This Week in Microbiology

With Vincent Racaniello, Michael Schmidt, and Michele Swanson

Episode 163: Saliva and sptR/S

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Vincent: This is TWIM, This Week in Microbiology, episode 163, recorded on October 26, 2017. This episode is brought to you by the Defense Threat Reduction Agency, part of the US Department of Defense. The agency's chemical and biological technologies department hosts the 2017 Chemical and Biological Defense Science and Technology Conference to exchange information on the latest and most dynamic developments for countering chemical and biological weapons of mass destruction. Find out more at cbdstconference.com. Hi everybody, I am Vincent Racaniello, and you are listening to the podcast that explores unseen life on Earth. Joining me today from Ann Arbor, Michigan, Michele Swanson.

Michele: Hello!

Vincent: Actually, you are not in Ann Arbor.

Michele: I'm not, I'm in our nation's capital.

Vincent: The heart of our nation, the lifeblood, the beating heart.

Michele: If not the soul, right now, but we won't go there.

Vincent: We won't go there. Also joining us from Charleston, South Carolina, Michael Schmidt.

Michael: Hello, everyone!

Vincent: Do you still have 80 degree weather out there?

Michael: No, it actually became October. It was 49 degrees as I was driving to work this morning.

Vincent: That's not typical.

Michael: No. It is a little cool and I am happy to have fall.

Vincent: But you have mild winters, right, down there?

Michael: Oh, extremely mild. There have been Christmases where it is 76 degrees outside.

Vincent: What is it like in DC, Michele, is it nice?

Michele: I would say it is maybe mid 60s and sunny.

Vincent: Same here, we have a lot of clouds, I think it's low 60s here or 20 Celsius, to be scientific about it. It turns out that yesterday, October 25, was the birthday of Antonie Van Leeuwenhoek.

Michele: Wow.

Vincent: How do you say it, Michele?

Michele: I try not to say it (laughs)

Vincent: And Anthony, our listener, sent a number of links from Facebook. Here's a Royal Society post, known as the father of microbiology, considered the first microbiologist, was born on October 25 1632.

Michael: Wow.

Michele: Did he coin the term animalcules?

Michael: He did indeed.

Vincent: He did, he wrote a letter to the Royal Society in September, 1683 describing animalcules, the first known description of bacteria. On his birthday, we unearthed exclusive photography of the letters in the original paper dealing with bacteria living in a range of environments. The colloquial heuristic style conceals the working of a startlingly original experimental mind. We used to, in the 60s and 70s, the science papers, we used to be colloquial, but not any more. You go back and look at old papers like Journal of Virology, they're much much different from today in terms of their writing. Not only that, but they have far fewer authors, as well. Okay, happy birthday, Antonie. Do you think he is the father of microbiology? I think he was important, right?

Michele: He definitely was an early leading figure, I'll give him that.

Vincent: Because without a microscope we wouldn't be able to see them, but I think it's always more than one person. But anyway, that's cool, thank you Anthony for that. Alright, we have a snippet and a paper for you today, and the snippet is going to be published in MSphere, I believe, sometime early in November, by the time this episode is released it will be published. We have it ahead of press and if you remember, I am an editor at MSphere, which means that I look at the manuscripts that have been accepted and pick some for the variety of podcasts. We've done some on TWIV, we've done some here on TWIM, I don't think we have a TWIP Msphere yet but we are working on it. And so this one was picked out in that way and it is called "Novel Genes Required for the Fitness of Streptococcus pyogenes in Human Saliva." Today's theme of today's TWIM is saliva. (laughs)

Michael: And we started with the father of microbiology and the first microbial sample was indeed his saliva.

Vincent: He looked at his saliva.

Michele: And in fact that's why we chose these two papers, in anticipation of this.

Michael: Augh.

Vincent: Make us look smart, Michele. That's right.

Michael: You make us look smart.

Michele: Keep organized.

Vincent: This paper is authored by Luchang Zhu, Amelia Charbonneau, Andrew Waller, Randall Olsen, Stephen Beres, and James Musser and it is from Houston Methodist Hospital, Weill Medical College of Cornell University here in New York, the Animal Health Trust, which is in the UK, and the University of Cambridge, also in the UK. And the idea here is, first of all, Streptococcus pyogenes, a group A Streptococcus, an important human pathogen, the primary sites of colonization is the human oropharynx, and this bacterium causes 600 million cases of pharyngitis annually worldwide, 15 million in the US and big health care costs. It's also responsible for 100 million cases of other human infections, so the initial colonization of the oropharynx, and then it goes elsewhere and includes rheumatic fever and rheumatic heart disease, which is very bad. The most common cause of preventable pediatric heart disease globally.

We don't understand how this bacterium successfully colonizes the mouth and causes pharyngitis and persists, and that is partly what this person, what this paper is addressing. They would like to know what genes are important for Strep pyogenes to be able to persist in saliva and saliva of course is something that we produce in our mouths and we swallow, and you'll hear more about that from our paper today. But the question here is how does this bacterium persist in saliva?

Michele: And if I could interject, this is a topic that this group has been working on for a while. They did a nice job in their introduction describing some of the background work, including identifying a key two component regulatory system that is important for group A strep to persist in saliva, and this two component regulatory system they named as a Strep pyogenes transcriptional regulator and sensor, and the acronym is SPTR/S or spitters.

Michael: Here it comes.

Michele: (laughs)

Vincent: You like that, don't you.

Michele: Yeah.

Vincent: Spitters.

Michele: Spitter and spittus. Spit. Spitter and spittus.

Vincent: That's great. Spitters. That's just great. Actually, I hadn't picked that up.

Michele: That would be on line 110. 110 and 111.

Vincent: Yeah, I see it now. I tend to look at these acronyms and go rapidly by them.

Michael: You don't pronounce them out loud.

Vincent: I don't pronounce them out loud, you're right. So they definitely tailored that. Okay.

Michele: I don't think that was a coincidence

Vincent: No no no, definitely not. Well, when you can, make an interesting acronym, that's true. We'll try and make a title that includes that, right? (laughs)

Michele: I wonder what gene spitters regulate. I wonder if there is a way to figure that out, Vincent.

Vincent: I think you probably could, right?

Michele: How?

Michael: Which is the intent of the paper.

Vincent: Well, here's the paper. So the idea here would be to make a lot of gene mutations in this bacterium and ask which genes are important for the ability to grow in saliva, and so they make a transposon insertion library where you introduce into the bacterium a plasmid including the transposon which will then integrate randomly into the DNA and then it will copy itself and insert somewhere else and hop around. And so this is a common way to make random mutations in many many genes. So they start with a strain of Strep pyogenes and they generate 140,000+ unique transposon insertions.

Michele: That was amazing.

Vincent: And how do they know that?

Michele: Well.

Vincent: I think there are a couple of ways you could do it, but probably sequencing would tell you that, right?

Michele: And that's what they did for this method. So it is kind of a next generation of signature tag mutagenesis, they actually sequenced out form each transposon and could therefore map each transposon to the site, and I think they said on average they had a transposon every like 13 nucleotides. So, saturation.

Vincent: 93% of the genes, 1,720 out of 1,841 have at least one transposon insertion. So they have pools of colonies with these mutations and then they can ask who can grow well in the presence of saliva. So the way they do this, they get saliva from human volunteers, and they mix it with bacteria and then they plate it on agar and they look at 12, 24, and 48 hours later after growth on the agar. So they mix with the saliva and put it on agar and then 12, 24, and 48 hours, then scrape off whatever is growing, and then you sequence, and you see what genes have decreased frequency in the population. In other words, the bacteria are not growing as well as you would expect them to. And so you can look at this entire library and ask what is needed to grow in saliva. I should say, and I might be wrong here, but these are not necessarily specific for saliva, but they are needed to grow in saliva.

Michele: True.

Vincent: Because they didn't look to see if it would affect growth in other conditions, not saliva conditions.

Michele: Yeah, like they didn't do a counter screen.

Michael: Well, they had 140,000 clones, so it's not equivable.

Vincent: No, it's okay, but they do focus in on a few at the end. At some point they're going to have to do that, right. It's an interesting question, to know if there are any saliva specific genes, right. So they identified 92 genes whose disruption by a transposon affects growth in human saliva. And these contain genes with 3 prevalent categories, genes involved in carbohydrate transport and metabolism, amino acid transport and metabolism, and inorganic ion transport and metabolism. There are others as well, but those are the major three categories. And they had previously done some work addressing the same question with non human primates, in synamalgous macaques, and they asked what genes are expressed during oral pharyngeal infection of these animals, and of the 92 that they call saliva fitness genes that they identify in this paper, 74% of them were also expressed during infection of these macaques, oral pharyngeal infections. So that's good.

Michele: That does speak a little bit to your question. Although it doesn't rule out or document that it is specific for saliva.

Vincent: So the next thing they do is they pick 6 genes are these are genes that have not been shown to participate in saliva fitness, and they in the core of all the known group A strep genomes, and they were expressed in the pharynx of all these non human primates and they are involved in a variety of pathways, including transporters, carbohydrate metabolism, pyrimidine and arginine synthesis, amino acid metabolism, and phosphate transport. So they take a parental strain, they inactivate again each of these genes specifically, so now you have an isogenic strain, you have the parent and you have a mutant strain. They confirm that in fact disruption of these genes caused reduction of growth in saliva, impaired fitness in saliva, I should say. But they don't, and here that would have been a nice time because there are only 6 compared growth in rich medium or something else, right?

Michele: Yeah, I think they did say that they don't have growth defects in rich media, but that doesn't get at your question of whether they are specifically induced.

Vincent: So these genes, as I said, all these group based strep genomes have been sequenced, they are highly conserved in terms of where they are located, the sequence is conserved, and these ABC transporter genes are in fact downstream from another gene, CAR B that they have shown to be important for fitness and is in fact one of these that they specifically disrupted. CAR B encodes a carbamoyl phosphate synthetase and that compound is a precursor for pyrimidine and arginine synthesis. Another gene that they looked at, PSTS, which you could probably pronounce in a way that invokes spitting in some way, it encodes a phosphate binding protein involved in phosphate uptake. So you see all of these are involved in transport acquisition of nutrients and metabolism, and I suppose the bottom line here is these may enable the bacteria to acquire nutrients in the oral cavity. Now you have to go on and figure out exactly what they are doing and how specific they are and so forth, but now we have this library of mutants that people can use to study persistence in the oral cavity and function and so forth. So this is actually a perfect snippet because there is no mechanism here. There are suggestions, of course, but it is the first step.

Michele: It is also a really great illustration of the power of this library and this new method that they applied, this TRODIS, is that how we pronounce that? Transposon with deep sequencing?

Vincent: Works for me, yeah. And again, the idea is you simply sequence after your selection to see the representation of the genes, so you can look at the whole population They did this in pools so you could look at the whole population, it's very good.

Michele: Yeah. And then deduce who is needed. Anything that is missing must have been important.

Vincent: So yeah, it's a cool paper. Any other thoughts, Michael, did you like this?

Michael: I'd like to commend them on their figures, and even though this is only audio, you will be able to see these figures because MSphere is actually published in the public domain. And so you can actually take a look at their figures and the one that I was most intrigued with was the one that has the Venn diagram in it, where you have overlapping circles and you can begin to see which genes are overlapping between 12 and 24 and 48 hours and you can see which ones are common. I found that figure especially compelling and most easy to digest as we were going through this great paper in terms of sheer volume of materials that they were looking at. This is going to give them I think some insight into how this organism is able to account for as many infections as it does indeed cause throughout the globe.

Vincent: Do you know, there must be inhibitors of bacteria in saliva, right?

Michael: There are.

Vincent: Peptides and so forth, right?

Michael: Peptides, all sorts of other things, some of the salivary proteins, it is a complex milieu controlling the dynamics of the microbes and you don't consider group A strep as being a predominant organism in the oral cavity, but it is in the oral pharynx, and mostly when you think of the oral cavity you are thinking of Strep mutans and some of the more common cariogenic microbes,

but here again the genes that were important for fitness were principally associated with carbohydrate metabolism, and if you think about it, that is exactly what streptococci are doing to initiate the cariogenic response or cause cavities, and for better or worse the most prevalent infection on the globe is caries, infecting our teeth with streptococci responsible for dissolving the wondrous crystal hypoxyapatite that is our tooth.

Vincent: So I didn't see any genes that would be involved in resistance to say, peptides or other inhibitors. However, quite a number of genes that were disrupted have an unknown function, so maybe they could be involved in resistance to an antimicrobial peptide, for example.

Michele: I also appreciated that the point they made in their discussion that this really emphasizes the intimate linkage between metabolism and persistence. So in the early days of microbial pathogenesis, we tended to fixate on secreted proteins that were easy to get our hands on and study biochemically like toxins, and then studying factors that as you pointed out overcome the immune system. But the ability to grow is obviously really important for any infection and we are learning that more and more as we use these whole genome methods for identifying key pathways that microbes need to establish infection. So we are having to go back to our microbial metabolism to understand infection.

Vincent: Michael, what fraction of the population carries Strep pyogenes in the oral pharynx?

Michael: I think everyone.

Vincent: Everyone does? Okay.

Michael: Everyone does, it is quite common and I think Michele brings up a really interesting point about the genesis of how we begin to digest pathogenesis. When we began to look at pathogenesis, it was always pure culture biology and today, because of our advances in sequencing technology, we know that nothing occurs in the pure state of a single organism in the context of the host, it is a very complex dynamic that we have in the oral pharynx and the oral cavity, and the two are indeed connected, so, what is going on that these authors are trying to elicit and thinking about the genes that were revealed that were important for the strep to be able to compete in this dynamic equilibrium where we feed them on an irregular schedule and if you eat between meals you are changing the sugar balance and you are doing all sorts of unique things to the carbohydrate mix, it is really this technique and the mutants or the transposons that they have generated will really begin to help them take things apart.

And as you point out, we don't understand how this microbe is responsible for the rheumatic events that we see which are not an infection but a reaction, our immune system is reacting to a protein of the microbe that happens to cross react with one of our heart proteins, and it's a second area. So. It's really pretty neat that they are offering this technology to the greater community to begin to think about how to dissect some of these things.

Michele: And presumably their two component regulatory system, spitters, will help.

Michael: Oh no, and that I think is the key, the two component system of what is turning certain genes on and off in the context of where the organism is.

Vincent: Spitters and saliva, right? That's a good title.

Michael: They're linked.

Michele: Can't have one without the other.

Vincent: You cannot. Let me tell you about the sponsor of this episode, the Defense Threat Reduction Agency. Imagine an everyday inexpensive drone you could by online, modified by terrorists to spread chemical or biological weapons over a crowded football stadium or a holiday parade. Plague, sarin, weaponized flu, how could we prevent a scenario like this from happening? How would we treat the victims? How would we counter the effects? Join us in Long Beach, California, November 28-30 for the 2017 Chemical and Biological Defense Science and Technology Conference to exchange information on the latest and most dynamic developments for countering chemical and biological weapons of mass destruction. Collaborate with over 1,500 scientists, subject matter experts, military service members, industry partners, and academic leaders from across the globe who are committed to making the world safer by confronting chem and bio defense challenges.

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Michael: This is a paper that appeared in last Friday's Science magazine, and it is entitled "Ectopic colonization of oral bacteria in the intestine drives TH1 cell induction and inflammation" and it is authored by 26 individuals and that should give you an idea of how intense this paper actually is. It only has four figures but it has a tremendous amount of supplemental information that I think as you begin to unpack this paper you will find very rewarding, because they are easy experiments to understand and you can actually understand the logic flow behind how they came to the conclusion that is really summed up in the title of this paper.

Vincent: Hey Michael, four figures but they have like ten panels each or more.

Michael: This is true. This is true. They are very intense. So here is the bottom line up front for this very cool study, which is a cross between a paper for this week and for microbiology and if you will our initial foray into This Week in Immunology. So the intestinal colonization by bacteria of oral origin has been correlated with several negative health outcomes, including inflammatory bowel disease. And as everyone knows since we just left talking about Strep pyogenes in the oral cavity, the oral cavity is a very complex habitat with over 600 prevalent taxa at the species level, and the average human will consume about 1.5 liters of their own saliva per day. If you recall that saliva has a concentration of 10^9 bacteria per mL, that means you are literally consuming 1.5 x 10^12 bacteria from your oral cavity and they are ending up in your GI tract each day.

Michele: Michael, is that for people who floss or people who don't floss? (laughter)

Michael: It's for everyone, because the flossing, this is, I spent last week at the American Dental Association, so that's why I threw this paper over the transom, I was with a bunch of dentists last week and it was actually a fascinating meeting and as I was sitting in one of the meetings, I was looking through the titles of Science and that's how I stumbled into this. But it is indeed true, what flossing does is it tightens up your periodontium so that you don't get as many microbes in the pockets, that's what flossing is good for. So the causal role of oral bacteria that are ectopically, and ectopically means that these organisms are in the wrong spot, and the causal role of the oral bacteria colonizing the intestine remains unclear. But what these authors did was they used notobiotic techniques, which are effectively animals that are raised in the absence of normal microbial constituents, and they showed that strains of Klebsiella isolated from the salivary microbiome were strong inducers of TH1 helper cells when they colonized the gut, and that these Klebsiella strains were resistant to multiple antibiotics and they only tended to colonize the intestine when the intestinal microbiota was dysbiotic, that it was disturbed, and this elicited the severe gut inflammation in the context of a genetically susceptible host.

And so their findings are extremely intriguing and suggest that the oral cavity may serve as a reservoir for a potential intestinal pathobiont. This is a word I had never heard of before and I may be pronouncing it wrong, and these are microbes that are proposed as pathobionts are associated with chronic inflammatory conditions. And unlike opportunistic pathogens which often cause acute infections like Michele was talking about in the early days of pathogenesis, we were always looking at the acute infection, these things are typically acquired from the environment or other parts of the body. So I learned a new word, the pathobionts, and they can exasperate intestinal disease. Any of you who are listening who know people who have Crohn's disease or inflammatory bowel disorder or any of the other inflammatory conditions associated with the digestive system, you know how devastating these diseases can be. So any insight that we may gather from looking at this can help us out.

So as background for some of our listeners who may not be card carrying immunologists, I found a handy cheat sheet that I am going to ask Ray to put in the show notes, so you can figure out the importance of various T cells as we begin to talk about the immunology. And it is by Chen Dong and Gustavo Martinez that was published in Nature Immunology Reviews, and it is freely available so it is not behind the Nature paywall, so you are able to download it and delve into what all these magical T cells do as you are reading the literature or just listening to a TWIV or TWIM or TWIP where we begin to go down the immunology paths. So I thought I would put that out there.

So broadly speaking, T cells can be grouped into various subsets based on their effector functions and molecular phenotype. They function by directly secreting soluble mediators or through cell contact dependent mechanisms and there may be various transcription factors. And the reason I am bringing this up is the one that we are going to focus on in today's discussion is the T helper 1 cell, or TH1 cell. And as you will see from the cheat sheet or poster, it has got a whole variety of receptors but we are not going to talk about them. It has got transcription factors, we're not going to talk about that. We're just going to talk about the things that it secretes, namely the interferon Gamma which is a soluble dimerized cytokine which is critical for innate and adaptive immunity against viral and some bacterial and protozoal infections, will activate macrophages, but for our discussion today, you need to

appreciate that too much interferon gamma is associated with a number of these autoinflammatory or autoimmune diseases. So that is one of the things they are measuring and they are doing it by flow cytometry, where they are harvesting the effector molecules, the white blood cells if you will, they are running them through a fax machine which will be able to tell how many are there and they are able to tag them as to whether or not they are indeed producing interferon gamma.

So the story starts with the authors mining their in house data sets of 16s ribosomal RNA sequences asking who is present in the oral cavity and what they have learned is that there were several species. There were things like rothea, Streptococci neisseria, prevotella, gamella, all of which are aerotolerant microbes, and they were significantly more abundant in patients with ulcerative colitis, primary scolosing coliongitis, gastroesophogeal reflux, a disease you have probably heard of as GERD, and they also found it associated with alcoholism as well. So that's the effective part of their first story. They display the aggregate relative abundance of these microbes and what you see is healthy individuals have little blips of bacteria. They are not anything one over the other. But as you go into these inflammatory things, you see spikes of things popping up.

Michele: And these things, Michael, just to be clear, are microbes that are normally in the oral cavity but they are popping up in the gut of these patient populations.

Michael: So a healthy individual has a diverse community in their stool, not anyone is sticking out like a sore thumb, but in these chronic inflammatory conditions there are organisms that actually pop up and become as high as 55% in the ulcerative colitis. So you can see in this particular figure of who is actually popping up. And then this takes us to the premise of their work or their hypothesis, namely that a subset of the oral microbes may supplant themselves from the oral cavity and colonize and persist in the intestine under certain circumstances to then abberantly activate the intestinal immune system, resulting in these chronic inflammatory diseases that we just listed.

And they tested this hypothesis by using an elegant approach of simply taking transplanted saliva samples from patients with Crohn's disease and they actually then placed them into C57 black 6 germ free mice and they just simple gavaged it in. So in other words, they collected the saliva from the patient and then they just pipetted it down the throats of these mice or by gavage, and then each mouse was housed in a separate notobiotic isolator because remember, mice eat their own stool, so the stool is infectious so you can move organisms around so they try to control for this. And then after 6 weeks of allowing the oral gavaged microbes to effectively take hold, they then sampled the small intestine and the colonic lamina propria, and the immune cells were examined.

In the mice receiving a saliva sample from a Crohn's disease patient, there was no significant changes to the intestinal cells. And they only did two saliva samples, which is really remarkable, but in the second patient, there was a profound difference. And the profound difference as we saw this spike of interferon gamma producing CD4 positive cells, namely these T helper 1 T cells in the intestinal lamina propria. And their next step was then to figure out what was unique about the community of microbes present in the saliva associated with patient 2.

And here again they go back to 16s ribosomal RNA gene sequencing and they compared the community composition of the saliva microbes before administration to the germ free mice and then the fecal microbiota of the colonized individuals. And they create a very colorful plot, and although

the saliva samples of both patients contained similar microbial communities, the fecal samples differed markedly between saliva 1 sample, which showed no interferon gamma, and saliva 2 which had a boatload of the stuff in there. They then learned what was unique to saliva 2 sample, which was only a minor component of the salivary microflora, offering them their first insight into how the oral microbiome could be implicated in disease that this bacterial species that was constituting a small fraction of the oral cavity could expand and colonize in the gut, and a subset of these oral species could then induce the accumulation of the intestinal TH1 cells.

Michele: So far we just have correlations though, right? Cause and effect we don't get at just yet.

Michael: So the next step they are going to do is they are going to try to go after Michele's fulfilling Koch's postulate. So they want to isolate the TH1 cell inducing bacteria. So they anaerobically cultured the cecal contact, and that is very important because most of the organisms in the digestive system of animals are anaerobes, so you don't want to cultivate them aerobically, you want to cultivate them anaerobically. And they cultivated them, and they picked 224 different colonies with different colony appearances.

They then sequenced them to learn their identities and they learned that the colonies contained 8 strains from diverse genera. We had gamella, which is an oral phoro organism, we had Bufidobacterium, Streptococcus escherichia, fusobacterium, enterococcus, as well as Klebsiella. These 8 broadly represented the major members of the gut microbiota colonizing the gut from saliva sample 2. So next on their path to address Michele's question, to demonstrate the causal relationship between disease, was to ask how these isolated strains had a TH1 cell inducing capability. And again, they just simply looked for the production of this interferon gamma.

So they cultured these 8 groups separately, made a cocktail, introduced them into the germ free mice, and asked, did it recapitulate the original observation of the saliva experiment? So instead of having the complex mixture from saliva, they just grew up these 8 organisms, mixed them all together, fed them back to the mice, and they observed the efficient induction of TH1 cells in the colonic lamina propria of the mice with a magnitude comparable to what they originally saw. SO again, it is still correlative, as Michel would say. So then the authors ask you to compare panel B and panel D, and they do indeed look remarkably similar.

Michele: And the first author, Koji Atarashi, told me that was an especially exciting day in the lab when they plotted out that data, when they started with a really complex sample from saliva of a patient and then in a matter of I don't know, weeks, were able to identify a particular bacterium from saliva that accounted for this spike in interferon gamma production.

Michael: And Michele sort of tumbled to their result, because they did a bunch of other experiments ruling out fusobacterium and glamella, because these two microbes were implicated in inflammatory bowel disease pathogenesis, and they didn't do much of anything. They didn't induce the interferon gamma, and what they ended up discovering is there was one microbe, Klebsiella pneumoniae 2H7, that was responsible for this interferon gamma spike, and they did the appropriate control experiment where they had 7 organisms without the klebsiellla and they were able to illustrate that it looked just like a germ free animal's normal gut lamina propria.

So you can understand how they were most excited, and the effect of this Klebsiella, or they abbreviate it KP2H7, was relatively specific for TH1 cells, and they have a tremendous number of supplemental figures, but it really takes you through the story. What was remarkable was that there was no increase in the percentage of TH1 cells in the oral tissues. Either the palate or the tongue, and that's where this microbe came from. Remember it came from the saliva of a patient with Crohn's disease, and the increase in TH1 cells was observed in another strain of mice, and here is where they demonstrate that it is dependent upon the genetics of the host. So there are two other strains that it recapitulated the results in, and those are hidden in the supplemental figures that you can take a look at.

But now the question is, how is it able to establish itself? Here is where the microbiology comes into play. As our listeners know, Klebsiella is a notorious microbe often acquiring resistance to multiple antibiotics, with the scariest one now being KPC, which is the Klebsiella pneumonia that has a carbapenemase resistance factor in it. Here, their isolate KP287 was resistant to multiple antibiotics including ampicillin, tylosin that many of you may not have heard of because it is typically not used in people, this is a feed additive that they use for supplementing the diet of animals to lessen the farm to table time. It is nothing more than a macrolyde. The organism was also resistant to spectinomycin, which is an aminoglycoside, and it was also resistant to metroniasol. So that is a pretty broad list of antibiotics.

Their next experiment was to ask whether or not the animal's gut was required to be dysbiotic, that is to say it was disrupted in order for the Klebsiella to displace the normal flora and then effectively bloom and recruit the TH1 cells. The experiment, as you might imagine, was using now the normal mice as we would call them in the lab, or specific pathogen free mice, which have a normal immune system, a normal digestive tract, they have their full complement of bacteria, and they were untreated or continuously treated with ampicillin, tylocin, spectinomycin, or metrazionol in the drinking water for four days before the oral gavage of the Klebsiella pneumoniae. And the antibiotic naive mice were resistant to colonization by the Klebsiella but the amp treated or tylosin treated animals allowed the Kp287 to persist within the animal's intestines. But the spectinomycin and metraniazol, it didn't matter, the organism wasn't able to displace it. So it was unique to those two antibiotics. Amp and tylocin.

They show a nice figure clearly delineating what is actually going on. And the percentage of TH1 cells amongst the colonic lamina propria was analyzed again by flow cytometry and they are able to recapitulate that it is indeed in the presence of the tylosin or the ampicillin, they are still generating the same level of interferon gamma production that was going on. The reason I am taking you through this first figure so carefully is they replicate this series of experiments throughout the paper in order to get at their final conclusion.

Michele: Before you go on, I found it really interesting that treatment with two antibiotics allowed this Klebsiella to colonize but the other two did not, and they were able to deduce from that that there must be some species of antibiotics normally in the gut that provide resistance and if they get wiped out by a particular drug, then the Klebsiella can establish a foothold. I think that is going to give them an entree into figuring out what microbes in the healthy gut prevent these Klebsiella from the mouth from getting in there and causing problems. I found that hopeful.

Michael: Which is very important as we are trying to begin to understand this whole genesis of these inflammatory digestive diseases.

Michele: And how do we protect ourselves? We can't just keep taking antibiotics to get rid of these Klebsiella in the mouth, so what other levers to we have to pull?

Michael: It's about this dynamic equilibrium that is actually indeed going on, and the authors contribute that because microbial and host factors both contribute to pathogenesis of inflammatory bowel disease, they then took them to test the influence of this Klebsiella colonization strain in the colitis prone mouse, which is an IL10 knockout animal. So here the experiment was a germ free wild type mouse along with a knockout, and they were each orally administered, either the Klebsiella pneumoniae strain or an E. coli strain or a mixture of the other 6 strains, and as you know both Klebsiella and E. coli are members of the enterobacteriaceae family, and both have been implicated in IBD pathogenesis.

So here they learned from this particular experiment 1 week after colonization, again a more potent induction of the TH1 cells was observed in the knockout animals given the Klebsiella pneumoniae in the other groups, and there was a greater induction of the colonic LP IL17 positive interferon gamma positive CD4T cells as well as epithelial tumor necrosis factor in MRNA expression in the knockout animals, and here the histological evaluation illustrated that KP, this Klebsiella pneumoneae that was prone to colonization, induced a more severe inflammation than the E. coli or the 6 mix in the proximal colon of these knockout animals. So again, the data affiliated with this first figure and the supplemental data they have been displaying all along as they are taking you through this story led the authors to propose/conclude that this Klebsiella 2H7 acts as this gut pathobiont in the context of this genetically susceptible host. And organisms proposed as this pathobiont are then associated with these chronic inflammatory conditions unlike the opportunistic pathogens which often cause just simply an acute infection.

Vincent: I think it is important to point out that up until this experiment, the Klebsiella could recolonize and induce TH1s but they didn't cause inflammatory changes in wild type mice. Only when you look at these knockouts does that happen, which is really interesting.

Michael: And so they are beginning to take, they are sneaking the immunology into their story on this because the beauty of using the notobiotic mice is that we do have a large family of knockout animals from which we can begin to dissect, is it the host immune system or is it the organism itself going on? Because it is a complex mixture in the gut of microbes and you don't know if it is the KP or some other minor actor. But it is still, the Klebsiella pneumoniae is really beginning to do this thing. And so that is where they take us.

Michele: But in a healthy gut, you have got plenty of good microbes that wont let the Klebsiella in and then we have got cells that make the cytokine IL10 that dampen the immune response. So normally we are protected. But I agree.

Michael: That's the beauty of IL10.

Vincent: That's why you can swallow 10^12 bacteria a day and you're okay.

Michael: And not have a chronically inflamed system. And they take us through and they show through some beautiful immuno fluorescence that this KP2H7 isn't invading. It is not invading the system. They do a really neat experiment where they use heat killed KP2H7 again, this is, if it is just an antigen response where you are throwing in all these things, but there is actually biology going on because what they were able to show in that particular experiment using dead KP and live KP, only the live KP induced an increase in interferon producing gamma CD4 positive T cells. And so it is actually very satisfying to see what is actually going on with that particular system. Now the intensity of induction was independent of bacterial load and was not accompanied by inflammation, and they have a grading scale of TH1 induction, of strong, medium, and weak, and then they began to correlate the multi locus sequence typing, K typing, or simply phylogeny, and again this is buried in the supplemental tables of what genes are responsible or what is actually going on.

Michele: This is bacterial genes.

Michael: These are bacterial genes and the comparative analysis of the whole genomes revealed that 61 orthologous groups of genes were positively correlated with the TH1 induction. So we have 61 groups of genes and these included genes to encode a homolysin correlated coregulated protein, enzymes involved in fructose, galactose, mannose, and long chain fatty acid metabolism, related uptake and metabolic pathways. So you can see this is not a simple throw a Klebsiella in and see what happens. This organism is unique to being able to cause this sort of pathology, and these genes have been reported to be enriched in the fecal microbiome of patients with inflammatory disease and have been suggested to have immunomodulatory effects and therefore may contribute to the induction of the TH1 cells, which Michele sort of stumbled into in the beginning, she says is there gonna be hope for us?

So then they take us down the path again trying to drive home the Koch's postulates to confirm the link between oral derived bacteria and TH1 induction, they obtained additional saliva samples, but now this time, from two healthy donors and two patients with active ulcerative colitis. Rather than Crohn's disease, we are now looking at ulcerative colitis. They reran the experiments that we just talked about and they ran these in germ free wild type B6 mice and as you might expect, the TH1 cells accumulated in the colonic lamina propria of mice inoculated with a saliva sample from the ulcerative colitis patient and it was again only one particular patient showed up. So it is not cause and effect, it is, not all saliva samples will have one of these unique microbes.

Vincent: That was the healthy patient, right?

Michael: And there was one that was a healthy patient, there was one with an ulcerative colitis and there was one from a healthy patient. So healthy patients that don't yet have this disease, you can be swallowing this organism, but unless you have a dysbiotic event of some triggering event, and that's what we don't know is going on. And the paper takes us through more of this rerunning it and they fished out a strain like the first experiment. They cultured the cecal contents the same way, anaerobically, from the inducer strain, they isolated 13 strains, they orally administered them. So you now understand why there are 22 authors. There is an incredible amount of work.

They replicated the phenotype they saw for the ulcerative ccolitis patient in terms of TH1 induction, and then among the 13 strains they recovered, they found one that was an Enterococcus faecium and a Klebsiella aeromobilis, and that drew their attention because both of those microbes have been implicated in IBD pathogenesis and have been reported to be important multi drug resistant pathobionts. And again, following the routine of germ free mice gavaged with either Enterococcus faecium or the Klebsiella aeromobilis or the mixture of the 11 other strains, they confirmed that it was the Klebsiella aeromobilis that induced the TH1 cells in the colon comparably to the 13 mix where the Enterococcus faecium and the 11 mix failed to do so.

So that takes us through their third set of figures, and then as they begin to wrap up their story, they talk about, they analyzed this new species of Klebsiella and learned that its colonization resulted in severe inflammation in the IL10 knockout mice, suggesting that this orally derived strain may act similarly to KP2H7, and likewise the aeromobilis Klebsiella did not attach to the surface and again they demonstrated that by the same FISH technology that they used in the first figure. And so their outlier as Vincent already tumbled into was their discovery that the healthy donors also could have one of these bad actors. They isolated this strain and it was a Klebsiella pneumoniae strain, KP40B3, and this strain also induced marked T cell accumulation in the colon of the mice. Their final experiment was they mined the 16s RNA sequencing data sets used in the analysis of figure 1 and found that the relative abundance of members of the Klebsiella were significantly higher in patients with Crohn's disease, this coliongitis, alcoholism, and they are contrasting with the control group.

And then they went off to other databases looking at inflammatory bowel disease and Crohn's disease, respectively, looking at the MassGen hospital PRISM database, and the Crohn's disease was from the University of Pennsylvania cohort. And again, they see that this Klebsiella actor is a bad actor. So summing up--

Vincent: Let me just say that that experiment is so important because it is okay in mice to show that these Klebs do something but if people with the disease don't have them that would really be the end of it, right? So that's key that these people with CD, alcoholism, etc, have these strains.

Michele: I thought it was really impressive that we have these two public databases.

Vincent: That's pretty cool.

Michele: So these are, as they say, these registries where they have now cataloged who is in the gut microbiota of patients with IBD or with ulcerative colitis and now anybody can query those and learn something more, so it is really a tribute to public research.

Michael: The subtlety is they use the Mann Whitney test to do the statistics, and that means that they are not normally distributed. So again, this is why in not all saliva samples from patients with this disease had this particular microbe or this particular family. So we don't know, as Elio would say--

Vincent: There might be others, right?

Michael: There might be others.

Vincent: So if you took, maybe if you had all these salivas, like dozens, you'd find other bacteria that could do similar things.

Michele: Almost certainly. And then it would be interesting to see if it is a different bacteria with a different name but it still has that subset of metabolic pathways that they pulled out.

Vincent: I'd bet it does, yeah.

Michael: That's the power of this paper. They had a readout for the immune system responding, namely the production of the interferon gamma that was fairly replicative, and they had this power of DNA sequencing and they did the mixing and matching experiments to demonstrate or fulfill the Koch's postulate that this organism alone was the actor or triggering even that actually caused it. Their concluding statement, the data suggests that the oral cavity may serve as a reservoir for Klebsiella pathobionts and a point of fact, the oral cavity or its microbiota contains the highest relative abundance of the enterobacteriaceae compared with other mucosal sites. So it may not always be Klebsiella, it may be one of the other enterobacteriaceae species that as Michele said have this cluster of genes that can actually trip this expansion of the TH1 helper cells that produce these inflammatory mediators.

Vincent: Do you think, Michael, if you have patients with these diseases and you find that they have Klebsiella, do you think as they suggest get rid of them with therapeutics, would that correct the disease? Is it a matter of continuous infusion of these Klebsiella into the gut or where once you stop the inflammation goes away, or is it just a trigger and it goes on on its own?

Michael: That's the autoimmune part. Once you have trained the immune system to react, it will react, because it is the adaptive arm of the immune system and it has memory.

Vincent: I guess you could do a clinical trial and just treat people to eliminate the Klebsiella and see if it corrects their IBD or whatever else, right?

Michael: And this is where I think, when I was at the American Dental Association meeting last week, there were a number of vendors selling probiotics for the oral cavity that were specifically, you would ingest these organisms, you would pop the pill, and they were enterically coated so they could withstand the stomach acid, but you are adding these organisms to your gut and I didn't quite get the connection of how organisms in the gut were gonna connect back to the mouth because I hope it is a one way trip. You eat something, it goes down, and it goes out. I don't try to regurg my food unlike a cow, I'm not chewing my cud.

Michele: I'll also say the idea that we should just get rid of the bacteria, that just seems like hopeless. Instead I love the idea of figuring out which microbes in our gut provide colonization resistance and then by changing our diet and making sure that we have those microbes, we could be naturally protected. That's actually the route that Koji said they are most interested in pursuing now, trying to identify which beneficial bacteria in the healthy gut prevented these Klebsiella from triggering a TH1 interferon gamma production as a step toward therapy.

Vincent: I think that you are absolutely right, but it is a long term thing because a lot of these gut microbes are not easily cultured, and in the meantime people with IBD would like to have some relief. Maybe the antibiotic treatment is…

Michele: Or fecal transplants, and we'll figure out later who is doing it. Yeah. So Koji is on the faculty, a member now, but let me tell you his background. He got his bachelor's of science at Kiyoshi University in Japan in the School of Sciences, and then did his PhD at Osaka University where he began to study the intestinal immune system. He is now an associate professor in the department of microbiology and immunology at Keio University School of Medicine and as we saw from this paper, he is primarily interested in the interaction between the gut microbiome and the immune system. He said a really exciting day in this project was when they did the experiment and first identified out of the complex mix of microbes in saliva of patients that there was one Klebsiella that could account for the majority of TH1 cell stimulation, and as I said, they are most interested in now in identifying beneficial bacteria that can prevent that from happening. So the advice he has for junior scientists is to be curious, be patient, and enjoy science.

Vincent: You bet. That's the key.

Michael: That's the key!

Michele: As I thought about this paper, it reminded me of some comments that people have made in the early days of the microbiome that oh, it's just cataloging, we're just learning who is there, it is just correlations. Here we see now in a single paper that they took a patient sample, were able to identify a response using a mouse model, they then took advantage of a number of genetic mutants in mice and were able to identify the key players in the immune system, and they could do the 16s analysis and identify not only the culprit bacterial species but then go deeper and actually correlate particular metabolic pathways of the bacteria that are doing this, and then test their model by taking yet more human samples and recapitulating the biology. They did this in one paper (laughs) so it is amazing how fearless they were and ambitious, and I should add, I love the cherry on top where they went to this registry of patients from Mass General and UPenn and were able to say, actually, this is very consistent with the patterns we are seeing in patient populations. So to do all of this work and publish it in one paper, wow, we have really come a long way. And hats off to this group and their highly productive collaboration.

Vincent: That's why there is 26 authors, I guess. (laughs)

Michael: It's a service to the community because I think if you had to read, if they published this in pieces, because you could have published this in pieces, figure 1 as you point out had 10 panels in it. And it really made, you could get the 50,000 foot level.

Vincent: I also like that it is a perfect use of an animal model, right? And then you go into humans and you say, yeah. This looks right. A lot of people criticize mouse models, they say they are used, but it shows that they have a lot of value.

Michele: And you need specific information to then interrogate all of the clinical data and ask if it is consistent or not, so really beautiful the way they went back and forth between the clinical samples, the mouse model, the bacterial genetics.

Vincent: Yep, good job. Thank you, Michael.

Michael: Thank you.

Vincent: Alright, we are out of time, so I will skip the emails until next time. I just want to remind you, you can send your questions and comments to twim@microbe.tv. I also want to let you know we are starting a new immunology podcast.

Michele: Wow!

Vincent: It's called Immune, it is just called Immune, no more This Week. It is called Immune and you can find it starting November 1st at microbe.tv/immune. My colleagues in that venture are two immunologists, Cynthia Leifer from Cornell University and Stephanie Langel from Ohio State University. They are card carrying immunologists. So it will be authentic. You have to know immunology and microbiology to really understand, right, you can't just know immunology, you have to know about strains and anaerobic growth, etc. So it is tough to split it all up. Anyway, that's November 1st, microbe.tv/immune. Michele Swanson is at the University of Michigan, thank you Michele.

Michele: Thank you.

Vincent: Michael Schmidt is at the Medical University of South Carolina, thanks Michael.

Michael: thanks, everyone.

Vincent: I'm Vincent Racaniello, you can find me at virology.ws. I want to thank the ASM for their support of TWIM, Ray Ortega for post production, and the sponsor of this episode, the Defense Threat Reduction Agency. The music you hear on TWIM is by Ronald Jenkees. You can find his work at ronaldjenkees.com. Thanks for listening everyone, see you next time on This Week in Microbiology.

(music)

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