CodY upregulates SaeR/S two-component system activity by increasing branched-chain fatty acid synthesis

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Staphylococcus aureus is an opportunistic pathogen that colonizes up to 30% of the human population, and is known to cause skin and soft tissue infections, endocarditis, and bacteremia. The success of *S. aureus* as a pathogen is attributed to its repertoire of virulence factors that enhance host colonization and facilitate evasion from the host immune response. The production of these factors is controlled, in part, by a complex network of regulatory proteins such as CodY, which monitors nutrient availability. When *S. aureus* is deprived of certain amino acids, CodY becomes inactive and activates the SaeR/S Two-Component System (TCS). This in turn upregulates toxins and other virulence factors. Herein, we show that reducing CodY activity in *S. aureus* cells does not alter SaeR or SaeS expression levels. Rather, SaeS kinase activity is enhanced in CodY-deficient cell membranes. We discovered that, CodY- deficient cell membranes have increased levels of branched-chain fatty acids (BCFAs) compared to wild-type cell membranes. Blocking BCFA synthesis substantially reduced SaeR/S activity, which was complemented chemically and genetically. Single-gene knockouts and epistasis experiments revealed that CodY constrains Sae activity by repressing genes for de novo branched-chain aketo acid synthesis and by repressing genes that encode permeases for branched-chain amino acid import. The result is a two-pronged strategy to restrict flow of BCFAs to the phospholipid bilayer. These results reveal a novel method of post-transcriptional virulence regulation via BCFA synthesis, linking CodY activity to virulence regulation in *S. aureus*.

Development of Biomass Containment Devices (BCDs) to Study Bacterial Degradation of Complex Substrates

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Studying the microbial degradation of real-world insoluble substrates has several challenges that are currently hindering both fundamental and translational applications. For example, insoluble substrates are not compatible with spectrophotometry, and consequently rely on other methods that are costly, time intensive, and not amenable to high-throughput screening. Furthermore, attempts to solubilize an otherwise insoluble substrate are costly, require harmful chemicals, and often result in altered microbial development or metabolism. Alternative methods that use protein concentration or cell viability counts to measure bacteria growth using insoluble substrates are time and resource intensive. Therefore, we designed and fabricated customizable 3D printed biomass containment devices (BCDs) to facilitate rapid and reproducible growth measurements. BCDs are porous capsules which separate insoluble materials from microbial cells. We have also developed techniques that allow a researcher to capture very fine particulate material. Using BCDs, we are able to obtain physiological data differentiating between subtle growth phenotypes that would otherwise have been missed using current methods. BCDs can be used in high-throughput microplate physiological and enzyme assays to observe bacterial growth and substrate degradation in real-time. Overall, the use of BCDs greatly expands the types of insoluble substrates that can be used when studying microbial physiology and metabolism.

Identification of the essential functions required for bacterial utilization of recalcitrant insect biomass: A model developed with saprophyte *Cellvibrio japonicus* and the Brood X periodical cicada (genus *Magicicada*)

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The polysaccharides that comprise insect exoskeletons, in particular chitin, are abundant sources of carbon for microbes able to deconstruct these complex insoluble substrates. Bacterial degradation of polysaccharides is an essential part of the global carbon cycle, and more recently has been the focus of biotechnology applications searching for efficient methods of renewable fuel and chemical production. In order to better understand and model how microbes are able to effectively utilize recalcitrant substrates a multifaceted approach is required that combines 'omic technologies, bacterial genetics, and real world substrates. To generate the fundamental data that will enable the generation of sophisticated models and identify enzyme targets for synthetic biology applications we used model saprophytic bacterium *Cellvibrio japonicus*. Furthermore, the emergence of the Brood X periodical cicada (genus *Magicicada*) provided a rare opportunity to study bacterial degradation of a unique insect biomass, and our experimental approach combined transcriptomics and bacterial genetics to identify the essential features of insect biomass deconstruction by bacteria. Our RNAseq results identified several highly up-regulated genes that encode Carbohydrate Active Enzymes (CAZymes), as well as sugar transporters when the bacterium was actively degrading cicada molts. Mutational analysis determined that the *chi18D* gene product, predicted to encode a chitinase, was essential for cicada molt utilization. Ongoing work will continue to identify the essential genes for insect biomass utilization by bacteria, with the goal being the generation of a model for chitin metabolism that is predictive for bacteria of industrial or agricultural importance.

Hopanoid Lipids Regulate Membrane Fluidity in the Legume Root-Nodulating Bacterium *Bradyrhizobium diazoefficiens*

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Hopanoids are a taxonomically widespread group of bacterial lipids that are structurally similar to cholesterol. The precursor for hopanoids is squalene, which is cyclized by Shc to form diploptene. Diploptene can then be modified by either HpnH or HpnP to form extended or methylated hopanoids, respectively. In the legume symbiont *Bradyrhizobium diazoefficiens*, knockouts of hopanoid biosynthesis genes lead to symbiotic deficiencies including delayed nodulation, and increased susceptibility to environmental stress [1-3]. We hypothesized that these phenotypes may stem from the ability of hopanoids to modulate membrane fluidity, which can impact the efficiency of membrane-based processes. **Our goal was to investigate how hopanoids influence membrane fluidity on a subcellular level.** We stained cultures of wild type and three hopanoid mutants (Δ*hpnP*, Δ*hpnH*, and P_{cu}-*shc*, a cumate inducible *shc* knockout]) with DiIC12, a fluorescent probe with an affinity for thin, fluid membrane regions, and NADA-green, a fluorescent marker of peptidoglycan insertion. Cells were then imaged using STED super-resolution microscopy. In wild type and Δ*hpnP* strains, we identified high fluidity bands circling the non-growing pole in a helical pattern. In the Δ*hpnH* and *shc* depletion (P_{cu}-*shc* without cumate) mutants had high overall fluidity and lacked this helical pattern. Preliminary proteomics of high-fluidity membrane domains identified the Min protein complex in strains exhibiting helical fluidity, and this complex helps guide cell division and forms membrane-associated helices in other microbes [4]. These observations suggest a new link between hopanoids and cell cycle regulation, bringing us closer to a molecular-level understanding of hopanoid mutant phenotypes. [1] Belin B.J. *et. al, Nat Rev Microbiol*, 16, 304-315, (2018) [2] Belin B.J. *et. al*, *IS-MPMI*, 32, 1415-1428, (2019) [3] Schmerk C.L. *et. al, J Bacteriol*, 193, 6712-6723, (2011) [4] Aguilar J. et. al, *PNAS,* 107, 3758-3763, (2010)

Development and Mechanisms of Pyocyanin Resistance in *Staphylococcus aureus*

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Cystic fibrosis (CF) is an inherited genetic disease characterized by mucus buildup that results in chronic lung infections. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the most frequently isolated bacterial pathogens, and co-colonize 20-30% of adults with CF. This co-colonization is associated with decreased lung function and worse patient outcomes. *P. aeruginosa* produces pyocyanin (PYO), a redox toxin that has been found in CF lungs and is known to antagonize *S. aureus* growth, raising the question of how *S. aureus* adapts in the presence of *P. aeruginosa*. To address this question, we evolved *S. aureus* with PYO under different conditions. Resistant isolates from populations exposed to PYO in the exponential-phase had mutations in *codY,* a global regulator of metabolism and virulence, and targeted *codY* mutants showed increased PYO tolerance. Transcriptional analysis upon PYO exposure identified differential expression in the *codY* mutant, compared to the wild-type, of ATP metabolism, translation, and oxidative stress response genes, indicating that reduced cellular activity or increased resistance to oxidative stress may underlie PYO tolerance in *codY* mutants. Using an alternate approach, resistant isolates from stationary-phase populations exposed to increasing PYO concentrations had independent mutations in *qsrR*, a putative quinone-sensing regulator, and the promoter of *azoR*, a putative azoreductase. Disruption of *azoR* sensitized resistant isolates of both backgrounds to PYO, suggesting that QsrR likely represses *azoR*, and increased *azoR* expression leads to PYO resistance. Thus, our study has revealed novel adaptive pathways for *S. aureus* upon PYO exposure, and future studies will investigate the underlying mechanisms.

Genomic Surveillance of *Escherichia coli* in a large tertiary care hospital in the United States

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Extraintestinal pathogenic *Escherichia coli* (ExPEC) are the most common cause of bloodstream and urinary tract infections globally. Epidemiological studies estimate there are over 110 million urinary tract infections annually in the United States as the result of ExPEC infections. Here we present a longitudinal genomic analysis of all ExPEC *E. coli* isolated from a large tertiary care hospital in the United States. 2,076 isolates were collected from 1,777 unique patients between September 2019 and September 2020. The majority belonged to *E. coli* phylogenetic groups B2 or D with the most represented clonal lineages being ST-69, ST-73, ST-95, ST-1193, ST-127 and ST-131. Antibiotic susceptibility profiles varied, with 29% of the isolates classified as multidrug resistant (MDR) (non-susceptible to ≥3 families of antibiotics), while 64% were susceptible to all 18 antibiotics tested. Isolates carrying extended spectrum β-lactamase (ESBL) genes were found in all main phylogenetic groups, with various $bla_{\text{CTX-M}}$ alleles and diverse plasmid replicons identified. 41% ESBL positive *E. coli* belonged to the epidemic ST-131 lineage. To trace nosocomial transmission events, sequence data was used to identify clusters of genetically related isolates (≤10 allelic differences) from 2 or more patients. Notably, isolates involved in possible transmission events were just as likely to be phenotypically susceptible as isolates involved in sporadic infections. These findings highlight the importance of tracking *E. coli* isolates with diverse susceptibility profiles as outbreak investigations that focus on MDR or ESBL-producing isolates are likely to underestimate nosocomial transmission events.

Galactomannan utilization by *C. japonicus* relies on a single essential α-galactosidase encoded by the *aga27A* gene

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Plant cell walls are comprised of a complex mixture of polysaccharides, of which mannans can constitute up to 60%. Plant mannans are a hemicellulose component of lignocellulose that can have diverse compositions, and consequently, the degradation of mannan substrates require a cadre of enzymes for complete reduction to its substituent monosaccharides. One bacterium that possesses this suite of enzymes is the Gram-negative saprophyte *Cellvibrio japonicus*, which has 10 predicted mannanases. Here, we've utilized a systems biology approach to determine the essential components of the mannan degradation apparatus in this bacterium. Transcriptomic analysis uncovered a significant change in gene expression for most mannanases and many genes that encode Carbohydrate Active Enzymes (CAZymes) when mannan was actively being degraded. Comprehensive mutational analysis characterized 54 CAZyme genes in the context of mannan utilization. Growth analyses indicated that the *man26C*, *aga27A*, and *man5D* genes which encode an endo-mannanase, alpha-galactosidase, and mannosidase, respectively, were critical for the utilization of galactomannan. Our current model of galactomannan utilization in *C. japonicus* suggests that the removal of sidechains from galactomannan is essential for complete degradation and takes place before the degradation of the polysaccharide backbone. Furthermore, *C. japonicus*' ability to discriminate between varying types of mannans and its transport of mannose-based oligomers may have industrial applications in the pursuit of renewable fuels and chemicals derived from plant-based biomass.

The role of colonization factors CFA/I and CS21 in ETEC pathogenesis in the human enteroid model

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Enterotoxigenic *Escherichia coli* (ETEC) is a primary causative agent of diarrhea in young children in developing countries and of traveler's diarrhea. ETEC adhere to intestinal epithelia via colonization factors (CFs) and secrete heat-stable toxin (ST) and/or heat-labile toxin (LT), causing dysregulated cellular ion transport and water secretion. ETEC isolates often harbor genes encoding more than one CF and are prime targets as vaccine antigens. Many clinical isolates express CFA/I as well as CS21; however, a role for CS21 alone or with CFA/I has not been defined. We hypothesize that expression of both CFs confer increased adherence and toxin delivery to the human enteroid. Clinical strains expressing CFA/I and/or CS21 were evaluated, and CF-deficient mutants were engineered. After confirming CF expression using Western blotting and electron microscopy, adherence assays using the human enteroid demonstrated CFA/I was important as CFA/I-deficient mutants and strains pre-incubated with anti-CFA/I antibody had significantly reduced adherence than wildtype. In contrast, CS21 was not required as CS21-deficient mutants and strains pre-incubated with anti-CS21 antibody adhered at similar levels as wildtype. Delivery of ST by ETEC with CFA/I and CS21 was evaluated, and resulting cGMP levels were similar across wildtype and CF-deficient strains. These data demonstrate that targeting CFA/I alone in a CFA/I-CS21 strain is sufficient for adherence inhibition. Toxin delivery in enteroids was not dependent on either CF expression, which may reflect the lack of flow and stretch in this model. Overall, these data support the role of CFA/I in pathogenesis and as a main target for vaccines.

Suppression of nitric oxide production by mucormycosis-causing fungi during macrophage infection

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The recent surge of mucormycoses among COVID-19 patients has thrust the disease and lack of available treatments into the spotlight. We sought to characterize immune evasion mechanisms by Mucorales fungi. RNA-seq analysis of lungs extracted from mice infected with various *R. delemar* strains revealed a global repression of well-known immune pathways. One such pathway contains the *Nos2* gene which encodes an enzyme to produce nitric oxide (NO), a free radical which is toxic to many pathogens. To verify our results, we treated MH-S macrophages with LPS and IFN-γ in the presence or absence of fungal spores. At 8 HPI, LPS and IFN-γ induced a 2,000-fold induction of *Nos2* expression in MH-S cells, which was accompanied by robust NO production at 24 HPI. MH-S cells infected with Mucorales in the presence of LPS and IFN-γ showed similar levels of *Nos2* mRNA. However, these cells showed significantly reduced NO production by >90%. There was no significant difference in NOS2 protein levels between LPS and IFN-γ treated MH-S cells and MH-S cells infected with Mucorales in the presence of LPS and IFN-γ. These trends were seen with multiple Mucorales species and in other MØ cell lines and primary cells. MØs treated with *R. delemar* conditioned media showed a 70% reduction in NO production. These results suggest that *R. delemar* alters its surrounding metabolic environment and depletes macrophages of nutrients required to mount proper antimicrobial defenses. This suppressive activity represents a novel mechanism by which Mucorales evade the immune response to cause disease.

Defense Against Disease: The protective role of anti-inflammatory receptor NLRX1 and its recognition of spirochete *Borrelia burgdorferi* during Lyme disease

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Borrelia burgdorferi, the etiological agent of Lyme disease, is a bacterium of pressing concern in the fields of microbiology and immunology. Over the last 30 years, there has been a 15-fold increase in Lyme disease cases affecting an estimated 475,000 individuals per year. Of these cases, roughly 10-30% of patients will develop inflammation of one or more joints termed Lyme arthritis. Lyme arthritis is thought to result from the innate immune response to *B. burgdorferi* unique surface proteins, peptidoglycan structure, and nucleic acids. Ultimately, the mitigation of inflammation from these bacterial components has not been fully explored. In our innate immune system, Pattern Recognition Receptors (PRRs) serve as the first line of host immune defense against pathogens. PRRs operate by sensing conserved pathogen genetic patterns known as Pathogen-Associated Molecular Patterns (PAMPs) to modulate inflammation. However, the role that anti-inflammatory PRRs play in regulating Lyme arthritis has not been elucidated. Here, we studied the role of anti-inflammatory PRR NLRX1 during *B. burgdorferi* infection in novel *Nlrx1*$^{-/-}$ mouse models. We hypothesized that NLRX1 would decrease arthritis presentation in response to *B. burgdorferi.* After infecting wildtype and *Nlrx1*$^{-/-}$ mice, we found that NLRX1 significantly decreased arthritis severity in comparison with knockout mice. Through further studies of potential *B. burgdorferi* PAMPs, NLRX1 recognition of peptidoglycan (PG) and genomic (g)DNA significantly decreased inflammation. Ultimately, these results indicate that NLRX1 plays a protective role in mitigating Lyme arthritis, warranting a further need to define NLRX1's mechanism of *B. burgdorferi* recognition during Lyme disease.

Interplay between amoxicillin resistance and osmotic stress in *Helicobacter pylori*

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Rising antibiotic resistance rates are a growing concern for all pathogens, including *Helicobacter pylori*. We previously examined the association of specific mutations in PBP1 with amoxicillin resistance and fitness in *H. pylori* and found that a N562Y mutation was associated with resistance, but also resulted in a fitness defect in standard growth media. However, we also found that hyperosmotic stress increased the fitness of the N562Y strain relative to wild-type G27. The finding that an amoxicillin-resistant strain shows environmentally dictated changes in fitness suggests a previously unexplored interaction between amoxicillin resistance and osmotic stress in *H. pylori*. Herein, we further characterized the interaction between osmotic stress and amoxicillin resistance. Wild-type and the isogenic N562Y PBP1 mutant strain were exposed to amoxicillin, various osmotic stressors, or combined antibiotic and osmotic stress and viability was monitored. While sub-inhibitory concentrations of NaCl did not affect *H. pylori* viability, the combination of NaCl and amoxicillin resulted in synergistic killing; this was true even for the N562Y mutant strain. Moreover, similar synergy was found with other beta-lactams, but not with antibiotics that did not target the cell wall. Similar synergistic killing was also demonstrated when KCl was utilized as the osmotic stressor. Conversely, osmolar equivalent concentrations of sucrose antagonized amoxicillin-mediated killing. Taken together, our results support a previously unrecognized interaction between amoxicillin resistance and osmotic stress in *H. pylori*. These findings have interesting implications for the effectiveness of antibiotic therapy for this pathogen.