This Week in Microbiology

With Vincent Racaniello, Elio Schaechter, Michael Schmidt, Michele Swanson

Episode 172: Unfolding relaxases and soil malacidins

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Vincent: This is TWIM, This Week in Microbiology, episode 172, recorded on March 8th, 2018. I'm Vincent Racaniello and you are listening to the podcast that explores unseen life on Earth. Joining me today from Small Things Considered, Elio Schaechter.

Elio: Hello there!

Vincent: Welcome back.

Elio: Hi, everybody.

Vincent: Also joining us from Ann Arbor, Michigan, Michele Swanson.

Michele: Hello and happy International Women's Day to you all!

Vincent: Thank you, you too! And also joining us from Charleston, South Carolina, Michael Schmidt.

Michael: Hello, everyone!

Vincent: We have the entire US covered here, from coast to coast.

Michele: Yeah.

Michael: We do indeed.

Vincent: Including a little bit on the inside on the northeast, wow. The wonders of technology (laughs) that we could all be brought together to chat.

Michael: And Michele, since it's International Women's Day, I was at home last week and eating dinner, and CVS, since it's Women's History Month as well, CVS is doing a feature on women and one of the women scientists that it features while I was eating dinner was Jo Handelsman.

Michele: Oh that's great, that's wonderful.

Michael: They have been featuring women scientists, women involved in STEM efforts, women in education, women in business, but it was just serendipitous that our former TWIM alumnus Dr. Jo Handelsman popped up as one of the greats that they were featuring.

Michele: A well-deserved honor.

Michael: Yes.

Elio: Very well deserved.

Michele: The New York Times today is running obituaries that should have occurred but didn't.

Vincent: Wow.

Michele: So women dignitaries and accomplished people who, in this day and age, certainly would have been featured, so that is kind of neat. And could I also add, on a personal note, I am celebrating today thirty years of motherhood.

Vincent: Nice! Congratulations.

Michael: Wow.

Michele: Happy birthday, Hannah!

Michael: You can't be that old!

Vincent: (laughs)

Michele: Oh, I was a child bride, Michael.

Vincent, Michele and Elio: (laughs)

Vincent: Nice, congratulations.

Michele: Thank you.

Vincent: We have an email followup from Ben, who writes:

Dear TWIMers, I really enjoyed the snippet on TWIM 171 in which you discussed the mycobiome of stingless bees. It came only days after I read this report which suggests that the agricultural use of fungicides is among the leading causes of declining bumblebee numbers. And he sends a link to an article in the Guardian, Alarming Link Between Fungicides and Bee Declines Revealed.

Elio: It may be that, you know, the article that we talked about was specific to a kind of bees. The stingless bees.

Michael: Yes.

Vincent: That's right.

Elio: So I don't know if all bees have this connection with yeast.

Vincent: He continues,

Mycologist Paul Stamets is using his observations of bees feeding on mycelium to develop fungal treatments that might help the bees survive some of the current pressures they are under. Given that bees evolved in

forest environments and often make their nests in tree hollows where fungal species that grow on wood would also be present, it makes sense that they would develop intimate associations with specific fungi. I think the fungal parasites that frequently infect insects as discussed by Raymond St. Leger on Meet the Microbiologist, another ASM podcast, may also create a selection pressure for the bees to associate with beneficial fungi as a way to keep the pathogenic kind at bay.

Michael: That makes sense.

Vincent: I look forward to hearing more about bee fungi interactions. Yeah, makes total sense, that, right, thank you, Ben. And Elio, did you want to chat a little bit about MBio?

Elio: Yes. We live in a world where there is an avalanche of new publications, new journals. They stem from the rapacious, the sense of just being a way to try to make money, to the serious and decent and very good. And in that spectrum is a publication by the ASM, our sponsor, called MBio. For those of you who don't know it, MBio is a creation of essentially two people, Arturo Casadevall and Ferric Fang. And it has become an incredibly successful journal. It is a premiere journal in microbiology, possibly the best, and it has a character that is unique. It's nothing but the genius. Let me give you an idea. When you open it up, the beginning you find the following sections. Editorials, Perspective, Commentaries, Opinion/Hypotheses, Mini Reviews, Observations. And then come about 80 research articles. Now let me tell you, when I scan those research articles, which by the way include one by Michele, about Legionella in Michigan, when I scan this list of articles, every other one excites me, which is huge. Other journals, some of the newer journals that have appeared, when I do the same kind of scanning, I'm not excited by more than one in five, I'd say. Something like that. So this gives you an idea that in my personal view, this is really an absolute must. I find that I am satisfied, I am sated by going through a new issue of MBio. And you know, we should be very happy about it because it is good for the profession and it is also good for our society, the American Society for Microbiology.

Vincent: What do you think is the reason for its success, Elio?

Elio: Well, Arturo comes to mind.

Michael: Yes.

Vincent: (laughs)

Elio: He works his rear end off, obviously, and he is very very ingenious and gutsy and is not afraid of going in to new ways and so forth. But it is an extraordinary amount of hard work. You guys agree with me?

Michele: I do.

Michael: Yes.

Vincent: Yep.

Michele: One of the things Arturo does is not allow authors to be dragged on for months and months and months with requests by reviewers to do one more experiment. So he really tries to be an advocate for the author while maintaining just the highest standards of scientific rigor.

Vincent: Yeah, I love that. It's great because the other journals, you can go on for almost a year, sometimes. You look at the date submitted versus accepted. Sometimes it's a year. It's crazy.

Michele: Right.

Michael: And the most of the experiments that they ask for end up getting tucked in to the supplemental sections, and they don't even make it to the printed paper. It's in the supplemental but a lot of the experiments that they force folks to do to prove their satisfy the reviewer's curiosity end up as a supplement because the editor doesn't feel that it warrants the extra lines or the extra space for the additional figure that they had to generate. And for some---

Michele: And it won't change the conclusion, it won't change the primary conclusion of the paper.

Michael: Absolutely.

Michele: That's the criteria.

Vincent: Wasn't this the first open access journal at ASM?

Michele: It was.

Vincent: Yeah. That was one of the, one of the parts that was unique. And then of course, there is this fast way, well not fast, but authors can solicit their own reviews and get them all together and send them in. What is that called? Author Direct or something like that?

Michele: So that's M Sphere Direct. You can do that for MBio, they do have that opportunity available to people who are members of the American Academy of Microbiology.

Michael: That's one of the perks of being a fellow of the academy.

Michele: But make no mistake, you still go through rigorous peer review, and because the reviewers names then are attached, I like to go for the real thought leaders in my field to make sure that my work is correct, but also then I get their stamp of approval on my manuscript. So I think that is a way to kind of elevate it as well.

Vincent: What I think it does that is really important, it reviews a paper whereas at many other journals you can't even get a review, right? And this way you send in the reviews and they make a decision, yes or no, and you are way beyond the other journals which wont even look at it for whatever reason. That's important.

Elio: Well, I give you a flavor of what the MBio goes in the editorials in this issue. There is an article by Casadevall and Fang called Elegant Science. We all know that when we like an experiment, really really like it, we say it is elegant. And so they go into what does it mean, and it's a beautiful article, I would recommend anybody to read it for the fun of it.

Vincent: Cool. Alright, thank you Elio. Now onwards to a snippet.

Michael: A snippet! And that's me! I'm gonna take us through this journey today of a paper entitled "Culture independent discovery of the malacidins as calcium dependent antibiotics with activity against multidrug-resistant Gram-positive pathogens" that appeared recently in Nature Microbiology, and it is an open access publication, and it is by Bradley Hover, Song-Hwan Kim, Micah Katz, Zachary Charlop-Powers, Jeremy Owen, Melinda Ternei, Jeffrey Maniko, Andreia Estrela, Henrik Molina, Steven Park, David Perlin, and Sean Brady. And they are at the Laboratory of Genetically Encoded Small Molecules at the Rockefeller, as well as the Proteum Research Center also at Rockefeller, and the Public Health Research Institute at Rutgers. They had me at the

title with this paper. Culture independent discovery of malacidins, or should I say, the original name for malacidins was metagenomic acidic lipopeptide antibiotic cidins.

Vincent: Wow.

Michele: Yuck.

Michael: I was so glad they shortened it to something I could pronounce.

Michele: Plus mala, that's got a root, I don't know whether it is in Latin or what that means ill, right?

Michael: Yes.

Michele: Malady.

Elio: They tell you what the origin of the name is, it means metagenomic acidic lipopeptide antibiotic-cidins.

Michael: Yes.

Elio: So it's an abbreviation. MALA.

Michele: And a clever play on words.

Michael: So they start their story with a sobering statistic, that by the year 2050 the untreatable infections in the United States are expected to rise more than tenfold. Now in 2013, the CDC released a report telling us that each year alone the United States has more than two million people who will get an infection that is resistant to an antibiotic. And of those two million folks, at least twenty three thousand of them will die from that infection.

So if you just do the simple math, as a result, by 2050, a short approximately 30 years away, we are going to see a million, a minimum of twenty million antibiotic resistant infections with about a quarter of a million people dying. So now we all know the significance of this work. I mean, it's pretty incredible when you consider this sobering statistic. And we know that the golden era of antibiotics that started about in the 1950s with the discovery of new antibiotics and being able to treat anything with these wonder molecules was a consequence of individuals being able to culture microbes and recover their natural products that conferred these remarkable antimicrobial properties. And many of these natural products were worked up and then were converted into the antibiotics that we use today to treat infections.

So listeners of TWIM will appreciate that the microbial world is principally undiscovered, undescribed or even characterized with estimates ranging that we have only tapped about 5% or been able to culture about 5% of the microbes that presumably inhabit planet Earth. So we get about 95% of them out there that we know nothing about.

And more importantly, of the 5% we are able to culture, only just a fraction of the chemistry encoded by these cultured bacteria make detectable antibiotics in their fermentation and culture liquor, leaving the majority of the potential of the natural products literally hidden in the global microbiome. So the story in today's snippet is a journey of discovery to assess the potential or in the case here these hidden natural products. And so what the authors' take us through is they have developed a culture independent natural product discovery platform that involves something as simple as sequencing dirt, followed by informed bioinformatic analysis, then the heterologous expression of biosynthetic gene clusters captured from their DNA that they cleverly barcode, and then they extract from the environmental samples the genetic information so what they inferred in silico, they

can actually assess whether or not it indeed does encode a natural product that we potentially will be able to use as an antibiotic.

Elio: Michael, can I interrupt and ask you a question?

Michael: Sure.

Elio: Why did this escape previous discovery? I mean, people have been looking at the soils and dirt and so forth for new antibiotics forever in a very intensive way. In Japan, there is a whole institute designed to just do nothing but, in this country there are lots of labs, lots of pharmaceutical companies have been looking. How did this escape?

Michael: I think it is because it is the culture independence that enabled it to work. Condensing it down to the end.

Michele: And they're not even worried about purifying a particular species, it's just, let's take the whole population and search for sequences that we have good reason to believe could make a great antibiotic and then use DNA methods to, it's very cool.

Michael: So, if you will, they are effectively metaphorically asking Siri or Alexa whether or not a particular sample of dirt will be able to synthesize a novel antibiotic. So we have a commercial, hey Siri, is there an antibiotic in this dirt? That's what they are doing.

Vincent: (laughs)

Michele: Or the potential, the potential to make it.

Michael: They actually take it all the way--

Elio: You're being very funny today.

Michele: (laughs)

Michael: They take it all the way through. So in their query of this new series Alexa platform, and I think that's really the crux of their approach, is they actually did informed probing of the very complex environmental samples, and they had two thousand of them. So in their query, the malacidins are significant because they are active against multi drug resistant pathogens, and in their characterization of the compounds that came out of this screen, they observed that these calcium dependent malacidins were effectively able to sterilize.

So they take it all the way to asking the question, okay, we are going to make a product, will it have activity? Not in a Kirby-Bauer test where you put the antibiotic on a plate and you do diffusion, they take it straight to the animal and ask whether or not it is able to treat an animal. So they have a classic wound model that they inflict on rats and they punch out a hole in the rat and then they add 500 CFUs of bacteria to it and they add their compound that has come out. They ask the question, how many bacteria are there?

And what is remarkable is their malacidin was effectively able to sterilize methicillin resistant Staph aureus skin infections in an animal wound model. And then they further take it the next step, and granted they justify that this is only in the lab, and they did not observe any selection for resistance at this point in time.

Elio: Is there any reason for believing that is unique? I mean is it unique, why is it unique?

Michael: It's their approach. They've effectively taken a platform based approach to this.

Elio: I understand that, but I am asking why are these drugs, why have these bugs not become resistant to these drugs when they become resistant to every other drug?

Michael: I'll get to that, and I think it is a function of where the malacidin is in what it is actually inhibiting in the cell, because of where the malacidin inhibits, and it's principally jumping ahead at cell wall biosynthesis and moving the growing lipopeptide into the growing peptidoglycan. That's effectively its target.

Elio: Yeah, but known antibiotics do the same thing, or something analogous?

Michael: It's analogous to vancomycin.

Elio: Exactly.

Michael: If you recall, it took a long time for resistance to emerge in to vancomycin. So I think a resistance will emerge but it may take time. So let me talk a little bit about these calcium dependent antibiotics. So a good example of the calcium dependent antibiotic is daptomycin, which if you have ever had an individual who has needed daptomycin, it's about $360 per 500 milligram dose before compounding. So then they have to compound it so that by the time you get the bill at the hospital it's like a thousand dollars a dose. And so daptomycin is a lipopeptide antibiotic and it was originally isolated from Streptomyces roseosporus in the late 80s by the folks at Eli Lilly. And this drug ultimately became the founding antibiotic of the pharmaceutical company Cubist, which is now a merc subsidiary and is sold under the trade name Cubicin.

So the mechanism of action, I think this will get to your question, Elio, is that this class of antimicrobial works remarkably by disrupting multiple aspects of bacterial cell membrane function by inserting itself into the membrane, specifically at the phosphotidoglycerol level, where it aggregates, changing the shape of the membrane, which results in holes forming, resulting in ion leakage. So you collapse the membrane potential, which by itself kills the cell. And when you do that, the cell can no longer deal with the toxins that are being produced as a consequence of normal metabolism.

So you generate free radicals inside the cell that in turn cleave the nucleic acid in addition to having your cell contents leak out. So these calcium dependent natural product antibiotics were of interest to this group because individual family members of this class of antibiotics have been shown to have two distinct modes of action. Either by targeting the cell wall biosynthetic genes involved in sliding that peptide of an acetylmureinic acid with the peptide across the cell in the growing peptidoglycan chain, or like daptomycin disrupting the cell membrane.

So, any time there is new tech released to the community, there is always an unboxing video on YouTube. So today on TWIM we are going to unpack what these guys did with this new technology. And I think that some of the tricks they are using will help others inform how they may go hunting for these biosynthetic gene clusters that may do whatever people are looking for. So given the differences in the modes of action of these calcium dependent antibiotics, the authors hypothesize that there is a four peptide calcium binding motif and it confers aspartic acid any amino acid, aspartic acid and glycine, that might be indicative of a broader collection of uncharacteristic bacterial encoded antibiotics. So they have their target. And this is important because it is this observation that helps inform them how they are going to build a search algorithm so that if you will in the Siri Alexa metaphor, Siri knows what to hunt for out of this complex list of music that we refer to as the metagenome. So it is literally going to pull the song that the authors are looking for.

Elio: That's very good.

Michael: They have a lot of songs to pull from. So it's a pretty straightforward process. Step one, get some dirt. And this is their key. They undertook a sequence guided screen of a large number of environmentally ecologically and geographically or geologically I should say unique soil samples. So they got 2000 soil samples. Step two, you design the search parameters, and here again is where they are informed understanding of how this calcium dependent antibiotic works because they knew that these drugs are biosynthesized by non ribosomal peptide synthetases, which is this aspartic x amino acid, aspartic glycine.

So their approach was based on the strategy that relied on barcoding, and barcoding is the only way we are ever able to do any of these metagenomic assemblies of all this complex DNA that we are sequencing today. So they have barcodes of biosynthetic genes using generate polymerase chain reaction. And again, here is the trick because aspartic acid only has two codons and glycerin only has four codons, they can make redundant PCR primers to effectively help them to hunt, and the PCR primer is tagged with the barcode. So they can effectively zip it out.

So they surmised and they used a very conservative estimate, they said that your average dirt sample only has a thousand unique bacterial species per gram. Norm Pace postulated that the average good garden soil has about ten thousand, so they are really using a conservative estimate. And so, they sequenced it and what they learned is remarkably seventy five percent of their dirt had natural product sequence tags that mapped to at least one non ribosomal peptide gene. And they are specifically looking for this adenylation domain. And it is known for these, it is the hallmark of the calcium dependent antibiotic biosynthetic gene cluster. And I think the reason these large groups that have been screening soil samples is now we have enough sequence information that we are able to infer things and you know target or bias our selection or our hunt.

And so what they concluded from the sequence and the genetic analysis is that the majority of the lipopeptide antibiotics encoded by the global soil metagenome is likely uncharacterized. Even with their large soil collection, they were only able to capture a fraction of the biosynthetic diversity that exists within this calcium dependent antibiotic family. So step three, what did they catch? So again, using this non ribosomal peptide synthetase gene as their target, they tweaked the algorithm and then it happens of where they are because they are at the Rockefeller, the Rockefeller has this environmental surveyor of natural product diversity systems that was specifically developed to evaluate metagenomes derived for natural product sequence tags, and they were able to track the calcium dependent biosynthetic gene clusters.

And so their analysis revealed numerous clades not associated with known biosynthetic gene clusters. So they found something new, indicating the existence of uncharacterized calcium dependent antibiotics in the soil genome. So step four, you make the product. And as Michele already remarked, they are just using old molecular biology techniques to do this. They effectively have the barcode, they fish out the DNA, they clone it into a cosmid library, they have a 20 million membered library, they put it into microtiter plate, each well contains 20,000 unique clones, and here it goes back to why we can do it now. You could not have 20,000 unique clones on petri plates! You just don't have enough lab space anymore. But in a microtiter plate, you can. So to find the needle in the haystack, again the trick is the barcode.

So now they express the combination of the genes where they fish them out, and they screen for activity. So I hate to keep beating the Siri Alexa metaphor, but it is indeed the beauty of their system. They found that malacidin biosynthetic gene clusters were recovered on three overlapping cosmid clones. And then through the technology that has been around for probably 30 years, they compiled if you will each gene cluster from the three clones was effectively like a movement of a symphony. So they effectively put the three movements together and they concatenated them, cloned them into Strep albus, which is the standard cloning vehicle for these genes in order to get them properly expressed, and they simply asked the organism to play the symphony.

And what a symphony it was, because then they just ask, what did they learn about the antibiotic that they made? And that is where it gets really cool, because they did indeed make one of these calcium dependent antibiotics. And it's really pretty neat of how they put it all together and their figures are crystal clear, and they first show the malacidins are broadly active against Gram positive bacteria. They show the MICs against a large number of pathogens and they show the spectrum of activity of the malacidins against these nasty Staph aureus, multidrug resistant Enterococcus faecium, Strep pneumo, Strep mutans, B subtilis, all the Gram positives. It doesn't have very good activity against any of the Gram negatives, the only one they checked was E coli, or yeast for that matter. And so, the next tested the en vivo effectiveness of the malacidin class using the animal wound model that I have already described. And here, this is the really cool part. First they demonstrate calcium dependence and so as you increase the calcium concentration, the MIC levels drop, which is expected because it is calcium dependent. And the animal wound model, they add malacidin and it literally all the bacteria, the 500 they put on are literally gone within 24 hours. The vehicle continues to grow and daptomycin is also effective in killing them.

Elio: So we have really a possibility for a new class of antibiotics where resistance may appear slowly. Isn't that sort of the take home lesson?

Michael: Correct, that's the take home message summing it up.

Elio: Alright, that's great.

Michele: Michael, I was thinking about this strategy, I agree it is really elegant and it is laid out beautifully in their Figure 1. If I were about to embark on this, the bottleneck I was going to worry about was once I pulled out this unique piece of DNA from the dirt, how could I be certain that I could find a bacterial species that would express this whole locus and make a product? So you said that Streptomyces albus is a standard vehicle? So tell me more about that.

Michael: It's the standard vehicle used to express their overarching hypothesis is that they are dealing with soil microorganisms that produce antibiotics, and the class of microbe that produces most of the antibiotics is Streptomyces.

Michele: I see.

Michael: And so the institute has done a lot of work on developing systems to effectively do exactly what you are saying, since this was a snippet I didn't want to go into why this strain and how they moved the DNA in, which was principally through conjugation. You couldn't do this in E. coli.

Michele: But it's like the E. coli K12 of antibiotic producers? I see. Okay. So I also wanted to remind the audience, some people who are not microbiologists might be wondering, why did they start with soil? And of course, antibiotics evolved as tools to equip microbes to protect their niche from competitors. So antibiotics have been around long before pharmaceutical companies.

Michael: Absolutely.

Michele and Elio: (laughs)

Michael: And there are a lot--

Elio: I think we are going to hear much more about this, won't we?

Michael: I do. The beauty of the system, and since it's only a snippet I didn't go into the mechanism of how they are working, but they finish off the paper with how the mechanism of action of this antibiotic. So the summary is they developed a novel Siri/Alexa like platform to interrogate complex environmental metagenomes for uncharacterized antibiotics by simply tracking natural product sequence tags that differ from those associated with known antibiotic gene clusters. So.

Elio: Beautiful.

Vincent: Thank you, Michael. I have to tell you, my wife used to work for a major pharmaceutical company and when we, years ago, when we went on vacation she would bring a kit with her and she would sample soil wherever she went.

Michele: Cool.

Vincent: It came with a little shovel and a Ziplock and she would take the GPS coordinate of the sample and then bring it back and then they would try and culture something out of it, right, that's why this is different. And then if it was a hit they would have the GPS, they could go back and get more if they needed it.

Michele: That's so smart.

Vincent: They don't do that much anymore.

Michael: To get more dirt. Well, it's Selman Wakman who was the great pioneer, anytime you send people, you effectively came back with dirt and in fact in the early days of antibiotics, I heard stories about how you would write a colleague at a manufacturing facility and then you would try to culture the organism off of the envelope when he sent you the envelope back.

Elio: You put the envelope in a warm blender?

Vincent: Yes (laughs) Wonderful.

Michele: And we should point out, we still need people to go out and collect samples from all kind of weird environments because that is where we might find something new that hasn't been discovered previously.

Michael: That's the key. They had 2000 complex soil samples. 2000, that's a lot.

Vincent: Yep. Alright, thank you.

Michael: You're welcome.

Vincent: I want to tell you about the upcoming meeting called ASM Microbe, the annual meeting of the American Society for Microbiology. Anyway, this year it is in Atlanta, Georgia and ASM has a special opportunity for our podcast listeners. This is not food, this is not hard drives, it's not videos. What it is, get $50 off registration for Microbe 2018, which is June 7th-11th in Atlanta using the promo code ASMPOD. ASM Microbe 2018 connects scientists with their science and showcases the best microbial scientists in the world. Delve into your scientific niche in eight different tracks. Visit asm.org/microbe, that's asm.org/microbe and use the promo code ASMPOD, all one word, for $50 off registration. When you go to register, you will see, do you have a promo code? You type in ASMPOD. Now we have a paper from the Journal of Bacteriology.

Michele: And I will present this. The title is “Translocation through the conjugative Type IV secretion system requires unfolding of its protein substrate.” And it’s by Martina Trotker and Gabriel Waksman from the Institute of Structural and Molecular Biology and the Department of Biological Sciences at the University of London and Birkbeck. And it was published this month in the ASM Journal of Bacteriology. So as we heard from Michael--

Elio: Which is a reputable online journal that has been appreciated by microbiologists for generations and which is not getting quite the play that it ought to in term of submissions. So here’s a plug for, send your manuscripts to the Journal of Bacteriology.

Michele: You get really great reviews back from experts in the field. I can complain and attest to that (laughs)

Michael: (laughs) We all can. We all can.

Michele: So as we heard from Michael, antibiotic resistance is a huge problem and the number of deaths that we are seeing now and are worrying about in the future is due to the ability of this nasty trait to spread. And it can spread horizontally on mobile genetic elements, pieces of DNA that can efficiently move from one bacterial cell to another. So one type of mobile element that spreads antibiotic genes, antibiotic resistance genes, are so called conjugative plasmids. These are self sufficient DNA molecules that encode all the machinery that molecule that DNA molecule needs to get out of one bacterial cell and into a neighboring cell. And to perform this trick, these conjugative plasmids can direct their host cell to assemble a needle, a big apparatus, that traverses from the cytoplasm of the resident cell across four membranes into the cytoplasm of the neighboring recipient cell.

So the secretion machinery then builds a tunnel through which the plasmid DNA can slip from one cell to another. And we call this process conjugation and it is common for many gram negative bacteria, gram positive, and even some archaea. But what we know from a lot of genetic and biochemistry experiments is that these DNA molecules can’t make the trip by themselves. Instead the DNA cargo is directed through that needle by a large protein complex that has been called a relaxosome. So what actually slips through the tunnel is single stranded DNA molecule that is bound covalently to an enzyme that is called a relaxase. And that enzyme beautifully works both in the recipient cell and in the donor cell, so it is a very efficient way for the DNA to bring what it needs to function in the next cell.

So in a paper that Waksman and his colleagues published in Cell in May of 2017, they used sophisticated imaging and biochemistry to describe in detail how this relaxase enzyme interacts with its DNA cargo and performs this magical work of moving the DNA into the tunnel of the Type IV secretion system, so they know that one domain of this relaxase enzyme will bind to the target DNA at what is called the origin transfer. It will nick the double-stranded DNA and then by a nucleophilic attack attach itself to the five prime phosphate that has been liberated on that cleaved DNA. And then a second domain of another subunit of the same enzyme is a helicase so named because it can unwind the double-stranded DNA by first wrapping completely around it's target ori T DNA. Once this helicase is clamped on, it can untwist the target DNA at remarkable speeds. 1100 base pairs per second. (laughs) So at that rate--

Michael: That is incredible.

Michele: Isn’t that amazing? It’s hard to even wrap my head around. But that’s efficiency that the entire large conjugated plasmid can be unwound and transferred in just a couple of minutes. So the Waksman group in its previous work were able to use site directed mutants and look at 3D structures and figure out how pairs of this relaxase enzyme cooperate to export single stranded DNA bound to the relaxase through the needle and then reconnect the free ends of the single stranded DNA to reform the plasmid.

So because we know from genetic experiments that this relaxase activity is absolutely required for transfer of DNA and therefore antibiotic resistance from one cell to another, this could be a great target for drugs that block the spread of antibiotic resistance. So that is part of the motivation for trying to understand this in mechanistic detail. Not only that, but a lot of bacteria use similar Type IV secretion machines to manipulate the host cell that they either are bound to or that they are inside of. So for example, the gram negative bacterial pathogens Legionella pneumophila and Francisella tularensis, both can manipulate the cell biology of their hosts by using Type IV secretion systems to put bacterial proteins into the cytoplasm and alter the host response. Or Helicobacter pylori, Bordetella pertussis, they can use Type IV secretion systems to deliver protein toxins to host cells. And then one of the best studied is Agrobacterium tumefaciens which is a plant pathogen and it can trigger formation of crown gall tumors on its host plant.

So these are really widespread machines and we know that they can do a lot of damage and transfer antibiotic resistance, but there are still some details that if we understood we might have a better opportunity to design rationally drugs that interfere with the process. So in this new paper, Waksman are tackling the mystery of whether this tunnel of the Type IV secretion system can accommodate the globular relaxase enzyme or whether like its DNA cargo the bound enzyme first has to be unfolded so it can slither through the needle. And knowing this confirmation would then allow them to deduce some of the mechanics of this tunnel that is able to spread antibiotic resistance. So that's the question they are after. Any questions at that point or comments?

Vincent: Michele, so if a plasmid does not encode its own machinery for transfer, how would it get, would it utilize the host?

Michele: One way is if the bacterial cell is lysed or naturally lyses and then DNA could be picked up by natural competence.

Vincent: Okay.

Michael: Or sometimes the plasmid can integrate into the genome of the organism and then depending on the number of IS elements in the cell and transposases, then you have transposon facilitated transfer.

Michele: Right. So instead of a plasmid, a transposase could pop, yeah. So as a tool to try to better understand what form the proteins are when they slither through this tunnel, they used a well studied conjugated plasmid R388 and they designed a really clever genetic assay so that they could quantify transfer of the protein from one cell to the next. And the trick was, they started with recipient cells that carry a gene that encodes resistance to chloramphenicol, but in the middle of that chloramphenicol resistance gene is another antibiotic resistance gene that confers tetracycline resistance. So these bacteria are resistant to tetracycline but sensitive to chloramphenicol. However, the tetracycline resistance cassette is flanked by sites called LoxP sites, which are recognized by a recombinase enzyme that will cut it out and release that tetracycline resistance gene, and it this case by doing so it would restore the function of the chloramphenicol resistance gene so the cell would now flip to being sensitive to tetracycline but resistant to chloramphenicol. So it sets up a plate assay for quantifying the percent of cells in a population that go from being tet resistant to now becoming chloramphenicol resistant.

Michael: And our molecular biology users have probably heard of the Cre Lox recombinase technology that a lot of labs have used in neuroscience and in other, and in fact the Cre Lox system has been used by a lot of neuroscientists, I've sat through many a neuroscience seminar listening all about the wonder of Cre Lox not knowing that it came from bacteria.

Michele: Yeah. And I think this is a really elegant application. So the way they exploited it is that they designed chimeric genes that encode both the Cre recombinase and then they fuse it to their enzyme of interest, which

is this relaxase that is key for conjugation. And therefore they could measure how efficiently relaxase is delivered by scoring appearance of chloramphenicol resistance. So first they had to make sure that their fusion protein worked as they intended, so they made three different versions, either the Cre was fused at the front, in the middle, or at the end of the relaxase enzyme, and that is shown in figure one.

And then to test those, they put the genes encoding each of these fusion proteins directly into E. coli by just direct transformation, and verified that in fact all three when expressed in the recipient could liberate that tetracycline resistance cassette and restore chloramphenicol resistance. So they knew that the tool would work if it was expressed in the cell, and that is shown in figure 1B. But then the real experiment was to ask, can these proteins, fusion proteins, be translocated by conjugation?

And what they found is that two of the three versions were able to transfer by conjugation their fusion protein, and that is shown in figure 1C. So that allowed them to now use this as a tool to alter their reporter fusion protein by adding domains that would either allow it to unfold or prevent it from completely unfolding into a skinny protein that could slip through the tunnel. So the strategy that they used took advantage of some proteins that had been previously characterized. One is gfp, which is well known to retain its 3 dimensional structure when it is in cells. And the other is a smaller protein, ubiquitin. And the beauty of using ubiquitin is, number one, it's small, so you don't worry about it perturbing 3D structure of the reporter. But there are also, it's well known to hold its structure very well, so being very stable. And a couple of mutants had been described that destabilized the protein so it could unfold when it is expressed in a cell.

So they had a ubiquitin that resisted unfolding and another ubiquitin variant that would unfold. So they popped these ubiquitin modules into their reporter with the Cre recombinase and the relaxase, and those constructs are shown in figure 3A, and again they verified that addition of the ubiquitin or GFP domain did not perturb the recombinase activity, and they then could do their experiment. So they asked, when the bacteria translocate or attempt to translocate, the Cre fused to the relaxase protein. If it was able to completely unfold, they saw that 10% of the recipient cells had received the protein. But if they used the reporter that could not unfold because of just two amino acids were changed in the ubiquitin moiety, now the relaxase and recombinase activities were translocated a hundred thousand times less efficiently. So they conclude then--

Michael: That's the beauty of their system, they are measuring the efficiency down to ten to the -7.

Michele: Orders of magnitude sensitivity in this assay. It's beautiful. They're just pouring colonies on a plate. It's very elegant and a great way to teach these concepts I think in a bacterial genetics class.

Michael: The other thing they use is they graph properly. They're using semi log plots.

Vincent: (laughs)

Michael: That's one of my pet peeves is because this is a population effect. You have to respect the math.

Elio: (laughs) That's true.

Vincent: Yeah.

Michele: Yeah, and they do have statistics. It's very convincing data.

Michael: Oh, yes.

Michele: Now, because they were using a somewhat artificial system with these fusion proteins, they wanted to test their interpretations more rigorously by going back to the native relaxase protein. So they used a similar strategy but now use the native protein as the template. And this is shown schematically in figure 4C. I'm going to focus on that. They again used these two different ubiquitin motifs, one that could unfold and the other which held its shape. And again they find about a thousand fold less frequent transfer of the relaxase protein if it was unable to completely unfold.

So again, consistent with their interpretation that not only must the DNA unwind to slip through this tunnel, but the covalently bound relaxase enzyme also must unfold. So that is the mechanistic conclusion they were able to draw from this series of really elegant bacterial genetics experiments that relied on using these clever fusion proteins and then also drug cassettes to rapidly score whether or not the protein had been translocated. So I will just add that this now gives them an opportunity to focus on the relaxase enzyme and better understand how its function can be perturbed for therapeutics and also I want to emphasize that a similar conclusion was reached by a study of a different type 4 secretion system, and that is work from Ralph Isberg's lab who studies Legionella pneumophila. And they also found in a paper published in Infection and Immunity that addition of domains that don't readily unfold will also inhibit translocation of virulence factors that Legionella deposits into the host cell cytoplasm and it will interfere with replication of those pathogenic bacteria. So in a completely different system, they came to the same conclusion that the cargo proteins need to be in an unfolded state in order to slip through this type 4 secretion needle and spread.

Elio: It's quite a paper, actually.

Michele: I would call it elegance but I will have to read Arturo's definition of elegant in MBio to make sure I'm not misusing it.

Vincent: (laughs)

Elio: I also think the take home message should be for people who are not necessarily in the field?

Michele: I think this is a great example of how you can use the combination of bacterial genetics and sophisticated imaging technology to deduce how these tiny machines work, and these tiny machines are really major contributions not only to the spread of antibiotic resistance but also the capacity of bacteria to cause disease. So if we want to design a different class of therapeutics, first we need to understand how these machines work, then we can pick targets and begin to use rational drug design to try to interfere with them.

Vincent: Michele, why not instead of having to unfold the protein, why not make the secretion system bigger?

Michele: That's probably a question for somebody who can do energetics calculations (laughs)

Michael: And membrane real estate because, remember, most of the membrane is dealing with the electrical load of the cell. You have to, because they are putting a million to ten million electrons into that membrane and so they need the square footage to deal with their electrical load. And so they want to be very economical in their export process, and about forty to fifty percent of the protein in the cell is actually destined for someplace other than the cytoplasm. And so it has got to be very efficient. And in this case because it is not only you are moving a protein but you are moving nucleic acid, the fitness benefit is actually quite remarkable.

Vincent: So is the relaxase refolded upon entering into the cell?

Michele: It is, and what is convenient of course is the DNA molecule has got the enzyme that it needs to rejoin its raw ends. It has got it covalently attached so that it increases the efficiency of that reaction in the recipient cell.

Vincent: That's very cool.

Michael:: And because that it is single stranded DNA that is coming in, RecA immediately begins to coat that single stranded DNA as it comes in and then it effectively protects it especially if it hasn't been tagged with the right restriction pattern, so the restriction and the nucleases that are normally the if you will very primitive immune system don't go trashing this new information that has come into the cell. So there is a reason single stranded DNA is ideal for moving information, because RecA will coat it and then once RecA coats it it will then replicate the second strand which then tags the DNA as self, if you will, and you can effectively acquire the information in this recombination system and again, a Rec dependent process.

Vincent: RecA being a single stranded DNA binding protein, right.

Michael: Yes.

Vincent: Which I remember from my grad student years.

Michael: Yeah, because all your cloning strains were effectively, I mean RecA drove me crazy.

Vincent: There were mutant strains lacking RecA, right.

Michael: Yeah! Yeah. Recombination.

Vincent: Because you want to allow any DNA to get into the cell, right?

Michael: Right, exactly. You just have to open one of your favorite cloning catalogs that sell you restriction enzymes and look at the genotypes of the strains that you are using to clone your DNA and it's a good way of refreshing some of your genetic knowledge every now and then.

Vincent: Very cool, as you say it's a lovely way of assaying this, just great. You would think oh, we have to do physical measurements, but you don't. You can make readouts that are beautiful.

Michele: And then deduce the biology from the patterns.

Vincent: It's very cool.

Michael: Well, Jon Beckwith pioneered the use of fusion proteins to do a lot of really elegant bacterial genetics work, and I was glad to see fusion proteins coming back to study something a little bit different. And they did it so beautifully.

Vincent: I used to have a colleague here who came from Beckwith who used to do that, Howard Shuman.

Michele: Howard Shuman, yeah.

Vincent: Alright, thank you, Michele.

Michele: My pleasure.

Vincent: Elio, do you need to go?

Elio: I will say goodbye.

Vincent: Alright.

Elio: I will see you next time.

Michael: Thank you, Elio.

Vincent: Thank you, Elio. We have some email. The first one is from Shishir who writes:

Hi to all the TWIM presenters. I enjoy TWIM immensely and use the information in my teaching activities. The discussion of food washing was interesting, as were other topics. This reminded me of one of our studies on coriander, cilantro, where we detected Salmonella and Shigella in unwashed specimens. The paper is under publication in BMC Research notes, soon to see the daylight. The advantages of nanoparticles as vaccine vector will be an enormous boost to vaccination in countries like Nepal. We eagerly look forward to the development. Keep the good things coming.

Shishir is in the Manipal College of Medical Sciences in Nepal. Cool.

Michele: Nice to hear from you.

Vincent: We have a reach, coriander. You have to wash it because sometimes you don't cook it, right?

Michael: You don't.

Michele: Most times.

Vincent: Most times you put it on salads.

Michael: It's in salsa.

Michele: Tacos.

Michael: That's what gives salsa its salsaliciousness.

Vincent: Yeah, so that's a problem with things like bean sprouts, you don't cook them. You get problems with that. Michael, can you read Neeraj?

Michael: Dear TWIMers, this is Neeraj, a long term listener of TWIM but first time emailer. I've been more active on TWIP and TWIV as far as writing to them is concerned. I just listened to the enlightened discussion on flu vaccine that you folks had on TWIM 170. After hearing it too, I resonate with Elio's disappointment at the fact that even after years of toiling, we still don't have a long lasting and effective universal vaccine against flu. This year has been particularly bad and I have some close friends who have been really hit hard by it, which is quite sad. But even after reading about the recent developments and really novel approaches taken to make a more robust and broadly protective vaccine, it is somewhat frustrating to note how poorly protective at times these vaccines are. I do realize it isn't a trivial problem to overcome, but given the experience and expertise and technical advances, the scientist in me thinks that I am sure we can do better than what we have. And in this

regard, it is absolutely great to listen to what is being done in the field through the scrutiny of TWIM, thus in addition to the paper discussed about the nanoparticle based flu vaccine, there is another study that recently caught my attention which I am sharing a link below.

I'm sure Vincent and Ray will put it into the show notes and it was a paper that was in Science.

So unlike the story that was discussed circling around generation of nanoparticle based flu vaccine, the authors of this study did cite directed mutagenesis to generate flu strains which are inept at suppressing interferon production, thereby helping in mountings an efficacious immune response, and in doing so they have utilized all the knowledge we have gathered over the years and understand the mutation landscape that the flu virus scans to overcome immune surveillance in vivo. Although these studies are important and provide novel insight academically, oftentimes the method of generation for them can be highly tedious and practically nonscalable, and thus it is something being part of the vaccine development company we constantly worry about.

Don't get me wrong, I don't mean to put a damper on these efforts, but just to point out the reality in manufacturability of products which takes a whole new dimension once you start engaging with it. Personally, I hope someone some time in the near future we will be able to generate if not one then maybe a concoction of these vaccines that could help generate broadly neutralizing antibody. In the end, even though I am obviously biased, I think vaccines are the most important or the most natural way to boost our immunity and confer protection, and being an eternal optimist, I am confident that it is similar to many challenges that the human race has endured, flu shall be conquered too. So with that, here's wishing everyone a big fun weekend ahead, and hopefully the markets will settle down a bit, too. Stay warm and drink cold beer. Best, Neeraj.

So Neeraj is a scientist too at SutroVax.

Vincent: So I did see this paper, Neeraj, and it's really interesting and maybe we will do it someday, either here or on TWIV. There's so many good things to do.

Michael: Flu I think is really caught the attention of everyone this year. We were especially hard hit here in South Carolina and I know simply because I've been teaching a class of 100 and watched how many of the young people have actually succumbed to this virus this year. And I've been lucky, the vaccine and me seemed to have protected me somewhat even though I've been exposed many times with the students coming down. So I've been thankful.

Vincent: Yeah, the vaccine is not great, we all know that, but it's what we have at the moment. It does make disease less serious, there's no doubt about that, so you should take it. But you know, uptake in this country is less than 50% and I don't understand it because really a lot of people are going to die this year and most of them are not vaccinated, so.

Michele: Wow. And it's gotten much easier to get your flu vaccine, right? Go to the local drugstore.

Vincent: Yeah.

Michael: You don't even need a prescription any more.

Vincent: Go to the drugstore, yep.

Michael: Just walk in.

Vincent: So Anthony writes, was the Black Death caused by a virus? This also argues against spread by rats. He sends an article that appeared in the New York Times which is very nice long article by Mark Derr. New theories link Black Death to Ebola-like virus. (laughs) But at the end of the article, a quote by Joshua Lederberg. Yersinia still seems to me the most reasonable assignment as the cause of the Black Death, but I say that with less than unshakeable conviction. Now this is an article from 2001.

Michael: So Josh actually wrote this response, unfortunately Dr. Lederberg has passed but in 2001 he was still around.

Vincent: Yeah, we talked about that last time, I guess that's what brought this to Anthony's mind. Michael or Michele, why don't we think that a bacterium caused the Black Death? That's the leading theory, right?

Michael: I think there is evidence from bones that suggests that Yersinia certainly was there. The question is we can never recapitulate it today we know that Yersinia pestis does indeed cause plague, I mean we see plague out west in the United States and of course in Madagascar and other places, and it is around, and in fact pneumonic forms have been reported. So I think the disease that is described in the literature of the times certainly fits Yersinia pestis.

Michele: Do you think a parallel is thinking about the 1918 flu and all the deaths? We used to think it was purely caused by the virus, and certainly the virus was a big part of it, but if you had pneumococcal infection then your death went up, so it could have been some interaction between the two. But you're right, we can't go back, maybe we can, maybe there are samples available to screen by PCR and get some idea of the virome.

Vincent: I think people have found in mass graves, right, they found evidence of Yersinia but it doesn't prove it, right.

Michele: But also now look for some Ebola like virus.

Vincent: The problem is with RNA viruses, the nucleic acid is gone.

Michael: It degrades.

Michele: It's not stable.

Vincent: It's not stable at all. So Michele, I don't know if you were here, we talked about a paper where they implicated human to human flea transmission as spreading that.

Michele: Right, right, yeah.

Michael: That was the last TWIM.

Michele: I found that convincing.

Vincent: Yeah, it was good.

Michele: Jerry writes,

Greetings from Philadelphia! I have been listening to TWIP for a couple years and recently got lured over to TWIM.

Michael: Yay!

Michele: Last week's episode finally convinced me that I need to write in. First off, the snippet was interesting to me because I briefly worked on an enzyme, dopamine beta hytroxylase, that contained cobalt.

Vincent: Looks like it, yep.

Michele: Also, I have a family connection to toxicology, so the idea that an element that is both essential and toxic is fascinating to me. But the main paper on extremophiles living in the Arctic is what really stimulated the imagination. I love it when different experiences overlap each other. Here is another example. Yesterday I listened to a TED talk by Karen Lloyd about microbes that live in the muck at the bottom of the ocean. She talked about the different energy requirements and multiplication rates of those organisms, especially compared to 'normal' microbes that live on the planet's surface. You raised a similar point when talking about how organisms seem to be spending all their energy just surviving and not multiplying. Dr. Lloyd's view is that with such a small amount of energy available to these organisms, they grow at a much slower longer time scale where surface organisms need to grow relatively fast, super fast, to outcompete their neighbors for energy, these organisms grow on a longer time scale since there is less energy to go around. Amazing food for thought. You should look at some of her papers, too.

And he provides some links.

I'm a lapsed bench scientist. While I no longer do basic science for a living thanks to an amazing group of teachers in my academic career, I still think like a scientist. Thanks for a chance to once again have a journal club. If you are giving a book away, please enter me in the contest, I'll enjoy the chance of winning, but if I am lucky enough to actually win a book, please give it to another deserving winner. All I need is bragging rights. Finally, Jerry Salem.

Michael: So we are not giving away a book this week but we can tell him he won, and then he can have the bragging rights.

Vincent: and Michele: (laughs)

Vincent: That's right. We will give one away in the future, for sure. Alright, let's do one more from Sandi.

Hello, I recently started listening to TWIM and now am obsessed. I don't have a background in microbiology, I didn't find out until about a year ago that it was my long lost love. Better late than never. I am wondering what you fine crew can tell me about Ethanoligenens harbinense, which, according to a recent gut sequencing test from the company Viome, is the most abundant bacterium taking up real estate in my gut microbiome.

And Sandy sent a list of all the wily microbes that were detected. Any idea where it may have come from? I can't find much information about this guy on the internet. Any input?

Michael: It's a Gram positive non spore forming mesophilic motile rod, and so the rule of thumb that I always use in the gut is that it is gram positive because Gram positive organisms are less inflammatory than gram negatives, is probably okay and you probably have a good gut. But if it's Gram negatives with all their evil LPS (laughs) it's bad.

Vincent: So this is an interesting--

Michele: Can we tell how abundant it is and whether it's colonizer or it's just passing through?

Vincent: That's a very good question.

Michele: Based on these tests?

Vincent: I've received other similar emails about this company on TWIV because they can tell you what viruses are there and one young lady wrote in and she was very worried that she had a lot of plant virus RNA in her fecal sample, you give them fecal samples.

Michele: And she's a vegetarian. (laughs)

Vincent: I told her it's just passing through, it's probably a plant virus RNA and she had a lettuce virus RNA and a pepper virus so it's just passing through. They don't grow in us. So I guess you could ask the same about the bacteria, right?

Michele: And it's just the DNA, we don't know if there were viable cells.

Vincent: Of course, absolutely.

Michael: But it said it was the most prevalent in it's taking up real estate and the most abundant bacteria, taking up real estate, and its in the family ruminococcaceae, and so it's probably involved in processing the bulk of you know it's a ruminant so it is processing the bulk, it's dealing with bulk. It's effectively harvesting whatever it can out of the system.

Vincent: I searched for this and I found that it is, it can be isolated from sewage which makes sense because it is coming out of the human gut. And it has been used to make bio hydrogen, which makes sense that it would be--

Michael: Hence it's name.

Vincent: Yeah. Anyway, interesting that there are companies that will tell you what's in your gut, right?

Michael: Yes.

Vincent: It's just great times we live in. Alright, that is TWIM 172, you can find it at ASM.org/twim. And if you listen on your phone or your tablet, you use some kind of a podcast player, just subscribe so you get every episode as we release them. And if you like what we do, consider supporting us financially. You can go to microbe.tv/contribute. We have a number of ways you can use to help us out. Send us your questions and comments to twim@microbe.tv. Michele Swanson is at the University of Michigan, thank you Michele.

Michele: Home of the big ten champs in basketball, thank you.

Michael: I knew that was coming.

Michele: (laughs)

Vincent: I was just going to ask you, I saw a tweet or something from you to that effect. Congratulations, very good. More to come, right?

Michael: Well, Michigan's going to the dance, they're going to the dance.

Vincent: The NCAA, right?

Michael: That's right.

Vincent: Do they go every year?

Michael: They try.

Michele: Not every year, but.

Vincent: Well have fun and enjoy, good luck. Michael Schmidt is at the Medical University of South Carolina, thank you Michael.

Michael: Thank you, and we are still undefeated in basketball by the way. We don't have a team, but we're still undefeated.

Michele: (laughs)

Vincent: Congratulations. I'm Vincent Racaniello, you can find me at virology.ws. Thanks to ASM for their support of TWIM and Ray Ortega for his technical help. Also thanks to Ronald Jenkees for the music you hear, he is at ronaldjenkees.com. Thanks for listening everyone, see you next time on This Week in Microbiology.

(music)

Transcribed by Sarah Morgan.