Project Title: Identifying the metabolomic shift of the pediatric airway colonized with multiply-drug resistant Gram negative organisms

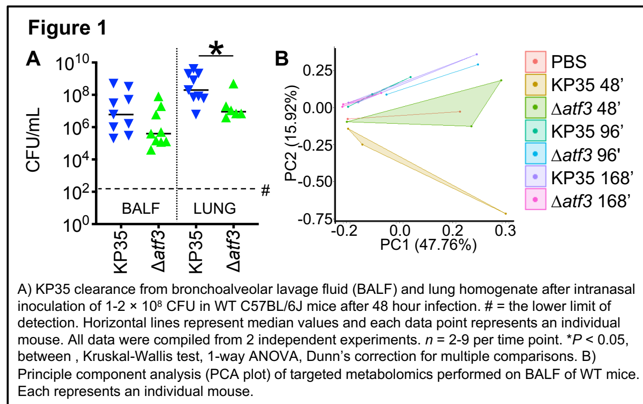
Background and rationale: Chronically ventilated children with tracheostomy tubes are frequently hospitalized and are at risk for death as a result of acute on chronic respiratory failure. Respiratory illness and infection are a major reason for hospitalization for acute illness and mortality in this population[1,2]. In a single institution cohort, 63% were found to have Gram negative rods grown from their tracheostomy and had significantly more hospitalizations, longer length of stay in the hospital and in the intensive care unit[3]. It is not surprising that the airways of these children are frequently colonized with bacteria, including multiply-drug resistant organisms due to the lack the protection of the upper airway with its ability to trap and clear antigens, the protection of the local bacterial flora, and the production of antimicrobial peptides. In the context of viral infection and bacterial colonization during an admission for acute on chronic respiratory failure, clinical decision making in the treatment of chronically colonized children is challenging. On one hand, broad spectrum antimicrobials could prevent progression of tracheitis or parenchymal lung disease, reducing morbidity and mortality, but on the other hand, treatment may continue to promote the selection of multiply-drug resistant bacteria. To solve this problem, it is important to understand how colonizing bacteria alter the host environment in both acute illness and baseline state of health. The overall success of the colonizing bacteria has largely been attributed to the expansion of antimicrobial resistance elements and biofilm formation. However, less well known are the metabolic changes that occur between the host and bacteria in the shift from colonization to clinically significant illness. There remains a critical need to understand the mechanisms by which this symbiotic relationship is altered in the airways of children with tracheostomies in sickness and in health.

*The overall goal of this project is to identify universal mechanisms acquired by multiply-drug resistant Gram negative organisms (**MDR GNOs**) that alter the metabolic environment in pediatric patients with tracheotomies and lead to chronic colonization and clinically significant disease.*

Hypothesis: We hypothesize that a subset of MDR GNOs alter the host metabolic environment and lead to chronic colonization and clinically significant disease in pediatric patients with tracheostomies.

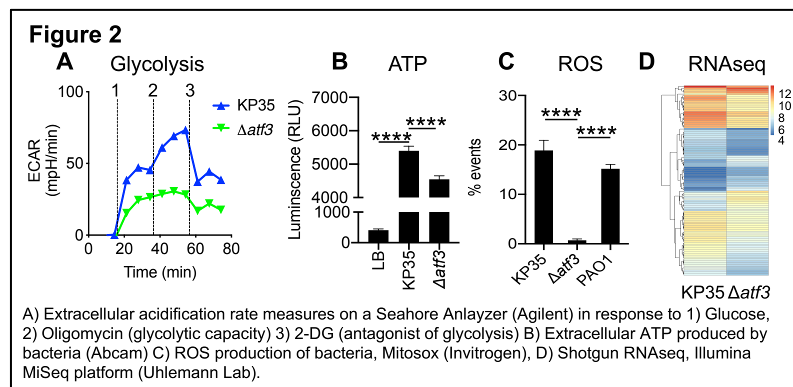
Innovation: The adaptation of MDR GNOs to the host airway has largely been attributed to drug resistance and biofilm formation. Recent work has highlighted the important contribution of the metabolic environment by immune cells and by bacteria, but the evolving symbiotic metabolic relationship of host and bacteria has not been well studied. This project aims to define the metabolic profile of organisms during chronic colonization and clinically significant disease. This approach will ultimately lead to either identification of therapeutic targets for eradication or a better understanding of when to utilize last resort antimicrobials.

Significance: Children with tracheotomies are frequently colonized with Gram negative organisms, and with repeated exposure to both antimicrobials and the host immune system, these organisms become more resistant to available therapeutics. Unveiling the metabolic signature that signifies clinically significant disease will lead to improved outcomes for this vulnerable population.



A) KP35 clearance from bronchoalveolar lavage fluid (BALF) and lung homogenate after intranasal inoculation of 1-2 × 10^8 CFU in WT C57BL/6J mice after 48 hour infection. # = the lower limit of detection. Horizontal lines represent median values and each data point represents an individual mouse. All data were compiled from 2 independent experiments. n = 2-9 per time point. $^*P < 0.05$, between , Kruskal-Wallis test, 1-way ANOVA, Dunn's correction for multiple comparisons. B) Principle component analysis (PCA plot) of targeted metabolomics performed on BALF of WT mice. Each represents an individual mouse.

Preliminary Results: Our approach to solving this clinically important question has stemmed from our studies of carbapenem-resistant *Klebsiella pneumoniae* (CRKP), an epidemic and deadly cause of ventilator associated pneumonia. We previously showed that a representative CRKP isolate (**KP35**) was genotypically and phenotypically very different from well-studied laboratory reference strains[4]. We further identified a novel acyltransferase superfamily 3 (*atf3*) unique to KP35 and found that this gene was enriched in CRKP genomes as compared to all *K. pneumoniae* (62% v 29%). We first used a mouse of model of acute pneumonia, comparing KP35 infection with an isogenic mutant lacking *atf3* (Δ*atf3*), constructed using Crispr-Cas9 technology (in collaboration with Dr. AC Uhlemann). We found increased bacterial colonization in the airway (**Fig 1A**) and increased weight loss without a difference in immune cell recruitment or cytokine production when *atf3* was present. The effect of this gene being present in the bacteria led to a complete change in airway metabolome (**Fig 1B**), with globally increased utilization of carbohydrates, lipids and amino acids. When examining the contribution of the



A) Extracellular acidification rate measures on a Seahore Anlayzer (Agilent) in response to 1) Glucose, 2) Oligomycin (glycolytic capacity) 3) 2-DG (antagonist of glycolysis) B) Extracellular ATP produced by bacteria (Abcam) C) ROS production of bacteria, Mitosox (Invitrogen), D) Shotgun RNAseq, Illumina MiSeq platform (Uhlemann Lab).

bacteria alone, we found that when *atf3* was present, CRKP was more glycolytic and generated more ATP and ROS (**Fig 2A-C**). RNA seq analysis (**Fig 2D**) showed that expression of enzymes involved in glycolysis and the TCA cycle were increased when *atf3* was present. We hypothesize that this finding is either due to acylation of a key enzyme or ATP transporter that drives glucose utilization and downstream energy production. Our preliminary data clearly demonstrate that we have all of the methods to identify genes of interest in multiply-drug resistant bacteria and characterize their influence on the airway metabolome.

Investigational Approach: Our overarching goal is to identify genes that alter the metabolic properties of MDR GNOs and promote persistence in the airway of patients with tracheostomies. Our approach is a comprehensive genetic and metabolic analysis of isolates obtained from these patients. I will apply my expertise in microbiology, biochemistry and molecular biology techniques to patient samples to address this very important clinical question.

Aim 1: To identify metabolic genes upregulated in MDR GNOs to bacterial persistence in pediatric patients with tracheostomies

We *hypothesize* that MDR GNOs that persist in the airway of pediatric patients with tracheostomies have acquired genes that give them a metabolic advantage. ***Experimental Strategy***: Since 2014, clinical isolates have been collected by the clinical microbiology lab under the supervision of Dr. AC Uhlemann. In this bank, 440 isolates have been collected from hospitalized pediatric patients and multiple isolates were acquired from the same patient over the course of a prolonged hospitalization. First, we will begin by performing RNAseq on clinical isolates from patients with prolonged intubation and chronic colonization for MDR GNOs (IRB AAAS6672, approved 9/18/19). Second, we will concurrently prospectively collect clinical isolates from pediatric patients with tracheostomies admitted to the pediatric intensive care unit (PICU) for acute on chronic respiratory failure and patients seen in the pediatric ENT tracheostomy clinic who are in a relative state of health (IRB AAAS9103, in progress). Non-hospitalized patients that are colonized with MDR GNOs will serve as controls. Antibiograms, bacterial growth rates and biofilm formation will be performed as control experiments. Bacterial isolates will be defined as persistent in the airway for greater than a 3-month period regardless of antimicrobial treatment. Concurrent clinical data will be recorded for each patient including antimicrobial treatment. Functional assays for common downstream metabolic effects such as reactive oxygen species production (Mitosox, Invitrogen) and ATP production (Abcam) will be measured. ***Expected Outcomes***: We expect to see that 1) MDR GNOs that are persistent in the airway upregulate the expression of genes involved in the bioenergetics such as glycolysis and the TCA cycle. Characterizing how these pathways are upregulated will allow identification of therapeutic targets for the eradication of these colonizing organisms. 2) Isolates that were higher producers of ROS and ATP will correlate with increased severity of illness as defined by longer duration of increased ventilatory support, increased length of stay in the PICU and increased use of antimicrobial therapy. ***Anticipated difficulties and alternate plans***: To normalize the variability in growth conditions of the host airway and to allow for robust yield of RNA, clinical isolates will be grown in nutrient rich media such as Luria-Bertani (LB). In case we see a large variability in gene expression from frozen retrospective clinical isolates and prospectively collected bacterial samples, we will take an alternative approach. Though more technically challenging, likely requiring extra enrichment steps and rRNA depletion due to low RNA yield, we will prospectively collect bacteria from tracheal aspirates of hospitalized and immediately extract RNA for gene expression analysis.

Aim 2: To determine if the airway metabolome of pediatric patients colonized with MDR Gram negative organisms predicts severity of illness

We *hypothesize* that patients with clinically significant tracheitis or parenchymal lung disease will have an alte airway metabolome from healthier control patients. ***Experimental Strategy***: To test this hypothesis, we perform targeted metabolomics on recovered tracheal aspirate samples from two groups of patients: 1) children admitted to the PICU and 2) children attending the pediatric ENT tracheostomy clinic. Since 2012, Dr. T Connors, a member of the Pediatric Critical Care Division and mentee of Dr. D Farber, has been collecting endotracheal aspirate samples of virally infected patients, intubated in the PICU (IRB protocol AAAI5912)[5,6]. Of the 121 patients enrolled, 29 patients eventually went on to grow bacteria from endotracheal aspirate cultures. Metabolomics will be performed on the banked BALF supernatant. In parallel, we will collect tracheal aspirate samples form PICU patients with tracheostomies admitted with acute on chronic respiratory failure. Patients seen in pediatric ENT tracheostomy clinic in relative states of health will enrolled as controls (IRB AAAS9103, in progress). Concurrent clinical data will be recorded for each patient including length of increased ventilatory support, maximum oxygenation indexes, antimicrobial use, pediatric sequential organ failure assessment (pSOFA) scores, routine laboratory data (WBC, inflammatory markers), radiology results and length of hospitalization. Statistical analysis will be performed with the assistance of the CTSA. ***Expected Outcomes***: We expect to detect a shift in the airway metabolome in pediatric patients that exhibit clinically significant tracheitis or parenchymal disease. Moreover, we expect that patients that demonstrate more severe disease will have increased substrate utilization. ***Anticipated difficulties and alternate plans***: With this approach, we do not take into account the phenotype of the immune cells recruited to the site of infection and cytokines produced that may also play a major role in the clinical course of these patients. Therefore, BALF supernatant for cytokine profiling and immune cells for characterization will be frozen for future analysis.

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