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Integrative Functional Genomics to Enhance Bioenergy Stress Tolerance of *Zymomonas mobilis*

By

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Chapter 6: Strategies of a Bacteria Therapist

Helping a Biofuel Bacterium Overcome Stress

I wrote this chapter as part of the Wisconsin Initiative for Science Literacy (WISL) Award for Communicating PhD Research to the Public. The Summary section provides an analogy-based overview of my thesis research. I adapted this summary from a script I wrote and presented for the 2022 UW-Madison Three Minute Thesis® competition and expanded the script to introduce results from later work in my thesis. The remaining sections describe my PhD research journey in greater detail. I am grateful to WISL and Professor Bassam Shakhashiri for the opportunity to include this chapter in my thesis and to Elizabeth Reynolds and Cayce Osborne for their guidance and editing.

Summary

I am a therapist — for bacteria.

You're probably wondering if there's actually a job market for that, but think about it: there are only seven billion people in the world. Meanwhile, there are an estimated five-million-trillion-trillion bacteria. And they are all just as stressed out by life as the rest of us.

Take my current patient, for example. It's called *Zymomonas mobilis*, or *Z. mobilis* for short, and it's a bacterium that helps us fight climate change.

How? By making biofuels. If we feed it renewable plant material – this could be prairie grass, or leftover bits of corn plants – *Z. mobilis* gobbles that up and turns it into eco-friendly fuel.

But making biofuels is stressful work. Plants (understandably) don't like being eaten by bacteria, so they produce toxins that stress the bacteria out. On top of that, *Z. mobilis* is forced to rapidly switch between different oxygen levels when it is pulled from storage and tossed into plant extracts. Although *Z. mobilis* can grow in the presence or absence of oxygen (we call this type of bacterium "aerotolerant"), switching between the two is a lot of work. And the biofuels themselves? They can harm the membrane, or the skin of bacteria, causing even more stress.

So *Z. mobilis* came to me and said, "Amy, I am so stressed out. I'm working as hard as I can, but I still can't produce enough biofuel to compete economically with fossil fuels. Can you help?"

Now, *Z. mobilis* can't speak, but I can tell it's stressed because it grows slower. And slow-growing bacteria means less biofuel production.

So, reader, let me ask you this: What do you do to relax? Do you take a bubble bath? Exercise? Well, bacteria have their own ways of dealing with stress, and they are encoded in

their DNA as genes. Instead of relaxing in a bubble bath, they switch on certain genes that help them cope. The problem is, I don't know which genes will help *Z. mobilis* during biofuel production, and since it's never done this job before, neither does *Z. mobilis*.

So, how do I as a therapist figure that out? Fortunately, I have a genetic tool to help me. It's called CRISPR interference, or CRISPRi (pronounced "crisper eye"), and it essentially lets me turn off genes, one at a time.

Now, imagine if I took away *your* favorite de-stressing activity, and you just got more and more stressed, until you became sick. That's what CRISPRi does. I turn off a gene, and I see how *Z. mobilis* responds. This strategy might seem harsh, but taking away its coping mechanisms is one of the fastest ways to figure out which genes help the most. So, if *Z. mobilis* grows slower after I turn off a gene, then I know that gene was important for dealing with stress. Plus, sometimes we discover genes that *Z. mobilis* is actually better off without.

Z. mobilis has been my patient for a few years now, and we've worked through several stressful situations together. First, I used CRISPRi to find genes that help it cope with the various oxygen levels it faces at work. Next, I tackled the stress caused by plant toxins and those membrane-stressing biofuels. Along the way, I found some surprising coping mechanisms that we wouldn't have known about otherwise. The following sections provide a closer look at the experiments I conducted to uncover these hidden strategies, and how this knowledge could help us build a more sustainable energy future.

What are biofuels?

Biofuels are liquid fuels made from renewable plant material. The plant material is fed to microorganisms that turn the sugars from the plants into fuels that we can use. When I entered the Microbiology Doctoral Training Program, I knew I wanted to study biofuel producing bacteria because of their important role in meeting global sustainable energy demands, ensuring energy

security through strong supply chains with reduced dependence on oil, and supporting agricultural economies by providing new markets. For these reasons, I was thrilled to embark on a research project with the Great Lakes Bioenergy Research Center, a cross-institutional research hub funded by the U.S. Department of Energy that is dedicated to creating economically viable and environmentally sustainable biofuels and bioproducts.

The most well-known biofuel is bioethanol. When you go to the gas station to fill up your vehicle, you may see a sticker on the gas pump that says, “contains up to 10% ethanol.” That ethanol is biofuel that was usually made by feeding corn to baker’s yeast (*Saccharomyces cerevisiae*) which convert the corn into ethanol by fermentation. These days, researchers are particularly interested in making other, more energy-dense biofuels besides ethanol that could power jets and large ships. While electric cars are commonplace, electrification of larger vehicles isn’t yet possible, so liquid biofuels are expected to fill a key role in this part of the transportation sector. Energy-dense biofuels that could power these large vehicles include another alcohol called isobutanol, as well as lipids (a group of molecules that includes fats and sterols, like cholesterol) that are called isoprenoids.

Meet the biofuel producing bacterium *Zymomonas mobilis*

Z. mobilis was chosen for biofuel production because of its remarkable natural qualities. In particular, it produces large quantities of ethanol very quickly. Though baker’s yeast is also a rapid ethanol producer, *Z. mobilis* is even faster, so scientists hope we can produce higher biofuel yields by taking advantage of its inherent talents. *Z. mobilis* also naturally produces lots of isoprenoids to make its outer membrane (“skin”), and scientists are working to reroute its remarkable ethanol production to make isobutanol too. Another advantage of *Z. mobilis* is that it can grow in either the presence or absence of oxygen, which is called aerotolerance. When humans breathe, we respire oxygen to make energy to survive. Many bacteria also need to respire oxygen to live, but others, like *Z. mobilis,* can make energy in other ways. This flexibility

is useful because scientists can easily work with *Z. mobilis* in normal air, and it saves a lot of money for biofuel production facilities because they don't need to pump oxygen into the tanks.

Challenges of microbial biofuel production

One benefit of making biofuels from corn is that its energy is stored in sugars and starches that are easily digested by microbes. However, corn is a major part of our food and feed supply, so it would be better if we could make biofuels from other plants, especially native plants that can grow in nutrient-poor soils that are less desirable for food agriculture. The problem with other plants is that their energy is tied up in complicated molecules called lignin and cellulose that make the plant structure difficult for microbes to break down. To release these sugars for microbes to eat, scientists chemically deconstruct the plant material using various pretreatment methods which may include soaking it in acids or solvents, treating it with high temperature and pressure, and using enzymes to break apart the plant structure. This deconstruction is often called hydrolysis, so the resulting plant-based feedstock is called hydrolysate.

However, the plants, pretreatment methods, and microbial fermentation itself result in chemicals that are toxic to microbes, slowing down fermentation and reducing biofuel yields. Plant toxins include aromatic compounds that plants produce naturally to ward off bacteria that can make them sick. Examples of aromatic compounds include vanillin, which gives vanilla its distinctive flavor, or cinnamic acid, which is found in cinnamon. Other toxins encountered during biofuel production include leftover pretreatment solvents, acids like acetic acid (vinegar), and even the desired fermentation products themselves, such as ethanol and isobutanol. To get microbes to produce enough biofuels to meet energy demands, we need to find ways to help them cope with the stress of growing in these toxins (bioenergy stress) so that they can be more efficient producers.

Functional genomics reveals bacterial coping mechanisms

The goal of my thesis work was to perform experiments that could tell us how to help *Z. mobilis* cope with bioenergy stress. Once we know the genes that are critical for handling bioenergy stress, we can use that information as a roadmap to modify the DNA of *Z. mobilis* to make it more resilient in these conditions, enabling it to make more biofuel.

To do this, I performed a series of experiments called functional genomics. As the name suggests, functional genomics can be used in many areas of research to understand the functions of genes. To perform functional genomics in bacteria, researchers use genetic tools to perturb the activity of the gene, often by either turning the gene off or by increasing the activity of the gene, and then testing the result of that genetic change. Bacteria with these types of genetic changes are called mutants. Since I needed to understand the function of specific genes that mediate bioenergy stress, I tested *Z. mobilis* mutants in conditions that are representative of the challenges it faces during biofuel production, including different oxygen levels, individual toxins, and hydrolysates, and evaluated how each mutation affected growth.

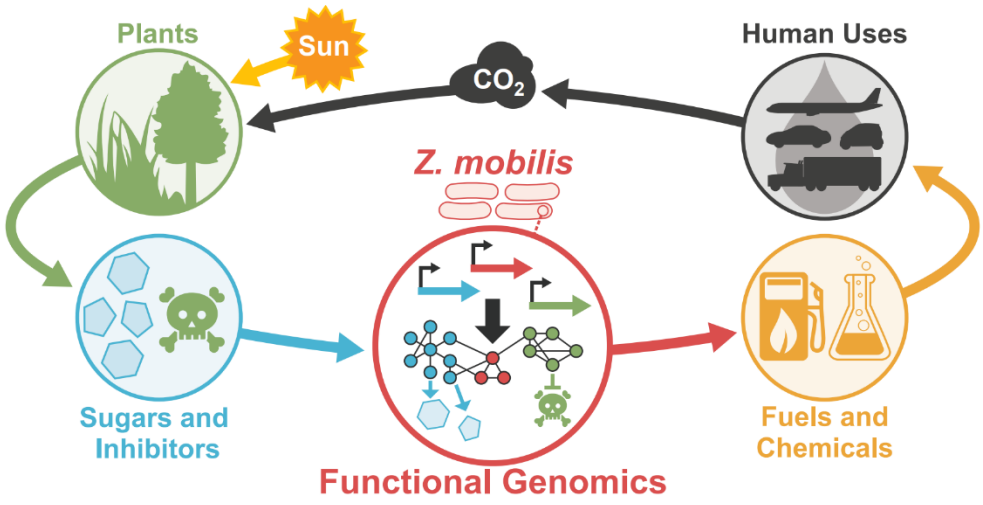

Figure 1. Functional genomics is a powerful tool to enhance sustainable biofuel production by *Z. mobilis*. Many thanks to Matt Wisniewski for creating this image.

What's amazing about the genetic tools available today is that we can use them to perform functional genomics on a large scale. *Z. mobilis* has approximately 2,000 genes total, and although this is only about a tenth of the number of genes that humans have, this is still a lot of genes! It would be impossible for one person to test each gene one by one during the span of a PhD research project. Fortunately, current tools let us make what we call pooled mutant libraries. Libraries are collections of mutants, each with a single mutation, that are mixed together or "pooled." Each mutant has a unique barcode – a small piece of DNA that acts much like the barcodes that a librarian would scan to keep track of titles in a book library. Scientists can use DNA sequencing to count the number of each barcode (mutant) in each sample, which tells us which mutants are growing exceptionally well and which ones are struggling or disappearing.

In my dissertation work, the main genetic tool that I used was CRISPR interference (CRISPRi), which allows me to turn off genes. This tool is based on traditional CRISPR approaches that are used for gene editing. Traditional CRISPR uses a molecule called Cas9 that acts like a pair of scissors to cut DNA at precise locations. CRISPRi is similar, but it uses a broken pair of scissors called dCas9 ("dead" Cas9). Instead of cutting, dCas9 grabs onto the DNA and sits there, physically blocking it from doing its job, like forcing a light switch to stay in the "off" position. CRISPRi is especially important for functional genomics in bacteria, because bacteria don't have the ability to repair their DNA and survive after their DNA is cut by traditional CRISPR without a researcher providing individually designed pieces of "repair template" DNA that act like bandages to patch each specific cut, which isn't possible for 2,000 genes at once.

One of my favorite parts of doing CRISPRi-based functional genomics to understand bioenergy stress in *Z. mobilis* was the surprisingly good smells. Some bacteria have a reputation for smelling terrible, but *Z. mobilis* has a somewhat yeast-like metabolism, which

means that it smells a bit like fresh baked bread. And thanks to the aromatic compounds like vanillin and cinnamic acid in the plant feedstocks, the hydrolysates smell good too!

Z. mobilis strategies for coping with bioenergy stress

By performing functional genomics, I identified many genes that were important for dealing with bioenergy stress, some of which were surprising. I found genes that are important for *Z. mobilis* to survive in the absence of oxygen and the presence of plant toxins, which could be used to engineer more resilient biofuel production strains. A key takeaway from my results is that the growth strategies used by *Z. mobilis* sometimes differ from those used by other species of bacteria.

Genes required for growth without oxygen

Since a strong advantage of *Z. mobilis* as a biofuel producer is its ability to switch between growth with oxygen (aerobic) or without oxygen (anaerobic), we wanted to understand the genes that enable it to do this. Given that growth without oxygen supplementation provides cost savings during biofuel production, I was particularly interested in the genes that are required for anaerobic growth. To investigate this, we performed functional genomics in the presence and absence of oxygen and identified mutants that grew well in one condition but not the other. For example, we discovered that genes responsible for making components of the Rnf complex were critical for growth without oxygen, since turning off these genes with CRISPRi caused the mutants to dwindle from the pool. At first, we weren't sure why the Rnf complex would be required for anaerobic growth. Bacterial genes are usually named for the function they perform, and Rnf stands for *Rhodobacter* nitrogen fixation. *Rhodobacter* is another type of bacterium, and *Rhodobacter* uses these genes to make essential nitrogen building blocks when there aren't any available in the environment. However, our experimental conditions provided plenty of these building blocks, so we inferred that *Z. mobilis* was using these genes for a different purpose in this case.

By collaborating with another lab on campus, we analyzed the molecules produced by these mutants and discovered that they had a defect in isoprenoid lipid production. Isoprenoids are valuable biofuel precursors, and they are also required for *Z. mobilis* survival. The defective isoprenoid production by these mutants provides an explanation for their reduced growth in the absence of oxygen and makes an exciting connection between the Rnf complex and production of valuable biofuel molecules. It's possible that future work could modify Rnf function to boost isoprenoid yields.

Genes that alter resilience to plant toxins

Next, I set out to determine which genes were important for growth in hydrolysate toxins. All these experiments were performed without oxygen to mimic bioenergy-relevant growth conditions. Alongside a team of four other researchers, I tested our pooled mutant library in toxins including solvents used to break down the plant material, individual aromatic compounds that are found in hydrolysates, and fermentation products like ethanol and isobutanol, which are also solvents.

We discovered a particularly interesting result by growing our library with a chemical called ferulic acid, an aromatic chemical found in plants. We discovered that genes responsible for producing the cytochrome bc_1 complex, a group of proteins required for some bacteria to respire oxygen, have a surprising effect during anaerobic *Z. mobilis* growth with ferulic acid. We found that CRISPRi mutants of cytochrome bc_1 genes grew better with ferulic acid than without it. This means that removing these genes from *Z. mobilis* could be an effective strategy for improving growth and biofuel production in hydrolysates containing ferulic acid. We also measured the proteins produced by *Z. mobilis* when it was grown with or without ferulic acid, and we found that ferulic acid treatment resulted in changes to proteins in the membrane. This

made sense given that previous work by other groups has shown that ferulic acid can harm bacterial membranes.

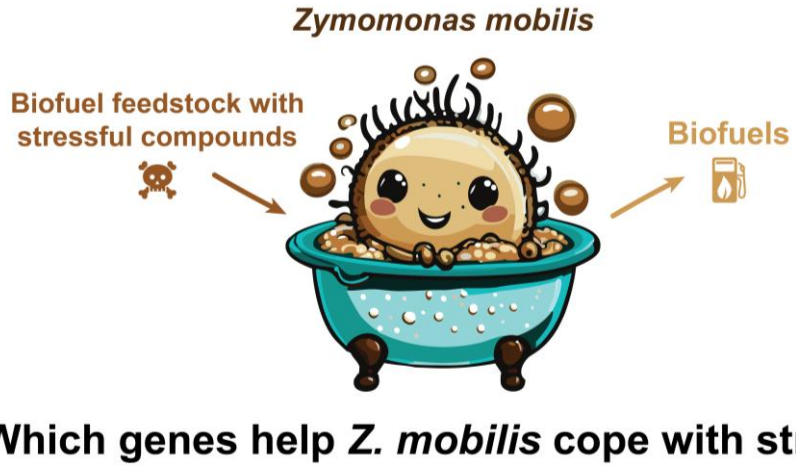

Figure 2. In my thesis, I identified genes that help *Z. mobilis* cope with stress to find ways to make it a more resilient and productive biofuel producer. This image was created using generative AI in Adobe Illustrator.

Finally, I reported results from an extended panel of growth conditions including additional solvents and various hydrolysate feedstocks. Although we didn't observe the same improved growth of cytochrome bc_1 mutants in the hydrolysates we tested, it was likely because these hydrolysates had lower concentrations of ferulic acid and its chemical siblings than we used in the earlier experiments. However, we also observed improved growth of these mutants in various solvents, which are known to cause stress by pulling apart bacterial membranes. Combined, these results give us a stronger understanding of the membrane-targeting stress that *Z. mobilis* experiences during biofuel production and a genetic toolkit to help it cope with that stress.

Conclusion and reflections

I have always been fascinated by the enormous impact that tiny microorganisms can have on our world. Through this work, I've also come to appreciate how ubiquitous stress is to

any living organism, even energy heroes like *Z. mobilis*. By using CRISPRi-based functional genomics, we identified genes that help this bacterium survive the harsh conditions of biofuel production, laying the groundwork for engineering strains that are more resilient and productive. These insights bring us closer to making biofuels a more viable alternative for powering transportation methods that can't yet run on electricity, like airplanes and cargo ships.

Beyond the science, one of the most rewarding aspects of this work was how collaborative it was. From teaming up with other researchers to tackle months of functional genomics experiments, to working across lab groups to interpret complex metabolic pathways, to partnering with data scientists to develop custom software for analyzing large datasets, this project was a huge team effort. That collaboration not only made the research more insightful, but also a lot more fun, and I am deeply grateful to the many cross-functional team members who brought this work to life.

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