives in many other ways. Unless we regularly brush our teeth, bacteria growing in our mouths produce acid that causes cavities.[4] Commensal bacteria on us and inside us help to digest food, train our immune system, and protect us from pathogens.[5, 6] Bacteria also play harmful and beneficial roles in agriculture and environmental maintenance. Plant pathogens cause disease that can wipe out crops,[7, 8] but other bacteria are essential players in collecting (or "fixing") nitrogen from the environment to help feed legumes and naturally fertilize soil.[2] Furthermore, bacteria break down dead organic material to return nutrients to the soil that eventually find their way through the food chain back to us.[9, 10] Not only do bacteria break down material to provide nutrients, but they can also be employed to clean up chemically contaminated natural environments in a process called bioremediation.[11, 12] Lastly, bacteria play significant roles in

industrial processes. For the pharmaceutical industry, many drugs are molecules that bacteria make for their own purposes and we have co-opted for our uses.[13] The food industry relies heavily on bacteria for the production of yogurt, cheese, and many other products.[14] However, industries also have to overcome challenges of bacterial contamination that spoils products and the formation of sessile bacterial colonies (or "biofilms") that clog and corrode pipes.[15] As we can see, bacteria are small but amazingly complex organisms that play key roles in practically every aspect of human life. Therefore, bacteria are certainly worthy of study to uncover their myriad functions and behaviors and leverage these discoveries for human well-being.

Table 1.1. Selected intersections of bacteria with human life.

Importance of bacteria	Reference
Cause pathogenic infections	3
Oral bacteria cause cavities	4
Improve digestive health and train immune system	5, 6
Destroy crops	7, 8
Fix nitrogen for legumes	2
Break down dead organic material	9, 10
Break down toxins in environment	11, 12
Synthesize life-saving drugs	13
Produce foods (e.g., yogurt and cheese)	14
Impede industrial processes	15

In the earlier days of microbiology, it was believed that each bacterial cell functioned on its own as an individual organism (i.e., each "factory" from the illustration above works on its own in isolation from other factories). Increasingly, we are instead finding that they often function as communities that are more productive when they cooperate with one another.[16, 17] In the case of troublesome bacteria, this "increased productivity" of communities could be a bad thing (for example, communities of bacteria form biofilms that are difficult to treat in human infections and on industrial equipment[15]). In the case of beneficial bacteria, however, the increased productivity makes them more helpful (for example, communal nodules of bacteria fix nitrogen in the roots of

legumes[2]). Therefore, within the study of bacteria, the sub-study of how bacteria function within communities has grown increasingly important.

This introductory chapter will address a few fundamental conceptual questions about bacterial communication. We will then shift to briefly explain three central questions in the study of bacterial communication that are addressed in the subsequent chapters of this thesis: (1) do bacteria of different species "speak the same language?"; (2) how can we artificially enter into the dialog?; and (3) can bacteria become resistant to our interference with their communication (as bacteria frequently become resistant to antibiotics)?

1.2 Fundamental Questions Regarding Bacterial Life in Communities

1.2.1 Why would bacteria live in communities?

Across many levels of living organisms, we frequently observe life in communities. At least two main reasons are useful to explain why living in communities can be more beneficial than living alone. First, division of labor can make a community of workers more efficient than each worker performing all the tasks without specialization. Division of labor was a key aspect of Adam Smith's revolutionary economic philosophies at the dawn of the industrial revolution,[18] but the concept is much older than that. Communities throughout history comprised people who specialized in farming, cooking, battling, raising children, or making useful tools.[19, 20] The comforts of our society today are, in part, due to the fact that we specialize at given tasks. An accountant doing excellent accounting, a farmer engaged in excellent farming, a manufacturer building excellent tools, a chef cooking delicious food, an entertainer providing exceptional enjoyment, a child care-provider providing nurturing care and education for children, and a physician providing excellent healthcare combine together for a richer and more secure existence than individuals building their own shelter, finding their own food, and healing their own

illnesses (**Figure 1.2A**). What may come as a surprise, however, is that humans are not alone in specialization of labor. When many animals mate, one parent gathers food while the other stays home to protect offspring. Even insects display clear division of labor: ants have queens and males for reproduction, and minor and major workers to perform the various tasks of maintaining the colony.[21]

A second clear advantage of communal living is that certain behaviors are only possible when performed in larger groups because a threshold strength is needed. The example of carrying a piano is illustrated in **Figure 1.2B**, but one could also consider protection from attackers or even workers unions gaining strength in numbers. Even if every member of a community is performing the same task, often a single member is not capable of completing the task on his/her own, and therefore a community is needed.

These two advantages of communal living are also displayed in bacteria. Some bacteria have been observed to benefit from division of labor. For example, many bacteria form miniature dandelion-like "fruiting body" structures in which some bacteria turn into spores (like the fluffy white seeds of a dandelion) and other bacteria form a sporulation stalk (like a dandelion stem) to hold the spores in the air for greater dispersal to distant fertile environments.[16] This division of labor of the bacteria, where some become spore cells and others become stalk cells, enables the bacteria to colonize distant environments that they could not otherwise reach as individuals (**Figure 1.2C**). Secondly many bacteria are known to require a threshold amount of strength to successfully complete a task. Often this manifests itself in the secretion of molecules that modify the environment around the bacteria (**Figure 1.2D**). For example, bacteria need the element iron in order to grow well, so if they are in an environment that has low levels of iron, they actually produce and secrete molecules called siderophores that will diffuse away, bind tightly to iron atoms, and if they passively diffuse back to the cell, the cell will import the siderophore-bound

iron. Since the siderophores can freely diffuse, this behavior is only beneficial if the bacteria are fairly dense in their environment. Otherwise, the siderophore bound to iron may never return to the cell. As an illustration, imagine we go fishing by shooting fish with darts, and after the fish has been shot, it floats to the surface. The problem is that the fish can float away from you quickly, so it is practically useless to shoot a fish when you are alone in a wavy ocean (like it is useless to try to lift a piano by yourself in **Figure 1.2B**). However, if you were in a tiny pond packed tightly with boats of other people, it is very productive for all of you to shoot fish. Even if the fish you shot floats away from you, a fish that someone else shot will float to you, so everyone should end up with fish. Therefore, the dense community of fishers enables the feeding of each person. Likewise, a dense community of bacteria enables the accession of iron via siderophores.

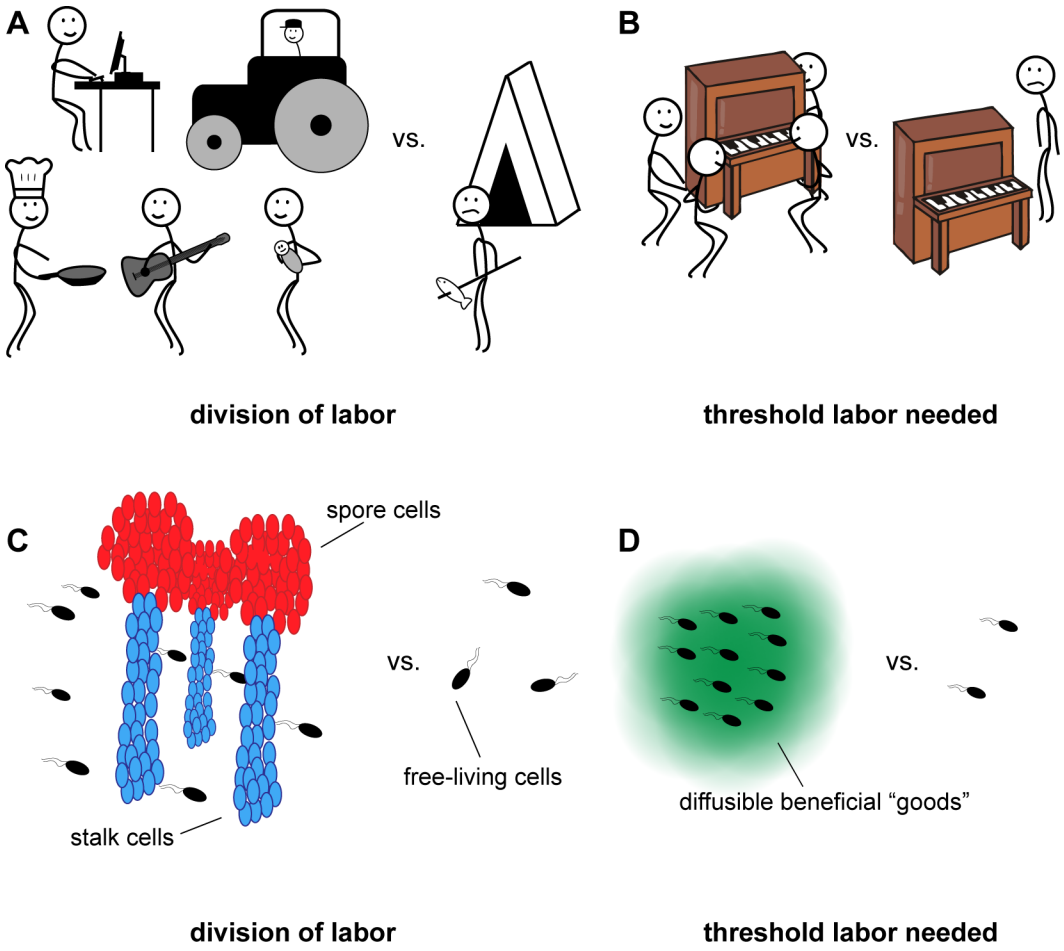

Figure 1.2. Benefits to living in communities.
(A) Humans benefit from communal living by adopting division of labor, which is more efficient than living as individuals. (B) Living in community also allows humans to perform tasks that require greater strength than an individual can muster (like lifting a heavy piano). (C) Bacteria likewise benefit from division of labor. Illustrated here are bacteria that differentiate into reproductive spore cells and non-reproductive stalk cells that hold the spore cells at a higher elevation to promote greater dissemination. (D) Bacteria also benefit from communal living by densely producing shared diffusible resources, like siderophores, which would be present at sub-functional concentrations if the bacterial density was low.

1.2.2 Why would communication be important?

We have established that communal living can have at least two major benefits for humans, bacteria, and other organisms. However, organisms are often capable of surviving on their own when necessary. They simply need to change the way that they behave. In every example in **Figure 1.2**, the organisms must discern whether to function as a community or not, and they use

communication to make this decision. For the case of division of labor, an accountant who is the lone survivor of a plane crash in the wilderness should not start crunching numbers—that would be a waste. Instead the accountant must alter his/her behavior to meet the necessities of life until he/she reaches a community again. Similarly, a few bacteria should not try to form a stalk when no dispersal spores are around. For the case of a necessary threshold strength, a single person should not try to lift a piano—he/she would waste energy and likely get hurt. Likewise, a single bacterium should not exert the metabolic cost to produce and secrete siderophores or other diffusible factors if the bacterium is not in a dense environment with other organisms that are also secreting these products. In all of these situations, the actors need to know if there are enough others around who are willing to cooperate in order to make their effort worthwhile. Communication is thus critical. Communication is the means by which the organisms can discern if sufficient cooperators are present to warrant community behavior. To borrow a word from governance proceedings, organisms use communication to determine if they have reached a "quorum" before acting. Hence this process in bacteria is called "quorum sensing."[22]

1.2.3 How do bacteria communicate?

Communication methods that enable humans to decide whether community behavior is prudent involve our five senses (**Figure 1.3**). We hear sounds and we see images to determine if other people are present, if they seem willing to work together with us, and if they have a good plan. Simpler organisms appear to rely more on other senses, such as smell and taste. For example, insects leave chemical trails that attract one another.[21] Smell and taste are analogous to the mechanism by which bacteria sense their quorum. Our noses and mouths have special nerve cells that contain receptor proteins, which each selectively bind specific odor and flavor molecules (like the malodor and perfume molecules shown in the right panel of **Figure 1.3A**).

Upon binding its specific molecule, a receptor protein changes shape and causes the nerve cell to send a signal to the brain indicating the presence of that specific odor or taste molecule (like a lock is selectively activated by a specific key, **Figure 1.3B**). Bacteria also have proteins that are selectively bound and activated by molecules that enable them to "smell" or "taste" the presence of other bacteria. If many bacteria are present, a high concentration of "scent" molecules is present, and thus the bacteria "know" they should cooperate (**Figure 1.3C**). A memorable analogy of how bacteria communicate their density is to consider blindfolding and gagging sweaty people and asking them to pick up a piano. A group of lifters will smell each other and know they should be able to lift the piano, but a lonely lifter will realize from the lack of odor that he/she should wait for more help to arrive. Likewise, if bacteria "smell" a quorum density of sibling bacteria, they "know" it's worth secreting costly, but beneficial, diffusible nutrient-acquiring factors.

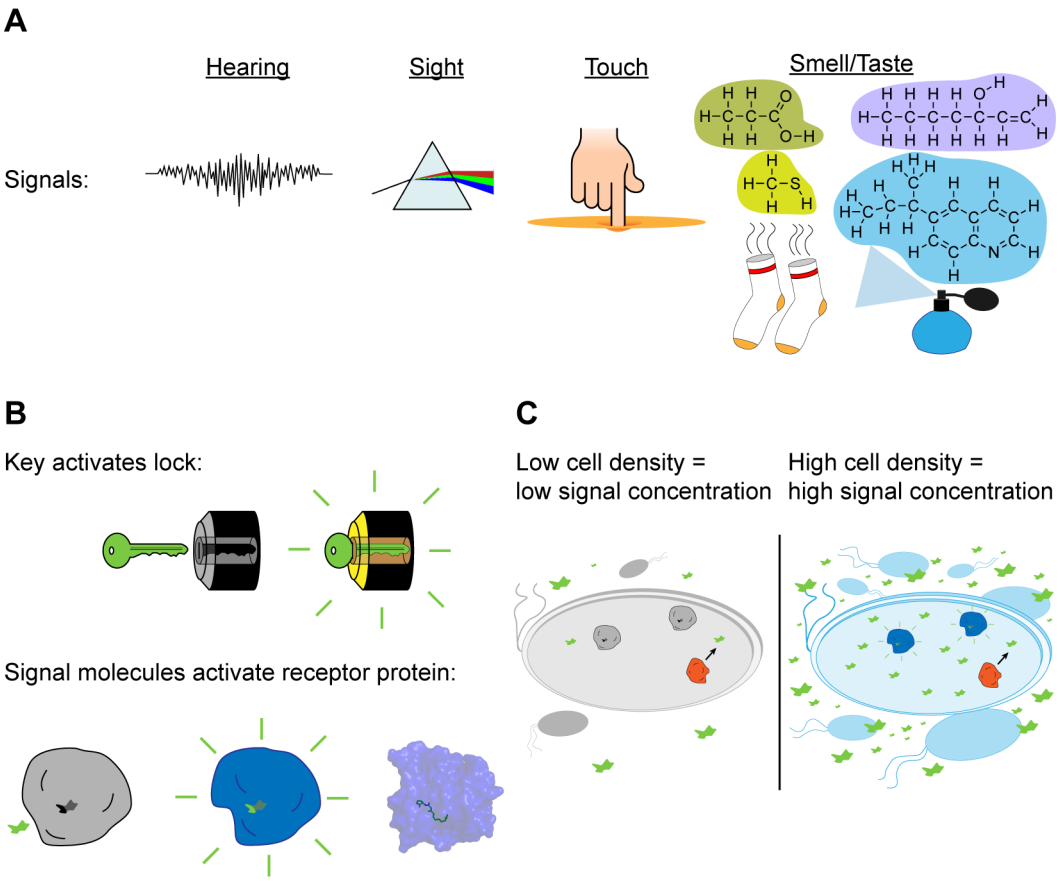

Figure 1.3. Mechanisms of perceiving communication signals.
(A) Humans perceive communication signals by hearing sound waves, seeing electromagnetic waves, feeling physical forces, and smelling and tasting specific molecules. The two molecules above the socks are malodors that resemble body odor and decaying matter, whereas the molecules above the perfume bottle are scents that resemble lavender and wood. (B) Molecules are smelled and tasted by humans, bacteria, and other organisms via specific receptor proteins that are activated when they bind their cognate signal molecule (like a lock is activated when it binds the correct key). To the right is a real molecular-resolution picture of a receptor protein (blue, LasR from *Pseudomonas aeruginosa*) bound to its signal molecule (green, *N*-(3-oxo)-dodecanoyl L-homoserine lactone). (C) Illustration of bacterial quorum sensing. A protein in bacteria (orange) synthesizes signal molecules (green), whose concentration correlates to the bacterial density. When the bacterial density reaches a threshold, the signal (or "scent") concentration is high enough to bind to and activate their receptor proteins (grey to blue), which causes the bacteria to change behavior.

1.3 Specific Research Questions

1.3.1 Do bacteria of different species "speak the same language?"

Many different kinds of bacteria exist, so how do they sense if they are surrounded by their siblings or by others (possibly even ones that could hurt them)? The answer is relatively simple:

different species make different molecules and respond primarily to their unique molecule (like humans not understanding a dog's barks, or more subtly, humans not understanding different languages). Interestingly, some bacteria respond to their own signals *and* also those produced by others, but other bacteria only sense and respond to their own.[23-28] An analogy is my brother only understands English, my sister-in-law is fluent in English and Spanish, and my friend regularly communicates in English, Malay, Korean, and three very different dialects of Chinese. Different bacterial species likewise display varying degrees of multilingual fluency (**Figure 1.4**). We were able to develop a model to explain how some bacteria distinguish between the different signals and others do not. Furthermore, we proposed a method for predicting if less studied species will be "multilingual" or not. This work is presented in **Chapter 6**.

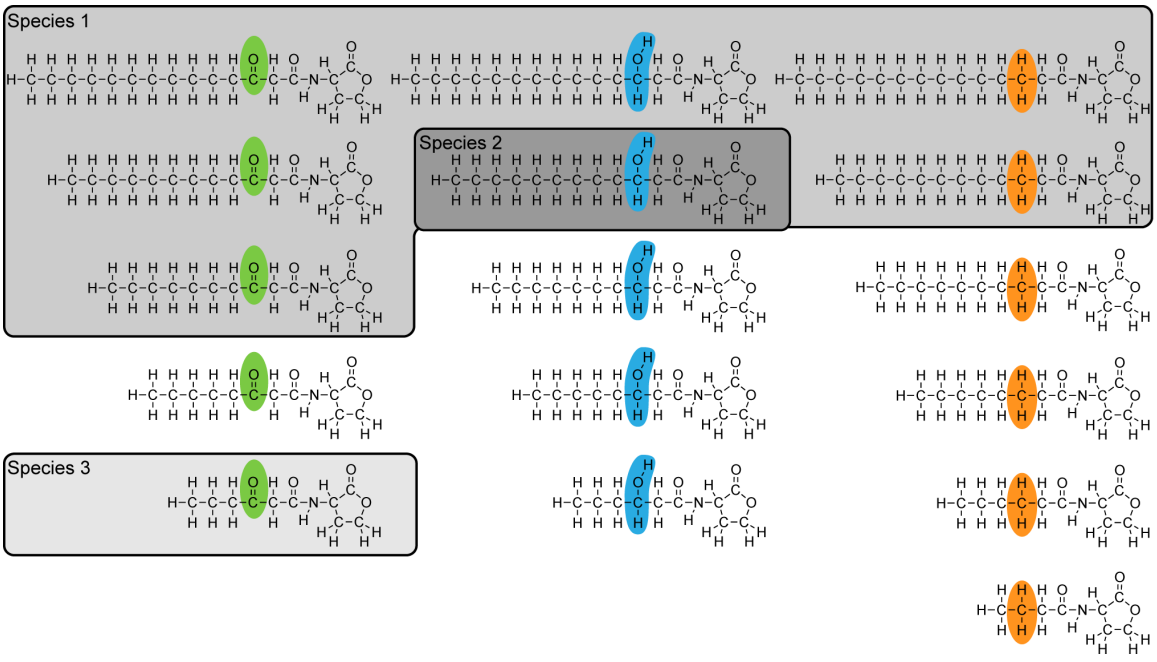

Figure 1.4. Examples of chemical "languages" used by bacteria.
Several signaling molecules are displayed that vary in length and in composition at the circled position. Species 1 is "multilingual" in that it can perceive many different signal molecules, but the two other species can only recognize one signal (i.e., they are "monolingual").

Interestingly, there are important benefits and harms to responding to signals from other organisms (**Figure 1.5**). Bacteria can have three different responses to signals from another organism: (1) respond to the alternative signal as if it were their own signal and perform cooperative activity, (2) ignore the signals made by others and only perform the cooperative activity if enough identical members are around, and (3) be inhibited by the signal made by another organism and stop the cooperative activity—even if a quorum of identical members are around. Each of these cases can be beneficial in certain scenarios (**Figure 1.5**). If the other bacterium is the same species or a closely related one that will cooperate, then it is good to respond to its signal positively and work together—like multilingual people who work together to lift a piano. If the other bacterium is a different species that has a neutral impact, then it is best for the initial bacterium to simply ignore the neutral bacterium's signal and only perform the cooperative behavior if there is a quorum of cooperative partners. In the piano-lifting example, this would be like having some calm, friendly dogs around—they will not help lift the piano, but they also should not be too much of an impediment to piano lifting, so its best to just ignore them. Lastly, if the neighbors will actually have a detrimental impact on the initial bacteria, the bacteria should respond in a negative manner to this competing neighbor. Even if they have sufficient numbers to perform their task, they should not do it. This case is akin to having sufficient help to lift a piano but then noticing a ferocious bear nearby. Even though you can lift the piano, it is better to leave the piano and run away. Since we see bacteria that respond positively, negatively, and neutrally toward signals from other bacteria, we suspect that the bacteria have evolved to respond properly to "good," "neutral," and "bad" neighbors. There is likely much more to discover about communication between different bacterial species, as we are just beginning to identify the complex mixtures of bacterial species that live together in varied environments (e.g., in our digestive systems, in the soil, and even deep in the sea[29]). Our work will help lay a

foundation for characterizing how these complex mixtures of organisms communicate with each other.

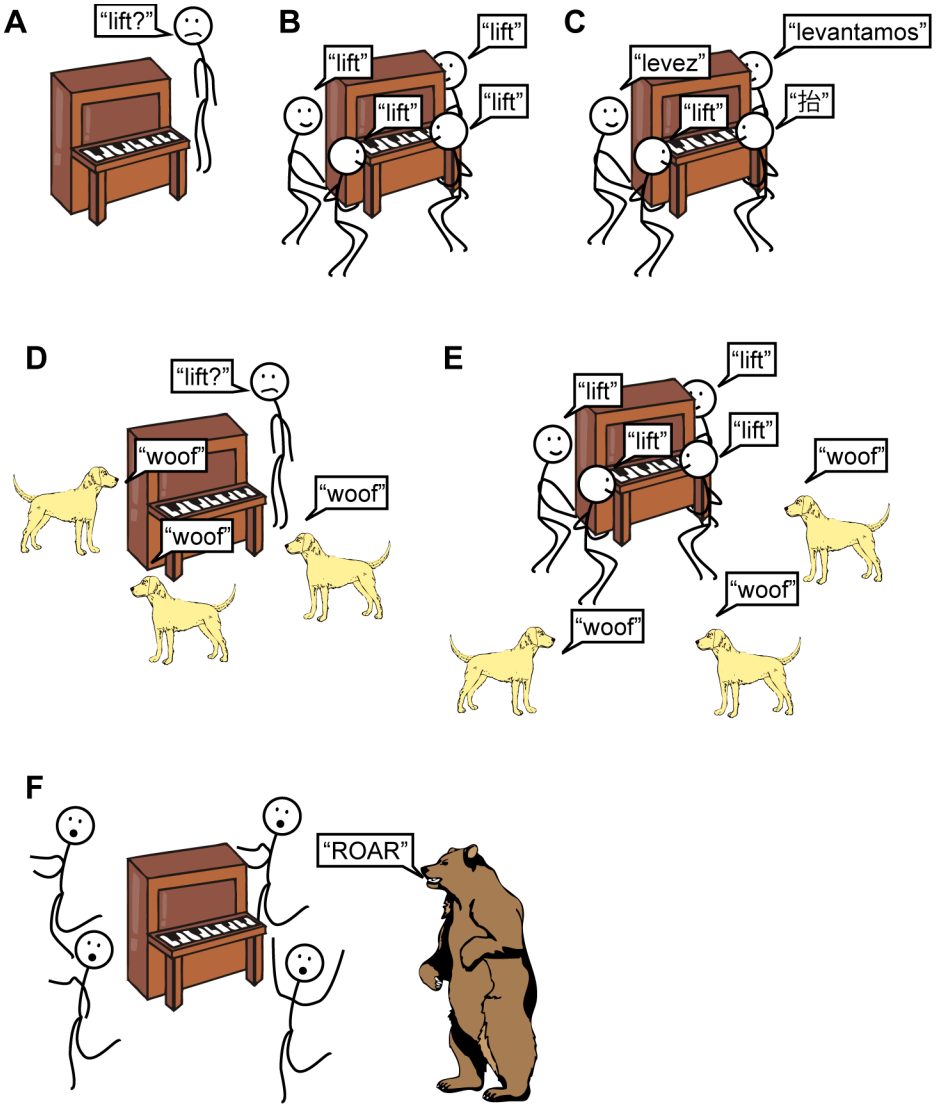

Figure 1.5. Examples of healthy responses to different types of neighbors.
(A) When alone, it is best to not waste energy lifting a piano. (B,C) When with a quorum of cooperative partners (whether they speak identical languages or not), it is possible to successfully lift a piano. (D) When surrounded by only unhelpful partners, it is best to not waste energy lifting a piano. (E) When a quorum of cooperative partners and several unhelpful (but harmless) partners are present, it is best to ignore the unhelpful partners and lift the piano. (F) When a harmful partner is present, it is best to not lift the piano (but instead focus on protection), even if a quorum of cooperative lifters is present.

1.3.2 How can we artificially enter into the dialog?

As stated above, bacteria communicate via quorum sensing in order to determine whether to perform communal behavior that often has harmful or beneficial impacts on humans (e.g., harmful biofilms that are difficult to clear from infections and industrial equipment or beneficial nitrogen-fixing nodules on legume roots). If bacteria used speech to communicate, we would try to find the right frequencies of sound to prevent them from hearing each other. If bacteria used vision to see each other, we might try to blind them with light. However, since bacteria communicate using chemical signals that bind selectively to receptor proteins, we can design specific molecules to bind to the receptor proteins to disrupt their communication. To illustrate, we return briefly to the analogy of sweaty, blindfolded, and gagged people who are asked to lift a piano. They are willing to lift the piano when they smell many others because they trust that together they can succeed. However, if we spray strong air freshener in the room, the people will no longer realize that they have a quorum that is sufficient to lift the piano, so they will leave it resting on the ground. This illustration is bizarre, but on a molecular level, it is not too different from our efforts to inhibit bacterial quorum sensing. Smell occurs when an odor molecule binds to a scent receptor protein in the nose (like a key fits into a lock) and activates the protein to send a signal to the brain. Air fresheners can function by binding a receptor protein slightly differently in a way that blocks it from binding the scent molecule, but does not activate the receptor protein (like an almost correct key getting stuck in a lock). We seek the same goal of blocking receptor proteins in bacteria without activating them (**Figure 1.6A**). Instead of preventing sweaty people from lifting a piano, however, our laboratory's main focus has been on preventing pathogenic bacteria from forming biofilms, migrating to infect new tissues, and producing toxins that destroy host cells.

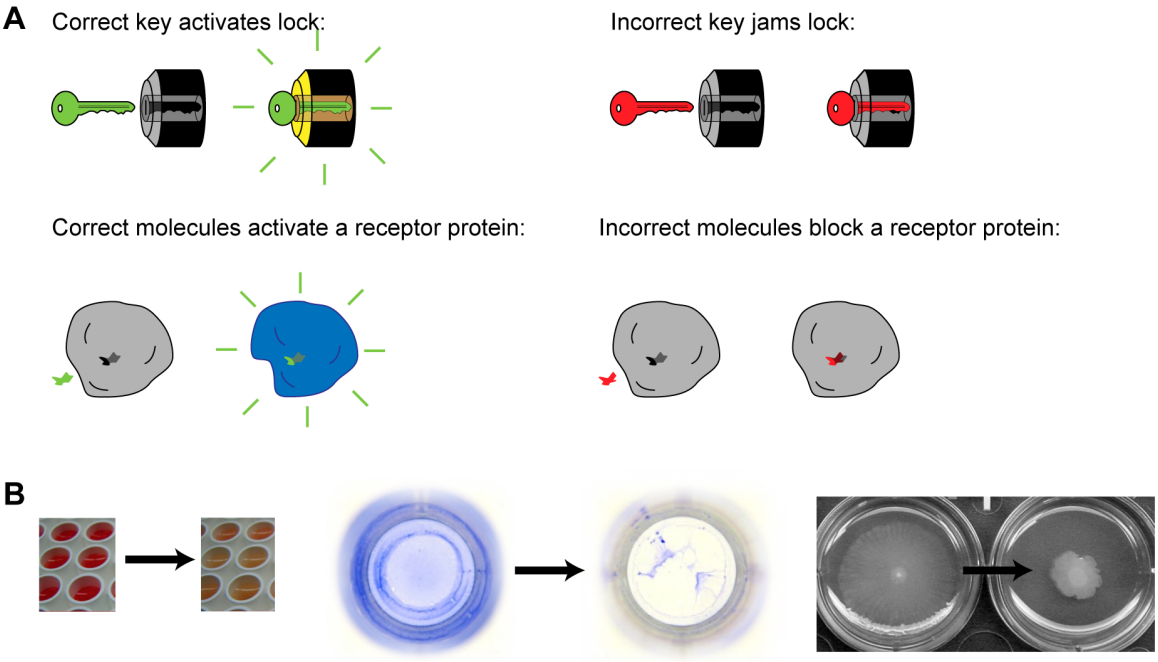

Figure 1.6. Blocking quorum sensing receptor proteins inhibits virulence of pathogens.
(A) Bacteria sense that they have a quorum density when signal molecules (green) bind to and activate receptor proteins (grey to blue) like a key binds to and activates a lock. Just like an incorrect key can fit in a lock and jam it in an inactive state, alternative molecules (red) can be synthesized that block a receptor protein from binding its signal and therefore prevent activation. (B) Images of bacterial virulence prevented by addition of quorum sensing inhibitors (left to right): lysis of red blood cells by *S. aureus* (image from Tal-gan, et al.[30]), biofilm formation by *P. aeruginosa* (image from Frei, et al.[31]), and swarming motility by *A. baumannii* (image from Stacy, et al.[32]).

As **Figure 1.6B** shows, our laboratory has successfully inhibited quorum sensing in multiple bacteria to prevent virulent behaviors (toxin production by *S. aureus* in the left image, biofilm formation by *P. aeruginosa* in the middle image, and swarming motility by *Acinetobacter baumannii* in the right image). However, many of our molecules that inhibit quorum sensing have drawbacks. Most are not very stable and therefore block communication for only a short period of time.[33-35] Other molecules are not very potent,[36] and therefore would require unreasonably large amounts of material to be used in real-life applications outside of controlled laboratory conditions. Also, for any treatment of disease in animals or plants, molecules must be found that cause no appreciable harm to these host organisms. All of these challenges provide the

opportunity to develop improved inhibitors of quorum sensing. To develop improved inhibitors, a better understanding of how the current ones function and their shortcomings is crucial.

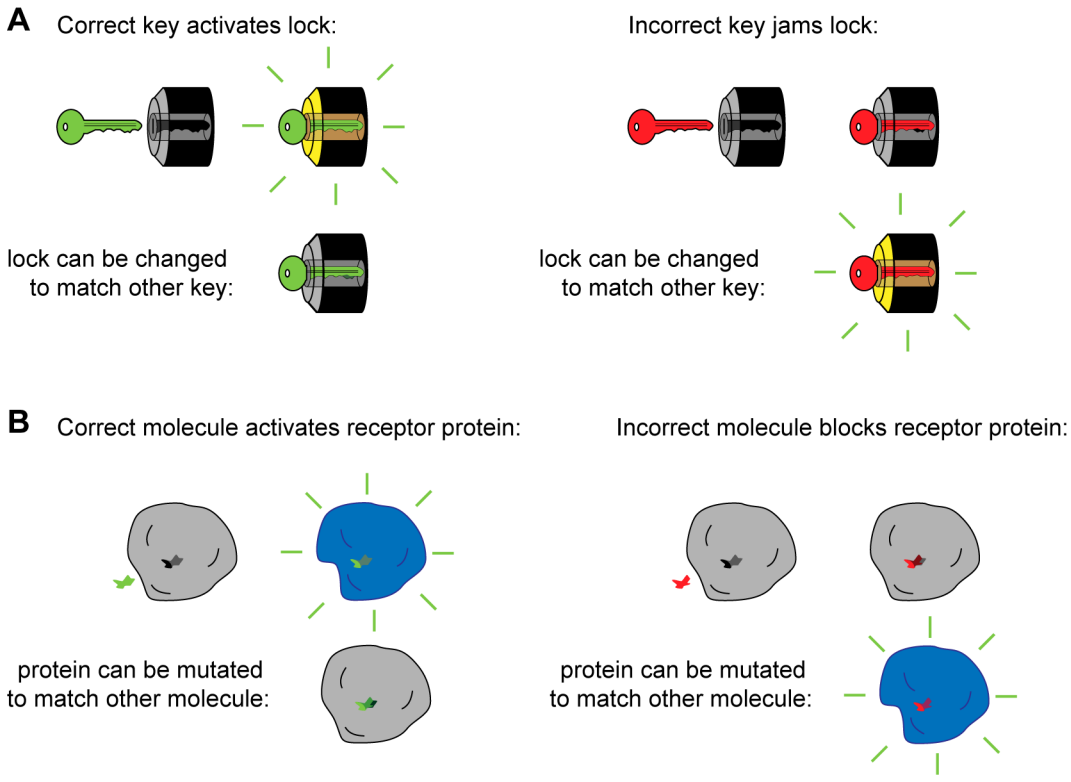

Figure 1.7. Mutation of quorum sensing receptors can allow activation by an inhibitor. (A) If an incorrect key jams a lock, the lock could theoretically be altered to properly fit this "incorrect" key and instead be activated by it. (B) Similarly, receptor proteins can be mutated to accommodate for different signal molecules and actually be activated by molecules that used to be inhibitors and be inhibited by molecules that used to be activators.

In **Chapters 3** and **4** of this thesis, we discovered why certain molecules are good at inhibiting quorum sensing and why other molecules are good at actually mimicking the natural communication signals to activate quorum sensing (i.e., function as proper keys that activate the lock). Three major techniques allowed us to make these discoveries. First, we studied 3-dimensional pictures that show how the receptor protein binds its signal molecule (like seeing a blueprint of a lock with the proper key inside of it). These images are the result of X-ray

crystallography, which is a challenging process of forming a crystal of the protein of interest (not unlike crystals of salt or sugar) and then shooting X-rays at the crystal and analyzing the pattern formed by the X-rays when they exit the crystal. However, on its own, this method neither tells us which aspects of the signal molecule are most important for activating the receptor protein (like certain peaks and valleys on a key could be more important than others), nor does it tell us what an ideal inhibitor would look like. To address those questions, we took a second approach and synthesized a set of different molecules that are similar to the signal but are subtly different in ways that could prevent activation (like making several keys that miss a certain peak or valley present in the true key). We then tested the activity of the molecules, and saw some that completely lost activity. These molecules likely cannot bind the protein, like a key that will not even fit in the lock. We also observed molecules that still activate the protein well, which indicate that the altered portion is not very important for binding and activating the protein. Lastly, we observed molecules that block the activity of the protein. These molecules likely bind the protein, but do not activate it, like a wrong key stuck in a lock. These are the communication blockers (or "quorum sensing inhibitors") that can prevent the harmful behavior of pathogens. The third technique used to confirm that certain aspects of a molecule-protein interaction are important for activating or blocking the protein was as follows: we modified (or "mutated") the protein and observed how those mutations changed the ability of the molecule to activate or inhibit the protein. We discovered mutations to the protein that cause it to be *inhibited* by a molecule that *activates* the original protein, and we found mutations that cause the protein to be *activated* by a molecule that *inhibits* the original protein (**Figure 1.7**). Therefore, we confirmed that those molecule-protein interactions (like interactions between a lock and specific ridges on a key) are important for activation and inhibition. By combining all three of these techniques (investigating structural images, testing different molecules for protein activation and inhibition, and testing the

activation and inhibition of mutated proteins), we discovered many crucial interactions that determine activation and inhibition of a quorum-sensing receptor protein in the pathogenic bacterium *P. aeruginosa*.

In **Chapter 5** of this thesis, we investigated another factor that determines whether a quorum sensing inhibitor will be active. Many of these communication-blocking molecules need to enter inside the bacterial cell in order to carry out their function, and unfortunately, we found that many of these molecules are excreted or "effluxed" (i.e., spit out) of the cell before they can perform their function. This situation is akin to giving a baby medicine. It could be an amazing medicine that will completely heal the baby, but if the baby keeps spitting it out, the medicine will not stay in the baby long enough to work. Although most quorum sensing inhibitors were strongly effluxed from the pathogenic bacterium *P. aeruginosa*, we found that some molecules were effluxed far less than others and therefore should be more active. The clear next step is to find molecules that are *both* very good at blocking the receptor *and* stay inside the cell. Other researchers in our laboratory are continuing this work to develop optimized quorum sensing inhibitors that exhibit both of these critical traits.

1.3.3 Can bacteria become resistant to our interference with their communication?

In the future, the interference of bacterial communication might be used to control infections in humans and plants, and could also be used to prevent biofilms in industry. If they see widespread use, the question arises of how bacterial populations will respond to these treatments over time. The pervasive use of antibiotics has caused antibiotic resistance to arise and spread through bacterial populations rapidly (See **Figure 1.8A**). Would resistance likely arise and quickly spread in the case of quorum sensing inhibition? This is an important research question for the field.

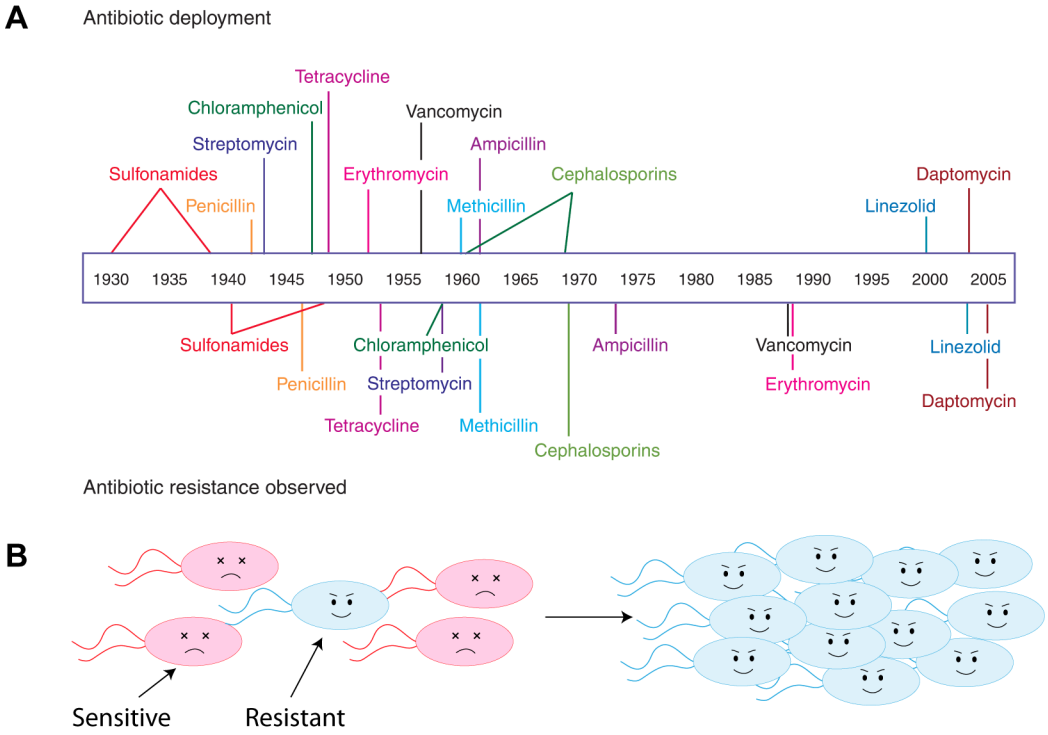

Figure 1.8. Spread of resistance to antibiotics.
(A) Diagram demonstrating the quickness with which resistance to new antibiotics has been observed (image from Clatworthy and Hung[37]). (B) Antibiotic resistance spreads quickly because only the resistant bacteria (blue) survive and reproduce.

When we consider antibiotics, the cause of their rapid spread is very clear. Antibiotics prevent the growth of all the bacteria except the resistant ones, so only the resistant bacteria multiply and eventually take over their local population and then disseminate widely (**Figure 1.8B**). Many different mechanisms of resistance exist: bacteria can start strongly effluxing antibiotics, the proteins that are blocked by the antibiotics can mutate to no longer be blocked, or the bacteria can even develop the ability to degrade the antibiotics. Acquiring a resistance mechanism is a very rare occurrence, so resistant bacteria are initially present at extremely low levels. However, because only the resistant ones can grow, that tiny fraction of resistant bacteria quickly overtakes the entire population.

We expect bacteria to also gain resistance mechanisms (at a very low rate) against molecules that inhibit quorum sensing. However, after quorum sensing inhibitor resistant bacteria arise, they would need to outcompete the non-resistant bacteria in order for resistance to spread. Unlike antibiotic resistant bacteria (which spread readily), we did not expect the quorum-sensing inhibitor resistant bacteria to spread. The experiments in **Chapter 2** demonstrate two obstacles that prevent the spread of resistance to these inhibitors of bacterial communication (**Figure 1.9**). The first obstacle is due to the fact that resistance should initially arise at low levels. If only a single bacterium is resistant, all the other bacteria are still blocked from communicating. This communication blocking not only prevents the other bacteria from sensing each other's signals, but it also prevents them from making signal molecules. So, although the single resistant bacterium is capable of sensing other bacteria, insufficient signal is present to activate its receptor proteins. As an illustration, this scenario is akin to blindfolded and gagged people being asked to lift a piano, and all but one of the people are incapable of smelling *and producing* body odor. Even though there are sufficient people present to lift the piano, and even though one "resistant" person can smell, he/she does not know there are enough others to lift because they are not producing odor.

The second obstacle to the spread of resistance is that the behaviors regulated by communication are often communal behaviors that are susceptible to "cheating." If one child brings a lunch to a room of hungry children and then leaves to use the restroom, it is quite possible that he/she will not have much of a lunch remaining upon returning to the room. Similarly, if a single bacterium is resistant and can now secrete siderophores into the environment, these siderophores bind iron ions, but then freely diffuse and can be taken up by other bacteria. Therefore, the resistant bacterium incurs a cost to make the siderophores, but only gets a fraction of the beneficial iron; whereas the neighboring communication-blocked bacteria

get iron without any cost. Thus, *the resistant bacteria can actually be in worse shape* than the bacteria that are still sensitive to the quorum sensing inhibition—the opposite of the case of traditional antibiotics. Experiments reported in **Chapter 2**, in which we grew mutants of the pathogen *P. aeruginosa* together and observed which one outcompetes the other, provided strong empirical support for the existence of these two barriers.

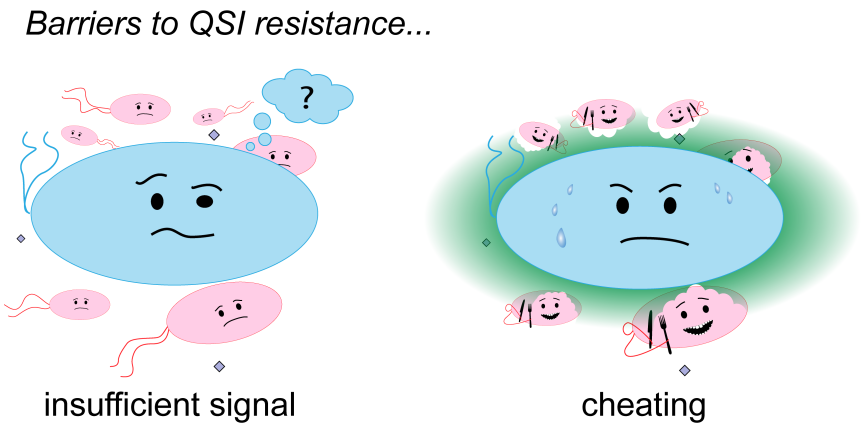

Figure 1.9. Obstacles that prevent the spread of resistance to quorum sensing inhibition (QSI). (Left) When only a few resistant bacteria (blue) are present, they cannot produce enough signal (green diamonds) to induce a quorum sensing response. (Right) When the quorum sensing response of a resistant bacterium does turn on, it secretes shared resources (green halo) that benefit non-resistant bacteria around it. Since the non-resistant bacteria benefit but have no cost to produce the resources, they are termed "cheaters." The cheaters can be more fit than the resistant bacterium, and they prevent the spread of the resistant one.

1.4 Summary

- Bacteria often live in communities and primitively communicate to regulate behavior.
- Different bacteria use different chemical "languages"—some bacteria are multilingual.
- Bacterial communication regulates behavior that has beneficial and harmful impacts on humans.
- By artificially entering into the dialog, humans can control bacterial behavior for our benefit –for example, developing infection treatments that are robust against the spread of resistance.

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