TRANSCRIPT OF PROCEEDINGS

IN THE MATTER OF:)
)
BACTERIOPHAGE THERAPY:)
SCIENTIFIC AND REGULATORY ISSUES)
PUBLIC WORKSHOP)

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BEFORE THE DEPARTMENT OF HEALTH AND HUMAN SERVICES
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IN THE MATTER OF:)
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BACTERIOPHAGE THERAPY:)
SCIENTIFIC AND REGULATORY ISSUES)
PUBLIC WORKSHOP)

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Rockville, Maryland

Tuesday,
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The parties met, pursuant to the notice, at 8:30 a.m.

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C O N T E N T S

P R O C E E D I N G S

(8:30 a.m.)

DR. RANALLO: Good morning, everybody. Sorry about that. Good morning. I hope everybody's up. Okay. So we're going to start on time today. My name is Ryan Ranallo, I'm a program officer here, at NIAID, and I'm going to be your moderator for the entire day today, something the organizing committee didn't tell me before they signed me up for this. Nevertheless, hopefully we'll get through it all day today.

So one thing that I wanted to just note is how in two years, how things have changed significantly since the last time we've held a phage therapy meeting, and so, with that, I think we have a couple of large buckets of topics today, phage engineering being one of them, and essentially looking at phage for different uses, including, you know, transmission and decolonization.

So, with that -- oh, the only other thing I would say is if you have any questions about whether or not your slides have been loaded for speakers, please check in the back. Marcus has been great all day yesterday, and certainly today as well. So for the first talk, it's a tag team talk of Col. Zapor and

Lt. Col. Tyner.

Col. Zapor is the deputy commander of operations at the Walter Reed Army Institute of Research, and Lt. Col. Tyner, who I first met actually when I was a post-doc at NCI and -- in Building 37, is the director of bacterial diseases branch, which just actually happens to be my old department where I spent 10 years at Walter Reed working on enteric vaccines. So, without further ado, I'm going to introduce Col. Zapor and Lt. Col. Tyner for our first talk.

DR. ZAPOR: Okay. Good morning, everybody. Thanks to the organizers for inviting me to speak at this conference. Unfortunately, I'm only here for the morning session because of conflicting obligations, as well as secondary to car problems, but -- so I'll be here until lunch and then depart after that.

As you heard, I'm splitting my 30-minute block with a colleague, Lt. Col. Tyner, so I'll be cognizant of the fact that I have 15 minutes to speak to ensure that he has 15 minutes as well.

So the purpose of this talk, I was asked to speak about potential therapeutic indications for bacteriophages and first thought we would kind of address some of the limitations of the current -- antibiotics and the current problems.

So antibiotics of course have been the mainstay of therapy in the -- for the treatment of infections for decades, but there have been some unintended consequences. Everybody of course is familiar with the issue of the emergence of multidrug-resistant organisms, in some cases extremely drug-resistant organisms, or even pan drug resistance.

Moreover, antibiotics, as effective as they are, are not 100 percent specific. In the parlance of my profession, we unfortunately see considerable friendly fire, especially with the broad-spectrum antibiotics such as the carbapenems, and so oftentimes the antibiotics are effective in eradicating the intended target, but have the unintended consequence of killing benign, or even beneficial, bacteria as well.

This is evidenced, for example, by the emergence of *C. diff* colitis in patients who are on broad spectrum antibiotics.

Other limitations with antibiotic use of course include the emergence of drug resistance. I've already spoken to that. Some types of infections are less amenable to treatment than other types. So infections which involve abscesses or other sequestra, antibiotics generally don't penetrate abscess fluid

very well, some less well than others. Rifampin works fairly well, but there are many other antibiotics that are inactivated in abscess fluid. Aminoglycosides come to mind.

Additionally, the presence of a foreign body can make infections difficult to treat. Foreign body -- we've seen a considerable number, a very large number, of war wounded coming back from Iraq and Afghanistan status post blast injuries with retained foreign bodies. Some of these can be removed surgically, some cannot. Some are intentionally left in place.

Each of these FBs becomes a potential nidus for infection. They get colonized with bacteria, oftentimes bacteria that elaborate glycocalyces or make a biofilm, and there are very few antibiotics that can reliably sterilize biofilms.

Other considerations include patient anatomy. So I gave the analogy or offered the example of war wounded. Patients who have had blast injuries oftentimes have interruptions in their blood supply, they have interrupted vasculature, and all the tissue prior, distal to the injury becomes ischemic, starved for oxygen, starved for blood, and antibiotics can only work where they're delivered, and if antibiotics

are not delivered to vascularized, oxygenated tissue, then they don't work very well. It's very common for us to see patients who have ischemic limbs, necrotic tissue, retained foreign bodies, and antibiotics just don't work very well. More often than not the intervention of choice for those patients is cold steel, for example, amputation, rather than medical therapy alone.

And then there are other considerations such as the rapid metabolizers. We know that there are some patients who just inherently metabolize and inactivate antibiotics and other drugs more rapidly than other patients.

And then we always have to be cognizant of patients who have drug allergies or some other contraindication to antibiotics. So, examples that come to mind, beta lactam allergies, which are fairly common, nephrotoxicity associated with aminoglycosides, associated with vancomycin and so forth.

So, for all these reasons, antibiotics, as effective as they are, as reliable as they have been, they do have their limitations, and, as a consequence, we're forced to explore alternatives.

So what are some of the pros and cons of

using phages as therapy? This is a table I put together with which you may or may not agree. In the pro column for phages there's long history of use. Everybody knows that phage has been used in Eastern Europe for many years, and at one point in time early in the 20th Century, phages of course were available by prescription in this country.

Phages are ubiquitous, they're fairly easy to isolate, they're much more specific than our antibiotics. We don't see that friendly fire, so to speak. Phages potentially are active against MDROs. Probably benign, as far as the patient is -- patient goes.

Phages are bactericidal. At least the lytic phages are. Phages, I think, are gaining acceptance. Certainly in Europe, both East and Western Europe, phages are getting more use and have a wider acceptance. And then phages also provide an opportunity to present an opportunity for us to pave the way in publishing evidence-based, peer-reviewed articles supporting their use.

On the con side, although phages have been used for many years in Europe, there is a paucity of high quality literature. Much of this literature has not been translated. We've got some folks over at the

WRAIR, at the Walter Reed Army Institute of Research, we've asked to translate some of this literature.

Phages need to be propagated under controlled environmental conditions. Phages are highly specific, and so just as that may be an advantage, that could be disadvantageous as well if we're looking at patients with polymicrobial infections, or if we have a phage that's only specific against a particular species or strain, then we may be forced to look at cocktails in order to sufficiently treat a patient with an infection.

Bacteria can acquire resistance to phages. We don't yet know what the host response will be. You know, the role of antibodies formed against phages, for example. I know over at the WRAIR there is a lot of concern about phages being lysogenic rather than lytic, and I know that's a concern from a regulatory standpoint as well.

Phages are viewed skeptically in the United States. I will tell you, as an infectious diseases physician, that a lot of my colleagues are very critical about phages. You know, they see this group as a little eclectic, and phages are a little bit like voodoo.

I don't mean that to sound pejorative or

facetious, but, you know, I'm here trying to tell you from a clinician's perspective how I think we can get a wider acceptance of phages therapeutically. But I know that I've engaged some of my colleagues over at the hospital where I spent 12 years, engaged some of my colleagues over at the hospital about clinical trials, and I get this kind of raised eyebrow response. So that poses a challenge.

So my opinion, for whatever it's worth, probably worth about two cents, we don't know if *in vitro* activity yet portends *in vivo* activity. In other words, if phages will behave or will perform for us in the laboratory as they do -- in the clinic rather, or in the operating room as they do in the laboratory.

Moreover, we don't yet know what the clinical indications might be. I don't imagine there are many people in this audience who are arguing that phages will be effective against every infection conceivable. Rather, what we need to do is identify those in particular clinical indications for which there is a use for phages.

But that said, my perspective at least is the issue of emerging drug resistance forces us to consider modalities and therapeutics that maybe we

wouldn't have considered years ago. So I think our backs are up against the wall, figuratively speaking.

I think that phages amongst the clinical community are most likely to be accepted and considered useful if we offer them as adjunctive therapy: to be used with antibiotics, perhaps with surgery, to be used in situations in which medical management alone is problematic or antibiotics might be ineffective or contraindicated, or -- and I think this is a big selling point, and I know, I believe there's at least one surgeon in the room -- if we can tell the surgeons we have a therapy that may potentially obviate the need to remove infected hardware.

I can tell you, as an infectious diseases physician, I spend a lot of time consulting, or providing consultation with orthopedic surgeon colleagues, and the last thing they want to hear from the ID doc is the hardware has to come out.

That poses technical challenges, both with the removal and subsequent replacement of hardware. And so if we can tell the surgeons we have a modality which may enable the patient to retain the hardware, I think then you're going to get some buy-in from the surgeons.

And lastly, look, whether or not phages live up to their expectations, at least we'll be able to do -- you know, with the experiments we're doing, at least we'll be able to say, you know what, we studied these rigorously, we subjected them to the rigorous scientific method, unfortunately, phages don't work, but we know we did the experiments right, controlled studies, and these were our conclusions.

So what are some of the potential indications? Abscesses and other infections in which antibiotics have limited activity. So one that comes to mind, for example, is osteomyelitis, right? Bone infections.

Mainstay of therapy for osteomyelitis is place a PIC line, give the patient six weeks of intravenous antibiotics, take the patient to the OR, debride the infected bone, all right? And if there's hardware involved, the hardware may have to be removed. More often than not, it has to be removed.

So, boy, it would be great if we could offer phages for the treatment of osteomyelitis. Now there's some intrinsic limitations to that. Ischemic bone is not vascularized and, you know, it may have an issue getting phages there in the first place, but that remains to be seen.

Pocket device infection -- the one that comes to mind would be something like a pacemaker infection. Pacemakers are very common in this country. They're placed in a small pocket over the pectoralis muscle over the chest. When they become infected they generally have to be removed because untreated pocket device infections are potentially very dangerous, as you can imagine.

With pacemaker leads, these go into the myocardium, the heart muscle, and the last thing you want to do is have infected pacemaker leads leading into the myocardium, and so they have to be removed.

I think, as far as this goes, we may end up really looking more for a prophylactic role for phages than a therapeutic role because I think we would be hard-pressed -- we'd have a difficult time selling the cardiologist on retaining an infected pacemaker, you know, while we inject phages into the pocket.

I think it may be an easier sell to say at the time you place the pacemaker in the pocket, why don't we add some phages that are active against the common culprits at gratis: *Staph aureus*, right, or coag-negative *Staph*.

Orthopedic hardware-associated infections such as patients with intramedullary rods, external

fixators, plates and screws -- very common. Since the wars in Iraq and Afghanistan in 2003 and 2001, respectively, the commonest reason for consultation at Walter Reed for ID has been 20 something year-old male, status post blast injury, traumatic amputation, placement of hardware, now with a hardware-associated wound infection.

As I mentioned earlier, telling the surgeons that the hardware has to come out is usually not met, you know, with a good reception. Boy, it would be great if we could introduce a therapeutic that would enable us to salvage hardware.

Burn infections. These are typically associated with very drug-resistant, slimy, gram-negatives such as *Pseudomonas aeruginosa* and some other related GNRs. Maybe there's a role there. Essentially, anything with biofilms. Catheter-associated urinary tract infections. We know that every patient with a catheter in his or her bladder eventually will acquire bacteriuria. That is bacteria in the urine. Many of those patients, most of those patients over time will go on to have catheter-associated urinary tract infections.

How do we treat those? We remove the catheter, we give them antibiotics, and we put another

catheter in, and so it's only a matter of time until they become re-colonized and re-infected. Maybe there's a role for phages there, obviating the need.

The other one I want to address quickly is mesh infection. Surgical mesh, right? You go and you have your herniorrhaphy, you have your hernia repair, surgeon puts in nylon mesh or Gore-Tex mesh, that becomes infected.

Removal is very difficult. It's not as simple as just snipping some sutures and just plucking it out because it gets epithelialized, the tissue grows over that mesh, and now you're looking at an *en bloc* resection. Maybe there's a role for phages there.

And then other potential indications include patients with cystic fibrosis, right? These patients have lung infections, chronic recurring pulmonary infections characterized by very drug-resistant, gram-negative bacteria such as *Burkholderia cepacia*, *Pseudomonas aeruginosa*, and so forth. Extremely drug-resistant -- multidrug-resistant organisms. Some other indications I'm not going to address may be the treatment of patients with bacillary dysentery.

Our priorities at the WRAIR. Right now we're interested in looking at orthopedic hardware-

associated infections. I'm going to hand off to my colleague in a second to talk about some of the experiments we're doing there, and also perhaps using phages to treat patients with *Shigella*, shigellosis, bacillary dysentery.

So, look, this is a 39-slide presentation. Yesterday I narrowed it down to 29 slides. I'm only on five. I think I'm out of time. I told you I'd be cognizant of my time, so I'm going to stop here. I will be here until the lunch break. If anybody would like to discuss this further, I'll happily stick around for a bit. Otherwise, I'm going to hand it off to my colleague, Lt. Col. Tyner.

DR. TYNER: Good morning. Hi. I'm Steve Tyner. Those of you that were here for Schooley's talk yesterday probably saw my name in one of his slides, and I think my phone number, too. Joke's on him, though. I didn't turn -- I haven't activated my voice mail.

So I'm going to try to run through this quickly. I think Col. Zapor did a great job, and other speakers have done a good job of highlighting where the problems are. This is just to emphasize that my group works on primarily two areas: militarily-relevant wound infections, and we'll talk a

little bit later about bacillary dysentery, or shigellosis.

You guys know that. So we have two different approaches. One of these approaches, which is this library-to-cocktail approach that you're going to hear from Dr. Biswas and Dr. Regeimbal later, is really a collaboration with the Navy. We interact with the Navy with this to help evaluate the therapeutics that they develop. We do not develop precision cocktails on the Army side.

What we do work on *de novo* in-house, Dr. Mikeljon Nikolich who is participating in this workshop, is -- fixed cocktails. So these cocktails are what we call sort of a broad host range, which is really kind of a misnomer, but essentially it's an expanded host range phage, so it targets more strains within a bacterial species than some of the other phages.

I'm going to talk about fixed cocktails first. These are a number of the different studies that Dr. Nikolich has been working on, he and his team. We partnered with Eliava to look at Sb-1, which is a *Staph aureus* phage.

We've expanded that host range in our lab. We've been isolating phages for ESKAPE pathogens, and

then beginning to try to select phages for biofilm degrading properties, as well as engaging with one of the other departments that I lead, which is the wound infection department, to look at phages and antibiotics and whether or not there's synergy or not.

We recently were recipient of an award with JCVI, and I think Dr. Fouts is here, in the back. We're going to be a partnering institute with them.

Pre-clinical studies. We've been looking at *aeruginosa*, so, phage against *aeruginosa* in a wound model. And then, more importantly, clinical studies. We were a partnering organization with AmpliPhi in a phase one safety skin trial study that was held at the Walter Reed Army Institute of Research, or the WRAIR. That was done last year.

This is an example of some of the phages that we've found against *Shigella*. Actually found 50 lytic phages. They're active against a bunch of different species of *Shigella*.

In fact, the best three phage cocktail was active against 90 percent of the strains from the panel of *Shigella* isolates from the Armed Forces Research Institute of Medical Sciences. That's that acronym that says AFRIMS. They're located in Bangkok, Thailand. That's an Army lab in Bangkok.

So we're beginning to look at assessing our best cocktails for shigellosis in our pre-clinical models which are mouse, guinea pig, and non-human primates, which we all have internal at Walter Reed.

So for fixed cocktail I'm going to shift now to some of the work which is a little bit more in-depth with precision cocktails. Again, this is a collaborative effort with the Navy.

Not to belabor the point, I'm sure Dr. Biswas is going to go into much more, and better, detail for the system that he's created than I can, but essentially what they are doing is developing synergistic phage cocktails so that when you lose activity with one phage in this cocktail, another one is still active against the particular bacteria that you are targeting.

This work that was published in *AAC* is a collaborative effort between Dr. Regeimbal, who is sitting in the second row over here, and one of my scientists, Dr. Anna Jacobs, who's the second author on this, in which we looked at a five-member phage cocktail and assessed it in a skin and soft tissue infection model. This was against a MDR *Acinetobacter baumannii* that we isolated from a war wounded subject from Walter Reed in 2010. What this graph shows is

that the -- there was a phage they call AB Army 1 which was very active against capsule positive *acinetobacter*.

It basically removed all the capsule-positive organisms, and resultant organisms that were left that were resistant were capsule-negative, and so we went back and the Navy found four more phages that were active against the capsule negatives. So, in combination, this eradicated the *baumannii* phage infection.

Just briefly, this is the model. These are CP-treated, or cyclophosphamide-treated, animals. These are mice. Then they follow up with three treatments. After the dorsal wound punch, there's a treatment about four hours after, and then for a couple days. Then we measure the wound and we do *in vivo* imaging.

On the left you can see the phage cocktail by day five by IVIS has basically removed the wound pathogen, and on the right you can see the biofilm on the occlusive dressing is much less robust in the phage cocktail-treated animal than the animal that was with PBS.

So the cocktail resulted in a reduced bio-burden, prevention of wound expansion, and a decrease

in biofilm formation. So we were very excited about this because this is a great proof of concept for the process that Dr. Biswas and team have developed.

So we wanted to move further with this, and so we started thinking, where can we innovate? Where we need to innovate is in areas, because we're the military, that are militarily-relevant. I think Col. Zapor did an excellent job of identifying some areas that have cross-over civilian military potential.

The top on the list for us is orthopedic hardware-associated infections. These are mainly biofilm-mediated. The principal organism that's causing this is *Staph aureus*. Then we also have an effort looking at enteric infections. So we believe that phages in this setting are going to be an adjunct to antibiotics, and we want to understand how they work in pre-clinical models.

So I'm going to walk you through the orthopedic hardware-associated infections. We did this in collaboration with the U.S. Army Institute of Surgical Research which is located down in San Antonio, Texas. That's where the Army Burn Center is located. They do a lot of trauma research there, and so they have a very well-developed rat femur pin infection model where they look at therapeutic

adjuncts to prevent orthopedic hardware-associated infections.

So in this animal, day zero, the animal has a cut down, and then there's a non-union segmentation done in the femur, and it's spanned with a wire. Then *Staph* is added into the wound at that time. The wound is closed. Six hours later they open it back up, they wash it with nine liters of isotonic saline, and then they debride it, much like we would any other service member that's in a -- that's been injured. When they first arrive to the first surgical facility, that's how they're treated.

We treat then at six hours, and then 24, 48, 72 hours. At that point we stop treating, and then we wait for 14 days, and then the animals are euthanized and we evaluate whether or not there's been a reduction in CFU.

So off the top this is -- for those of you that are phage guys you're looking at this and saying why aren't you treating all the way through, and there's a good reason for that. The reason is there's a boatload of information, published information, that this organization has done with this model. We need to have a baseline of where we need to begin before we can start modifying the pre-clinical model and

modifying how we add therapeutic adjuncts into their system.

So this is a very challenging model, and you're getting ready to see some data that's not overwhelming, but I don't want to take the wind out of the room. All right. So this is the data. The inoculum was one times ten to the five CFU.

Phage treatment. We did local, as well as systemic. You can see the different doses that we did there. We did local only, systemic only, and local and systemic, and what we had is we had a slight reduction at day 14. Remember, that's 11 days after the last treatment with phage of *aureus* in the bone, as well as on the hardware.

So it's slightly encouraging. It's encouraging particularly because this is a very challenging model. It's also an extremely challenging organism to treat, and it's in a biofilm.

So there's a number of different things that encouraged us, and we're moving forward and trying to come up with our next steps, one of which is to modify this model so that we shorten the time and we're able to take earlier time points and begin to look at the effectiveness of phage much earlier in the system.

But I like to focus on the positive. What

this did for me, if you're going to look at orthopedic hardware-associated infections, then you need to evaluate your phage activity against biofilms. They are evaluated against biofilms, but the process by which the precision cocktails, and I think the fixed cocktails for the most part, are derived are phage are isolated against organisms that are pretty fat and happy.

They're planktonic organisms. *Staph* itself changes its extracellular receptors quite substantially when it's in a biofilm as opposed to planktonic state.

So if we're actually interested in clinical problems where biofilm is the problem, and that's the reason why it's challenging to treat, then we need to think about how we're isolating phage or how we're assessing phage activity against biofilm.

In this model there was concomitant antimicrobial use, and we need to assess phage activity with concomitant antimicrobials. I think some papers have recently come out. There was one in January that looked at *in vitro* phage plus antimicrobials.

I don't think phage is going to work with every single antibiotic, and we need to assess and

understand how well they work both *in vitro* and *in vivo* as we're moving forward because, unlike a basic science lab, I'm not interested in studying the phage. What I'm interested in doing is building a therapeutic.

So I'm looking at making different efforts that we can plug and play and add into a therapeutic development pipeline. The precision cocktail is in collaboration with our Navy partners.

And then, of course, you know, how phages are administered is an important point, but I think it's less important early than the phage activity against biofilms and with concomitant antibiotics.

I have been charged to get us back on time. So I've got one more -- I think one more slide that I'm going to show you.

This is a biofilm assay that Dr. Jacobs has been working on where we're looking at a phage cocktail, I think this is a precision cocktail, against a biofilm. So it's the *Staph* biofilm. You can see there there's a nice dose response against phage. The biofilm was grown in TSB, plus one percent sodium, plus one percent glucose.

The literature suggested that this was one of the more accepted ways to grow a *Staph* biofilm.

Literature's a little all over the place, I think, in terms of how people grow these.

TSB is in no way a non-nutritive media, it is a nutritive media, so that's one caveat, but there is a dose response to phage. So the biofilm was grown for 24 hours, remove all planktonic cells, so it's a fairly -- it's a mature biofilm, from an *in vitro* perspective. We add phage for 24 hours, then we do CFU and look at absorbance.

You see a nice dose response, and then you see about a log, log and a half reduction, almost two log reduction, in CFU after treatment. So the phage work in *in vitro* setting against biofilm.

So what's going on *in vivo*? Why is it so difficult to treat *in vivo*? I think it's a whole ‘nother hurdle that we're trying to come up with a technical solution for.

So, with this, I'd really like to thank my colleagues. I really have to thank my colleagues not just at the Walter Reed Army Institute of Research, but at the Naval Medical Research Center, in particular, BDRD. And some of those colleagues are sitting here, in the second row, and then Cmdr. Stockelman's over here, three rows back.

Without their engagement, their input, their

energy and intelligence, it would have been very hard to get to this point. Thank you.

DR. RANALLO: Okay. Thanks, Stu, and thanks, Col. Zapor. I appreciate it very much. So we're going to transition a little bit to the next talk. It's by Dr. Breck Duerkop who just recently started his lab in 2016. He post-doc'ed with Lora Hooper at UT Southwestern. His talk is going to focus on *Enterococcus* and receptors and resistance mechanisms.

DR. DUERKOP: All right. Good morning. So I'd like to first start out by thanking the organizers for giving me an opportunity to spend a little time talking about my fledgling laboratory that I just started at the University of Colorado, where we're interested in a number of different aspects of phage biology, one of them focusing on receptors that phage utilize to infect and kill gram-positive pathogens like *Enterococcus*. All right.

So just a little bit of background on phage receptors in gram-positive bacteria. So there's a number of different moieties on the surface of gram-positive cells that can be targeted by phage, and these include standard polysaccharides that coat the surface of the cells, peptidoglycan which, you know,

obviously forms a thick layer around the body of the gram-positive bacterial cell, and then other more interspersed polysaccharides like wall teichoic acid, lipoteichoic acid.

Our interest has primarily been in membrane proteins that are, you know, embedded in the cell wall of gram-positive bacteria, and how phage target these receptors.

So I would argue that gram-positive receptors are kind of understudied in comparison to receptors in gram-negative bacteria, especially in classic organisms like *E. coli*, but, due to the fact that we're interested in the potential for therapeutics for phage, I think there's a need to better understand the gram-positive cell surface in terms of how phage interact with that molecular body.

Interestingly, I kind of didn't realize this, but there's a lot of interest in phages in the dairy industry for industrial applications due to the fact that large dairy fermentations can usually be destroyed by organisms that are utilized during fermentation by phage such as *Lactobacillus* and *Lactococcus*. All right.

So the focus of my lab is really looking at *Enterococci*, and so these are facultative anaerobic

gram-positive bacteria, and they're natural commensals that are found both in the intestine and in the oral mucosa. *E. faecalis* and *E. faecium* represent the most common drug-resistant versions of this genre, and they can, under certain environmental perturbations, like antibiotic treatment, go on to form intestinal dysbiosis that can lead to sepsis.

So over the last several years we've been collecting phage from wastewater. This is just an image showing the Dallas/Ft. Worth water reclamation facility where we've sampled a lot of different areas. What we found is that wastewater, as many of you know, is a very abundant source of phage, and specifically for *Enterococcal* phage.

So we can find these phage in fecal-contaminated water sources, whether this is primary effluent coming directly out of the flow at the facility, or even some of the, you know, more processed water further down the line.

These sewage phage are actually quite effective at killing *E. faecalis*, and so we've been isolating these over time from these samples and purifying them to high purity to then study their interactions with *E. faecalis*.

So I'm going to talk to you today primarily

about one phage, but what we found was that we have two more or less identical phage at the genetic level. They have some polymorphisms that, you know, make them a little bit different at the nucleic acid level, but primarily these phage are about 97 percent identical. We call them VPE25 and VFW.

I'm just showing you here the genetic organization of these phage. They're modular, as many phage organize their genomes in terms of organizing different regions of the genome in terms of their gene content.

So we've been interested in kind of exploring these *Sipho* phages as potential targets that can be used to manipulate *Enterococcal* communities, but the first question we really wanted to answer is how do they actually interact with the cell surface of *E. faecalis*?

So what we did is we grew *E. faecalis* in the presence of these phage over time, and we just isolated resistant colonies that came out of these growth cultures. What we found after doing some genomics to basically map resistant genome reads to our reference strain, we found that phage resistance mapped to a membrane protein that was encoded by a gene called EF0858.

EF0858 is a homologue of two different proteins that have been described in the literature, one called UEB and *Bacillus subtilis*, which is known to be involved in phage absorption for a particular phage called SSP1, and then in *Lactococcus lactis* it has been termed PIP for phage infection protein, and so we kind of went with that nomenclature for our *E. faecalis* homologue.

So what I'm showing you here is a cross-streak, and you're going to see several of these throughout the talk. Really what this is is we just take the bacteria of interest, we streak it in one direction on a plate, we take our phage of interest and counter-streak that, you know, vertically, and we can look for the presence, or absence, of killing.

What we see is that with VPE25, it can effectively kill wild type *E. faecalis*. If we knock out PIP by making a clean deletion, you can see that you're no longer susceptible to infection. And we can complement this. So this shows that PIP is sufficient for infection of *E. faecalis*.

So we wanted to learn a little bit more about PIP. So not much had been really, you know, studied in the literature, other than the fact that it was involved in phage infection. So, due to the fact

that we have many genomes now available, we kind of compiled a number of these PIP homologues across the *Enterococci*, specifically in *E. faecalis*, and we just aligned these proteins.

What we found was that the N- and C-termini of these proteins are actually quite conserved; however, there's a large extracellular -- there's a large variable region in the center of this open reading frame. What we found was that this variable region, or this region of high diversity, actually maps to a predicted extracellular domain that would, in theory, be on the outside of the cell.

So we were curious if this diverse region actually played any role in the biology of *E. faecalis* during phage infection. So what we did is we took our two phages and we did cross-streaks -- I'm just showing you this here on a very crude heat map -- where we looked for the sensitivity, or the resistance, of these different phage based on whether thy could be infected by one phage or the other.

What we found was that a number of strains could be infected by both phages, and some phages could actually only infect one strain or another. When we actually did alignments of this variable region in PIP, what we found was that they clustered

identically to their susceptibility pattern.

So what you can see here is all the strains that cluster in black are susceptible to both phage, whereas the ones in blue are only susceptible to VPE25, and then, vice versa, the ones in red are only susceptible to VFW. So what this told us is that the diverse region in PIP likely drives phage tropism for the surface of the *E. faecalis* cell.

So we wanted to test this genetically, so what we did is we took a strain called E1Sol *E. faecalis*, and if you just, you know, reference the map on the right, E1Sol is actually resistant to VPE25, but susceptible to VFW.

So if we actually express the V583 version, which is our standard wild type strain that we work with in the lab, in E1Sol on a plasmid, you can change tropism. So that's what we're showing on the second from the top cross-streak.

And then if we cross that V583 version of PIP into the chromosome and make a clean insertion onto the genome, we get a similar phenotype.

But I think the most important thing is if we actually engineer a plasmid that only has the variable region from V583 that's different from E1Sol -- so this is the last, the very bottom cross-streak

you're looking at -- that's sufficient to drive tropism change.

So what this tells us is that the variable region in the surface protein is likely driving the specificity of VPE25 for the surface of the *E. faecalis* cell and, most likely, the infectivity of those phage.

So then we asked another question. Can we actually, you know, go outside of *E. faecalis*, and can we expand this to related organism such as *E. faecium*? This became a little bit more I guess muddy in the sense that when we over-expressed wild type V583 PIP in *E. faecium* we saw a somewhat mild killing effect on our cross-streak assay, as you can see there.

If we actually spike this phage into growing culture, what we found was that it could inhibit growth, but it didn't actually collapse the culture in terms of, you know, real robust killing like we see with wild type *E. faecalis*.

So we wanted to learn a little bit more about this. So what we actually did is we actually looked at phage transcription, and we looked at a number of genes -- and I'm just showing you one open reading frame here -- in the presence, and absence, of phage in the different strains.

What we saw is that there's, you know -- after 30 minutes there's a large transcriptional up regulation of this ORF123 in our wild type *E. faecalis*. You can see in our delta PIP mutant that there's virtually no transcription below baseline, or above baseline.

However, in *E. faecium* what we saw is we saw kind of an intermediate transcriptional phenotype in the wild type version, and then when we expressed PIP in *E. faecium* we saw that this was elevated by several logs.

But we were never able to actually recover phage from these cultures. So you'd add phage to these cultures, it would slow their growth, but when you titered those cultures you were never able to get more phage out than what you put in.

So what we determined was that these phage are actually infecting, they're replicating inside of *E. faecium*, but they can't actually get out of the cells. So that's what I'm showing you here, in this bottom graph on the right.

So we basically took these cells and we lysed them by sonication, and then we were able to liberate a number of different -- a number of phage from these bacteria. So what this tells us is that *E.*

faecium actually has a receptor that is sufficient to promote infection, but that once the phage get inside the cell and replicate, they can't actually get out. So that means there's something defective about the holin, or the lysin that doesn't allow the cell to actually be lysed from within.

So I think this is something that should be considered in terms of when we're thinking about engineering phage. If we don't see infection, it doesn't necessarily mean that -- or killing, it doesn't necessarily mean infection is not happening, it may just be that the -- a downstream mechanism has been blocked.

So then of course we wanted to try and apply these phage to an animal model to see if we could decolonize *E. faecalis* from an environment where it's a native organism, and so we've set up some experiments using germ-free mice. So I come from Laura Hooper's lab. We study -- most people study epithelial cell interactions in the microbiota, so we have many germ-free mice that are accessible to us.

So what we did is we took germ-free -- male germ-free mice, we colonized them initially with E. *faecalis*, and then six hours later we gave them a single phage treatment. Then we monitored

colonization levels at 24, 48, 144, and 216 hours. So six days, and nine days.

We observed a number of interesting things. So initially, at 24 hours we see a modest reduction in the colonization levels of *E. faecalis*, about roughly a log decrease; however, over time we saw that these levels came right back to levels similar to untreated animals.

And when we actually monitored the phage abundance in these animals over time, we saw that the PFU recoverable from the feces actually decreased considerably.

So we were interested to know whether or not this was due to the fact that maybe the phage were not getting access to the bacteria or if we had the outgrowth of resistant bacteria.

So we looked at bacteria that were coming out of these feces and we sequenced a number of these PIP alleles in *E. faecalis* -- in these strains coming out of the mouse feces, and what we found was that by 48 hours we were upwards of 75 percent non-susceptible strains coming out of the mice, and by six days we were virtually at 100 percent of the isolates were receptor-deficient *E. faecalis*.

These were receptors that had not evolved

changes in the variable region, but they were mostly truncations, or insertion mutants, or polymorphisms that led to the generation of stop codons.

So the next question, and this is kind of really where I think we're starting to take some of this work, is I kind of talked to you about this protein called PIP, but, you know, what does it do? I mean it probably did not evolve as a protein that's meant for phage to infect.

So one of the things that we're interested in is identifying novel surface receptors using phage to better understand proteins in gram-positive bacteria that might be utilized for lifestyle.

So if you look at the domain organization of PIP, it has several interesting domains. So obviously it has this variable region in the center, but at the N-terminus it has this YhgE PIP domain which is actually conserved in some type 7 secretion proteins that are considered to be part of the potential apparatus of the type 7 secretion system in *Staph aureus*, and then at the C-terminus, interestingly enough, there's a major facilitator super family domain.

These domains are largely involved in transport of small molecules either inside or outside

of the cell.

So the fact that PIP is highly conserved across the *Enterococci*, not just in *E. faecalis*, but *E. faecium*, and the fact that phage use this to actually infect the bacteria probably suggests to me that, or it suggests to me that this is likely an important protein for some component of its lifestyle.

So we set up an experiment where we took wild type *E. faecalis* and our PIP mutant that was marked with a tetracycline cassette and we just did a co-colonization in antibiotic-treated mice.

What we found was that by comparing the competitive indices, so the ratio of the wild type to the delta PIP, over time, during colonization we found that the wild type outcompetes the PIP mutant by about -- after about two weeks. We see about, you know, roughly, on average, about a log out competition.

So what this tells us is that PIP may be involved in niche adaptation, it may be involved in some aspect of colonization, and so we're going to spend some time now in the future to really kind of run down whether or not this plays any specific role in colonization.

Okay. So I've talked to you so far about phages that infect through a PIP mechanism. So what

about phages that infect in a PIP-independent manner. So there's a phage that some of you may be familiar with. It's a very old phage. It's called NPV-1. It was originally isolated by Gary Dunny's lab from wastewater back in 1990. It's a *Sipho* phage that has a non-contractile tail and a prolate head, and it has -- compared to our VPE25 and VFW phages, it has a very limited host range.

So you can see here that it only infects, out of at least the collection -- the strains that we tested from our collection, it only infects two: OG1RF and JH1. It infects in a PIP-independent mechanism because it can kill OG1RF delta PIP mutant and it can also kill the wild type, but it can't kill V583.

So, again, we wanted to, you know, use genomics to figure out what the receptor is for NPV-1, and so we did -- we used a similar strategy to what I described to you earlier in the talk. We came up with one isolate that we call OG1RF-C. It's an NPV-1-resistant strain, and it was generated from a confluently lysed agar plate of OG1RF delta PIP.

We did whole genome sequencing on this strain, and we found three polymorphisms. We found a polymorphism in *epaR*, which is a sugar transferase,

bgsB, which is a glycosyl transfer, or glycosyltransferase, and then *iolA*, which is a malonic semialdehyde dehydrogenase that's involved in inositol metabolism.

So we were interested in the first two because these are actually enzymes that would be involved in changing, potentially, the surface of the bacterial cell. So the *epa* cluster in *Enterococcus* has been well-characterized by Barbara Murray's group in Houston over the last decade or so, and it's a polysaccharide that's composed of numerous carbohydrates, including rhamnose, glucose, and others.

So we went in and we made an in-frame deletion of *epaR*, and what we found was that if you delete *epaR*, similar to the OG1RF-C strain, you get resistance to NPV-1.

We also made an in-frame, a single in-frame deletion of *bgsB*, and this did not result in resistance to NPV-1, but it doesn't necessarily mean it's not involved in resistance because, if you can see, OG1RF-C tends to be a little bit more resistant than the delta *epaR* mutant, so these may actually work together in some way dur -- to promote a fully-efficient infection.

So, in conclusion, what I've told you is that some lytic *Enterococcal* phages use a conserved membrane protein that we call PIP-EF, or the exopolysaccharide Epa, in *E. faecalis*, an extracellular variable region actually determines phage specificity for *E. faecalis* hosts, and that phages can temporarily reduce *E. faecalis* abundance in the mouse intestine, yet resistance is rapidly re -- acquired, suggesting that, you know, cocktail methodologies might be more applicable in this situation.

And then PIP-EF conservation among the *Enterococci* may be linked to efficient intestinal colonization.

So kind of some of the future directions where I kind of see some of this work going and how, you know, this will contribute to the phage -- to the field of phage biology, and also phage therapy, is we're in a good position now to expand the repertoire of virulent phages that infect *E. faecalis*. I know there's a number of them out there, and I'm learning more and more every day.

So we're returning to wastewater to -- for new virus discovery. We've actually received 20 *Enterococcal* phages from the Navy from Biswajit Biswas

who generously provided those for us, and we're going to spend a significant amount of time looking for putative receptors for a number of those phages, and then we've started to establish methods for the genetic modification of existing phages to alter receptor specificities and CRISPR technology.

So I guess, from a broader perspective, can we actually use phages to identify conserved proteins that might be indispensable for *Enterococcal* lifestyle? So phages target surface proteins that are conserved, and sometimes these are important for, you know, the viability of the cell.

So, for instance, PIP-EF looks like it is involved in colonization, but also, the *epa* cluster of polysaccharide genes has been shown to also be a colonization determinant, so this may be a useful method that we can use to identify novel proteins that could be targeted for other types of medical applications or drug applications.

And then I think a broader, more kind of hand waving direction is what are the physiological effects of phage predation in the intestine? That's something we're very interested in. You know, does phage predation have an effect on the global community of commensals that are in that environment, and how

does that impact the host? Does phage predation select, you know, on select bacteria actually influence the biology of the mammalian host, such as impacting innate immunity, adaptive immunity, things of that nature?

So, with that, I need to acknowledge a number of people. I need to acknowledge, of course, my lab, which has just started at the University of Colorado.

I really need to acknowledge Dr. Kelli Palmer at UT Dallas who's been an active collaborator throughout the course of all of these studies, my former mentor, Laura Hooper, for allowing me to take a phage project in a direction that was very different from what the lab traditionally works on, and then some of my new colleagues that I've started collaborations with here.

So I thank you for your time, and I can take questions if there's any time left. Thank you.

DR. RANALLO: Yeah. So we do have time for questions if anybody has any questions for either of the speakers this morning. I apologize. I didn't give you guys time for questions.

AUDIENCE MEMBER: I'll ask a question. So in your resistant mutants, I mean, so you look at just

kind of killing in liquid and that kind of sensitivity. Do you do like adsorption rate experiments or anything like that to see if it's gone down or absent?

DR. DUERKOP: Yeah. So we've done some adsorption experiments, especially with the PIP mutant, and there's no adsorption difference. So I just didn't have time to show that data, but that data's published. The phage adsorb fine, but what we think is happening is that the -- is that PIP actually promotes DNA entry into the cell.

MR. DIXON: Morning. I'm Dennis Dixon from NIAID. I'm an interloper from the other room. I wanted to come in and commend Col. Zapor for his point on reluctance of the infectious diseases community when actually confronted with phage as an experimental possibility.

I do see the same disconnect between give us something new, we have to have some alternative and something innovative, and then when you present the community with this as an option, would you be interested in moving forward with this, well we don't know, it looks so different and we don't know if it'll work, even though you have a DSMB in place that's monitoring safety, and you have all the steps you need

to determine evidence to guide your decision so you will know.

So I liked your idea about specialty populations, where maybe the mainstream ID doc doesn't seem them on a recurring basis, moving to things like spinal cord injury, where you know the consequences of repeated catheter insertion, or the plates and implants from surgery, because surgeons generally have no reluctance to give something such as antibiotic, even if it's not exactly an antibiotic.

So that might be worthy of further discussion, on how to start to have discussions to engage the community that's going to be necessary to buy in to any clinical evaluation. Thanks.

MR. CHEN: Yeah. Good morning. Rong Chen from Phagelux. I have question to Dr. Tyner. I am very interested in your wound model. I found it very interesting to see on the slide, it looks like the topical application is better than systematic, right? Its look like at least similar, or even better. That's my understanding.

So, and another question is that did you found any difference between -- in the systemic use between IP, IV, and SC?

DR. TYNER: Okay. Thanks. You're right.

It looks as if perhaps putting the phage into the -- you're talking about the rat model with the orthopedic heart? Yeah.

MR. CHEN: Yeah.

DR. TYNER: So it looks like that might be a little bit more effective, but the N is so small and the effect right now is not large enough to really draw a definitive conclusion.

The other delivery was IP. We did not -- we have not yet tried IV or SC, but we have to solve the -- part of the issue with the effect of the therapeutic on reducing the biofilm, before we start looking at the delivery method, although delivery method is important. You're right.

MR. CHEN: I notice there's a difference on the dose between your topical and the systemic. It's 2.5 and 1.75. They're different because of dose, or difference is because of route?

DR. TYNER: That's a good question. I'm happy to discuss that with you after. We probably should rope Dr. Jacobs in for that discussion. Thanks.

AUDIENCE MEMBER: Hi. Nancy from Phagelux. I just have a couple of questions here for your prosthetic joint infection models.

I was wondering if you had looked at the activity of phages if you pre-treat your nail or your implant versus if you do the post-treatment after the infection has started.

DR. TYNER: That's an excellent question. We have not done that yet. Might also be interesting to look at whether or not if we deliver antimicrobials first, then add phage, if there's a difference than if we add phage first and then do antimicrobials.

AUDIENCE MEMBER: And maybe a follow up question on that. Well, maybe more a follow up question on what Rong was discussing. Have you tried to do the phages intramedullarly? So you would just make a hole inside of your tibial cavity, put the phages in it, and see how the infections would result. Think it might be very different from --

DR. TYNER: That's an excellent point. No, we have not tried that yet.

AUDIENCE MEMBER: Okay. Thank you. Thanks.

DR. RANALLO: Okay. Thank you. Let's move on to our next speaker, Dr. Paul Turner from Yale University. We heard a little bit about Paul's work yesterday, but we're going to hear much more in-depth detailed information about how selective pressures can reduce virulence and sensitize against antibiotics.

So the -- Paul's talk is using phage to select for evolution or reduce virulence in pathogenic bacteria. Thanks, Paul.

DR. TURNER: All right. Good morning, everybody. Pleasure to be here. I'd like to thank the organizers for inviting me. So what I'm going to do today, first talk will be a little bit about my background and the mission that we have in my laboratory.

I have a very broad interest in the evolution of microbes, and we focus a lot on viruses, so on the left are very familiar pictures for this audience of phages and bacteria, but we also look at other types of viruses, especially mosquito-borne viruses. So we do evolution experiments on dengue virus, and chikungunya virus, and some other human pathogens.

So what I want to do today is demonstrate for this one project how there was a nice move from basic research, longstanding interest of mine in evolutionary biology, that in a very short period of time led to, you know, we're on the cusp now, we hope, of investigational new drug status and continuing to pursue that for phages, especially a phage that we found in a lake in Connecticut that -- you heard a

little bit about that yesterday from my colleague Deepak. Okay.

We like to address big questions, and here's kind of a big question. Why are there so many species on Earth? As an evolutionary biologist, it's very obvious to me that evolution involves compromises.

So one of the most misunderstood concepts in biology, unfortunately by the lay -- public, is how evolution occurs.

So what is not at all controversial and what Darwin first, and best, articulated is that organisms interact with their environment, and the variants that leave more progeny, are the ones that end up being enriched in those populations, and the traits that they have end up dominating populations through time. So the only controversy is how much people want to believe that that happens in humans.

But the main point is that natural selection often leads to trade-offs, and I'm finding that trade-offs in my career are a very prevalent thing that we observe in our research.

Essentially, it works this way. If you improve in one trait, it doesn't necessarily mean that you're going to improve in other traits simultaneously, and often you sort of give up the

ability to perform another trait well. This opens up niche space for organisms that do the opposite. So, in this way, you have, through eons of time, species diversity evolving on the planet.

The gentleman on the right is one of my colleagues at Yale, Steve Stearns, and he is very famous for life history theory, which is this general idea that traits cannot be simultaneously maximized. An interesting general trade-off, this is a talk for a different day but you see this also in viruses, is that survival versus reproduction is something that is a difficult thing to maximize on both sides.

This, I would say, is one of the cornerstones of evolution by natural selection, and you can demonstrate it in *Drosophila* populations, but also in viruses. That if they evolve greater reproduction, it might take away from their stability, and vice versa.

So I want to step back a little bit to a system that is not a phage of humans, but it is one of the first phage systems, virus systems, that I started working on in the 1990s.

So this is a phage called phi-6 that infects *Pseudomonads*, especially *Pseudomonas syringae* pathovars, and it's a well-characterized system with a

segmented genome. I started working on it because of its segmentation and RNA genome because it mimics genetics of human pathogens like influenza and hanta viruses.

So you have a cartoon of the familiar lytic infection cycle, and in the middle here there's a picture of these phage particles, visible as little, white spheres, that are lined up along the type 4 pilus of these bacteria.

So this is the initial receptor site for this phage in nature, and the type 4 pili are also what these bacteria use to twitch across a leaf surface and enter into the stomata. So this is absolutely essential as a structure for these bacteria to get inside of a plant and to be pathogens. And, not surprisingly, you see this a lot in phage biology and other virus systems. These viruses have evolved to use as a receptor something that is absolutely essential to their hosts.

What we have seen in the laboratory is that the resistance to the phage *in vitro* easily occurs if the bacteria simply shed these pili. They get rid of the type 4 pili.

Now, this is a bacterial pathogen of some interest in agriculture. It causes halo blight

disease, which is a big deal in crop production of beans. So if they had this option in nature they would be out of luck in terms of bacteria surviving in their natural environmental.

If the pilus loss occurs, they cannot get inside of the leaf, as I mentioned. So I would call that a conditional virulence factor, meaning that if you simply took the bacteria and you put them in a plant, they will happily function as pathogens.

So what I would assert here is that the interaction of the phages with these bacteria demonstrates that the bacteria can easily be forced into an evolutionary trade off. If they evolve resistance to the phage, then this lowers their pathogenicity.

You know, I'd seen this for a very long time, since the mid-'90s, and it was of interest to me simply because I was using this phage in experiments. Maybe about four or five years ago, really in earnest, my group started looking at this property in phages of humans in human -- phages of human-associated bacteria, of course.

So could you use the same principle to drive our thinking in developing, or at least finding, better candidates for phage therapy. So here, the

general question is can phage therapy also exploit evolutionary trade-offs?

By now, at this point in the conference, this is a little familiar to people, but firstly, antibiotics are becoming less useful, MDR bacteria are on the increase, *Pseudomonas aeruginosa* is particularly worrisome for CF patients, severe burn and immune-compromised patients.

So what we've focused on are efflux pumps, which I think are these fascinating complexes of proteins that span the inner and the outer portion of the cell of bacteria like *Pseudomonas aeruginosa*. These efflux pumps are transport proteins that help the bacteria efficiently remove a wide variety of drugs from the cell.

They have a lot other properties as well. They function in host colonization, evasion of host immunity, and biofilm formation, but obviously this is a big problem in *Pseudomonas aeruginosa*. That if you throw an antibiotic at it and it manages to get in, it can be very effectively pumped out.

So efflux pumps are typically chromosome encoded, they're genetically conserved -- that turned out to be important in the study that I'm going to focus on, and I'll try to remember to get back to that

later -- they are generally found in gram-negatives, and for many antibiotic classes, but not all, these are the major determinants of how the resistance would occur for the antibiotics.

So kind of a useless slide at this point. Phage therapy is amazingly interesting, and we should invest in it further.

So here is another cartoon to help illustrate a point that really is the core of this project. So this is a lytic infection cycle, very obviously. If you use a phage to target a bacterium, then, in essence, I would expect, as an evolutionary biologist, you're going to get the same problem that often occurs any time an organism faces a selective challenge. It's going to be selected to change.

So now I'm showing the bacteria in this cartoon. It is now presenting different-colored -- blue-colored proteins now that is not able to be used by this phage to enter and initiate the infection cycle. So if I throw a phage at a bacterium, the natural consequence is it's going to select for increased phage resistance.

So wouldn't it be cool if that came along with increased antibiotic sensitivity? That's not only cool, but that's also the take home of my talk as

well otherwise I wouldn't be suggesting it. So this genetic trade-off between phage resistance and antibiotic sensitivity would of course improve antibiotical -- antimicrobial therapy options and would extend the lifetime of our current antibiotic arsenal.

And I want to really emphasize that. So if you have drugs that are approved currently and they're in use, if you can use phages to interact with pathogenic bacteria and convert them into genotypes that are susceptible to something that's already approved by the FDA, then you have a faster track to being able to use phages, I would say, in therapy.

So we found such a phage. It's abbreviated as OMKO1 for outer membrane knockout one. It's in the family of *Myoviridae*. It's a lytic phage that binds to that outermost protein in many of the very commonly found efflux pumps in *P. aeruginosa*, these Mex system efflux pumps.

We confirmed that using a mutant knockout library that we got from University of Washington. So we know that when the genotype that has the *oprM* gene knocked out, that is the only strain that this phage cannot infect.

So we discovered in sequence this phage

which has a pretty whoppingly large genome, but we found that in 2016, and it does force this genetic trade-off that I mentioned. The phage-sensitive bacteria can efflux antibiotics, but they're killed by the phage, and the phage-resistant mutants have an impaired ability to efflux antibiotics. So that demonstrates the interaction. Again, that was found in a contaminated lake in Connecticut called Dodge Pond.

So probably obvious to many people in the room, but I want to make sure you understand the core thing that we're measuring in the table that I'll show in a moment. So what you should keep in mind is that the evolution of *P. aeruginosa* resistance to this phage causes sensitivity to certain drugs.

So how you easily measure sensitivity to drugs for bacteria is through a MIC assay, minimum inhibitory concentration. So this agar plate has a lawn of bacteria growing on it, and imagine you've got a strain that is in that lawn that grows up right next to a Kirby-Bauer disc that you had placed on the lawn, and that has antibiotic leaching out from it. If it doesn't care about the antibiotic, it grows up right to the edge of the disc.

Well, what I'm emphasizing is that strains

of these bacteria that become resistant to the phage no longer have that property. So they are one mutational step away from having a much larger killing zone and a much greater sensitivity to antibiotic.

So I'll show you that in the following table that was sort of a compilation of the data that we presented in the 2016 paper.

So let's begin first with -- efflux pump literature does implicate certain antibiotics and antibiotic classes for which efflux pumps function, and it's pretty rock-solid evidence.

So if we begin with tetracycline and erythromycin, you can see that the isolate MIC has the number shown in the third column, and when these bacteria -- and basically what I should emphasize, that this table is kind of a compilation of data from multiple bacteria, but I'll get into that more in a moment.

So the phage-resistant isolate MIC changes dramatically. You'll see in the final column there's a fold increase drug sensitivity that's a very impressive number.

Now we move on to -- efflux pump is associated with these other four antibiotic classes, but the evidence isn't as rock-solid. Nevertheless,

you get a change in the isolate MIC versus the phage-resistant isolate MIC. It's not as dramatic of an increased drug sensitivity, but the asterisks are showing you how these agree with break points for clinical importance. So it has now changed the bacterium to a clinically relevant resistance to susceptibility instead.

And finally, efflux pumps are not involved in penicillin class antibiotics. Moving them out of the cell. This is due to other types of mutations that happen in the chromosome. You can think of this last example here as a control, and, not surprisingly, we saw no change in the fold increase drug sensitivity.

So everything agrees with my assertion that the interaction of the phage with the efflux pump protein is placing selection pressure on these bacteria to change, and they change in a way that makes them a better outcome for humans in terms of our ability to treat them with existing drugs.

I'll now show you a bit of the unpublished data in my talk. I think I have time for this. Not very many slides of it.

So this is a cartoon that probably you can figure out this is a bacteria biofilm. The problem

with these little, red, I guess they're circles, trying to get through that biofilm at the bacteria is that a biofilm is very resilient to antibiotics getting in.

If you have the phages that are interacting with the biofilm and they can disrupt it and allow those cells to become exposed to the antibiotic, then you can get a synergistic activity of killing for the phages and the antibiotic.

So what we thought is really the promise of this phage and, frankly, why it worked in a patient -- and I'll talk about that more in a moment -- is that there's a synergistic interaction that is expected.

So here are some unpublished data where -- focus on the taller bars in each one of these examples. I'm kind of in shock and awe that there's very little in the literature on commonly-used substrates that you place in the human body and the ability of bacterial biofilms to form.

We know this, surgeons know this very well, and yet you don't see very much in the literature of the ability of, say phages versus antibiotics to tackle that problem. So these data illustrate that point.

The three bars on the right in each case

show you that in a control versus these two antibiotics, there's really no action of the antibiotic in disrupting the biofilm and reducing cell density, whereas the phage alone, which is the bar on the left-most in each one of the categories, this is this phage and its ability to break apart the biofilm.

The asterisks show you the cases of where the combination of the phage and the bacteria -- I'm sorry -- and the antibiotic are doing a better job at killing the bacteria than the phage alone, and in the majority of the cases, that's what we observe. So that's a very promising result.

So I said that the data that I showed you quickly from the '16 paper were for a variety of strains. Indeed, this worked for laboratory model strains PA01, PA14. It worked on clinical isolates from multiple sources.

It also worked on environmental isolates, bacteria that we pulled directly from an estuary, and also from human homes in the Louisville, Kentucky area. Everybody, if you don't know this, you generally have *Pseudomonas aeruginosa* growing at least in your kitchen sink, if not in your bathroom sink as well.

So the objective is to examine the impact of

this phage on a much larger set of isolates, and that's what we have as submitted grants to NIH, as well as to the Cystic Fibrosis Foundation.

The objective is, with FDA approval, we would use this phage to treat chronically-infected human volunteers. So yesterday you did hear about this one case presented by Deepak where we did successfully treat an MDR *P. aeruginosa* biofilm infection that was associated with aortic arch replacement. That case study is still in review, but we are optimistic that it will come out soon.

Nevertheless, we were able to talk about this publicly, so we mentioned it in media presentations, on public radio international, People's Pharmacy, and Carl Zimmer, the science writer, had a very nice piece on this late last year, so you can go look for it on the web, if you choose.

The objective for the future work is to test the safety and efficacy of this in animal models. So I think this is a very interesting project, where it went to discovering something that was found through a natural product sort of pipeline, to bring something interesting that might be useful for translational medicine, and quickly we found a patient and we helped the patient, and now we're doing, I would say, a lot

of backfill.

So we were awarded an NIH pre-clinical services award, where there's a contract to a team at University of Louisville who are testing the safety and efficacy in a mouse model for lung pneumonia in immunocompromised patients. So that study is still underway. I can't tell you very much about it.

Some of the controls in that study had to be repeated, so the entire thing is being repeated next month, but I found this data set to be pretty interesting. What that laboratory at Louisville did was, even though the experiment has to be repeated, they sent us tissue samples from the mice in this three day experiment that -- we were able to retrieve phage from the animal tissues that were subjected to phage trying to control the infection.

So UNC-D is this pathogenic strain that they use in their pneumonia model, and focus on the data set in gray there, the left-most one. It's showing the efficiency of our phage that we sent them and its ability to grow on that pathogenic strain relative to our typical lab strain that we would use to enrich it, PA01. And they don't grow as well on the pathogenic strain, but they grow on it.

So after only three days, in the vast

majority of these cases, the phages we isolated from those tissue samples are remarkably better by orders of magnitude in growing on the target bacterium.

As an evolutionary biologist, I will tell you that impresses the heck out of me because this is a DNA phage, and I think it is demonstrating if you put it in this very novel environment of a mouse -- animal -- an animal with -- that is used in the experiment, there is strong selection pressure on it to do its job very well in targeting the bacteria that are there and present for it to grow on.

So my point is that strong selection can happen *in vitro*, and even stronger selection can happen *in vivo* in some circumstances.

So I'll finish up by saying that we want to continue with our clinical application of OMK01, and we did acquire the IND in 2016 for compassionate use. We have a teleconference, I found out only yesterday so I didn't put it on this slide, with FDA next month to talk about the possibility of this phage going into clinical trials.

The targeted diseases are ambitiously, hospital-acquired pneumonia, CF-associated pulmonary infections, catheter-associated UTIs, and burns.

We thought we would make faster strides in

agriculture. I'll have you -- I'll just be completely transparent and honest about that. So we know that a lot of agricultural systems we rely on to feed an ever-hungry world are having just as big a problems with antibiotic-resistant bacteria: the shrimp industry and many leafy plants, so the development of phages for bio-control and agricultural systems, I think, has amazing promise as well, and that's something we would like to get into eventually.

So I'd like to acknowledge the folks who actually did the work because all I do is look over people's shoulders and make them nervous. I really have to credit my lab group for being very bold about taking on risky projects, and also bold about me showing embarrassing pictures of them from the murder mystery party that we have annually.

The individual in the middle, I don't know if you can see him, this is the patient who was treated who is now back to work, and this is Ben Chan -- he was the primary person on this project -- to the right. He's a research scientist at my lab group.

We're in that picture showing, or we're giving a thank you card to the patient, as well as a phage plush toy. I don't know if you can see that, but that's what he's holding.

So I'd like to thank Deepak, as well as John Wertz, another one of my longstanding collaborators at Yale, and the funders for the project. Thank you for listening.

DR. RANALLO: We have plenty of time to take a few questions.

AUDIENCE MEMBER: Hi. Nancy from Phage Lux. I have a question. We find in our lab that the presence of *Pseudomonas* usually inhibits the way that *Staph aureus* bacteriophages are able to infect *Staph aureus*, and I was wondering if you would expect the same results on polymicrobial biofilms, or if you would expect the same kind of selection pressures. Or would it be different in polymicrobial models?

DR. TURNER: So I don't know your data because I haven't seen them, but maybe one possibility is if you have a phage that you're using against a target bacterium but it has maybe an ability to passively bind to something else, especially another bacterium, it's probably going to weaken the ability of the phage to do its job. So you could have in a polymicrobial setting sort of a weakened ability for the phage therapy to work.

We haven't seen that with this particular phage, but I would agree that that's just one of the

very many interactions of the phage therapy candidate with a diverse community of bacteria that we need to address and study further. I guess that's my only answer to that.

AUDIENCE MEMBER: Two quick questions, Paul. First, have you tried selecting for resistance changes in the pump that would give you resistance? Because they should be in the external loops of the --

DR. TURNER: Right.

AUDIENCE MEMBER: Have you tried that yet?

DR. TURNER: No, we have not tried that yet. Yeah. It's all been kind of just what is phage doing to interact with the bacterium, and what's the mutational spectrum of the bacterium response.

AUDIENCE MEMBER: Right. And you also said the phage didn't grow as well on the pathogenic strain.

DR. TURNER: Right.

AUDIENCE MEMBER: So when you say that, is that just reduced EOP or what --

DR. TURNER: Correct. Correct. Just reduced EOP.

AUDIENCE MEMBER: So it's likely to be a restriction escape?

DR. TURNER: I'm not sure what's at the root

of it, but it's kind of remarkable that this phage grows very well on a wide variety of genotypes of *Pseudomonas aeruginosa*, so --

AUDIENCE MEMBER: Yeah, but if it's got a restrictions problem with that strain, then it'll just take one escapee.

DR. TURNER: Exactly. So we have to examine that. You know, it's kind of reminding me, ambitiously, of if you had a phage that transcends all genotypes of a species and it doesn't infect other species, then you do have a species-specific drug in phage therapy. So I'm not claiming that that's what this is, but maybe a modified version of this phage would be closer to that. But I hear what you're saying. Yeah.

AUDIENCE MEMBER: My question's kind of similar. I was wondering whether you had tried selection with an antibiotic that you're trying to re-sensitize to and phage at the same time.

DR. TURNER: Right.

AUDIENCE MEMBER: Try to generate those mis-sense mutations and understand the resistance frequency. Whether you really are going to reduce the barrier to resistance by maybe co-dosing using it as an adjunctive therapy.

DR. TURNER: Yeah. I guess maybe the way I could have answered the prior question is we are trying some of those experiments, and, you know, I'm not sure why, but there's kind of a remarkable inability of the bacterium to regain antibiotic resistance when it sees this phage.

I think what is going on is it's placing selection pressure. We're looking for mutations in *oprM*, and we're actually not finding them. I think that there's something else epistatically happening to make them more resistant to the phage, and then when you remove the phage -- we've cultured them for up to 10 days afterwards in the absence of phage and they don't go back to being antibiotic-resistant, so that suggests there's something going on.

That they're happily growing, but they're sort of -- they lost the ability to have a toggle switch that moves back. It's not like efflux pump repression and --

AUDIENCE MEMBER: Right. So you're not co-administering, you're first selecting for resistance to the phage and then later looking for --

DR. TURNER: Oh, I see what you're saying. Correct. Yes.

AUDIENCE MEMBER: Because the eas -- it's a

un -- it's a non-essential protein. The easiest way to get resistance is to knock it out. And it's not going to revert back on its own without selective pressure.

DR. TURNER: I agree.

AUDIENCE MEMBER: It's similar to what we saw with the PIP protein with the *Enterococci* where the strains that were not susceptible had point mutations, but when you select for resistance, all you get is knock out after knock out out of it.

DR. TURNER: Right. Right. So I have to admit we have to look at that further, but, anecdotally, I would have predicted we would have seen a lot more of that by now, and we're not. So I think there's something interesting going on there that maybe has not been shown biologically in phages. I just don't know.

AUDIENCE MEMBER: Okay. It's important because if you're going to go into the clinic and do the co-administration adjunctive therapy to antibiotics, you want to know what that resistance --

DR. TURNER: Completely agree. Let me emphasize, though, when we did treat the patient, we put a useless antibiotic in at the same time, okay? So that worked.

AUDIENCE MEMBER: Okay.

DR. TURNER: All right. Yeah.

AUDIENCE MEMBER: So, nice presentation.

DR. TURNER: Thank you.

AUDIENCE MEMBER: I have a specific question for you. You mentioned that you like to expand it for environmental uses, the phage.

DR. TURNER: Uh-huh.

AUDIENCE MEMBER: So how do you isolate it in the environmental application? Because selected pressure on used phage, it will, you know, generate resistance population. So how you overcome those resistance bacteria in the environmental situation?

DR. TURNER: Right. So what I should have said, I didn't want to confuse, is we have other phages that do the same thing for different target bacteria, and I would say they're actually not that hard to find. So we found them for cholera, *Klebsiella*, *Shigella*, et cetera. I think it's more a matter of looking for them in the right way.

So your question is if you deploy it in a large scale in an agricultural field, what will happen?

AUDIENCE MEMBER: Yes.

DR. TURNER: I would think you're going to

get resistance to it, and it may fail ultimately. An intriguing basic research question is whether you can run through the co-evolution in the laboratory and, in a sense, get a cocktail that is, you know, the ghost of evolution future or something like that, right, and then you use that.

I think that that's an intriguing idea. I have no idea if it will work because evolution can take many paths, right? But --

AUDIENCE MEMBER: But --

DR. TURNER: Yeah? Go ahead.

AUDIENCE MEMBER: I agree with you, but my problem is that if phage is that effective, and if we can make a broad spectrum cocktail to prevent all these things, all of these phages are present in the environmental situation --

DR. TURNER: Yes.

AUDIENCE MEMBER: -- but we don't see the phage has eliminated all the bacteria on the surface of the Earth right now. So I think, my -- this is my personal opinion, that phage can be used as like antibiotic, but it cannot be used as disinfectant.

DR. TURNER: I agree. Yeah. Yeah. I'm a big believer in spatial models, and you have local sort of, you know, pros and cons to things in biology.

So, yeah, I see exactly what you're saying, but I am not worried that we would change the landscape of bacteria on this planet with selection pressure due to phages because they've existed together for billions of years on the planet.

AUDIENCE MEMBER: No, no, I'm not worried about that, I'm worried about the effectiveness of that phage application, because within a couple of hours, the resistance population will start over dominate the system --

DR. TURNER: Yeah. We should talk more further because I -- yeah -- I have lots of ideas about ways to test it in the field, and I know exactly where you're coming from.

AUDIENCE MEMBER: I have, first, one question, and then one comment.

DR. TURNER: Sure.

AUDIENCE MEMBER: The question, have you tried much working with small cell variants like you tend to find in the cystic fibrosis lung? I've been particularly curious, also, about small cell variants of *Staph*.

DR. TURNER: Right. Not yet. So that is in the realm of these large repositories of strains that we're trying to acquire to test the generality of this

phenomenon for clinical isolates coming directly from CF patients, okay? So we can kind of get at that variation through those experiments.

AUDIENCE MEMBER: By the way, we did once work with 200 CF strains from Univers -- from Children's Hospital in Seattle, and we were able to find phage against all but about eight of them, and of those, four actually turned out not to be *aeruginosa*. We checked them using the 16S ribosomal marker.

DR. TURNER: Yeah.

AUDIENCE MEMBER: You do find them working in other parts of the world as well. When I first got started with phage back in '97, then -- or started with *Pseudomonas* phage, I should say -- I'd always worked with *E. coli* -- we got a bunch of strains of phage from Tbilisi that had been isolated against wounds and burns, and they worked against all of -- all but one of the 18 strains of cystic fibrosis we got at that point.

So from a completely different use and comdip -- completely different part of the world, they worked.

And, again, the one that they didn't work on turned out later -- not actually to be *aeruginosa* when we did --

DR. TURNER: Yeah, yeah.

AUDIENCE MEMBER. So that's something to think about tied in with it.

In terms of how low you can get them, in the oceans they're -- it's completely controlled by phage in terms of what the high levels are. What they do is you do -- it's like the red tide situation. They are at such low levels, about 10 to the fourth per ml, and so are the bacteria below that, and it's only when they get higher than that that the phage can find them enough.

So if you get a sudden bloom of e. coli O157, as we saw in sheep models, then you can activate the phage that are naturally there --

DR. TURNER: Oh, I see. Because they're in the system already is what you're saying.

AUDIENCE MEMBER: They're in the system already --

DR. TURNER: Yeah. Yeah.

AUDIENCE MEMBER: And they work, actually, better. That seems to be what's going on in livestock to keep them in balance.

DR. TURNER: Right. So a radical idea would be whether you can decrease antibiotic administration to CF patients by at least giving them a lower dose of

antibiotic and a phage which helps their quality of life, and the phage is sitting around in case a variant emerges. That kind of a thing.

AUDIENCE MEMBER: And to keep them lower in that kind of way.

DR. TURNER: Yeah. Yeah. I agree.

AUDIENCE MEMBER: So nice work. Keep it up.

DR. TURNER: Thank you. Thanks, Betty.

AUDIENCE MEMBER: Thanks.

DR. RANALLO: Okay. So I just want to thank the morning speakers. I am going to take programmatic liberty and give us a 25 minute break, so we'll be back here at 10:30 for the next set of speakers. Again, thank you.

(Whereupon, a short recess was taken.)

DR. RANALLO: So we have a little bit of a change in our agenda. Frank Ramig had a personal emergency and is unable to make our conference, our workshop today, so we're going to start off with Dr. Roy Stevens from Temple University.

Dr. Stevens is a professor of endodontology at Temple University's Kornberg School of Dentistry, as well as a professor of microbiology at Temple University's Katz School of Medicine.

Roy is going to talk to us a bit about

engineering phage and phage products to disrupt *Enterococcus faecalis* biofilms.

DR. STEVENS: Okay. Well thanks --

(Away from microphone.)

(Pause.)

DR. STEVENS: Okay. That's better. Well I'm still delighted to participate in this wonderfully informative workshop, so thank you for organizing this.

So this morning I'd like to speak to you about a phage genetic engineering strategy that we've been exploring in my laboratory. What you see on the screen here are a couple of phages that we've isolated in our laboratory.

Since my laboratory is located in a dental school, as Ryan alluded to -- endodontology, by the way, for those uninformed in that area, is root canal treatment. I don't hear any moans, so that's good.

So my laboratory is located in the dental school so it shouldn't come to anybody's surprise that the phage that we've isolated infect oral bacteria.

So, for example, the *Siphoviridae* phage on the left infects strains of *E. faecalis* and was originally isolated from a root canal of an infected tooth -- an infected root canal of a tooth.

The *Myoviridae* phage on the right was -- infects strains of the periodontal pathogen *Aggregatibacter actinomycetemcomitans*, and this was originally isolated from dental plaque of a periodontally-diseased tooth.

Most of my discussion this morning is -- about genetic engineering is going to be directed towards the *E. faecalis* phage.

So to start out I think I should say a little bit about a rationale for genetically engineering phage for phage therapy. So what I have on the screen here is a simplistic schematic view of the conventional paradigm for isolating phage that are used in phage therapy, and this is going to be very familiar to everybody in the audience here.

Typically, phage are isolated from the environment, whether it's sewage, or water sources, or animal effluents and so forth. The isolated phage are typically tested for host range.

In the last 60 years or so phage are also characterized morphologically by EM to describe the morphotype, and then in the last 20 years or so phage that have been isolated and planned for use in phage therapy often are sequenced, and then, typically, there may be some clinical trials or animal studies

prior for use in phage therapy.

This approach has been -- the overall success of this approach is largely due to the rate abundance of phage in the natural environment. However, there are limitations to the -- to this approach, and some of them I have listed on this slide.

So using this approach, basically there's a random isolation of phages. It's a relatively hit or miss approach. The saving grace again is the fact that phage are so abundant, plentiful, so that it makes it possible for, in most cases, the process to succeed in any event.

Using randomly isolated phages for phage therapy run the risk of employing a virus with an unpredictable, or even undesirable, property, so, obviously, we wouldn't want to do that. Randomly isolated wild type phages may, in fact, lack qualities that would improve their therapeutic performance, so just using a wild type phage, we may be missing some advantages.

Genetic manipulations of virulent phage may be problematic. Of course there's no convenient way for selecting for recombinant mutants, or positive selection of desired recombinant mutants with the

desired characteristics.

And finally, as we see over and over, what's necessary to be used in phage therapy are basically phage cocktails because of -- the host range limitations of any one specific phage may necessitate using cocktails, and this may complicate safety evaluations needed for clinical development.

That's not to say that there are no genetic strategies for modifying virulent phages, and I have several of these strategies listed on this slide, but even in these cases the same issue applies, or the same issues apply. There isn't really any good, positive selection system available, recombination rates are relatively low, and *in vitro* manipulation of a large, synthetically-assembled DNA molecule is tep -- technically difficult.

So we are looking for an alternative way of modifying a phage to make it perhaps more useful in phage therapy, and our strategy essentially involves starting out with a prophage of a temperate virus and winding up with a recombinant phage of a virulent virus.

Basically what we do is it allows us to use conventional bacterial genetic strategies to make modifications in the genome, in the prophage genome,

and ultimately change the region of the genome that controls lysogeny such that the resulting virus is no longer capable of lysogeny. So we convert it into a virulent phage after we do whatever other recombination work we want to do in the prophage.

So by doing this we actually have sort of an oxymoron. We have a prophage of a virulent virus, which to most phage people probably wouldn't make sense, but this is basically what we are able to achieve.

So it's a three step process, in which we initially make -- we replace, or delete genes in the prophage that we wish to change. In the second step we use a second allelic exchange mutagenesis to delete lysogeny-related genes and replace the wild type promoter that drives lytic cycle functions with an exogenous inducible promoter.

And so what we're -- in doing these manipulations we can easily select for lysogens that contain the recombinant prophage by simply plating the reaction mixtures on antibiotic-resistant plates and recovering the recombinant lysogens.

In the final step we can induce the phage using appropriate inducing agents to produce the virulent version of the original temperate virus.

So let me give you an example of how this works using one of the phages that I showed you earlier in the talk. This is the *E. faecalis* phage that we isolated in our laboratory from an infected root canal.

This clearly is not a phage that anybody in their right mind would consider as a candidate for phage therapy in its wild type state. Upon isolation it was identified as a temperate virus. It's weakly lytic, and it has a narrow host range.

So the isolation procedure for this phage was nothing very unusual. We isolated it, again, from an infected root canal; that is, we isolated *Enterococcal* strains from an infected root canal, we plated these out on selective media for *Enterococci*, we got -- we recovered *E. coli* clones, we picked clones and we induced with mitomycin c, and then we test the resulting cell-free culture medium for plaques against the panel of *E. coli* strains, and this is what you see. Small, somewhat turbid plaques.

If you grow them up and purify the phage and -- you can do EM analysis, and this is what the phage looks like.

So when we purified the virus we further analyzed the genome. After sequencing the genome we

found that this virus has a genome consisting of 42,822 base pairs, distributed among 65 open reading frames. And that's many -- as has been mentioned by other speakers here today, typical of many, many other phages. The genes are arranged in functional modules, as you see illustrated in this diagram.

We focused on one region of the genome, which you see here, and it appears that the apparatus that determines lysogeny, or lytic functions, are found within this region of the genome. That is, the establishment and maintenance of lysogeny is basically determined here.

Now if we look at this in a little bit more detail we can see that there's open reading frame 31 which is predicted to code for an integrase, open reading frame 36, which is predicted to code for a *cI*-type repressor, and open reading frame 37, which is predicted to type for a *cro* type repressor. In between 36 and 37 there is a regulatory region, which we'll look at in a little bit more detail.

And, as we'll see shortly, transcription in the right direction results in lytic infection, transcription in the left direction results in lysogeny.

This is that region between 36 and 37. You

see that there is a stem loop structure, and to the right there's a promoter that controls transcription of *cro* and the remainder of the lytic functions, and to the left is a promoter that controls transcription for the *cI* repressor and the lysogeny functions.

So how do we go about doing this? We design a vector in which there are homologous regions upstream and downstream of the lysogeny genes, and between these two homology regions we have an antibiotic resistance marker and we have a inducible exogenous promoter. In this case it's the nisin promoter.

So upon homologous complementation, this will permit complementation between the vector and the prophage, and ultimately, in a small fraction of the cases, there will be an allelic exchange, and the result of that will be a pro phage that now has the antibiotic resistance marker and the nisin-inducible promoter in place of the lysogeny genes and the wild type promoter that was in the original prophage.

So this actually represents, as I mentioned before, a -- now a prophage of what is now a virulent pha -- virus. The lysogens that now contain this construct can easily be selected on antibiotic-resistant plates, in this case with erythromycin, and

those clones can then be induced using the appropriate inducer, in this case nisin, and you can get the phage out, and that phage will have the properties of the virulent virus.

So what we've done by doing this is to cause the deletion of all the lysogeny-specific genes of the prophage and replacement of the wild type promoter with an exogenous inducible promoter, in this case the nisin promoter, and this will yield a virulent variant that is incapable of lysogeny since it has none of the genes needed for lysogeny, and, furthermore, it's not sensitive to repressor repression since it has an exogenous promoter that's not sensitive to repressor.

So we've changed this genome on the left from the wild type to the recombinant genome you see on the right.

If we compare the wild type to the genetically-modified as you see in this slide, you can see that there is a noticeable change in the host range. We have -- the wild type had a very limited host range. As you can imagine, the wild type temperate virus is subject to repressor repression, whereas the genetically-engineered version is not sensitive to repressor, and so it can, in fact, infect other lysoge -- lysogenic strains.

If we take that genetically-engineered phage and we inf -- and use it to infect biofilms, we can see a very dramatic result. On the left you see controlled biofilms of two strains of *E. faecalis*. JH2 is a vancomycin-sensitive strain, V583 is a vancomycin-resistant strain.

This is a live dead stain, and you can see a very rich biofilm that was formed in this system. In the phage-treated biofilms you see almost complete elimination of the biofilm in the JH2 strain, and an almost as complete elimination wi -- in the vancomycin-resistant strain.

In fact, what's -- where I found interesting in this is that if you do a cut through the biofilm, you can even see the death of the cells throughout the depth of the biofilm.

This is actually a concern of -- in terms of being able to deal with biofilms. It's been, you know, postulated that cells at the depth of biofilms are protected in certain ways from agents that are going to be used for treating them, and yet here we see bio -- in a biofilm all the way to the bottom of the depth of the biofilm predominantly dead cells.

If we want to take the recovery, we can see that in both the cases of the JH2 strain and the V583

strain there is a substantial diminution of the recovery, there's basically a two log drop, at least, in the recovery, and the amount of detectable residual cells recovered is quite small.

In addition to testing a biofilm formed on a glass slide, it turns out, for all the non-dentists in the audience, that infected root canals also produce biofilms inside the tooth, the -- depending on what kind of infection it is, and so we fabricated a dentin infection model in order to test the effects of phage on infections of the dentin.

So here, in this model, we fabricated a cylinder made out of the de -- the root of a tooth, which is basically all dentin. This dentin cylinder is then sealed inside the encasement of a disposable needle cap, and then that is put -- assembled inside a -- the cap of a needle and buffer can be placed in the lower portion of the cap, and either this -- the *E. faecalis* can then be injected into the root canal, which you see in the center of the dentin cylinder.

After incubation for a period of time, the phage can also be introduced into the root canal. The result of that is -- to the remaining bacteria is shown on the next slide.

You can see with the vancomycin-resistant

strain there's a dramatic drop in recovered *E. faecalis* from these infected root canals. For a reason I -- we not quite clear about yet, the decrease in the J -- in the vancomycin-sensitive strain is not very impressive. We're curious about that, and we'll probably be looking at that further.

So it appears that you can genetically alter phage to change its properties and make the phage more usable and useful in -- as an antimicrobial agent.

Now, in addition to looking at the phage itself, we also looked at products of the phage. In this portion of the phage genome you see a series of genes that appear to be related to the lysis of the cell. There are -- there is a lysin, a holin -- I'm sorry -- a endolysin, and the ORF28 gene product appears to be an amidase-type lysin.

So what we did was we PCR-amplified the ORF28 gene using the phage DNA as a template, we cloned the gene into an expression vector in tandem to a GST tag, and so we got this vector as you see on the right.

If we transform that into an *E. coli* cell and express the gene and then make a sonic extract of the *E. coli* where the gene is being expressed, we get this mixture of, basically a gemisch of all of the

components of the *E. coli* cell, including the produced ORF28-GST fusion product.

We can put that through a glutathione affinity column which will bind to the GST, the glutathione, as transferase protein, which is associated, or attached, or fused to the ORF28 lysin. Then, by adding excess of glutathione, we can elute off that protein.

In the gels that you see in the lower left of this slide, you can see that after putting it through a column several times, we get basically a homogenous preparation of a protein of 72 kilodaltons. And you'll notice that the ORF28 gene product is predicted, or the sum of the ORF28 gene product and the GST fusion tag is 72.5 kilodaltons, so this appears to be a purification to just about homogeneity.

What's interesting is if you take -- if you spot some of this material onto a lawn of any of the -- or many of the strains of *E. faecalis* that we have in our collection, you can see that it produces a very noticeable and distinctive lytic zone in these different *E. faecalis* strains, including vancomycin-resistant strains.

So out of 99 strains that we've tested so

far, just a little over half of them are quite sensitive to this lysin. Of the 99 strains, two of them, two of the *E. faecalis* strains are vancomycin-resistant, and both of those are sensitive to the lysin. So vancomycin resistance, as in VRE strains, does not pose a problem to the lysin.

Adding this substance to a suspension of the *E. faecalis* strains causes a very rapid clearing of the suspension. In about 15 minutes you can start to see a precipitous drop of the turbidity of *E. faecalis* suspension, so the reaction occurs quite rapidly.

Again, you can use that purified lysin that we got from the phage on a *E. faecalis* biofilm. On the right you can see what the biofilm looks like after it's been treated with this lysin. The left is, of course, a control. You can see an obvious difference.

And you can see -- if you quantitate the recovered -- the recov -- the cov -- recovery of the residual cells from the treated versus the controlled biofilm, you see that there's about a two log drop, and to very low levels. So the lysin, as well as the phage is very active in disrupting *E. faecalis* biofilms. Okay.

So as we've seen in other presentations

today, there are many, many other *E. faecalis* phages that have been isolated and characterized, and out of many of these, the lysins have also been identified, sequenced.

We've compared the sequence of the ly -- the ORF28 lysin that we're -- that we got from our phage to each of these other lysins, and, surprisingly enough, when we look -- when we do a BLAST analysis, we see only very moderate homology between the EF11 ORF28 lysin, which is what we've been working with, and each of the lysins of the other *E. faecalis* phages.

So I'm no -- I won't go through each one individually, but you can see easily that there's only a modest percentage of identity between these two lysins.

In another phage, phage 1 -- this one was I think the one used by Fischetti's group in isolating the lysin that they published on -- again, you can see only a moderate degree of homology between our lysin and the lysin of the phage 1, and so on and so forth for each of the other *E. faecalis* phages that we analyzed, and so this is sort of a summary of that.

If you, you know, go down the list, there appears to be only 10 to 20 percent identity between

the lysin that we obtained from this phage and any of the other *E. faecalis* phages, which is curious to me because these are all *E. faecalis* phages and, presumably, they all have to lyse the same, or very similar, cell walls in order to go through a lytic cycle, and yet they are obviously different.

So one thing that we would like to do is actually compare the host range, if you will, of the lysin that we have to the host range of many of these other phage lysins and see if there's an overlap or not.

So, with that, I'll conclude my talk. We're trying to produce a super phage that will be super useful in phage therapy.

Before we close up shop I have to thank the -- all the contributors to this work. Hongming Zhang is a research scientist working in my lab. Tina Buttaro is a professor at the medical school who set up all of the biofilm assays. She's done a lot of work in *E. faecalis* biofilm analysis.

Derrick Fouts who is here, in the audience, somewhere in the back I think, helped. He is a staff scientist at JCVI, and he was -- played a major role in the sequencing and annotation of our phage genome. Lastly, but not leastly, Justine Tinoco was a graduate

student who did many of the assays that you saw in this presentation.

So, with that, I thank you.

DR. RANALLO: Okay. So we have some time for questions if anybody has, anybody has any questions.

AUDIENCE MEMBER: It's an interesting observation that you don't see a lot of homology between your lysin and those of other phages. I'm just curious what the identity -- if you exclude yours and look at how similar those other lysins are to each other, is there also dispar -- is there disparate relationships between those as well?

DR. STEVENS: I haven't really done that. That would be interesting to do as well. I mean you can do each permutation of each of them against all of the others and see.

But, again, I know that you're very interested in cell wall structure, and, you know, I'd be very interesting -- interested in learning more about the potential binding sites for the lysin. That may also be something you're interested in as well, whether each of these lysins have a different target on the cell surface or not. We just don't know that.

DR. RANALLO: Actually, I had a question.

Is it -- did I understand correctly that the antibiotic resistance marker, once you're done, is still present? You know what --

DR. STEVENS: Right. It's a great point. I did not mean to imply that the genetic engineering is completed. This is mark two or three of the manipulations that we've been doing. Before this could be used in a patient certainly, you're absolutely right, we would have to use a different strategy for just eliminating the antibiotic-resistant marker, and there are markerless methods of doing that as well. Yeah.

AUDIENCE MEMBER: So, yeah, just one quick question. Have you looked at using Hidden Markov Modeled -- Modeling -- predictive modeling for the structures of the various lysins that you're looking at to see if there's an overall structural fold that's held in common?

DR. STEVENS: No. Haven't done that.

DR. RANALLO: Okay. So we're going to continue on with the engineering theme with Dr. Timothy Lu from MIT. Tim is a rising star at MIT. He's an associate professor and leader of the synthetic biology group in the department of electrical engineering and computer science and

department of biological engineering at MIT. Tim's going to talk to us about engineered phages for the dia -- for diagnostics and therapeutics. Take it away, Tim.

DR. LU:: All right. Thanks a lot for the opportunity to be here. I think it's a really exciting forum to be able to talk at. I also want to thank Dr. Stevens earlier for basically introducing why we want to engineer bacteriophages. So I'm going to walk through some of the work that we've been doing in our own group to try to engineer bacteriophages for a variety of applications. I think they're pretty interesting, you know, chassis to play with.

Before I start, you know, I'm involved with several companies involved in sort of commercializing bacteriophages -- I wanted to list them here -- including BiomX, Eligo, and AmpliPhi, as well as Sample6.

So my lab is really focused on synthetic biology. Really what we're excited about is really this exponential increase in our ability to genetically engineer stuff. That might include viruses, it might include cells. Today I'm going to focus primarily on viruses.

So we're excited, really, by this

exponential improvement in our ability to read and write DNA, and how can we leverage that to modify organisms or viruses for useful applications.

So this has been a challenge for us since I was doing my Ph.D., and one of the questions that I started off with was could we try to engineer bacteriophages for therapeutic, as well as diagnostic applications.

Initially we were inspired by this challenge which I think we've heard about already, which is that can we get away from this idea of using broad spectrum antimicrobials and move to a paradigm where we use narrow spectrum antimicrobials to either treat infections or, I think, actually, a potentially even more exciting opportunity, or at least equally exciting opportunity, is to modulate the microbiome.

If we're going to do this we need strategies that allow us to do diagnostics and therapeutics. So if you have a narrow spectrum antimicrobial but you can't quickly tell whether an infection is going to be susceptible to it, from a clinical perspective, it's going to be really hard to deploy.

So I think we've been focused on trying to develop tools to allow you to build rapid diagnostics. So can you engineer phages as a diagnostic tool? So

I'll tell you briefly about that effort, and then for the remaining time I'll tell you about some of the effort to now engineer bacteriophages in a variety of different ways, primarily using them as gene therapies for bacteria, and how we can then use that to modulate bacterial populations in targeted fashions.

So I'll start off with the diagnostic application. Sort of alluded to this earlier. Really, what we want to ultimately do is enable precision therapy, right? So we do precision therapy increasingly for cancer. Why don't we do that for infectious diseases?

Well one of the things we need to enable that is a rapid diagnostic platform, and, ideally, something that's relatively easy to use, point of care, and can give us information about what bacteria we're actually going after.

If we can do that, then we can potentially couple that with narrow spectrum antimicrobials. In some cases that might be phage therapy on its own. I think we've heard a lot of great examples here about combining phages with other antimicrobials. I think that's a very potentially powerful way to go about it, especially if you start coupling some of the strategies we heard earlier from Dr. Turner and

others.

So we're excited about coupling the two together, and so a -- you know, after my Ph.D. we decided to try to see if we can actually try to solve the first part of this problem. Can we develop diagnostic tools that allow us to rapidly diagnose the presence of microbes?

So here's the basic idea. And this is an idea that the field has worked on for the last 20, 30 years in terms of building reporter phages, but I think we're quite excited that we've been able to now commercialize this and actually do the genetic engineering of these phages at a point where it's actually applicable at industrial scale.

So the idea is really simple. We know that phages can be narrow spectrum, and so we can identify phages that are selective for certain bacterial populations, and then we can genetically engineer those bacteriophages to basically force the cells that they infect to produce some sort of reporter.

So in this particular case we're engineering the bacteriophages to deliver some sort of reporter gene, like a very strong luciferase, and basically what happens is the bacteria get infected by the phage, they start generating luciferase, and now, with

a reader, you can basically detect whether there's light coming from your bacterial population.

This has allowed us in -- to build diagnostic tools that give us readouts of presence of bacteria in a population in a few hours.

So the initial application for this technology we started off with was actually for the food industry. It was a little bit lower hanging fruit for us initially when we started the company. So we started off going after *Listeria* -- so *Listeria* is one of the major food pathogens -- and subsequently we have tests for *Salmonella* and *E. coli* sort of in the pipeline.

Longer term I think this technology, especially as we get better and better at engineering these bacteriophages, has a broad range of applications in the clinical space, being able to do, potentially, rapid diagnostics for other clinically-relevant systems.

So here's just a comparison for the sort of phage technology we've developed to compare it to sort of conventional assays that are used in the food industry. Like PCR or immunoassays, those can be quite slow, primarily because they require a primary enrichment step.

So both of those methodologies require you to grow the bacteria for a period of time so that it's -- the test is either sensitive or specific enough. Number one, most food companies don't want to be growing large amounts of pathogens on site so they often ship that out, that adds additional time, and then the enrichment time itself adds time to the actual assay.

So our goal was to try to see, can you develop a test that you can run on site that's easy enough to use, that you can basically take non-trained biologists, basically, you know, potentially high school or college-trained technicians, teach them to run this assay on the factory, and be able to get a result on the same day. So you can come in the morning, do an assay, see if the food has, for example, bacteria or not, and then you'll make a decision on what you do with that.

So I'm happy to tell you that we spent a few years and developed actually a test that's, frankly, pretty simple and easy to use. So basically one version of this test looks as follows:

We have basically a sponge they use to swab some sort of surface. You then put a bit into a bag, you add the bacteriophages, you let it sit in the

incubator for about six hours.

Then you take a little bit, an aliquot of the liquid there, stick it into a very simple luminometer, and then you basically read is there light or not? Based on that information, you can make a diagnosis of whether there was a particular bacteria, in this case *Listeria*, in the sample that you were taking.

The system is quite easy to use, and so we've been able to deploy this in a variety of sort of large food processing plants where people basically -- we don't need to train technicians, as you might need if you're performing PCR-based assays to carry this out.

In addition to this, we don't really have time, but the cool thing about sort of having a cheap and easy to use diagnostic is then you can couple that with analytical tools. So we've now developed methods where you can sort of geo-locate where assays are being taken on the factory floor and really build sort of analytical cloud-based tools to see where contaminations are happening on your factory floor and how you stop that from happening.

So one of the reasons we started off with the food application is because you can go through and

get a test that's industrially used in a relatively short order. So we got the certification from this AOAC institute for the detect test.

But now we've also been working on a variety of therapeutic, sort of clinically-relevant sensors. Here's just an example. I don't have sort of like the more finalized data to show you, but just thought I would just point out some, you know, that this can work to detect bacteria in other formats.

So in terms of saliva, urine, and blood, we've done this sort of testing. In this case we have *Salmonella* that we can detect pretty -- relatively quickly. In about a few hours you can detect down to about one or 10 CFUs/ml. So I think there's app -- sort of potential applications for this technology that you can envision beyond just the food industry.

So I think I told you about some more efforts to try to develop rapid diagnostic tools with phages. I think they're very useful and already sort of making an impact in the industrial space. I'll spend the rest of the time talking about some of the therapeutic applications that we've been envisioning and what we've been focused on over the last, I would say decade.

So I think we've heard about this

previously. I think one of the areas that we're excited about is this idea that, potentially, we can engineer, or evolve, antimicrobial agents in a -- to keep pace with the evolution of resistance, bacterial resistance.

I think the challenge with going after bacteria is they're, you know, probably going to outsmart anything we throw at them eventually, but if we can keep -- at least keep pace with them in the development of novel antimicrobial agents, maybe we can at least keep pace with their development of resistance. So if they take a step forward, can we take a step forward ourselves with a counter-measure.

So we've developed a variety of phage engineering-based technologies to do this. We heard a lot yesterday and today about engineered bio -- about bacterial biofilms and how they can be a problem.

From my simplistic engineer's perspective, I sort of think of biofilms like fruit Jello, where the fruit is sort of like the bacteria and they make this gelatinous matrix that makes it very hard to clear out the bacterial contamination, so it poses a challenge for any antimicrobial agent you're trying to develop.

Similarly, biofilms can be associated with antimicrobial resistance. I think we've heard a lot

about the challenge of going after antimicrobial resistance so I'll skip over this.

In our lab we're primarily focused on going after the gram-negative pathogens. I think we're particularly focused on this because of the great need for novel antimicrobial agents, especially going after these specific pathogens.

So early on -- actually, this is some of the Ph.D. work I was doing together with Jim Collins. We started to think about how do we develop target therapies to go after biofilms, right? We know that biofilms are involved in a lot of medical-related issues, and as well as in the food or industrial space, biofilms are part of a major sort of burden on industry. Current methods for going after this including mechanical disruption or chemical-based methods are not necessarily the most effective.

So one of the strategies we came up with, this was back in 2007 when we published this, was this idea that we could engineer bacteriophages to express biofilm-degrading enzymes. If you actually look at natural phages, some phages actually carry these enzymes with them to allow them to access biofilms or to degrade polysaccharides.

What we tried to do is to demonstrate that

you could actually synthetically encode the expression of these enzymes into an artificial phage. So in this particular case we took a model phage T7, showed that you could genetically modify it to express a biofilm-degrading enzyme, and the idea is if you could then sneak just even a little bit of that bacteriophage into the biofilm, you could generate this loop where you generate more enzyme, it breaks up the biofilm, and hopefully help propagation of the engineered bacteriophages.

So we showed in this particular experiment that if you compare the engineered bacteriophage, which has dispersin B, an enzyme that is known to disrupt certain types of biofilms, with a control or untreated, we could get, in general, two to four orders of magnitude increases in our ability to eradicate these biofilms, even with a very small dose of bacteriophages to start.

In addition to that, so we -- you know, that was like the initial work that we did, and we quickly realized that perhaps the bacteriophages could then be extended to other sorts of applications. So could we use the bacteriophages not just to degrade bacterial biofilms, but to potentially synergize with other treatments that are already in use.

So one of the strategies that we started to look at was whether we could actually engineer phages sort of like gene therapy vectors for bacteria to deliver payloads into bacterial populations that allow them to have an effect on antimicrobial resistance, for example.

We heard a lot earlier about sort of diverse mechanisms by which bacteria can become resistant to antibiotics, including sort of discrete mechanisms like exporting the antibiotic or degrading the antibiotic. In addition, there are sort of bacterial defense mechanisms, for example, the generation of reactive oxygen species and sort of the triggering of certain response pathways inside the cell, that could be potentially targeted with an engineered phage if you think about it really -- not the phage as a sort of direct killer, but as a gene therapy device.

So here's a very simple schematic of some of the work that we did. If you envision antibiotics inducing DNA damage that induces some sort of let's say a DNA repair response that allows a cell to survive, what if we could try to potentiate that sort of strategy by engineering a phage. In this case, this is a phage that's not lytic, it's lysogenic, that potentially can deliver a gene inside of the cell.

Here we used a particular protein, LexA3, which suppresses the SOS response. The idea was we wanted to test whether we put these two strategies together, can you get a potentiation of killing.

So we actually looked at this engineered bacteriophage, phage lexA3 which is shown here in blue, in comparison -- sorry -- in combination with three different classes of antibiotics: quinolone antibiotics, which in this case is ofloxacin, aminoglycoside, gentamicin, as well as a penicillin class drug, and showed that in all cases, if we combined the bacteria -- engineered bacteriophage together with the antibiotic we -- you got a potentiation of killing by several orders of magnitude. This was simultaneous treatment.

We also looked at what happens if you can take sort of bacteria that are already resistant to drugs. So here's an example where these bacteria were already resistant to ofloxacin.

We showed that, you know, normally if you just apply ofloxacin on its own, really, these bacteria don't really get affected by very much, maybe an order of magnitude of killing. Combine this together with the engineered bacteriophage, we again get a very significant potentiation of the killing

effect.

So we then went on to test this in an animal model of infection. We basically took *E. coli*, infected the bac -- infected mice, and then tried to treat with either antibiotic alone, which is shown in black, the solid black line, or the combination therapy, the engineered phage, plus the ofloxacin antibiotic, and showed here in blue we can -- found sort of increased survival with the combination type approach.

So I think, moving forward, it would be quite interesting to explore how, you know, engineered -- phages can be engineered in this fashion to try to synergize with antibiotic -- particular antibiotics, or, as I'll show you a little bit later, where we might be able to engineer phages to try to directly re-sensitize bacteria to antibiotics or kill selectively antibiotic-resistant pathogens.

So that sort of leads us to this next story. So when I first started the lab I had two very talented students, Rob Citorik and Mark Mimee, who wanted to take this to the next level and think about, can we build even more targeted strategies as antimicrobial agents.

So we started off thinking about, again,

this problem of broad spectrum antimicrobials, which generally address either protein-based targets or other sort of, you know, cell wall synthesis type mechanisms. What if we could actually develop antimicrobials that act at a very different level, right?

So if we want to really realize this dream where we can make a new antimicrobial base really quickly, then one of the best ways, potentially, to do that is if we can just make sort of sequence-specific antimicrobials. Because we can make -- we can sequence DNA really easily, and then we can make -- we can print DNA really easily, right? It's a lot easier for us to do that rather than develop a new drug with a target-specific protein.

So what if we could actually enact specific targeted pressure against undesirable genes at the level of DNA? So in order to do this we actually started off using zinc finger and TALE factors, but quickly realized that the CRISPR system was a more powerful way to do this.

I'm sure everyone here knows how CRISPR works, but just briefly to mention that we sort of think about the Cas9 enzyme, which is shown here, this Mickey Mouse structure, as a molecular scissor, it's

directed by what's known as guide RNA, to target a specific location of DNA and cause cutting.

In human cells people use this for genome editing because the cutting event leads to repair pathways that repair the DNA in a specific way. In bacterial systems that lack, you know, very robust repair systems, this can induce cell death.

So the idea here would be very simple. What if you could actually engineer a bacteriophage -- we also did this with bacterial conjugation in sort of like a -- we could talk about this later, if you're interested -- we -- sort of like in a gene drive type methodology. You can imagine spreading sort of self-transmissible plasmids everywhere that contain this. But here, because this is a bacteriophage meeting, I'll just focus on the phage-based data.

So what if you could make a phagemid, right? So this is not a propagating phage, it's just a virus structure that contains a piece of DNA that just delivers the DNA. So, again, this is really thinking about phages as a gene therapy vector.

So what if we could package the Cas9 system into a phagemid, and then use that to deliver this Cas9 system into targeted bacteria? The idea would be that in bacteria that contain a specific gene that you

don't like, like a resistance gene, antibiotic resistance gene, you could cause DNA cleavage, cause those cells to die, but in a related bacteria that doesn't have that sequence, they would be fine.

So here's the experiment we did. We took a bacterial cell. This is, in this case, *E. coli* that contains a genomic target. Here we tried two different settings. So we had wild type *E. coli*, as well as *E. coli* with the gyrase A mutation that confers quinolone resistance, and then we developed two different RNA-guided nucleus type phagemids, one that targets the *ndm-1* beta lactamase gene -- this is -- as well as one that targets specifically this mutation, *gyrA*.

So in the base case, in the wild type cells, you basically transduce them with this phagemid, and it basically showed there's no really toxicity that you can see with this sort of approach, but if you then follow up with using this engineered, what we call the sort of RNA-guided nuclease phage, or the CRISPR phage, to deliver into these cells, we can get a very selective killing of the bacterial population with the gyrase A mutant, but not the *ndm-1* targeting mutant.

So this, in effect, shows us that we can

actually -- if we can achieve efficient delivery of payloads into a specific bacteria either using phages, or conjugative methods, or other methods of these Cas9 type elements or other CRISPR systems, could be useful for causing site-specific cleavage, as well as then cell death.

Because of time I won't show you some of the other data we generated in this paper. You could take a look. We also show that you could actually target plasmid-borne targets. Depending on the context of that plasmid, you could either just cure the cells of the plasmid without affecting, really, toxicity against the cells, or cause cells to die.

Other applications of this technology potentially using the CRISPR system as a diagnostic tool. So here, this idea is, again, very simple. If we can engineer these phages to cause cleavage, the cleavage event, at least in some bacteria, triggers the SOS system, and then if you have a reporter that turns on some sort of GFP or luciferase, you could then use this for very sequence-specific diagnostic tools.

We did two examples of this. Here, one where we have *E. coli* with the *ndm-1* plasmid from a clinical isolate. We show that with the cognate RNA-

guided nuclease you see an increase in GFP fluorescence in that case, and similarly in the *E. coli* that contains the gyrase A mutation, you get a very selective increase in GFP fluorescence.

So, again, I think if you're going to build very specific killing tools, you need very good diagnostics that go with them. There's a lot of improvements we can do upon this, but this is a proof of concept that you might be able to use this methodology for sequence-specific diagnostics of bacteria based on their genomic sequence.

So I think one of the things I want to follow up on is, you know, we've talked a lot in this conference, I think almost every talk in this conference has really been about infectious disease applications of bacteriophages, and, indeed, that's the traditional way of thinking about phage therapy, and then -- we are quite also excited about that potential approach.

I do think that actually one way for phages to maybe have a broader usage is to sort of think about phages as a way of modulating the microbiomes, right? So when we're using bacteriophages to target a population, oftentimes the bacteria that we're going after are not just like sort of the dominant player

there, they might be one small member of a sort of consortia of bacteria that have a wide range of effects on our immune system or health, and so if we're going to go and try to modulate the microbiome, we need tools that are very narrow spectrum and targeted to be able to modulate specific members of the microbiome.

Right now the tools that we have are relatively crude. So we have fecal transplants, which is like taking an entire ecosystem and trying to slam it onto another ecosystem, we have antibiotics that sort of act as like sort of a nuclear bomb on your microbiome.

So I think if we're thinking more precisely about replacing or delivering things into a microbiome we need tools like phages, potentially, or other sort of narrow spectrum antimicrobials that can be useful for this type of approach.

So I would advocate that thinking -- going forward, as a field, we should think not only about the infectious disease applications, but let's think about the microbiome-type applications. There's a couple advantages here that we can talk about maybe later in the forum, one of which is that potentially you can avoid sort of only talking to the ID docs.

No offense to ID docs, but really expanding upon the indications that you can go after with engineered phages or, you know, natural phages for microbiome-associated diseases which are now being implicated in a variety of different areas, including GI health, neurodevelopment, et cetera. Really opens up the scope of what this powerful tool is that we all have a lot of interest in.

So we've been thinking about how do you start modulating microbiomes, and can you test the specificity of this. I'm just only going to show you *in vitro* stuff because it's the stuff that's been published so far, but we're certainly doing a lot of *in vivo* work in this now.

Here's an example using the CRISPR phage. It's to target a three-member consortia, right? So all through these bacteria here, blue, purple, and orange are all susceptible to the phagemid, they can all be infected by the phagemid, but only -- they all have different genomic signatures.

So, for example, if we apply phage B, which targets the B gene in this population, we only want to kill the bacteria and we want to leave the other guys happy, right? So imagine if you had a bunch of *E. coli* in your body. One of them was bad, you want to

get rid of that, but affecting everyone else. Similarly, we do that with this phage A gene.

So here's an example where we have the three member community. If we apply the very specific *ndm-1* bacteriophage, we can basically knock down the *ndm-1* tool -- sorry -- the *ndm-1*-containing bacteria, and similarly with the gyrase A mutation.

So we're starting to think about these phages not just as like antimicrobials, but, really, can we do what RNAi did for genetics. But think about this at a population level. Can we build phage-based tools or other conjugative-based tools that allow us to do specific knock downs of a bacteria in a population. All right.

So this is a summary of this particular approach. I sort of mentioned sort of genomic targeting, but the paper actually describes other stuff. Targeting plasmids, for example. And we're sort of thinking about this like gene drives, where people try and eliminate mosquitoes in a population. What if you could eliminate bacteria also, similarly in a way. This is kind of radical, we haven't figured out the regulatory path, but I think, technically, it's actually very doable. All right.

So in the remaining time, I know we have

lunch afterwards, I thought I'd tell you some -- just finally, the other things we're working on in the lab. I think we've quickly realized after all this work that phages are a really useful tool. We can engineer them to deliver all sorts of cool payloads into the bacteria. The challenge ultimately, and this is similar to the gene therapy field, is that like delivery is still the challenge.

So how do we achieve delivery? How do we get the bacteria that we want, and be able to engineer the phages to do what we want, and deliver the right thing to the right place? So we heard about this earlier from the previous speaker, about sort of classic phage hunting. I think there's a lot of benefit for that side of -- type of approach. The primary benefit is that you come out with natural phages, and, potentially, the regulatory hurdle I think is lower with those sort of things.

But I think there is a great opportunity for thinking about phages as an engineerable biotechnology. So one of the things we've been interested in doing is to try to adapt this idea of the antibody, but conceptualize this with the phage scaffold.

So what if you could take a phage and keep

most of the phage the same and simply switch out some parts of that phage to redirect its activity against other bacteria? If you could do this, there might be some advantages.

First, most of the phage scaffold is the same. It just makes genetic engineering of that phage scaffold really easy, right? We can develop one set of tools and just use it over and over again.

Secondly, manufacturing potentially could be easier, right? So if we don't have to worry about sort of manufacturing 20 different phages that are completely different from each other, if we have one phage that's quite uniform and simply tweak, for example, the tail fiber to change a spectrum, that might be beneficial.

So this idea of the phagebody we did some work on a few years ago, and we continue to do stuff now. I have a couple papers in review on this idea. Basically, we wanted to show that you could actually take phages and swap tail fibers, right? So it's previously been shown in -- from the literature that phages can make hybrids, for example, and you can change specificity based on that strategy.

Could we develop an engineering pipeline to enable that tail fiber swapping more efficiently? So

here's a very simple concept. Can we take the red phage, which normally goes after the red bacteria, and instead make it go after the blue bacteria. The way we do that is by giving the red bacter -- phage the blue bacteria phage's tail, right? So we swap them. All right.

So we heard earlier about some of the challenges of engineering bacteriophages. One of the challenges with engineering bacteriophages, especially lytic ones, is they kill bacteria, and most of the tools that we have for engineering anything is reliant on sort of bacteria staying alive.

So we had to come up with an alternative strategy. Fortunately, you know, the folks at JCVI, and others, have developed tools based on yeast that allow you to do very efficient genome engineering.

So what we realized is if you could capture the phage genome into a yeast artificial chromosome, you know, the phages are pretty happy in terms of living there because they don't really kill the yeast, and now you can propagate the yeast, do whatever you want with it.

The other cool thing about yeast is it's really good at DNA assembly. So you can make different fragments genomically and assemble them

together. You can then extract the phage genome out and then transform your bacteria, and then you can then, in many cases, actually boot up viable phages. It's pretty cool.

So here's an example where we did this. We basically wanted to just recapitulate some of the experiments that had been known previously on T7, T3 hybrids. So here's an example where we basically took T7, and we grafted the GP17 tail fiber protein from T3 onto the T7. We built two different constructs, one where we swapped the whole gene 17, we also swapped just a portion of gene 17 between the two phages, and we showed that this basically was sufficient to cover host range switching activities.

So basically, the T7 with the T3 tail fiber basically looks like T3 in terms of its host range, the T3 with the T7 tail fiber looks like T7 in terms of its host range.

We've also now done this with other bacteria -- sort of targeting other types of bacteria. Here, this is a very simple example where we simply mutated several point mutations in the T3 genome to make T3 phage now go after *Yersinia*.

We've also done other experiments where we actually start swapping tail fiber components between

bacteria that target different species. So here we took *Klebsiella* K11 and we swapped several components onto the T7 scaffold.

So one of the things we're trying to explore is really how modular and flexible this strategy is going to be. Initially, we were hoping that the tail fiber itself was going to be sufficient for conferring this host range switch. With K11 we realized that this was not actually going to be possible, and so we ended up having to swap the GP11-12 structure which really composes this sort of tubular structure, as well as the tail fiber, in order to get sufficient tail fiber swapping.

Here's just a demonstration that the T7 with the *Klebsiella* tail components infects *Klebsiella*, the K11 phage with the T7 components infects *E. co*li. Here's both on plaque assay, as well as based on killing. The T7, K11 shown here in the green does have about three or four orders of magnitude of killing, although it's not as good as the native bacteriophage, and we're trying to figure out why that might be the case.

Similar as what I showed you earlier. The ultimate goal here is can you produce a population, a cocktail of these bacteriophages, whether directly

having lytic efficacy or delivering some sort of payload, but go after a population of bacteria.

And so here, again we just show that you could actually make cocktails of these bacteriophages, for example, T7 plus T7 containing the K11 tail fiber -- so basically, these phages are majority similar -- except of the tail fiber component -- and show that when you put these things together, you can then eliminate one or more species in a sort of rational way from a microbial population, as we show here.

So, with that, sorry to run a little bit over time. I think I just want to reiterate this idea that, you know, infectious diseases is great, we should continue going after that, but I think, personally at least, we're very excited about sort of the opportunities here for modulating microbial communities and thinking about microbiome type applications.

This wouldn't be possible without a very good lab and talented group of people. So Rob Citorik and Mark Mimee did the work on the CRISPR phages, Sebastian Lemire, as well as Hiroki Ando, did the work on the engineered phage bodies. With that, I'd like to thank you for your time and take any questions.

DR. RANALLO: So that was a lot to unpack,

Tim, but I'm sure we have -- we certainly have time for questions if anybody has any questions. Oh, and just for my FDA regulatory colleagues, in terms of engineering, that pain in your head, that's called a new headache.

(Laughter.)

AUDIENCE MEMBER: Hi. That was a super, super cool presentation.

DR. LU: Thanks.

AUDIENCE MEMBER: I have a couple of questions for you. Have you tried using nanoswitches for your bio detection assays? Sort of a similar approach, but with a nanoswitch.

DR. LU: What do you mean by a nanoswitch? I'm not familiar with the term.

AUDIENCE MEMBER: You could use your bacteriophage, couple it with your whatever, luminescent release thing, and when the bacteria binds, it will make a conformational change that will release that light.

DR. LU: I see. So you're saying that instead of like forcing the cell to express some sort of payload, to have some sort of reporter that, based on its conformational change, when the phage binds, it sort of switches on activity?

AUDIENCE MEMBER: Yeah.

DR. LU: No, we haven't done that. That's an interesting concept. Potentially could be a faster way of doing the detection.

AUDIENCE MEMBER: It would be. And did you think about coupling your bacteriophages with nanodots or nanoparticles? And so you would amplify your signal and get a real time detection.

DR. LU: Yeah. No, I think that's a great idea. Think it's worth thinking about. Thanks.

AUDIENCE MEMBER: Okay. That's my comment.

DR. LU: Okay. Thanks.

DR. TURNER: That was really nice. I have a question about -- the evolution of modularity does help you with the swapping of tail fibers, and then you found out that you had to kind of go deeper into the structure to make it work.

What about the mode of replication inside of the cell, where there is kind of a phage that used to a stamping machine, order of replication has to do something else. Do you have any evidence yet that you can take the approach and move into a very distant cousin, you know, a very distant relative, and what are the challenges?

DR. LU: Yeah. So I think the challenge is,

as you mentioned, if you want the phage to replicate, it sort of goes -- you not only need to be able to bind and deliver DNA, but you need that sort of DNA to be functional.

So in this particular experiment we actually started off with phages that we knew could replicate in the target host. So, for example, we just took *Klebsiella* phage DNA, electroporated it into *E. coli*, it could boot up.

I think that's going to be a challenge with some more distantly related applications, so I think there we're going to need to be able to identify chassis that potentially either have broad host range capabilities in terms of replication capacity, or you might have to build sort of cocktails that are based on sort of nearly related bacteriophages.

The one area that we are quite excited about is actually not thinking about the phages as totally a lytic tool, but, really, just delivery. So if you could just deliver stuff and you could have some other mechanisms of action, you know, based on the DNA that you've encoded, then I don't have to necessarily worry about the phage having to replicate in order for us to have the activity.

So sort of more thinking about them as sort

of purely just biocapsids that you can swap around is another -- is actually one of the big areas that we're trying to move this technology into. It's a little bit simpler.

DR. TURNER: Nice.

DR. LU: Thanks.

AUDIENCE MEMBER: Excellent presentation. So this engineering things is very attractive, but my question is if the receptor starts changing when you use this engineered phage, what is your remedy? Because you spend too much time and money to develop this engineered phage.

DR. LU: Yeah, yeah, that's a great ques -- so I didn't show you here because the paper is currently in review right now, but we have some strategies to engineer phages and -- sort of at a high level, build very dense libraries of bacteriophages that you can then easily find sort of new vectors that overcome that. So I think, at a vague level, that's what we've been able to do.

I think certainly phage evolution, I mean bacterial evolution is always going to be a challenge for I think phage-based approaches, whether you're using it for lytic applications or non. So I think -- I'm not going to say that you're going to ever be able

to deliver -- sort of have a methodology that's going to always work universally, but I think we need high throughput strategies to keep up with the pace of evolution. I think that's the only way we're going to keep up with bacteria.

So, but they're always going to try to outrun us in terms of their ability to evolve resistance to whatever we're throwing at them.

AUDIENCE MEMBER: So your concept is developing an engineered phage library to tackle all the problems.

DR. LU: Yeah. I think there's a lot of regulatory questions about how that might be applied, but like if we can get these phage-bodies sort of to work and we have a common scaffold, but we're simply changing certain components to get around the bacteria -- keep pace with as the bacteria resist, then, from a technological level, we can definitely do it. From a regulatory perspective, I'm not sure how they would view that.

But from -- if we can do it technically, I'd rather show that first, and then maybe figure out the details of the regulatory afterwards.

AUDIENCE MEMBER: Right. It look ready for the venture capital aspect because you can take the

patent on those, you know, modified phages, but nature already prepared these phages into the 10 to the 31 titer. You need to just harvest them and use it.

DR. LU: Well I think that's certainly an interesting -- yeah. So I think there's sort of like the natural phage groups, and then there's a engineered phage group.

I think the challenge, at least from my perspective, if you want to enhance the natural capacity of some of the phages through genetic engineering at least, if you're going to make a cocktail of like 50 different, well even just like five really diverse phages, it just becomes really hard to genetically engineer those in any industrially-relevant way, and so we're trying to set up methodologies where you sort of have well-defined things that you can manipulate over and over again. It just makes it a lot easier to commercialize. Yeah. Yeah. Thanks.

DR. RANALLO: Okay. So we have ample time for lunch. We're going to be back here -- oh. Thank you, morning speakers, very much. It was excellent. We're going to have some more discussion on engineered phage and natural phage during the panel discussion. I hope you guys can all join us.

We're going to come back here a little over an hour, at 12:40. So we're off for lunch until 12:40. Thanks.

(Whereupon, at 11:35 a.m., the meeting in the above-entitled matter was recessed, to reconvene at 12:40 p.m. this same day, Tuesday, July 11, 2017.)

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A F T E R N O O N S E S S I O N

(12:40 p.m.)

DR. RANALLO: So we're going to get started again. In interest of our speakers, for the last three speakers, I did give everybody a little bit more time for lunch. So -- not that much time. So we're going to get started here again with this last session on future directions.

Andrew, or Andy Camilli is going to start us off about -- talking about prophylactic use of bacteriophages against cholera. Andy Camilli is a Professor in the department of molecular biology and microbiology at Tufts University School of Medicine, and is also an investigator with the Howard Hughes Medical Institute.

I'm really excited to hear his talk on prophylactic use of bacteriophage against cholera, so, without further ado.

DR. CAMILLI: All right. Good afternoon. I guess this is the dangers of being the first talk after lunch. Hopefully I'll also keep you awake. So, yeah, I'm going to talk -- so I guess I'm one of the rare talk about using phages for disease prevention.

I think, you know, this is a -- kind of a unique example, cholera, as I'll tell you about, but I

think it's interesting to keep in mind that there could be some other diseases and some other situations where phages could potentially be used prophylactically.

Some other, I think, unique parts of my talk compared to what you've heard so far is, you know, we've heard a lot about the traditional paradigm of finding phages from the environment, from sewage, and finding ones that are active against the bacteria you want, and so one of the important parts of my talk is that we get our phages from the same environment where you want them to work, and I'll try to point out why I think that's important.

So just conflict of interest statements. So, along with two of my post-docs, we founded a company called PhagePro, and I'm currently a scientific advisor.

Okay, so the science. So cholera, as you probably all know, is this profuse watery diarrhea and vomiting disease. Virtually all cholera in the world and in the previous pandemics that we're able to have data on the strain have been caused by this O1 sera group. That's an LPS type.

There's about 150 different serogroups known for this species, so it's interesting that virtually

all cholera is caused by this O1 type. That's important because I'm going to show you later that this is a receptor for many phages, this O1 LPS.

The secretory diarrhea and vomit are filled with *Vibrio cholerae*, and this is a highly transmissible bacterial pathogen, as I'll show you in the next couple of slides. It's got a high death rate, so prompt treatment with rehydration therapy is very important.

There is an oral whole cell killed vaccine for cholera, but it only gives partial, short term immunity. There's a lot of research on trying to come up with better vaccines.

So we've talked -- we learned a lot about d'Herelle yesterday. He was the one who first discovered cholera phages, and he noted that often he would find these virulent phages coming out in cholera patients' stool samples, so it's fun to a hundred years later still be working on, you know, I wouldn't say rediscovering what he's done, but making use of it in modern times.

So this slide kind of shows the classic view of the life cycle of cholera. So a susceptible person drinks contaminated water, they get cholera. The bacteria colonize the small intestine, make cholera

toxin, and they get this profuse watery diarrhea that results in what are called rice water stools. This contaminates the water further and this -- you get this vicious cycle.

But it's been appreciated for a long time, but there's been some recent studies that have really pointed out this tremendous problem of rapid household transmission.

So some recent papers have shown that the infection rate jumps two orders of magnitude, from about 2.5 per thousand via water-borne to about 230 per thousand if you're in a household where somebody comes down with cholera, so this means that about 23 percent of the households are exposed.

The peak incidence of these secondary cases in the household is two to three days after the index case. that's a huge problem during cholera outbreaks. There's not enough time to go in and vaccinate the household contacts.

So this is one idea we had, is perhaps we could use phages in a prophylactic manner to protect these, you know, the household contacts. The idea is that maybe by doing this very efficiently, we could perhaps blunt outbreaks.

So in thinking then about this idea of

prophylaxis using phages, we had a number of questions that we have come up I'm sure in other people's minds as well.

So the first is are there *Vibrio cholerae*-specific lytic phages that are virulent in the human small intestine where the cholera is happening? I think this is an important point.

We all screen for phages in the laboratory but it's been known for many years from many pathogens that they alter their surface properties during infection as opposed to growth in a flask in the laboratory, and so perhaps the receptors change. I'm going to talk a bit about that today for some of the phages we're going to talk about.

So if there are such phages, what's the biology of these phages? What receptors do they use? What insights can you get from looking at the arms race between the bacteria and the phages? What are the mechanisms of *Vibrio cholerae* escape from these phages, because of course they will escape. And, importantly, do escape mutants remain infectious? Then finally I'll address this last question, can phages protect from cholera in an animal model.

So this work on this started off a number of years ago with a former post-doc, Kimberley Seed.

She's now an assistant professor at UC Berkeley. In collaboration with my collaborators in Dhaka, Firdausi Qadri, and in Boston, Stephen Calderwood, we took advantage of this great collection of glyceroled and frozen rice water stools that Firdausi Qadri's been keeping in her freezers for years.

So we did this respective study just going back and getting a little bit of a frozen stool sample and then screening for phages in the stool sample.

So these are three different stool samples per year, and what you can see is we found plaques in a number of these stool samples, and we were able to isolate the phages, sequence them, and put them into families and learn a lot about what these phages were.

But for this slide, what's important is once we had the sequence and we saw how highly conserved these phages were, we were able to design PCR primers to go back and screen these stool samples in a more sensitive manner. When we did that we found something surprising.

So there was a much more prevalence of these phages in these stool samples, and this one, IC -- we call ICP1 was omnipresent. It was in every patient's stool sample, which is really -- to us, was shocking. I'll say that these three phages are still around to

this day. Even last year and this year's sampling shows that they're still the phages we find in cholera patients' stool samples.

So this kind of goes against that idea that there's a huge diversity of phages. This is a bacteria that lives out in the environment, that infects people. You'd expect a lot -- a huge diversity of phages. But it's not true. Apparently there's selection for phages that are really fit during this -- these epidemics and going into humans.

Now you might ask why do we get plaques in some cases and no plaques in others, and I'll say that a lot of these stool samples have a high titer of phage in them. And the reason is we look for plaques -- we isolate a single colony from that stool sample and use that to screen for plaques, and so the reason is that because often the *Vibrio cholerae* in that stool sample is an escape mutant that's resistant to the phage.

So starting with that first phage, ICP1, that phage that's omnipresent, we then asked basic questions. What's the receptor? It turns out if you mix this phage with *Vibrio cholerae* in the lab, you very quickly get escape mutants.

The escape mutants are truncations, or

alterations, to the LPS O1 antigen. That's the receptor for the phage. So this is what the O1 antigen looks like.

Vibrio, as I said, high frequency escape, and the reason -- the way it does this is two of the genes within the biosynthetic locus for this O1 antigen have this run of *A*s, and so at a very frequency you get slip strand mispairing during replication and you get a frameshift mutation.

When you get a frameshift mutation there's stop codons downstream of these poly *A* tracts that now become in frame and you make a truncated product. The result, and this is work that Kimberley Seed did, is for the *manA* frameshift mutants you have a less dense O antigen on the surface, the phage don't like that, and the *wbeL* frameshift mutants are missing this tetronate modification and the phage can't plaque on them either.

So this high frequency escape is apparently evolved as a built-in mechanism within the bacteria, but we don't see these escape mutants, these frameshift mutants coming out of cholera patients. The reason for that, and this I think is an important principle that's been mentioned a couple of times in other talks, is that the receptor in this case is an

important virulence factor.

The O1 antigen, this was shown by Matt Waldor and John Mekalanos years ago, is critical for infectivity, and so these frameshift mutants are anywhere from 10 to a thousand-fold attenuated for virulence in, in this case, an infant mouse model of colonization. You can revert these frameshift mutants back and they regain virulence.

So this is why we don't see these high frequency frameshift mutants coming out of cholera patients, is they're -- they lose virulence. Yet some of those stool samples that I showed you where there was a circle, you can detect the phage by PCR, but not by plaque, the *Vibrio* strain in that stool sample is resistant.

So we then asked, well what's the mechanism of resistance of those *Vibrio cholerae* clinical isolates, and we used whole genome sequencing to show that these contained this unique island. It's an 18 kilobase island called the PLE, for phage-inducible chromosomal island-like element.

You can see, here's a strain with the PLE. It's resistant, and it's called phage-inducible because it's been shown in analogous phage-inducible islands in gram-positives that upon phage infection,

these things pop out of the genome as a circle, replicate, steal packaging material of the helper phage, and that's how they're transmitted.

So we designed these outward-facing PCR primers to be able to detect this excision and circularization of this element and, lo and behold, within five minutes of adding ICP1 phage, we can detect this circle.

This excision and replication is not induced by other phages, and it gives immunity to this phage only. So it's kind of a phage-specific immunity system that the bacteria have evolved. And it turns out there's four different versions of this PLE in circulating clinical isolates. And Kimberley in her own lab is trying to figure out how these PLEs give resistance to ICP1.

But what we do know is that it works very well as a defense mechanism against this phage. So it reduces -- in a culture can reduce production of phages by five orders of magnitude.

Now we did occasionally come upon a stool sample from a patient where the *Vibrio cholerae* in that stool sample had a PLE, and yet ICP1 could still form plaques on it, so that there was more going on there. To figure that out, we just sequenced the

phage isolates, and what we discovered is that this -- these phages that could plaque on a PLE plus host had their own CRISPR/Cas system. We published that a few years ago.

This shows the Cas genes. So this is, as far as we know, the only phage-encoded, naturally encoded CRISPR/Cas system. So here's the Cas genes. There's two CRISPR arrays, and here's four different isolates with CRISPR arrays. Upon sequencing and looking at these spacers we immediately learned the mechanism, because all these spacers in this green color are perfect matches to proto-spacers in the PLEs, either PLE1, 2, 3, or 4.

In fact, this phage from 2011 has spacers that target all four of the known PLEs, and so this phage is the first component of a phage cocktail I'm going to tell you about in a minute.

So the next question was is this CRISPR/Cas system fully functional? Can it acquire new spacers, which would be an amazing property for a phage. And, indeed, it can. If we delete spacers so it can't target, and we infect *Vibrio cholerae*, we'll get rare plaques where the phage has acquired new spacers against the PLE. This just shows some of those newly-acquired spacers.

So this is a phage that has an adaptive immune system that it can use against *Vibrio cholerae*, which I think is going to be a unique aspect of a phage cocktail.

So I have time to talk about one other phage real quickly. So ICP2 was not -- wasn't omnipresent, it was more scattered. We still find it. We found it last year, and in this year as well. It's also in Haiti, and we're working on a manuscript right now for that.

This is a completely different phage, and what we found is its receptor is not the LPS, but a surface protein, a porin called OmpU. So this is just a predicted structure of this porin that sits in the outer membrane. There's these loops sticking out to the surface of the cell.

What we found is that in some patients that were shedding *Vibrio cholerae* and ICP2, we found isogenic escape mutants of *Vibrio cholerae*. And sequencing them we saw that they had mutations, precise -- it's not deletions or stop codons, it's amino acid changes in these two outer loops, and so we hypothesized that the phage tail fiber probably interacts with this. And we have some unpublished data that confirms that. That this is what the phage

tail fibers engage with.

So these types of point mutations are kind of hard to get, if you think about it numerically. It's much easier to delete a gene or mutate it in other ways that just knock out the function.

But it turns out that OmpU is critical for virulence of *Vibrio cholerae*. During infection it switches the major porin from a porin called OmpT to this one called OmpU.

So, again, during infection in the presence of this phage, *Vibrio cholerae* is between a rock and a hard place. It needs to express OmpU, it needs to express its O antigen, and yet these phages are using those as receptors. So I think that, or we think that that's part of the reason for the success of these three phages.

Now some patients will be shedding these point mutants, but what's interesting is when we look at the publicly-available database of cholera strains that have been sequenced from many patients from many parts of the world, we don't see these point mutants.

They don't become fixed in the population. We just see the wild type and variant *ompU* sequence. And we have some data to show that these point mutants do have a subtle fitness cost, and we think that

that's why they -- there's probably evolutionary pressure to revert these mutants back.

Now some patients were shedding escape mutants against ICP2 that made a normal OmpU, at least by gene sequence, and so we went in and figured out how these are escape mutants. It turns out they have null mutations in a gene called *toxR*, and this just shows a few examples: stop codons, mutations, and critical residues.

Now why would mutations in *toxR* give escape? Well it turns out that *toxR* is a positive regulator of *ompU* during infection. Again, *Vibrio* has this switch from OmpT to OmpU during infection.

What's interesting is *toxR* is also a major virulence regulator. It regulates the cholera toxin genes, it regulates pilus that's needed for colonization, and so these escape mutants are rendered avirulent. We wanted to show that taking some of these point mutants that we got out of human patients, and showing that they're highly attenuated in animal models. So this is now two examples of this where escape mutants can escape the phage, but they're attenuated, or have fitness costs.

So we've put together this cocktail of these three phages that we find -- year in and year out

we're finding in cholera patients in Bangladesh and tested them out for prophylaxis. Again, keep in mind this idea of preventing household transmission.

So this is not a novel idea. Of course, phage therapy was -- back in the 1920s and '30s was tried for cholera, but a lot of those studies weren't well-controlled. It's not clear if it worked or not. I'd like to point out that this well-controlled study, clinical study that was done in 1971 unfortunately showed that a phage cocktail did not have efficacy in a -- again, in a well-controlled study.

And I would think nowadays cholera is such an acute disease where, really, it's rehydrating the patient and giving them antibiotics. That's the treatment. I don't foresee therapy being used, at least not in and of itself, in treating cholera patients.

The idea of prophylaxis is an old one as well for cholera, but recent studies haven't shown that it works. So this study from the Sarkar lab used an adult rabbit model of cholera, and they basically showed it didn't work. The phages lost orders of magnitude titer within a few hours, and it didn't bode well, but we forged ahead, thinking that maybe our phages, which I'd like to think have evolved to be

virulent in the context of the cholera small intestine, maybe they will work.

So I'm going to show you some data from Mimmin Yen who's here with us and another post-doc, Lynne Cairns, where we've tried out this idea. And we've recently published this this year.

So we have two animal models for cholera: the infant mouse, the infant rabbit. Cholera will not infect adults, except for adult humans, which we have no data for.

So first the infant mouse model. So a typical experiment is we'll give them 10 to the seventh pfu of single phages, or the cocktail, we wait three hours, that's the transit time of liquid through the small intestine, then we challenge them, and then we'll determine the load of *Vibrio cholerae* 24 hours later.

So here we see that the load in the no phage group of mice is very high, and with single phages we get different levels of reduction of the load of the bacteria. ICP1 not so good. Not surprising. I showed -- told you that's this high frequency frameshift mechanism. The cocktail worked the best, and ICP3 worked the best.

Now when we look at the *Vibrio cholerae* that

are still in these animals at 24 hours, we see escape mutants. That's no surprise. We see escape mutants for ICP1, 2, and 3. The concentration of phage in these animals generally reflects the load of the bacteria. The more bacteria there are, the higher the load of phage, and that's -- of course you'd expect that.

So based on this first experiment showing the cocktail seemed to work pretty well, we asked, well how long do the phages last in the intestinal tract? So we gave them about 10 to the seventh, 10 to the eighth of these individual phages and looked at retention. What you can see is they were retained pretty well out to 24 hours, although ICP3 really starts to go away by 24 hours.

So I'm going to show you some prophylaxis experiments where we give the cocktail and we test longer times, up to 24 hours, the idea being, for humans, they could drink the phage cocktail once or twice a day.

So when we look at longer times of prophylaxis we see a different story. The bacteria do colonize. So we see this bimodal protection at six and 12 hours between giving the phage cocktail and challenging them. Within 24 hours, all of the animals

are colonized. I'll note that the load is about eighteen-fold lower.

When we go in and look at the bacteria that are colonizing these animals, many of them are escape mutants. They're escape mutants that have lost the receptor, and so they’re -- they have lost virulence.

So now I'm going to switch to this infant rabbit model. The infant mouse model's a model of colonization, they don't really get profuse diarrhea like humans do, but infant rabbits do get profuse diarrhea like humans.

So we give the infant rabbits 10 to the tenth pfu -- that's the combination of the three -- we wait three or 24 hours, and then we challenge them. So what you can see is the rabbits that don't get phage are -- have a high titer, they're very sick, they lose a lot of body weight. We have to euthanize them once they lose 10 percent of their body weight.

The three and the 24-hour prophylaxis times were protected to varying degrees. Again we see a bimodal protection for the three-hour prophylaxis, and in the 24-hour, just like in the infant mice, they're all colonized, but here, the load is about 300-fold lower. And, again, if we go -- and we've done exhaustive studies on what *Vibrios* are still there.

Many of them are escape mutants to one, more rarely two, of the phages, but we don't see escape mutants to all three of the phages in these populations, and a lot of these escape mutants we see are avirulent.

So, again, I think it's part of the reason for the success of these phages in nature. And so -- that these animals are colonized, but with mostly avirulent strains, the hope would be, well they don't have disease. Indeed, if we look, we don't see any symptoms of cholera in these animals.

If you go and look at the percent body weight, there's no significant loss of body weight. So no phage, no *Vibrio cholerae* challenge. They lose a little bit of body weight because they're away from their mothers for this duration of this experiment.

The no phage prophylaxis group, I mentioned they're all very sick. These had to be euthanized much earlier than any of these other animals. But, again, no body weight loss, and that's consistent with the lack of seeing any symptoms.

So we're hoping that this can work in a similar manner in humans by reducing the load of the bacteria, or perhaps preventing the bacteria from colonizing. I point out that here we administer a huge dose of the bacteria. During household

transmission I would -- we don't really know the dose that people are exposed to, but hopefully it's not tremendous numbers.

So the last thing I'll tell you is these three phages, we've look at other gram-negatives, they appear to be very specific for *Vibrio cholerae*, but we wanted to show that they don't alter the gut microbiome, and so we did this experiment that I'll mention quickly where we have a heat-killed phage group, a group of adult mice that got the live phage cocktail, and then as a positive control for a change, vancomycin.

Now we looked at phage coming out in the stool pellets and it kind of declines, but even at 60 hours we still see phages. So we looked at the microbiome at zero, one, and two days, and the bottom line is the antibiotic-treated group going from *T* zero to one, to two days has this tremendous change in the fecal microbiome, as you'd expect, but our heat-killed and our phage-treated all cluster together. There is no substantial change. If you blow this up, there's no pattern of change in the microbiome.

So we expected this, but it's nice to show this, that the phage cocktail does not alter the gut microbiome.

So just to summarize, we have these three virulent phages that we find repeatedly coming out in cholera patients naturally, and all three phages use receptors that are essential virulence factors so this limits escape within humans. When there is escape you have these avirulent mutants, and so probably this is reducing the pathogenesis in some humans that are asymptomatic or have mild symptoms.

One of our phages has its own CRISPR/Cas system, an adaptive immune system that can keep pace with *Vibrio cholerae*'s PLE defense system. This phage continues to be prevalent today with the CRISPR/Cas system, so it's part of its success.

Then I showed you that a cocktail of these three phages can be used to prevent infection and reduce infection in a high-dose challenge in animal models. And then finally, that the cocktail, as would be predicted, doesn't substantially alter the intestinal microbiome.

So I mentioned the people that did the work during the talk. I should also mention Andrea Wong's working on ICP2 receptor work, and Dave Lazinski's the senior researcher in my lab who has a hand in a lot of this stuff. And I thank my international collaborator here, Firdausi Qadri. Thanks. Happy to answer any

questions.

AUDIENCE MEMBER: Hi. So do you see any changes in expression of cholera toxin in your escape mutants?

DR. CAMILLI: Yeah. So the *toxR* escape mutants that come out of some humans with this phage in their stool are avirulent in animal models, and they don't express the cholera toxin in the entire *toxR* regulon, which inc -- has many virulence factors.

Other alterations to cholera toxin, we haven't seen that yet. The other escape mutants, like the LPS rough mutants, they still have the virulence regulon intact, but they're avirulent for another reason. They need their LPS for colonization.

AUDIENCE MEMBER: Two questions. One is is the PLE induction purely a matter of gene dosage or are there also genes actually turned on? Secondly, what -- can phages -- escape PLE, and, if so, how do they do that?

DR. CAMILLI: So we don't yet know what induces the PLE to excise and replicate. We know it's specific for ICP1. And Kim, I can't -- I mean she -- I saw her recently. She has some data where she kind of is figuring out what causes excision, and she hasn't told me the details so I can't tell you, but

it's some --

AUDIENCE MEMBER: So it's gene dosage clearly goes up, and as the --

DR. CAMILLI: Oh, you mean their excised element?

AUDIENCE MEMBER: Yeah --

DR. CAMILLI: Oh, that thing replicates to a copy number of about a thousand, which is tremendous.

AUDIENCE MEMBER: Oh. So it's going to be a huge dose of whatever it is it's delivering.

DR. CAMILLI: Yeah. But once the signal has been given to excise, then it just takes off replicating.

AUDIENCE MEMBER: And so if -- phages can escape PLE, can they not? I mean I saw it's 10 to minus six or something like that when you --

DR. CAMILLI: But it's only through the CRISPR mechanism.

AUDIENCE MEMBER: So you can't get point mutations in the phage that doesn't have a CRISPR.

DR. CAMILLI: We have no mutants, other than the CRISPR/Cas system, that can overcome the PLE defense system. That being said, have we looked very hard?

AUDIENCE MEMBER: I was going to say, you

can't repeal the law of phage genetics, right? It'll find a way, I would think.

DR. CAMILLI: Yeah. I mean there -- maybe there's isolates in our, but the dominating is the CRISPR/Cas systems. CRISPR/Cas.

AUDIENCE MEMBER: I was just wondering, of the 77 percent of the household contacts that don't get it, have you or your collaborator looked to see if they have signs of having some of your phages?

DR. CAMILLI: There are now some NIH-funded projects to start to look at that, look at the -- look more at this household transmission, and why do some people get cholera and others don't.

I would hypothesize, it's speculation, that sometimes they're the lucky ones that got a dose of phage at the same time they encountered the bacteria, but that's pure speculation. D'Herrelle was on to this stuff, you know, a hundred years ago, hypothesizing similar things.

AUDIENCE MEMBER: There are obviously several parallels between the PLE and SaPIs. Do you have any evidence that they might be packaged by the ICP1 machinery?

DR. CAMILLI: Yeah. So the SaPIs in *Staph aureus* are these chromosomal islands that pop out.

They steal packaging material. They're transmitted at a high frequency that way. They don't -- they interfere with the helper phage a little bit, but not much. *Vibrio cholerae* PLE, there's no homology with the SaPIs, other than an integrase gene.

So we don't know if there's a common ancestor, but they knock down phage infection almost completely, so we think they're different in that sense.

Vibrio cholerae -- so the bacteria lyse and release those thousands of circles, and *Vibrio* is naturally competent. That's probably the major mode of transmission of this element. But Kim Seed, my former post-doc, does have some evidence that there is some packaging, very low level packaging, and she's trying to work out the details of that.

DR. RANALLO: Quick question. So do you ever see ICP1, 2, and 3 in the same stool? Have you been able to detect that?

DR. CAMILLI: Yeah. So we see -- rarely, we'll see two of the phages in a stool sample, but we've never seen all three in a stool sample. Because it's a good question. Why -- well it would be -- it would not be in the phage's best interest to prevent cholera. They need it for dissemination.

So it could just be a predator/prey. Like the household contacts that don't get cholera, maybe they have all three.

DR. RANALLO: Okay. So we heard a little bit about Tom Patterson's story yesterday a few times. We're going to continue that with the next talk.

Dr. Biswajit Biswas from the Naval Medical Research Center is a phage team leader at the Biological Defense Research Directorate at NMRC in Fort Detrick, and his title is rapid emergence of phage-resistant bacteria during phage therapy of a terminally-ill patient who was infected with a multidrug-resistant *Acineto baumannii*.

DR. BISWAS: Hello. Good afternoon, everybody, and thanks the organizers to allow me to present my data of the recent phage therapeutic applications in human.

So my topic today is rapid emergence of phage-resistant bacteria during intravenous application of phage therapy of a terminally-ill patient who was infected with the multidrug-resistant *A. baumannii*.

You know, you hear the -- you heard all the story yesterday from Dr. Schooley. Today I'm going to mainly discuss about the bacterial mutation leading to

the phage resistance during this therapy.

So this is the disclaimer. I have to show it. I have no conflict of interest to declare.

So I work for U.S. Navy at Biological Defense Research Directorate at Fort Detrick. Currently, our phage-based programmatic efforts are the -- can be, you know, explained in three different part. There are therapeutic applications of phages, prophylactic applications, and diagnostic applications.

Our therapeutic applications, we are generally working with natural phages. Prophylactic applications, we try to use some lambda phage to modify to make vaccines. In this aspect, a long time back when I used to work for a company, I prepared a vaccine, cancer-based vaccine for using phage display technology which is in phase 1 clinical trial currently at BDRD. We are making some vaccine specific for targeting for malaria and prevention.

So for diagnostic applications, we are currently developing some rapid diagnostic process for using phage. So for therapeutic applications, we are currently working with MRSA, VRE, and *Klebsiella, Pseudomonas*, and *baumannii*. So these are all based on natural phage applications.

For engineered phage side we are developing some sorts of, you know, delivery systems to deliver some lethal genes to neutralize the bacteria which are mainly in stationary phase, because stationary phase bacteria is very difficult to treat with, you know, phages.

So, lastly, the phage components which we are trying to clone is like some source of some endolysins and lysozyme genes. This is ongoing projects.

So 2013 -- you know, I joined the BDRD at 2010. During that time I was working to develop natural phage therapy for *Bacillus anthracis*. That was very interesting work.

But in 2013 we got some seed money to develop some therapy, natural phage therapy, for *Acinetobacter baumannii* and *Staph aureus*, so we joined with Navy Wound Department and Army Wound Department to develop some animal model to use to develop phage therapy for *Acinetobacter baumannii* infections. Mainly wounds infections.

So why we are interested for this? Because during the Iraq War we saw the type of -- last Iraq War we saw 30 percent of -- 35 percent of clinical infection was caused due to *A. baumannii* infections.

Currently, WHO prioritized *A. baumannii* as their priority number one organisms for antibiotic resistance problem.

You see that there are near about 60,000 to 100,000 infections reported at USA and 13,000 in all five European, you know, countries. This data is part ER reported cases. So there are a lot of *A. baumannii* problems.

So when I thought about these projects, how to develop these, we were thinking about different approach. So I talked about using a very broad spectrum monophage because previously we develop such type of treatment for VRE bacteria at NIH. So we thought that probably it is possible to find a monophage.

Then next one was to -- what about a cocktail, fixed cocktail with phage therapy? Then we thought about to make some engineered phage also. Soon we realized that none of these things will probably work for *A. baumannii* treatment because *A. baumannii* is very, very diverse. The clinical isolates are very diverse.

So monophage -- find a monophage is very -- prospect of monophage is very difficult. Also the -- if we try to use cocktail, the -- probably resistance

will pop out. Engineered phage is a lucrative idea, but it take long time and lots of manpower.

So we lastly thought about to use natural phages and to direct -- this was towards more than personalized and precision approach. So we start harvesting phages, lot number of natural -- large supplies of natural phages from environmental samples.

So the process is very simple. I think yesterday somebody asked what is the process? How you isolate the phages from the nature? It's very, very easy. We get sewage water, and then near about 300 ml of sewage water we put tryptic soy agar powder, just the raw powder, and then inoculate them with a little bit, 200 microliters of actively growing culture. In this case it's *A. baumannii* against which we are looking for phages.

So in this primordial soup everything start growing, and the smell is not pleasant. You know, the whole lab starts smelling horrible. But anyway, so after that, within the -- within six to -- six hours toward 18 hours later, we harvest samples from there, we filter-sterilize those samples or chloroform treat to deactivate all other bacteria, and then we plate them against the bacteria against which we are looking for these phages -- this way we can find many phages

simultaneously, sometimes for many different bacterial isolates -- and we make our phage collection libraries.

So right now we are near about 208 *A. baumannii* phages in our collections.

So recently we have opportunity to test the strength of -- about this natural phage library. This is specifically that case which associated with UCSD. You know, the UCSD -- one of the USCD case. This case actually, the case history was reported yesterday by Dr. Schooley, but for the newcomer, I'm just presenting it again.

The patient was a 68 years professor psychiatrist from UCSD, and he was traveling to Egypt during Thanksgiving time. He developed pancreatitis in Luxor, and he was hospitalized. During time -- that time, probably he was infected with this multidrug-resistant *A. baumannii*.

They transfer him in Frankfort where they found this multidrug-resistant *baumannii* from his pancreatic pseudocyst, and he was evacuated, ultimately, to UCSD, his home station. Home hospitals.

So here you can see the -- these pictures were provided by Dr. Schooley. You can see the growth

of the abscess in the biliary duct. So I'm avoiding these slides because we don't need to put it there.

Previously, also, we developed our unique system to evaluate all the natural phages simultaneously to find out their therapeutic efficacy. In this process we actually use microwell plates, 96-well microwell plates. We diluted the phage serially, and then we used some control, bacterial control and media control, and then we infected all of these wells with the same number of bacteria.

During this time we also -- in the media we add a dye called tetrazolium dye. So during active bacterial respiration, tetrazolium dye start to reduce, and during this process the dye start changing color. So the color change from light yellow to a very dark purple.

So we scan these plates in a machine called OmniLog, TM system. In this machine a camera every 15 minutes take a live picture of these plates. So this is actually a graph which produce from every 15 minutes monitoring the bacterial growth.

So here you see that when we collect the data from the machine and plot it, you see the growth rate of different bacter -- same bacteria in presence of different phage. So this is the bacteria control.

You can see it. So it is actually you are monitoring the phage-bacterial interaction in real time.

So when we receive this, you know, request from UCSD to provide some phage for treatment, we immediately pull out 98 *A. baumannii* phage from our collections, we very rapidly use our robots to distribute all the phages, and then we inoculate it with the patient's isolates, whatever we receive from patient.

So within 16 hours -- 16 to 18 hours, we found 10 of the phages which are active against this patient's bacteria. So that particular isolate we call TP isolate because the person who was -- from whom we gave this, you know, isolates, is -- his name was Dr. Tom Patterson.

So now the question is how we select this personalized phage. You know, phage for this personalized phage therapy. We found four phages, I mentioned, and then we monitor their activity in the BioLog system. We see all these phages are very virulent.

So we didn't have a chance to monitor their receptors activities or anything like that because the time was short, so we selected these four phages, and then we studied and we found that they can combinely

reduce the bacterial growth completely. This is the control bacteria.

So we pull out all these four phages from our collections, and then we make a small-scale lysate, then we grow a large-scale lysate. From there we -- this is near about a 3.8-liter culture. We purify it through tangential flow filtration systems, and this is actually a diafiltrations where we exchange the media against buffer, and that also helps to reduce the LPS, some extent.

So then it goes through the continuous cesium density gradient purification process, and then we isolate the phage bands. So here you can see the phage bands. These phage -- after we collect these phage bands, generally the titer is 10 to 11 per ml during this time, and we dialyzed it very rapidly, filter-sterilize, and then, you know -- this was done separately.

Then we combined all those phages together and did a sterility test and produce investigational drugs for personalized cocktail, for use.

So I like to mention for this therapy the source of the therapeutic phages came from two different places. So phages provide by the Center of Phage Technology in Texas A&M Universities are AC4,

CP12, CP21, CP24. AC4 actually came from AmpliPhi.

Here, in Biological Defense Research Directorate, we produce four phages, which are Ab phage 1, 4, 71, 97. Later also, we provide another phage that is AbTP3 phage 1. I will talk about it little later.

So you can see that -- here is the phage therapy dose per day. This is actually our cocktail phage, what was used intravenously. The phage administration start two days before, but that was for the inter-cavitary wash.

Seventeenth March, Dr. Schooley start giving this phage intravenously, and this is the number of time he injected it. So you can see that -- how many times he give this -- use this phage.

So I like to mention, also, that our phage was never used directly for inter-cavity wash, so always this phage was used for intravenous administration.

So during this process Dr. Schooley also harvested the bacteria from the patients. So those bacteria call -- we -- those isolate we named as TP1, TP2, TP3. TP1 was isolate before giving our phage, and TP2, TP3, and TP4, TP4.1, all these things was isolated after giving phage therapy.

So the source of all these bacteria is mainly from pancreatic drain. You see their date when they're isolated, 21, 23rd, 9th May. Like that.

So we were very interested to see what is going on into the -- into bacterial side, you know, so we use BioLog system to monitor this -- our phage activities on these different TP1 isolate -- TP isolates. So we see that before the phage was given, the isolate which we call TP1 isolate, the -- all phages are very, very active.

So after the phage therapy, which was done at 17 March, and this isolate TP2 was harvested 21st March, we see the phage is not that much active on this isolate anymore. We see the -- all these four phages are not that active like, you know, what was -- they were very active before, or very virulent.

So we took the TP3 isolate and we ran it in our BioLog machine, and we see they are completely resistant. So we did the same thing with the A&M phages. We see that they have also, in initial stage of TP1, before given phage, they were partially active.

Because all these phage can make plaques, but they are not that very virulent like what they were -- our phages were. But later on we study this.

In the TP2 isolate, you see they are still little bit active, and then TP3, they're completely inactive. So this is the composite profile. So this is actually phage came from the Texas A&M, and this is a phage came from -- used by Navy. U.S. Navy.

So the resistance pop out. So what is the solution? So we thought about to find another phage immediately, and this time we went to environment directly, environmental samples, and we used this resistant bacteria to find out another phage, and here you see this phage. This phage is a unique halo former, so you can see the halo around this phage, clear phage spot.

We tested that new phage on original isolate, parental isolate, and also the resistant bacteria. So we see that, you know, these particular phage, which we call AbTP3 phage 1 is very active TP1, and also TP2 and TP3. TP2 figures are not given here. So that means this phage is very active, its parental isolates and the resistance population.

So we need to produce another cocktail so we run the BioLog assay using this new phage. You can see these phages can active up to seven hours, but then after that, resistance start popping out. So we thought, what about to pick up another phage from our

previous cocktail, which is AB71, and combine these two. When we combine, we see there is a complete remission of bacterial growth.

So we prepared a new phage cocktail, which call -- which we call Navy phage cocktail 2, using AbTP31 and Ab phage 71. So these are the phages which we used from our side. These are phage -- electronic photograph of those phages.

All these phages are *Myoviridae*. This is phage cocktail 1. Probably they had the same phage and they are using same receptors. And this is the *Podoviridae*, which is AbTP3 phage 1, which can kill the parental and the resistance isolate.

So what is going on in the bacterial side? Is it -- we thought -- first we thought that it may be the capsular difference between the parental and the phage-resistant bacteria because previously we developed another model for *A. baumannii* AB 5075 for wound infection. This one for wound infection in mouse model.

So we have five phages that time we used: AB phage *A, B, C, D, E*. We observe the AB phage A can produce plaque on the AB 5075 bacteria, but other phages, AB phage *B, C, D, E* has no effect. So you see here the AB phage A can, you know, prevent the

infection up to six to seven hours. Then the resistance pop out.

But when we mixed any of these -- any of the other phages with AB phage A, we see complete remission of bacterial growth. So, but this phage alone, this phage cannot make any plaques on this AB 5075. Surprisingly, when you mix *AbB, C, D,* and *E*, they cannot prevent the bacterial growth. Here is the curve.

So to understand what is going on, we collect the bacteria after phage exposure, and then we monitor their surface, using the Raman spectroscopy, and we see that there is a specific peak appear if the bacteria has capsule. Is the peak appear in 979. But if bacteria lose capsule, then it become plain.

So we realize that after exposure to the AB phage A, the bacteria, you know, the selection pressure move the bacteria from live variant, to capsular variant, to a smooth variant, and that smooth variant then can be infected with the other phages, which are *AbC, D,* and *E*.

So we realize that AB 5075 is cap-positive. When we expose them in AB phage 1, they become AB 5075 cap-negative, and they can -- then they can be killed by other phages.

So we thought the same thing is probably happening here. So we monitor the TP1, TP2, TP3, you know, with Raman spect and we found that there is not much difference. They are all same in 900 peak.

So then we though that let's stain the capsule itself. We stained the capsule and we found some difference in the thickness of the capsule. Here is the three pictures. So this is actually TP1, this is TP2, and TP3. You see that TP3, the capsules is less thick.

So, to understand better, we sequenced the whole genome of all these TP isolates, TP1, TP2, TP3, and you can see here this, you know, comparison of all these three different bacteria. This is compared to TP1, TP2, and TP3. The outermost ring is TP2, the innermost ring is TP3, and these are the reference bacteria.

You can see that these TP1, TP2, TP3, as compared to each other, they are very similar, whereas in reference bacteria they are very different. This indicate the heat map. The blue means, you know, they match properly.

So we look deep and we found that insertion of two mobile elements in TP3 disrupt the gene for a cell surface protein. Excision of mobile elements

that is present in TP1 joins two hypothetical protein sequence into one TP2 -- in one, TP2 and TP3.

Genes for the outer membrane protein CarO is truncated in TP3 and missing one amino acid -- missing several amino acids that would form a surface-exposed loop. Maybe that loop is contributing in the receptor. Within capsular biosynthesis region TP1 and TP3, glycosyl transferase genes also differ.

So all these findings, this one for CarO was very interesting to us, and also the glycosyl transferase gene, because it can change the thickness of the capsule.

So we further analyze that one, and here you see the CarO proteins in TP3 is missing, this part, and this cause a loop formation. CarO protein was also responsible for carbapenem resistance. So here you see the glycosyl transferase protein involved in capsular biosynthesis. There you see the gap of the two SNPs.

So we are investigating this more, and we don't know exactly what is causing this phage resistance yet, but we will going to dig it more.

So just to inform you that when we produce this Navy phage cocktail 1 and 2, we also estimate the LPS, and our LPS for cocktail 2 was 10 to the three EU

per ml, and this titer was near about 10 to 11 to 10 to 12, so when we diluted it we maintained the FDA-recommended guideline 5 EU per kg per hour recommended per dose. So it is possible to make, you know, LPS-reduced phage prep using the cesium density gradient.

So we also estimate the plasma phage concentration. Here you see after just giving the phage, phage titer goes 1.8 times 10 to the four per ml of blood, but is rapidly reduced. It's mainly probably the liver and spleen entrapment of the phage in human body.

So phage stability. We also study the phage stability in Ringer’s solution because they diluted the phage in Ringer’s solution. So you see phage is very stable in the Ringer’s solution, and there is no difference between this in the buffer and the Ringer’s solution titer.

So we also monitor -- because Dr. Schooley reported that the phage be -- I'm sorry -- the bacteria become minocycline-sensitive, so we monitor their activity against minocycline and phage combined. So you see the minocycline, one microgram per ml, you know, is not -- cannot prevent the bacterial growth completely, but when it -- and the bacteria -- and the phage alone cannot prevent the bacterial growth, but

when we mix phage and minocycline together, you see that its diminish the bacterial growth. So there is some synergistic effect.

So we study that effect before also with some other bacteria, and we can see very eas -- very clearly that for *Staph aureus*, gentamicin, nafcillin, and cefoxitin work very well with phage and antibiotic. So here you see the bacteria and antibiotic, here you see the bacteria and phage, but when we mix bacteria and antibiotics, and phage, you see the complete, almost, inhibition of bacterial growth. All these study was done simultaneously in a BioLog system.

So recently we do -- did a -- you know, investigate the effect of meropenem in antibiotic-resistant *K. pneumoniae*, and we see that very little amount of phage and antibiotic can prevent the bacterial growth.

So we exposed near about four microgram per ml of meropenem and carbapenem-resistance *K. pneumoniae*, and you see that bacteria completely growing in presence of antibiotics, but in presence of very little phage, it's even in 0.0 -- 0.01 MOI, the phage and antibiotic can prevent the bacterial growth. So the phage and antibiotic, some antibiotic, can

produce a very strong synergistic effort.

So outcome of the phage therapy. Phage therapy was started -- actually, Dr. Schooley described all those things, but I'm just reading the slide here again. Phage therapy started as inter-cavitary installation at day 109, which were continued at six, 12 -- six to 12 hourly intervals. During this time, patient was unresponsive and -- to commands and had developed renal failure.

So over the next 36 hours clinical condition was stable, but he remained comatose. He needs pressors, and his renal hepatic functions was declining. After 36 hours of infection of inter-cavitary installations of the phage cocktail, phage therapy was introduced through intravenous route and five times 10 to the nine phage was given intravenously. That's our Navy cocktail.

After intravenous administration -- the patient tolerated that intravenous administration very well. After that, he came out from his coma. After intravenous application he came out from his coma, and then he start talking with his family, and for the first time in several weeks, that things happen. He was sick for last three months, almost.

So Dr. Schooley describe all those

phenomenon yesterday. I'm not going to go very details of that. So finally what happened, over the ensuing three weeks patient's mental status continued to improve and he was fully conversant and lucid. He was weaned off the ventilators, and his pressors were gradually weaned and were discontinued.

So the conclusion from my side is -- from our study, that modified OmniLog system is an ideal platform for studying phage bacterial interaction because you can monitor many phage-bacterial interactions simultaneously in real time with using this system.

Precision phage cocktail suppress emergence of phage resistance. Phage therapy can resensitize bacteria to antibiotic against which it has previously acquired resistance. Different phage-resistant phenotypes are observed depending on the phage-host combination studies. Antibiotic phage therapy synergy is possible.

So here you see the patient before given phage. Post-phage treatment and he's reading cards. Here you see he's watching and telling that science saves lives.

Just a couple of slides. This is the acknowledgment. This whole things was possible

because our support from our captain, Dr. Mateczun, and LCDR Theron Hamilton. He's actually my boss, and he is a very fine Navy officer. So -- no, he is really brave. He actually activate me to do these things.

Our lieutenant commander, Luis Estrella, Mr. Matthew Henry, and Mr. Javier Quinones worked day and night to make this preparation, phage preparation to send it to UCSD. I also like to mention that we are currently working with Adaptive Phage Therapeutics to develop this system further to provide it for general public.

Dr. Carl Merril who is actually -- is my mentor also, I worked previously at NIH with him for a long time. So from the Food and Drug Administration I like to give thinks to Cara Fiore who actually approved the eIND process. This is the end of the story.

DR. RANALLO: Any questions?

(No response.)

DR. RANALLO: Okay. SO, with that, we have our last speaker. Jimmy Regeimbal is going to talk to us about phage and personalized medicine. The essence of his talk is to look at a well-characterized library to build personalized cocktails.

So Lt. Regeimbal is currently stationed at the Navy Medical Research Unit in Lima, Peru, where he's expanding the isolation of natural phages from remote and unique environmental samples. The title of his talk is phage therapy against MDR strains: Overcoming the double-edged sword of phage specificity.

Jimmy, it's all yours.

DR. REGEIMBAL: Okay. Good afternoon. Once again, my name is Lt. Jimmmy Regeimbal. I'm stationed at the Naval Medical Research Unit No. 6 in Lima, Peru, but prior to that I was at the Naval Medical Research Center in Silver Spring, Maryland, which is where a lot of the work I'm going to be talking about was actually done.

I'm also very aware that I am the last presenter of the last session on the last day of what is a very packed meeting, and it is tempting my natural ability to be reckless a little bit, so I might actually be a little bit more provocative than I was originally planning on being. Sorry about that.

So I tend to beat a fairly specific drum, which is this idea that -- well first let me get through my disclaimer because I have to do that. Although I am a uniformed service member, I'm speaking

on behalf of only Jimmy at this moment. These are my opinions. I am not speaking on behalf of the Navy or the DoD.

So within the Naval Medical Research and Development enterprise, it's really a collection of labs all over the planet. The Army has one as well, and so we work in very close partnership with them.

So, actually, I should say just from the very beginning that everything we have been doing, and everything that we are doing, has been in very close collaboration with the Army, specifically the Army Wound Infections Department, but also the Bacterial Diseases Branch, in general, at the WRAIR, and all of our animal model data, for example, will be -- was worked out in collaboration, in very close collaboration with that group.

But, generally, on the Navy side, we have groups that are interested in population level cocktails and engineered phages, phage diagnostics, phage vaccines. Obviously the project that I was most associated with was the natural phage therapy developing, using a library-to-cocktail approach.

I sort of think this is one of the most durable and robust ways of generating a phage-based therapeutic, and, really, to wrap your head around

what I really think this product actually is is I think you need to view it through the paradigm that the product is actually the library, and the application of that product to any individual case is actually the cocktail.

So our product is a little different. It's a little bigger. I think it's important to view it through that paradigm to really understand what it is I'm trying to do, or what we are trying to do.

So I'm not a very sophisticated person so I wanted to start back from the very bottom and ask the question of what are we actually trying to do when we try to use phages as therapeutics? Really, you're exploiting a predator/prey interaction. It's a horrible, but extremely helpful, analogy.

What you're doing is you're actually trying to generate an artificial situation. And I use that word purposefully. It's an artificial situation in which a collection of phages can drive a contained and local bacterial population to near extinction. That's what you're asking it to do, and that's actually a fairly big ask. It's kind of hard to get phages to do that. To ask a phage cocktail to do that not only in one person, but in every person at a population level is an enormous ask, in my opinion.

So if you do this sort of *reductio ad absurdum* thought experiment and then you imagine you have a phage on the planet, or a cocktail, or let's just say it's one, and it can kill every single strain of *Pseudomonas aeruginosa*, imagine a world where that existed.

What would happen over a period of time? That broad spectrum phage would eventually kill all the *Pseudomonas aeruginosa*, and then you would not find that phage anymore because it ran out of its host and it hit a biological dead end. But that's what people are actually trying to do when they're looking for truly broad spectrum stuff.

So that situation's almost selected against in nature because it would result in a biological dead end. So I think it's much more advantageous to just realize that exploiting that predator/prey interaction involves asking the phage to do something that, anthropomorphically, they don't want to do, and so you have to engineer that situation in which that phage cocktail can drive a local bacterial population to near extinction.

So a lot of my talk is how we arrived at that. It's going to seem comically simplistic, but I'm doing that on purpose. So when you -- when -- the

first way you try to engineer that artificial situation is you use a ridiculously large population of bacteriophage, at like 10 to the seventh, or 10 to the tenth, 10 to the eleventh.

These are numbers we use all the time, but that's actually an enormous number of individuals at a population level. With that enormous number of individuals comes a whole lot of sequence diversity and a host range, and those are related to each other, but they're not exactly the same.

So I have here a sequence diversity. In any bacterial population you're actually going to have a consensus sequence and some distribution around that consensus. This is grossly oversimplified, but it helps me illustrate my point.

This is actually going to vary in at least four dimensions. Not smooth distribution around the consensus, but you have four nucleotides, you have indels, you have rearrangements, and so what you actually have is a cloud of closely-related bacteriophage.

Then you're taking that cloud of closely-related organisms, the N-dimensional cloud, and smashing it into a bacterial population which itself is an N-dimensional cloud of closely-related bacteria,

and the collision between those two populations is actually your therapeutic. So population dynamics really matter.

If you talk to microbial ecologists, a lot of them don't even consider -- I'm a biochemist by training. It's just -- to give that disclaimer, I'm not a phage biologist. It gets me into trouble with phage biologists.

But population microbial ecologists don't even sometimes view phages as being predominantly bactericidal, they view them as agents that can introduce bacterial diversity with antibacterial populations, and one of the major mechanisms for doing that is by killing off huge swaths of local bacterial populations and allowing those resistant mutants to outgrow, and so that's already happening in nature all the time, and we're actually trying to fight against that. We're trying to get them to drive the population all the way to extinction.

So what happens when you infect a phage into a bacteria, right? We've gone through this over and over again, where you basically have a phage that infects. Over a certain amount of time you're eventually going to get resistance. So it starts off where your sequence diversity is enough to cover your

strain of interest and that strain resides into the host range of that particular phage.

Eventually, resistance is going to pop out, it's going to pop out outside of the host range, and the sequence diversity is no longer enough to cover it.

So how do people get around that? Well, they go let's build a cocktail. That gives you a lot more sequence diversity to play with, you have a larger aggregate host range to deal with, and so when you treat the bacterial infection with those -- with that phage cocktail, it might take a longer period of time, but eventually, you're still going to get resistance.

This will happen every single time a phage interacts with a bacterial population, even a cocktail of phages, and so eventually you're going to get a host, or a bacterial strain that pops out and is now resistant.

But if your product was the cocktail, what do you do now? What do you do if you started with a cocktail and you have a whole bunch of strains that are -- just lie outside of coverage from that fixed cocktail in time?

If you started with a library you have far

more sequence diversity to play with, if you build your library correctly you can have far more aggregate host range to play with, and now it's a question of finding the correct phages in your library that could cover any clinical-relevant -- clinically-relevant strain that comes in to the lab.

So what you do is you have an arrayed library that's characterized -- I'll get into that in a second -- you screen using robotics and an algorithm for screening, which we have developed on the Navy side of the house, and you have to feed that through an assay that Dr. Biswas just recently talked about, but I'll come back to it in a second.

What this assay does is it actually helps you find what we are terming as synergistic cocktails, cocktails that show internal synergy between the phages.

So a more traditional cocktail is all the phages interact with, and infect, the parent strain of an infection, you get a several log reduction, sometimes up to four logs and so it could be really huge, then -- but eventually you're going to get resistance, and that will happen every single time at some frequency. Some low frequency.

When we generate our synergistic cocktails

through our iterative screening process, what we can actually do is find a collection of phages that work together, whereas one phage in the cocktail will infect the parent strain of the infection, you'll get several log reduction, that strain will become resistant so that phage will no longer work -- that's what you see here, in the middle -- eventually another phage in the cocktail which now didn't infect before now can infect that emergent strain, and so you have these phages working in series to drive a bacterial population to near extinction, even if the phages cannot go in reverse and infect the previous iterations of the phage.

Sorry. The phage cannot infect the previous iterations of the strain.

What we're also seeing, just like everyone else is noticing, is that when you get phage resistance, which finally will emerge even against our synergistic cocktails, those bacteria are usually way lower, they have reduced virulence, and they're often more sensitive to antibiotics. So that's also a mechanism that these cocktails are using to drive bacterial populations to extinction.

So when we go back in these synergistic cocktails and we ferret through our workflow, another

controversial aspect of this that I think is actually important whenever possible is to actually manufacture the phages you're trying to use therapeutically to the degree possible on the target strain. Everyone hates that idea because you'll be using an MDR clinical isolate to manufacture, at some level, phages.

The reason why I think that's actually kind of important to think about is because any time a phage interacts with a bacterial culture you're going to get some level of host adaption. That host adaptation will happen every single time.

Again, what you can imagine is imagine you have a consensus sequence of your phage and it's perfect for infecting your target, but then you manufacture that strain, or that phage on a manufacturing strain.

What if the sequence is optimized here for infecting the manufacturing strain? What will happen is the -- when you grow that phage the new consensus sequence will shift. The sequence that was optimized for the manufacturing strain will become the new consensus sequence of that new emergent population of phages.

That *in vitro* might be completely undetectable. In a diffusion-controlled environment

you might not even notice that ever happened, but *in vivo*, when you put it back into an animal, for example, what we've noticed is that you have three-dimensional architectures. You have an immune system that's constantly trying to remove those phages.

That might be massively consequential, and you didn't really know it at the time. You could have shot yourself in the foot and shifted your population away from being optimized to your target, even though *in vitro* you can't even detect that shift. So if this is really possible, I think you should manufacture in the target strain if you can.

So that's sort of the way we envisioned how this would -- could work, and then we actually went and did it. So the way we build our libraries is we go to some of the worst places you would ever want to go.

We go to wastewater treatment facilities, we go to standing cesspools. This is the training population in Fort Benning Georgia where guys are swimming in a pond. There's phage to *Staph* there.

It turns out that ships are probably a pretty good way to look for phages because of the way they deal with what's called brown water -- you can use your imagination for what that is -- and it's in a

really big tank on the ship. So that is probably a good place to go.

In Peru, this is one of our favorite spots. We have five spots that look just like this, and they are filled with household refuse, diapers and fecal matter, food waste, trash. Animals water here. We've actually found a dead dog in it several times. It's very unpleasant. It's actually downstream of a local hospital, so you get hospital runoff.

Actually, the best place to find phage, or the best time to find phages is right -- is about a day or two after a rain storm because this would become, essentially, a static culture. Couple days after it rained you get this churning event, you get new stuff introduced in the environment. We find a burst of phages about two days after a good rain storm.

We have about five sites like this. I actually wrote a grant to try and do global phage harvesting at every place that DoD has a lab. I don't know if it's going to be funded yet, but what we want to build is one of the most robust libraries against all the clinically-relevant ESKAPE pathogens that the world has ever seen. That's what we're trying to do with the infrastructure of the United States Military,

but I don't know if it's going to be funded.

Once you build your library, again, Dr. Biswas talked about how you would isolate phages. What we're currently doing is we'll use clinically relevant strains of the ESKAPE pathogens, for example, that are local to the site of phage isolation because we want to get the best -- that would be the best soil to sort of grow your phages in from that region.

What we're trying to do is build a diverse phage library against clinically relevant ESKAPE strains. So once you get the -- a culture supernatant that's rich in phages that you care about, this red arrow is extremely important because that's going to be the arrow that is the characterization that is required to transition your phages from just environmental isolates to what is needed to be an arrayed library, for inclusion in that library.

So that arrow is probably going to be very expensive, it's probably going to involve sequencing, but a lot of the characterization requirements aren't even worked out yet. Eventually, what we want to build is an arrayed phage library in that way.

What we're going to be doing is iteratively screening it on a per person basis to come up with a personalized therapeutic cocktail. The way you do

that is you have a phage library, you're not sure which phages are going to be used, but a strain comes in from the clinic -- so this was the example of how we demonstrated this in an animal model.

Our target organism is *A. baumannii* I5075, which is a clinical isolate from an osteomyelitis patient. We have a version of it that expresses luciferase. It's got the lux cassette.

So the idea would be you would screen this phage library using -- against your target pathogen using our iterative process in the assay -- the BioLog assay that Dr. Biswas just presented, and what it helps you do is find phage that work synergistically, but you don't have to know the underlying mechanism of that synergy.

We've figured it out in this case, and it has to do with capsule production. So the Army actually had a great phage which could infect 5075 and it causes a lag in growth at about six hours, and then you get a resistant population that pops up. That resistant population is uncapsulated.

Then the Navy had four phages that infected that version of *A. baumannii* 5075, the uncapsulated version, very well. You blend them all together and you get a complete killing event that lasts way past

20 hours. It goes well out to over 36, even maybe 72. When you do see resistance, which will pop up eventually, it's stochastic. It doesn't happen in every version of the culture.

So essentially what you have is four phages that basically do nothing. They have no detectable activity against this isolate, you have one phage that sort of just delays its growth for about six-ish hours, but when you blend them together you have a possible therapeutic that gives you a complete killing, or at least as near as we can come to complete killing.

You've engineered the artificial situation in which you're almost driving a bacterial population to extinction.

Although we know it here, you don't necessarily need to know the underlying mechanism for that, which would allow you to screen through potentially dozens of these kinds of events without having to know the underlying mechanism for how that synergy's working as long as you know the phages you're starting with are safe.

And so we tried this in a mouse animal model. I think Col. Tyner presented this this morning, so I'll just go through it as quickly as I

can. It was a 60-animal study. The only reason I show this busy aggregate picture is because if you look in the PBS groups, what you see is that we had some adverse events.

We don't want to use death as an endpoint in this model, but sometimes it happened accidentally. There were also two cases in which we had to euthanize PBS-treated animals because they developed paralysis. The location of the wound is on the back of the mouse. We got tissue invasion that led to hind limb paralysis, and so we had to euthanize those animals. But we never saw those adverse events in any of the phage-treated mice.

So to give you a cleaner picture to look at, essentially, this is an aggregate picture, or a representative picture. You have a PBS-treated group on days one, three, and five. The group treated with just the Army's phage that -- it's the capsulated version of the *baumannii*, and then the full five membered cocktail.

In the PBS-treated group, again we saw about five fatalities. All those animals lost way more weight, and they all developed eye infections. So it's frequent that they start to groom each other again, and they all had massive eye infections. These

animals were very sick.

We never saw any of those events in any of the phage-treated mice, and in -- basically, in the full five membered cocktail we were able to lower bioburden by IVIS signal, and we were also able to lower bioburden not only by intensity, but by area.

So you can't really get better than the surgical wound, but you can get far worse if the bacteria invade neighboring tissue, which happened in the PBS control cases and didn't happen in the full five-member cocktail. The cocktail actually can -- restrained the bacteria to only being in the original surgical wound.

We also had no detectable necrosis in the phage-treated mice. Again, in the PBS-treated group it advanced outward and you got necrosis in the surrounding tissue, and that didn't happen. The wound never got larger.

So then, as a result of that, the phage-treated wounds got -- remained smaller and closed faster, which allowed them -- basically, we concluded that the -- this proof-of-concept cocktail was able to treat a multidrug-resistant infection in mice. This technology development was actually the foundation for the work that was then used to compound a cocktail in

the eIND case in California.

What we also noticed, which is also the same thing that everyone is noticing, is that phage can push around bacterial populations. One of the ways the -- our phage cocktail could push the bacterial population was to become less virulent.

So 5075, when you -- so we have a very simple *Galleria mellonella* model, that was worked out again by the WRAIR, the Army side of the house, from the wound infections department, and basically, you have a wax worm, you inject it with a bacteria.

If the wax worm shrivels up and dies, the bacteria was virulent. It's a very easy assay to do. So if you inject wax worms with wild type 5075, the capsulated version of the bacteria, all the worms shrivel up and die by four days.

You can use any number of controls that don't make a capsule, and any of the mutants that popped up from our synergistic cocktail also had -- were uncapsulated. If you inject those into the wax worm, they essentially survive.

So you've basically taken a phage therapeutic and was able to render a bacterial infection, or render a bacterial isolate less virulent. This is happening in lots of different

cases. There's lots of ways we've even seen that today, where bacteriophage can alter bacterial virulence in the emergent resistant populations. We can do that as well with just phage you might find in the sewer outside of this building, as long as you compound the cocktails correctly.

Again, we also see, which Dr. Biswas went over just a second ago, is that our phages -- the phages -- the kind of phages that we're finding can also synergize with antibiotics. So not only can we develop cocktails that have an internal synergy amongst the phages, but the phage, like everyone else is noticing, can synergize with antibiotics.

This is an example of *Kleb*. I think he just actually went over it so I'll just briefly go over this. We can see, even with low concentrations of phage in the presence of meropenem, you can reactivate the activity of meropenem in some way in the presence of antibiotic, or in the presence of phage.

So it could be that a strategy for phage therapeutics maybe to start is that you're never going to convince a clinician to stop using an antibiotic, so maybe we should just embrace that and say the first application for a phage could actually be, and the way to augment antibiotic therapy, and possibly even

reactivate an antibiotic that hasn't even been used in 20 years. That could be a potential strategy, assuming that we can get it to work.

So just generally speaking, the Navy phage therapeutic program, in my opinion, I think a phage therapeutic that's based on a library-to-cocktail approach is actually the most robust and the most durable way of generating phage cocktails that will actually be efficacious in the clinic.

I think it makes -- and we've actually demonstrated this. We've showed it in animal models, we've shown it in a human compassionate use case. We can show that we can alter virulence, we can show that we can alter antibiotic sensitivity. Essentially, it's all based on phages that can be found all over the planet in the wild.

So what we're limited now by is just the availability of wild phages that we can then characterize, do the correct husbandry, and build the -- a library the world has never seen. I think we're poised to be able to do that.

So, in thinking about some of those issues, I think there's probably some -- numerous regulatory concerns, because that would mean lots of things that -- would be different about this kind of technology.

The first is we have to really figure out what is required to move a environmental isolate of a phage into a phage library and have that be called safe.

What does that mean? Do you require full genome sequencing? Does that genome have to be closed? Are draft genomes okay? Can we use PCR in certain cases? I also think the library will probably have to be iteratively updated.

So I heard yesterday people were talking about, well what if I have a fixed cocktail and I want to swap out a phage over time? And if you start to think about that, and if your product was the cocktail and you want to already start swapping out phage, that starts to sound a lot like a library-to-cocktail approach, just with a very small library.

So I would invite you to come over to the dark side and just embrace the library-to-cocktail approach. It would mean you have to change a lot of things, potentially, but it's a very robust idea, I think.

So, as clinically-relevant strains drift, we'll constantly have to be updating our library. There will no such thing as even a fixed library. Maybe you have to do it every year, every six months, every two years. It's hard to say.

In terms of manufacturing, I understood -- you know, I think it's a good idea whenever possible to grow the bacteria on the MDR strain, the target strain of interest, so that you host adapt to the correct and most appropriate strain.

If that is your strategy, then your scale up isn't a 300 or 1,000-liter fermenter making a lot of GMP phages. What you're doing is it's a question of scale-up according to bandwidth. How often can you compound a personalized phage for individuals per unit time? So that scale-up is a little different than the way you would currently think about normal CMC for drug manufacture.

That would also mean that every time I compound a cocktail and I grow it on the target pathogen of interest, I would never give those phages to anybody else. They would be one-offs. It's just how many times can you do those one-offs per unit time.

And, again, this would also affect clinical trials. I think that the clinical trial caveats for phages have been beaten to death, so I can just sort of skip over that.

Finally, I'd just like to say that when I first joined the Navy four years ago I had no idea I'd

be working on phage. I'm actually a biochemist by training. The people that I've had a chance to work with have been fantastic, both in the Navy side, the Army side.

And now, down in Peru, we actually have a very eager team because in Peru, for example, and, actually, all over South America, MDR *Pseudomonas*, MDR *baumannii* is an extremely massive problem. You hear cases in the newspaper all the time of a young girl, for example, who goes in for appendicitis, she gets an IV line placed two days before for some reason, she got a *Pseudomonas* infection, and then three days later they had to cut off her arms and her legs because nothing would work.

I mean there -- this problem is everywhere. It might not be as visible in the U.S., but it's everywhere. It's a particular problem for the military because our wounded service members were coming back with some of the most severe injuries that you could think a human could survive, and they did, and then they got an infection which required even more surgery and more removal of tissue.

That just sort of can't happen, so we have to come up with a solution for this problem. Personally, I think a library-to-cocktail approach

using natural phages is one of the most robust I've seen as a potential solution for this.

So thanks again for everybody on the list. They're awesome. Doing science with them is a lot of fun. If you have any questions, I'd be happy to answer them.

Yes, sir?

AUDIENCE MEMBER: If I could ask a question with respect to someone who's run a successful phase 2 trial with a fixed cocktail. Well, yeah, the only one. Agreed. But the only one. Two things.

First, antagonistic co-evolution. Your fixed *X* will drop outside the circle, but the circle will then spread to find it again. I've got a really good chapter written for a book I'm editing right now by Brockhurst on that. It is a fact, and it does happen. Phages aren't fixed the way a chemical is.

DR. REGEIMBAL: No. No.

AUDIENCE MEMBER: You know, that is an argument that I've used many times in the past. Second, you don't, I agree, expect a phage to eliminate its dinner. That's not what it does. That's not what it does ecologically.

DR. REGEIMBAL: Right.

AUDIENCE MEMBER: And, again, I've said that

many times. But if you can get the number of phage down below quorum sensing, down below pathogenic effect, down below -- sorry -- bacteria, down below pathogenic effect tissue damage, then you have got responses in the body which will help to clear it. Not only the adaptive, but the non-adaptive immune response. Even physical clearance, cilia in the ear, cilia in the lungs.

So is elimination actually required? I mean most antibiotics won't eliminate but they'll drop it down below the pathogenic threshold and the body can then cope, to quote me. Isn't that the possibility with a cocktail, regardless of the outlying *X*s?

DR. REGEIMBAL: So I would answer my ques -- your question this way. I am not ready to down-select any modality. I was meaning to be sort of tongue-in-cheek provocative, but I don't think anyone who's in the room ready to down-select what modality we should use.

I do think fixed cocktails would have lots of clinical applications. But when you use a fixed cocktail you're making, you know, several hopes, or maybe assumptions is an easier way to say that. Your assumption is that you can -- your cocktail will cover enough clinically-relevant strains to give you some

sort of efficacy.

You're hoping that your cocktail will knock down the infectious target in all people to a degree that can show clinical efficacy, you're hoping that your emergence of resistance is infrequent enough to give you clinical efficacy, and you're hoping that it can do all of those things for a long enough period of time to make it economically viable to sink the $50 to $120 million in your product you just sank.

So while that is possible, I do think we should all desire a better alternative, and I think that's not a mysterious alternative. It already exists, it's just a lot more complicated to bring to the market, which is to start with a library and just personalize as best we can.

So I fully admit, for example, retrospectively, after people compound personalized cocktails for a while you might empirically discover that a fixed cocktail in that dataset is great, and so every time you're making a *baumannii* library you have the same handful of phages in all those cocktails, so just start with those.

But I think that should be decided empirically downstream, not today when there is no commercialized product in the U.S., for example.

AUDIENCE MEMBER: Or you could take it the other way and go with the cocktail to start with, and people who come through that get the personalized approach.

DR. REGEIMBAL: Fair enough.

AUDIENCE MEMBER: Because the people in Peru living in that alley you showed won't have the resources to do the personalized approach, I don't think.

DR. REGEIMBAL: Well, fair enough. I understand the argument. And, like I said, I meant to be a little bit provocative. I obviously am not ready to down-select anything. I just wanted to be a champion -- or not a champion, that's the wrong word, an advocate for this kind of idea because I think it's seen as the sort of weird fringe in a world of weird fringe.

AUDIENCE MEMBER: They're all weird, but everywhere you get a Sith, you get a Jedi.

DR. REGEIMBAL: Fair enough.

AUDIENCE MEMBER: I have two -- one question and one point to make. So it sounds like the Navy may take care of *Acinetobacter baumannii*, and we won't -- I mean, look, this is a governmental intervention at that point. So if the Army would do one, and the

Marine Corps would do one, Air Force would do one, and the Coast Guard would do one, we'd have six of the ESKAPE pathogens and there would be no commercialization, it would be provided by the military, and I think that's a great idea.

Secondly, there is a problem with the -- I mean I -- and we did it under the time pressure and so did -- and that is growing your therapeutic cocktail on the pathogen itself. We have seen in multiple cases if you infect a bacterial strain, you will induce prophages.

DR. REGEIMBAL: Yeah.

AUDIENCE MEMBER: So that's one of the problems there's going to be. Of course, in an eIND situation, that's a risk you just take, right? But trying to put it, when -- to non-eIND situations, I think you would have to make sure that the pathogen is not going to induce prophages carrying the very toxins that made the patient sick already.

DR. REGEIMBAL: Well I would ask this question. If you're going to use phages, in general, in a person, whether you grow that phage in the target strain of interest --

AUDIENCE MEMBER: A numbers game. I mean you're --

DR. REGEIMBAL: I understand that. But you're going to get burst events downstream within the human, and so whether that happens five minutes before or five minutes after you push it into the IV -- I'm not sure I understand the --

AUDIENCE MEMBER: In the liter culture or three-liter culture you're growing, you're going to have a lot -- an awful lot of those phages, and they can -- phages can lysogenize way beyond the domains where they can make plaques or grow virulently. Just something to be concerned about. Because we've seen phages become -- one percent of the total phage population is induced prophages when you're super-infecting with a virulent --

DR. REGEIMBAL: Yeah. And that's why I would also just add the additional caveat that if I -- if you were to do that, you could never use those phages in anybody else. It would be a one-off. Those phages that grown on that target pathogen would only go back into that person in an attempt to limit those kinds of outlying events, or side events.

DR. TURNER: That was an intriguing talk. I guess the comment, in defense of evolutionary biology, is that I don't think any species wants to go extinct, but the vast majority of them have in the history of

the planet. A phage doesn't want to eliminate its dinner, it just doesn't mean it won't happen.

So I guess I just want to make sure the audience understands that, you know, humans drove smallpox virus into extinction, and it certainly wasn't in the interest of that virus, variola virus, to have that outcome, okay? But let's just put that comment aside.

It was intriguing what you said about, you know, if you do groom the phage on the patient strain you may get adaptation that is specific to it. I agree with that. But another core principle in evolutionary biology is correlated response to selection.

You could just as easily groom it on that, and it's actually very good on other strains as well. Because that explains how this gets into humans very readily, you know. It was not groomed on humans. So I think it's an open question --

DR. REGEIMBAL: Absolutely.

DR. TURNER: And that bears more research.

DR. REGEIMBAL: Absolutely. So, in my opinion -- well, again, this is Jimmy talking, this isn't Lt. Reigembal. There's a lot of work that has to be done to bring this kind of product to the next

step. I mean we would have to show that -- whether manufacturing on the host versus a manufacturing strain would actually make a difference. It might be that you get clinical efficacy without the need to doing that.

But what I'm saying is that what everyone needs to realize is that regardless of your modality, though, you are smashing two populations together, and those population dynamics really matter. Most people just talk about -- well I don't want to -- it's a gross characterization.

But frequently what you hear about is lytic spectrum, and host range, and that kind of stuff, but, really, you're going to -- all of molecular biology is selecting for the rare event. That's like all you ever do. That rare event can happen weirdly at any time if you're mixing any kind of 10 to the eleventh population with a local population that's in equal numbers.

So my goal was to bring some of those kinds of ar, or those kinds of issues to the table. But, no, I don't think that tomorrow I necessar -- well it depends on how sick I was because I saw it work. But I think there's a lot of work that still needs to be done in this space of personalized therapeutics.

DR. TURNER: Yeah. I didn't want to sound hypercritical because I think you're raising a lot of interesting questions that need to be studied.

DR. REGEIMBAL: Yep.

AUDIENCE MEMBER: I like your idea to be able to bank for the whole world. I think it sounded like a very big task.

DR. REGEIMBAL: Yeah. Yes, it is. That's why I might not get funded. But I try.

AUDIENCE MEMBER: The clinically relevant bacteria are changing, so, from your experience, how often you have to monitor to ensure you're current?

DR. REGEIMBAL: I mean that's an open question because what does it mean to monitor? Are you monitoring only *in vivo*? Sorry. Are you monitoring only *in vitro*, or you're monitoring *in vivo* using some sort of animal model?

AUDIENCE MEMBER: If you built a bank that either covered the whole world, you have to ensure for each country all the clinical-relevant bacteria is covered --

DR. REGEIMBAL: Yeah, but -- yeah, and that's -- obviously it's a -- but that's a problem of scale, it's not a problem of techno -- if you have engineering solutions, if you have other kinds of

solutions -- I mean that's a -- it seems to me like even though it's big, it's not difficult. It's not easy, but it's not difficult. It's just you have to get larger in scale.

AUDIENCE MEMBER: And I'm interested to know how far you been on that road now.

DR. REGEIMBAL: In terms of trying to build a large library? So we have -- right now I have med students from Penn State in Peru harvesting phages for me in some of the worst places you would ever want to go. We have gone into Honduras, we've gone into a lot of -- basically in Central and South America we have lots of sites that we're now going to.

I'm trying to go international over into southeast Asia, as well as Africa. We have -- the military has infrastructure there, both Navy and Army labs. But the problem now is funding. It's not even willing partners. There's people with those labs ready to go. They want to be involved in this effort.

I think it's an effort that if we build a diverse enough library, it will -- it might be great source material for people who think that a therapeutic phage cocktail that's fixed could be the best modality to go with. I might have a whole bunch of interesting phages you might want to try.

But we're just -- really, it's limited now by funding. I'm just waiting to see if that happens.

AUDIENCE MEMBER: Okay. Last one is can your bacteria or your -- or the information about these bacteria be shared?

DR. REGEIMBAL: I don't know the answer -- which bacteria? The phage or the --

AUDIENCE MEMBER: What you got in your bank. Can that --

DR. REGEIMBAL: I'm not sure about that. I can't speak to that because I'm not sure if it can be shared outside the DoD or with our partners. I don't want to say the wrong answer. I have to ask nine layers of people before I can almost make any decision, so, but I can figure --

AUDIENCE MEMBER: I shouldn't ask here. I'll ask --

DR. REGEIMBAL: Yeah. Yes, sir.

AUDIENCE MEMBER: First of all, it's incredibly exciting to see the world having gone from basically two phages being looked at in some detail to people all over the world doing this kind of enormous amount, and I want to say that's incredible.

How can one get, for example, students various places involved in doing things that could be

helpful and other people involved? What suggestions do you have in ways like the Phage Hunters program, but going to ones that are perhaps more broadly useful?

DR. REGEIMBAL: I mean I don't know that we have -- like, so the military, to my knowledge, does not have a common repository in that way, and I'm not even sure that you would want the military to be that kind of repository.

AUDIENCE MEMBER: I'm not saying necessarily the military, but for guidance, just encouragement.

DR. REGEIMBAL: Oh, I mean I guess word of mouth at this point is the only place I know to go. I mean the students that came down to work with us, they were planning on working on something else, a clinical study, and I just said, well you could do this idea, and all of them wanted to do it because they all saw, hey, this is unique, it's getting out into the lab, but it's also getting out into the -- to doing some of the more grimy field stuff. I mean really grimy field stuff.

So it appealed to them on that level. It's a way of doing tropical medicine and mixing it with sort of a laboratory setting. So advertising it, I guess. I have no other answer for that.

AUDIENCE MEMBER: Have you published anything about how you're doing that that one could get their hands on?

DR. REGEIMBAL: No, ma'am. No. We're still in our stages of -- like, so all my phages are sitting in a freezer in a fridge in Peru, waiting to figure out the correct export for that.

DR. RANALLO: Okay. So we're at the end of our presentations. I have a couple of announcements. One, the organizers would like me to at least investigate the possibility of making the presentations that we've heard over the past two days publicly available, so I'd ask speakers who are still present to reach out to one of the organizers. I'll just mention them by name: Roger Plaut, Scott Stibitz, Paul Carlson, and Randy Kincaid. Those are the only, at least off the top of my head.

So, as I said, I'd like to ask the organizers, or the speakers to consider that with the, you know, with the possibility of perhaps doing some small redaction.

And then the last thing is, again, we're ready for our panel here. We have until 3:00, and I don't have on my agenda that there's a break, so we're going to just transition really quickly into a panel.

This panel is with our speakers today, as well as with Scott Stibitz from CBER. So we're going to get that going so stick around, please. We'll only be a few minutes getting speakers up here.

(Pause.)

DR. RANALLO: Just also to round the bases, I've been up here all day, I'm fairly exhausted, but I can tell you that there are a few areas that we heard today that I'd like the panel to opine on and perhaps address specific questions from the audience.

So we heard, you know, we heard talks on novel uses and future uses, so specifically looking at prophylactic or preventative use of phage. I think that, to me, is very intriguing, and an area that we have not discussed in terms of -- we haven't covered that.

Another is in terms of phage engineering and looking at how we can serve to, you know, genetically modify phage to make them more useful or to have them as tools to study bacterial populations.

So, with that, I don't have any specific questions for the panel. I certainly think, like I said, I would like -- maybe I could start off and, Andy, maybe press upon you a little bit, again, thinking about cholera phages and just trying to

understand how in a high event situation such as household transmission of cholera one could, I won't say run a clinical trial, but at least, you know, conceptualize how that might be done.

DR. CAMILLI: Yeah. I mean, you know, in Bangladesh and in India there's two outbreaks per year. Pretty reproducible. During those outbreaks there's certain places, like the icddr,b hospital in Dhaka gets thousands of patients a day.

So they've run a number of household studies over the years, various investigators, various groups, and so the mechanism's there to do this, where they would -- they could incorporate into a household study where they go and teach them about transmission, and cleanliness, and chlorination of water, et cetera.

They could do a trial with a small number of households where they -- the household contacts take the phage cocktail. That's the ideal field trial. We need to get the money to do that. We have the product ready. And then you would look. This high rate of transmission, 23 percent, is a easy target. Do you lower that or do you not?

DR. RANALLO: Is there a role in a -- and I don't know the status or -- in a human challenge model or something like that, or -- and I think I mentioned

this perhaps earlier, just the idea of an attenuated strain just to look at the dynamics of how this would occur and what the rate of excretion would be?

Because I think you mentioned that there was a tenfold -- two log increase in transmission rates. Can that be predicted by the excretion of a rice water stool in a clinical setting? Is that a first step or is that --

DR. CAMILLI: Well with animals you can mimic this transmission. You know, with the infant rabbit model, they will transmit it either naturally, just have the baby rabbits together, or you can take some of the stool and transmit it so that you can easily model that in the laboratory.

In households it's not clear how the cholera is being transmitted within the household. I mean you can imagine somebody comes down with cholera and it gets all over the place, maybe it gets in the water that they're drinking, but nobody really knows. Nobody's looked at that yet.

AUDIENCE MEMBER: Maybe just an idea for these -- the people in this room. Based on the things we have had to do all with the individual case treatment, we have been discussing that with AmpliPhi Bio Science already, and I wonder if would not be

possible to set up some kind of a database with a standard format, simple format, of all the patient cases that we are starting to treat to try to harmonize the information on these patients treating with phages in a way to provide more information and to get that through the -- information available to the regulatory authorities in USA and in Europe.

DR. STIBITZ: I think that's very hard to do. You have to ask the question of who's going to fund it. I think it's -- you're talking about, for example, a database entry for each case where phage therapy has been attempted --

AUDIENCE MEMBER: Yeah.

DR. STIBITZ: -- with certain minimal details. I mean I think that the FDA lacks the regulatory authority to do that unless it were under an IND. I think it's something that a group of concerned scientists and/or citizens could organize, and I think there would be great value in the sense that it would capture the denominator.

Currently, we hear about the successes, and I think Dr. Gorski's presentation I think was certainly an eye-opener for me for somebody who's really, you know, kept the records so that we are getting a -- estimates for the efficacy of phage

therapy at least in one modality. He's standing right behind you, so he might want to respond.

DR. GORSKI: -- would be to reduce the dose patient within clinicaltrials.gov, even though clinicaltrails.gov is restricted for clinical trials. For example, we did so. We did not update the information -- I'm sorry about it -- but our, not trial, but our experimental therapy is registered within clinicaltrials.gov.

I don't know if it's legally possible. That's another question. But if it is, why not?

DR. STIBITZ: Right. I think I'm on pretty safe ground saying, and I can look for nods from my colleagues, but I don't think that's something that the FDA could mandate or be that instrumental in doing. I'm not seeing nods, so maybe they'd like to respond.

DR. TYNER: So I have something just briefly to add perhaps for consideration is I like the idea of having a database. I think the important part of a database is some level of harmonization of the data you collect.

Previous life I was a malaria researcher, and the malaria community realized when it did meta analysis that they couldn't compare one study to the

next, and so maybe it's incumbent upon some folks, perhaps in this room, to sit down and talk about what it would be -- what are the things we would want to collect and at what time points, et cetera.

I realize it's not a clinical study, but if there was some level of agreement, consensus on the information that you collect, it sure does make it a lot easier to compare as you're beginning to put all these different eIND cases which are disparate enough and different enough that they're hard to compare in the first place.

DR. STIBITZ: Before I let Jay ask the next question I just wanted to add there's -- it seems to me in this scenario there would be a very strong incentive to report positive data and a very weak incentive to report negative data.

AUDIENCE MEMBER: Hi. So I want to ask you to focus for a minute on a different area of anxiety. Not so much efficacy, but safety, perhaps in a more global perspective. A number of you touched on it, but I guess I didn't feel like I really got a fully developed response, especially from the phage engineering folks.

Certainly in Dr. Duerkop's discussion he mentioned the great anxiety that the dairy industry

has about phage gone wild, and that's a major concern in that universe of the dairy industry and fermentations that you don't want to get extinguished.

I don't think anybody's talking about a phage that gets out in the world and destroys every gram-negative on Earth and unleashes other horrible problems, but certainly in our microcosms that we work with, not so much patients, but maybe hospitals and other little microorganism universes, can you imagine any adverse effects in those universes that you should worry about, especially with an engineered product, not necessarily a product that's been co-evolving with these organisms for hundreds of millions of years.

Just fantasize about your worst nightmare and then we can -- then we could just -- after you verbalize it, we could just sleep better. How about that?

DR. LU: Right. I mean I could write a Hollywood script on it. I mean I guess it's the same question as asking, you know, any genetically-modified organism: can you accurately assess every possibility that could happen, right?

So I think we have the same debate over GM foods or, you know, oncolytic viruses that are engineered in a variety of different ways. So I'm not

sure that I can *a priori* predict to you all the bad things that can happen with engineering. I think we can probably take off things that we might expect to look for, right?

So we don't want to be able to transduce genes between organisms at a greater rate than we might naturally be able to do that. We might want to test that our engineered phage, as a well-defined spectrum, it doesn't hit -- won't go commensal, or good bacteria or bacteria that we're worried about in the dairy industry, for example.

I think if the requirement's going to be that we have to take an engineered phage to a standard where I can prove to you with a hundred percent certainty that there is zero risk, I think that it's sort of an impossible bar for an engineered construct across.

AUDIENCE MEMBER: No, no, no, no. But somebody's going to ask you to do an environmental impact assessment for sure of some sort, and I think -- I just think the conversation is worth having. I think it's worth having among scientists rather than in the --

DR. LU: Yeah. So I think doing an impact assessment of spectrum and transducing capability of

an engineered phage versus its natural counterpart could be worthwhile to do. I'm not personally concerned that engineered phage would be any worse in that particular context, but I think -- I mean I -- certainly we can define assays that we can all agree make sense, but I don't want that to necessarily turn into like GM is necessarily bad.

I think it's always a risk/benefit trade-off of, you know, sometimes it makes sense to do it, in other cases the natural phages make a lot of sense. If you can get great efficacy with the, "natural phages," that have been, frankly, evolving for a very, very long time, then why not go ahead with that?

AUDIENCE MEMBER: Just to add a little bit to that, so our center is collaborating with a large pharmaceutical company to generate phages to treat Pierce's disease which is destroying the wine industry in California, and there's no way we'll ever be able to use engineered phages.

The California EPA has already said never, ever, ever, and then the -- and our EP -- the U.S. EPA doesn't seem to be quite as negative, but the California EPA says no way.

DR. LU: Yeah. So I think certainly environmental applications is probably a very, very

difficult way of using engineered phages. I think if we're talking about serious human disease, then I think the bar is probably different.

DR. RANALLO: So, yeah. One of the things that I've been thinking about over the last -- this -- today here is this idea of phage cocktail and the utility of phage cocktails for dealing with resistance generation. Do you think that there's a specific engineered solution to that in terms of engineering phage to not – I guess my question is a phage cocktail almost always going to be the solution, or is – do you feel that there's an approach that –

DR. LU: Yeah, I think the engineer – I think the phage cocktails make a lot of sense currently, given the data. I think we have some stuff in the works that shows that you can integrate certain properties into single phages that are quite interesting.

So I don't want to say too much about that right now, but I think there is the possibility of integrating multiple properties into a single phage, and -- but I think, you know, the cocktail approach seems to do pretty well in most cases, so I'm not saying that that should be thrown out the window.

DR. RANALLO: And -- sorry. Yes?

DR. BISWAS: So I just like to mention one thing. Also I prefer natural phages, as engineered phages are great to attack stationary phase bacteria. Because stationary phase bacteria, to kill them with natural phage is very difficult sometimes. So something to deliver lethal gene or something in those bacteria using engineered phage is a very good idea.

DR. RANALLO: Yeah. And I guess that's my question for developers, in general. You know, we heard Scott say yesterday basically on this topic is that engineering is not bad, it's just you have to explain why and prov -- and likely incumbent upon the developer to develop assays, or at least to have some way to address the issues at least that are brought up, or that, you know, that -- I'm sorry.

DR. LU: Yeah. No, I agree. I mean I think we have to justify why we're doing it and -- in some sense certainly to the regulators, but also to our own time. Like why am I spending all this time doing it? So I think there are certain properties that we would need to be able to demonstrate why the engineered phages make sense.

I think the other thing, to address sort of some of the comments brought up earlier, I think one of the reasons to go to an engineered construct is if

you can perhaps remove the replicative ability of these phages and sort of simply use them as delivery. I think that's an alternative way we've been thinking about sort of hopefully addressing some of these concerns about sort of freely replicating genetically-modified mutant viruses.

DR. RANALLO: Okay.

AUDIENCE MEMBER: Sorry. Real quick. I don't think it's ironic that you two are sitting right next to each other. I think when you talk about diagnostics and you talk about personalized medicine, can you just kind of touch quickly on like the time-sensitivity of having to deliver these therapeutics pretty rapidly and how important like the complement of each other are.

DR. BISWAS: So your question is how we can mark this diagnostic approach with personalized phage cocktail, right?

AUDIENCE MEMBER: Yeah. So like say you need to identify what the pathogen is pretty rapidly, and then you need to come up with a cocktail just as rapidly. I guess what stage of -- you know, is it in hours, or is it in days, or is it in weeks right now, and at what point do you think that --

DR. BISWAS: So currently, the systems which

we developed, we can figure out within six hours, almost, that bacteria is going to be infected with those particular group of phage. If you don't know the bacteria, what is the bacteria if you have a group of phage, you can use them to find out that -- what is that bacteria is -- actually.

Not only that, you can also study the antibiotic-resistance pattern of the bacteria in presence of phage because you can use the antibiotic, you know, in the bacteria, and then let them grow, and then use phage to see that they are affected or not.

The bacteria inhibit the -- if the antibiotic inhibit the bacterial growth, then you will not see the signal. The phage will not multiply, and they will not produce any signal. So you can do all these things in one single run, actually.

AUDIENCE MEMBER: And on the personalized medicine side, how quick do you think that we can kind of get cocktails together that can be effective to treat --

DR. BISWAS: Oh, okay. So last time when we prepared these we need three days. Three to four days. Three and a half days, almost. People took much more time to transfer the phages. But in real-time scenario, if we have previously prepared phage

already, and cesium chloride and LPS, you know, removed phage, then we can use it right away.

But if we are looking at, like Colonel Regeimbal mentioned, that we need to grow it into the patient's bacteria, then we -- it takes little bit longer time. So that -- in that case, probably three and a half days will be fine.

But its depends. If we are -- and the people working the night shift, we can do it within two days.

AUDIENCE MEMBER: Since we're being controversial this afternoon, I'll just pick up on one of Tim's comments. Removing the ability to replicate. When you use a therapeutic dose of penicillin you use about 10 to the 19 molecules. Now, penicillin isn't a single hit kill, and phage can be. I appreciate that.

But equally, penicillin, 350 daltons, gets through things easily. Phage, pick a dalton range, doesn't. So maybe those two balance out. So you -- maybe you need to use the same number.

If they can replicate, they can produce what they need, but if they can't replicate, okay, great, if you're putting it onto a situation where you can see the infection. You can dump on the amount of non-replicating phage you need.

But if you're relying on it passing through wax, or body surfaces, or mucus, or whatever, I've done a few mathematical calculations on this, and I know Steve Abedon disagrees with me, but I come up with a dosing level, depending on the size of the phage, of somewhere between 400 and 1,000 kilograms.

So is not removing the ability to replicate going to be slightly problematic in some situations?

DR. BISWAS: Definitely.

DR. STIBITZ: Well I think I can get partly there. I mean ampicillin doesn't kill with a single molecule per cell.

DR. RANALLO: Okay. So in -- with regard to finishing on time, what I wanted to do is maybe -- unless we have any other questions for the panel?

(No response.)

DR. RANALLO: Seems like we got them all out during the day. So I'd like to, you know, thank the -- well I'd like to thank everybody who came up for the panel, and I'd like to thank all our speakers today. I think it was a wonderful day. Then we're going to conclude with Dr. Mike Kurilla who opened the meeting yesterday with some concluding remarks. So thank you, panel members, thank you, speakers.

Mike, you're up for concluding remarks.

DR. KURILLA: Well we've come to the end and -- of the workshop. I hope that the comments I've received over the last two days from staff and from individual participants are representative.

I'm always encouraged by the sort of crude marker I use that at the end of the meeting, if the density of occupancy of seats is similar to what it opened with, that obviously a lot of people have found a lot of useful things to stick around.

I have to say, personally, you know, this is now our second phage workshop, and I can tell you that, having been an undergraduate student back in the late '70s and actually had the honor of meeting Max Delbruck who introduced, I think, molecular biology to the world by studying phages, I never anticipated in a medical career that this would be something that would be realistically considered, and the amount of interest and focus that is being applied is very heartening.

So Dr. Marks from the FDA opened the workshop, and he noticed -- he remarked on the importance of history, phages being a little over a hundred years old, but that, with a little bit of effort, you know, they could be an example of the old becoming the new new, and I think that's been clearly

evident in what's gone on.

For that new new it's going to require a continuous input, both in terms of guiding developers on the current and evolving regulatory perspectives, which was a major focus on what we discussed here for the last two days, as well as encouraging continued investment in the scientific foundations that are needed to fill the gaps of knowledge, as well as to identify new, and potentially exciting opportunities that phages can offer us.

So the first and second sessions of the workshop focused on the clinical use of phages and regulatory perspective and, really, in terms of their applications to what we're seeing as probably the -- one of the most critical in developing unmet medical needs, that is, antibiotic resistance.

While antibiotics may be considered one of the real gems in terms of 20th-century medicine, the 21st century may see a much more limited utilization because we know that they are so easily overused.

In addressing these unmet needs we can't underestimate that phages clearly offer us, potentially, a new solution set, but it's still going to require quite a bit of effort in order to establish that regulatory guidance that defines product

expectations, as well as the types of clinical studies and trials that are going to be needed in order to achieve regulatory approval.

The third session gave us a forecast of future possibilities and emphasized the value of incorporating lots of different perspectives in terms of looking at phages, and we're beginning to appreciate the understanding of co-evolutionary relationships and trade-offs that impact bacterial resistance and are likely to inform strategies for future development of phages.

We can also appreciate the newly-realized powers of genomics, bioinformatics, synthetic biology that will shed new light on phage evolution and prospects for useful and clinically-relevant modifications that will be desirable.

We saw some of the perspectives drawn from military medicine and ongoing challenges seen due to injury and exposure to initially unique, but becoming more commonplace infectious agents.

So these are just examples of the kind of cross-fertilization that these types of conferences afford us going forward, as well as just the overall value of communication with different communities, all with the goal of treating and preventing infectious

disease.

So, on my part, we and my FDA colleagues would like to thank all of you for making this a successful conference. We really want to encourage everyone to continue the communication, momentum, and collaboration that is building towards developing phage-based solutions for the future. Thank you very much.

(Whereupon, at 2:55 p.m., the meeting in the above-entitled matter was concluded.)

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REPORTER'S CERTIFICATE

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HEARING DATE: July 11, 2017

LOCATION: Rockville, Maryland

I hereby certify that the proceedings and evidence are contained fully and accurately on the digital recording and notes reported by me at the hearing in the above case before the Department of Health and Human Services, United States Food and Drug Administration.

Date: July 11, 2017

/s/

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