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# Structural and Functional Studies of Prepilin Peptidase PilD:
# An antivirulence target in *Pseudomonas aeruginosa*

By

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# Chapter 1: The Persistent Struggle: Rethinking How We Fight Bacteria

Wisconsin Institute for Science Literacy (WISL) Dissertation Chapter


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## Introduction

I believe that scientists have a contract with society that, in return for funding support and a level of independence to choose the research questions we pursue, we have an obligation to ensure the benefits of research flow out of the lab and into the community. I chose to pursue my graduate education at UW–Madison because I deeply believe in the Wisconsin Idea that "education should influence people's lives beyond the boundaries of the classroom." Additionally, most of my graduate research work has been supported by public funding. I wrote this chapter because I feel science should be accessible to anyone interested, especially those whose tax dollars have helped fund the research work, and because I feel an obligation to live out the Wisconsin Idea.

After graduate school, I aspire to run my own lab at a land-grant university. I hope to focus my research at the intersection of chemistry and microbiology, leveraging fundamental insights into nature to inform new approaches to tackling the looming public health challenge of antimicrobial resistance. Nothing would be more fulfilling than conducting science to uphold the land-grant ideal of translating knowledge from the university out into society to reinvest public support into social good. I plan to be active in efforts to re-engage our public universities with their communities through research, outreach, and government engagement. I aim to embody the model of a civically engaged scientist, shaping a future where scientific expertise not only continues to improve society but also informs and enriches public policy.

**The Persistent Struggle: Rethinking How We Fight Bacteria**

Each year, the number of people who die from infections that no longer respond to antibiotics increases. In hospitals across the world, including here in Wisconsin, doctors increasingly encounter bacteria that are resistant to not just one drug, but often to many—or all—of the antibiotics available. These bacteria cause infections that make surgery riskier, childbirth more dangerous, and everyday injuries potentially life-threatening. They are particularly dangerous to those with suppressed immune systems, such as those undergoing treatment for HIV/AIDS or cancer.

Antibiotic resistance is not a future crisis. Headlines may speak of the end of a golden era, the coming wave, or an impending antibiotic apocalypse, but resistance is not new. It is not a fluke or a failure. It is biology. The evolutionary pressure that leads bacteria to resist our drugs is the same force that once helped them survive against competing bacteria and fungi in the environment. Drug resistance is likely not a war we can win, but the good news is that we are also not doomed to lose our antibiotic drugs. Drug resistance should be thought of as a chronic challenge that will require new and innovative approaches to address.

*Disarming, Not Destroying*

For decades, the model for treating infections has been straightforward: the best way to clear an infection is to kill bacteria—often the bad and the good. This approach has saved millions of lives, and it remains an essential tool in modern medicine. But it also comes with a cost. Trying to kill bacteria creates a strong evolutionary pressure for them to survive—and over time, some cells inevitably do. Resistance emerges, spreads, and erodes the effectiveness of our drugs.

Not only is antibiotic resistance inevitable, but it can also emerge rapidly. Resistance to penicillin emerged within 1 year of it being used as the first broad spectrum antibiotic. The bacteria *Staph. aureus* developed resistance to the antibiotic methicillin the same year the drug was introduced. On average, it only takes 2–3 years for resistance to emerge to a new antibiotic drug.

Recently, researchers have begun asking a different question: what if we didn't have to kill bacteria to stop them from making us sick? What if we could disarm them instead? That is where my research comes in. At the most basic level, I study the structure and function of proteins in bacteria. That might sound like very fundamental research far away from a new drug dispensed at your local pharmacy. The protein I study, however, we think could be a new kind of drug target. Instead of finding a new way to kill bacteria, what if we could disarm them? It's part of a growing shift in how scientists think about treating infections.

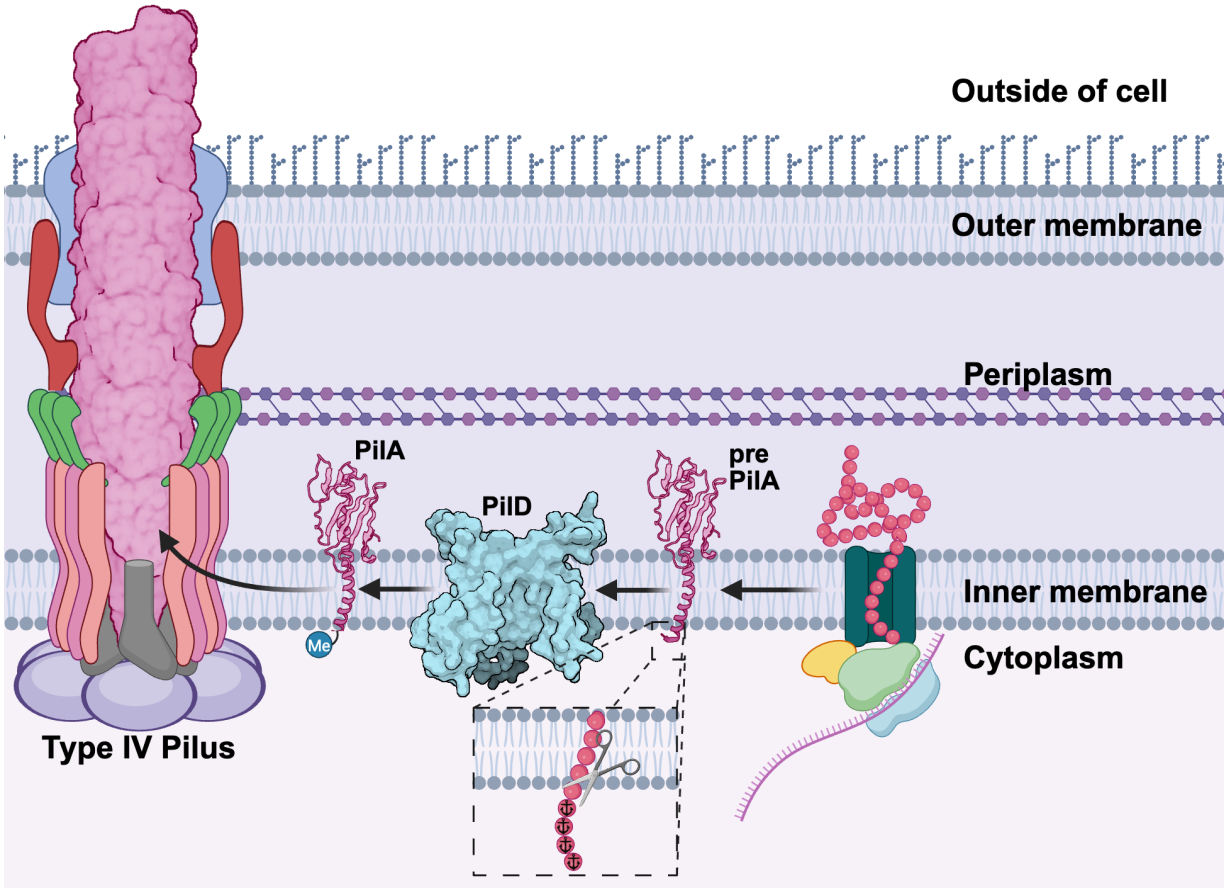


**Figure 1.1 The role of PilD in processing T4P and T2SS subunit proteins for assembly.** This cartoon depicts PilD (blue) cleaving the leader peptide (circles with anchors) from the main T4P subunit "pre pilA" after it has been synthesized and inserted into the inner membrane of the cell. PilD also sticks a methyl group in the new end of PilA (blue circle with "Me" label), which is then incorporated into the growing pilus structure. The same enzymatic activity is done to the other subunits of the T4P and T2SS.

In my work, I study a prepilin peptidase protein called PilD in the bacterium *Pseudomonas aeruginosa*, a pathogen that frequently causes drug-resistant infections in burn patients, those with chronic wounds, people on ventilators, and individuals living with cystic fibrosis. PilD does not help the bacterium grow. Instead, it helps it build two tiny molecular machines—called Type IV Pili (T4P) and the Type II Secretion System (T2SS)—that it uses to move, attach to cells, and release virulence factors that make it dangerous during an infection. Each of these systems are composed of thousands of small proteins called pilins (for the T4P system) or endopilins (for the

T2SS) that look like lollipops or helium balloons. When these pilins/endopilins are made in the cell, they get inserted into the cell's inner membrane with a highly charged tail section that acts like an anchor—like the plastic weight that's tied to a balloon to prevent it from floating away (figure 1.1). PilD acts like a pair of scissors and clips that anchor off to allow each pilin/endopilin to leave the membrane and be assembled into the nanomachines (figure 1.1). In addition to its scissor-like cleavage function, PilD also performs a second transformation of its protein substrates, attaching a methyl group of one carbon and three hydrogen atoms to the new end of pilins/endopilins after their anchor sections are removed (Figure 1.1). Without PilD, *P. aeruginosa* can survive—but it becomes much less dangerous.

This makes PilD an ideal target for what we call an "antivirulence" drug: one that knocks out the bacteria's ability to cause harm without necessarily killing it. The goal is to reduce the bacteria's threat level enough that it either is never able to establish an infection in the first place or the body's immune system can clear the infection on its own. Because antivirulence drugs are not trying to kill the bacteria, there is less selective pressure for drug resistance to evolve.

To develop these new antivirulence drugs, scientists need to understand the targets they are aiming for. This often means having a clear picture of the structure and function of the protein or enzyme (a special kind of protein that speeds up a chemical reaction like a tiny molecular machine) they are interested in so that they have a good hypothesis for how a drug might interact and bind. Despite PilD being identified as central to *P. aeruginosa* virulence over thirty years ago, however, its structure and function have remained only partially understood. When I started my graduate research

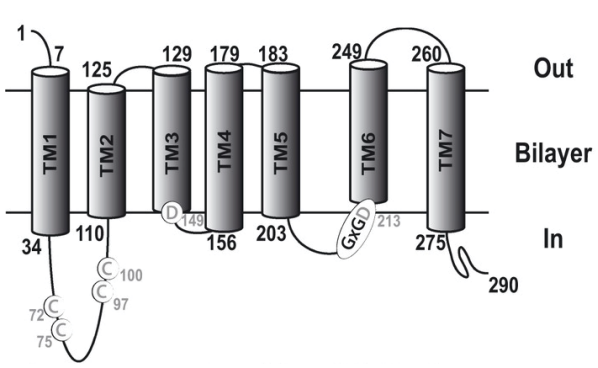


**Figure 1.2 The best working model of PilD when I began graduate school.** PilD is represented by a cartoon of lines and cylinders. Cylinders represent the segments of PilD predicted to be in the membrane. Residues important for PilD's activity are represented by their single letter code in circles. Reproduced from Aly *et al.* 2013.[1]

work, the model of PilD and related prepilin peptidases proteins called homologs in other bacteria was limited to a cartoon illustration of the general components without any high confidence hypotheses about its 3-dimensional structure (Figure 1.2).[1] Because of this limited knowledge about the structure of bacterial prepilin peptidases, they had also not really been investigated as possible antivirulence drug targets.

In my graduate research, I have worked to better understand the structure of PilD and how it works at the molecular level. Aided by advances in artificial intelligence, which enabled me to more accurately predict the likely structure of PilD, I have examined how it evolved, how its parts fit together to make it work, and how it might be inhibited to lay the foundation for future antivirulence drug development.

Artificial intelligence (AI) and machine learning has transformed many aspects of our lives over the past few years. We can now generate images based on a single sentence prompt, write computer code without knowing what a compiler is, and get creative recommendations for how to use the ingredients in our pantries to make a tasty dinner. AI has similarly ushered in a revolution in science. During my time in graduate

[1] Aly KA, Beebe ET, Chan CH, Goren MA, Sepúlveda C, Makino SI, Fox BG, Forest KT. 2013. Cell-free production of integral membrane aspartic acid proteases reveals zinc-dependent methyltransferase activity of the *Pseudomonas aeruginosa* prepilin peptidase PilD. MicrobiologyOpen 2:94–104.

school, a tool called AlphaFold2 was released that allowed researchers to predict the likely structure of proteins with greater accuracy than ever before. Historically, scientists could only be confident in the structural model of a protein after the often-long process of experimentally determining its structure. With AlphaFold and other AI-powered prediction tools, it now takes minutes, not years, for scientists to generate high-confidence models of a protein's structure. Advances in these AI protein structure prediction tools, including the release of AlphaFold3 and other tools, now enable scientists to not just confidently predict the structure of a protein alone but to predict how proteins might form complexes with each other and/or with DNA and small molecule compounds.

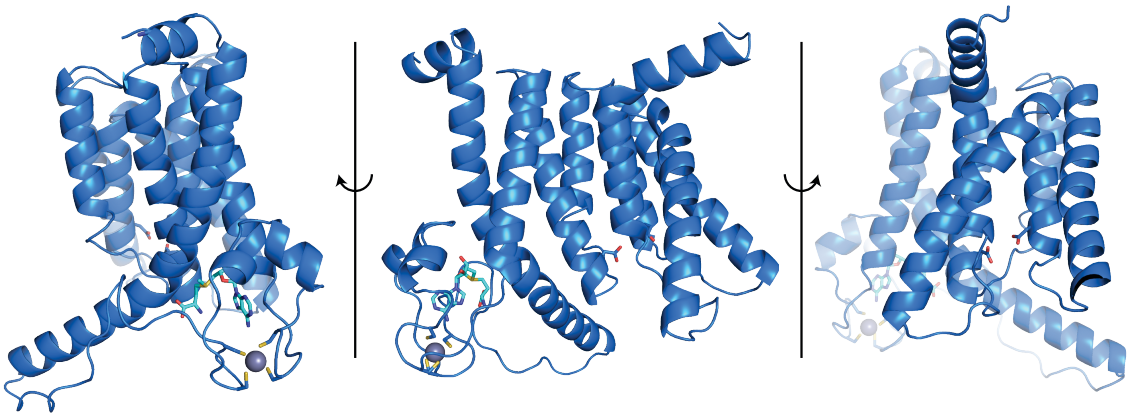

**Figure 1.3 The AI-predicted structure of PilD gives us a high-confidence 3-dimensional model for the first time.** The predicted structure of PilD (dark blue) is shown in cartoon representation for simplicity. The residues important for cleavage activity and for binding a Zn ion (grey sphere) known to be important for methylation activity are shown as sticks. The aspartic acids residues that perform protein cleavage can be identified by their oxygens, which are colored red. The cysteine residues that help bind Zn can be identified by their sulfur atoms, which are colored yellow. These same residues are either D (aspartic acid) or C (cysteine) in circles in figure 1.1 The compound (cofactor) that donates the methyl group that PilD attaches to the end of cleaved substrates is called S-adenosylmethionine (SAM) and is shown as sticks in light blue. The right panel shows the architecture of the peptidase (scissors) active site. The left panel shows the architecture of the methylation active site and how SAM is positioned to donate its methyl group.

Using these new AI protein structure prediction tools, I was able to generate models of PilD alone (Figure 1.3) and in complex with the pilin/endopilin proteins it cleaves and methylates (Figure 1.4). These models, for the first time, provided insights into the likely architecture of both the cleavage and methylation active sites and have

given us a better working model for how PilD likely interacts with and binds the protein substrates it acts on. These models also allowed us to predict where S-adenosylmethionine (SAM), the compound that donates the methyl group for PilD's second activity, likely binds to the protein, which was previously unknown.

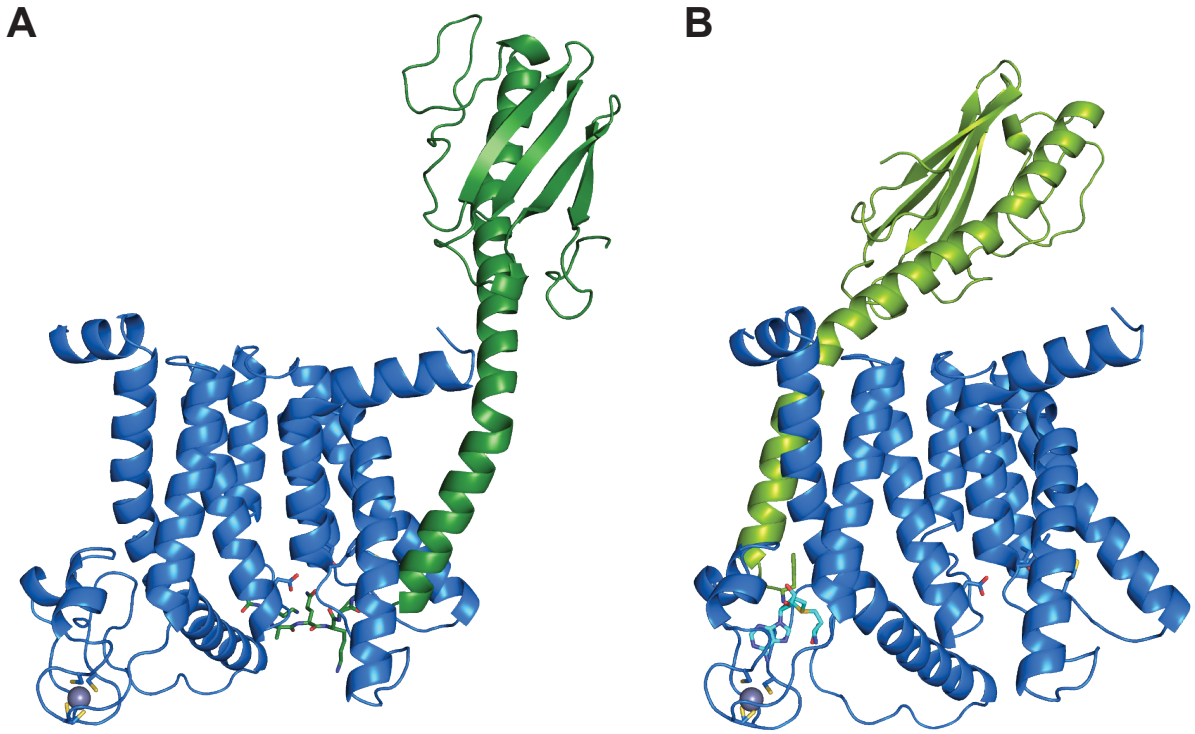


**Figure 1.4 The AI-predicted models of PilD in complex with its protein substrates.** A) The predicted structure of PilD (dark blue) in complex with prePilA (dark green) positioned for cleavage to remove its highly charged tail (shown as sticks). B) The predicted structure of PilD in complex with PilA (light green) post tail cleavage positioned for its end (shown in sticks) to be methylated by SAM (light blue sticks). These two protein complexes show how the two active sites of PilD are separated from each other and how PilD's protein substrates might bind to each one. The catalytic aspartic acid residues and Zn-binding cysteines are shown as sticks, and Zn is shown as a silver sphere as in Figure 1.3.

I was even able to embed these models in a mock membrane and simulate how they might behave using computational analysis (figure 1.5). These simulations allowed us to evaluate whether the models generated by AI held up when we applied the principles of physics to every atom in the proteins, the lipids in the membrane, and even the water molecules in the system. These simulations take a lot of computer power calculating and tracking the motion of thousands of atoms, so we could only simulate

very short timeframes (50 nanoseconds, or 50 1 billionths of a second). Nevertheless, this is often long enough to know if a model or system is unstable or modeled poorly. It turned out the models of PilD were in fact quite stable, and we could watch how PilD interacted with zinc (Zn) and its protein substrates. Because of this modeling and simulation work, we have been able to move from cartoons and arrows to molecular movies to represent how PilD behaves inside the cell.

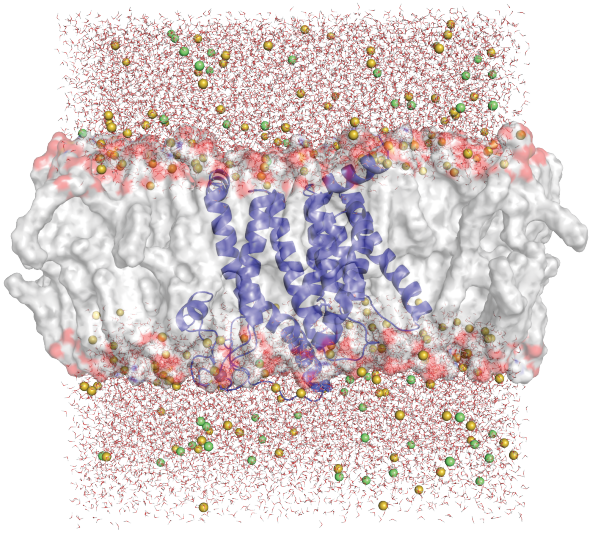

**Figure 1.5 PilD embedded in a model bacterial inner membrane provides a starting model for simulations.** This is the starting system model for the simulation of PilD embedded in a membrane. PilD is shown as a blue cartoon. The membrane lipids are shown as a translucent grey blob. Water molecules are shown as sticks, and solvent ions are shown as green and yellow spheres. The motion and interactions of every single atom in this system was simulated over 50 nanoseconds. Other PilD-protein complex models were simulated in the same membrane.

I was also able to map the conservation of amino acids, the building blocks of proteins, across related prepilin peptidases to the structure of PilD to identify residues that might be important for substrate and co-factor binding. Substrates are the proteins (pilins and endopilins) that PilD acts on to cleave and methylate. Cofactors are the ions and small molecules ($Zn^{2+}$ and SAM) that also bind to PilD and are used in the reactions PilD caries out. Residues that play a role in catalysis (the reactions an enzyme performs), structural stability, and co-factor binding are more likely to be preserved across related proteins because any changes could lead to the enzyme effectively breaking or even falling apart. By identifying highly conserved residues, I was able to then individually change them and

observe the effects these changes had on *P. aeruginosa*'s ability to move and secret virulence factors. I was able to explicitly identify the two residues necessary for cleaving the tails of pilins and endopilins (the two blades of the scissors) in PilD. These are the aspartic acid residues depicted as Ds in circles in Figure 1.1 and as blue sticks with red oxygen atoms in later figures. Researches thought these were likely the two blades of the PilD scissors, but they had not been experimentally confirmed. I also identified residues likely important for substrate binding for both active sites.

I was also able to leverage the explosion in predicted protein structure databases to examine the evolution of PilD and prepilin peptidases over time. T4P, T2SS, and other related nanomachines called Type 4 filaments are widespread across two of the three domains of life, bacteria and archaea, and all these systems utilize a prepilin peptidase. In fact, it is hypothesized that the prepilin peptidase family of enzymes is an ancient family that existed before archaea and bacteria split from each other. While scientists have been able to map the evolution of Type 4 filaments, an outstanding question remained: what was the evolutionary trajectory of the prepilin peptidase family? While some prepilin peptidases, like PilD, are bifunctional, others only perform cleavage activity.-Did the ancestral prepilin peptidase have both functions, or did the methylation function arise later, and if so, when?

Because prepilin peptidases can be shared between Type 4 filaments (*e.g.* PilD is used by both the T4P and T2SS in *P. aeruginosa*), and the genes encoding these enzymes are known to be passed between bacteria, traditional genomic approaches to studying the evolution of this enzyme family have failed. Instead of looking at how the DNA that encodes prepilin peptidases may have evolved over time across bacteria and

archaea, I examined how the likely structure of prepilin peptidases evolved. At the end of the day, it is not the As, Gs, Cs, and Ts of the DNA that make an enzyme do its job, it's how the protein folds and the structure it forms. Often, related proteins in different organisms will slowly evolve away from each other to have different encoding DNA sequences and amino acid compositions, but the most important residues and structural elements essential for the enzyme's function remain the same. I was able to compare the likely structure of over 1,000 prepilin peptidases in bacteria and archaea and compare the regions that have evolved away from each other and the regions that have stayed the same over eons. What I found was that the ancient prepilin peptidase, like PilD, was very likely a bifunction enzyme that had both peptidase (scissor-like) activity and methylation activity and that the methylation domain was lost several times in various lineages of bacteria. The prepilin peptidases in archaea seem to represent an even more recent evolution within this family. These insights helped us better understand the relationship among prepilin peptidases and may enable researchers to develop more targeted drugs that are specific for a subgroup of prepilin peptidases.

When developing new inhibitors and drugs, often large libraries of compounds are screened against the specific target. When I began my graduate research work, however, there was no prepilin peptidase test established to enable a large number of compounds to be screened as possible drug candidates. I worked to develop a test that would measure PilD cleavage activity and indicate when a compound might be inhibiting PilD. I took inspiration from nature and the fact that PilD acts on proteins to

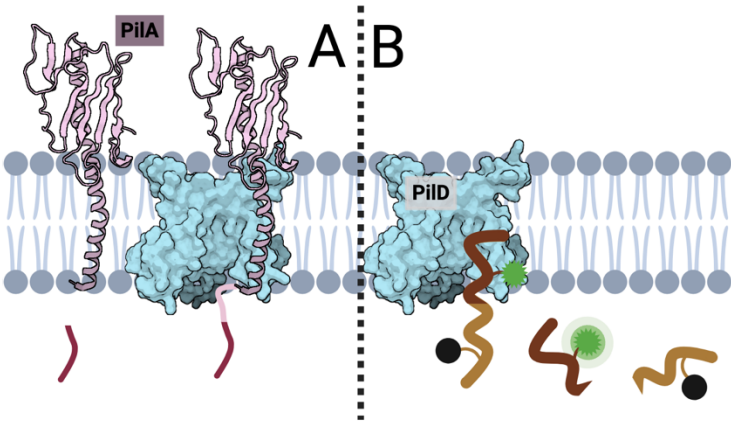


**Figure 1.6 The design principle for the PilD cleavage assay.** A schematic of PilD in the memebrane is shown twice. A) PilD processing of native prepilin or pre-pseudopilin substrate (pink, represented by PDB: 1OQW) by cleaving a short N-terminal leader peptide that acts like a weight on a helium balloon (dark pink). After cleavage, the subunit can rise through the membrane and be incorporated into T4P or T2SS filaments. B) PilD processing the FRET peptide substrate with the fluorophore (green star) and quencher (black circle) across the bond that is cleaved. Before cleavage, the fluorophore is quenched by the proximity of the quencher. After cleavage fluorescence increases.

design a short mimic peptide that looked exactly like the region PilD cleaves. Attached to this peptide was a fluorophore—a compound that shines brightly when excited with light—and a quencher—a compound that dampens the fluorophore when they are close together. When PilD cleaved this reporter peptide, the fluorophore and quencher became separated, and the fluorophore was no longer dampened (Figure 1.6). If PilD was inactive or was inhibited from cleaving the peptide, however, the fluorophore would stay dampened. While I was able to generate all the components of this test and show that PilD could cleave the reporter peptide, I was never able to make the test reproduceable enough to begin screening inhibitor compound libraries.

Even when we identify good targets for new drugs, progress is often slow. Drug development is expensive, high-risk, and failure-prone. It can take years to even develop a good test to screen for drug candidates and years more to optimize those candidates for use in the clinic. Promising hits do not even always translate to viable treatments. That does not mean the work is not worth doing. Every step forward—even a failed experiment—adds to our understanding. Over time, these steps lead to breakthroughs.

*Looking at the Bigger Picture*

My work in the lab is just one small piece of the larger puzzle of tackling antibiotic resistance. When we talk about resistance, there is often a temptation to blame individuals: people who do not finish their antibiotics, or who take them for viral infections like colds. While misuse matters, it is only part of the story. The deeper causes of resistance are structural—rooted in how we fund science, regulate drugs, farm animals, and build global health systems. Addressing any cause in isolation will never move the needle on tackling antibiotic resistance.

I became aware of the broader scope of the challenge when I went to Washington, D.C. with the American Society for Microbiology to advocate for antimicrobial stewardship funding as the ASM Fenwick Fellow for the Advancement of Civic Science. There, I heard firsthand how antibiotic resistance is already affecting real patients, some of whom have infections resistant to every antibiotic available at the hospital, and how the threat is underreported in much of the world. Pathogens do not respect national borders, and the spread of drug resistance anywhere threatens health systems everywhere around the world. Testing, detection, and surveillance of resistance, however, is costly, and many countries have an incomplete view of drug-resistant infection rates in their hospitals.

In this way, resistance is not just a scientific challenge—it is a structural and political one. Unlike other major health threats like cancer or rare diseases, antibiotic resistance does not have a patient advocacy lobby. There is no awareness ribbon or annual walk, and that invisibility makes it harder to understand the magnitude of the

challenge. That is why it is not enough to just do the science. We also must advocate for the policies and regulations that make research useful.

*Toward a Smarter Fight*

We will never "win" the war on resistance. But we can get smarter about how we fight. That means rethinking what a drug can or should do. It means investing in basic and translational science as well as global surveillance systems. It means treating antibiotic resistance as a civic and policy issue not just a medical one.

As a scientist trained at a land-grant university, I feel that responsibility to not only do good research but to consider the policy implications of my work. The Wisconsin Idea holds that the work of the university should extend beyond the classroom and laboratory to serve the people of the state—and beyond. For me, that means recognizing that antibiotic resistance is not just a molecular puzzle to solve. It is a public health challenge that demands innovative approaches that ask not just what is scientifically possible, but what is socially necessary.

My hope is that this chapter helps reframe the way we think about infectious disease and antibiotic resistance—not as an arms race to be won, but as a persistent struggle we can manage with innovative research, new tools, and the right policies. Research into new antivirulence strategies is one step. Surveillance systems, drug stewardship programs, and effective science communication are others. Even small acts like completing a prescribed course of antibiotics when sick or encouraging your elected officials to fund research endeavors add up. No step, alone, is a silver bullet, but together, efforts in the lab, in the clinic, in government, and in the community can work together to put up a smarter, more effective fight.