Recurring Microbial Infections: Recrudescence – The Persister State

Mum: teacher told us today microbes can sometimes survive a course of antibiotics. How do they do that?

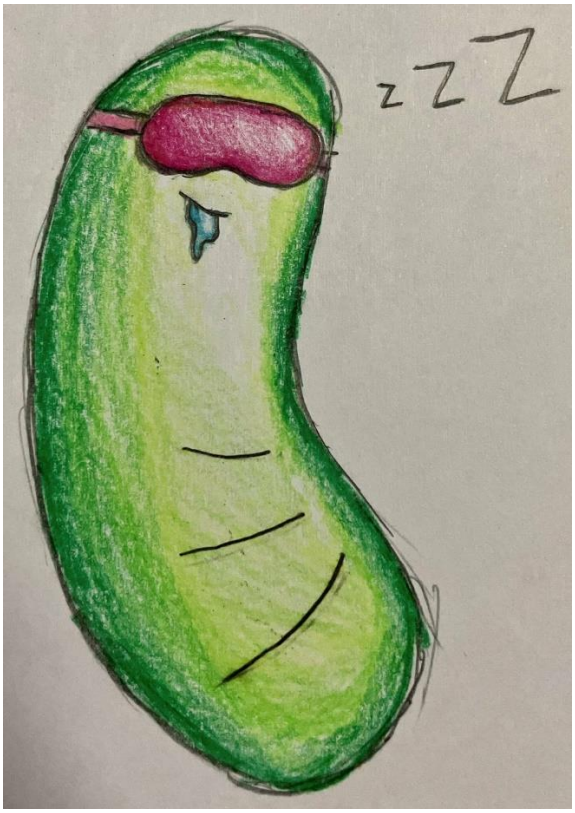

Illustration by S. N. Wood

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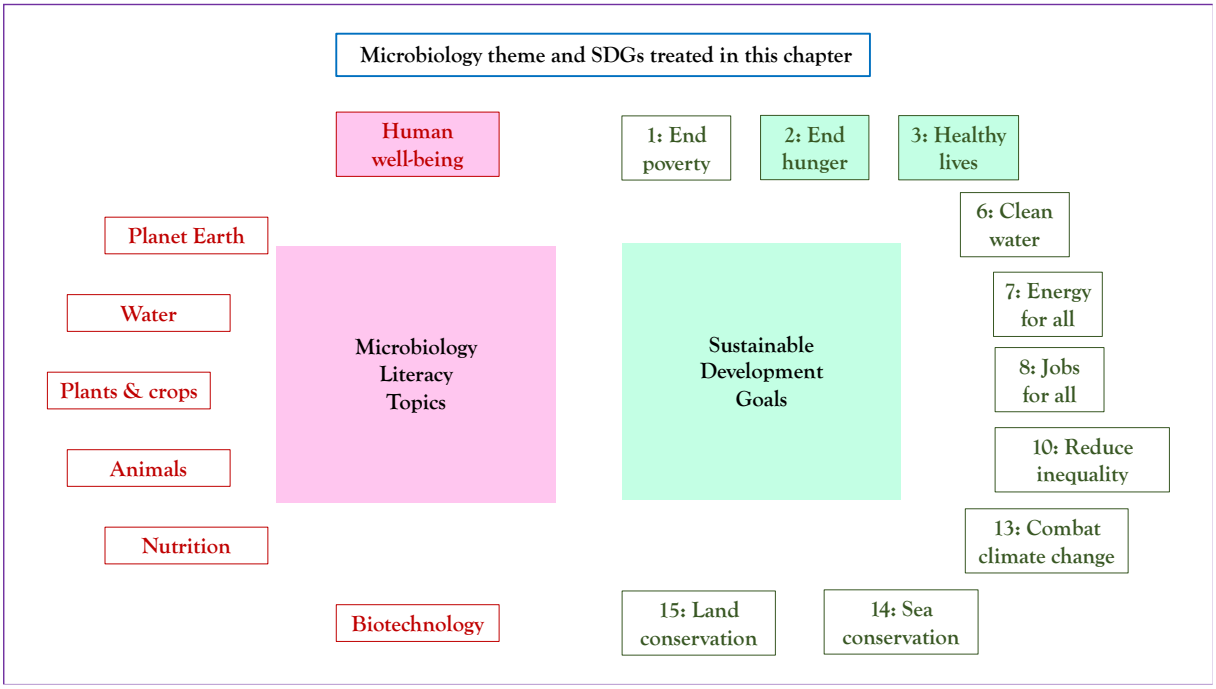
Recrudescence: The Persister State

Storyline

Most microorganisms are beneficial, but some, known as pathogens, don't have our best interests in mind and cause infections. Antibiotics, like penicillin, were discovered in the 1920s, and are a powerful means to treat bacterial infections; the lives of millions of people have been saved. However, currently, over a million people a year die from microbial infections all over the world, making bacterial infections the main cause of death. This illustrates that we do not have enough tools to always control infections. There are several reasons microorganisms survive antibiotic treatment including resistance, where the bacteria undergo changes in their DNA to thwart the antimicrobials, and persistence, where the bacteria become metabolically inactive (i.e., sleep). Because antibiotics inhibit metabolic activities, most can only kill actively-growing microbes, so inactive ones usually survive the treatments. Notably, all microorganisms can enter a dormant state and sleeping pathogens can subsequently wake up and restart infections. Therefore, for human health, it is important to understand persistence and to be able to treat bacteria if they are hiding in this dormant state. This requires that we distinguish resistance from persistence and identify new compounds that are effective against persistent microorganisms.

The Microbiology and Societal Context

The microbiology: microbial pathogen survival after antimicrobial treatment; infection; antibiotic resistance; microbial response to environmental stress; dormancy; stress signaling through alarmones. *And, peripherally for completeness of the storyline*: food safety; human cancer treatments. *Sustainability issues:* health; food.



Recrudescence: the Microbiology

1. ***What is recrudescence?*** Recrudescence is an undesirable thing, like a toothache, that happens repeatedly. Unfortunately, regarding human health, microbial infections can reoccur since the underlying cause, microorganisms, may not be eradicated after an antibiotic treatment. Examples include recurring ear infections in small children and urinary tract infections in adults.

2. ***Resistance vs. Persistence.*** The main difference between resistance and persistence is that resistant cells have mutated (i.e., changed their DNA) to survive antimicrobial treatment, whereas the persister cells have no changes in their DNA. Also, resistant microorganisms are immune to the antibiotic and can grow in their presence. In contrast, persistent microorganisms do not grow in the presence of antibiotics.

a. *Resistance.* The changes in DNA that lead to resistance may also take the form of genes added to the bacterial DNA; these new genes encode enzymes that destroy the antibiotic. An example is some bacteria obtain the enzyme penicillinase to destroy the first antibiotic, penicillin. Or, the changes in the DNA may activate bacterial pumps that remove the antibiotic faster than it gets into the microbes (like using a bucket to remove water faster than it enters a boat with a small hole in it). In addition, the changes in the DNA may alter the nano-machines in the cell that make proteins – the ribosomes – such that they are not affected by the antibiotic, which often target ribosomes. Hence, the DNA of the microbe is changed, rendering the antibiotics ineffective.

An important lesson here is that whenever we add compounds, like antibiotics, that reduce the growth of microorganisms, they will find a way to grow in a different way, because there are just so many of them and they are always changing their DNA to adapt to new conditions and chemicals. So, their high numbers favor the microorganisms, not our antibiotics. Such is the way of life for microbes where faster growth is handsomely rewarded!

b. *Persistence.* In contrast, persister cells survive antibiotic treatment without changing their DNA. Instead, they just stop growing, becoming metabolically dormant, and wait for a better time to grow, when conditions are more favorable. So persister cells are incredibly patient (up to 100 million years!) and wait until the antibiotic stress is over. Persister cells stop growing by shutting down their protein-making machinery, their ribosomes. Since you, me, and microorganisms are mostly protein, stopping protein production stops growth.

Notably, persister cells are an extremely small fraction of the total microorganism population and usually have numbers far less than 1% of the total population. So, the vast majority of cells die when antibiotics are applied (unless the microorganisms are resistant) and only a small number of persister cells survive treatment.

This protective response of the persister state is universal in that it has been found in all bacteria tested. Moreover, it is a general stress response since it not only holds for the stress created by antibiotics but also holds for other stresses, too, like the stress created by a lack of nutrients or too much heat or attack by our immune system. Since all microorganisms experience a lack food sometimes, and live in a stressful world, this persister state is arguably more important than the state of rapid growth. Therefore, it behooves us to understand it.

3. ***Formation of persister cells.*** All forms of life, from bacteria to trees, have the ability to shut down their ribosomes by putting two of them together to make a dimer that is protected during the stress created by the antibiotic treatment or lack of nutrients, etc. This ribosome doublet puts the two ribosomes into a state of hibernation, like hibernating bears (if two of them were to hug each other while they sleep in the winter!). When conditions are favorable, special enzymes convert the ribosome doublet back into two robust protein-making machines, so that

when the microorganism wakes, it has the nanomachines already assembled and is ready to make the most important commodity, protein.

a. *Alarmones.* For microorganisms, external stress, like the presence of antibiotics, is communicated to the inside of the cell using special signals, which are RNA-building blocks that are decorated with phosphate known as alarmones. These phosphorylated bits of RNA turn on enzymes that stop individual ribosomes from working and combine the ribosomes pairwise to hibernate them. Alarmones are the equivalent of fire alarms for us: the classroom may be a hive of activity one minute, but when a fire alarm goes off, it quickly empties and no longer has any activity. The energy currency of the cell, ATP, is also reduced to make doubly sure that protein is not synthesized since making protein requires large amounts of energy in the form of ATP.

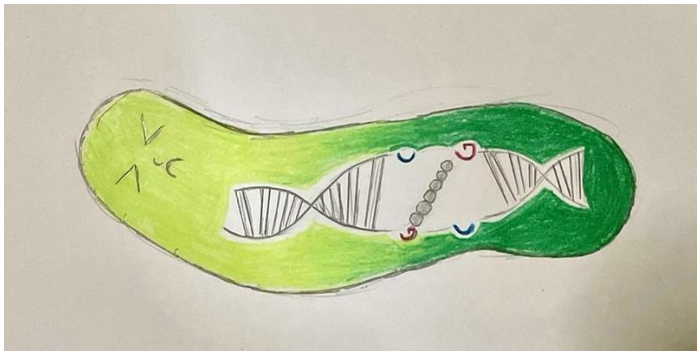
4. ***Resuscitation of persister cells.*** Persister cells do not wake from their slumber spontaneously. Instead, they wake when the stress, like antibiotics, is removed and nutrients are present. In effect, the process of persister cell formation is reversed: the concentration of the alarmones is reduced (the fire alarm stops), the enzymes that hibernate ribosomes are switched off, and a new enzyme is activated that converts the dimerized ribosomes into two active ribosomes that are ready to make protein.

The nutrients are sensed by proteins on the outside of the cells that are used for importing compounds like sugars and that are used by the cell to move toward food. So as the nutrients wake the sleeping cells, they begin to move to the food source. And like you after a slumber, waking microorganisms are hungry since they need to replenish their energy stores in the form of ATP.

5. ***Compounds that kill persister cells.*** Most, if not all, antibiotics have been identified based on their ability to kill actively-growing microorganisms. However, persister cells are dormant so new kinds of antimicrobials need to be identified with an emphasis on killing non-growing cells. This represents a paradigm shift in how we identify compounds to treat infections.

Basically, there are three broad approaches to killing persister cells: (i) kill them as they sleep, (ii) wake them and then treat them with traditional antibiotics to kill them, and (iii) prevent the formation of persister cells in the first place. It is important that these anti-persister compounds function without requiring cell activity; in other words, the anti-persister compounds must be able to passively enter the cell while it sleeps, since there is no energy available to import the compounds, and the compound must be able to corrupt some cellular process, without relying on active assistance, once the anti-persister compound is in the cell.

Some notable successes have been achieved in killing persister cells by using government-approved anti-cancer compounds to kill them by crosslinking their DNA while they sleep. Compounds like cisplatin and mitomycin C enter sleeping cells and destroy persister cell DNA in this way. Since they are somewhat toxic, their use is best for skin surface (topical) applications where they may be used below toxic concentrations.



Adding fresh medium wakes persister cells so, in this trivial way, persister cells can be converted into actively-growing cells which are susceptible to antibiotics. Also, some cell signals like *cis*-decenoic acid and brominated furanones have been found that wake persister cells without acting like nutrients. In addition, persister cell formation has been limited to some extent by masking the way microorganisms talk to one another.

As more is learned about persister cells and how to convert large populations into persister cells, some groups are now screening directly for the ability of compounds to kill sleeping pathogens, rather than focusing on actively-growing cells. For example, 10,000 compounds were screened directly for persister cell killing, and a substituted indole known as NPIMA (5-nitro-3-phenyl-1H-indol-2-yl-methylamine hydrochloride) was identified that kills a wide range of pathogenic persister cells. Hence, there is hope for killing sleeping pathogens.

6. ***Persisters and disease recrudescence.*** Since microorganisms live primarily in slime layers known as biofilms, persister cells are frequently found in biofilms, for it is within biofilms (microbial 'houses'), that microorganisms weather adverse conditions. Biofilms are everywhere there is liquid water: they are the plaque that dental hygienists remove every six months, and they are what makes rocks feel slippery in rivers and lakes. Biofilms are important, then, since most human infections occur because of biofilms, and it is the persister cells in biofilms that are most difficult to kill with antibiotics.

For treating microbial infections of the body, such as those of the ear, wounds, and stomach (e.g., ulcers), most of our antibiotics have been focused on killing actively-growing microbes, but many microbes in biofilms are not actively-growing and are, instead, in the dormant persister state. Hence, a course of antibiotics often leaves these dormant cells unaffected. Unfortunately, the unharmed persister pathogens may wake to restart the infection. Since actively-growing cells always mutate, these pools of sleeping cells that wake and reconstitute infections often lead to resistance. Hence, it is important now to be able to detect these unharmed, sleeping microbes and to develop pharmaceuticals that eliminate both growing and non-growing cells.

This situation is especially critical for prosthetic devices like knee replacements, where 25% of failed knee replacements are due to bacterial infection. In these cases, no amount of currently-used antibiotics can rid of the infection, so the prosthetic knee must be replaced, which poses an extraordinary strain on the patient who has to undergo rehabilitation again.

Persistence is also related to human cancer since long-term relapse for myriad cancers is common; for example, relapse occurs in 20% of breast cancer patients. Analogous to the metabolically-dormant bacteria that do not respond to antibiotics known as persister cells, dormancy for cancer cells occurs when the growth of the cells is temporarily stopped during cancer therapy; the surviving cancer cells may then initiate a new primary tumor that is often more difficult to treat. Therefore, like eliminating bacterial persister cells, eliminating cancer persister cells should be an inherently critical part of cancer treatments.

7. ***The interplay of treatment and host defenses in the infection wars.*** Host defenses play an important part in eradicating infections. For example, our bodies employ white blood cells to kill invading pathogens. These specialized cells engulf pathogens and rid our bodies of infection by secreting strong oxidizing agents like hydrogen peroxide to kill the invaders. You may have encountered hydrogen peroxide being used to kill bacteria in small cuts in our skin. Unfortunately, although nearly all of the pathogens are killed by hydrogen peroxide and white blood cells, some microbes may survive by becoming persister cells. Moreover, the pathogens have learned to recognize the hydrogen peroxide used by the white blood cells and use it as a signal to induce dormancy. Even more devious, some pathogens have learned how to not only

survive as persister cells but also how to wake and live inside the white blood cells as slow-growing bacteria.

Pathogens have also learned how to outwit our white blood cells by forming specialized biofilm homes. This occurs in urinary-tract infections, which are notoriously difficult to treat and are the primary reason individuals seek help from physicians. The microbes in these biofilm infections form a ball of cells called a pellicle, and this pellicle is made from the tough building block of trees, cellulose. The bacteria hide in a dense, fibrous shell of cellulose in the pellicle, and this shell shields them from antibiotics and white blood cells. Once the antibiotic is cleared from our systems, the pathogens leave the pellicle and re-new the infection.

8. ***The importance of completing antibiotic treatments.*** It is important to note that there has been microbial life for at least two billion years, and during this nearly-incomprehensible passage of time, microbes have adapted to the antibiotics produced by their neighbors. So, it is not surprising that there are many evasive techniques microbes have mastered over this timespan to escape antibiotic treatment, such as becoming resistant and forming dormant persister cells, as already outlined in this chapter.

Another aspect of recrudescence that should be mentioned is that individual microbial cells do not behave the same way, even if they are the same kind of microbe. For example, in the soil, there may be a billion cells in every gram of dirt, and the cells of each species of microbe do not behave exactly in the same way. (This diversity of behavior in populations of the same organisms is also seen for example with a flock of birds: whereas most are usually clustered together, there are always a few separated from the flock, behaving a bit differently. Or children returning to class after the bell rings signaling the end of a break: there are often one or two well ahead of the majority and always a few stragglers.)

In a population of bacterial cells, these behavioral differences may be caused by the slightly-different environment each cell experiences due to slightly different positions in their biofilm home, as well as due to subtle changes in the set of chemical reactions known as metabolism in each cell, even when the conditions are identical. Because of these differences, it will take different amounts of time to kill microbes with antibiotics. Therefore, given the diverse coping mechanisms of microbes, and these differences in time required to kill microbes, it is imperative that each of us finish a complete course of antibiotics to ensure we kill as many, and perhaps all, of the invading pathogens. If we do not, then we may have unintentionally helped to create stronger microbes that are even more difficult to treat with existing antibiotics.

Relevance for Sustainable Development Goals and Grand Challenges

The microbial concept of recrudescence of persister cells relates primarily to Goals 2 and 3 of the SDGs.

- **Goal 2. End hunger, achieve food security and improved nutrition and promote sustainable agriculture** (*end hunger and malnutrition, increase agricultural productivity).* About one in six people all over the world endures food poisoning every year from microbial pathogens (e.g., 48 million in the U.S.), so it is imperative to improve conditions related to food in grocery stores, restaurants, and food preparation facilities. To this end, procedures are required that more effectively remove persister cells. In addition, better testing mechanisms are required to detect the presence of persister cells in food and on food-preparation surfaces.

- **Goal 3. Ensure healthy lives and promote well-being for all at all ages** (*improve health, reduce preventable disease and premature deaths).* The inability to clear microbial infections affects the well-being of us all since microbial infections are the main cause of death. Therefore, significant funding needs to be secured to identify new antimicrobials capable of killing not only growing microorganisms but also capable of killing persister cells, too. These compounds may also have relevance for treating human cancers. Therefore, persistence has profound economic consequences for health budgets.

Potential Implications for Decisions

1. ***Individual***

a. After wounds or operations, patients should always be on the lookout for signs of infection including swelling, redness, drainage, and fever, and should consider seeing a physician as soon as possible when these occur.

b. If most of our current antibiotics fail to kill microbial persister cells, patients should consider asking their physicians about whether there are compounds they could take that are effective with persister cells.

c. For the treatment of cancer, patients should consider asking their physicians about whether there are compounds they could take that are effective in killing cancer persister cells, to prevent relapses.

2. ***Community Policies***

a. Public education relating to reduction of misuse of antibiotics which increases the development and spread of antibiotic resistant microbes

b. Public education about completing antibiotic treatments to help completely

eradicate a pathogen causing an infection

3. National Policies

a. How can the safety of our food supply be improved? Emphasize the need to look for sleeping bacteria as well as active ones in food preparation sites, grocery stores, and home.

b. How can cancer treatments be improved by killing cancer persister cells?

c. Policies relating to the non-clinical use of antibiotics in food animal and fruit production

d. policies relating to research and development of new prophylaxis and treatment options for infections and cancers which, because of persisters, are difficult to eradicate.

Pupil Participation

1. Class discussion of the issues associated with microbial viability

a. **Biofilms**: Ask the class if they have ever picked up a rock from a river and what did it feel like? Explain the slimy layer is a biofilm of microbes cemented to each other and the rock. Explain this is what a dental hygienist removes from our teeth.

b. **Persistence:** Ask the class if they have ever surprised a turtle. Explain a turtle withdrawn into its shell is like a persister (dormant) microbe. Like the turtle once it is over the surprise (or after the dog leaves), persisters wake up when food is presented and grow.

c. **Dead microbes**: Ask the class if they have ever picked up a seashell on a beach. If there is nothing inside, then this is the way dead microbes often appear in a microscope. Live but sleeping microorganisms (i.e., persisters) are not empty inside but have all components of normal cytosol (DNA, RNA, protein, etc.).

d. **Microbes and food poisoning.** Discuss the need to detect dormant bacteria in food to prevent food poisoning.

e. **Antibiotic resistance and personalized medicine.** Discuss the need to discontinue the use of antibiotics on farms and on humans with viral infections to prevent selecting for resistant bacteria.

2. Pupil stakeholder awareness

a. Antibiotics are great for curing infections. Ask the class if they can see the negative effects, too (like killing the beneficial microbes in our GI tract).

b. If antibiotics are not always effective and result in infections causing death more than anything else, ask the class how they can check for the signs of recurring infections.

The Evidence Base, Further Reading and Teaching Aids

Song, S., Gong, T., Yamasaki, R., Kim, J. S. & Wood, T. K. Identification of a potent indigoid persister antimicrobial by screening dormant cells. *Biotechnol Bioeng* 116, 2263-2274 (2019).

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Glossary

Alarmone: a highly-phosphorylated piece of the building blocks of RNA, like guanosine tetraphosphate, that signals to the inside of the cell that conditions are dire outside the cell (this signaling is analogous to our sense of touch and how heat and cold outside our bodies is relayed to our brains by nerve cells).

ATP: adenosine triphosphate, energy source that powers enzymes in cells, like a battery for a toy.

Antibiotics: compounds that stop the growth of microbes or kills most, but not all, of them (remaining live cells are persister cells).

Biofilms: sticky microbial homes in which the cell is cemented to a solid surface and to other cells. Frequently called slime.

Cancer: unregulated and unwanted growth of cells in our bodies.

Dormant: sleeping microbes that stop making the primary building block of you and microorganisms, protein.

Infection: pathogenic microorganisms can make us sick by producing toxins in our bodies.

Mutation: changes in DNA including the addition of genes, removal of genes, and changes in existing genes.

Pathogens: ill-tempered microorganisms that cause us harm.

Persistence: dormant state in which microbes sleep through a course of antibiotic treatment or other environmental stress, like what you do at night, if you did not get taller when you sleep.

Pellicles: Tough, fibrous, biofilms that are made from the building blocks of the woody part of trees.

Recrudescence: an undesirable thing, like a toothache, that occurs again.

Resistance: active state in which microbes can grow in the presence of antimicrobials due to genetic changes, like when you wear protective equipment to avoid injury in sports.

Ribosomes: nano-machines that assemble proteins from 20 different amino acid building blocks, like making a Lego structure from 20 different-colored blocks where DNA is the set of instructions for the structure (protein).