Calcium Regulates Quorum Sensing in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is an opportunistic pathogen that causes numerous infections in humans, including severe respiratory infections in cystic fibrosis (CF) patients. Since CF patients have elevated levels of calcium in their saliva, nasal and lung fluids, it is important to understand the effect of calcium levels on the production of virulence factors in *P. aeruginosa.* Quorum-sensing (QS) molecules guide cell-to-cell communication, which controls the expression of numerous genes, including those that encode virulence factors. The synthesis of QS molecules in *P. aeruginosa* relies on three main synthases: LasI, RhlI, and PqsA. To measure the changes in the expression of the QS molecule-encoding genes, *lasI, rhlI,* and *pqsA*, in response to 5-mM calcium, a promoter activity assay was used, and the luminescence produced under the control of the genes' promoter regions over a 14-hour incubation time, was evaluated. Results showed that elevated calcium induced the expression of the QS synthases. To identify the component(s) of *P. aeruginosa* calcium signaling system responsible for this activation, deletion mutants lacking genes encoding the calcium channel (CalC), calcium sensor (EfhP), or calcium-induced two-component transcriptional regulator (CarR) were tested. Promoter activity assays for *lasI, rhlI,* and *pqsA* in the mutants at varying calcium conditions showed that CalC appears to be responsible for calcium-dependent upregulation of QS synthases in *P. aeruginosa*.

Metatranscriptomic Characterization of Microbial Community Functions in Chronic Human Infections

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Polymicrobial infections often harbor different bacteria with a wide range of metabolic capacities which often encourages the establishment of several synergistic relationships among the community members. Several of these polymicrobial communities have been profiled by metagenomics and 16S rDNA sequencing, however, literature is lacking on chronic bacterial infections, such as cystic fibrosis and chronic diabetic wounds, and the functions in these microbial communities. To address this knowledge gap, we analyzed 109 previously published metatranscriptomes collected from chronic pulmonary (24.77%) and wound (75.22%) infections to identify key bacterial members and community functions in these chronic polymicrobial infections. Our initial samples had an average of 128,419,168 reads; however, we found the majority of our reads were human reads, with a mean of 12.5% of the reads (mean 16,080,907) attributable to microbes following data cleaning and prepossessing. Community composition analysis of the prokaryotic reads with MetaPhlAn4 revealed that bacteria in the phylum Firmicutes, Proteobacteria, Actinobacteria, Bacteroides, and Fusobacteria made up more than 50% of our metatranscriptomes. We found our sputum samples to be more diverse than wound samples with a mean of 13.96 and 7.53 species identified, respectively. Gram positive bacteria dominated our samples at 59% (48.7% of sputum and 68.6% of wound samples) at the species level. While members of the genera *Staphylococcus*, *Streptococcus, Porphyromonas and Anaerococcus* dominated the wound samples, *Rothia spp.*, *Streptococcus spp.,* and *Prevotella spp.* were the most prevalent in our sputum samples. Strict anaerobes made up 46.7% of the 46 distinct genera identified with a mean of 34.45% suggesting that these communities are hypoxic. Members of the Gram positive anaerobic cocci (GPAC) group made up 19.4% of the identified genera in our wound samples and these were present in at least 62.1% of our wound samples and had a negative correlation (ρ = -0.25) with *Staphylococcus*, a known key member of chronic wound infections, suggesting a competitive relationship. Ongoing analyses are identifying critical community functions in each infection site using SAMSA2, HUMAnN3 and MetaPro. Compositional and functional profiling of chronic bacterial infections is underlying to understanding the molecular mechanisms that aid microbe-microbe interactions between the active members of these communities and how these interactions contribute to microbial persistence or delayed immune responses and, ultimately, the slow healing process often observed in clinical settings.

Metabolism and Ontogeny of Alveolar Macrophages Contribute to Peripheral Trained Immunity and Confer Protection against *Mycobacterium tuberculosis*

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Trained immunity is a nonspecific memory-like response that can be induced predominantly in innate immune cells. This process is characterized by altered cell activation states, and is mediated by both metabolic and epigenetic reprogramming. β-glucan, a cell wall component of fungi and yeasts, is well-known for its capability of inducing robust systemic trained immunity. However, whether trained immunity can be induced at specific mucosal sites, particularly in the lung, is unknown. We hypothesized that directly exposing β-glucan to the lung environment can induce localized trained immunity in lung resident macrophages, and confer protection to *Mycobacterium tuberculosis* (Mtb) infection. We observed that AMs in mice treated with β-glucan intranasally acquire sustained and enhanced activations as well as metabolic reprogramming. Using a lineage tracing mouse model, we found that β-glucan leads to a CCR2-dependent influx of monocytes and promotes the generation of monocyte-derived AMs, which exhibit canonical features of trained immunity, including enhanced cellular activations, an altered metabolic shift towards glycolysis, increased cytokine production, and enhanced phagocytic activity. Notably, IFNγ appears to be essential for these responses. Importantly, β-glucan treatment significantly reduces Mtb burden in the lung, associated with enhanced AMs functions. Similarly, monocyte-derived AMs likely play a pivotal role in mediating such protection as they produce significantly higher levels of TNF-α, IL-6, and IL-1β upon *ex-vivo* Mtb challenge compared to tissue-resident AMs. These results suggest that β-glucan may offer a unique vaccine strategy to Mtb by harnessing local trained innate immunity in the lung and highlight the importance of macrophage ontogeny as a novel determining factor of trained immunity.

Abstract

Negative Regulation of the *Yersinia pestis* Plasminogen Activator Protease (Pla) by the PhoP/PhoQ Two-Component System

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Background: *Yersinia pestis* is a gram-negative bacterium that is the causative agent of bubonic, pneumonic, and septicemic plague. *Y. pestis* harbors a number of virulence factors for establishing infection in the mammalian host including the plasminogen activator protease (Pla). Pla is an outer membrane protease that cleaves plasminogen into plasmin to promote the degradation of fibrin clots, thereby facilitating dissemination of bacteria during bubonic plague. During pneumonic plague, Pla acts as an adhesin that contributes to suppression of early innate immune responses, and as a protease that facilitates bacterial survival via an unknown mechanism. Two-component regulatory systems (TCS) are involved in bacterial adaptation to environmental stressors, such as changes in pH, changes in ion concentration (Mg^{+2}), and the presence of cationic anti-microbial peptides. TCS generally consist of a membrane bound sensor kinase that detects environmental stressors, and in turn activates a response regulator that coordinately alters expression of bacterial genes. The PhoP/PhoQ TCS is well established and is known to regulate virulence factors in a variety of pathogenic bacteria including *Escherichia coli* and *Salmonella* species. *Salmonella* contains a homolog of Pla, PgtE that is positively regulated by the PhoP/PhoQ TCS. Our laboratory recently identified a putative PhoP binding site within the -10 box and the +1 transcription start site of *pla*. We therefore sought to determine if Pla is regulated by the PhoP/PhoQ TCS.

Methods: WT or Δ*phoP* CO92 *Y. pestis* strains were cultured in BHI and grown at 37°C to equivalent OD_{600} values. Cultures were then treated with identical, sub-lethal concentrations of various anti-microbial peptides or in low pH media to induce the PhoP/PhoQ TCS. Following treatment, samples were pelleted and resuspended in Trizol for RNA isolation and processed for qRT-PCR.

Results: Our qRT-PCR results suggest PhoP acts as a repressor of *pla* transcription under our tested conditions.

Conclusion: Overall, our data suggest *pla* is negatively regulated by the PhoP/PhoQ TCS in *Y. pestis*, and this negative regulation occurs under PhoP/PhoQ inducing conditions. This work demonstrates the intersection of a critical *Y. pestis* virulence factor and a well-established bacterial TCS for the first time.

Yucatan Minipigs as a Model for Influenza A Virus Infections

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Influenza A viruses (IAVs) are zoonotic pathogens that cause respiratory illness with high morbidity and low mortality. They are known to have high mutation rates, allowing them to evade host immunity and sometimes cause pandemics. Influenza-related respiratory disease causes major economic losses and several hundred thousand deaths annually worldwide. Here, we evaluate Yucatan minipigs as a model to further understand influenza-host interactions. To establish this model, nine minipigs were assigned to three groups, infected with 10^4, 10^5, or 10^6 $TCID_{50}$ of pandemic H1N1 IAV (strain A/CA/04/2009; intranasal). Two additional minipigs were included as non-infected controls. Body temperature, clinical signs and body weight were observed to determine severity of clinical disease. All minipigs were euthanized and necropsied 14 days post-infection (dpi). Body temperature increased at 1 dpi in the group infected with the high dose. Body weight decreased by 4 dpi in the high and mid dose infected animals; nonetheless, weight gain was restored by the end of the study at 14 dpi. Nasal viral shedding was present in all infected minipigs until 5 dpi, with significantly higher titers in the high dose group at 1 dpi. After 7 dpi, virus isolation was negative in nasal swabs, bronchioalveolar lavage, and lung tissues. Seroconversion determined by HI titers was observed in all infected animals by 14 dpi. Macroscopic and histologic lung lesions were still present at 14 dpi mainly in animals infected with mid or high dose, with a statistically significant difference observed between minipigs infected with the mid and low dose. In conclusion, Yucatan minipigs are a suitable animal model to study viral replication, viral shedding, pathology, and immune responses during IAV infections. This model will allow the evaluation of IAV intervention strategies such as vaccines or therapeutics, or support epidemiological data observed in swine and human populations.

Coxiella burnetii Alters Human Macrophage Metabolism and Mitochondrial Physiology

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Coxiella burnetti (C. burnetii) is a Gram-negative obligate intracellular pathogen that is transmitted to humans by aerosols. After inhalation by a mammalian host, the pathogen is phagocytosed by alveolar macrophages. *C. burnetii* subsequently replicates within a lysosome-like parasitophorous vacuole (PV) that forms by transit through the phagolysosomal maturation pathway. This replication niche requires modulation of numerous host cell processes. *C. burnetii* uses a Dot/Icm type IV secretion system (T4SS) to secrete effector proteins into the host cell that modulate infection events, including autophagosome recruitment to the PV and prevention of apoptosis. However, little is known about the impact of *C. burnetii* on host cell metabolism. In this study, we investigated the metabolic state of human macrophages during *C. burnetii* infection and probed involvement of the T4SS in regulating host cell metabolism. We investigated the central metabolic pathway using mitochondrial stress and glycolytic rate Seahorse assays. We also assessed macrophage polarization during *C. burnetii* infection to link host metabolic signatures to macrophage antibacterial activity. Our results indicate that macrophages infected with wild type *C. burnetii* maintain normal oxidative phosphorylation (OXPHOS) activity, whereas T4SS-defective bacteria trigger decreased OXPHOS activity. When OXPHOS is inhibited, *C. burnetii* is unable to form prototypical PVs. Furthermore, mitochondrial morphology is altered during wild type, but not T4SS-defective, *C. burnetii*-infected macrophages. Overall, this study demonstrates the involvement of mitochondrial OXPHOS and altered metabolism in *C. burnetii* parasitism of human macrophages.

Human WDR7 Functions as a Host Factor for Rift Valley Fever Phlebovirus Infection

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Rift Valley fever phlebovirus (RVFV) is an emerging zoonotic pathogen that causes Rift Valley fever (RVF) in livestock and humans. Currently, there is no licensed human vaccine or antiviral drug to the control of RVF. Although multiple species of animals and humans are vulnerable to RVFV infection, host factors affecting susceptibility are not well understood. Here, we performed CRISPR-Cas9 knock-out screen in human A549 cells to identify host factors essential for RVFV replication. From our screen, we found approximately 900 genes putatively involved in host interaction with RVFV infection. Further evaluation of the effect of six genes on viral replication using siRNA-mediated knockdowns found that silencing two genes (WDR7 and LRP1) significantly impaired RVFV replication. We focused on *WDR7* for further analysis as the role of *LRP1* in RVFV replication was previously reported. Knock-out A549 cell lines were generated and used to dissect the effect of *WRD7* on RVFV and another bunyavirus, La Crosse encephalitis virus (LACV). We observed significant effects of *WDR7* knock-out on both intracellular RVFV RNA load and viral titers. At the intracellular RNA level, *WRD7* influence on virus replication was observed at a later phase for RVFV (24 hours post-infection (hpi)) compared to an earlier phase for LACV (12 hpi). Overall, we describe an important role of *WDR7* as a host factor for the replication of two relevant bunyaviruses, RVFV and LACV. The mechanism by which *WDR7* allows/facilitates arbovirus replication will be investigated in future studies.

Quorum Sensing Regulation by the Nitrogen Phosphotransferase System in *Pseudomonas aeruginosa*

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The opportunistic bacterial pathogen, *Pseudomonas aeruginosa* is a major cause of hospital-acquired infections. *P. aeruginosa* uses a population-density dependent gene regulation system called quorum sensing to regulate expression of hundreds of genes, including many that contribute to virulence. *P. aeruginosa* virulence is also regulated by a nitrogen-phosphotransferase system (PTS^{Ntr}). This system is a conserved phosphotransfer system found in many gram-negative bacteria, which is poorly studied but believed to be important for integrating information on carbon and nitrogen availability to alter metabolism. We and others have demonstrated *P. aeruginosa* strains with null mutations of the first PTS^{Ntr} gene *ptsP* have enhanced quorum sensing activity as well as increased production of pyocyanin, a quorum sensing controlled toxin. The purpose of this study is to investigate the mechanism of PtsP-dependent quorum sensing activation. To carry out this objective, single and double deletions of the three PTS^{Ntr} genes were prepared and their effects on quorum sensing activity were assessed. We also constructed mutations affecting predicted phosphorylation sites of the PTS^{Ntr} proteins. Our genetic studies support the idea that the unphosphorylated versions of PtsO and PtsN proteins elevate quorum sensing activity in a *ptsP* mutant. We were able to identify specific phosphorylation sites on the PtsN protein that are important for activation, although the PtsO phosphorylation sites remain elusive. Results also show that this regulation occurs through modulation of LasR, for example by altering the levels of LasR in the cell. These results together suggest that phosphate flow through the PTS^{Ntr} system is important for regulating quorum sensing. Deleting *ptsP* blocks phosphate flow, leading to unphosphorylated PtsO and PtsN and subsequent activation of quorum sensing. Results of our studies may reveal new insights into *P. aeruginosa* biology and could ultimately inform the development of anti-*P. aeruginosa* therapeutics.

***Klebsiella pneumoniae* Exhibits Spontaneous Resistance to the Beta-Lactam, Mecillinam**

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Bacteria must be able to sense and to adapt to everchanging environmental conditions. Shifts in environmental pH are known to impact a variety of bacterial properties, ranging from cell size to antibiotic response. Acidic environments are known to protect *Escherichia coli* cells from the bactericidal effects of Beta-lactam antibiotics that inhibits cell wall synthesis. To determine if acidic pH protects against Beta-lactam killing in other organisms, we assayed how pH alters the minimal inhibitory concentrations (MIC) of Beta-lactams needed to kill *Klebsiella pneumoniae. K. pneumoniae* is a rod-shaped, encapsulated, Gram-negative pathogen capable of causing a wide range of host infections that are becoming increasingly difficult to treat due to the rapid emergence of antibiotic resistance. Surprisingly, when exposed to the Beta-lactam antibiotic, Mecillinam, *K. pneumoniae* exhibits a spontaneous resistance phenotype in MIC assays. We demonstrate that this spontaneous resistance is a pH-dependent phenotype and is not due to genetic mutations that enable growth in high drug concentrations. Time-lapse microscopy reveals drastic morphological changes are associated with survival in high Mecillinam concentrations. RNA sequencing of Mecillinam-resistant cells shows that genes involved in the breakdown of two compounds, ethanolamine and 1,2-propanediol are highly upregulated. Mutants in these pathways demonstrate altered morphological responses to Mecillinam and a loss of the spontaneous resistance MIC phenotype. Given that ethanolamine and 1,2-propanediol are common constituents of bacterial membranes and capsules, we are exploring the role that cell envelope remodeling plays in the ability of *K. pneumoniae* to survive in high Beta-lactam concentrations.

Determining the Molecular Mechanisms of *Pseudomonas aeruginosa* Interactions with Host Cells

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Pseudomonas aeruginosa is an opportunistic human pathogen which causes acute and chronic infections in immunocompromised individuals. In cystic fibrosis (CF) patients, *P. aeruginosa* is the predominate cause of death. CF is a genetic disease resulting in increased levels of Ca^{2+} in the nasal and pulmonary fluids. *P. aeruginosa* has been classified as an extracellular pathogen, however recent studies have shown *P. aeruginosa* can invade the host cell. Since Ca^{2+} levels change drastically from millimolar concentrations in the extracellular space to micromolar concentrations within the host cytosol, *P. aeruginosa* is exposed to these major transitions during internalization. Therefore, we hypothesize the pathogen's adaptation to intracellular mode of survival is at least in part regulated by Ca^{2+}. Previously, our group has shown that Ca^{2+} regulates production of multiple virulence factors in *P. aeruginosa,* as well as its antibiotic resistance and ability to cause infection in plant and insect models. Since invasion could be a strategy used by the pathogen to evade the host immune system, we aim to study the molecular mechanisms used by *P. aeruginosa* during host cell interactions and their regulation by Ca^{2+}. For this, we will characterize the transcriptional response of the pathogen during invasion as well as escape from the host cell by using gentamicin protection assays followed by genome wide RNA-seq analysis. We have optimized the gentamicin protection assay using A549 host cells and collected RNA samples from *P. aeruginosa* adhered to and invaded A549. We also collected RNA samples from the bacteria not adhered to the host cells and those incubated without cells, to be used as controls. To understand if the key components of the Ca^{2+} signaling system earlier identified in *P. aeruginosa* play a role in invasion, we used RT-qPCR. We observed the increased expression of the genes encoding Ca^{2+} sensor EfhP, Ca^{2+}-regulated protein CarP, and Ca^{2+} channel CalC in *P. aeruginosa* during invasion. Once completed, this study will help identify important regulatory and signaling pathways used by *P. aeruginosa* during infection and determine the regulatory role of Ca^{2+}.

Phase Separation Facilitated by a Viral Protein Underlies Protein-Protein Interactions with the Host

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Liquid-liquid phase separation (LLPS) is a process in which a single-phase solution dissociates to form a dilute phase and a droplet phase. Biomolecules concentrate into these droplets, forming membraneless bodies, such as stress granules or the nucleolus. It is well-known that host proteins involved in translation repression, RNA metabolism, and stress responses localize into these membraneless organelles. Viral proteins have also been shown to partition into these droplets throughout the course of infection. *Pea Enation Mosaic Virus 2* (PEMV2), a plant virus, is a single-stranded, positive-sense RNA virus that encodes a movement protein, p26, that forms large cytoplasmic condensates in addition to partitioning into the nucleolus. In these studies, the model yeast *Saccharomyces cerevisiae* was used to identify the cellular proteins sequestered into p26 condensates. Green fluorescent protein (GFP)-tagged p26 was cloned into an inducible expression plasmid, pYES2, and transformed into *S. cerevisiae* (BY4741). Fluorescent confocal microscopy was utilized to visualize droplet formation *in vivo* which revealed that p26-GFP forms two to three distinct droplets per cell following induction. Differential centrifugation was used to enrich p26 condensates and was followed by affinity purification to immunoprecipitate p26 complexes. Samples were analyzed using liquid chromatography and tandem mass spectrometry (LC-MS/MS). p26 condensates were enriched with fibrillarin, a previously validated p26 interactor, as well as several translation-associated factors. Given the inhibitory role p26 plays in the co-translational nonsense-mediated decay (NMD) pathway, p26 condensates could function to sequester ribosomal proteins and disrupt cellular translation or associated RNA decay pathways. Finally, systemic movement of viral RNAs by p26 is incompatible with translation and suggests that p26 condensates could function as a switch to favor virus trafficking during the later stage of infection.

Facilitating Mouse Studies of Post-Acute Sequelae of COVID-19 (PASC)

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Abstract

COVID-19, caused by SARS-CoV-2, and its chronic form, Post-Acute Sequelae of COVID-19 (PASC), remain significant public health concerns. Transgenic mice are an effective model for acute COVID-19 research, but PASC studies are currently lacking due to the prohibitive costs of performing such studies in an Animal Biosafety Level 3 (ABSL-3) containment setting. The goal of this study was to determine the timing of clearance of the SARS-CoV-2 virus from mice and establish a protocol for transfer of animals from ABSL-3 to ABSL-2 for PASC studies. We hypothesized that infected mice would clear viral infection by 16 weeks post infection (WPI) and that sentinel mice would become infected after being exposed to inoculated mice. Six- to 18-week-old, B6.Cg-Tg(K18-ACE2)2Prlmn/J (hACE2) mice (N= 78 females + 75 males) were intranasally inoculated with a pre-alpha strain of SARS-CoV-2 in ABSL-3 containment. Cohorts of surviving mice were necropsied at 4, 8, and 16 WPI when lungs were collected. Naïve, female, homozygous hACE2 sentinel mice (N =5) were exposed to previously inoculated mice at 0 DPI (N=3) and 8 WPI (N=2), and euthanized 2 weeks post-exposure, when lungs were harvested. RT-qPCR and RT-PCR were used to assess for the presence of viral RNA in lungs. Lungs remained positive for the virus on PCR up to 16 WPI, but none of the sentinel lung samples were positive, raising the possibility that PCR positivity does not indicate infectivity.

H-89 Pharmacological Inhibition of Host Signaling during *Chlamydia trachomatis* Infection

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Chlamydia trachomatis is an obligate intracellular pathogen and the most common leading cause of bacterial sexually transmitted disease in the United States. Every year 3 million cases of sexually transmitted bacterial infection cases are recorded in the United States. *C. trachomatis* can be treated by antibiotics, but post-clearance the pathogen leaves long term damage to the host that results in many complications such pelvic inflammatory disorder, ectopic pregnancy, and infertility, among others. Given its intracellular nature it is known to depend on the host for its replication and survival; however, the extent of host cell signaling modulation is not known. The long-term goal of this project is to determine PKA phosphorylation of both host and chlamydial substrates during infections

PKA is an important host kinase regulating transcription, protein expression and cell survival. During *C. trachomatis* infection, we have previously shown that PKA contributes to extrusions production and increased phosphorylation of PKA substrates in late infection. We hypothesize that PKA is important for *C. trachomatis* growth and exit. To confirm the role of PKA, we tested a known PKA pharmacological inhibitor, H-89. We were interested in looking at how different concentrations of this inhibitor affects the viability of the host cells and how it affects the inclusion development and infection forming units post-infection with *C. trachomatis*. PKA manipulation during infection is important but how changes in PKA activation benefits *C. trachomatis* is not clear. We anticipate that the results obtained with further study will help us understand to what degree is the host cell being manipulated during infection and in developing effective future strategies for prevention and control of long-term sequelae like pelvic inflammatory disease and excessive scarring after *C. trachomatis* infection.

Genomic Diversity and Associated Phenotyping of *Escherichia coli* Isolated from Poultry in the Southern United States

Abstract

Escherichia coli (E. coli) are typically present as commensal bacteria in the gastro-intestinal tract of most animals including poultry species, but some avian pathogenic E. coli (APEC) strains can cause localized and even systematic infections in domestic poultry. Understanding the genomic diversity with their associated phenotyping, including host species, age, sites of collection, and clinical diseases is the key to designing an optimal vaccine candidate and to developing an effective vaccination program. In this study, we collected 188 E. coli isolates from the sick poultry samples submitted for routine diagnostic testing between May 2017 and July 2021, which cover diversity in various epidemiological factors. Phylogenetic analyses results showed that our samples have a large variation and are distributed in a large range of the whole phylogenetic tree contenting 1,463 E. coli sequences downloaded from GenBank. Genes from Genomic Islands, and those identified as potential virulence factors and antimicrobial resistance genes were encoded as sparse features. All the 188 E. coli samples were classified into 5 age groups, 4 host specie groups, 8 site groups, and 6 disease groups. LASSO model was used to explore the most important genetic features of each of the above groups. Results showed that those selected genetic features had specific molecular functions and could help to understand the connection between the genomic diversity and associated phenotyping of E. coli.

Title: **Retrospective Surveillance of SARS-CoV-2 in White-tailed deer using Formalin Fixed Paraffin Embedded Tissues Collected for Chronic Wasting Disease Surveillance**

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Abstract Body:

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the cause of the coronavirus disease 2019 (COVID-19) pandemic. Historical evidence has shown that interactions between humans and spillover animal species play an important role in transmitting SARS-like coronaviruses. SARS-CoV-2 has been shown to naturally and experimentally infect a variety of animal species, including white-tailed deer (WTD, *Odocoileus virginianus*). WTD are an abundant and widespread wild ruminant species in the US, and their health can have an impact on the ecosystem and economies across the country. WOAH and the USDA recommend surveillance of SARS-CoV-2 in spillover species throughout the US; thus, identifying convenient and reliable ways for surveillance of SARS-CoV-2 in wild animal populations is a critical need.

Formalin fixation, followed by paraffin embedding, is a well-established method for tissue preservation/archiving, making them an excellent resource for retrospectively analyzing samples collected over extended periods of time. In this study, we evaluated the suitability of formalin-fixed, paraffin-embedded tissues (FFPET) originally prepared for chronic wasting disease (CWD) surveillance as a sample source for the surveillance of SARS-CoV-2 in deer populations. Through collaboration with AAVLD-accredited diagnostic laboratories in Louisiana (LADDL) and Kansas (KSVDL), a collection of 690 FFPET representing retropharyngeal lymph nodes and tonsils were obtained, and tested for the presence of SARS-CoV-2-specific RNA by RT-qPCR. The samples were obtained between 2019 and 2021, which allowed for data collection both before and during the COVID-19 pandemic. In the present study, viral RNA was isolated from FFPET using a previously validated protocol that utilizes a single tube de-

paraffinization and tissue digestion protocol, followed by a rapid automated magnetic bead nucleic acid extraction. Using the CDC RT-qPCR for SARS-CoV-2, we detected viral RNA in 2 out of 470 (0.426%) blocks from Louisiana and 2 out of 308 (0.649%) from Kansas. In conclusion, here we demonstrate that FFPET samples can be used to retrospectively study the prevalence of SARS-CoV-2 in white-tailed deer populations.

Selecting high-yield vaccine candidates directly from epidemic influenza viruses using machine learning

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Abstract

Despite substantial effort, the effectiveness of influenza vaccines remains suboptimal. Engineering an antigenic matched, high-yield vaccine seed strain in a timely manner is critical to the success of influenza vaccination. Here, by targeting the hemagglutinin receptor-binding site of the poor growth A/California/04/09(H1N1), we generated a pool of 189 mutants with diverse growth and glycan receptor binding properties. Through machine learning, we are reporting a set of high-yield signatures to improve virus growth in both embryonated chicken eggs and MDCK cells without changing their antigenicity, and changes at these residues diversify virus binding to different sialylated glycan receptors. Based on these features, a genomic sequence-based machine learning model was developed and applied in selecting influenza vaccine strain from 2009 H1N1 viruses with unique HA sequences (n = 11,424) for the 2009-2020 influenza seasons. Compared to sporadic viruses in earlier influenza seasons, the proportion of high-yield 2009 H1N1 viruses significantly increased in the years of 2019 (11.65%) and 2020 (43.13%), and such a phenotypic trait appears to emerge randomly and are distributed across multiple genetic lineages and geographic orders. Four vaccine candidates (2016-2020) selected by the model were synthesized and validated to antigenically match vaccine strains and with high growth properties in both cells and eggs. The computational model from this study can be used to select high-yield influenza antigenic variants based on genomic sequences.

RNA Aptamers with Specificities for Different HIV-1 Capsid Assembly States and Their Effects on Viral Infectivity

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The HIV-1 capsid protein (CA) has critical roles in both early and late stages of viral replication. The mature capsid core has a fullerene cone structure composed of approximately 250 CA hexamers and 12 CA pentamers and participates in several replication steps, including reverse transcription, cytoplasmic trafficking, nuclear import, uncoating, integration, and evasion of host immune responses. As part of the Gag polyprotein, CA also plays important roles in viral assembly and maturation. While there has been progress in understanding capsid's roles in HIV infectivity, there are limited tools available to differentiate among CA assembly states (i.e., CA hexamers or the assembled lattice) to evaluate their roles in replication events. Aptamers are functional nucleic acids that bind molecular targets with high affinity and specificity, and they could offer key insights into the biological functions of capsid assembly states or capsid-host factor interactions. To select aptamer subsets that are either specific for the lattice form of CA or that bind both lattice and hexamer, differentiation selection was performed on a pre-enriched aptamer library with affinity for the assembled CA lattice. The resulting aptamer libraries were subjected to high-throughput sequencing and bioinformatic analyses. Upon screening candidate aptamers for binding to CA lattice and hexamer, 17 of 23 aptamers (~74%) demonstrated binding specificities consistent with predictions based on their enrichment profiles within the selection trajectories. Importantly, some aptamers were lattice-specific, while others bound both lattice and hexamer forms of CA. Further analysis focused on three aptamers representing the most abundant sequence motifs within the lattice-specific aptamer population and on one aptamer that bound both lattice and hexamer forms. Biochemical and bioinformatic analyses identified RNA sequence and structural features required for capsid binding. We further tested effects of these aptamers on HIV-1 replication in cells and observed that aptamers from distinct structural families had different effects on viral infectivity. In particular, we have aptamer phenotypes that range from inhibitory to inert for both producer and target cell assays. These results suggest that the aptamer motif families may have structure-specific interactions with capsid during viral replication.

Determining Mechanism(s) by Which HSV-1 ICP0 Counteracts Innate Immune Repression

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Abstract:

Herpes simplex virus 1 (HSV-1) is a highly contagious virus that infects 70% of the world population. Infected cell protein 0 (ICP0) is a multifunctional HSV-1 protein that plays a crucial role in promoting the lytic infection and productive reactivation from latency. Several studies suggest that one of the strategies ICP0 uses to enhance viral replication is by impacting (directly or indirectly) HSV-1 chromatin and viral gene expression. ICP0 stimulates viral gene expression, in part, by impairing the antiviral effects of interferon (IFN)-β, a component of host innate defenses. Mechanisms of how ICP0 counteracts a pre-existing IFN-β response are largely unknown. Previous studies from our lab indicate that IFN-β induces repressive heterochromatin on multiple viral promoters in the absence of ICP0. To determine which host cellular factors are targeted by ICP0 to disarm the IFN-β repression on viral chromatin, we will use CRISPR/nuclease-deficient Cas9(CRISPR/dCas9)-targeted chromatin-based purification strategy with tandem mass spectrometry (TMS), termed “CLASP” (dCas9 locus-associated proteome). This approach will allow us identify host factors that are associated with viral chromatin with or without IFN-β pre-treatment. Once target proteins have been identified, we will perform Western blot and ChIP assays to determine whether and how ICP0 counteracts these cellular factors on viral chromatin via IFN-β. Results from our study will provide a better understanding of how HSV-1-host interactions influence the HSV-1 life cycle.

Assessing the function of the cyclic-di-GMP binding protein, PlzC, in *Borrelia turicatae*

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Relapsing fever (RF) is a globally distributed, vector-borne disease caused by Gram-negative spirochetes belonging to the genus *Borrelia*. Infection with RF spirochetes results in recurrent febrile episodes that coincides with high bacterial loads in the blood. Despite its continuous presence in developing countries and increasing prevalence in developed countries, our knowledge concerning the molecular pathogenesis of RF *Borrelia* is significantly limited. Bacterial dinucleotide second messenger signaling systems are critical regulatory systems contributing to growth, adaptation, survival, and pathogenesis in diverse environments. Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), is an important dinucleotide second messenger found in Gram-negative bacteria, including RF spirochetes. While the c-di-GMP system is well-studied in many other pathogenic bacteria, its contribution in RF spirochetes is unknown. Our objective is to determine if the c-di-GMP pathway of RF *Borrelia* is required for infection and to understand how this system contributes to pathogenesis. We used allelic exchange mutagenesis to inactivate the gene encoding the c-di-GMP-binding protein (*bt0733/plzC*) in *Borrelia turicatae*. We then infected Swiss Webster mice to assess the ability of the *plzC* mutant to infect mammals. We further investigated key in vitro phenotypes in the *plzC* mutant to identify altered phenotypes that may be responsible for impaired infectivity. We conducted assays to evaluate general growth, swarm plate motility, oxidative stress sensitivity, human serum resistance, and sensitivity to whole human blood. Infection of Swiss Webster mice with the *plzC* mutant resulted in attenuated infection. Although the *plzC* mutant exhibited no growth defect or increased sensitivity to human serum, the *plzC* mutant was observed to have reduced motility, increased sensitivity to oxidative stress, and impaired growth in human whole blood. *B. turicatae* PlzC influences motility and resistance to oxidative stress and is essential for survival in human blood. These observed phenotypes may explain the attenuated infection phenotype and define previously unknown roles for this c-di-GMP binding protein. Future studies are required to identify PlzC-regulated genes in the c-di-GMP pathway that contribute to these key phenotypes.

Chlamydia trachomatis and the Characterization of its Inclusion Membrane Protein CT226

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Background: *Chlamydia trachomatis* remains a significant human pathogen. *Chlamydia*'s biphasic lifecycle has its replicative stage inside of a parasitophorous vacuole termed an "inclusion". *Chlamydia* secretes inclusion membrane proteins (Incs) into the membrane of the inclusion to interact with host cell components. This study focuses on the Inc CT226 which has been shown to have multiple interacting partners including LRRFIP1, FLII, and TMOD3. TMOD3 is known to bind to and modify the actin cytoskeleton of the host cell while LRRFIP1, LRRFIP2 and FLII are negative regulators of the inflammasome. We hypothesize that Inc CT226 modulates the immune response during *Chlamydia trachomatis* infection.

Methods: A CT226 mutant (L2ΔCT226) was generated using an allelic exchange and the mutation was confirmed via whole genome sequencing. A comparison of L2 (wildtype) and L2ΔCT226 infection in HeLa cells was performed to examine recruitment of LRRFIP1, LRRFIP2, FLII and TMOD3 to the inclusion by immunofluorescent microscopy. L2 and L2ΔCT226 mutant was further used to compare infectivity and inflammation in the reproductive tract of mice using a murine cervicovaginal infection model. After infecting the mice with both the L2 wild-type and the L2ΔCT226 mutant, swabs were taken and used to count shed Infectious Forming Units (IFUs) from each mouse for a period of 3 weeks. Reproductive tracts were removed for histopathology analysis.

Results: A comparison of L2 (wildtype) and L2ΔCT226 infection in HeLa cells by immunofluorescent microscopy showed a loss of recruitment of FLII by L2ΔCT226 to the chlamydial inclusion and altered/more diffuse recruitment of LRRFIP1 and TMOD3 surrounding the inclusion. Recruitment of LRRFIP2 was identical between L2 and L2ΔCT226. In the murine cervicovaginal model infectious forming units were higher in the L2ΔCT226 mutant than in the L2 wild type for the duration of infection.

Conclusion: The deletion of CT226 has an effect on both recruitment of its interacting proteins as well as an increased level of shed bacteria as observed in the cervicovaginal infection model. This study confirms and an important role for CT226 in Chlamydial infection.

$FtsZ_1$ is an antagonist of proper FtsZ-ring formation during cell division in *Agrobacterium tumefaciens*

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Agrobacterium tumefaciens possesses two paralogs of the division protein FtsZ: *$FtsZ_{AT,}$* and *$FtsZ_1$*, enabling the formation of a heteropolymeric Z-ring. At present, the function of the heteropolymeric Z-ring is unknown since *$FtsZ_1$* is dispensable and cell division proceeds with homopolymeric rings comprised of *$FtsZ_{AT}$*. *$FtsZ_{AT}$* possesses the three canonical domains of FtsZ molecules: the GTPase, C-Terminal Linker, and C-Terminal Peptide Domains. In contrast, *$FtsZ_1$* is solely comprised of a GTPase domain. Here, we explore the function of the truncated, non-essential *$FtsZ_1$* protein by overexpressing it from a replicating plasmid. Interestingly, overexpressing *$FtsZ_1$* results in notable cell swelling such that *A. tumefaciens*, a rod-shaped bacterium, appears as a sphere. To assess if the *$FtsZ_1$* overexpression phenotype is GTPase-dependent, we constructed and overexpressed two GTPase-null mutants of *$FtsZ_{1,}$* in wildtype cells. We predict that one mutant will bind but not hydrolyze GTP enabling it to join but not leave the Z-ring. The other mutant should not bind GTP; thus, we expect this mutant to function similarly to the deletion of *$FtsZ_1$*. Preliminary results suggest that *$FtsZ_1$* mutants are no longer toxic, suggesting that the phenotype requires GTPase activity. Next, to better understand the cell swelling phenotype, we overexpressed *$FtsZ_1$* in a hyperactive *FtsW** strain which does not require activation to produce septal peptidoglycan during cell division. Preliminary results suggest that cell swelling is due to misregulated cell wall synthesis. These characterizations of the *$FtsZ_1$* overexpression phenotype are expected to help understand how heteropolymeric Z-rings function in cell division.

A multivalent protein subunit vaccine elicits balanced immune response that protects against *Pseudomonas aeruginosa* pulmonary infection

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Keywords: *Pseudomonas*, T3SS vaccine, L-PaF BECC438, Nanoparticle vaccine, Transcriptome, Innate response, Adaptive response.

Abstract.

The opportunist, *Pseudomonas aeruginosa* (Pa), can cause severe nosocomial infections, especially in immunocompromised individuals and the elderly. Increasing drug resistance, absence of a licensed vaccine and increased hospitalizations due to SARS-CoV-2 has made Pa a major healthcare risk. To address this, we formulated a candidate subunit vaccine against Pa (L-PaF), by fusing the TTSS tip and translocator proteins with LTA1 in an oil-in-water emulsion (ME). This was mixed with TLR4 agonist (BECC438b). Lung RNA sequencing showed that the formulation activates genes from multiple immunological pathways eliciting a protective Th1-Th17 response following IN immunization. Following infection, however, the immunized mice showed an adaptive response, and the PBS-vaccinated mice experienced a sudden onset of inflammatory response. The latter displayed a hypoxic lung environment with high bacterial burden. Finally, the importance of IL-17 and immunoglobulins were demonstrated using KO mice. These findings suggest a need for a balanced humoral and cellular response to prevent the onset of Pa infection and that our formulation could elicit such a response.

Biomarkers Selection for Population Normalization in SARS-CoV-2 Wastewater-based Epidemiology

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ABSTRACT

Wastewater-based epidemiology (WBE) has been one of the most cost-effective approaches to track the SARS-CoV-2 levels in the communities since the COVID-19 outbreak in 2020. Normalizing SARS-CoV-2 concentrations by the human fecal biomarkers in wastewater can be critical for interpreting SARS-CoV-2 load, comparing viral load between sewage samples over time, and identifying the vulnerable communities. In this study, five population biomarkers, caffeine (CAF), its major metabolite paraxanthine (PARA), creatinine (CRE), 5-hydroxyinoleacetic acid (5-HIAA), and pepper mild mottle virus (pMMoV) in the wastewater were investigated for their utility for normalizing the SARS-CoV-2 loads. Their utility for estimating the real-time population contributing to the wastewater was also assessed for future WBE applications. The best candidate identified from this study was further assessed for its capacity for improving the correlation between normalized SARS-CoV-2 loads per capita and the clinical cases reported in the City of Columbia, Missouri, a university town, and a community with a constantly fluctuating population. Our results showed that PARA is the most reliable population biomarker for determining the SARS-CoV-2 load per capita due to its higher accuracy, lower variability, higher stability, excellent recovery rates and higher temporal consistency to reflect the change in population dynamics and dilution in wastewater. Both direct and indirect normalization approaches derived from the regression functions allow accounting for the changes in wastewater dilution and differences in relative human waste input over time without the requirement of flow rate and population at a given WWTP. The PARA also demonstrates its excellent utility for real-time assessment of the population contributing to the wastewater. Finally, the correlation between SARS-CoV-2 load per capita and prevalence data per capita normalized by the PARA-estimated population was significantly improved (rho=0.5878) suggesting that the population dynamics determined by a reliable biomarker should be always taken into the consideration for the SARS-CoV-2 WEB. The chemical marker PARA offers an excellent alternative to the currently recommended pMMoV genetic marker to help us understand the size, distribution, and dynamics of local populations for forecasting the prevalence of SARS-CoV-2 within each sewershed.

The Calcium-Regulated Protein, CarP, Mediates Calcium Regulation of Virulence and Metabolism in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa, a Gram-negative opportunistic pathogen, is notorious for its ability to adapt to different environments, such as in the lungs of individuals with Cystic Fibrosis (CF), where it resides and leads to multidrug resistant infections. CF is associated with a dysregulation of ion levels, including the increase in Ca^{2+} concentrations in the lungs and nasal secretions. Previously, we have shown that *P. aeruginosa* responds to elevated Ca^{2+} via the calcium-responsive two-component regulatory system, CarSR, leading to an alteration in gene transcription and virulence. One of the known regulatory targets of CarSR, *carP*, encodes a predicted inner-membrane 5-bladed β-propellor with putative phytase-like and nucleotide-binding domains. We have shown that CarP plays a role in regulating intracellular Ca^{2+} levels, contributes to the regulation of pyocyanin production and antibiotic resistance, and is involved in oxidative stress tolerance. However, the function of CarP remains unknown. Due to the prediction of a phytase-like domain, two approaches were used to identify its function. The enzyme activity assays using a recombinantly expressed and purified CarP revealed no activity towards phytic acid, indicating that CarP is not a phytase. Global untargeted metabolomics showed that several major metabolic pathways were perturbed in Δ*carP* which included membrane biogenesis, amino acid, and purine metabolism, suggesting that CarP plays a central role in the shaping of general metabolism of *P. aeruginosa*. To verify the topology of the protein, we have generated a dual PhoA-LacZα reporter translationally fused to CarP. To explore the role of the putative nucleotide-binding domain, we are currently generating a complementing construct encoding *carP* devoid of the domain to subject to phenotypic assays. With these new results and predictions, we hypothesize that CarP mediates calcium regulation of virulence and metabolism *via* transcriptional regulation.

12/15-Lipoxygenase Activity Promotes Efficient Inflammation Resolution in a Murine Model of Lyme Arthritis

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Lyme arthritis is a major sequela of disseminated Lyme disease that can persist despite antibiotic clearance of the etiologic agent Borrelia burgdorferi. In the murine model of Lyme arthritis (mLA), infected C3H mice develop an inflammatory arthritis characterized by robust cellular infiltrate and synovial hyperplasia in the tibiotarsal joint, followed by spontaneous resolution starting around 21-28 days post-infection (p.i.). While the timeline for mLA resolution usually coincides with antibody-mediated spirochete clearance, inhibition of lipid mediators and their signaling can prevent or delay resolution despite effective bacterial control. Indeed, we have previously reported that disrupting eicosanoid biosynthesis via inhibition or genetic deletion of the enzymes COX-2 or 5-LO results in a resolution defect in our mLA model. In other inflammation models, late-phase PGE_2 skews the 5-LO eicosanoid biosynthesis pathway away from LTB_4 production and towards proresolving LXA_4 production via 12/15-LO activity. As 12/15-LO is also involved in the production of several specialized proresolving mediators, we hypothesize that 12/15-LO activity is required to facilitate efficient mLA resolution. To investigate this, we characterized mLA in 12/15-$LO^{-/-}$ mice and found that these mice develop arthritis to the same degree as wild-type (WT) mice. However, we observed a pronounced resolution defect in 12/15-$LO^{-/-}$ mice, as evidenced by significantly higher ankle swelling and inflammatory cell infiltrate by day 35 p.i. as compared to WT controls. This resolution defect is not due to uncontrolled infection, as 12/15-$LO^{-/-}$ mice developed robust *B. burgdorferi*-specific IgM responses, successfully class-switched to IgG, and efficiently cleared *B. burgdorferi* burdens in the joint. *In vitro* experiments indicate that persistent cellular infiltrate in the joint may be due to a defect in efferocytosis of 12/15-$LO^{-/-}$ neutrophils. These results suggest that 12/15-LO products may play a critical role in promoting inflammation resolution in mLA. To determine if LXA_4 alone could boost mLA resolution, we treated WT C3H mice intraperitoneally with LXA_4 on days 18-20 p.i. and tracked mLA resolution parameters. On day 21 p.i., ankle edema was significantly reduced, and macrophage populations were skewed towards a reparative phenotype. However, LXA_4 treatment did not alter arthritis severity in WT mice by D35 p.i. These findings implicate 12/15-LO and its products as key factors in inflammation resolution in mLA.

Evaluation of the Susceptibility of Human ACE-2 Knock-in Rats to SARS-CoV-2

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Distinguishing features of coronavirus disease 2019 (COVID-19) in humans that present in animal models facilitates investigation into SARS-CoV-2 pathogenesis and therapeutic discovery. In this study, a codon-optimized human ACE2 cDNA was integrated into the first coding exon of the rat *Ace2* gene locus using a CRISPR-based strategy. Rats expressing human angiotensin-converting enzyme 2 (hACE2) were evaluated as a preclinical model for SARS-CoV-2. The susceptibility of hemizygous and homozygous knock-in rats to the ancestral Wuhan-like SARS-CoV-2 USA-WA1/2020 strain was evaluated for 14 days following intranasal infection, with *post mortem* evaluations performed at 3, 6, and 14 days post challenge (DPC). Both hemizygous and homozygous knock-in rats were permissive to SARS-CoV-2 infection and demonstrated moderate weight loss by 3 DPC. Viral RNA was detected by RT-qPCR in both, nasal washes and oral swabs at high levels (>10e9 copy number [CN] viral RNA/ml), and in lesser amounts (~10e5 CN viral RNA/ml) in rectal swabs. There was no significant difference in the amounts or kinetics of viral RNA shedding between the two rat genotypes. The rats shed viral RNA from the upper respiratory tract for less than 10 days (tested positive up to 7 DPC), while rectal swabs remained positive until 5 DPC. Fresh tissues (lung, forebrain, and hindbrain) collected at necropsy harbored high levels of viral RNA in the lung at 3 DPC (>10e10 CN/ml), with slightly lesser amounts detected at 6 DPC (>10e9 CN/ml), and significantly less RNA detected at 14 DPC (10e4 CN/ml), with no discernable differences between genotypes. In addition, viral RNA (10e2-10e3 CN/ml) was recovered from the forebrain and/or hindbrain at 3 DPC and 6 DPC. Pathological evaluation demonstrated mild, multifocal, necrotizing rhinitis involving the respiratory and olfactory sensory epithelium and moderate to severe, necrotizing, bronchointerstitial pneumonia at 3 DPC with persistent and progressive pneumonia at 6 DPC. IHC for Viral antigen was detected most prominently in the respiratory epithelium at 3 DPC. This work describes the development and characterization of a preclinical COVID-19 animal model using hACE2 knock-in rats. This rat model is highly susceptible to SARS-CoV-2 infection and exhibits similar pathology as COVID-19 in humans, and may be relevant for COVID-19 countermeasure development.

Core Laboratories of the Chemical Biology of Infectious Disease COBRE

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The University of Kansas Infectious Disease Assay development core (IDAD) is a state-of-the-art facility dedicated to enabling researchers in identifying and evaluating compounds that modulate various classes of infectious disease targets. The core provides assay development and high throughput screening optimizations using diverse biochemical and cellular formats. KU-IDAD laboratory is innovative and flexible in providing superior service to the drug/probe discovery research community, including assay development using all available platform technologies, screening, compound profiling and data mining. IDAD lab also provides training and consultation services, helps in generating pilot data and support documents for grant submissions. The optimized primary assays are validated against a collection of 40,000 compounds and hits from screens are prioritized based on secondary selectivity and cytotoxicity assays.

The IDAD laboratory is supported by 1P20GM113117 (PI: Scott Hefty).

The University of Kansas Computational Chemical Biology Core (CCB) provides the computational resources and expertise to enhance the productivity of researchers studying infectious diseases. The CCB is able to provide or assist with virtual screening, protein-small molecule docking, binding site prediction, protein modeling and design, prediction of protein stability changes upon mutation, fragment based probe design, as well as preparation of presentation graphics. The core utilizes the KU Community Cluster at the Advanced Computing Facility for its high-performance computing needs. The KU Community Cluster offers 458 compute nodes with a total of 8,568 compute cores, including 17 nodes that offer GPU-accelerated computing. The CCB specializes in initial hit identification of non-traditional drug targets such as protein-protein or protein-RNA interfaces by offering high-throughput virtual screening via pocket optimization with exemplar screening at protein-protein interfaces and hotspot pharmacophore mimicry of protein-RNA interactions.

The CCB Core is funded by the NIH NIGMS grant 1P20GM113117 (PI: Scott Hefty).

Synthetic Chemical Biology Core (SCB), a part of the Center for Chemical Biology of Infectious Diseases (CBID) and Center for Molecular Analysis of Disease Pathways (CMADP), strives to provide comprehensive synthetic chemistry capabilities to investigators under one roof.

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GPID Abstract

Title

Media Matters: *Agrobacterium tumefaciens* Requires a Global Stress Response for Growth in Complex Media

Authors

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Abstract

Many bacteria are routinely grown in undefined, complex culture media to favor rapid growth. While this is a well-established laboratory practice, it does not promote balanced growth and may even be considered a stressful environment for many microbes. Here, we find that growth on Lysogeny Broth (LB) or 2x Yeast Extract-Tryptone (2YT) activates a global stress in the bacterial plant pathogen *Agrobacterium tumefaciens*. In the Alphaproteobacteria, the ChvG and ChvI (ChvG-I) two component system is well conserved in and its activation is protective against several environmental stressors. Within the Rhizobiales, an order that contains several pathogens and symbionts, activation of ChvG-I promotes host invasion in response to the acidic environment. Curiously, ChvG-I is also essential to grow on low-carbohydrate, peptide-rich complex media. Here, we report that loss of the response regulator ChvI results in an inability to grow on complex media agar plates. Furthermore, Δ*chvI* mutants grown first in the defined glucose-based medium ATGN display limited growth when transitioned to LB liquid and, to a lesser extent, in 2YT liquid. Instead, Δ*chvI* mutants form a robust biofilm to survive. These observations indicate that growth in rich media represents a stressful environment potentially due to pH changes, oxidative stress, osmotic pressure, membrane permeability, or the accumulation of toxic metabolites. To better understand the advantage conferred by the ChvG-I system during growth on complex media, we used adaptive laboratory evolution to generate a Δ*chvI* suppressor strain that restores the growth phenotype in 2YT media without robust biofilm formation. This result demonstrates that while ChvG-I is essential for growth in complex rich media, adaptive mechanisms and genetic mutations can compensate for the loss of TCS function. Future investigations will focus both on identifying the stress that is detected during growth on complex media and clarifying the specific defects of the Δ*chvI* mutant that makes it susceptible to complex media. Overall, these studies will reveal why growth on complex media activates the ChvG-I global stress response in *A. tumefaciens* and may provide insights which can be extended to other microbes.

Mechanism of Quorum Sensing Regulation of Antibiotic Resistance in the Bacterium *Chromobacterium subtsugae*

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In many Proteobacteria, LuxI-R-type quorum-sensing (QS) systems regulate genes in a cell density-dependent manner. *Chromobacterium subtsugae* is a nonpathogenic soil bacterium related to the pathogen *Chromobacterium violaceum*. In *C. substugae*, quorum sensing is carried out by the LuxI-family signal synthase CviI, which synthesizes hexanoyl-homoserine lactone (C6-HSL), and the LuxR-family signal receptor CviR, which regulates genes in response to C6-HSL by binding to a *lux* box in the promoter of target genes. Recently, we showed the CdeAB-OprM efflux system contributes to antibiotic resistance in *C. subtsugae* and that this system is controlled by the CviI-R QS system. We also showed that the *cdeAB-oprM* genes are repressed by an adjacently encoded TetR-family transcriptional regulator, CdeR. Here, we sought to delineate the mechanism of CviR activation and CdeR repression of the *cdeAB-oprM* genes. We use heterologous *Escherichia coli* to demonstrate that the activation of the cdeA promoter requires CviR, C6-HSL and a *lux*-box-like sequence in the *cdeA* promoter. We also demonstrate that purified CviR can bind to the *cdeA* promoter, but not the *lux*-box-mutated promoter, and cause a mobility shift *in vitro.* We added CdeR to our recombinant *E. coli* system and show that CdeR can repress the activation of *cdeA* by CviR. We identify a conserved TetR-family binding site sequence in the *cdeA* promoter about 92 base pairs upstream of the *lux*-box-like sequence that is required for CdeR repression of the *cdeA* promoter and show that mutating this site abolishes repression by CdeR in our heterologous *E. coli* system. Together, our results support the idea that CviR and CdeR both regulate the *cdeA* promoter by binding different sites in the promoter. These results will ultimately have implications for understanding how antibiotic resistance is regulated by QS and how LuxR-family proteins interact with other regulators to control gene expression.

Linkage of Outer Membrane Proteins to the Bacterial Cell Wall Confers Protection Against Osmotic Stress in *Agrobacterium tumefaciens*

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Many proteobacteria use outer membrane proteins (OMPs) known as lipoproteins to link the outer membrane (OM) to the peptidoglycan (PG). In contrast, the bacterial plant pathogen *Agrobacterium tumefaciens* has highly abundant β-barrel proteins in the OM. Since β-barrel proteins are linked to the peptidoglycan by LD-transpeptidase (LDT) enzymes, we hypothesize that β-barrel proteins may be important for membrane integrity and survival during osmotic stress. Based on phylogenetic and structural predictions, we hypothesize that two LDTs, Atu_3332 and Atu_3631, function to crosslink the β-barrel protein AopB to the cell wall. In addition, since AopB is suspected to be essential under stress conditions, we predict that deletion of *aopB* will cause outer membrane defects and increased susceptibility to stressors. We have constructed a Δ*aopB* strain and are exploring whether the absence of this β-barrel protein or decreased linking of AopB to PG by LDTs impairs survival during various stress conditions including heat and EDTA. These studies will allow us to identify the LDTs responsible for the crosslinking of AopB and further characterize the role of AopB in cell envelope integrity. Preliminary data for Δ3332, Δ3631, Δ3332Δ3631, and Δ*aopB* indicate a higher sensitivity to both EDTA and higher temperatures when compared to WT cells. We are currently expanding our study to include functional characterization of mutants lacking other β-barrels proteins. Overall, we expect our research to provide a greater understanding of non-canonical OMP-PG linkages in bacteria.

HSV-1 ICP0 Dimer Domain Adopts a Novel β-barrel Fold

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Infected cell protein 0 (ICP0) is an immediate-early regulatory protein of herpes simplex virus 1 (HSV-1) that possesses E3 ubiquitin ligase activity. ICP0 transactivates viral genes, in part, through its C-terminal dimer domain (residues 555-767). Deletions in this dimer domain result in reduced viral gene expression, lytic infection, and reactivation from latency. Since ICP0's dimer domain is associated with its transactivation activity and efficient HSV-1 replication, we wanted to determine the structure of this specific domain. ICP0 was purified from bacteria and analyzed by X-ray crystallography to solve its structure. Each subunit or monomer in the ICP0 dimer is composed of nine β-sheets and two α-helices. Interestingly, 2 adjacent β-sheets from one monomer "reach" into adjacent subunit during dimer formation, generating 2 β-barrel-like motifs. Additionally, from the crystallographic analyses, a tetramer structure is formed from 2 β-sheets of each dimer, creating a "stacking" of the β-barrels. Structural protein database searches indicate the fold/structure adopted by the ICP0 dimer is novel, and its dimer is held together by an extensive network of hydrogen bonds. Computational analyses reveal that ICP0 can either form a dimer or bind to SUMO1 via its C-terminal SUMO-interacting motifs but not both. Understanding the structure of the dimer domain will provide insights into the activities of ICP0.

Genetic Adaptation to Tobramycin Alters the Antibiotic Susceptibility of Quorum Sensing (LasR)-Null Mutants in *Pseudomonas aeruginosa*

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The LasR-I quorum-sensing system contributes to *Pseudomonas aeruginosa* virulence and has been shown to increase resistance to the antibiotic tobramycin. Paradoxically, *lasR*-null mutations are commonly isolated from chronic infections of patients treated over sustained periods with tobramycin, suggesting there may be some mechanism allowing the *lasR* mutants to persist under antibiotic selection. We explore the hypothesis that adaptive mutations conferring tobramycin resistance change the role of *lasR* in tobramycin resistance. We identified that a single-nucleotide mutation in the translation elongation factor gene *fusA1* G61A (FusA1^{A21T}) confers a specific advantage to *lasR* mutants under tobramycin selection, a phenomenon known as sign epistasis. Under tobramycin selection a *lasR*-null mutation decreases fitness in the ancestral PA14 parent strain, but this same mutation increases fitness in a *fusA1* G61A mutant background. This mutation resides in a motif of *fusA1* known as the Walker-A P-loop, which binds phosphoryl groups and is essential for the GTPase function of FusA1. Analysis of alternate mutations spanning *fusA1* highlights the importance of this specific motif in the altered fitness of the *lasR* mutant. In infection isolates, *fusA1* mutations are common and known to increase tobramycin resistance through activation of an aminoglycoside-specific efflux pump called the MexXY pump. We demonstrate that in *fusA1* G61A mutants, *lasR*-dependent changes in antibiotic resistance were dependent on MexXY and the *mexXY* regulator, ArmZ. Overall, these results highlight the importance of adaptation on the evolutionary trajectory of quorum sensing and may explain how quorum sensing-null mutants persist in antibiotic-treated patients.

Determining the Role of Innate Lymphoid Cells in Brucellosis

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Brucellosis is a globally significant zoonotic disease in which humans can develop mild flu-like symptoms as well as more severe complications including arthritis and neurobrucellosis. Innate lymphoid cells (ILCs) are a group of tissue-resident innate immune cells that include the cytotoxic natural killer cells (NK cells) and helper-like ILCs (ILC1, 2, and 3). The purpose of our study was to uncover the role that ILCs play in the pathogenesis of brucellosis. Mice either possessing or lacking ILCs were inoculated with *Brucella* and following euthanasia, bacterial burdens and histology of infected organs were examined. When compared to mice with ILCs, mice lacking all ILCs exhibited significantly higher bacterial burdens in the joint, brain, blood, and spleen, and worsened swelling in the tarsus. Mice lacking ILCs also showed a significantly higher likelihood of developing neurologic signs. When investigating individual helper ILC groups, we found that NK cells, and to a lesser extent ILC1 cells, did contribute to the control of infection, but ILC subsets appear to play synergistic or compensatory roles in controlling bacterial colonization. Our findings indicate that ILCs play an important role in preventing focal complications throughout *Brucella* infection.

Differential Effects of Unique Mutations in Mouse Hepatitis Virus Suggest Multiple Roles for CoV Macrodomain

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All coronaviruses (CoVs) contain a macrodomain, also termed Mac1, in nonstructural protein 3 (nsp3) that binds and hydrolyzes mono-ADP-ribose (MAR) covalently attached to proteins. Despite several reports demonstrating that Mac1 is a prominent virulence factor, there is still a limited understanding of its cellular roles during infection. Currently, most information regarding the role of CoV Mac1 during infection is based on a single alanine mutation of a highly conserved asparagine residue, which is known to largely eliminate Mac1 ADP-ribosylhydrolase activity. To determine if additional Mac1 activities contribute to CoV replication, we compared the replication of murine hepatitis virus (MHV) Mac1 mutant D1329A to the previously mentioned asparagine mutant N1347A. Both mutations resulted in progeny virus that replicated poorly in bone marrow-derived macrophages (BMDMs), were inhibited by PARP enzymes, and were highly attenuated in vivo. However, D1329A was significantly more attenuated than N1347A in all cell lines tested but retained an ability to block IFN-β transcript accumulation compared to N1347A, indicating that these mutations have different effects on Mac1 functions. Combining these two mutations resulted in a virus that was unrecoverable, suggesting that the combined activities of Mac1 are essential for MHV replication. We conclude that Mac1 has multiple functions that promote the replication of MHV, and that these results provide further evidence that Mac1 is a prominent target for anti-CoV therapeutics.

Identification of a Coronavirus Macrodomain Inhibitor That Impairs Virus Replication in Cell Culture

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Coronaviruses (CoVs) are well-known to emerge from zoonotic sources and cause severe human and veterinary diseases, including recent outbreaks of MERS-CoV and SARS-CoV-2. All Coronaviruses (CoVs) encode for macrodomain protein (termed Mac1) that binds to and removes ADP-ribose from protein. Mac1 is essential for pathogenesis, indicating that it is a potential target for anti-viral therapeutics. Murine-Hepatitis virus (MHV), a model CoV that can be easily studied at BSL-2 levels, is ideal for testing Mac1 inhibitors for their ability to target virus replication because Mac1 is essential for replication of this virus. However, we lacked high-throughput methods to identify inhibitors of MHV infection. To create a more efficient drug screening system for MHV, we created a luciferase-expressing virus, which can rapidly measure virus replication. Previous screens identified a novel compound, MCD-628, that inhibited Mac1 activity *in vitro* at low micromolar levels. Interestingly, MCD-628 had no impact on virus replication, and we hypothesize this was due to an acidic side chain that could prevent entry into the cell. We then tested a compound with an ester at that site, termed MCD-617, and then found that this compound impaired MHV replication by ~ 2-logs without significant cytotoxicity at the concentrations tested. To validate that MCD-617 specifically targets Mac1, we are passaging virus in the presence of MCD-617 to identify drug-resistant mutants. Deep sequencing will be performed on drug-resistant mutants to identify any Mac1 or off-target mutations and explore potential mechanistic interactions.

GPID 2022 Abstract

Host Metabolic Variations in Different Phases of *Brucella melitensis* Infection

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Brucella melitensis is characterized as a select agent of a substantial threat to human and animal health. *Brucella* can invade almost every organ or system in the host, indicating adaptability for diverse metabolic conditions to replicate. Here, murine spleens, livers, and female reproductive tracts were analyzed by GCMS/MS to determine how tissue-specific metabolic changes affect the pathogenesis of brucellosis. In addition, blood and serum were analyzed by LCMS/MS to potentially identify biomarkers of infection. Tissues were harvested at multiple timepoints accordingly to disease development in mice; with 7 days post-infection (dpi) representing the peak of infection, 14 dpi as the peak of inflammation, and 28 dpi as a starting point of chronic infection. The most remarkable changes were observed at 14 dpi when compared to uninfected tissues; 42 out of 329 metabolites in the reproductive tracts were significantly altered by *Brucella* infection, while in the spleens and livers, 68/205, and 139/330 metabolites were significantly changed, respectively. Several identified metabolites were linked to the TCA cycle, GABA shunt, and Arginosuccinate shunt. Additionally, analysis of blood and serum revealed more than 90 significantly altered compounds, some of which may have the potential to distinguish between acute and chronic infection. Overall, multiple metabolic pathways were shown to be altered in tissues during experimental brucellosis, which brings insight into how *Brucella* manipulates and/or takes advantage of changes in host metabolism for replication and maintenance. Furthermore, metabolic profiling of blood or serum has the potential to discriminate between infected and uninfected animals.

Keywords: Brucella, metabolism, immunopathogenesis,

Adaptation of seasonal Influenza A(H3N2) virus to a mice model

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Each year, 290,000 to 650,000 estimated deaths are caused by influenza worldwide. In the United States alone, the estimated average annual economic burden of seasonal influenza to the healthcare system and society is $11.2 billion. Being complementary to other animal models, the mouse model has been an economic and effective research and preclinical tool widely used for developing vaccine and pharmaceutical interventions for influenza prevention and control. However, like many other subtypes of influenza A viruses, seasonal influenza A (H3N2) viruses do not naturally infect mice, and thus, development of mice-adapted H3N2 strains, particularly for contemporary viruses, is necessary. In this study, a mice-adapted strain of A/Switzerland/9715293/2013(H3N2) (SWZ/13) was developed through a combination of serial passages in DBA/1J and BALB/c mice. Viral titration showed that one mice-adapted strain (SWZ/13-miceP) purified from plaque assays had titers of $6.32x10^4$ and $5.02x10^2$ LD50/mL in DBA/1J and BALB/c mice, respectively. Next generation sequencing analyses revealed the evolutionary pathways for the SWZ/13 virus during mice adpation. Although amino acid substitutions in the hemagluttinin (HA), neuraminidase (NA), and/or ribonucleoprotein complex were associated with increases in viral replication and pathogenesis during mice adaptation, those substitutions in HA predominantly enhanced viral pathogenesis in the lethal mice adapted strain SWZ/13-miceP. Of interest, parallel passaging of A/Hong Kong/4801/2014(H3N2) viruses failed to obtain any mutant with replication ability in mice. In summary, this study reports a unique set of mutations across HA, NA, and RNA polymerases that facilitated mice adaptation for a seasonal A(H3N2) virus, and our results clearly showed that the ability of mice adaptation varied among seasonal A(H3N2) virus.

Influenza A viruses showed distinct replication ability in the CMP-sialic acid transporter deficient human A549 cells

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The sialic acids (Sias), particularly α2,3-linked (Sia2,3Gal) and α2,6-linked Sias (Sia2,6Gal) are known as the primary receptors of influenza A viruses (IAVs). An effective recognition between the viral surface glycoprotein hemagglutinin (HA) and the sialylated cellular receptor is required to initiate viral infection. However, recent studies suggested that non-sialylated glycans can bind to IAVs but their roles in viral infection are not fully understood. Additional studies suggested that virus receptor-binding specificities may not be static and evolve rapidly, even affecting antigenic properties. In humans, the SLC35A1 gene codifies for the transporter protein, responsible for transferring CMP-sialic acid to the medial- and trans-Golgi apparatus, and therefore plays an important role in Sia2,3Gal and Sia2,6Gal expression on the cell surface. In this study, using the human lung epithelial cell line A549 as the template, a knockout (KO) mutant ΔSLC35A1, was generated using the CRISPR/Cas9 gene-editing tool. The elimination of CMP-sialic acid transporter expression observed by Western Blot resulted in a reduction of both Sia2,3Gal and Sia2,6Gal on the cells surface, which were shown by lectin binding analyses. Mass spectrophotometry was performed to describe the N-glycans that covered ΔSLC35A1 and the A549 wild type cells, showing a lacking Sias profile on the surface of the ΔSLC35A1 cells compared to the A549 wild type cells. Growth kinetic analyses for human and avian IAVs showed a significant reduction in the level of virus replication, compared to those in A549 wild type cells. Of interest, not all viruses tested were affected in their replication in ΔSLC35A1 cells, and the variations were associated with whether the seed virus was prepared in eggs or cells. Future studies will be performed to determine the specific mutations in viruses and the alternative non-Sia receptors responsible for the replication in ΔSLC35A1 cells. In summary, this study showed that non-sialylated glycans may contribute to virus infection.

Increasing Antimicrobial Susceptibility of Multidrug-Resistant *Salmonella* With 1-(1-Naphthylmethyl)-Piperazine, an Efflux Pump Inhibitor

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Salmonella is a leading cause of human foodborne illness, and emerging drug resistance in non-typhoidal *Salmonella* is a prevalent issue in the United States. Each year, *Salmonella* infections occur in an estimated 1.35 million people and cause 420 deaths. Despite its societal impact, *Salmonella* is difficult to prevent due to its ability to commensally colonize in the intestine of food animals including cattle, poultry, and swine. While human medical treatment is uncommon for *Salmonella* infections, a large amount of time and money is expended in preventative measures in poultry and livestock. Recent human outbreaks have involved *Salmonella* isolates resistant to many antimicrobials (multidrug-resistant, MDR) including those that have been commonly used in animal production such as tetracycline. This is predictably due to over usage of these antibiotics. Tetracycline-specific resistance in *Salmonella* is primarily due to the efflux mechanism although ribosomal protection and enzymatic inactivation are available. Alternative treatments to make existing antimicrobials effective again are needed, and one possible solution is the usage of compounds that prevent efflux pump activity, or efflux pump inhibitors (EPIs). Efflux pumps are a common mechanism in Gram-negative bacteria to reduce antimicrobial sensitivity. In particular, pumps of the Resistance-Nodulation-Cell Division (RND) Superfamily are often responsible for bacterial resistance to a broad spectrum of antimicrobial compounds. Therefore, we evaluated 1-(1-Naphthylmethyl)-Piperazine (NMP) and phenylalanine-arginine β-naphthylamide (PaβN), two EPIs that target RND efflux pumps, with tetracycline. The goal of this study was to determine if a synergistic relationship between the antibiotic and the efflux pump inhibitor can reduce bacterial resistance. Each EPI reduced tetracycline resistance by up to 32-fold in several highly resistant strains. For example, in an isolate from the 2017-2019 *Salmonella* Infantis poultry outbreak, the tetracycline MIC was 64μg/mL, but in the presence of NMP, this MIC dropped to 4μg/mL which is below the CLSI breakpoint (≥16μg/mL) for tetracycline resistance in *Salmonella*. While PaβN was effective at lower concentrations than NMP, the higher cost outweighed this benefit. These findings suggest that EPIs may represent a possible strategy to reduce tetracycline resistance in MDR *Salmonella* isolates and potentially other tetracycline-resistant bacteria.

Characterizing the Oral Mycobiome of Domestic Dogs

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Understanding the oral and gut mycobiome is a relatively new field of study in humans and animals. The goals of this area of research is to identify potential pathogens as well as commensal organisms present in the body in order to learn how they affect health and disease. The purpose of this study is to characterize the oral mycobiome of domestic dogs.

Over 253 buccal swabs were obtained from dogs at Kansas State University's veterinary center. Swabs were struck onto a chromogenic fungal growth media that distinguishes between fungal species based on colony color and morphology. After isolating and harvesting single colonies, DNA was extracted from each species. PCR was used to amplify a fungal-specific variable rDNA region of the genome, which was then sent for sequencing. Sequencing results were input into the NCBI BLAST database to identify each species present from the oral swabs. The protocol provides information not only on which species are present in individual dogs, but also provides an approximate frequency with which the different fungal species appear in the mouths of domestic dogs.

After the fungal isolates have been collected, cultured, and speciated, antifungal drug susceptibility testing is conducted on each isolate. These drug testing surveys will indicate the level of drug resistance to common antifungal drugs, such as fluconazole and ketoconazole, present in the oral mycobiome of domestic dogs.

Exploring the oral mycobiome of dogs as well as the corresponding drug susceptibility will allow veterinary and medical researchers to assess the appropriateness of antifungal use as it relates to drug resistance in animals and humans. These findings will offer pertinent information to the fields of mycology, veterinary medicine, and human medicine by helping improve our understanding of the microorganisms within our pets, as well as organisms that humans are commonly exposed to through our canine companions.

Arabinose-Inducible MtParE1 Toxin Expression Drives Changes in Arabinose Metabolism

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The ParDE toxin-antitoxin (TA) system is encoded by a small bicistronic genetic element found in the genomes of many prokaryotes, such as *Pseudomonas aeruginosa* and *Mycobacteria tuberculosis* (Mt). In this type-II TA family, ParE toxin and ParD antitoxin are both proteins, and the toxin inhibits DNA gyrase when not complexed via protein-protein interactions with the cognate antitoxin. As gyrase is inhibited, the resulting accumulation of dsDNA breaks can lead to the death of the bacterial cells, or potentially be repaired by DNA repair pathways to allow the resumption of growth of bacterial cells.

Our lab previously has performed viability assays to determine *E. coli* MG1655 cell survival as a function of arabinose induction level of MtParE1 toxin. Surprisingly, we found a fraction of cells survived MtParE1 toxin overexpression, and further, were resistant to secondary induction. The survival despite MtParE1 induction persisted even after re-transformation with the wild type plasmid, ruling out possible mutation of the original plasmid. Additionally, a construct encoding the fluorescent protein mCherry under control of an arabinose-inducible promoter was not expressed in these phenotypically resistant cells even at the highest induction concentration. Therefore, we hypothesized that arabinose uptake is impaired in response to overexpressing the highly potent MtParE1 toxin. On-going experiments will constitutively express the arabinose transporter AraE to see if this complements the observed phenotypic resistance to MtParE1 induced expression. Based on these studies, it seems likely that bacterial cells will take the "path of least resistance" to escape the potent toxicity of TA system proteins.

Molecular Mechanism of N155H/E92Q Resistance to HIV-1 Integrase Inhibitors

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Abstract

HIV-1 integrase inhibitors (INIs) are currently recommended in first-line AIDS therapy. However, as with other antivirals, resistance mutations can emerge among patients receiving INIs. The dual mutations N155H/E92Q confer resistance to all approved INIs. The molecular mechanism of the N155H/E92Q dual mutation resistance remains unknown, partly due to the unavailability of a high-resolution crystal structure of HIV-1 IN in complex with DNA and INSTIs. Here, using computational tools including hybrid quantum mechanics/molecular mechanics (QM/MM), molecular dynamics (MD) simulations, *in vitro* 3'-end processing and strand-transfer assays, we show that N155H/E92Q mutations impart a long-range impact on the INSTIs' binding through water molecules that bind the divalent cations at the active sites. The biochemical data show that N155H/E92Q mutations affect DNA binding affinity ($K_{d.DNA}$) of HIV-1 IN (~4-fold) and reduce the 3'-end processing and strand-transfer activity (~4-fold). Collectively, our results suggest that resistance to INSTIs by the N155H/E92Q mutation is likely due to the disruption of the coordination of water molecules near the active site, their electronic properties, and reduced inhibitor binding due to the reduced DNA binding.

[¶]Equal contribution

Phosphate and Calcium Regulated Protein, PcrP (PA2803) Contributes to Polymyxin B Resistance in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a ubiquitous opportunistic human pathogen and the leading cause of morbidity and mortality in immuno-compromised patients such as patients suffering from cystic fibrosis (CF). It has rapidly become one of the six top pathogens causing deaths due to their multi-drug resistance, exhibiting resistance to nearly all antibiotics, including the last resort drug polymyxin-B (PMB). PMB resistance in *P. aeruginosa* is known to be multifactorial, however the molecular pathways responsible for high-level resistance commonly observed in clinical settings are not well understood. Changes in levels of divalent cations such as calcium (Ca^{2+}) have been shown to impact the bacterial susceptibility to several antimicrobials during infections. Here, we show that elevated Ca^{2+} levels commonly detected in airways of CF patients, enhance PMB resistance in *P. aeruginosa* through novel Ca^{2+}-dependent mechanisms. By using random chemical mutagenesis, we identified three genes contributing to Ca^{2+}-induced PMB resistance, including *PA2803* that encodes putative phosphonatase. The transcription of this gene is regulated by both Ca^{2+} and PhoB-mediated phosphate starvation. Therefore, it was designated as PcrP, phosphate and calcium regulated protein. During phosphate starvation, PcrP provides Ca^{2+}-dependent growth advantage to *P. aeruginosa*. We have also observed that this protein undergoes phosphorylation and exists as a dimer in native conditions. The recombinantly-expressed and purified PcrP showed no catalytic activity as phosphonatase, congruent with our sequence-based predictions. To explore possible protein binding function of PcrP, we carried out pull-down assays and identified several putative binding partners including PqsB and Acp3. These interactions are currently being validated using a bacterial two-hybrid system. Further, we showed that elevated Ca^{2+} causes several Ca^{2+}-dependent changes in *P. aeruginosa* membrane properties, such as increased permeability of the outer and inner membranes and alterations in the LPS, proteomic and lipidomic profiles. We hypothesize that these membrane changes may also contribute to the Ca^{2+}-induced PMB resistance independent of PcrP. Current work aims to understand the mechanistic role of PcrP in *P. aeruginosa* PMB resistance.

Detection by Death: a novel way to detect living microorganisms

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In many real-world situations, one seeks to detect viable (live) microorganisms present in samples where it dead microorganisms are known/expected to be also present. An example of the same is the need to detect viable cells of *Mycobacterium tuberculosis* (the organism that causes Tuberculosis (TB)) in the sputum of patients suspected of having an active infection because dormant M. tuberculosis cells may be present in cases of "latent" TB, or previous treatment may have left behind some dead cells of M. tuberculosis.

Because DNA based methods like PCR and antibody based approaches like ELISA would give false positives, automated culture based systems like the Mycobacteria Growth Indicator Tube (MGIT) from BD, and Trek-ESP from Thermo-Scientific currently serve as the work-horses of the microbiology laboratory for these applications.

In trying to detect viable microorganisms, these automated culture-based systems (MGIT, etc.) ask "do they metabolize and/or proliferate? ", and try to detect signatures of bacterial metabolism/ growth (changes in pH, solution-conductivity, O_2/CO_2 levels, etc.). They are thus limited by the metabolism rate and doubling time of the microorganisms present. For *M. tuberculosis* that has a very low metabolic rate and a doubling time of ~24 hours, these system can take weeks to detect. (Cultures are typically deemed negative after 6 weeks of incubation)

In contrast, we ask, "can they be killed"? Since only living entities can be killed, an answer in the affirmative should confirm the presence of live entities (microorganisms). Doing so makes our time-to-detection (TTD) dependent not on the metabolic-rate of the microorganisms, but on how fast they are killed. Since one can kill microorganisms (even very slow growing ones like M. tuberculosis) in hours, this makes our TTDs dramatically shorter (hours instead of weeks).

Competitive Impact of Hydrogen Cyanide Produced via Acyl-homoserine Lactone-dependent Eavesdropping by *Chromobacterium subtsugae*

Important words: hydrogen cyanide, eavesdropping, quorum sensing

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Many soil saprophytic bacteria use LuxR-I-type acyl-homoserine lactone (AHL) quorum-sensing systems to activate production of antimicrobials that can be utilized to compete with other bacteria in polymicrobial communities. LuxR signal receptors specifically interact with cognate AHLs produced through LuxI to cause changes in gene expression. Some LuxR-type AHL receptors have relaxed specificity and are responsive to non-cognate AHLs. The soil saprophyte *Chromobacterium subtsugae* has a single AHL circuit, CviI-R, which produces and responds to *N*-hexanoyl-HSL (C6-HSL). The AHL receptor CviR can also respond to a variety of AHLs in addition to C6-HSL. In prior studies, we showed that CviR allows *C. subtsugae* to compete with another saprophyte, *Burkholderia thailandenis,* by responding to *B. thailandensis* AHLs by eavesdropping. In this study, we use RNAseq to show that eavesdropping activation of CviR induces dozens of genes, including genes coding for production of hydrogen cyanide (HCN). We show that *C. subtsugae* produces a maximal concentration of ~3 μM cyanide (CN^-), which is dependent on both the cyanide biosynthetic genes and the quorum-sensing receptor CviR. We also demonstrate that this concentration is more than sufficient to kill *B. thailandensis.* We use genetic mutants to demonstrate that hydrogen cyanide is needed for *C. subtsugae* to compete with *B. thailandensis* in response to *B. thailandensis* AHLs in laboratory cocultures. Our results demonstrate that *C. subtsugae* uses hydrogen cyanide for interspecies competition in response to non-native AHLs via eavesdropping and establish a laboratory coculture system that can be used for future studies on the role of eavesdropping in interspecies competition.

Title: Evolution and Transmission of *SARS-CoV-2* in Rural Missouri

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Abstract
Background
SARS-CoV-2 has been rapidly evolving with over 2,100 identified lineages. While variants are often first detected in urban centers, the origins of these variants and their patterns of spread remain unclear. Further, little is known about how the virus emerges and spreads among rural communities. During late 2020, rural areas experienced higher incidences of COVID-19 disease and mortality compared to urban regions, suggesting an increased pandemic burden in rural populations. Despite this, disease mitigation efforts are largely informed by urban-centric data. Here, we study the urban-rural evolution and transmission patterns of SARS-CoV-2 variants in Southwest Missouri.
Methods
COVID-19-positive nasopharyngeal swab samples were collected from CoxHealth, a large medical center in Southwest Missouri. To understand the early evolution and transmission dynamics of SARS-CoV-2 in a vaccine-naïve population, our study period was set to July through December 2020 to exclude biases from the limited testing during the early pandemic, the variations of vaccine distribution and intake, and the sweeping predominance of the variants of concern. We analyzed 1,161 geocoded SARS-CoV-2-positive samples, 980 of which contained sufficient viral load for genomic sequencing (435 rural, 545 urban). Evolutionary dynamics and transmission patterns were studied using Bayesian inference phylogenetic and phylogeographic analyses.
Results
Our results revealed high genomic diversity within our study population, encompassing 51 recognized lineages. From the primary circulating lineages, five novel variants emerged in rural communities, and four emerged from urban communities. Within the eight predominant lineages containing at least 10 urban and 10 rural samples (B.1, B.1.1, B.1.1.337, B.1.2, B.1.234, B.1.240, B.1.311, and B.1.509), 30% (n=21) of statistically significant transmission events (Bayes factor ≥ 3, posterior probability ≥ 0.7) originated from rural areas.
Conclusion
In this study, we demonstrated that rural communities have become an important source of new SARS-CoV-2 variants and of transmission to both rural and urban areas. These results suggest that rural communities have a critical role in SARS-CoV-2 evolution and transmission. Genomic studies should strengthen sampling from rural communities, and granular geographic metadata should be integrated into the analyses to better our understanding of the evolution and disease burdens of SARS-CoV-2.

LDBF cross protect mice against different *Shigella* serotypes after early exposure to pathogen.

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Abstract

Shigellosis is a severe diarrheal disease caused by members of the genus *Shigella*, which results in 90 million cases annually around the world. The *Shigella* spp. type III secretion system (T3SS) is a specialized secretion system that is a primary virulence factor used by *Shigella* spp. to infect the colonic epithelia. The type three secretion apparatus (T3SA) proteins IpaB and IpaD, as well as the genetic fusion, DBF, have been demonstrated to protect mice from *Shigella* spp. infection in a lethal pulmonary model. In our previous study, we fused LTA1, the active moiety of lethal toxin from enterotoxigenic *E. coli* to DBF to produce a self-adjuvating vaccine candidate L-DBF, which cross-protected mice against four serotypes of *S. flexneri*, and *S. sonnei*. Here, we exposed mice with one or two sublethal doses of *S. flexneri* 2a to identify whether the immune response induced by L-DBF in host would be affected by early infection. We demonstrate that pre-infection with two sublethal doses of *S. flexneri* 2a did not elicit cross-protection against *S. sonnei*, while vaccination with L-DBF did. Our results indicate that L-DBF is a feasible vaccine candidate that offered cross-protection against *Shigella*'s different serotypes in a pathogen exposure condition.

Evaluation of the Susceptibility of Human ACE-2 Knock-in Rats to SARS-CoV-2

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Distinguishing features of coronavirus disease 2019 (COVID-19) in humans that present in animal models facilitates investigation into SARS-CoV-2 pathogenesis and therapeutic discovery. In this study, a codon-optimized human ACE2 cDNA was integrated into the first coding exon of the rat *Ace2* gene locus using a CRISPR-based strategy. Rats expressing human angiotensin-converting enzyme 2 (hACE2) were evaluated as a preclinical model for SARS-CoV-2. The susceptibility of hemizygous and homozygous knock-in rats to the ancestral Wuhan-like SARS-CoV-2 USA-WA1/2020 strain was evaluated for 14 days following intranasal infection, with *post mortem* evaluations performed at 3, 6, and 14 days post challenge (DPC). Both hemizygous and homozygous knock-in rats were permissive to SARS-CoV-2 infection and demonstrated moderate weight loss by 3 DPC. Viral RNA was detected by RT-qPCR in both, nasal washes and oral swabs at high levels (>10e9 copy number [CN] viral RNA/ml), and in lesser amounts (~10e5 CN viral RNA/ml) in rectal swabs. There was no significant difference in the amounts or kinetics of viral RNA shedding between the two rat genotypes. The rats shed viral RNA from the upper respiratory tract for less than 10 days (tested positive up to 7 DPC), while rectal swabs remained positive until 5 DPC. Fresh tissues (lung, forebrain, and hindbrain) collected at necropsy harbored high levels of viral RNA in the lung at 3 DPC (>10e10 CN/ml), with slightly lesser amounts detected at 6 DPC (>10e9 CN/ml), and significantly less RNA detected at 14 DPC (10e4 CN/ml), with no discernable differences between genotypes. In addition, viral RNA (10e2-10e3 CN/ml) was recovered from the forebrain and/or hindbrain at 3 DPC and 6 DPC. Pathological evaluation demonstrated mild, multifocal, necrotizing rhinitis involving the respiratory and olfactory sensory epithelium and moderate to severe, necrotizing, bronchointerstitial pneumonia at 3 DPC with persistent and progressive pneumonia at 6 DPC. IHC for Viral antigen was detected most prominently in the respiratory epithelium at 3 DPC. This work describes the development and characterization of a preclinical COVID-19 animal model using hACE2 knock-in rats. This rat model is highly susceptible to SARS-CoV-2 infection and exhibits similar pathology as COVID-19 in humans, and may be relevant for COVID-19 countermeasure development.

Characterization of *V. cholerae* ParE toxin proteins from ParDE TA systems

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DNA gyrase functions in regulating DNA topology by temporarily rendering double-strand breaks when cells undergo DNA replication or transcription. Therefore, it is essential and exclusive to bacterial cells. Compounds capable of stabilizing gyrase-mediated double-strand breaks are valuable antibacterial therapeutics but these also impart higher rates of mutations and thus development of resistance. Therefore, developing new forms of gyrase inhibitors may yield additional antibacterial strategies.

In bacterial TA systems, the ParE family of toxins have been confirmed to poison DNA gyrase. Importantly, the inhibition is different to known gyrase inhibitors, including the well-characterized ccdB toxin and quinolone type antibiotics; however, the molecular mechanism of the inhibition remains elusive. Therefore, the overall goal of this study is to deduce the inhibition mechanism of the two ParE toxins from *Vibrio cholerae* as these have been previously characterized as potently toxic. Since inhibition of gyrase triggers SOS response, which potentially increases mutation rate, the bacterial cells might gain antimicrobial resistance through ParE inhibition. Our previous studies have confirmed a dose-dependent bactericidal activity to the host *V. cholerae* cells and determined the dosage for investigating the possibility of ParE-induced mutagenesis. Our result confirms that induction of *Vibrio cholerae* ParE1 and ParE2 significantly increase the mutation rates as compared to the wild type cell. Despite the higher frequency of mutations, the antibiotic susceptibility to clinically useful antibiotics is only modestly impacted as inducing the expression of ParE1 and ParE2 toxins. On-going work is to purify the recombinant ParE toxin proteins for structural studies. The outcome of these studies is expected to reveal a new modality for the inhibition of DNA gyrase, thus contributing to the development of new antibacterial strategies.

Nucleocapsid substitutions R203K/G204R of SARS-CoV-2 elevate innate immune responses and increase viral pathogenicity

Running title: Nucleocapsid substitutions enhanced pathogenicity of SARS-CoV-2

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Abstract

The paired substitutions R203K/G204R on the nucleocapsid protein (NP:R203K/G204R) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) comprises ~48% of viruses being sequenced. The substitutions were minimal in the Delta variants and then became predominant in the Omicron variants. Here two human isolates with identical proteomes except NP:R203K/G204R, were used to determine the clinical impact of NP:R203K/G204R. Virus and genome replication analyses demonstrated that NP:R203K/G204R did not increase viral replication efficiency but did enhance genomic/subgenomic RNA productivity, leading to elevated cytokine/chemokine responses, which in turn antagonized virus growth in human lung Calu-3 epithelial cells but not in type-I-interferon deficient Vero E6 cells. In a hamster model, NP:R203K/G204R mutations mediated a higher body weight loss and enhanced pro-inflammatory cytokine/chemokine expression in the respiratory tract. This study suggests that SARS-CoV-2 variants with NP:R203K/G204R can enhance host pro-inflammatory responses and consequently viral pathogenicity by manipulating viral RNA production instead of infectious viral load.

Isolation And Characterization Of Mammalian Orthoreovirus From Bats In The United States

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Abstract

Mammalian orthoreovirus (MRV) infects many mammalian species including humans, bats, and domestic animals. To determine the prevalence of MRV in bats in the United States, we screened more than 900 bats of different species collected during 2015 to 2019 by a real-time RT-PCR assay; 4.4% bats tested MRV-positive and 13 MRVs were isolated. Sequence and phylogenetic analysis revealed that these isolates belonged to four different strains/genotypes of viruses in serotypes 1 or 2, which contain genes similar to those of MRVs detected in humans, bats, bovine, and deer. Further characterization showed that these four MRV strains replicated efficiently on human, canine, monkey, and swine cell lines. The 40/Bat/USA/2018 strain belonging to the serotype 1 demonstrated the ability to infect and transmit in pigs without prior adaptation. Taken together, this is evidence for different genotypes and serotypes of MRVs circulating in U.S. bats, which can be a mixing vessel of MRVs that may spread to other species, including humans, resulting in cross-species infections.

Keywords: Mammalian orthoreovirus (MRV); bats; infection and replication, pathogenesis and transmissibility

Pathogenicity of a novel reassortant H1N1 virus from swine responsible for deaths in sows in the US

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An H1N1 A/swine/Kansas/17211/2017 (KS17) was isolated from a commercial sow farm in which 1000 sows showed clinical signs of respiratory disease and 5 sows died. The farm had previously tested negative for porcine reproductive and respiratory syndrome virus and mycoplasma. Sequence and phylogenic analysis revealed that the KS17 H1N1 virus was a reassortant virus containing the HA gene from the 2009 H1N1 pandemic (H1N1pdm09) virus and the remaining genes from North American triple reassortant swine influenza viruses. We investigated the pathogenicity and transmissibility of this virus in pigs and ferrets using the H1N1pdm09 A/California/04/2009 (CA09) virus as the control. The KS17 H1N1 virus was more pathogenic than the CA09 H1N1 virus in pigs in terms of macroscopic and microscopic lung lesions and virus nasal shedding, although there was no difference between the 2 viruses in virus titers present in the lungs of the principal infected and contact pigs. The KS17 H1N1 virus displayed similar viral replication kinetics and transmission capacity as the CA09 H1N1 virus in ferrets. Taken together, the reassortant KS17 H1N1 swine influenza virus is pathogenic and transmissible in both pigs and ferrets and may be a potential threat to public health if it spills over to humans.

Bat Influenza M2 Shows Similar Functions as Those of Classical Influenza A Viruses

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Abstract

Influenza A virus (IAV) could infect many kinds of animals, include human, and cause disease even death. According to the glycoprotein (haemagglutinin, HA and neuraminidase, NA) on the virus surface, IAVs are divided to multiple subtypes. In 2013, two bat influenza viruses were found, the HA and NA subtypes of IAV are expanded to 18 and 11, respectively. Novel bat influenza viruses show different features in contrast to classical influenza A viruses (IAVs). The M2 of IAVs functions as the ion channel that plays an important role in virus entry, viral assembly and release as well as is the antiviral target. To date, whether bat influenza M2 functions as the ion channel like classical IAV M2 remains unknown. Herein, we analyzed bat influenza virus sensitivity to antivirals targeting M2 ion channel and impact of critical amnio acids for ion channel on virus replication. Results show that antiviral drugs, targeting M2 ion channel, could inhibit bat influenza recombinant viruses and WT H18N11 bat influenza virus. And through mutate bat WT M2 to a non-functional M2, H17-M2/W41A and H17-M2/H37G W41A recombinant viruses were failed to be rescued; H17-M2/H37G, H18-M2/H37G, H18-M2/W41A, and H18-M2/H37G W41A recombinant viruses grow much slower than WT M2. The results indicate that the proton conductance channel function of bat influenza M2 is essential for viral replication and survival.

Key words: bat influenza M2, antiviral resistance, ion channel

Development and Characterization of Monoclonal Antibodies against SARS-CoV-2 Nucleocapsid Protein: Implication for Blocking ELISA Development

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The global pandemic of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), poses a significant threat to public health. Besides humans, SARS-CoV-2 can infect certain animal species, including wild/domestic cats and white-tailed deer. Highly sensitive and specific diagnostic reagents and assays are urgently needed for rapid detection and implementation in quarantine and the elimination of infected animals. In this study, we developed a panel of monoclonal antibodies (mAbs) against SARS-CoV-2 nucleocapsid (N) through hybridoma technology. The mAbs were initially screened by immunofluorescent assay using *in vitro* expression system, followed by further characterization by various assays. Testing live virus-infected cells reveals broad reactivity against different variants of SARS-CoV-2. Epitope mapping result demonstrates that this panel mAbs recognize specific B-cell epitopes spanning different regions of N protein. Cross-reactivities with other human and animal coronaviruses were also evaluated. To explore the potential use of mAbs for serological diagnostics in all animal species, a mAb-based bELISA was developed and optimized using a set of internal control serum standards. The assay demonstrates high repeatability as determined by the coefficient of variation to be 7.23%, 6.95%, and 5.15% for between-runs, within-run, and within-plate, respectively. Initial test validation on a set of known-status serum samples collected from a variety of (experimental) animals, including cat, ferret, mink, and deer revealed an optimal percentage of inhibition (PI) cut-off value of 17.6% with diagnostic sensitivity of 97.8% and diagnostic specificity of 98.9%. A time course study of experimentally infected cats showed that the bELISA was able to detect seroconversion as early as 7 days post-infection. Retrospective surveillance of US domestic cats revealed an overall 8.9% sera-prevalence, with the year of 2022 being the highest (13.8%) and 2020 the lowest (6%). In summary, the panel of mAbs generated in this study provides a valuable tool for SARS-CoV-2 diagnostics and research. The mAb-based bELISA has the potential to be used as a serological test in aid of COVID-19 surveillance in animals.

Structural Insights into the Type IV Secretion System of *Legionella pneumophila*

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Legionella pneumophila, the causative agent of Legionnaires' disease, is a gram-negative bacterium that utilizes a type IV secretion system (T4SS) for the translocation of ~300 effector proteins to subvert host cell processes during infection. Many pathogenic bacteria use T4SS to establish a pathogen-host interaction, transfer toxic effectors in a host cell, & exchange genetic material & antibiotic-resistance genes between bacteria. The system comprises ~ 30 protein components that are embedded in the membrane, and thus it has been very challenging to isolate & purify this multiprotein translocation complex. Recently a T4SS structure from R388 plasmid has been published, but it is significantly different from the T4SS complex of *Legionella pneumophila* in terms of protein components, size, substrates, etc. Furthermore, five novel T4SS proteins have recently been identified using single-particle cryo-EM, but the intact structure of the native T4SS complex and the mechanism by which it translocates the proteins across multiple membranes is yet to be discovered. Therefore, this research aims to elucidate the structure, dynamic changes & translocation mechanism of the T4SS complex by using a combination of structural biology, genetics, biochemical and biophysical approaches. To investigate the translocation machinery at a mechanistic level, we will isolate & purify the intact T4SS from the native membrane of *Legionella pneumophila* by using a construct with a multifunctional affinity tag that will enhance the stability, purity, and yield of the complex. The purified T4SS complex can then be used for structure determination via Cryo-EM and will set the stage for our future functional studies. This study will provide a model system for a T4SS virulence complex to get fundamental new insights into the mechanism of survival and replication of an intracellular pathogen inside a eukaryotic cell.

Durie, C. et al. (2020). Structural Study of the Legionella pneumophila Dot/Icm T4SS Using Cryoelectron Microscopy. *Microscopy and Microanalysis, 26*(S2), 1814-1815.