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Detection Of The Lung Environment By Multi-Drug Resistant Gram-Negative Bacterial Pathogens

Graham Geier Willsey
University of Vermont

DETECTION OF THE LUNG ENVIRONMENT BY MULTI-DRUG RESISTANT GRAM-NEGATIVE BACTERIAL PATHOGENS

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Graham Geier Willsey

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Dissertation Examination Committee:

Matthew J. Wargo, Ph.D., Advisor
Jay R. Silveira, Ph.D., Chairperson
Mary J. Dunlop, Ph.D.
Aimee Shen, Ph.D.
Cynthia J. Forehand, Ph.D., Dean of the Graduate College

ABSTRACT

Nosocomial lung infections are a growing concern in the United States, with more than 300,000 cases reported annually. More than 30 % of which are caused by the Gram-negative bacteria, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Similarly, Gram-negative bacteria establish chronic infections in individuals with cystic fibrosis (CF) that are difficult or impossible to eradicate. *P. aeruginosa* has historically been one of the most prevalent pathogens of adults with CF. However, as antipseudomonal therapy has improved, more antibiotic resistant species have taken hold, including *Stenotrophomonas maltophilia*, which now colonizes more than 10 % of individuals with CF. Regardless of the cause or source, Gram-negative respiratory infections are becoming increasingly difficult to treat due to the rising incidence of multiple drug resistance among these organisms. To aid in the development of new therapeutics, a greater understanding of how these organisms transition from the environment to the host lung is needed. Here we utilized a combination of transcriptomics and molecular genetics to examine how *P. aeruginosa, K. pneumoniae,* and *S. maltophilia,* recognize and exploit the host lung milieu during the initiation of infection.

One of the first components of the host lung environment that aspirated bacteria are exposed to is pulmonary surfactant (PS). This phospholipid-rich substance coats the distal airways of the lung and is thought to contain molecular cues that facilitate lung colonization by pathogenic bacteria. Here, we characterized the transcriptional response of *K. pneumoniae* to purified PS to examine how this organism interreacts with the host lung during colonization. This work revealed numerous virulence and colonization-related genes that are expressed by *K. pneumoniae* under these conditions. We also tested the contribution of other surfactant-induced transcripts to *K. pneumoniae* pathogenesis using engineered gene deletion strains and a mouse model of pneumonia. This work revealed the polyamine efflux pump, MdtJI, and glycine betaine transporter, ProU are required for *K. pneumoniae* virulence.

Phosphatidylcholine is the primary constituent of PS. *P. aeruginosa* is capable of completely metabolizing the phosphocholine head group of this lipid, and readily does so when exposed to PS. We previously observed that the most highly expressed genes in *P. aeruginosa* in response to PS were those involved in the catabolism of a downstream choline metabolite, sarcosine. Although our group had previously characterized the choline catabolic pathway of *P. aeruginosa*, the transcriptional regulation of sarcosine catabolism was not known. We utilized a genetic screen to identify the regulator controlling the expression of the sarcosine catabolic genes in *P. aeruginosa*. This regulator, which we named SouR (Sarcosine oxidase utilization Regulator) is the first sarcosine-responsive regulator to be characterized.

The thick, viscous mucus (sputum) that accumulates within the CF lung serves as the primary nutrient source for microbes colonizing the CF lung. Here, we characterized the transcriptional responses of three *S. maltophilia* strains during growth in synthetic CF sputum media (SCFM2) to gain insight into how this organism interreacts with the host lung. We also compared the SCFM2 transcriptomes of two *S. maltophilia* CF isolates with the SCFM2 transcriptome of the acute infection model strain, K279A. This revealed CF isolate-specific signatures in gene expression that reflect adaptation to the CF lung.

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CHAPTER 1:

Introduction

1.1 Multiple drug resistance: A serious threat to public health

The spread of antibiotic resistant bacteria poses one of the greatest current threats to global health (1, 2). More than 2 million drug resistant infections are reported in the United States each year that are directly responsible for more than 23,000 deaths (3). Antibiotic-resistant infections cause a significant financial burden on the United States healthcare system due to their association with extended hospital stays, longer courses of treatment, additional hospital visits, and increased mortality (2, 4). The additional medical costs associated with treating antibiotic resistant infections has been estimated at more than 20 billion dollars in the United States annually (5).

As current antibiotics continue to lose their effectiveness, treatment options are becoming increasingly limited. The steady rise of multidrug-resistant (MDR) Gram-negative bacteria is particularly worrisome, especially among healthcare-associated opportunistic pathogens (6, 7). To highlight the MDR organisms posing the greatest threats to public health, the Infectious Disease Society of America devised the acronym, "ESKAPE" to distinguish these bacteria (8). Four out of the six "ESKAPE" pathogens are Gram-negative species, including: *Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumannii,* and *Enterobacter spp.(8)*.

Nosocomial infections are the sixth leading cause of death in the United States, claiming nearly 100,000 lives each year (9, 10). Infections of the lower respiratory tract (pneumonia) are the most dangerous type of hospital acquired infection and were attributed to the death of 36,000 individuals in the United States in 2002 (10), the most recent year such data has been compiled. Nearly 300,000 cases of nosocomial pneumonia are reported in the United States annually, of which *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are responsible for more than 30 % (7, 11, 12). Infections with either species are often complicated due to drug resistance against multiple classes of antibiotics.

The emergence of MDR in *K. pneumoniae* was first observed in the early 1980's with clinical isolates harboring plasmids carrying multiple antibiotic modifying enzymes including extended spectrum β-lacatamases (ESBLs) that confer resistance to cephalosporins and penicillins (13, 14). A 2013 report from the Center for Disease Control suggests that more than 20 % of *K. pneumoniae* clinical isolates in the United States now harbor at least one ESBL (14, 15). Carbapenem resistance in *K. pneumoniae* has spread throughout the world at an alarming rate through horizontal transfer events mediated by plasmids and transposons (16-19). As of 2013, 11% of *K. pneumoniae* infections in the United States were carbapenem resistant (15). Infections with carbapenem-resistant *K. pneumoniae* (CRKP) are associated with extremely high treatment costs and mortality rates approaching 50 % (20, 21). CRKP strains are frequently resistant to nearly all classes of antibiotics, and are often only treatable with the "last resort" drug, colistin (22, 23). Unfortunately, the overuse of colistin has resulted in resistance and lead to the emergence of *K. pneumoniae* infections that are no longer

treatable (22, 24, 25). Resistance against colistin in *K. pneumoniae* is most often mediated through mutations within genes that control LPS lipid A remodeling (26, 27), although plasmid-borne resistance was detected several years ago (28) and has since spread at a terrifying rate (29).

More than 13 % of all *P. aeruginosa* infections are considered MDR in nature due to resistance to three or more classes of antibiotics (3). *P. aeruginosa* is intrinsically resistant to numerous antibiotics largely due to a combination of low outer membrane permeability, a chromosomally encoded β-Lactamase (*ampC)*, and numerous multidrug efflux pumps (30). Drug resistance in *P. aeruginosa* is frequently acquired through mutations within the promoter regions of antibiotic resistance genes that result in elevated expression (31). This phenomenon is often observed in genes encoding the Mex-family multiple drug efflux pumps, which result in acquired resistance to cephalosporins, meropenem, and fluoroquinolones (32-34), as well as *ampC,* which confers resistance to cephalosporins and penicillins when over-expressed (34, 35). *P. aeruginosa* resistance to fluoroquinolones is also mediated through mutations within DNA topoisomerases that inhibit the binding of these antibiotics (36). Furthermore, mutations that result in the loss of *oprD* expression confer resistance against numerous antibiotics, including imipenem (37). Aside from mutations, *P. aeruginosa* strains also acquire multi-drug resistance through horizontal transfer of plasmids that harbor antibiotic-modifying enzymes, including carbapenemases (18, 31).

Other environmental MDR Gram-negatives are also emerging as serious nosocomial respiratory pathogens, including *Stenotrophomonas maltophilia* (38, 39). *S. maltophilia* is intrinsically resistant to nearly all classes of antibiotics due to low outer

membrane permeability, coupled with an armament of ten multidrug efflux pumps, two broad spectrum β-lactamases, and several aminoglycoside modifying enzymes that are all encoded within this organism's core genome (39, 40). Due to the extensive antibiotic resistance profile of *S. maltophilia*, therapeutic options for these infections are extremely limited, with trimethoprim/sulfamethoxazole (TMP/SMX) being the treatment of choice (39, 40). Unfortunately, resistance to these drugs is emerging via efflux pump overexpression and the spread of antibiotic modifying enzymes carried in class 1 integrons (41, 42).

Recent reports have indicated that the susceptibility of *S. maltophilia* to TMP/SMX has dropped to 90 % in some regions (40, 43). Similarly, pan antibiotic resistant strains of *P. aeruginosa* and *K. pneumoniae* have been isolated in the clinical setting (23, 44, 45). The emergence of untreatable Gram-negative pathogens highlights the urgent need for new therapeutics-the development of which could be greatly facilitated through characterizing the initiation of infection by these organisms *in vitro*.

1.2 Opportunistic respiratory tract infections associated with MDR pathogens

MDR Gram-negative species typically possess lower virulence potential than their non-MDR counterparts and mostly cause respiratory tract infections in individuals with chronic lung disease or weakened immune systems following antibiotic treatment. These infections are common in the nosocomial setting and in individuals with Cystic Fibrosis (CF). In each type of infection, the host exhibits specific immune deficiencies that promote lung colonization and infection by distinct MDR Gram-negative species. An

overview of the innate immune defenses within the respiratory tract that must be overcome by these organisms to cause infection is briefly summarized below in section 1.21. This is immediately followed by brief descriptions of nosocomial pneumonia and CF that center on the immune deficiencies within each host that promote infection by MDR pathogens.

1.2.1 The innate immune defenses of the respiratory tract

Following deposition into the lung, inhaled or aspirated bacteria must overcome detection and clearance by the innate immune system in order to colonize and establish an infection. This includes killing mediated by numerous antimicrobial peptides, the complement system, phagocytic cells, and alveolar epithelial cells. These components of the innate immune system are briefly discussed below in the context of Gram-negative respiratory tract infections, and are also summarized in **Figure 1.1.**

The surfaces of the respiratory tract are coated with numerous antimicrobial peptides that are secreted by alveolar epithelial cells, macrophages, and activated neutrophils that serve to kill or inhibit the growth of aspirated or inhaled pathogens (46, 47). Notable antimicrobial peptides within the lung include lysozyme, defensins, cathelicidin, which are thought to act synergistically to kill invading microbes through permeabilizing or disrupting bacterial cell membranes (46, 48). Lactoferrin is also found within the respiratory tract and serves to inhibit bacterial growth by sequestering iron cations (48, 49).

The complement system is a major component of the innate immune defenses against pathogenic microbes that enter the lung and serves to detect and promote bacterial clearance via opsonization and also coat bacteria to be targets of the membrane attack complex (MAC) once they breach the endothelial barrier (50). This proteolytic cascade is activated by three distinct biochemical processes, including the classical (antibody mediated), alternative, and lectin-mediated pathways (50). Regardless of the mechanism of initiation, in the lung lumen complement activation stimulates a proteolytic cascade that leads to neutrophil recruitment and the opsonization of Gram-negative bacteria (47, 50).

Several notable lectins are found within the lung milieu that promote opsonization and stimulate the compliment cascade. Two of the most critical effectors within the lung are the pulmonary surfactant-associated collectins, SP-A, and SP-D, which are pattern recognition receptors (PRR) that bind to carbohydrate structures on bacteria to promote opsonization by alveolar macrophages or recruited neutrophils (47, 51). Other important PRR's of the innate immune system within the respiratory tract include mannose binding protein and ficolin, which recognize polysaccharides on bacterial cell surfaces to promote their opsonization and stimulate the classical compliment cascade via C-reactive protein (CRP) (47).

Alveolar macrophages are likely the first immune cells to recognize inhaled or aspirated bacteria through detection of pathogen-associated molecular patterns (PAMPs) mediated by TLRs that are expressed on their cell membrane (52). Macrophages specifically recognize Gram-negative bacteria through TLR4 and TLR5-mediated signaling in response to lipopolysaccharide (TLR4) and flagellin (TLR5), which in turn

activates these cells (52). Once activated, macrophages release pro-inflammatory cytokines that recruit neutrophils, increase their own phagocytic capacity and induce reactive nitrogen species production, and stimulate alveolar epithelial cells to secrete antimicrobial peptides (47, 52).

Neutrophils infiltrate the lung in response to cytokines and proinflammatory signals released by activated alveolar macrophages. These cells serve a critical role during respiratory tract infection and act as the primary leukocytes responsible for killing extracellular bacteria. Neutrophils kill bacteria through a variety of mechanisms, including phagocytosis, respiratory bursts, degranulation of secretory vesicles containing proteases, and through the generation of neutrophil extracellular traps (NETs) (47, 53).

Epithelial cells within the lung also play several distinct roles in preventing bacterial colonization of the respiratory tract. The mucociliary escalator consists of ciliated epithelial cells that line the bronchioles and serve a critical clearance function through synchronized uni-directional cilia beating that serves to transport inhaled or aspirated bacteria bound to mucin from the peripheral airways, through the central airways, and up through the trachea where these secretions can be expectorated or swallowed (54). Furthermore, alveolar epithelial cells express TLR4 and TLR5 (55), and secrete antimicrobial peptides in response to signaling mediated by alveolar macrophages (47). Type II alveolar epithelial cells form lamellar bodies that secrete the phospholipid-rich substance, pulmonary surfactant, which coats the alveolar surfaces (56). Aside from lowering the surface tension within the lung, pulmonary surfactant contains the SP-A and SP-D collectins and limits airway inflammation (57, 58).

1.2.2 Nosocomial pneumonia

Pneumonia is the primary cause of death due to bacterial infection in the United States (59, 60). Approximately 300,000 cases of nosocomial pneumonia occur annually, which are associated with crude mortality rates ranging from 23-50 % (59, 61, 62). Nosocomial pneumonia is defined as pneumonia developing at least 48 h following admission into the hospital (63). Ventilator-associated pneumonia (VAP) is a specific subset of nosocomial pneumonia that typically arises 48-72 h following endotracheal intubation, and is associated with increased mortality rates (63). More than 50 % of all antibiotics prescribed in the intensive care unit (ICU) are administered for treating respiratory tract infections (12). Not surprisingly, infections with MDR Gram-negative species are common in nosocomial pneumonia and are typically "late-onset" in nature, occurring 4-7 days following hospital admission (7, 63, 64).

Mechanical ventilation is the greatest risk factor associated with developing nosocomial pneumonia due to inhibited lung clearance, potentially damaged/torn epithelium resulting from intubation, and prolonged non-ambulatory periods (7, 61). Other risk factors associated with developing MDR pneumonia include: antibiotic treatment within 90 days prior to hospital admission, prolonged hospital stays (>5 days), pre-existing immunosuppressive diseases/therapies that compromise the immune system, and extremes in age (61, 63). Early-onset nosocomial pneumonia is most often caused by *Staphylococcus aureus, Haemophilus influenzae*, and drug-sensitive Enterics, including: *K. pneumoniae, Escherichia coli, Enterobacter spp.*, and *Serratia spp.*. Late-onset pneumonia is most often caused by MDR Gram-negative species, such as: *P. aeruginosa,*

K. pneumoniae, A. baumannii, and *S. maltophilia* (7). The prevalence of each species in nosocomial pneumonia are listed in **Table 1.1.**

1.2.3 Cystic Fibrosis

Cystic Fibrosis (CF) is the most common lethal genetic condition affecting the Caucasian population and afflicts more than 70,000 individuals worldwide (65). The disease is characterized by mutations within the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) that result in impaired chloride and bicarbonate transport across the epithelial cells lining several organ systems (65, 66). Within the respiratory tract, lack of functional CFTR manifests in reduced airway surface water activity that impairs mucociliary clearance and causes the accumulation of viscous mucus in the airways, ultimately creating an environment that permits colonization by aspirated or inhaled microbes (65, 67). Furthermore, defective ion transport results in a hypertonic environment within the CF lung that impairs innate immune function through disrupting the activity of antimicrobial peptides (68). Individuals with CF also elicit a chronic neutrophil-driven inflammatory response to pathogens that is characterized by the excessive infiltration of these cells into the lung coupled with their impaired clearance (69, 70). Unfortunately, these neutrophils exhibit defective phagocytic killing capabilities and invoke substantial proteolytic damage to the respiratory tract (69, 70). Collectively, these environmental conditions promote chronic respiratory tract infections in individuals with CF that lead to progressive lung damage and declining respiratory function that eventually lead to death (54, 66).

Respiratory tract infections in individuals with CF begin shortly after birth and are initially caused by professional pathogens, such as *H. influenzae* and *S. aureus* (71). However, by adulthood, more than 50 % of CF patients become chronically infected with *P. aeruginosa,* and these infections are closely associated with declining lung function and ultimately death in their third decade of life (72, 73). Other MDR Gram-negative bacteria also emerge later in CF disease progression and establish chronic infections, including: *Burkholderia cenocepacia, Stenotrophomonas maltophilia,* and *Achromobacter xylosoxidans* (65, 74). The prevalence of *S. maltophilia* within the CF population has more than tripled over the last decade, largely due to this organism's extensive drug-resistance profile and the success of antipseudomonal therapies (38, 39). Recent reports have indicated that more than 13% of the worldwide CF population is infected with *S. maltophilia* (74, 75).

1.3 MDR pathogen interactions with the host

1.3.1 *Pseudomonas aeruginosa*

1.3.1.1 Nosocomial pneumonia

P. aeruginosa is most often encountered in the soil and a range of aquatic environments. Under these diverse environmental conditions, *P. aeruginosa* evolved an arsenal of weapons to evade or kill single-celled predatory protists. This machinery serves an important secondary purpose in *P. aeruginosa* and permits immune disfunction and virulence during acute infections in immunocompromised hosts.

P. aeruginosa encodes several secretion systems that transport a variety of lytic enzymes and toxins during acute respiratory tract infections. The type III secretion system serves as this organism's primary virulence determinant and functions through forming a needle-like apparatus that directly injects effector proteins into epithelial cells lining the respiratory tract and phagocytic cells of the innate immune system (76). Four effector proteins have been characterized that are transported by this system, including three toxins (ExoS, ExoT, and ExoY) which disrupt actin polymerization and stimulate apoptosis in target cells, and the phospholipase ExoU which elicits a pro-inflammatory response and causes target cell lysis (76, 77). Regulation of this system is complex, and subjected to multiple levels of control primarily in response to host cell contact and calcium levels in the environment (78, 79). Downstream of these signals, transcription of the type III secretion apparatus is primarily regulated by the AraC family regulator, ExsA (78). The expression of ExsA is autoregulated, and positively regulated by the TetR-family regulator, PsrA in response to long chain fatty acids (80), as well as negatively regulated by ExsD and PtrA (79).

P. aeruginosa also utilizes two type II secretion systems to deliver at least 14 cytotoxic effector proteins into the lung milieu that invoke tissue damage, increase inflammation, and disrupt immune function (81). Exotoxin A is one such effector, that inhibits host elongation factor EF2 resulting in cell death (82). Other well-characterized effectors include the quorum-regulated LasB elastase and Protease IV, which degrade the SP-A and SP-D collectins in pulmonary surfactant and disrupt phagocytosis by alveolar macrophages (83, 84). The hemolytic phospholipase C, PlcH, is also transported via type II secretion and degrades phosphatidylcholine and sphingomyelin in pulmonary

surfactant, leading to increased inflammation and disrupted surfactant function (85). *plcH* expression is influenced by the PhoPQ two component system in response to magnesium availability (79), and positively regulated by the AraC-family transcription regulator, GbdR, in response to the choline catabolites, glycine betaine and dimethylglycine (86). The degradation of sphingomyelin within the respiratory tract by PlcH liberates the potent antimicrobial sphingosine, which is also degraded by *P. aeruginosa* through the activities of enzymes positively regulated by the AraC-family regulator, SphR (87).

P. aeruginosa also synthesizes several quorum-regulated cytotoxic molecules that contribute to pathogenesis during acute respiratory tract infection (79, 81). These include the phenazine, pyocyanin, which gives *P. aeruginosa* characteristic blue-green pigmentation and causes detrimental effects on cells of the innate immune system through inducing oxidative stress and disrupting catalase activity, inhibiting macrophage phagocytosis, and inducing neutrophil apoptosis (88, 89). *P. aeruginosa* also synthesizes hydrogen cyanide during acute infection that is also thought to contribute to host cell cytotoxicity and immune disfunction (90).

The expression of type 4 pili and flagella are subjected to quorum and cyclic-di-GMP mediated regulation in *P. aeruginosa* (79, 91). Aside from their respective functions in twitching and swimming motilities, flagella and pili also facilitate host colonization and biofilm formation through mediating adhesion to host epithelial cells and mucus within the respiratory tract (81, 91). Flagella are highly immunogenic (92) and are also required for type III secretion function during acute infections (76, 78). Moreover, biofilms are generated by *P. aeruginosa* during chronic respiratory tract infections that are associated with decreased antibiotic susceptibly and enhanced

resistance against clearance by the host immune system (81). A summary of the interactions between *P. aeruginosa* and the host during acute pneumonia is provided in **Figure 1.2.**

1.3.1.1 Chronic CF infection

Numerous studies have examined the phenotypic and genotypic changes that occur within *P. aeruginosa* over the course of chronic CF infection. Collectively, this work revealed that *P. aeruginosa* adapts to growth within the CF lung to become less inflammatory, less virulent, slower growing, and more drug resistant as a result of selection pressures imposed by host immune system and repeated antibiotic therapy (93). This adaptive process is facilitated through the accumulation of mutations within DNA repair genes that result in the development of hypermutator strains (94). A hallmark of CF adapted *P. aeruginosa* strains is the adoption of hypermucoid phenotypes that often result from mutations within the antisigma factor, *mucA* which de-repress envelope stress response genes and promote the overproduction of the exopolysaccharide, alginate (93). As a result, hypermucoid strains are less susceptible to recognition and clearance by the immune system. Furthermore, disruption of *mucA* results in the elevated expression of stress response genes that confer enhanced resistance against reactive oxygen and nitrogen species (ROS & NOS) (93). Diminished biofilm production also arises in *P. aeruginosa* CF isolates resulting from the loss of flagella and twitching pili due to the highly immunogenic properties of these structures. In addition, CF-adapted *P. aeruginosa* strains become less cytotoxic over the course of chronic infection through the loss of type III secretion (95), which is thought to be driven by the immunostimulatory properties of

the effector proteins as well as the inability to adhere to target cells following the loss of flagella and twitching pili (96).

1.3.2 *Klebsiella pneumoniae*

In contrast with *P. aeruginosa, K. pneumoniae* utilizes a different approach to overcome the host immune defenses within the lung. Rather than kill or disrupt the activity of resident leukocytes, *K. pneumoniae* evades detection by the host defenses. *K. pneumoniae* increases capsule production during infection to avoid recognition through toll-kike Receptor (TLR)-mediated signaling and the complement system (14, 97). Environmental selection pressures resulting from interactions with their opportunistic hosts, phage, and protists has resulted in tremendous variation in the glycans expressed by *K. pneumoniae* (98), with nearly 80 serotypes being recognized clinically(14). Nevertheless, nearly 70 % of hypervirulent *K. pneumoniae* strains express capsule with either K1 or K2 serotypes that produce sialic acid-containing capsule saccharides which serve to impair detection by phagocytes by mimicking the sialic acid glycan linkages that coat alveolar epithelial cells (99, 100). Capsule synthesis in *K. pneumoniae* is primarily regulated by the RcsAB two-component phosphorelay system which detects perturbations within the cell membrane that can be invoked by a variety of environmental stressors, including antimicrobial peptides (101, 102). In addition, capsule production is also influenced by the iron and oxidative stress responsive transcription regulator, IscR (103). Other regulatory systems also impinge on the expression of the *cps* gene cluster and are associated with hypercapsule production, including the plasmid-borne *rmpA* and

rmpA2 transcription regulators that respond to environmental stimuli including glucose and iron (104-106).

K. pneumoniae can further evade detection by the host through modifying the structure of LPS to forms not recognized by receptors of the innate immune system. Within the respiratory tract, *K. pneumoniae* has been shown to alter its lipid A structure to include a 2-hydoxyacyl modification that reduces the inflammatory response (107). The addition of 4-amino-4-deoxy- l -arabinose (Ara4N) to lipid A also occurs in *K. pneumoniae* during respiratory tract infections and provides resistance against cationic antimicrobial peptides that is required for virulence (101). Lipid A modification in *K. pneumoniae* requires crosstalk between the PhoPQ and PmrAB two component systems in response to low pH, magnesium, phosphate, and iron availability (108, 109). However, the environmental signals and transcription regulators that dictate modification specificity are largely unknown. Nevertheless, lipid A hydroxylation was recently shown in *Salmonella enterica* to be directed by Fnr and ArcA in response to oxygen availability (110).

Iron is a limiting nutrient that is required for bacterial growth. Within mammalian systems, extracellular iron is bound to the transport protein, transferrin, that also serves a secondary innate immune function through sequestering this cation from potential pathogens (49). Nevertheless, pathogenic bacteria secrete iron-scavenging siderophores to steal this vital resource from the host. However, the host secretes iron transport proteins with higher affinity during infection such as lactoferrin to restrict bacterial growth (49). Neutrophils within the respiratory tract also secrete lipocalin 2 during infection which serves to further starve bacteria of iron by binding to, and inactivating,

siderophores (111). To circumvent these host defenses, *K. pneumoniae* secretes multiple structurally distinct iron-chelating siderophores including enterobactin, which has a higher affinity for iron than lactoferrin, and yersiniabactin, which is not inhibited by lipocalin 2 (112, 113). Furthermore, hypervirulent, hyper-encapsulated strains of *K. pneumoniae* isolated from respiratory tract infections often express a third siderophore, aerobactin (114). As is the case with other Enterics, iron acquisition is transcriptionally regulated by IscR and Fur in *K. pneumoniae,* in response to iron availability (103).

Interactions with the host and indwelling devices have been shown to induce type 3 fimbriae-mediated biofilm formation in *K. pneumoniae*. Type 3 fimbriae, or Mrk fimbriae, have been extensively studied in *K. pneumoniae* and facilitate cell adhesion to a range of biotic and abiotic substrates including type IV & type V collagen, silicone, and hard plastics (115-118). Although type 3 fimbriae are not directly involved in *K. pneumoniae* virulence, their requirement for colonization and persistence in catheter-associated urinary tract infections (CAUTI) has been demonstrated by multiple groups (119, 120), and these structures are believed to play analogous roles in ventilator associated pneumonia (VAP) (14). Transcriptional regulation of the *mrk* locus is not well understood, but is dependent on the intracellular accumulation of the secondary messenger, cyclic-di-GMP and the activities of multiple integrated regulatory networks (121-127). Extensive research efforts have determined that the LuxR-family transcription regulator MrkI directly interacts with the PilZ-domain containing MrkH protein upon cyclic-di-GMP accumulation to stimulate type 3 fimbriae locus transcription, while the MrkJ phosphodiesterase degrades cyclic-di-GMP to repress transcription from the *mrk* promoter (121, 124, 126, 127). Surprisingly however, the environmental signals and

diguanylate cyclases acting upstream of MrkH/I and MrkJ to promote type 3 fimbriae expression are still largely unknown, particularly in the context of infection. Recent reports however, have identified oxidative stress and iron-responsive transcription regulators that indirectly influence the expression of these adhesins (123, 125, 128). The known virulence factors of *K. pneumoniae* are summarized in **Figure 1.3.**

1.3.3 *Stenotrophomonas maltophilia*

1.3.3.1 Nosocomial pneumonia

Little is known regarding the molecular interactions that occur between *S. maltophilia* and the host during infection. *S. maltophilia* infections are often associated with underlying malignancies and prior broad-spectrum antibiotic therapy in severely immunodeficient individuals (40). Several groups have used mouse models of acute pneumonia to examine the pulmonary immune response mounted against *S. maltophilia*. These studies collectively revealed that *S. maltophilia* elicits an excessive proinflammatory response that results in prolonged neutrophil recruitment and activation (129-131). This aberrant response is thought to be driven by TNFα overproduction by alveolar macrophages resulting from enhanced TLR4-mediated signaling due to structural heterogeneity within the LPS of *S. maltophilia* (130). TLR5-mediated signaling is also thought to substantially contribute to this proinflammatory response (131), which is predicted to further exacerbate immune function in patients with CF.

The low virulence of *S. maltophilia* in mammalian models (129, 132) has hindered research efforts into understanding pathogenicity mechanisms as a lung

infection model that supports net bacterial growth does not exist. Nevertheless, tissue culture-based approaches have been useful in identifying virulence-associated genes and characterizing their potential contributions to pathogenesis. *S. maltophilia* clinical isolates cultured on airway epithelial cells demonstrated that this organism secreted enzymes with lytic and cytotoxic capacities (133). The activity of these enzymes was later determined to be dependent on the expression of the Xps type II secretion system of *S. maltophilia* (134), which secretes the StmPr1, StmPr2, and StmPr3 proteases that cause cell rounding, actin rearrangements, and cell death of airway epithelial cells *in vitro* (135, 136). Although the *in vivo* contribution of these secreted proteases to virulence is uncertain, serological evidence indicates that the StmPr1 protease is expressed by *S. maltophilia* during acute and chronic respiratory tract infections (130), and is likely to contribute to airway inflammation (136).

The ability of *S. maltophilia* to generate biofilms on a range of biotic and abiotic surfaces is thought to contribute to pathogenesis through facilitating deposition into the respiratory tract, increasing resistance to antibiotics, and inhibiting killing/clearance by the immune system (38, 39). *S. maltophilia* readily adheres to and generates biofilms on surfaces associated with the respiratory tract, including cultured human bronchial epithelial cells (137), CF-derived bronchial epithelial cells (138), and mouse tracheal mucus (139). While biofilm formation in *S. maltophilia* is poorly understood, adherence to cultured respiratory tract epithelial cells and mouse endotracheal mucus has been shown to be flagella-dependent (138-140). Several studies have examined the environmental conditions influencing biofilm production in *S. maltophilia*, and identified mildly acidic pH, temperatures between 32 ˚C and 37 ˚C, and aerobic conditions as

strong stimulators of biofilm formation (141). Similarly, the concentrations of phosphate, iron, and chloride ions have also been shown to influence biofilm production in this organism (39, 142). Cellular motility and biofilm formation in *S. maltophilia* was recently determined to be controlled through cyclic-di-GMP-mediated signaling via the BmsRT two component system, with additional regulatory input from several other factors that have not yet been identified (143). Not surprisingly, cell motility and biofilm formation are also influenced by quorum signaling, which is mediated through a DSF (diffusible signal factor) system in *S. maltophilia* and other *Xanthomonads* via RpF-1 in response to the fatty acid, cis-11-methyl-2-dodecenoic acid (144). **Figure 1.4** summarizes the known or suspected virulence factors of *S. maltophilia*.

1.3.3.2 Chronic CF infection

Like *P. aeruginosa*, *S. maltophilia* is believed to adapt to the CF lung over the course of chronic infection. *S. maltophilia* is also thought to lose motility and the capacity to form surface-attached biofilms within the CF lung, most likely due to selection pressures associated with the immunogenic properties of this organism's flagellin. Furthermore, *S. maltophilia* clinical CF isolates exhibit higher mutation frequencies relative to strains of environmental origin (145), and also generate hypermutator phenotypes during chronic infection through mutations within mismatch repair genes including *mutS*, *mutL,* and *uvrD* (145-148). The development of such strains is believed to promote intrastrain phenotypic diversity and increased antibiotic resistance that typifies *S. maltophilia* CF infections (148). Multiple lines of evidence suggest that like *P.*

aeruginosa, S. maltophilia also decreases the expression of virulence factors over the course of chronic infection. Studies examining the virulence phenotypes of *S. maltophilia* clinical CF isolates have identified numerous strains lacking protease activity that often correlate with frameshift and nonsense mutations in the genes encoding the StmPr1 and StmPr2 secreted serine proteases (146, 147). These observations are also backed by serological evidence, as antibody titers against the StmPr1 protease have been shown to diminish over time during chronic CF infection (130). Interestingly however, *S. maltophilia* does not adopt the hypermucoid phenotype that characterizes CF-adapted *P. aeruginosa* strains, which likely contributes to the increased airway inflammation and systemic neutropenia associated with these chronic infections.

1.4 The host as a nutrient source

The ability to obtain nutrients from the host is critical to the success of pathogenic bacteria during infection (149). Despite this long-standing knowledge, the metabolic requirements of bacteria during infection remains poorly understood (150). This is especially true among extracellular opportunistic pathogens of environmental origin that have evolved outside of their incidental host, and are capable of metabolizing an enormous variety of carbon and nitrogen sources for growth.

As antibiotics continue to lose their effectiveness clinically, the need for alternative therapeutics becomes more and more imperative. One promising treatment strategy involves designing therapeutics that alter or disrupt the metabolism of pathogenic bacteria during infection through either inhibiting growth or increasing the

efficacy of antibiotics. The potential success of such treatments was recently demonstrated *in vitro* in *P. aeruginosa,* where the authors observed that the addition of fumarate to bacterial culture media greatly enhanced this organism's susceptibility to the antibiotic, tobramycin-the drug of choice for treating chronic *P. aeruginosa* CF infections (151). Below, the host-derived molecules that are known to be metabolized by *P. aeruginosa, K. pneumoniae,* and *S. maltophilia* during acute and chronic respiratory tract infections are briefly summarized.

1.4.1 *P. aeruginosa*

P. aeruginosa actively degrades phospholipids and proteins within pulmonary surfactant during acute infection. Phosphatidylcholine comprises roughly 70 % of the dry weight of pulmonary surfactant, making it one of the most abundant extracellular nutrient sources available to bacteria growing within the lumen of the lung. During infection, *P. aeruginosa* secretes the hemolytic phospholipase C, PlcH, to liberate phosphocholine (81), which is then further degraded to obtain the osmoprotectant, glycine betaine (86, 152). *P. aeruginosa* is also capable of further catabolizing glycine betaine as a carbon and nitrogen source, and a transcriptional profiling study with purified pulmonary surfactant demonstrated that this occurs *in vitro* (87). PlcH activity, in combination with the neutral ceramidase, CerN, within the lung also degrades sphingomyelin found in pulmonary surfactant, leading to the release of the antimicrobial, sphingosine. To circumvent sphingosine-mediated killing, *P. aeruginosa* expresses *sphA* and *sphBCD*, which are thought to prevent sphingosine-mediated disruption of the cell membrane and degrade

this sphingosine, respectively (87). Aside from metabolizing phospholipids within surfactant, *P. aeruginosa* also secretes LasA, LasB, and Protease IV during acute respiratory tract infection that degrade the four surfactant-associated proteins found within pulmonary surfactant (83, 84).

Hypoxic microenvironments exist in mucus plugs found within the CF lung due to continuous reactive oxygen and nitrogen species production by activated neutrophils (153, 154). Chronic respiratory bursts mediated by these lymphocytes have been shown to generate nitrate as a biproduct, which is abundant in the CF lung, and actively utilized as a terminal electron acceptor by *P. aeruginosa* to permit anaerobic growth in these anoxic microenvironments (155, 156). The capacity of *P. aeruginosa* to utilize nitrate for anaerobic respiration within the CF lung has been suggested to promote persistence and survival against clearance by the host immune system (155, 157). In addition, transcriptional profiling studies performed with native and synthetic CF sputum revealed that the amino acids: alanine, proline, and arginine, are likely to serve as the preferred nutrient sources of *P. aeruginosa* during growth within the CF lung (158, 159). Moreover, the importance of alanine catabolism to *P. aeruginosa* fitness has been demonstrated in a rat model of chronic lung infection (160).

1.4.2 *K. pneumoniae*

Few investigations have examined the metabolic requirements of *K. pneumoniae* that permit colonization and growth during infection. Outside of the lung, the use of allantoin as a carbon and nitrogen source has been linked to *K. pneumoniae* virulence in a

liver abscess model of infection (161). Within the respiratory tract, an *in vivo* transposon-based genetic screen revealed the necessity of endogenous branched chain amino acid production to *K. pneumoniae* fitness during acute pneumonia (162). In a separate study, a Pld1-family phospholipase D was demonstrated to be required for *K. pneumoniae* virulence in a mouse model of acute pneumonia (163). While the substrate of this enzyme has yet to be conclusively identified, thin layer chromatography, bioinformatics, and trans complementation experiments suggest that this phospholipase participates in the metabolism of phosphatidylglycerol and cardiolipin (163). Phosphatidylglycerol is prevalent within the respiratory tract as a major constituent of pulmonary surfactant (58), which could be an important nutrient source for *K. pneumoniae* during infection.

1.4.3 *S. maltophilia*

Perhaps unsurprisingly, the metabolic requirements and nutritional preferences of *S. maltophilia* during infection have never been investigated. Since *S. maltophilia* is a methionine auxotroph and obligate aerobe (39), it is assumed that aerophilic conditions and the ability to obtain this amino acid from the host are prerequisite to this organism's success during infection. Since *S. maltophilia* lacks canonical virulence factors, targeting the metabolic pathways used by this organism during infection could be a promising avenue for developing alternative therapeutics.

1.5. Dissectible models of the lung for examining host-pathogen interactions

Opportunistic pathogens require specific conditions within their immunocompromised hosts to cause disease, which can be difficult and sometimes impossible to replicate in *in vivo* animal infection models. Such is the case for modeling the damaged, inflamed conditions of the adult CF lung that exists prior to the establishment of chronic *P. aeruginosa* or *S. maltophilia* infections. Similarly, accurate mouse pneumonia models do not exist for examining the pathogenesis mechanisms of bacteria with low virulence potential, including *P. aeruginosa* and *S. maltophilia* which fail to grow in the lungs of healthy mice. Nevertheless, mouse pneumonia models that measure relative bacterial clearance rates as a proxy for virulence have proven useful for identifying genes and metabolic pathways that influence the pathogenesis of these organisms.

Despite their utility, animal lung infection models often fail to reveal the mechanistic contribution of virulence-associated genes to the pathogenesis of these bacteria (150). As a result, we often know the identity of many of the genes that permit virulence within organisms like *P. aeruginosa*, but have a limited understanding of their function, or how interactions with the host influence their expression *in vivo*. Bacterial transcriptomics-based studies using molecularly defined components of the host lung environment have been particularly effective in elucidating the bacterial response to the host and identifying the host-derived signals that promote pathogenesis (86, 87, 157-159, 164, 165). Below, pulmonary surfactant and synthetic CF sputum are described as dissectible models of the lung infection milieus for examining bacterial interactions with the host during pneumonia and chronic CF infection.

1.5.1 Pneumonia: purified pulmonary surfactant

Pulmonary surfactant serves as an initial point of contact for aspirated or inhaled pathogens upon deposition into the lung. This phospholipid-rich mixture coats the alveolar surfaces at the air-liquid interface and serves to reduce surface tension within the lung to prevent collapse following expiration (58, 166). Pulmonary surfactant also modulates the activity of inflammatory cells and directly participates in the innate immune response through the activities of the surfactant-associated collectins, SP-A and SP-D (57, 167, 168). Extracellular bacteria growing within the lung are constantly exposed to pulmonary surfactant, and this substance is thought to serve as a nutrient source for these organisms during colonization and early in infection.

Pulmonary surfactant contains nearly one hundred unique components, including four surfactant associated proteins (SP-A, SP-B, SP-C, SP-D), as well as a much larger lipid fraction comprising the bulk of this substance. Dipalmitoylphosphatidylcholine and mixed-tail phosphatidylcholines are the major lipid constituents of surfactant, making up nearly 70 % of the total lipid content, followed by phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, and sphingomyelin. Pulmonary surfactant additionally contains various free fatty acids, triglycerides, and neutral lipids such as cholesterol that make up the remainder of this substance **(Figure 1.5)** (56, 58, 169).

Several purified pulmonary surfactant preparations are commercially available that are used clinically to treat infant respiratory distress syndrome (170, 171). In addition to their clinical use, purified pulmonary surfactant preparations have proven their value to researchers seeking to understand how respiratory pathogens interact with the host at the

site of infection. Previous transcriptional profiling studies by our group with the purified surfactant preparation, Survanta, led to the determination that the detection of sphingosine and the metabolism of the choline moiety of phosphatidylcholine by *P. aeruginosa* are both independently required for full virulence in a mouse model of acute pneumonia (86, 87, 152). In a thematically similar study, Ishii *et al.* determined that fatty acids within pulmonary surfactant invoked a membrane stress response in *Staphylococcus aureus*, and also identified a novel virulence determinant implicated in this process (165). Together, these studies demonstrate the utility of purified pulmonary surfactant preparations for identifying and dissecting host-lung pathogen interactions in the context of acute infection.

1.5.2 The CF lung: Synthetic Cystic Fibrosis Sputum Media (SCFM2)

The thick, viscous mucus that accumulates within the CF lung is comprised of heavily glycosylated mucin, high molecular weight DNA, serum components, and cell debris resulting from chronic infection (158, 159). This substance is expectorated as sputum and serves as the primary nutrient source for microbes colonizing the CF lung. Moreover, sputum has been shown to contain many of the host-derived signals that promote the virulence-associated phenotypes of CF pathogens such as *P. aeruginosa* and *Burkholderia cenocepacia* (159, 164, 172, 173).

Several artificial sputum medias have been developed for use in CF pathogen-host interaction studies in attempts to circumvent the difficulties associated with obtaining, purifying, and standardizing sputum from patients with CF. Of these various formations,

synthetic cystic fibrosis sputum media (SCFM2) most accurately reflects native CF sputum (158, 174). This defined media was developed in two stages, and contains the average concentration of each amino acid, ion, and other carbon/nitrogen sources in CF sputum, as measured via mass spectrometry in sputum samples collected from twelve individuals with CF (158). Phosphatidylcholine, mucin, and extracellular DNA were later added to this media in order to more closely reflect the bacterial growth milieu within the CF lung (174). The composition of this media, referred to as "SCFM2," is shown in **Table 1.2**. SCFM2 affords a powerful, dissectible, model for understanding how CF pathogens interact with this critical aspect of the host lung environment.

1.6 Dissertation Overview

The increasing prevalence of MDR respiratory pathogens poses a serious threat to public health and has made it clear that new treatments are required to treat these infections. To aid in the development of new therapeutics, a greater understanding of how these organisms transition from the environment to the host lung is needed. The research described in the following chapters focuses on expanding our knowledge of how *P. aeruginosa, K. pneumoniae,* and *S. maltophilia* recognize, exploit, and adapt to the host lung environment. These chapters are introduced and briefly summarized below.

Extensive research efforts have revealed many of the virulence and metabolism-related genes within *P. aeruginosa* that influence pathogenesis in both acute and chronic respiratory tract infections. Despite this knowledge, many of the regulatory systems within *P. aeruginosa* that coordinate virulence and metabolism during infection have not

been characterized. As mentioned in above, phosphatidylcholine comprises the bulk of human pulmonary surfactant and serves as an important source of choline for *P. aeruginosa* during infection (58, 152). We previously observed that the most highly expressed genes by *P. aeruginosa* following exposure to pulmonary surfactant were those involved in the catabolism of a downstream choline metabolite, sarcosine. Although our group had earlier characterized the choline catabolic pathway of *P. aeruginosa,* the transcriptional regulation of sarcosine catabolism was not known. In Chapter 2 of this dissertation, we describe the identification and characterization of SouR as the first known sarcosine-responsive transcription regulator. We also identified a formaldehyde-responsive transcription regulator, GfnR, that controls the metabolism of formaldehyde released through the oxidative demethylation of sarcosine and glycine betaine.

In contrast with *P. aeruginosa,* the interactions between *K. pneumoniae* and the host lung environment are far less understood. In Chapter 3, we characterized the transcriptional response of *K. pneumoniae* to purified bovine pulmonary surfactant to gain insight into how this organism interreacts with this critical aspect of the lung environment. This work revealed that pulmonary surfactant invokes a transcriptional response in *K. pneumoniae* that supports host colonization, adaptation, and virulence *in vivo*. We also determined that pulmonary surfactant promoted type 3 fimbriae-mediated biofilm formation in *K. pneumoniae* and identified two components of pulmonary surfactant that drive this response (phosphatidylcholine and cholesterol). We also examined the contribution of metabolism-related surfactant-induced transcripts to *K. pneumoniae* pathogenesis using engineered gene deletion strains and a mouse model of pneumonia. In doing so, we identified the polyamine efflux pump, MdtJI, and the glycine

betaine transporter, ProU as required for *K. pneumoniae* virulence within the respiratory tract.

Finally, in Chapter 5 we characterized the transcriptional responses of three *S. maltophilia* strains during growth in synthetic CF sputum media (SCFM2) to examine how this organism interreacts with the host and utilizes nutrients within the CF lung. These efforts led to the identification of nearly 250 transcripts expressed by all three strains that largely reflect nutrient utilization by *S. maltophilia* during CF lung infection. In this chapter, we also compared the SCFM2 transcriptomes of two *S. maltophilia* CF isolates with the SCFM2 transcriptome of the acute infection model strain, *S. maltophilia* K279A. This revealed CF isolate-specific signatures in gene expression reflective of adaptation to the CF lung, including the repression of genes involved in cell motility and biofilm formation, and increased expression of oxidative stress-related genes as well as alternative cytochromes associated with growth in microaerophilic environments. Finally, we also demonstrated that these transcriptional changes correlated with phenotypes observed *in vitro*, as the *S. maltophilia* CF isolates failed to form surface-adhered biofilms in SCFM2 and were less susceptible to killing via oxidative stress than K279A.

This work collectively provides novel insight into the interactions occurring between *P. aeruginosa, K. pneumoniae,* and *S. maltophilia* and the host during the initiation of lung infection. Using a combination of molecular genetics and transcriptomics, we revealed novel transcription regulators involved in the detection of the host, as well as metabolism and virulence-associated genes within these MDR Gram-negative lung pathogens that will be the subject of future research endeavors.

1.7 Chapter 1 Figures

Figure 1.1 Airway immune defenses protecting against bacterial infections.

Aspirated or inhaled bacteria entering the lung must overcome significant immune defenses to colonize and initiate infection. Bacteria entering the lung become trapped within the mucus layer and cleared from the respiratory tract via the mucociliary escalator. Numerous antimicrobial peptides are also found within the airways that serve to lyse bacterial pathogens through disrupting their cell membranes. Bacteria within the lung are also recognized by the complement system and collectins found within surfactant, that result in opsonization and clearance by macrophages or infiltrating neutrophils. Flagella and LPS on the surfaces of Gram-negative bacteria are recognized by TLR's expressed by alveolar epithelial cells and resident macrophages that invoke a pro-inflammatory response. During infection, activated alveolar macrophages secrete cytokines and pro-inflammatory signals that recruit neutrophils to the respiratory tract to kill and clear these bacteria. Figure adapted from: *Gellatly & Hancock* (81) and reused with permission from the publisher.

Figure 1.2 Known virulence factors of *Pseudomonas aeruginosa*.

P. aeruginosa encodes a multitude of virulence factors that serve to disrupt host immune function and invoke tissue damage during acute respiratory tract infections. Following contact with host cells, *P. aeruginosa* injects several cytotoxins through a type III secretion system that impair phagocytosis and trigger apoptosis. *P. aeruginosa* also utilizes two type II secretion system to deliver a variety of phospholipases, proteases, and toxins into the lung milieu that cause significant tissue damage. These enzymes degrade

the phospholipids and proteins within pulmonary surfactant and damage epithelial cell membranes. *P. aeruginosa* also secretes pyocyanin during infection which disrupts the electron transport chain in host cells and impairs the immune response through inhibiting catalase activity and triggering neutrophil apoptosis. Flagella and type 4 pili are also used by *P. aeruginosa* during acute infection to adhere to host cells and trigger type III secretion activity. The immunostimulatory nature of these structures significantly contribute to inflammation during infection. Figure reused with permission from the publisher(81).

Figure 1.3 Known virulence factors of *Klebsiella pneumoniae*

K. pneumoniae possesses four types of virulence factors that allow this organism to generate biofilms on a variety of surfaces, elude detection by the immune system, and obtain iron from the host during infection. *K. pneumoniae* is able to adhere to a variety of biotic and abiotic surfaces via type 1 and type 3 fimbriae. *K. pneumoniae* is able to evade detection by the host during infection via capsule production and various LPS lipid A modifications. *K. pneumoniae* also secretes multiple structurally distinct iron-chelating siderophores that circumvent the hosts ability to sequester iron, including enterobactin, which has a higher affinity for iron than lactoferrin, and yersiniabactin, which is not inactivated by lipocalin 2. Hypervirulent strains of *K. pneumoniae* are distinguished from classical strains by the overproduction of K1 or K2 serotype capsules and the expression of additional siderophores, such as aerobactin and salmochelin. Figure reused with permission from the publisher (14).

Figure 1.4 Known virulence factors of *Stenotrophomonas maltophilia*.

Little is known regarding the molecular interactions that occur between *S. maltophilia* and the host during infection. However, flagella and fimbriae are expressed by *S. maltophilia* that facilitate colonization of various medical devices, deposition within the lung, and adherence to alveolar epithelial cells during infection. The expression of these appendages, coupled with the structural heterogeneity found within *S. maltophilia's* LPS, invoke an excessive pro-inflammatory response during infection that exacerbates neutropenia and inflicts tissue damage. In addition, *S. malophilia* utilizes a type II secretion system to deliver several proteases into the host lung milieu that induce alveolar epithelial cell rounding and apoptosis. The extreme intrinsic drug resistance exhibited by *S. maltophilia* is also thought to provide a competitive advantage against more virulent, drug-susceptible species that facilitates infection of patients following antibiotic therapy.

Figure 1.5 Composition of pulmonary surfactant.

Pulmonary surfactant contains nearly one hundred unique components, including four surfactant associated proteins (SP-A, SP-B, SP-C, SP-D), and larger lipid fraction that comprises nearly 90 % of the dry weight of this substance. Within the lipid fraction, dipalmitoylphosphatidylcholine (DPPC) and mixed-tail phosphatidylcholines (PC) are the major constituents, making roughly 70 % of the total lipid content. Other phospholipids are also found within pulmonary surfactant, including phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE), and sphingomyelin (SPM). Free fatty acids, triglycerides, and neutral lipids such as

cholesterol (Chol) are also found within this substance. Figure reused with permission (175).

1.8 Chapter 1 Tables

Table 1.1 Prevalence of the ten most common pathogens isolated from patients with nosocomial pneumonia.

The worldwide and regional incidence of pathogens isolated from more than 31,000 patients hospitalized with pneumonia between 2004 and 2009. This data was collected and compiled via the SENTRY Antimicrobial Surveillance Program. Table reused with permission from the publisher (7).

Table 1.2 Composition of Synthetic Cystic Fibrosis Sputum Media (SCFM2)

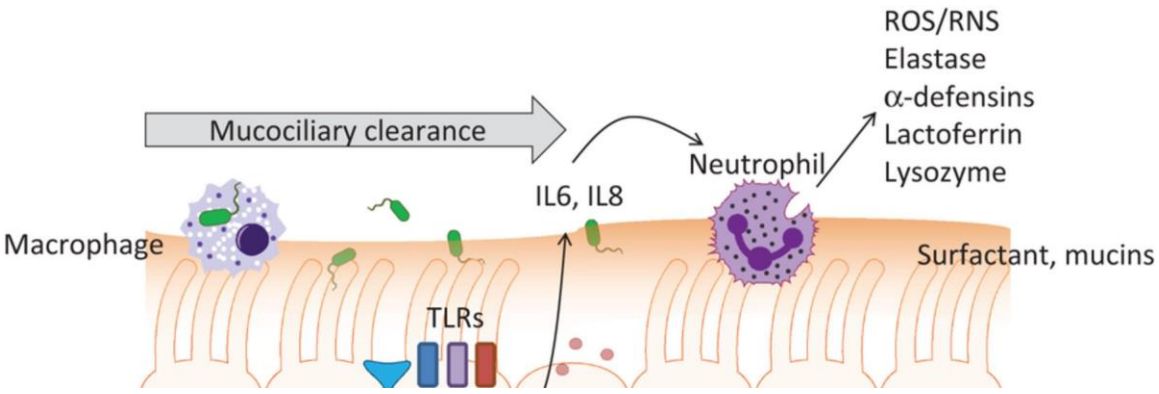

Figure 1.1 Airway immune defenses protecting against bacterial infections.

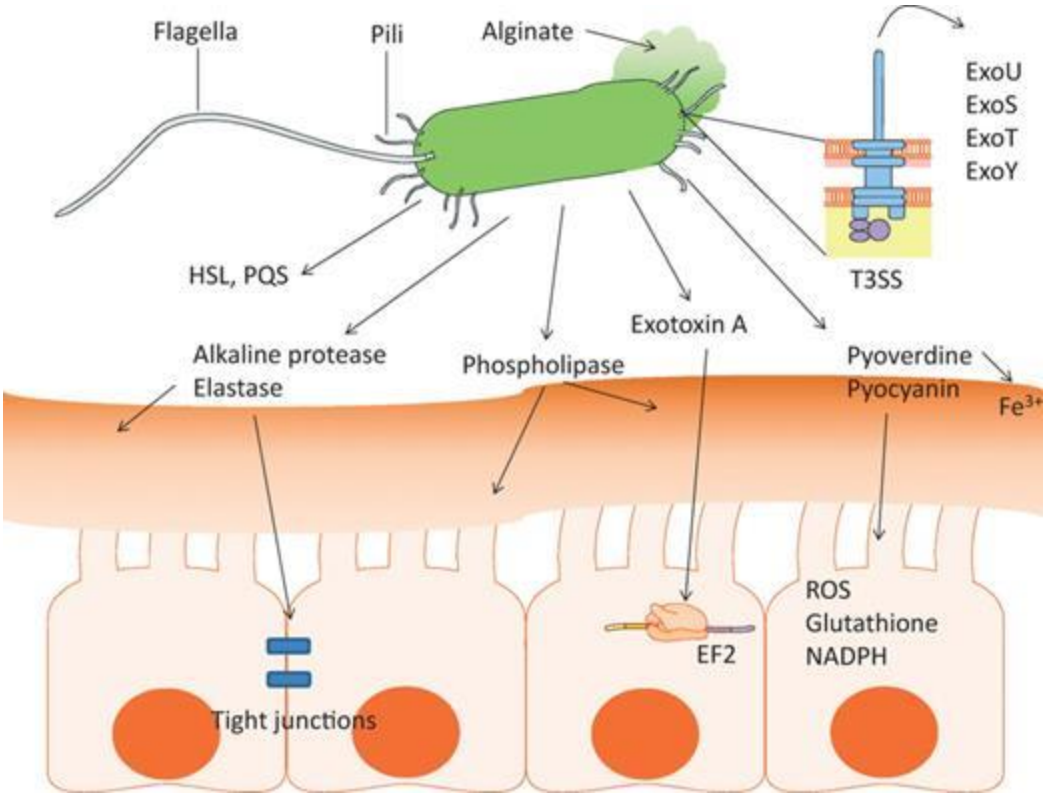

Figure 1.2 Known virulence factors of *Pseudomonas aeruginosa*

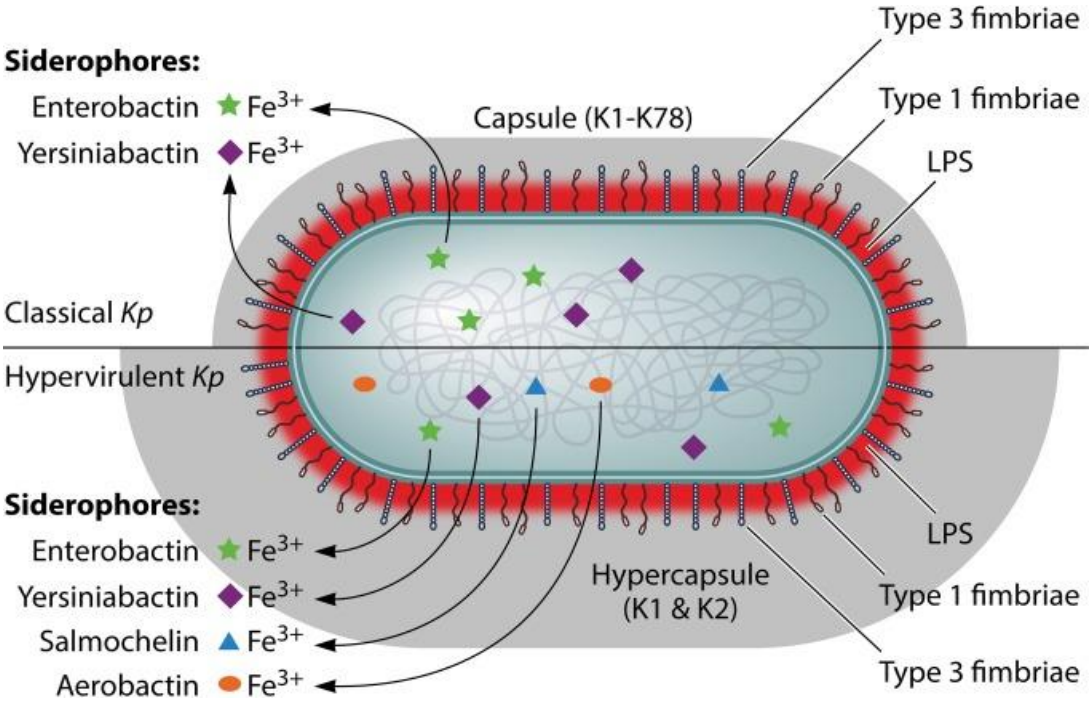

Figure 1.3 Known virulence factors of *Klebsiella pneumoniae*

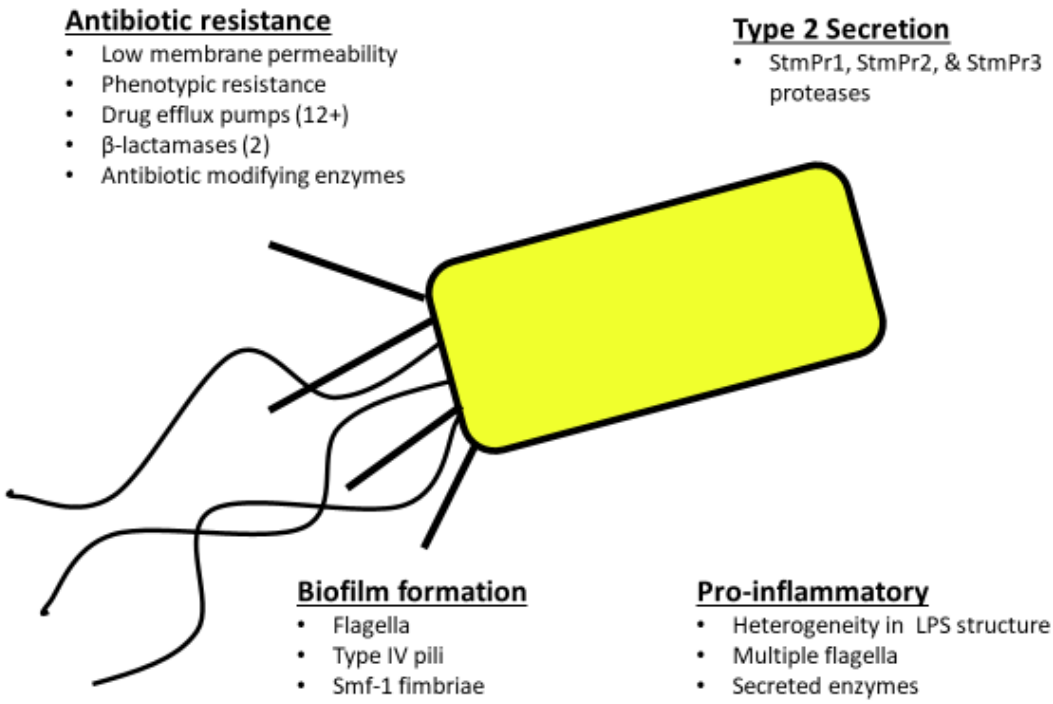

Figure 1.4 Known virulence factors of *Stenotrophomonas maltophilia*

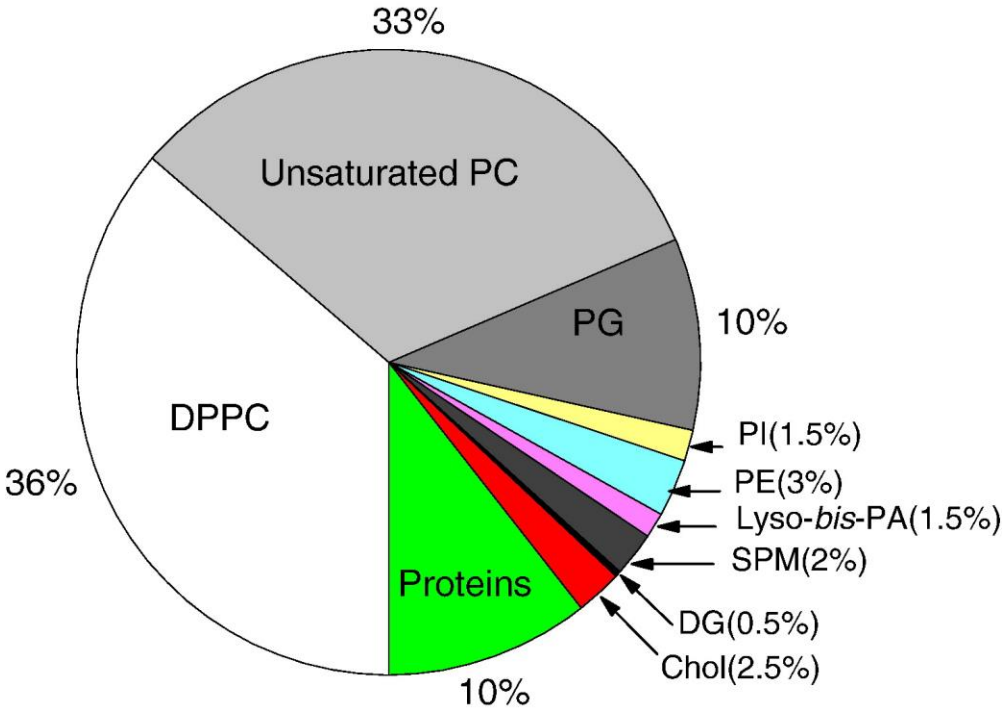

Figure 1.5 Composition of pulmonary surfactant

Table 1.1 Prevalence of the ten most common pathogens isolated from patients with nosocomial pneumonia

Pathogen	Incidence, %			
	All regions	United States	Europe	Latin America
Staphylococcus aureus	28.0	36.3	23.0	20.1
Pseudomonas aeruginosa	21.8	19.7	20.8	28.2
Klebsiella species	9.8	8.5	10.1	12.1
Escherichia coli	6.9	4.6	10.1	5.5
Acinetobacter species	6.8	4.8	5.6	13.3
Enterobacter species	6.3	6.5	6.2	6.2
Serratia species	3.5	4.1	3.2	2.4
Stenotrophomonas maltophilia	3.1	3.3	3.2	2.3
Streptococcus pneumoniae	2.9	2.5	3.6	2.4
Haemophilus influenzae	2.7	2.5	3.7	1.3

Table 1.2 Composition of Synthetic Cystic Fibrosis Sputum Media II (SCFM2)

Amino acids	(mM)	Ions	(mM)
Serine	1.4	Na^+	66.6
Threonine	1.0	K^+	15.8
Alanine	1.8	NH_4^+	2.3
Glycine	1.2	Ca^{2+}	1.7
Proline	1.7	Mg^{2+}	0.6
Isoleucine	1.1	Cl^-	79.1
Leucine	1.6	NO_3^-	0.35
Valine	1.1	PO_4^{3-}	2.5
Aspartate	0.8	SO_4^{2-}	0.27
Glutamate	1.5		
Phenylalanine	0.5		
Tyrosine	0.8	**Other**	
Tryptophan	0.01	Glucose	3.2 mM
Lysine	2.1	Lactate	9.0 mM
Histidine	0.5	$FeSO_4$	3.6 µM
Arginine	0.3	DNA	0.6 mg/ml
Ornithine	0.7	Mucin	5.0 mg/ml
Cysteine	0.2	Phosphatidylcholine	0.1 mg/ml
Methionine	0.6	GlcNAc	0.3 mM

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CHAPTER 2:

Sarcosine catabolism in *Pseudomonas aeruginosa* is transcriptionally regulated by SouR

Graham G. Willsey[1] and Matthew J. Wargo[1,2]

[1] Department of Microbiology and Molecular Genetics and [2] The Vermont Lung Center, University of Vermont College of Medicine, Burlington, VT 05405

2.1 Abstract

Sarcosine (N-methylglycine) is present in many environments inhabited by *Pseudomonas* and is likely most often encountered as an intermediate in the metabolism of choline, carnitine, creatine, and glyphosate. While the enzymology of sarcosine metabolism has been relatively well studied in bacteria, the regulatory mechanisms governing catabolism have remained largely unknown. We previously determined that the sarcosine catabolic operon (*sox* operon) of *P. aeruginosa* is induced by the AraC-family regulator GbdR in response to glycine betaine and dimethylglycine. However, induction of these genes was still observed in response to sarcosine in a *gbdR* deletion mutant, indicating that an independent sarcosine-responsive transcription factor also acted at this locus. Our goal in this study was to identify and characterize this regulator. Using a transposon-based genetic screen, we identified PA4184, or SouR (Sarcosine oxidation & utilization Regulator), as the sarcosine-responsive regulator of the *sox* operon, with tight induction specificity for sarcosine. The *souR* gene is required for appreciable growth on sarcosine as a carbon and nitrogen source. We also characterized the transcriptome response to sarcosine governed by SouR using microarray analyses and performed electrophoretic mobility shift assays to identify promoters directly regulated by this transcription factor. Finally, we characterized PA3630, or GfnR (Glutathione-dependent formaldehyde neutralization Regulator), as the regulator of the glutathione-dependent formaldehyde detoxification system in *P. aeruginosa* that is expressed in response to formaldehyde released during the catabolism of sarcosine. This study expands our understanding of sarcosine metabolic regulation in bacteria through the

identification and characterization of the first known sarcosine-responsive transcriptional regulator.

Importance

The *Pseudomonas aeruginosa* genome encodes many diverse metabolic pathways, yet the specific transcription regulators controlling their expression remain mostly unknown. Here we used a genetic screen to identify the sarcosine-specific regulator of the sarcosine oxidase operon, which we have named SouR. SouR is the first bacterial regulator shown to respond to sarcosine and it is required for growth on sarcosine. Sarcosine is found in its free form and is also an intermediate in the catabolic pathways of glycine betaine, carnitine, creatine, and glyphosate. The similarity of SouR to the regulators of carnitine and glycine betaine catabolism suggests evolutionary diversification within this regulatory family to allow response to structurally similar but physiologically distinct ligands.

2.2 Introduction

Pseudomonas aeruginosa and other bacteria from similar environments are capable of utilizing sarcosine (N-methylglycine) as a carbon and nitrogen source for growth (1-3). Sarcosine is present in many environments inhabited by *Pseudomonads*, and it is also produced as an intermediate in the metabolism of choline, carnitine, creatine, and glyphosate (**Fig. 2.1A**). Choline is abundant in many eukaryote-associated environments including clinically important sites of opportunistic infection by *P. aeruginosa,* such as the lung (4), where phosphatidylcholine constitutes an estimated 85% of the dry weight of human pulmonary surfactant (5). Within this environment, *P. aeruginosa* acquires choline from phosphatidylcholine via the virulence factors phospholipase C (PlcH) and phosphorylcholine phosphatase (PchP) (6, 7). Burns and deep lacerations also expose *P. aeruginosa* to readily available sarcosine precursors, including carnitine in muscle tissue and choline released from damaged cell membranes (7, 8). Furthermore, *Pseudomonas putida* and some isolates of *P. aeruginosa* can metabolize creatine to generate sarcosine (9-11), while other Pseudomonads obtain sarcosine through metabolism of the herbicide glyphosate (12-14).

Aerobic bacterial sarcosine catabolism proceeds via oxidative demethylation catalyzed by one of two classes of sarcosine oxidase. Monomeric sarcosine oxidases are the simplest form of these enzymes and produce glycine, hydrogen peroxide, and formaldehyde from sarcosine (15). In contrast, heterotetrameric sarcosine oxidases (TsoX) are more complex and assimilate the N-methyl group of sarcosine into the C1 carbon pool through a 5,10-methylenetetrahydrofolate intermediate instead of releasing it as formaldehyde (15, 16). In *P. aeruginosa* and a variety of soil bacteria, TsoX is

encoded in an operon as *soxBDAG* (**Fig. 2.1B**), along with a serine hydroxymethyltransferase *glyA1*, and the 10-formyltetrahydrofolate hydrolase *purU2* (17-19), which together function to transform sarcosine into metabolites used for energy production and biosynthesis. In the absence of sufficient tetrahydrofolate, TsoX demethylation of sarcosine releases formaldehyde (15, 16), and *P. aeruginosa* and other Proteobacteria encode a sarcosine-inducible glutathione-independent formaldehyde dehydrogenase (*fdhA)* adjacent to the *soxBDAG* locus that converts formaldehyde to formate and generates reducing potential through NADH synthesis (20).

Although the enzymology of sarcosine catabolism has been relatively well studied in bacteria, the regulatory mechanisms governing this process are largely unknown. We previously determined that expression of the *sox* operon of *P. aeruginosa* is induced in response to glycine betaine and dimethylglycine through the AraC-family regulator, GbdR (21, 22). Consistent with previous reports however (1, 2), we also observed induction of this operon in response to sarcosine in a *gbdR* deletion mutant, indicating that an independent sarcosine-responsive transcription factor also acts at this locus (21).

Here we report the identification and characterization of the first known sarcosine-responsive transcription factor, PA4184, which we have named SouR (Sarcosine oxidation & utilization Regulator). SouR regulates the *soxBDAG* operon in *P. aeruginosa*, and we have determined that it is necessary for appreciable growth when sarcosine is utilized as a sole carbon and nitrogen source. We further determined that transcriptional activation by SouR is specific for sarcosine and characterized the transcriptome response to sarcosine governed by this regulator. During this research we also characterized PA3630, which we have named GfnR (Glutathione-dependent

formaldehyde neutralization Regulator), as the regulator of the glutathione-dependent formaldehyde detoxification system in *P. aeruginosa* that is expressed during the catabolism of sarcosine.

2.3 Materials and Methods

2.3.1 Bacterial strains and growth conditions

P. aeruginosa PA14 wild-type, transposon mutants, and deletion strains (**Table 2.S1**) were maintained on Lennox Broth (LB) or Pseudomonas Isolation Agar (PIA) supplemented with 50 µg/mL gentamicin when appropriate. *E. coli* strains used in this study (**Table 2.S1**) were maintained on LB supplemented with gentamicin (7 µg/mL liquid, 10 µg/mL agar) or carbenicillin (100 µg/mL) when necessary. During genetic manipulations, selection for *P. aeruginosa* over *E. coli* was performed using PIA supplemented with 50 µg/mL gentamicin. Growth and selection conditions used in the genetic screen are described in detail below. Growth and transcriptional induction assays in *P. aeruginosa* were performed using MOPS (morpholinepropanesulfonic acid) minimal media (23) as modified by our group (8, 24, 25).

2.3.2 Construction of deletion strains, complementation constructs, and the sarcosine oxidase operon reporter

All amplifications and cloning steps were performed using Q5 DNA polymerase and restriction enzymes purchased from New England Biolabs (Ipswitch, MA). General nucleic acid procedures were performed using Qiagen kits unless otherwise noted. The

gene numbers generally referred to in this study are based on the PAO1 orthologs. Sequences for the primers used to generate each construct are listed in **Table 2.S2**.

In-frame chromosomal deletions of *souR (PA4184)* and *gfnR (PA3630)* were created using splice overlap extension (SOE) as previously described using pMQ30-based allelic replacement (26). Briefly, two ~ 1Kb regions directly upstream and downstream of the gene to be deleted were amplified from PA14 genomic DNA with primers PA14_9770KO_F1, PA14_9770KO_R1, PA14_9770KO_F2, PA14_9770KO_R2, and PA3630KO_F1, PA3630KO_ R1, PA3630KO_F2, PA3630KO_R2, ligated into pCR-Blunt (Invitrogen), and transformed into *E. coli* DH5α cells. After selection on kanamycin and plasmid preparation, overlap extension products were excised with XbaI and HindIII, gel purified, and ligated into similarly cut pMQ30 before being transformed into DH5α cells. Transformants were selected on LB with 10 μg/mL gentamicin, and plasmid DNA was purified from resistant colonies to generate the pGW008 *(Δ*s*ouR)* and pGW023 *(Δ*P*A3630)* deletion constructs. pGW008 and pGW023 were electroporated into the conjugative *E. coli* S17/λpir strain. Donor S17/λpir strains were mixed with recipient PA14 strains, and single-crossover mutants were selected for growth on PIA supplemented with 50 μg/mL gentamicin. Recombinants were verified by PCR after selecting for loss of *sacB* by growth on 5% sucrose LB plates lacking sodium chloride (26, 27) to yield strains GGW034 (PA14 Δ*souR*), GGW036 (PA14 Δ*gbdR* Δ*souR*), GGW076 (PA14 Δ*gfnR*), and GGW078 (PA14 Δ*gbdR* Δ*gfnR*).

The *souR* complementation construct included the *souR* open reading frame and native promoter cloned into pMQ80 using primers with engineered KpnI and HindIII restriction sites (PA14_9770_RescueF & PA14_9770_RescueR). This construct was

designated pGW007 (pSouR). Complementation of the Δ*gnfR* strain was achieved by chromosomal integration of the *gfnR* ORF and its native promoter at the *attTn7* site as described by Choi (28). Briefly, the *PA3630* gene and promoter region were amplified from PA14 genomic DNA using the primers PA3630_RescueF & PA3630_RescueR, which incorporated flanking HindIII and KpnI restriction sites. The amplified product was digested, ligated into similarly cut pUC18-mini-Tn*7*T-Gm, transformed into DH5α, and transformants were selected for gentamicin resistance. This construct was designated pGW024. pGW024 and pTNS2 were co-electroporated into the target strains as previously described (28, 29).

Chromosomal *soxB'-lacZYA-'soxG* operonic reporter strains were engineered through allelic replacement using a pMQ30-based strategy (26). Briefly, ~1 kb regions upstream of the *soxB* translational start site and ~ 1kb downstream of the *soxG* stop codon were amplified from PA14 genomic DNA with SOE-based primers (soxKO_F1, soxKO_R1, soxKO_F2, soxKO_R2) incorporating an engineered NcoI site into the overlap portion of the construct, ligated, transformed, and the resultant plasmid purified as described above. This plasmid was linearized between the *soxB* and *soxG* fragments with NcoI and treated with Klenow to generate blunt ends, which allowed ligation of *lacZYA* (obtained from pMW5 following KpnI & EcoRI digestion and Klenow treatment). Following transformation into DH5α cells, plasmid DNA was purified, digested with KpnI and HindIII to excise *soxB'-lacZYA-'soxG* for ligation into similarly-cut pMQ30, yielding pGW005. pGW005 was transformed into *E. coli* S17/λpir (GGW040) and mixed with PA14 recipient strains to create the chromosomal *soxB'-*

lacZYA-'soxG strains, which are effectively Δ*soxBDAG* and cannot grow on sarcosine, as *lacZYA* has replaced most of the operon.

2.3.3 Genetic screen to identify the sarcosine-responsive regulator of *soxBDAG* expression

Transposon mutagenesis was performed on PA14 Δ*gbdR soxB'-lacZYA-'soxG* (GGW039) via conjugation with *E. coli* SM10 harboring pBT20, a Mariner-based transposon, using methods modified from Kulasekara et al (30, 31). Briefly, the transposon donor was grown overnight on LB agar supplemented with 100 µg/mL of carbenicillin while GGW039, the recipient, was cultured on PIA. After 24 hours, cells of each species were scraped from the plates and resuspended in LB broth to final concentrations of 40 (donor) and 20 (recipient) OD_{600} units. For mating, equal volumes of each strain were mixed and 50 µl aliquots were spotted onto LB agar and incubated for two hours at room temperature. To simultaneously select for *P. aeruginosa* transposon integrants and conduct the screen, cells from the conjugation mix were resuspended in 2 mL of MOPS and 400 µl plated on PIA with 50 µg/mL of gentamicin, 100 µg/mL of X-gal, and in the presence or absence of 2 mM sarcosine. Colonies exhibiting low or no β-galactosidase activity were tested by Miller assay in liquid media (as described below), before identifying the transposon insertion sites using two rounds of PCR with a TnM specific forward primer (Rnd1-TnM20) and an arbitrary primer (Rnd1-PA-Arb-2), followed by a second round of amplification using the Rnd2-TnM20 and Rnd2-Arb-primer primer set as previously described (31, 32). Sequencing was performed using the

TnM specific primer, BT20TnMSeq (31) and reads were mapped to their respective loci within PA14 and PA01 genomes using the BLAST function on the *Pseudomonas* genome database (33).

2.3.4 Testing activation specificity of SouR

The small molecule specificity required for SouR-dependent activation was examined using β-galactosidase assays as described previously (7, 22). GGW039 (*PA14 ΔgbdR soxB'-lacZYA-'soxG)* was grown overnight at 37 ºC on a rotating wheel in MOPS supplemented with 25 mM sodium pyruvate and 5 mM D-glucose. Cells were collected by centrifugation, washed in MOPS, and resuspended in either MOPS 20mM pyruvate or MOPS 20 mM pyruvate plus 1mM of either glycine betaine, dimethylglycine, sarcosine, ethylglycine, or glycine. Inductions were then carried out at 37 ºC on a shaker set to 170 RPM for 3 hours before β-galactosidase activity was measured according to Miller (34).

2.3.5 Growth Assays

Growth assays were performed as previously described (8). Briefly, strains were grown overnight at 37 ºC on a roller drum in MOPS media supplemented with 25 mM sodium pyruvate and 5 mM D-glucose. Cells were collected by centrifugation, washed with MOPS (with no carbon source), resuspended, and added to 48-well tissue culture plates to a final optical density of 0.05 OD_{600} units in MOPS supplemented with 40 mM of sarcosine as the sole carbon and nitrogen source or 40mM of sodium pyruvate in

MOPS with ammonium chloride as the nitrogen source. Growth was measured by OD_{600} using a Synergy 2 Biotek plate reader.

2.3.6 MBP-SouR fusion construct and protein purification

A maltose binding protein-SouR fusion (MBP-SouR) was engineered into the pMALc2x vector as previously described for AraC-family transcription factors (22, 35). Briefly, *souR* was amplified from genomic DNA with primers (souR_MBP_F & souR_MBP_R) designed to exclude the start codon and incorporate flanking restriction sites to facilitate ligation in-frame with the MBP ORF, generating pGW015. Following cloning in *E. coli* DH5α, purified pGW015 was transformed into chemically competent T7 *lysY/I^q E. coli* (New England Biolabs) to generate the MBP-SouR expression strain, GGW47.

GGW47 was induced with 1 mM IPTG for 3 h at 37 ºC. Cells were collected by centrifugation, rinsed twice in MOPS, and resuspended in 3 mL of cold (150 mM) Tris HCl, (pH 7.2) containing Halt 1x protease inhibitor cocktail (Thermo Scientific). Cells were lysed using a French-Press, DNaseI treated, and sheared using a 21 gauge needle. Following centrifugation at 4ºC and 13,000 rpm, the soluble fraction was applied to a 3 mL amylose resin column (New England Biolabs). The column was rinsed four times with 6 mL of column buffer (20 mM Tris HCl, 150 mM NaCl, 1 mM EDTA [pH 7.4]) before protein was eluted in amylose elution buffer (20 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 10 mM maltose [pH 7.4]). Elution fractions were evaluated by SDS-PAGE. Fractions containing MBP-SouR were pooled and dialyzed against 20 mM Tris-HCl, pH

7.5 at 4°C in a 10 kDa molecular weight cutoff Slide-A-lyzer cassette (Pierce). Protein aliquots were stored frozen at -80°C until use.

2.3.7 Electrophoretic Mobility Shift Assays

EMSAs were performed as previously described (22) using DNA probes spanning the promoter of potential SouR regulon members. Probes were constructed by PCR amplification, where one of the primers was 5'-biotinylated (IDT), and were subsequently purified using Qiagen's PCR Clean Up Kit. EMSAs were conducted using the Pierce Lightshift kit following the manufacturer's instructions with changes made as previously described (22), and salmon sperm DNA (Invitrogen) substituted for poly(dIdC) at a final concentration of 500 ng/µL. Binding, electrophoresis, and detection were done as previously described (22). The sequences of each primer used in the construction of EMSA probes for *adhC (*PA3629-prom-5'-biotin, PA3629-prom-3'*)*, *glyA1* (glyA1-prom-5'-biotin & glyA1-prom-3'), *PA2762* (PA2762-prom-5'-biotin & PA2762-prom-3'), *sdaB* (cbcX-prom-5'-biotin & cbcX-prom-3'), and the negative control *dhcA* (PA1999-prom-3' & PA1999-prom-5'-biot) are listed in Table S2.

2.3.8 Promoter Mapping

To identify the SouR and GbdR binding region within the sarcosine oxidase operon promoter, full length and truncated P*glyA1* fragments were engineered into the

pMW5 *lacZYA* reporter vector. Briefly, the region upstream of *glyA1* was amplified from PA14 genomic DNA using primers that incorporated flanking HindIII and KpnI restriction sites (PglyA1_F1, PglyA1_50bp_del_F2, PglyA1_100bp_del_F3, PglyA1_150bp_del_F4, & PglyA1_R). Amplicons were digested and ligated into similarly cut pMW5 creating the plasmids pGW011 through pGW014. Following transformation into DH5α and verification, these plasmids were transformed into *PA14* wild type, Δ*gbdR*, Δ*souR*, and Δ*souR*Δ*gbdR* strains via electroporation. P_{glyA1} induction was measured in response to 1 mM pyruvate, sarcosine, or glycine betaine as described above, with the addition of 20 μg/mL of gentamicin, and β-galactosidase activity quantified according to Miller (34).

2.3.9 Growth conditions and RNA preparation for microarrays and quantitative RT-PCR

PA14 Δ*gbdR* and *PA14* Δ*gbdR* Δ*souR (and PA14* Δ*gbdR* Δ*gfnR for* qRT-PCR) were grown overnight in 3 mL of MOPS minimal medium supplemented with 20 mM sodium pyruvate and 5 mM D-glucose at 37 °C on a rotating wheel. Cells were collected by centrifugation, washed with pre-warmed MOPS, and resuspended in MOPS with 20 mM sodium pyruvate at an OD_{600} of 0.6. Six hundred microliters of each strain was then added to 12-well tissue culture plates containing 600 μL of pre-warmed MOPS with 20 mM sodium pyruvate and 2 mM sarcosine, or MOPS with 20 mM sodium pyruvate (no induction control) to achieve a final OD_{600} of 0.3. Inductions were carried out for 3 hours

at 37 ºC, shaking at 170 rpm. Following induction, cells were collected by centrifugation, resuspended in 400 µL of fresh MOPS, and mixed with 800 µL of RNA Protect Bacterial Reagent (Qiagen). The cells were again centrifuged, and the supernatant decanted before the pellets were frozen at -80 °C.

RNA was prepared using a Qiagen RNeasy kit following the manufacturer's protocol with the following changes. Prior to extraction, cell pellets were resuspended in 200 µL of TE supplemented with 3 mg/mL lysozyme and incubated at room temperature for 20 minutes. An on-column DNase I treatment was performed before the RNA was eluted in RNase-free water. Samples were then treated a second time with RNase-free DNase I (NEB) and incubated for 1 hour at 37 ºC before a second round of RNeasy column purification was performed.

2.3.10 Microarray methodology

Microarray analysis was performed by the Vermont Genetics Network Microarray Facility using Affymetrix *Pseudomonas aeruginosa* PAO1 gene chips and DNA probes generated by the NuGEN Pico system. Each condition was analyzed in duplicate, and signals from all probes for a given gene were averaged into one probe intensity using the Expression Console and Transcriptome Analysis Console software package version 2.0 (Affymetrix). Potential SouR regulon members were identified as those exhibiting at least a 2.5-fold change in detection between sarcosine induced and control cultures using RMA analysis and a p value <0.05. Array data is available in the GEO database under accession number GSE72613.

2.3.11 Quantitative RT-PCR

Growth conditions and RNA preparations were carried out as described above (in biological triplicate). cDNA was generated using Superscript IV with the 5′-NSNSNSNSNS-3′ primer previously described (36) and 20 ng of total RNA isolated from each strain under each condition. Quantitative PCR was performed with technical duplicates using Luminaris HiGreen fluorescein qPCR master mix (Thermo Fisher), and primers described previously (22). Due to difficulties in the amplification of *sdaB* with Taq-based Luminaris mix, quantitative PCR was performed with NEB's Q5 2x MM supplemented with SYBR Green I nucleic acid gel stain (Thermo Fisher) at a final concentration of 0.2X. For each gene, transcript abundance was determined using a five-step standard curve dilution series with cDNA from the Δ*gbdR* strain exposed to sarcosine (the highest responding strain and condition) as described previously (22). Each sample for each transcript was normalized to its cognate *rplU* abundance before conversion to relative expression based on the average expression level in the non-induced control sample (pyruvate) of each strain.

2.3.12 Formaldehyde susceptibility assay

PA14 WT, Δ*gfnR,* Δ*gfnR attTn7::gfnR,* and Δ*gfnR attTn7::EV* (empty site) strains were grown overnight at 37 ºC in MOPS media supplemented with 25 mM sodium pyruvate and 5 mM glucose. Cells were collected by centrifugation, washed with fresh MOPS media, and resuspended in 48-well tissue culture plates to a final optical density of 0.05 OD_{600} units in MOPS containing 20 mM sodium pyruvate and 5 mM glucose, or

MOPS with 20 mM sodium pyruvate, 5 mM D-glucose, and 0.75 mM formaldehyde. Susceptibility to formaldehyde was assessed by growth in the presence of formaldehyde through OD_{600} using a Synergy 2 Biotek plate reader. The concentration of formaldehyde utilized in this assay was arrived at by titrating the ability of PA14 WT to grow in MOPS media with 25 mM sodium pyruvate and 5 mM glucose supplemented with 0.25 mM, 0.5 mM, 0.75 mM, or 1.0 mM formaldehyde. The highest formaldehyde concentration that did not impede growth of PA14 WT after 24 hours under these conditions was then chosen for assessing the susceptibility of *gfnR* deletion and complementation strains.

2.4 Results

2.4.1 Identification of the sarcosine-responsive regulator of the sarcosine catabolic operon.

Our previous work demonstrated that while GbdR could control the sarcosine oxidase operon, *soxBDAG* could still be induced in a *gbdR* deletion strain in response to sarcosine (21), Indicating that an unidentified sarcosine-responsive transcription factor regulated the sarcosine oxidase genes. The sarcosine oxidase operon consists of *glyA1-soxBDAG-purU2 (PA14_71460-PA14_714530, PA5415-PA5420),* which we will refer to as the *sox* operon, and is controlled from the P_{glyA1} promoter. To identify the sarcosine-responsive regulator of the *sox* operon, an operonic *lacZYA* transcriptional reporter was engineered into the *sox* locus of a Δ*gbdR* strain, generating both a reporter and a simultaneous deletion of most of the operon (Δ*gbdR soxB'-lacZYA-'soxG).* This parent strain was mutagenized with the Mariner transposon from pBT20, and approximately

60,000 transposon insertion mutants were screened for their ability to cleave X-gal in response to sarcosine. In total, 23 colonies were identified that failed to induce β-galactosidase in the presence of sarcosine. Sixteen of these mutants carried unique insertions in the *lacZYA* locus, while seven unique insertions mapped within *PA14_09770* (*PA4184)*, predicted to encode an AraC-family transcription regulator. The unique insertion rate into *lacZYA* and *PA4184* suggests that the screen was saturated for identification of activators**.**

An ortholog search of *PA4184* against the *Pseudomonas* genome database (33) revealed the widespread conservation of this gene among sequenced *Pseudomonads*. Unique to *P. aeruginosa* however, *PA4184* is part of an operon with a gene *PA4183 (PA14_09780)* (**Fig. 2.1B**) encoding a protein of unknown function exhibiting modest structural homology with the glyoxylase I-family of enzymes. A reciprocal BLASTP search of PA4183 against the genome database failed to identify homologs outside of *P. aeruginosa*.

2.4.2 PA4184 is a sarcosine-responsive transcription regulator

The induction specificity of PA4184 was examined through β-galactosidase assays performed using the same reporter strain described above (Δ*gbdR soxB'-lacZYA-'soxG)* with sarcosine or structurally related compounds. Glycine betaine, dimethylglycine, glycine, and pyruvate failed to induce transcription of the *sox* operon, while incubation with sarcosine, and to a lesser extent, the synthetic compound ethylglycine, resulted in induction of β-galactosidase activity (**Fig. 2.2A**). This induction

was dependent on *PA4184*, as the same assay conducted in the *PA4184* deletion strains yielded no β-galactosidase activity (**Fig. 2.2B** and data not shown). Moreover, transcription from the *soxBDAG* operon in response to sarcosine was restored in a Δ*gbdR* Δ*PA4184 soxB'-lacZYA-'soxG* strain carrying *PA4184* on a plasmid under the control of its native promoter (**Fig 2.2B**). These results confirm that *PA4184* is required for transcriptional induction of the sarcosine oxidase operon in response to sarcosine. Furthermore, the ability of ethylglycine to stimulate transcription from this promoter implicates the necessity of the secondary amine moiety in the recognition of the inducing compound by PA4184. Based on this data, and the growth data reported below, we renamed *PA4184* as *souR* (**s**arcosine **o**xidation and **u**tilization **R**egulator), which encodes an AraC-family transcription regulator.

2.4.3 *souR* is essential for growth on sarcosine as a sole carbon and nitrogen source

P. aeruginosa can use sarcosine as a sole carbon and nitrogen source for growth (1). To assess the requirement for *souR* in the metabolism of sarcosine by *P. aeruginosa*, growth assays were performed with WT, Δ*gbdR,* Δ*souR,* and Δ*gbdR*Δ*souR* strains cultured in MOPS minimal media with sarcosine as the sole carbon and nitrogen source. Deletion of *souR* resulted in substantial growth defects compared to WT, and there was no detectable growth in the Δ*gbdR*Δ*souR* double deletion mutant (**Fig. 2.3A**). The necessity of *souR* for this activity was confirmed by trans-complementation with a plasmid carrying *souR* with its native promoter, which restored growth (**Fig. 2.3B**). All deletion strains grew similarly to WT when cultured in MOPS media supplemented with

pyruvate and ammonium chloride as carbon and nitrogen sources, respectively, indicating that the observed growth defects are sarcosine-specific (**Fig. 2.3**).

2.4.4 SouR and GbdR bind within the same region of P_{glyA1}

We previously determined that GbdR recognizes a binding site within the promoter of *glyA1* using an electrophoretic mobility shift assay (EMSA) with a maltose binding protein-GbdR fusion (22). Here we show that a maltose binding protein-SouR fusion also binds the promoter of *glyA1* and that this binding was sensitive to competition with unlabeled P_{glyA1} DNA (**Fig. 2.4A**). As previously reported for MBP-GbdR (17), the MBP-SouR DNA interaction was not affected by the presence of sarcosine (data not shown). Promoter mapping was used to determine where the SouR and GbdR binding sites were within P_{glyA1}. Serial truncations of P_{glyA1} were engineered into the pMW5 promoter-less *lacZ* reporter plasmid and transformed into Δ*gbdR* and Δ*souR* cells. In both scenarios, deletion of the region upstream between -210 and -158 bp from the *glyA1* translational start site resulted in loss of induction of β-galactosidase activity in response to sarcosine (in Δ*gbdR)* and glycine betaine (in Δ*souR*), indicating that SouR and GbdR require the same region of the promoter (**Fig. 2.4B**). SouR and GbdR appear to function independently at this promoter and either can support induction in response to their cognate inducing molecule (**Fig. 2.4C**). The full promoter deletion series in each of the four strains shown in **Fig. 2.4C** are presented in **Fig. 2.S1**. These data demonstrate that the minimal requirements for induction by GbdR or SouR are present between -210 and -158.

2.4.5 Characterization of sarcosine-induced transcripts and determination of the SouR regulon

Using Affymetrix *P. aeruginosa* microarrays, we characterized the transcriptional response of *P. aeruginosa* Δ*gbdR* and Δ*gbdR*Δ*souR* in the presence and absence of sarcosine, which allowed us to distinguish SouR-dependent transcriptional changes from the total cellular response to sarcosine. Potential SouR regulon members were those transcripts exhibiting at least a 2.5-fold induction in the Δ*gbdR* strain (*souR* intact) and no induction in the Δ*gbdR*Δ*souR* strain in response to sarcosine.

The Δ*gbdR* and Δ*gbdR*Δ*souR* strains revealed no statistically significant differences in their expression profiles during exposure to MOPS pyruvate media (not shown, see GSE72613). In contrast, the transcriptional responses of the two strains to sarcosine were markedly different. As expected from the results of the genetic screen, transcription of the *sox* operon *(PA5415-PA5420)* and the glutathione-independent formaldehyde dehydrogenase (*fdhA*) were induced in the strain expressing SouR (Δ*gbdR*), when compared to the MOPS-pyruvate control (**Table 2.1**). Sarcosine also induced expression of the glutathione-dependent formaldehyde detoxification system encoded by *PA14_17410* and *adhC* (*PA3628* and *adhC)* in a SouR-dependent manner (**Table 2.1**). Since sarcosine catabolism by *Pseudomonas* species is known to generate formaldehyde (15), the expression of a second detoxification system was not completely unanticipated. In the *souR* deletion strain (Δ*gbdR*Δ*souR*), sarcosine failed to induce transcription of the *sox* operon, *fdhA,* or the glutathione-dependent formaldehyde

detoxification operon. Surprisingly, the dipeptide transport operon, encoding the *opdP* porin and associated ABC transporter genes exhibited a roughly four-fold increase in expression over the pyruvate control in the absence of SouR and the presence of sarcosine (**Table 2.1**).

2.4.6 Testing SouR binding to the promoters of potential regulon members

A short induction period (3 hours) was used in our microarray studies to limit the expression of genes involved in secondary processes downstream of sarcosine metabolism. Nevertheless, alterations within the transcriptome could reflect the response to metabolic intermediates generated during sarcosine catabolism, including formaldehyde, glycine, serine, and pyruvate (**Fig. 2.1A**). Therefore, EMSAs were performed with MBP-SouR and biotinylated probes from the *adhC* and *sdaB* promoter regions to determine if they were directly bound by SouR. Although expression of the serine dehydratase transcript *sdaB,* failed to surpass our 2.5 fold cut off (2.32 fold change), we included the promoter of this gene in our EMSAs because *sdaB* has previously been identified as a member of the GbdR regulon and plays a critical role in the conversion of serine to pyruvate during sarcosine metabolism (22). As shown in **Fig. 2.5**, MBP-SouR specifically bound to the promoters of *glyA1*, *adhC,* and *sdaB,* but not to the promoter region of *dhcA* (negative control (8, 22)).

2.4.7 Effects of SouR regulon members and sarcosine-induced genes on sarcosine catabolism

The genes encoded within the *sox* operon and their respective roles in the metabolism of sarcosine have been well characterized (3, 15, 17, 18). However, the contributions of the other genes in the sarcosine regulon: *PA4183*, the glutathione-dependent formaldehyde detoxification system *(PA3628 & adhC)*, and the sarcosine-induced dipeptide porin and transport system *(PA4501-06)* in the metabolism of sarcosine were unknown. To determine the requirement for these genes in this process, transposon mutants were selected from the PA14 transposon mutant library (37) and screened for their ability to grow in MOPS minimal media using 40 mM sarcosine as the sole carbon and nitrogen source. With the exception of *soxA::TnM*, all of the TnM disruption mutants from the sarcosine regulon tested were capable of utilizing sarcosine as a carbon and nitrogen source to some extent (**Fig. 2.6**). However, growth was significantly slower than the positive growth control strain, *dhcA::TnM*, for all strains except for *opdP::TnM* (**Fig. 2.6**). As a whole, this data indicates that the glutathione dependent formaldehyde detoxification genes, *PA4183, sdaB, souR,* and *gbdR* are not absolutely necessary for the metabolism of sarcosine but are important for achieving optimal growth under these conditions.

2.4.8 *PA3630* encodes the transcription regulator of the glutathione-dependent formaldehyde detoxification genes

Although EMSAs demonstrated a clear interaction between MBP-SouR and the promoter region of the glutathione-dependent formaldehyde detoxification operon *(PA3628 & adhC),* we suspected that the divergently transcribed LysR-family transcription factor encoded by *PA3630* (**Fig. 2.1B**) might also influence the expression of these genes in response to formaldehyde generated endogenously through the metabolism of sarcosine. Evidence for this function is supported by a search of *PA3628, adhC,* and *PA3630* using the String database (38), which revealed that the synteny of these genes is conserved among hundreds of Proteobacterial taxa. To test the role of *PA3630* in the cellular response to formaldehyde, an unmarked deletion of *PA3630* was generated. Growth of this strain was severely attenuated compared to WT when cultured in minimal media containing 0.75 mM formaldehyde. Moreover, integration of *PA3630* at the *attTn7* site restored growth of the deletion strain to wild-type levels (**Fig. 2.7**). These data suggest that *PA3630* encodes a formaldehyde-responsive regulator of the glutathione-dependent formaldehyde detoxification genes and we propose the name, GfnR (**G**lutathione-dependent **f**ormaldehyde **n**eutralization **R**egulator), to reflect this function.

2.4.9 Confirmation of SouR and GfnR regulon members

Quantitative RT-PCR was performed to confirm the expression of SouR regulon members identified through microarray, as well as to distinguish the regulatory

contribution of GfnR from SouR in the expression of the glutathione-dependent formaldehyde detoxification system in response to sarcosine. While the expression of *sdaB* and the *sox* operon was induced by sarcosine in a SouR-dependent manner, induction of *adhC* was more stochastic, as a greater than two-fold increase in expression was only observed in half of the Δ*gbdR* replicates exposed to sarcosine (3 out of 6 biological replicates) (**Table 2.2**). However, induction of *adhC* and *PA3628* was not observed in the Δ*gbdR*Δ*gfnR* strain in response to sarcosine (**Table 2.2**), indicating that the expression of the glutathione-dependent formaldehyde detoxification system is likely induced by GfnR in response to formaldehyde generated through sarcosine catabolism.

2.5 Discussion

Pseudomonas aeruginosa is ubiquitous in nature and is often described as an optimal exploiter of nutrient pulses largely as a result of the diverse metabolic potential encoded within its genome. Related to this metabolic flexibility, close to 10% of *P. aeruginosa's* genes are predicted to encode transcription factors (39), many of which likely allow this organism to sense potential nutrient sources and regulate enzymatic pathways to exploit a variety of metabolic niches. Sarcosine is present in a range of environments inhabited by *P. aeruginosa*, although it is likely encountered most often as an intermediate metabolite of glycine betaine, carnitine, glyphosate, or creatine catabolism (6-14, 22)(**Fig. 2.1A**). We propose the capacity to sense and metabolize sarcosine as providing *Pseudomonas* with a fitness advantage in certain environments

through the ability to fully catabolize a carbon and nitrogen source that competitors cannot.

In this study, we utilized a genetic screen to identify an AraC-family transcription factor SouR (PA4184) as the sarcosine-responsive regulator of sarcosine catabolism in *P. aeruginosa*. SouR is required for appreciable growth when sarcosine is utilized as a sole carbon and nitrogen source, and transcriptional induction is limited to sarcosine, a natural metabolite, and ethylglycine, a non-natural sarcosine analog. Together, these data support SouR as the first known sarcosine-responsive transcription factor. While previous work by Nishiya and Imanaka reported SoxR as a repressor of monomeric sarcosine oxidase in *Arthrobacter* spe4, the authors noted that sarcosine failed to relieve repression *in vitro* (40). Moreover, a follow up study determined that *soxR* and the monomeric sarcosine oxidase genes clustered with genes involved in the degradation of creatinine and creatine (41). Since sarcosine is generated during creatine metabolism, it is therefore likely that either creatinine or creatine acts as the inducing ligand of SoxR in *Arthrobacter*.

While all Pseudomonads sequenced to date encode clear orthologs of SouR, only *P. aeruginosa* isolates carry this gene as part of a two gene operon with *PA4183*. *PA4183* encodes a protein of unknown function that shares modest structural similarity with members of the glyoxylase I family of enzymes (PF00903). The lack of genus-wide conservation of *PA4183* outside of *P. aeruginosa* suggests that this gene is likely to play an accessary role in the metabolism of sarcosine, and growth assays performed with a *PA4183* transposon mutant support this theory (**Fig. 2.6**). However, we have no current hypothesis as to the role of PA4183 in *P. aeruginosa* sarcosine catabolism.

SouR is a member of the glutamine amidotransferase I-like transcription regulator (GATR) subfamily of the AraC regulator family (CD03137). Little is known about this group aside from their widespread distribution among Gram-negative taxa. Like other members of the AraC family, GATRs exhibit a two-domain layout with a C-terminal AraC-like helix-turn-helix DNA binding domain. Unlike other members of the AraC family, the amino terminal domain is a glutamine amidotransferaseI-like domain (42), likely involved in the recognition of the inducing molecules. *Pseudomonas* species encode a number of GATRs, with seven members conserved among the core genomes of sequenced and annotated *P. aeruginosa* isolates. Interestingly, multiple GATRs regulate glycine betaine acquisition and catabolism in *P. aeruginosa*, with the GATR member GbdR controlling glycine betaine catabolic genes in response to glycine betaine and dimethylglycine (21, 22), and the GATR member CdhR regulating the carnitine catabolic pathway in response to carnitine (8).

Evidence suggests that SouR and CdhR may be paralogs of GbdR that arose through gene duplication. SouR and CdhR display close homology to GbdR (58% and 62% similarity, respectively) and their phylogenetic distribution hints to shared common ancestry, as orthologs of SouR and CdhR are present only in taxa that also encode the glycine betaine catabolic pathway regulated by GbdR. In contrast, GbdR orthologs are widespread in taxa that lack clear SouR and CdhR orthologs. Here, we have shown that SouR and GbdR both regulate the expression of the *glyA1* promoter (the promoter of the *sox* operon), and we determined that they likely recognize the same binding region (**Fig. 2.4B-C, Fig. 2.S1**). We are currently investigating whether CdhR and GbdR also regulate genes from the same binding region in one or more promoters. Such co-regulation may

indicate a hierarchy of binding priority contributing to regulation as a means to control flux through the intermediate metabolite pools in the glycine betaine catabolic pathway.

Additional transcripts regulated by SouR were identified through microarrays, EMSAs with MBP-tagged SouR, and quantitative RT-PCR, which point to additional overlap between the GbdR and SouR regulons. The serine dehydratase, *sdaB* is a member of the GbdR regulon (22), and we were initially surprised that sarcosine failed to induce transcription of this gene above our expression fold-change cut-off in our microarrays, as the activity of this enzyme links sarcosine catabolism to central metabolism by converting serine generated from glycine and 5,10-methyltetrahydrofolate via GlyA1 (**Fig. 2.1A**), to pyruvate and ammonium. However, the expression of SdaB did increase 2.3-fold in response to sarcosine, hence we included the promoter of this gene in our EMSAs with MBP-tagged SouR. In doing so, we determined that SouR, like GbdR, could bind the promoter region of *sdaB* (**Fig. 2.5**). Furthermore, quantitative RT-PCR revealed that the expression of *sdaB* is induced by SouR in response to sarcosine (**Table 2.2**). Thus, the expression cut-off used in our microarrays (2.5-fold change) was likely conservative and additional less dramatically induced SouR regulon members, like *sdaB,* might exist.

Our microarrays also revealed that the dipeptide transport system (*PA4501-06*) was induced by sarcosine in the Δ*gbdR*Δ*souR* strain, but not in the Δ*gbdR* strain. Regulation of this system is complex and expression has been shown to be influenced by numerous dipeptides as well as the amino acid arginine (43-45). Similarly, the substrate specificity of the OpdP porin (PA4501) and associated transporter proteins has recently been examined and the system was found to be implicated in the uptake and metabolism

of over one hundred unique dipeptides (46). Growth assays performed with an *opdP* transposon disruption mutant revealed a wild-type growth phenotype when utilizing sarcosine as a sole carbon and nitrogen source, indicating that this system does not contribute significantly to the catabolism of this molecule (**Fig 2.6**). We instead hypothesize that the *opdP* operon might be induced in the Δ*gbdR*Δ*souR* genotype as a consequence of perceived nutrient deprivation and/or this strain's inability to metabolize sarcosine. In the latter scenario, we speculate that the accumulation of sarcosine within the cytosol might promote detection by the low specificity regulator governing expression of the dipeptide transport operon.

Glycine betaine and sarcosine catabolism in proteobacterial species generates formaldehyde in the absence of tetrahydrofolate (15, 17). However, these bacteria also encode a sarcosine-inducible glutathione-independent formaldehyde dehydrogenase (*fdhA)* that functions in converting formaldehyde to formate (20). Our microarrays revealed that a second formaldehyde detoxification system is expressed during sarcosine catabolism in *P. aeruginosa*. *adhC* and *PA3628* encode a glutathione-dependent formaldehyde dehydrogenase and formate esterase that are nearly universally conserved among Gram-negative bacteria. This system has been well characterized in Proteobacteria and has been demonstrated to function in protecting cells against the effects of intracellular formaldehyde (20, 47). Interestingly, while EMSAs revealed that MBP-tagged SouR is capable of binding to the promoter region of the *adhC* and *PA3628* operon, qRT-PCR data suggests that expression of these genes is likely influenced by a second regulator in response to formaldehyde production (**Figure 2.5A & Table 2.2**). Searching the *Pseudomonas* genome database, we identified an uncharacterized LysR-

family regulator (PA3630) that is divergently transcribed from the *PA3628* and *adhC* operon in all *Pseudomonas* genomes annotated to date (33). Moreover, a study in *Pseudomonas putida* revealed that the expression of this transcription factor is upregulated along with the (then uncharacterized) glutathione-dependent formaldehyde detoxification operon following exposure to formaldehyde (48).

Here, using formaldehyde susceptibility challenge with chromosomal deletion and complementation strains, we have shown that GfnR (PA3630) is required for optimal growth of *P. aeruginosa* in the presence of formaldehyde (**Fig. 2.7**). Alternative regulatory mechanisms have been described for the glutathione-dependent formaldehyde detoxification system in Proteobacteria, including the *frmR* repressor of *E. coli (47)* and the *fhlRS* two-component sensor system of *Paracoccus denitroficans (49)*. Nevertheless, a synteny search on the String database (38) revealed widespread conservation of *gfnR* orthologs in association with the detoxification genes among hundreds of taxa, indicating that the LysR-family regulatory mechanism is likely prevalent among Proteobacteria.

To summarize, this study has expanded our understanding of how sarcosine metabolism is transcriptionally regulated in *P. aeruginosa*. SouR is the first sarcosine-responsive transcription factor to be described and we speculate that this regulator arose from GbdR as a means for *Pseudomonas* species to independently detect this intermediate of glycine betaine and creatine degradation in the environment. Finally, we identified GfnR as the regulator of the glutathione-dependent formaldehyde detoxification system in *P. aeruginosa* and determined that homologs are widespread among proteobacterial taxa.

2.6 Funding Information

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2.7 Chapter 2 figures

Figure 2.1 Sarcosine catabolism in *Pseudomonas* species. (A) Diagram of sarcosine catabolism in *P. aeruginosa* and related species. Environmental sources of sarcosine that can be metabolized by *Pseudomonas* species are shown, along with structure of sarcosine and the names of each enzyme involved in the conversion of sarcosine into glycine, serine, and pyruvate. **(B)** Genomic depiction of the sarcosine catabolic operon in wild-type *P. aeruginosa* and the altered locus that functions as the transcriptional reporter strain in this study. **(C)** Genomic arrangement of the *souR* locus and glutathione-dependent formaldehyde detoxification system genes in *P. aeruginosa.*

Figure 2.2 Activating ligand specificity and necessity of SouR for sarcosine dependent induction of the *sox* operon. (A) Results from β-galactosidase assay of Δ*gbdR soxB'-lacZYA-'soxG* exposed in MOPS pyruvate (Pyr) to 1mM of either glycine betaine (GB), dimethylglycine (DM), sarcosine (Sarc), ethylglycine (EG), glycine (Gly), or no compound (Pyr) as a control. For convenience, the structures for sarcosine and ethylglycine are shown over their respective bars. **(B)** Results of β-galactosidase assay of Δ*gbdR*Δ*souR soxB'-lacZYA-'soxG* exposed in MOPS pyruvate (Pyr) +/- 1 mM sarcosine (Sarc) with the addition of the empty vector (pMQ80) or the plasmid carrying *souR* and its native promoter (pSouR). Statistical significance was determined using one-way ANOVA with Dunnett's post-test with the uninduced (Pyr) condition being the comparator for all other data. p-value summaries: n.s. = not significant; *** for $p<0.001$; **** for $p<0.0001$. The data shown are representative of three independent experiments and error bars represent standard deviation.

Figure 2.3 The role of *souR* during growth on sarcosine. (A) Culture density (OD_{600}) after 24 hours of growth in wild type, Δ*gbdR,* Δ*souR,* and Δ*gbdR*Δ*souR* cells in MOPS minimal media without nitrogen supplemented with either 20 mM pyruvate and 10 mM ammonium chloride (P), or 40 mM sarcosine (S). **(B)** Culture density (OD_{600}) after 24 hours of growth in Δ*gbdR*Δ*souR* transformed with the empty vector (pMQ80) or *souR* with its native promoter (pSouR). Statistical significance determined using two-way ANOVA with a Tukey's post-test for (A) and with a Sidak's post-test for (B). p-value summaries: n.s. = not significant; *** for $p<0.001$. The data shown are the summary of three independent experiments each with three biological replicates, therefore error bars represent standard errors of the means.

Figure 2.4 SouR interaction with the P_{glyA1} promoter. (A) Electrophoretic mobility shift assay (EMSA) performed with MBP-SouR (SouR) and biotinylated P_{glyA1} probe. Data is representative of four independent experiments performed with two separately purified batches of MBP-SouR. The presence (+) or absence (-) of unlabeled competitor (comp) P_{glyA1} probe is noted below each lane. **(B)** Results from β-galactosidase assay for promoter mapping to identify regions within P_{glyA1} required for *souR*- and *gbdR*-dependent induction. The Δ*gbdR* cells were exposed to 1 mM sarcosine (S, light bars) and Δ*souR* cells were exposed to glycine betaine (G, dark bars) and compared to controls with pyruvate. The size of each P_{glyA1} promoter construct is noted as the beginning position relative to the *glyA1* translational start site. Fold induction was calculated as a multiple of the pyruvate condition for each strain. **(C)** Results from β-galactosidase assay

for the -210 P_{glyA1} promoter in wild type, Δ*souR*, Δ*gbdR*, and Δ*souR* Δ*gbdR*. Cells were induced as in panel (B) and presented as Miller units. The data shown are representative of three biological replicates and error bars represent standard deviation.

Figure 2.5 SouR binding to promoters of potential regulon members. Electrophoretic mobility shift assays (EMSAs) with purified MBP-SouR and biotinylated probes of promoter regions from operons induced by sarcosine. Each panel represents a separate biotinylated probe, with *dhcA* included as a negative control. The MBP-SouR concentrations (nM) are listed below each lane. Data is representative of at least three independent experiments with two separate batches of purified MBP-SouR.

Figure 2.6 Role of SouR and sarcosine regulated genes during growth on sarcosine. Culture density (OD_{600}) after 24 hours of growth in MOPS minimal media without nitrogen supplemented with either 20 mM pyruvate and 10 mM ammonium chloride (top panel) or 40 mM sarcosine (bottom panel) for the transposon insertion mutants labeled on the x-axis. The *dhcA* insertion mutant is known not to have a role in this pathway and served as the positive growth control (no growth defect). Statistical significance determined using one-way ANOVA with Dunnett's post-test with growth in the *dhcA* mutant being the comparator for all other data. p-value summaries: n.s. = not significant; ** for $p<0.01$; *** for $p<0.001$. The data shown are the summary of three independent experiments each with three biological replicates, therefore error bars represent standard errors of the means.

Figure 2.7 The role of *gfnR* during growth in the presence of formaldehyde. Culture density (OD_{600}) after 24 hours of growth in MOPS minimal media 20 mM sodium pyruvate and 5 mM D-glucose in the presence (+) and absence (-) of 0.75 mM formaldehyde for wild-type, Δ*gfnR,* Δ*gfnR attTn7::gfnR*, and Δ*gfnR attTn7::EV* (empty site) strains. The data are representative of three separate experiments and error bars represent standard deviation.

Figure S2.1 PglyA1 promoter mapping of sarcosine or glycine betaine induced SouR and GbdR-dependent induction. Results from β-galactosidase assay of the four promoter truncation constructs (noted as bases upstream of the translational start site) in each of the four strains listed below the x-axis. The data shown are representative of three biological experiments and error bars represent standard deviation.

2.8 Chapter 2 tables

Table 2.1 Transcript changes (fold abundance) related to sarcosine and SouR.

Table 2.2 Effect of *souR* mutation on sarcosine regulation of regulon members.

Table 2.S1 Bacterial strains and plasmids used in this study.

Table 2.S2 Primers used in this study.

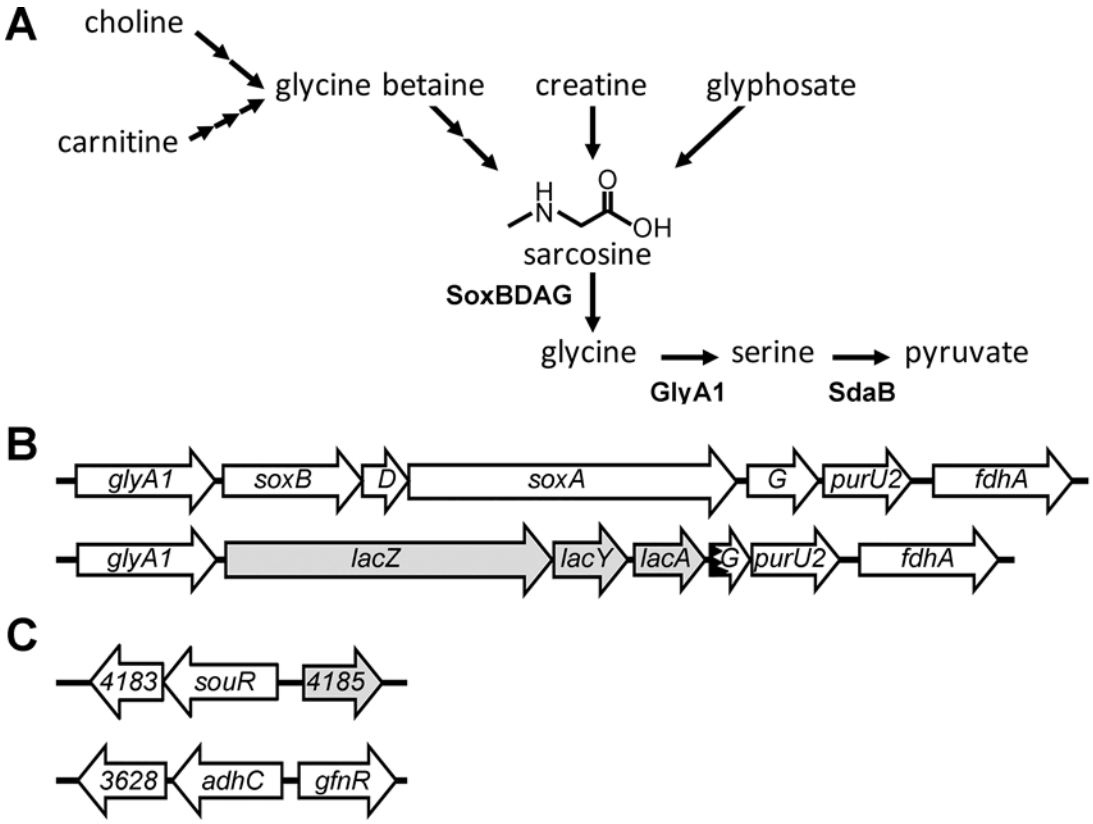

Figure 2.1 Sarcosine catabolism in *Pseudomonas* species.

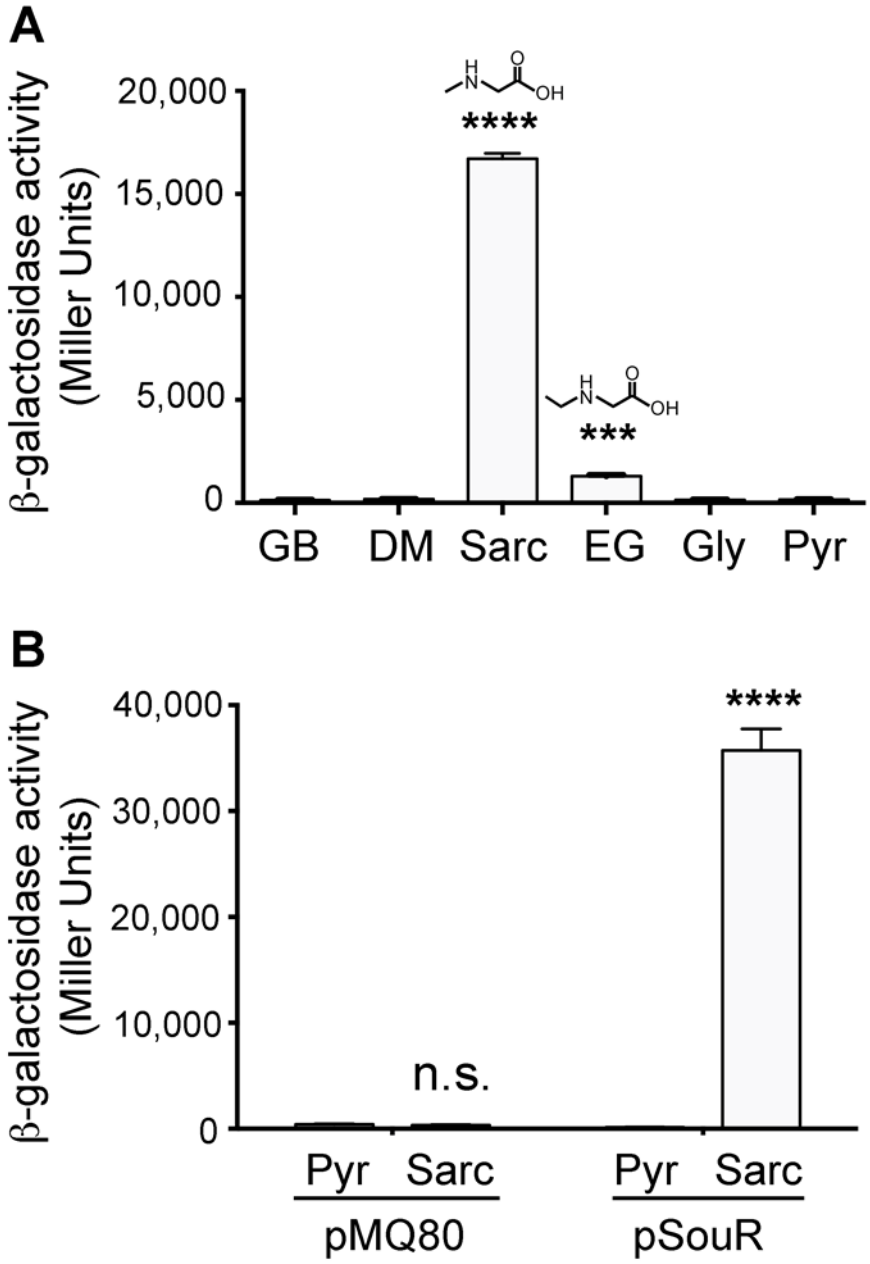

Figure 2.2 Activating ligand specificity and necessity of SouR for sarcosine dependent induction of the *sox* operon.

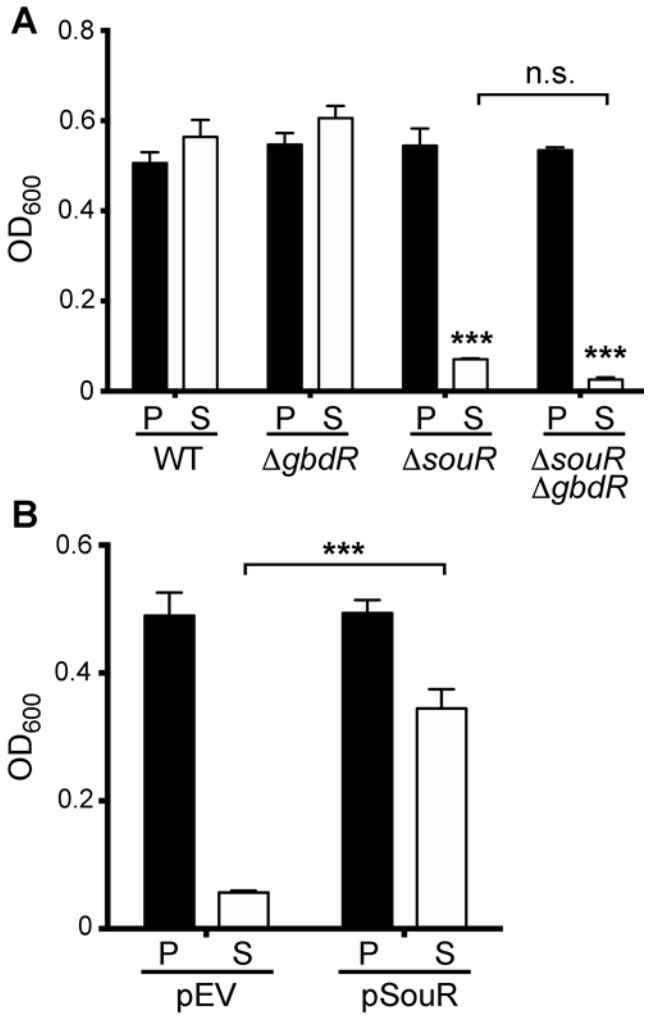

Figure 2.3 The role of *souR* during growth on sarcosine.

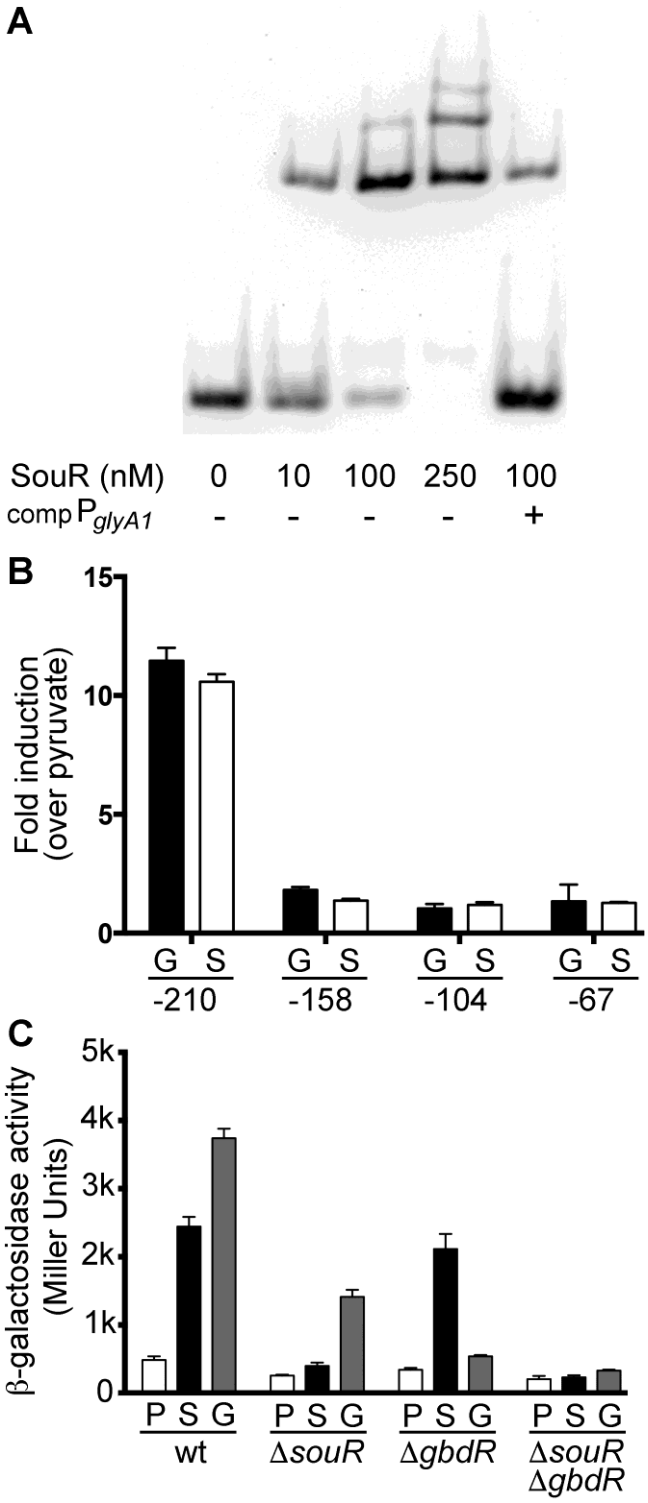

Figure 2.4 SouR interaction with the P_{glyA1} promoter.

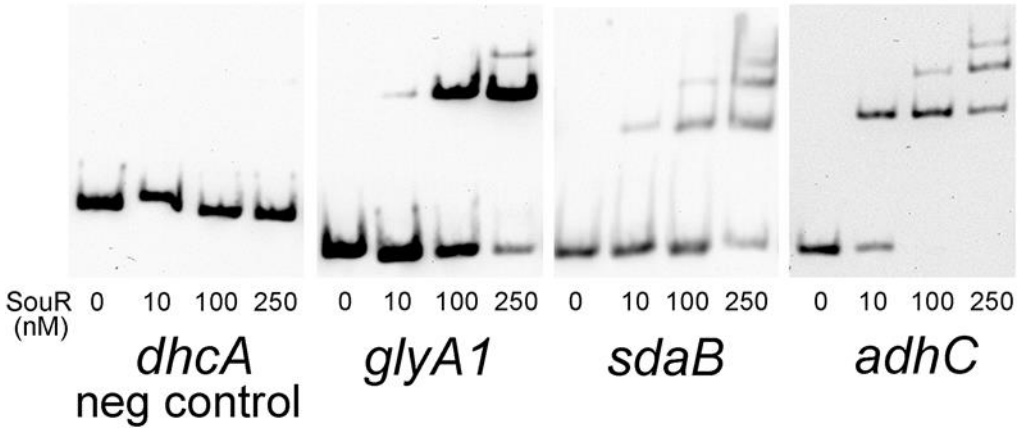

Figure 2.5 SouR binding to promoters of potential regulon members.

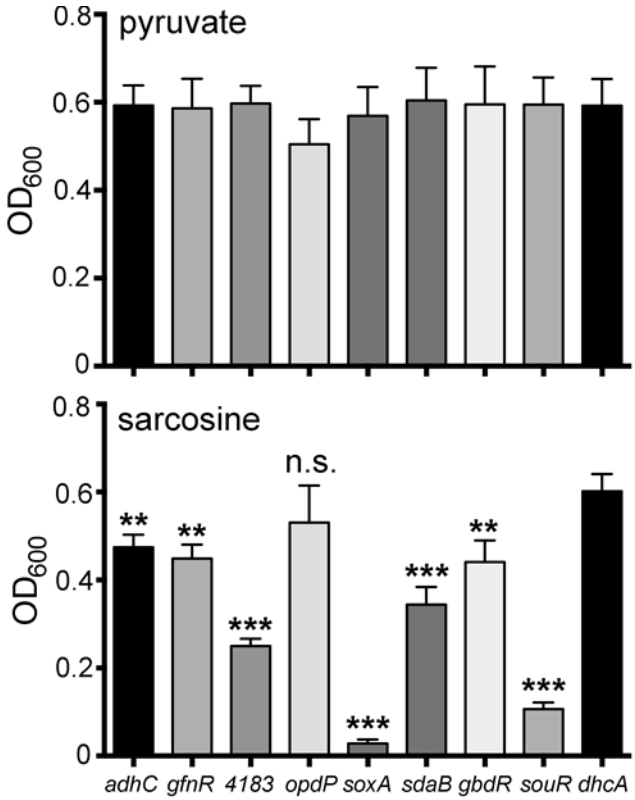

Figure 2.6 Role of SouR and sarcosine regulated genes during growth on sarcosine.

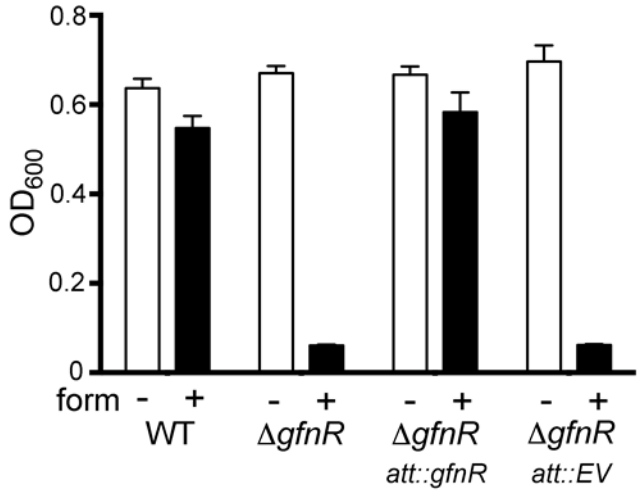

Figure 2.7 The role of *gfnR* during growth in the presence of formaldehyde.

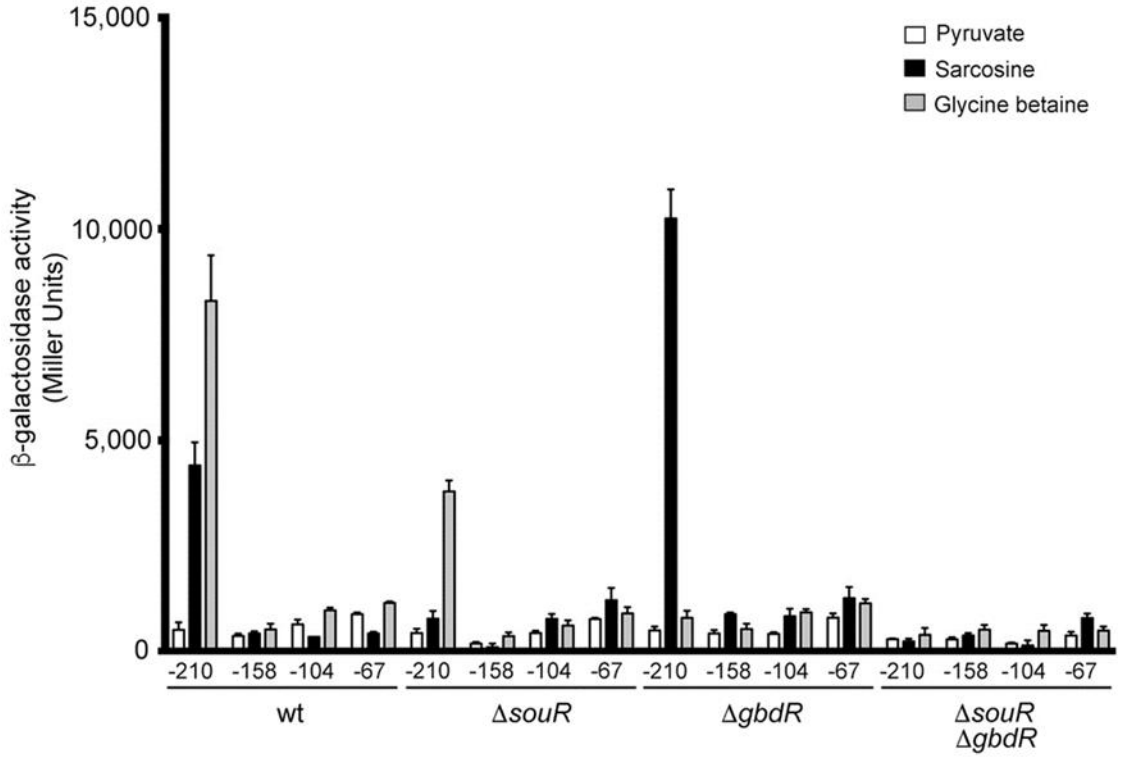

Figure S2.1 PglyA1 promoter mapping of sarcosine or glycine betaine induced SouR and GbdR-dependent induction.

Table 2.1 Transcript changes (fold abundance) related to sarcosine and SouR

Gene #	Gene Name	Δ*qbdR* [2] Sarc:Pyr	Δ*qbdR*Δ*souR* Sarc:Pyr	Sarc Δ*qbdR*:Δ*qbdR*Δ*souR*
Transcripts increased in abundance (fold induction over pyruvate):				
PA1168		-1.8	**-2.5**	-1.8
PA1247	aprE	1.6	**3.1**	-2.4
PA1250	aprI	2.4	**2.8**	-2.4
PA2513	antB	-1.9	**-12.2**	2.4
PA3628		**4.2**	-1.3	**5.2**
PA3629	adhC	**3.4**	-1.1	**3.6**
PA4385	*groEL*	1.1	**3.3**	-2.3
PA4386	*groES*	1.3	**3.5**	-2.2
PA4498	*mdpA*	1.2	**2.8**	-2.6
PA4501	*odpD*	1.4	**3.6**	**-3.4**
PA4502		1.1	**4.2**	**-4.0**
PA4504		1.4	**5.0**	**-4.3**
PA4505		1.1	**4.1**	**-3.9**
PA4506		1.1	**4.6**	**-4.6**
PA4761	*dnaK*	-1.1	**3.1**	**-2.7**
PA5415	*glyA1*	**2.7**	1.1	**2.7**
PA5416	*soxB*	**3.5**	1.1	**4.0**
PA5417	*soxD*	**12.3**	-1.1	**11.6**
PA5418	*soxA* [3]	**3.1**	1.0	2.1
PA5419	*soxG*	**8.0**	1.1	**8.2**
PA5420	*purU2*	**8.3**	1.1	**10.2**
PA5421	*fdhA*	**5.5**	1.1	**5.4**

[1] all bolded changes are >2.5 fold different and significant with p-values < 0.05.
[2] the top line denotes the fixed strain or treatment in the column, the bottom lists the comparison being made in the data. Abbreviations: sarcosine (Sarc), pyruvate (Pyr).
[3] signal from the *soxA* probe is low due to poor hybridization.

Table 2.2 Effect of *souR* mutation on sarcosine regulation of regulon members.

Transcript	*ΔgbdR*	*ΔgbdRΔsouR*	*ΔgbdRΔgfnR*
soxA	32.9 (8.0)** [2]	1.11 (0.2)	31.0 (11.0)**
adhC	3.60 (2.8) [3]	1.23 (0.2)	1.21 (0.3)
sdaB	5.40 (2.4)*	0.73 (0.1)	not determined

[1] Relative expression calculated based on the expression in WT pyruvate normalized to the *rplU* transcript.

[2] Data analyzed using one-way ANOVA within each transcript using a Dunnett's-corrected post-test with the pyruvate condition as the comparator. p-value abbreviations: * (<0.05); ** (<0.01); unmarked relative expression numbers are not statistically significant.

[3] the *adhC* transcript is stochastically induced under these conditions and while not different using the above parametric analysis, the data are not normally distributed. Analysis with the non-parametric Mann-Whitney test shows significance (p=0.026).

Table 2.S1 Bacterial strains and plasmids used in this study.

Designation	Genotype or description	Reference or Source
P. aeruginosa strains		
MJ101	PA14 wild type	(50)
MJ285	Δ*gbdR* in MJ285	(7)
GGW039	*soxB'-lacZYA-'soxG* in MJ285	This study
GGW034	Δ*souR* in MJ101	This study
GGW036	Δ*souR* in MJ285	This study
GGW076	Δ*gfnR* in MJ101	This study
GGW078	Δ*gfnR* in MJ285	This study
E. coli strains		
NEB5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NEB
S17-1 λpir	*thi pro hsdR- hsdM*$^{+}$ Δ*recA* RP4-2::TcMu-Km::Tn*7*	(51)
T7 Express	See manufacturer	NEB
GGW040	pGW005 in S17-1 λpir	This study
GGW031	pGW008 in S17-1 λpir	This study
GGW064	pGW024 in S17-1 λpir	This study
MJ500	pBT20 in SM10 λpir	(13, 30)
GGW047	pGW015 in T7 Express	This study
Plasmids		
pMQ30	suicide vector, Gm^{R}, *sacB*	(26)
pMQ80	High copy *Pseudomonas* vector, Gm^{R}	(26)
pUC18-mini-Tn7T-Gm	*attTn7* integration vector, Gm^{R}	(28)
pTNS2	carrying the *att*Tn*7* transposase	(29)
pMW5	*lacZYA* reporter plasmid Gm^{R}	(7)
pMALc2X	MBP-fusion vector, Ap^{R}	NEB
pKH10	MBP-GbdR expression vector	(22)
pGW005	*soxB'-lacZYA-'soxG* in pMQ30	This study
pGW007	*souR* complementation in pMQ80	This study
pGW008	*souR (PA14_9770)* in pMQ30	This study
pGW023	*gfnR (PA14_17380)* in pMQ30	This study
pGW024	*gfnR (PA14_17380)* in pUC18-mini-Tn7T-Gm	This study
pGW011	P_{glyA1} -210 to +1 in pMW5	This study
pGW012	P_{glyA1} -158 to +1 in pMW5	This study
pGW013	P_{glyA1} -104 to +1 in pMW5	This study
pGW014	P_{glyA1} -67 to +1 in pMW5	This study
pGW015	*souR (PA14_17380)* in pMALc2X	This study

Table 2.S2 Primers used in this study.

Primer Name	Sequence
SoxKO-F1	aagcttCTACCGGGCTGATCGACTAC
SoxkO-R1	AAGTACGAAGGCGACTCGACCATGGTGGTGGATGCTCCTGAACTGTT
SoxKO-F2	CCATGGTCGAGTCGCCTTCGTACTTACTTCTGGCTCTGGTTGCAG
SoxKO-R2	ggtaccTTGCACTGGAAAGTCGTCTG
PA14_9770 KO F1	CCG GGA GGT GGG TTA CTT TC
PA14_9770 KO R1	CTT CAG ACT CCG ACT GCC GCG CGC TGA AAC GCC TTC TTT CCA T
PA14_9770 KO F2	CGCGGCAGTCGGAGTCTGAAGGCAACGTCCGC CGACGAAATG
PA14_9770 KO R2	CCG CCG ACA GCG TAT AAG GA
PA14_9770 Rescue F	GCT CGG TAC CCA TGG AAA GAA GGC GTT TCA GCG CCG
PA14_9770 Rescue R	GCC AAG CTT TCA TTT CGT CGA GCG GAC GTT GCC
PglyA1_F1	TGT ATT AAG CTT GGT GTT CTC GCA AGA CGA AGA GC
PglyA1_50bp_del_F2	TGT ATT AAG CTT CCG CAT CGG TTG CCG AAT CCC AC
PglyA1_100bp_del_F3	TGA TAT TAA GCT TGC ATA GGC ATC TGG GCC GGC AGG
PglyA1_150bp_del_F4	TCG CTA TTA AGC TTC GCT GGC AAA GGG ACC GCG TGT
PglyA1_R1	ATA TCA AGG TAC CAT TCC GGC GCG GTT CCG GCG C
souR_MPB_F (Ecor1)	TTT CAG AAT TCG AAA GAA GGC GTT TCA GCG CCG C
souR_MPB_R (BamHI)	CTA GAG GAT CCT CAT TTC GTC GAG CGG ACG TTG CCG
PA3630KO_F1	CAT TCG GGC CCA TCC AGA AGA T
PA3630KO_ R1	CGCGCGGCCTTCGCAGGCTGCAACTGGTGGACTATCTCAAG GAA
PA3630KO_F2	AGG CCT GCG AAG GCC GCG CGG AGG AAA CGC CCA TGC GTT CG
PA3630KO_R2	TTG TCG TAG TCC TTC GGA TTG ATG
PA3630 RescueF KpnI	CATGGTACCGCGAAGGCGACGGCGGCACGGG
PA3630 Rescue R HindIII	CATAAGCTT G CGA CCA GAA CCT CAC CAG GTA G
PA3630 KO screen F	GGA CAG ACC TTC CTG CAA CA
PA3630 KO screen R	CAG AGG TAC ATG ACG CGT GG
PA2762_F1	GGCAAGTGGGAGGTGAACTA
PA2762_R1	AAGTACTTGCGCACCGTCTC
rplU_F1	GCAGCACAAAGTCACCGAAGG
rplU_R1	CCGTGGGAAACCACTTCAGC
sdaB_F1	CATGGAATGGGTCAACCTGT
sdaB_R1	AGATCGAGGCGTTCTTCTTG
soxA_F1	GTTCCTCAACCGGGTCTACA
soxA_R1	ATTCGGTCTGGTGGTACAGC
PA3630_F1	GTG CGG CAA GTG CAA ATT CT
PA3630_R1	GGG GCA TCC TTG GGA ATC TT
PA3629-prom-5'-biotin	5'biotin-CGC TCT TCC AGG CGG GCG ACC TGG C
PA3629-prom-3'	ATA GGT ACC GAT GGA AGC CGG CGG GCC G
PA2762-prom-5'-biotin	5'biotin-CAGGAAGGCAGTGGATGAAT
PA2762-prom-3'	CCTTTGCCTGTGGTGGAC
glyA1-prom-5'-biotin	5'biotin-GTGTTCTCGCAGGACGAAG
glyA1-prom-3'	CTTTGCCAGCGATGGTATG
cbcX-prom-5'-biotin	5'biotin-GAACTCCTCTGCAGGGTAAGG
cbcX-prom-3'	CCGGCAAAGACCACTATGAT
PA1999_prom_R1_KpnI	GGATggtaccCTCTTCCGGCTCTTGTGATT
PA1999_F_biot	GAGGCTTTCCTCCAGGCTCT
TnM5 amp F	TAC AGT TTA CGA ACC GAA CAG GC
SouR tnm R	GAA GAA CAG GCG AAC GCA TC
PA4183 TnM R	TTC TTG GTG CGC TTT GGT TG
GbdR TnM R	TGG CAT AGC CCC CAA TTT GT
sdaB TnM R	AGG CCT GCA GAT GAT GTT GG
PA2762 TnM R	CTC CCA GCG TTC GTA GTT CA

OpdP TnM R	TTG GGA ATG CGG AAG GTG AA
soxA TnM R	GTG GTG GTT CAT CCA GTC GT
GfnR TnM R	GGA CAG ACC TTC CTG CAA CA
adhC TnM R	TCT CGA CAT AGC TCG GCA AC
dhcA TnmR	GGA CTC GAC AAG CGA GTA GG
Rnd1 TnM20	TATAATGTGTGGAATTGTGAGCGG
Rnd1 PA Arb primer 2	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC
Rnd2 TnM20	ACAGGAAACAGGACTCTAGAGG
Rnd2 Arb primer	GGCCACGCGTCGACTAGTAC
BT20TnMSeq	CACCCAGCTTTCTTGTACAC

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CHAPTER 3:

Pulmonary surfactant promotes virulence gene expression and biofilm formation in *Klebsiella pneumoniae*

Graham G. Willsey[1,2], Sebastian Ventrone[3], Kristin C. Schutz[1], Aaron M. Wallace[3], John W. Ribis[1], Benjamin T. Suratt[2,3], and Matthew J. Wargo[1,2]

[1] Department of Microbiology and Molecular Genetics, [2] The Vermont Lung Center, [3] Department of Medicine, University of Vermont Larner College of Medicine, Burlington, VT 05405

3.1 Abstract

The interactions between *Klebsiella pneumoniae* and the host environment at the site of infection largely unknown. Pulmonary surfactant serves as an initial point of contact for inhaled bacteria entering the lung and is thought to contain molecular cues that aid colonization and pathogenesis. To gain insight into this ecological transition, we characterized the transcriptional response of *K. pneumoniae* MGH 78578 to purified pulmonary surfactant. This work revealed changes within the *K. pneumoniae* transcriptome that likely contribute to host colonization, adaptation, and virulence *in vivo*. Notable transcripts expressed under these conditions include genes involved in capsule synthesis, LPS modification, antibiotic resistance, biofilm formation, and metabolism. In addition, we tested the contributions of other surfactant-induced transcripts to *K. pneumoniae* survival using engineered isogenic KPPR1 deletion strains in a murine model of acute pneumonia. In these infection studies we identified the MdtJI polyamine efflux pump and ProU glycine betaine ABC transporter as significant mediators of *K. pneumoniae* survival within the lung and confirmed previous evidence for the importance of de novo leucine synthesis to bacterial survival during infection. Finally, we determined that pulmonary surfactant promoted type 3 fimbriae-mediated biofilm formation in *K. pneumoniae* and identified two surfactant constituents, phosphatidylcholine and cholesterol, that drive this response. This study provides novel insight into the interactions occurring between *K. pneumoniae* and the host at an important infection site and demonstrates the utility of purified lung surfactant preparations for dissecting host-lung pathogen interactions *in vitro*.

3.2 Introduction

Klebsiella pneumoniae is a Gram-negative opportunistic pathogen that causes an estimated 8-10% of nosocomial infections in the United States and Europe (1-3). *K. pneumoniae* is often found in the environment (4-6) and is also a frequent colonizer of the human gastrointestinal tract (7, 8). Infections by this bacterium occur in a range of tissues within immunocompromised individuals, with the urinary and respiratory tracts being the most prevalent (1, 2, 9). Pulmonary infections caused by *K. pneumoniae* are particularly concerning and are associated with high levels of morbidity and mortality. Unfortunately, treatment options for combating these infections are becoming increasingly limited due to the widespread development of drug resistance (10-12). The recent emergence of colistin resistance in *K. pneumoniae*, coupled with the increasing prevalence of extended spectrum beta lactamase (ESBL) and carbapenemase-producing strains suggest that new therapeutics are urgently needed (13-15). Despite the clinical significance of *K. pneumoniae*, little is known about its interaction with the host lung environment during infection. *K. pneumoniae* transcriptional changes occurring following inhalation and deposition into the lung are likely associated with adaptation and niche colonization. Therefore, characterizing this ecological transition is critical to our understanding of the infection process.

One of the first aspects of the host lung environment encountered by inhaled bacteria is pulmonary surfactant. This phospholipid-rich mixture coats the alveolar surfaces at the air-liquid interface and serves to reduce surface tension within the lung to prevent collapse following expiration (16, 17). Aside from this mechano-physical role, lung surfactant also modulates the activity of inflammatory cells and directly participates

in the innate immune response via two surfactant-associated collectins (SP-A and SP-D) (18-20). Lung surfactant contains roughly one hundred unique components, including a minor proteinaceous fraction consisting of four surfactant associated proteins (SP-A, SP-B, SP-C, SP-D), as well as a much larger lipid fraction comprising nearly 90% of the dry weight of this substance. Within the lipid fraction, dipalmitoylphosphatidylcholine and mixed-tail phosphatidylcholines are the major constituents, making up nearly 80% of the total lipid content, followed by phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, and sphingomyelin. Also present within the lipid fraction are fatty acids, free triglycerides, and neutral lipids such as cholesterol (17, 21, 22).

Pathogenic bacteria entering the host lung must generate an appropriate transcriptional response to successfully transition to this environment and avoid clearance by the innate immune system. Recognition of components within lung surfactant has been associated with the survival and virulence of several other opportunistic pathogens, perhaps unsurprisingly, given the locale of this substance at the respiratory surfaces of the alveoli and terminal bronchioles. Previous transcriptional profiling studies by our group with purified lung surfactant led to the determination that the detection of sphingosine and the metabolism of the choline moiety of phosphatidylcholine by *Pseudomonas aeruginosa* are both independently required for full virulence in a mouse model of acute pneumonia (23-25). Similarly, work by Ishii *et al*. concluded that fatty acids within lung surfactant invoked a membrane stress response in *Staphylococcus aureus* and identified a novel virulence determinant implicated in this process (26).

Based on these studies, purified lung surfactant represents a critical, yet experimentally tractable, aspect of the host lung environment that offers an attractive *in*

vitro model to examine host-pathogen interactions occurring during the onset of infection. Here, we characterized the transcriptional response of *K. pneumoniae* MGH 78578, a multidrug resistant clinical isolate (27), to purified bovine lung surfactant (Survanta). This transcriptomic-based strategy allowed us to determine that numerous characterized virulence and fitness-related genes of *K. pneumoniae* are expressed in response to lung surfactant, including those involved in capsule synthesis, biofilm formation, antibiotic resistance, LPS modification, and metabolism (1, 2, 9). We also tested the contributions of some of the identified genes to survival in a mouse model of acute pneumonia. We identified the MdtJI polyamine efflux pump and ProU glycine betaine ABC transporter as significant mediators of *K. pneumoniae* survival within the lung and confirmed the importance of endogenous leucine synthesis for *K. pneumoniae* survival during infection. An additional goal of this study was to identify the constituents within lung surfactant that induced expression of *K. pneumoniae* virulence-associated transcripts. Here, we have shown that at least two components of lung surfactant, phosphatidylcholine and cholesterol, promoted type 3 fimbriae expression.

3.3 Results

3.3.1 Lung surfactant alters expression of *K. pneumoniae* metabolism pathways and virulence factors

Our goal was to characterize the transcriptional changes occurring within *K. pneumoniae* resulting from exposure to purified lung surfactant. To accomplish this, we performed microarray analysis using a custom Affymetrix GeneChip designed for the *K. pneumoniae* MGH 78578 (ATCC 700721) genome and RNA collected from cells that were cultured in MOPS minimal media containing lactate as a carbon source with or without purified bovine lung surfactant (Survanta). Under these conditions, 89 transcripts exhibited more than a two-and-a-half-fold change in expression ($P<0.05$) between the presence and absence of lung surfactant. Eighty of these genes increased in expression in response to surfactant, while nine genes were repressed. A summary of these changes is shown in **Figure 3.1A & B**, with transcripts categorized into groups reflecting their known or bioinformatically predicted function (28-30). The 25 most highly expressed transcripts are also shown in **Table 3.2**, while a full list of the transcriptional changes occurring within *K. pneumoniae* in response to Survanta can be found in **Supplementary Table 3.1**.

Fifteen percent of the genes expressed by *K. pneumoniae* in response to Survanta are predicted to function in phospholipid and fatty acid metabolism. FadR regulon members are well represented among this group, with six β-oxidation-related genes (*fadBA, fadHIJ, fadE*) (31) exhibiting between 3.8- and 12.3-fold increases in transcript abundance in response to surfactant. In addition, the four genes within the *Kpn_02053-56*

operon displayed between 5 and 19.2-fold increases in abundance under these conditions. Encoded within this operon are a predicted citrate permease-like transporter and orthologs of genes found within the dehydroxycarnitine and 3-hydroxybutryate metabolism gene clusters of *P. aeruginosa* PAO1, reflecting their probable function in the uptake and metabolism of short chain fatty acids (32, 33).

Other changes within the *K. pneumoniae* transcriptome reflect global alterations in nitrogen metabolism. Glutamate synthase, *gltD*, the glutamine ABC transporter permease, *glnP*, and the nitrogen regulatory protein, *glnK*, exhibited between 2.6 and 3.1-fold increases in transcript abundance in response to lung surfactant, indicative of fluctuations in nitrogen pool homeostasis. Other transcriptional changes reflect the accumulation, metabolism, and excretion of polyamines during growth in lung surfactant. Notably, increases in the abundance of several putrescine-inducible transcripts (34, 35) were observed, including the *mdtJI* polyamine efflux pump and *yneI* succinate semialdehyde dehydrogenase [5.6 to 2.7-fold increase].

Exposure to lung surfactant also altered the expression of metabolic transcripts in *K. pneumoniae* in unexpected ways. Interestingly, Survanta stimulated the transcription of genes involved in the synthesis of branched chain amino acids (BCAA), including the valine-pyruvate transaminase, *avtA* [2.6-fold increase] and *leuABCD* leucine synthesis operon [2.9 to 2.3-fold increase]. In addition, repression of the phenylacetic acid (*paaCDFEFIK)* and histidine (*hutUIH)* catabolism gene clusters was observed [6.7 to 3-fold decrease].

Numerous oxidative stress-related transcripts were also induced by *K. pneumoniae* in lung surfactant, potentially in response to elevated reactive oxygen

species generated through the β-oxidation of fatty acids. Transcription of *nemAR*, encoding the oxidative stress-responsive regulator, NemR, and reactive-electrophile neutralizing N-ethylmaleimide reductase, NemA (36, 37), increased 2.8 and 7.4-fold, respectively, under these conditions. Other oxidative stress response genes were also expressed including, *ybbL* (2.5-fold increase) and a hydrogen peroxide-inducible gene of unknown function, *ybjM* [2.9-fold increase] (38, 39).

Other aspects of the *K. pneumoniae* transcriptional response to lung surfactant are reflective of metabolic/cytosolic stress. Notably, transcription of the multiple drug resistance and acid response regulator (40-42), *evgA* increased 3.5-fold in response to surfactant exposure. Transcription of the glycine betaine ABC transporter, *proVWX (43),* also increased 4-fold, suggesting that the lipid-rich environment of lung surfactant invokes osmotic stress in *K. pneumoniae*. Two tRNA nucleotide modification enzymes were also induced under these conditions, with *queC* and *gidA* exhibiting 5.4 and 2.8-fold increases in transcript abundance, respectively (44, 45). Finally, transcription of genes associated with antibiotic resistance were also upregulated, including the 23S ribosomal RNA methylation enzyme (*yfgB)* and aminoglycoside 3'-phosphotransferase, *strB* [2.6 to 2.7-fold increase] (46, 47).

Lung surfactant also induced transcriptional changes within *K. pneumoniae* associated with colonization, virulence, and immune evasion. Exposure to Survanta induced the expression of type 3 fimbriae encoded by the *mrkABCDF* gene cluster [6.7 to 2.6-fold increase] (48). Similarly, a 5.4-fold increase in transcript abundance was observed for a cyclic-di-GMP phosphodiesterase (KPN_01159) that has been implicated in promoting *mrK* operon expression *in vitro* (49). Increased transcription of genes

encoded within the capsular polysaccharide synthesis *(cps)* region were also observed following exposure to surfactant, including *ugd, Kp52D,* and *KPN_02483* [2.6 to 6.2-fold increase]. Two other capsule synthesis genes, *KPN_02503 & KPN_02506* (50), also exhibited statistically significant increases in transcript abundance, but failed to surpass our 2.5-fold change cutoff for inclusion in this study [2.0 & 2.1-fold increase]. Lung surfactant also invoked transcriptional changes within *K. pneumoniae* indicative of LPS modification. A 3.1-fold increase in transcript abundance was observed for *arnA*, whose product participates in conferring resistance to cationic peptides and polymyxin B through the addition of 4-amino-4-deoxy-L-arabinose (Ara4N) to lipid A (51-53).

3.3.2 Validation of Microarray Data

Quantitative RT-PCR was used to confirm the Survanta-induced transcriptional changes within *K. pneumoniae* that we identified through microarray and to determine conservation of these responses in KPPR1. To accomplish this, RT-PCR was performed on *K. pneumoniae* MGH 78578 and KPPR1 RNA collected from three additional Survanta induction experiments as described in the methods section. The relative abundance of seven transcripts were examined, representing nearly 10% of the genes identified through microarray as induced under these conditions. Genes for analysis were chosen to represent a range of cellular functions, including: fatty acid and phospholipid metabolism *(fadB & Kpn_02053)*, biofilm formation *(mrkA)*, branched-chain amino acid synthesis *(leuA)*, nutrient uptake *(proV)*, polyamine efflux *(mdtJ)*, and LPS modification *(arnA)*. As shown in **Figure 3.2**, all transcripts examined exhibited greater than a two-

fold increase in expression in response to Survanta, in close agreement with the microarray data. For the five primers that produced correct amplicons, all showed induction, with *leuB* and *arnA* showing higher relative induction in KPPR1.

3.3.3 Surfactant-induced transcripts contribute to *K. pneumoniae* fitness during lung infection

The contributions of other surfactant-induced transcripts to *K. pneumoniae* lung pathogenesis were explored using engineered *K. pneumoniae* gene deletion strains in a mouse oropharyngeal aspiration model of acute pneumonia. Due to the historic usage of KPPR1 as the model for Klebsiella lung infection, gene deletions were engineered into KPPR1 (ATCC 43816) (1, 54-58). KPPR1 and MGH 78578 share a high level of gene conservation with 88% of open reading frames being considered orthologous (59). More importantly, this genetic similarly is reflected within the surfactant microarray data where 81% of transcripts expressed by MGH 78578 under these conditions are also encoded within the genome of KPPR1.

Exposure to lung surfactant induced the expression of the leucine synthesis gene cluster (*leuABCD)* in both strains of *K. pneumoniae* (**Figure 3.2**). The importance of branched chain amino acid synthesis to *K. pneumoniae* during pulmonary infection was recently demonstrated through an *in vivo* genetic screen that recognized the *ilvADE* isoleucine and valine synthesis gene clusters as required for pathogenesis, and also noted that *leuABCD* disruption mutants displayed competitive fitness defects *in vivo* (60). Therefore, we generated a *leuABCD* deletion strain to determine if the defect was

absolute or only manifests in competition with the WT. Deletion of *leuABCD* resulted in a nearly a fifty-fold decrease in bacterial CFU in the lung compared to the WT strain (48.98-fold decrease in CFU, p value = 0.0048) **(Figure 3.3A)**. Interestingly, although the total number of immune cells in the bronchoalveolar lavage fluid (BALF) collected from mice infected with the *ΔleuABCD* strain were similar to those infected with KPPR1 WT, the composition of the infiltrating leukocytes differed. BALF collected from mice infected with the deletion strain demonstrated a reduction in neutrophilic response to the mutant strain, likely as a consequence of reduced bacterial CFU **(Figure 3.3B)**.

The *mdtJI* operon encodes a small multi-drug resistance (SMR-family) efflux pump that was first implicated in resistance to deoxycholate and SDS in *E. coli* (61). More recent reports have indicated that MdtJI primarily functions in the excretion of the polyamines spermidine and putrescine (35, 62). Polyamines have been recognized as important mediators of virulence in numerous bacterial genera including *Shigella, Salmonella,* and *Staphylococcus* (63), leading to our interest in exploring the potential contribution of MdtJI to *K. pneumoniae* fitness during infection of the lung. As shown in **Figure 3.4A**, deletion of *mdtJI* resulted in more than a ten-fold decrease in bacterial lung burden relative to the WT strain 24 h post inoculation (10.86-fold decrease in CFU). Interestingly, BALF collected from mice infected with KPPR1 *ΔmdtJI* contained significantly fewer infiltrating leukocytes, neutrophils, and macrophages than mice infected with the WT strain **(Figure 3.4B).**

The ProU (*proVWX)* ABC transporter was the most highly induced metabolite acquisition system expressed by *K. pneumoniae* following exposure to Survanta. The role of this transporter has been extensively studied in *Escherichia coli* and *Salmonella*

typhimurium and participates in the uptake of glycine betaine from the environment during periods of osmotic stress (43, 64). Phosphatidylcholine is the most abundant phospholipid in lung surfactant, and has previously been shown to serve as an important source of the osmoprotectant, glycine betaine, that is required for *Pseudomonas aeruginosa* fitness within the lung (24). We were therefore curious to determine if ProU-mediated glycine betaine uptake also contributed to *K. pneumoniae* fitness during lung infection. As shown in **Figure 3.5A,** deletion of *proV* resulted in a significant decrease in bacterial lung burden compared to the WT KPPR1 strain 24 H post inoculation (6.99-fold decrease in CFU). Examination of immune cells in BALF collected from these mice indicated that deletion of *proV* altered polymorphonuclear leukocyte recruitment **(Figure 3.5B).** It is important to note for the CFU changes reported for these three strains that although we suggest a survival difference, we have not enumerated CFU in the BALF or other body compartments and it therefore remains a formal possibility that localization is affected instead of, or in addition to survival.

3.3.4 KPPR1 isogeneic deletion strains exhibit wild type growth kinetics in TSB

Our *K. pneumoniae* gene deletion strains were assessed for general growth defects in nutrient rich media to ensure that the decreased CFU counts we observed *in vivo* were not a consequence of generalized growth defects. In order to address this question, the growth of KPPR1 WT and engineered gene deletion strains were measured in tryptic soy broth (TSB), the media used to culture the bacteria prior to inoculation into mice. As shown in **Figure 3.6,** the growth kinetics of all deletion strains closely mirrored the WT

strain. We were also interested in exploring the impact that deletion of these genes had on the ability of *K. pneumoniae* to grow in the presence of Survanta. However, both *K. pneumoniae* MGH 78578 and KPPR1 failed to effectively utilize Survanta as a nutrient source in a range of medias, including: LB, TSB, R2A, MOPS, and M63 (data not shown). Specifically, addition of Survanta to either these rich or minimal media formulations did not result in increase or decrease of CFU counts compared to the media without Survanta.

3.3.5 Some Survanta-induced transcripts are expressed in response to specific lung surfactant components

An additional goal of this study was to identify the molecules within lung surfactant that promoted *K. pneumoniae* virulence gene expression. The ability of individual constituents of lung surfactant to stimulate *mrkA, proV,* and *mdtJ* transcription was examined through quantitative RT-PCR. For these experiments, RNA was collected from *K. pneumoniae* MGH 78578 cells grown in MOPS minimal media and subsequently exposed to individual components found within lung surfactant, or lactate as a control. Compounds tested included phosphatidylcholine, diacylglycerol, palmitate, sphingosine, cholesterol, in addition to choline and ethanolamine, which have previously been shown to induce fimbriae expression in enterohemorrhagic *E. coli (65)*. Exposure to cholesterol and phosphatidylcholine stimulated transcription of *mrkA,* but none of the individual compounds tested significantly induced transcription of *mdtJ* or *proV* **(Figure 3.7).**

3.3.6 Surfactant-induced biofilm formation is mediated by type 3 fimbriae

Both our microarrays and subsequent qRT-PCR revealed that the type 3 fimbriae gene cluster *(mrkABCDF)* is expressed by *K. pneumoniae* MGH 78578 and KPPR1 following exposure to lung surfactant **(Figures 3.1, 3.2, and Table 3.2).** In addition, our gene induction experiments indicated that two constituents of lung surfactant, phosphatidylcholine and cholesterol, induced transcription from the *mrkA* promoter **(Figure 3.7).** We were therefore curious to determine if these observations were reflected through increased biofilm production. To address this question, we cultured *K. pneumoniae* MGH 78578, KPPR1 WT, and KPPR1 *ΔmrkABC* in minimal media in the presence and absence of Survanta, phosphatidylcholine, or cholesterol, then quantified the resultant biofilm material adhered to the plastic culture wells the following day through crystal violet staining assay.

As shown in **Figure 3.8A,** exposure to Survanta, phosphatidylcholine, and cholesterol resulted in significant increases in biofilm production in *K. pneumoniae* MGH 78578 and KPPR1. The biofilms generated by KPPR1 under these conditions were notably less robust than those produced by MGH 78578. These observations can be explained in part by the hypermucoid phenotype of KPPR1 (cps_{K2} serotype (56)) relative to MGH 78578 (cps_{K52} serotype(66)), since capsule production is known to negatively impact biofilm formation in *K. pneumoniae* (67, 68). Furthermore, deletion of the *mrkABC* fimbria genes in KPPR1 disrupted biofilm formation at the air liquid interface in the presence of lung surfactant, and resulted in a substantial reduction in adhered biofilm material compared to the WT strain in every condition tested **(Figure 3.8A).** These data

indicate that lung surfactant-induced biofilm formation is primarily mediated by type 3 fimbriae.

3.4 Discussion

Our understanding of the genetic factors influencing *Klebsiella pneumoniae* pathogenesis has significantly improved in recent years (1, 9). *In vivo* genetic screens and deep sequencing have been particularly effective in identifying genes associated with *K. pneumoniae* fitness during infection in a range of tissue types (42, 69-72). Bachman and colleagues recently applied this methodology to uncover numerous *K. pneumoniae* genes that contribute to pathogenesis within the lung (60). Despite these advances, there is still much that we do not understand regarding the role *K. pneumoniae's* response to the host environment plays in shaping colonization and pathogenesis.

Lung surfactant serves as an initial point of contact for inhaled bacteria entering the lung, particularly those in small aerosol droplets, and likely contains molecular cues that influence colonization and pathogenesis. Our group has demonstrated the utility of the lung surfactant preparation, Survanta, for dissecting host-lung pathogen interactions in *P. aeruginosa*. We previously showed that lung surfactant led to induction of transcripts involved in the detection of sphingosine and the metabolism of choline, and that both of these pathways were required for *P. aeruginosa* survival in a mouse model of acute pneumonia (23, 24). We also showed that the utilization of phosphatidylcholine metabolites in lung surfactant by *P. aeruginosa* promoted virulence factor expression

(25), and directly contributed to the loss of surfactant function during murine infection (73).

In this study, we expanded on our previous efforts with *P. aeruginosa* and characterized the transcriptional changes within *Klebsiella pneumoniae* MGH 78578 resulting from exposure to Survanta. We observed numerous alterations within the *K. pneumoniae* transcriptome that likely promote colonization, adaptation to the host, and virulence *in vivo*. Notable transcripts expressed by *K. pneumoniae* under these conditions include genes involved in capsule synthesis, LPS modification, antibiotic resistance, and biofilm formation **(Figures 3.1 & 3.2).** Furthermore, a sizeable fraction of the transcripts identified through this work indicate that the lipid-rich environment of lung surfactant invokes significant membrane, cytosolic, and oxidative stress in *K. pneumoniae* **(Figure 3.1B & Table 3.2).** These results parallel our earlier findings in *P. aeruginosa* (23, 25), and support similar observations in *Staphylococcus aureus (26),* suggesting that lung surfactant is likely to promote the expression of virulence and stress-related genes in a range of lung pathogens.

3.4.1 Lung surfactant-induced transcripts contribute to *K. pneumoniae* survival during acute murine pneumonia

We also demonstrated that lung surfactant-induced transcripts contribute to *K. pneumoniae* survival and resulting inflammation during acute pneumonia. For these experiments, we focused on metabolism-related genes induced by surfactant.

Our interest in BCAA synthesis and the role these genes play in bacterial fitness during infection stems from our observation that lung surfactant specifically stimulated transcription of the leucine synthetic operon (*leuABCD)* in *K. pneumoniae*. In contrast, the expression of other amino acid anabolic pathways were not altered by lung surfactant under these conditions. The mechanism driving this induction is unclear, but lung surfactant metabolism by *K. pneumoniae* could invoke a specific, previously unknown need for increased leucine synthesis. The ability to synthesize BCAAs during infection is known be critical for the survival and virulence of several bacterial lung pathogens, given its scarcity in the lung environment (60, 74-76). The necessity of BCAA synthesis for *K. pneumoniae* during pulmonary infection was recently highlighted through an *in vivo* transposon mutant screen that recognized *ilvADE* and *leuABCD* gene disruption mutants displayed competitive fitness defects in the murine lung (60). Results from our mouse infections with an engineered leucine auxotroph of *K. pneumoniae* support these earlier findings, and confirm that BCAA biosynthesis is required for both fitness and survival in the absence of competition during lung infection **(Figure 3.3)**, suggesting that, like in other bacterial lung pathogens, loss of leucine synthesis is deleterious to survival in the lungs**.**

Polyamines have been recognized as significant mediators of bacterial virulence and often have pleiotropic effects on pathogenesis (63, 77). Within enteric species, the accumulation of putrescine and spermidine has been shown to promote biofilm formation in *Yersinia pestis*, type 3 secretion system expression in *Salmonella typhimurium,* and increased resistance to reactive oxygen species in *Shigella flexneri* during macrophage infection (78-80). Surprisingly however, the influence of polyamines on *K. pneumoniae*

survival had not previously been explored. Here, we have shown that deletion of the genes encoding the spermidine and putrescine efflux pump, *mdtJI* (35, 62), resulted in a significant defect in *K. pneumoniae* survival relative to the parental WT strain in our murine model of pneumonia **(Figure 3.4).** We propose two potential explanations for these observations. First, putrescine and spermidine are present on the outer membranes of enteric species and have been shown to alter membrane permeability through modifying the charge and shape of porins in *E. coli* (81, 82). Likewise, the presence of these polyamines on the outer membrane surface of *P. aeruginosa* has been shown to protect against oxidative stress and antibiotic-mediated killing (83). Therefore, MdtJI-mediated polyamine efflux could similarly facilitate resistance against oxidative killing in *K. pneumoniae*. Second, polyamines secreted by bacteria and fungi have been shown to interfere with the innate immune response by disrupting polymorphonuclear leukocyte (PMN) function (84-87)(88).

Our lung surfactant lipid induction experiments failed to reveal any individual components within surfactant that stimulated transcription of *mdtJI* **(Figure 3.7).** The expression of this pump is primarily regulated by the intracellular concentration of putrescine (35). However, transcription of *mdtJI* has also been shown to be stimulated by deoxycholate and bile salts in *S. flexneri* (35), suggesting that this efflux pump could also be induced by membrane stress or other environmental cues. We predict that the expression of *mdtJI* in *K. pneumoniae* under these conditions could either be a consequence of membrane stress or the metabolism of multiple components within lung surfactant.

The ProU *(proVWX)* ABC transporter has been well characterized in *E. coli* and *S. typhimurium,* and functions in the uptake of the osmoprotectant glycine betaine under periods of osmotic stress (43, 64). Phosphatidylcholine is the most abundant phospholipid within lung surfactant and serves as a vital precursor of glycine betaine for *P. aeruginosa*, the accumulation of which is required for bacterial survival in the lung (24). The survival defect we observed in the *K. pneumoniae ΔproV* strain in our acute murine pneumonia model is in close agreement with these earlier findings, and suggests that the ability to obtain glycine betaine from phosphatidylcholine is likely important for other Gram-negative respiratory pathogens as well **(Figure 3.5).** Host-derived glycine betaine has additionally been shown to promote *K. pneumoniae* success at other sites of infection. An *in vivo* screen previously revealed that *proV* gene disruption mutants displayed a competitive fitness defect in the colon and liver (42), indicating that glycine betaine likely serves as a preferred osmoprotectant for *K. pneumoniae* during infection.

It is important to note that not all surfactant-induced transcripts expressed by *K. pneumoniae* contribute to bacterial fitness during lung infection. The products of the five gene *Kpn_02053-5* operon are predicted to function in the uptake and metabolism of short chain fatty acids (28, 29), and represent the most highly induced transcript expressed by *K. pneumoniae* in response to lung surfactant **(Figure 3.1 & Table 3.2).** Despite the dramatic increase in transcription of this operon in response to lung surfactant, the deletion strain exhibited no defect in bacterial lung burden compared to the WT strain 24 H post inoculation. Similar results were also observed in a *ΔfadBA* strain, indicating that the metabolism of fatty acids within lung surfactant does not directly contribute to *K. pneumoniae* fitness during acute pneumonia (data not shown). The lack

of a phenotype for these highly expressed transcripts is not unexpected, as there is no evidence of a direct relationship between gene expression and fitness phenotype in bacterial lung infections to date (89).

3.4.2 Lung surfactant promotes type 3 fimbriae expression and biofilm formation in *K. pneumoniae*

Exposure to lung surfactant induced type 3 fimbriae-mediated biofilm formation in *K. pneumoniae* MGH 78578 and KPPR1 **(Figure 3.8)**. Type 3 fimbriae (Mrk fimbriae) have been extensively studied in *K. pneumoniae* and facilitate cell adhesion to a range of biotic and abiotic substrates including type IV & type V collagen, silicone, and hard plastics (90-93). Although type 3 fimbriae are not directly involved in *K. pneumoniae* virulence, their requirement for colonization and persistence in catheter-associated urinary tract infections (CAUTI) has been demonstrated by multiple groups (94, 95).

Transcriptional regulation of type 3 fimbriae expression in *K. pneumoniae* is complex and governed by multiple integrated regulatory networks, including being dependent on the coordinated activities of MrkH and MrkI in response to the intracellular accumulation of the secondary messenger, cyclic-di-GMP (96, 97). Surprisingly, the environmental signals and regulatory networks acting upstream of MrkHI that drive type 3 fimbriae expression are largely unknown, particularly in the context of infection. Recent reports have identified iron and oxidative stress-responsive transcription regulators that modulate *mrk* fimbriae expression (98-100), and Chet *et al.* also identified bile salts as stimulators of type 3 fimbriae-mediated biofilm formation (101). Here, we

expand on these previous findings and report that at least two components of lung surfactant, phosphatidylcholine and cholesterol, promote type 3 frimbriae transcription and biofilm formation in *K. pneumoniae* **(Figures 3.7 & 3.8).**

3.4.3 Conclusions

In summary, we characterized the transcriptional response of *K. pneumoniae* MGH 78578 to the lung surfactant preparation, Survanta. This work revealed numerous transcripts expressed by *K. pneumoniae* in response to lung surfactant that reflect metabolic adaptation, stress resistance, virulence, and host colonization. We also demonstrated that some surfactant-induced transcripts contribute to bacterial survival *in vivo* in a mouse model of acute pneumonia. Through this effort we confirmed the necessity of BCAA synthesis to *K. pneumoniae* success during infection and provided novel evidence suggesting that glycine betaine uptake and polyamine efflux also contribute to *Klebsiella* survival during respiratory tract infection. Finally, we identified multiple components within lung surfactant that stimulate type 3 fimbriae-mediated biofilm formation. This study provides novel insight into the interactions occurring between *K. pneumoniae* and the host at an important infection site. This work, together with our previous studies in *P. aeruginosa* highlight the utility of using lung surfactant to uncover important aspects of host-lung pathogen interactions *in vitro*.

3.5 Methods

3.5.1 Bacterial strains and compounds

K. pneumoniae KPPR1 (ATCC 43816) and *K. pneumoniae* MGH 78578 (ATCC 700721) were maintained on Lysogeny Broth (LB), Lennox formulation, supplemented with 200 µg/mL of hygromycin B when appropriate. All cloning steps were performed with *E. coli* DH5α λpir, while *E. coli* S17-1 λpir was used for conjugation with *K. pneumoniae*. Both *E. coli* strains were maintained in LB, supplemented with 150 µg/mL of hygromycin B, when appropriate. The purified bovine pulmonary surfactant preparation, Survanta (Beractant, AbbVie, Lake Bluff, IL) was utilized for our surfactant-response microarrays and biofilm experiments. Survanta is an organic extraction of lung surfactant from cows and as such is missing the polar surfactant proteins involved in pulmonary defense (SP-A and SP-D) as well as most antimicrobial peptides (including defensins) and antimicrobial proteins present in the lung lining fluid (ex. lysozyme), thus it is composed of the lipids naturally present in lung surfactant along with the hydrophobic proteins SP-B and SP-C. Because it is an organic extraction product, physiological concentrations of salts and dissolved polar compounds are added back by dilution of this product into minimal media. Lung surfactant constituents used in our gene induction assays were purchased from Avanti Polar Lipids (Alabaster, AL) and Sigma-Aldrich (St. Louis, MO).

3.5.2 Construction of *K. pneumoniae* gene deletion strains

Gene deletion strains in *K. pneumoniae* KPPR1 were generated through allelic exchange facilitated by the suicide vector, pGW65. To create pGW65, pMQ310 and pMQ30 (102, 103) were first digested with NcoI and KpnI (New England Biolabs, Ipswich, MA). The 3.9 kbp fragment of pMQ310 carrying the hygromycin B resistant cassette and R6Kγ origin and the 4.6 kbp fragment of pMQ30 carrying the *sacB* counterselectable marker were gel extracted using Thermo-Fisher's GeneJET kit (Waltham, MA), and subsequently ligated together before transformation into chemically competent DH5α-λpir. Gene deletion constructs were engineered into this vector using the molecular cloning methodology previously described with pMQ30 (103, 104). Briefly, ~1 kbp fragments immediately upstream and downstream of the gene (or genes) targeted for deletion were amplified using the primers listed in **Supplementary Table 3.2**. For each deletion construct, tailed primers were used to facilitate the fusion of each fragment via overlap extension PCR as well as ligation into pGW65 through incorporated flanking restriction sites. Ligation reactions were then chemically transformed into DH5α-λpir cells and transformants were selected for on LB supplemented with 150 μg/mL of hygromycin B. Plasmid DNA was harvested from these colonies by Miniprep (Qiagen) and verified by restriction digest.

Deletion constructs were subsequently transformed into chemically competent S17-1 λpir *E. coli* and mobilized into *K. pneumoniae* KPPR1 via conjugation (105). Following overnight incubation at 37 °C, merodiploids were selected by plating on MOPS minimal agar supplemented with 200 μg/mL of hygromycin B and 25 mM sodium pyruvate. To select for the Δ*leuABCD* strain, 0.5% casamino acids was added to this

media. KPPR1 merodiploids that arose the next day were then restreaked onto this media to ensure S17-1 λpir *E. coli* cells were not carried over. A second round of recombination was then permitted by first growing hygromycin B-resistant colonies overnight in LB containing 200 μg/mL of the antibiotic, diluting the overnight 1:500, and growing to mid-log phase in LB in the absence of hygromycin B. Dilutions of this culture were then plated on low salt LB agar containing 6% sucrose and incubated overnight at 25°C as suggested (105). Sucrose resistant colonies arising 24 h later were screened via PCR for deletion of the gene(s) of interest with primers listed in **Supplementary Table 3.2.**

3.5.3 Growth conditions and RNA purification for microarrays/qRT-PCR

K. pneumoniae MGH 78578 was grown overnight at 37 °C in modified MOPS (morpholinepropanesulfonic acid) minimal media (106, 107), supplemented with 25 mM lactate and 5 mM D-glucose. The following day, cells were collected by centrifugation, washed with 1 mL of MOPS media, and resuspended in MOPS containing 4 mM lactate to achieve an OD_{600} of 0.6. These cultures were then mixed 1:1 with MOPS media containing 4 mM lactate, or the same media supplemented with Survanta (AbbVie, Lake Bluff, IL) at a dilution of 1:50 to reflect the physiological concentration of PS in the airway surface liquid (15 mg/mL). Cultures were incubated at 37 °C shaking at 170 RPM for 4 h, at which point the cells here harvested via centrifugation and immediately lysed in ~85°C RNAzol RT (Sigma-Aldrich, St. Louis, MO), and frozen at -80°C. RNA extractions were first performed using Zymo Research's RNA mini-prep kit (Irvine, CA) following the manufacturers provided protocol. The resulting RNA was then incubated

for one hour with DNaseI (NEB) before being re-purified using RNeasy columns (Qiagen) to remove small RNAs in preparation for microarrays as we have done previously (23, 108). The quality of each RNA sample was then assessed via Agilent BioAnalyzer and quantified through Qubit fluorometer.

3.5.4 Survanta microarray methodology

Microarrays were performed by the UVM Advanced Genome Technology Core using a custom Affymetrix chip containing probes specific to the genomes of *Klebsiella pneumoniae* MGH 78578, *Stenotrophomonas maltophilia* K279A, *Burkholderia thailandensis* E264, and *Pseudomonas aeruginosa* PA14 (109). Arrays were performed in biological duplicate, with RNA collected from two independent Survanta induction experiments that were performed on separate days. *K. pneumoniae* cDNA hybridization was performed simultaneously with a 1:1 mixture of Survanta-induced *Stenotrophomonas maltophilia K279A cDNA* (cultured under the same conditions) per manufacturer's recommendation. Each condition was analyzed in duplicate, with probes for each gene averaged into one probe intensity using the Affymetrix Expression Console and Transcriptome Analysis Console software packages (version 3.0). Surfactant-altered transcripts were identified as those exhibiting at least a 2.5-fold change in signal between the two conditions as determined using robust multi-array average (RMA) analysis and *P* value of <0.05. The array data has been submitted to the GEO database under accession number GSE110628.

3.5.5 Quantitative RT-PCR

Total RNA was prepared from three additional Survanta inductions with *K. pneumoniae* MGH 78578 as described above. Twenty nanograms of RNA from each sample was then utilized as template for cDNA synthesis using Superscript IV and random hexamers (Thermo-Fisher), per manufacturer's instructions. Quantitative PCR was performed using the resulting cDNA in technical duplicate with primers listed in supplementary table 1 and NEB's Q5 2x master mix supplemented with SYBR green I nucleic acid gel stain (Thermo-Fisher) at a concentration of 0.2x, as we have done previously (108). A standard curve dilution series was generated for each primer set to determine transcript abundance (110). Values for each reaction were normalized to *Kpn_04184*, which exhibited no change in expression between conditions in the Survanta microarrays. Fold change for each transcript was determined by dividing normalized surfactant-exposed values by their corresponding control condition values. Absence of reverse transcriptase during cDNA synthesis resulted in no product from any primer set using the isolated RNA.

3.5.6 Mouse Infections

Infections were performed as previously described (57, 73). Briefly, *K. pneumoniae* KPPR1 WT and isogenic deletion strains were grown in TSB overnight, normalized by OD_{600}, harvested via centrifugation, washed in 2 mL of PBS, and finally resuspended in PBS to achieve 2 x 10^3 CFU per 50 µL. For each strain, the actual input

inoculum was determined by serial dilution plating on LB agar. Eight to ten-week-old adult male C57Bl/6J mice (Jackson Laboratories, Detroit, MI) were briefly anesthetized with isoflurane and inoculated with 2 x 10^3 CFU of either KPPR1 WT or isogenic deletion strains through oropharyngeal aspiration. Twenty-four hours later, mice were euthanized via sodium pentobarbital delivered through intraperitoneal injection, Bronchoalveolar lavage fluid was then collected, and lungs were then quickly removed, placed into 1 mL of cold PBS, and immediately homogenized.

Serial dilutions of the resulting lung homogenates were plated on LB agar to determine bacterial burden by counting CFU. White blood cell content within the BAL fluid was enumerated manually. Infections were performed at least three times with 3-4 mice per strain per experiment. In each case, paired infections were performed with one gene deletion strain and the parental WT strain for comparison of lung CFU.

3.5.7 Growth Assays

Growth assays were conducted with *K. pneumoniae* KPPR1 WT and isogenic deletion strains as we have done previously with *P. aeruginosa* (104). Briefly, KPPR1 WT and our isogenic deletion strains were grown overnight at 37 °C on a roller drum in MOPS minimal media supplemented with 20 mM lactate and 5 mM D-glucose. In the case of KPPR1 *ΔleuABC,* 0.5% casamino acids was added to this media to permit growth. The following day, cells were collected via centrifugation, washed with 1 mL MOPS media, and resuspended in TSB at a final optical density of 0.05 OD_{600} units. Growth assays were performed three time, each with technical triplicates, in 48-well tissue culture

plate and growth was determined by OD_{600} using a Synergy 2 plate reader (Biotek). Growth assays in Survanta were conducted as above, except growth was quantified by serial dilution plating, as Survanta is a colloidal suspension and prevents growth assessment by OD_{600}.

3.5.8 Gene induction assays with components of lung surfactant

To identify the transcript-inducing molecules within lung surfactant, quantitative RT-PCR was performed on *K. pneumoniae* MGH 78578 RNA collected from cells exposed to 1 mM phosphatidylcholine, sphingosine, cholesterol, diacylglycerol, palmitate, choline, ethanolamine, or no compound as a control. For these experiments, *K. pneumoniae* was first grown overnight in MOPS minimal media as described above. The following day, cells were collected by centrifugation, washed in 1 mL MOPS, and resuspended in MOPS 20 mM lactate to achieve a final OD_{600} of 0.3. One milliliter aliquots of this culture were then added to a plastic culture dish with wells containing these compounds deposited via the evaporation of ethanol, and incubated for 4 h at 37 °C and 170 RPM. Following the induction period, RNA was purified from these cells, cDNA was synthesized, and quantitative PCR was performed as described above.

3.5.9 Biofilm assay

K. pneumoniae MGH 78578, KPPR1 WT, and KPPR1 *ΔmrkABC* were grown overnight at 37 °C on a roller drum in MOPS minimal media supplemented with 20 mM sodium pyruvate and 5 mM glucose. The following day, cells were collected by

centrifugation, washed in 1 mL of MOPS media, and adjusted to an OD_{600} of 0.1. Each strain was then added 1:1 to either MOPS media containing 20 mM sodium lactate in addition to the same media supplemented with Survanta to achieve a final surfactant dilution of 1:50. The OD_{600}-adjusted cultures were also diluted 1:1 in the same media (MOPS 20 mM sodium lactate), and added to wells of a 48 well dish containing phosphatidylcholine or cholesterol that were deposited the night prior through ethanol evaporation. These cultures were incubated for 18 h at 37 °C and agitated at 170 RPM to loosely reflect the continuous aeration and mixing of surfactant that occurs within the lung. Following the incubation, the cell suspension was removed from the wells and the remaining biofilm material stained using 0.1% crystal violet, followed by water rinse, and solubilization of the remaining crystal violet in 30% acetic acid (111). Biofilm was quantified by measuring A_{550} using a Biotek Synergy 2 plate reader. This experiment was performed four times with technical triplicates of each experiment.

3.5.10 Statistical analysis and data visualization

All statistical analyses and figure generation were performed using GraphPad Prism Version 7.0, unless otherwise noted. Microarray analysis and statistical assessment was performed through RMA using Affymetrix's Expression Console and Transcriptome Analysis Console software packages (version 3.0) as described above. Gene functional classification was done by manually combining related GO, COG, and KEGG predictions into more general functions.

3.6 Acknowledgements

This work was supported by AI103003 to MJW, AI117069 to MJW and BTS, pilot funding through the Vermont Center for Immunology and Infectious Diseases to MJW (GM118228), and internal funding from the Department of Medicine, Larner College of Medicine to BTS. GGW was supported by T32 HL076122 fellowship, awarded through the Vermont Lung Center. The funders had no role in experimental design, or the collection and interpretation of data.

3.7 Chapter 3 figures

Figure 3.1. *K. pneumoniae* MGH 78578 transcriptome changes in response to lung surfactant. (A) Volcano plot of transcripts detected through microarray as exhibiting more than a 2.5-fold change in expression ($P < 0.05$) following exposure to Survanta. (B) Survanta-regulated transcripts were categorized into groups reflecting their known or bioinformatically-predicted functions. The color coding of the categories is the same for both panels.

Figure 3.2. qRT-PCR validation of induced transcripts in *K. pneumoniae* following exposure to lung surfactant. The relative abundance of seven Survanta-induced transcripts detected through microarray were re-examined using quantitative RT-PCR with RNA collected from three independent Survanta induction experiments as described in the methods section. Genes for analysis were chosen to represent a range of cellular functions, including: lipid metabolism *(fadB & Kpn_02053* (*dhcA))*, biofilm formation *(mrkA)*, branched-chain amino acid synthesis *(leuA)*, nutrient uptake *(proV)*, polyamine efflux *(mdtJ)*, and LPS modification *(arnA)*. Raw transcript values were normalized to *Kpn_04184*, which exhibited no change in expression between conditions in our microarrays. We examined expression in both MGH78578 (M) and KPPR1 (K), though our primers designed for *fadB* and *Kpn_02053* in MGH 78578 were not usable in KPPR1 due to multiple products (noted with the # symbol). Statistical analysis was conducted via two-way analysis of the variance (ANOVA) with a Sidak's post-test analyzing in-strain changes comparing expression from survanta additions to the lactate-alone condition. The data shown summarizes three independent Survanta induction experiments, and statistical

significance is indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$.

Figure 3.3. Leucine biosynthesis by *K. pneumoniae* is required for virulence during acute pneumonia. (A) Adult male C57BL/6 mice were infected via oropharyngeal aspiration with either *K. pneumoniae* KPPR1 WT or KPPR1 *ΔleABCD*. Colony forming units (CFU) per lung were measured 24 h post instillation. (B) White blood cells were enumerated within the bronchoalveolar lavage fluid collected from each infected mouse. The total WBC, Macrophage, and PMN cell counts are shown. The data shown summarizes three independent experiments, with statistical significance being determined through unpaired t-test. The arithmetic mean is depicted for each strain, with counts from individual mice represented by individual points. Statistical significance is depicted as follows: *represents $P < 0.05$, **represents $P < 0.01$.

Figure 3.4. The MdtJI-polyamine efflux pump contributes to *K. pneumoniae* fitness during acute pneumonia. (A) Adult male C57BL/6 mice were infected via oropharyngeal aspiration with either *K. pneumoniae* KPPR1 WT or KPPR1 *ΔmdJI*. Colony forming units (CFU) per lung were measured 24 h post instillation. (B) White blood cells were enumerated within the bronchoalveolar lavage fluid collected from each infected mouse. The total WBC, Macrophage, and PMN cell counts are shown. The data shown summarizes three independent experiments, with statistical significance being determined through unpaired t-test. The arithmetic mean is depicted for each strain, with counts from

individual mice represented by individual points. Statistical significance is depicted as follows: *represents $P < 0.05$, **represents $P < 0.01$, and ***represents $P < 0.001$.

Figure 3.5. The ProU glycine betaine ABC transporter (*proVWX*) contributes to *K. pneumoniae* fitness during acute pneumonia. (A) Adult male C57BL/6 mice were infected via oropharyngeal aspiration with either *K. pneumoniae* KPPR1 WT or KPPR1 *ΔproV*. Colony forming units (CFU) per lung were measured 24 h following instillation. (B) White blood cells were enumerated within the bronchoalveolar lavage fluid collected from each infected mouse. The total WBC, Macrophage, and PMN cell counts are shown. The data shown summarizes four independent experiments, with statistical significance being determined through unpaired t-test. In each panel, the arithmetic mean is depicted for each strain, with counts from individual mice represented by individual points. Statistical significance is depicted as follows: *represents $P < 0.05$, **represents $P < 0.01$.

Figure 3.6. KPPR1 isogenic deletion strains exhibit wildtype growth kinetics in TSB. The growth of KPPR1 WT and gene deletion strains in TSB was measured via OD_{600} over an 18 h period. The growth curves shown are representative of three independent experiments, with error bars indicating standard deviation.

Figure 3.7. Constituents of lung surfactant stimulate *K. pneumoniae* gene expression. Gene induction assays were performed for 4 h with *K. pneumoniae* MGH 78578 in MOPS minimal media containing lactate, and individual compounds found within PS. RNA collected from these inductions was then used for quantitative RT-PCR, with raw

transcript values normalized to *Kpn_04184*. The data shown encompasses three separate experiments. Statistical analysis was performed via two-way ANOVA and Dunnett's multiple comparison test, using the uninduced (lactate-alone) condition as the comparator. For this analysis, the *Kpn_04184*-adjusted transcript values in the uninduced (lactate-alone) condition were first set to one for comparison. Statistical significance is depicted as follows: *represents $P < 0.05$, **represents $P < 0.01$.

Figure 3.8. Type 3 fimbriae mediate biofilm formation in response to lung surfactant. *K. pneumoniae* MGH78578, KPPR1, and KPPR1 *ΔmrkABC* were cultured in MOPS minimal media containing 20 mM lactate in the presence and absence of Survanta, phosphatidylcholine, and cholesterol. After 18 h, the extracellular material remaining adhered to the culture dish was stained with 0.1% crystal violet. (A) Representative crystal violet-stained biofilms generated by each strain under these culturing conditions. (B) Biofilm-adhered crystal violet was solubilized with 30% glacial acetic acid, and quantified through measuring the absorbance at 550 nanometers. The data shown is the summary of four individual experiments that were performed in technical triplicate, with error bars representing standard deviation. Statistical analysis was performed using the MOPS lactate condition of each strain as the comparator via one-way ANOVA and Dunnett's multiple comparison test for *K. pneumoniae* MGH 78578, and two-way ANOVA with Sidak's multiple comparison test for KPPR1 WT and KPPR1 *ΔmrkABC*. Statistical significance is specified as follows: *represents $P < 0.05$, **represents $P < 0.01$, ***represents $P < 0.001$, and ****represents $P < 0.0001$.

3.8 Chapter 3 Tables

Table 3.1. Bacterial strains and plasmids used in this study**.**

Table 3.2. Summary of the 25 most highly induced transcripts expressed by *K. pneumoniae* MGH 78578 in response to lung surfactant.

Table 3.S1. *K. pneumoniae* MGH 78578 transcriptional response to lung surfactant.

Table 3.S2. Primers used in this study.

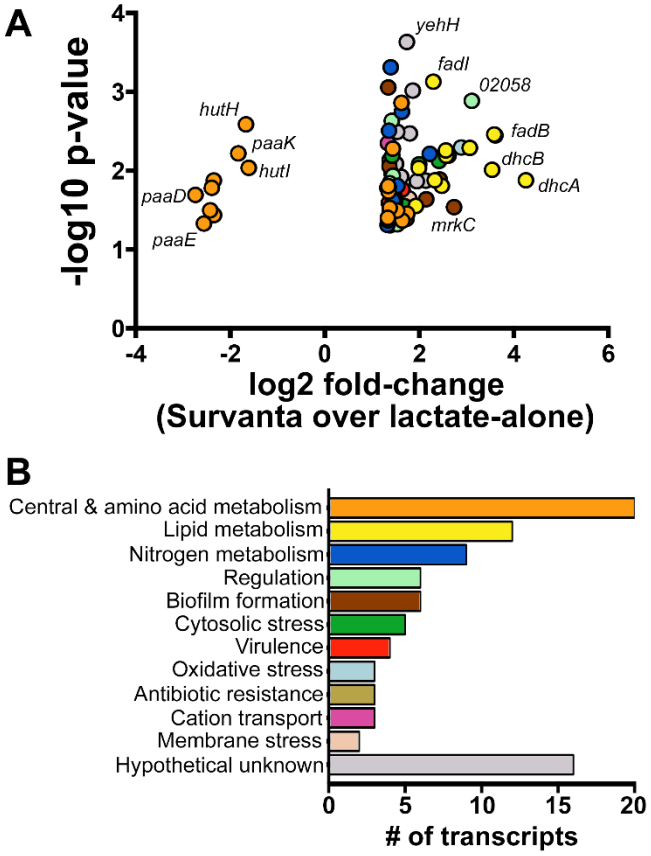

Figure 3.1. *K. pneumoniae* MGH 78578 transcriptome changes in response to lung surfactant.

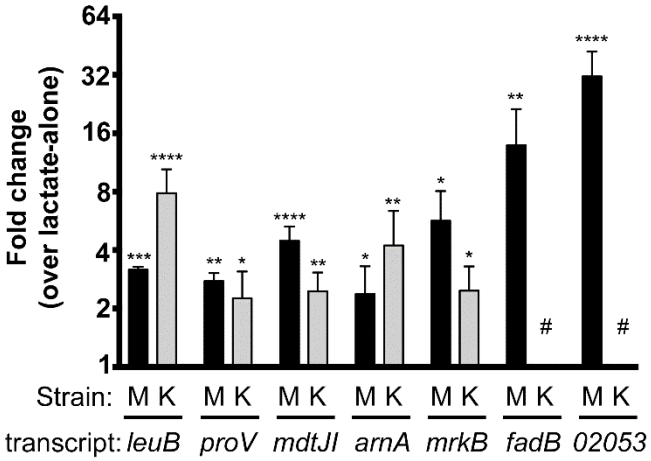

Figure 3.2. qRT-PCR validation of induced transcripts in *K. pneumoniae* following exposure to lung surfactant.

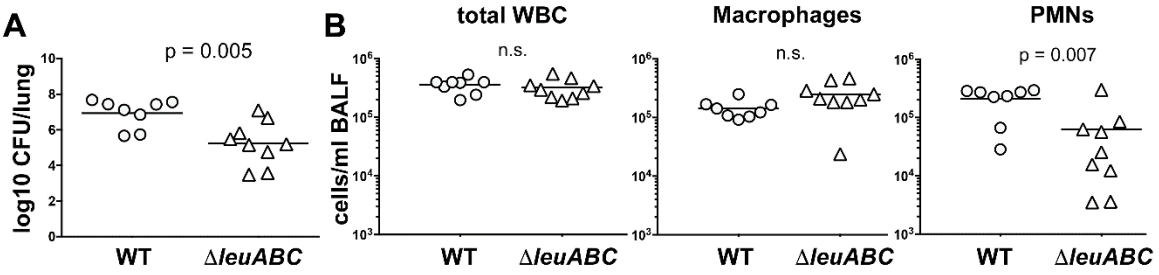

Figure 3.3. Leucine biosynthesis by *K. pneumoniae* is required for virulence during acute pneumonia.

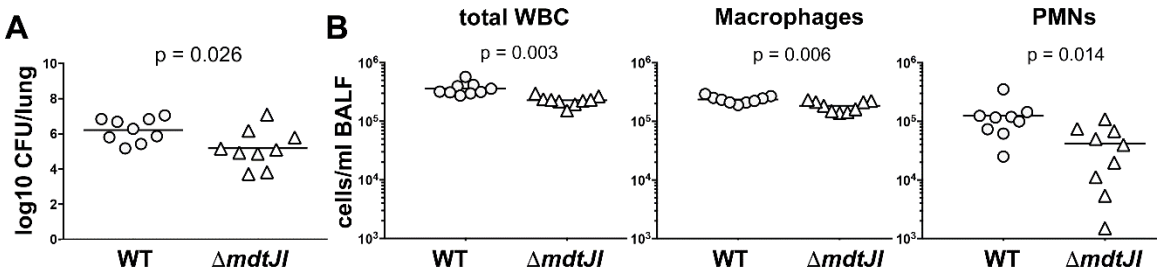

Figure 3.4. The MdtJI-polyamine efflux pump contributes to *K. pneumoniae* fitness during acute pneumonia.

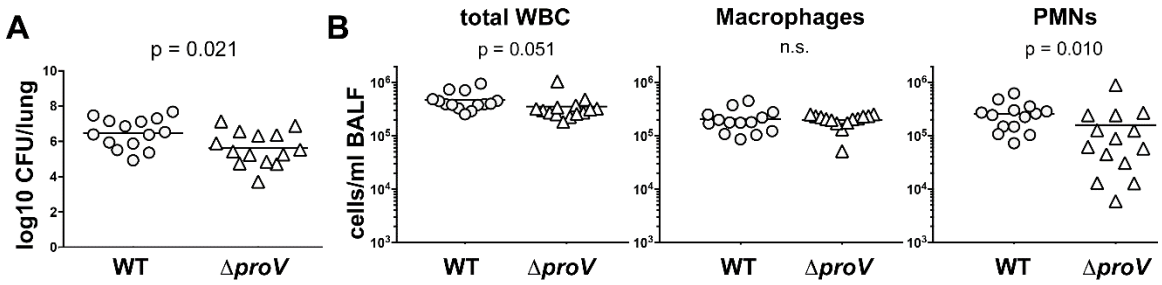

Figure 3.5. The ProU glycine betaine ABC transporter (*proVWX*) contributes to *K. pneumoniae* fitness during acute pneumonia.

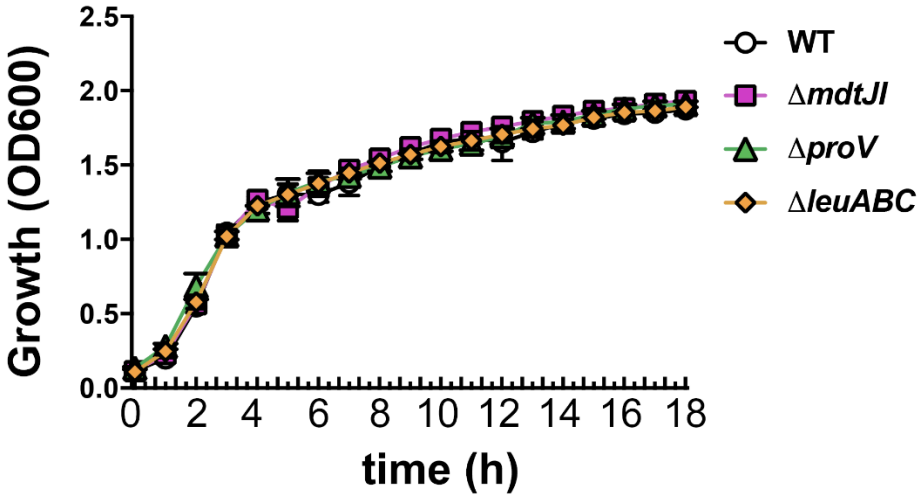

Figure 3.6. KPPR1 isogenic deletion strains exhibit wildtype growth kinetics in TSB.

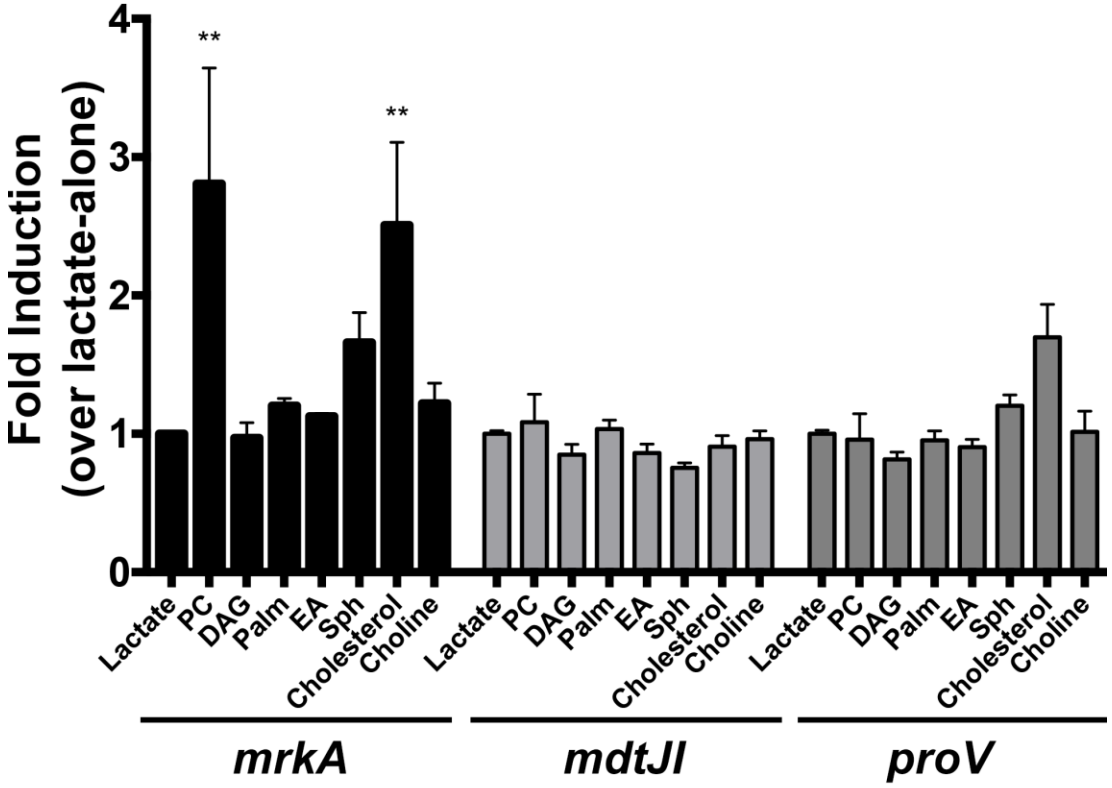

Figure 3.7. Constituents of lung surfactant stimulate *K. pneumoniae* gene expression.

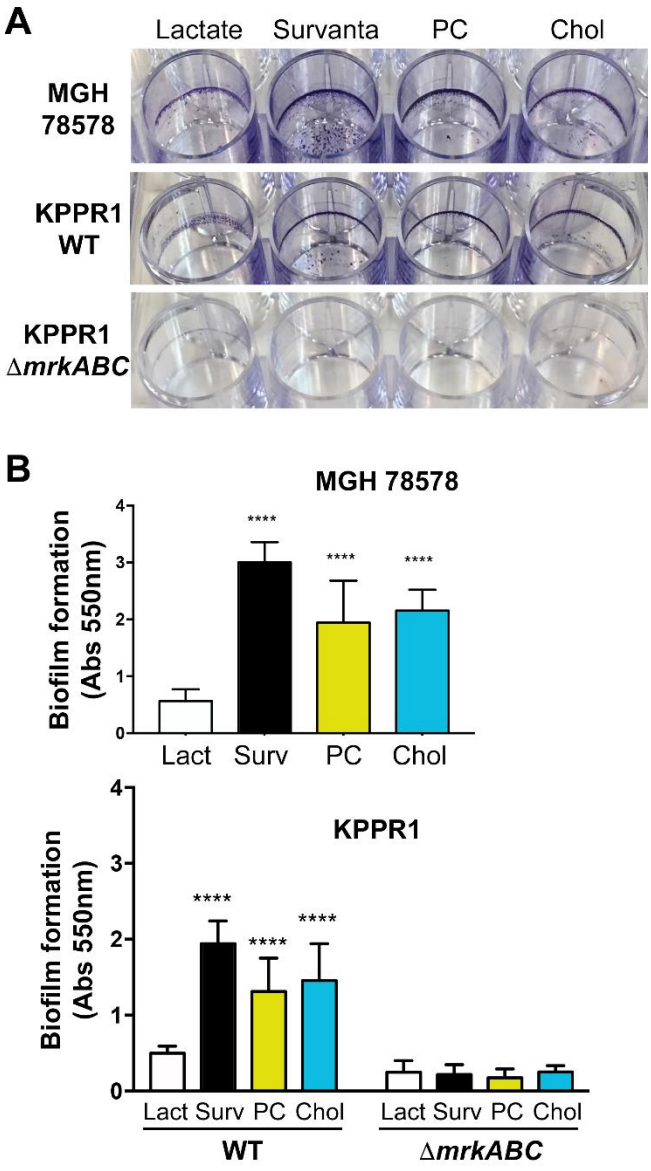

Figure 3.8. Type 3 fimbriae mediate biofilm formation in response to lung surfactant.

Table 3.1. Bacterial strains and plasmids used in this study.

Designation	Genotype or description	Reference/source
K. pneumoniae strains		
GGW112	MGH 78578	ATCC 700721
GGW231	KPPR1	ATCC 43816
GGW178	Δ*proV* in GGW231	This study
GGW180	Δ*leuABCD* in GGW231	This study
GGW192	Δ*mrkABC* in GGW231	This study
GGW194	Δ*mdtJI* in GGW231	This study
E. coli strains		
DH5α λpir	*sup E44 ΔlacU169 (ΦlacZΔM15) recA1 endA1 hsdR17 thi-1, gyrA96relA pir*	Bio-Rad
NEB5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NEB
S17-1 λpir	*thi pro hsdR- hsdM*$^{+}$ Δ*recA RP4-2::TcMu-Km::Tn7αλpir*	(112)
GGW166	pGW74 in S17-1 λpir	This study
GGW168	pGW76 in S17-1 λpir	This study
GGW172	pGW78 in S17-1 λpir	This study
GGW186	pGW79 in S17-1 λpir	This study
Plasmids		
pGW65	suicide vector, R6Kγ ori, Hm^{R}, *sacB*	This study
pGW74	*proV*-SOE in pGW65	This study
pGW76	*leuABCD*-SOE in pGW65	This study
pGW78	*mdtJI*-SOE in pGW65	This study
pGW79	*mrkABC*-SOE in pGW65	This study

Table 3.2. Summary of the 25 most highly induced transcripts expressed by *K. pneumoniae* MGH 78578 in response to lung surfactant.

Fold Increase	Gene ID	Alternate Name	Function
19.16	KPN_02053	*dhcA*	acetyl-CoA transferase subunit
12.25	KPN_04340	*fadB*	3-hydroxyacyl-CoA dehydrogenase
12.06	KPN_00235	*fadE*	acyl-CoA dehydrogenase
11.69	KPN_02054	*dhcB*	acetyl-CoA transferase beta subunit
8.68	KPN_02058		lysR-family transcription regulator
8.41	KPN_02055	*atoB*	beta-ketothiolase
7.41	KPN_01989	*nemA*	N-ethylmaleimide reductase
6.67	KPN_03278	*mrkC*	type 3 fimbrial assembly chaperone
6.16	KPN_02505	*Kp52D*	glycosyltransferase: capsule synthesis
5.96	KPN_04339	f*adA*	acetyl-CoA acetyltransferase
5.94	KPN_02057	*bdhA*	short chain dehydrogenase
5.58	KPN_01635	*yneI*	putative aldehyde dehydrogenase
5.40	KPN_01159		cyclic di-GMP phosphodiesterase
5.36	KPN_00406	*queC*	7-cyano-7-deazaguanine synthase
5.04	KPN_02056	*bdhB*	3-hydroxybutyryl-CoA dehydrogenase
4.93	KPN_02724	*fadI*	acetyl-CoA acetyltransferase
4.68	KPN_01565	*mdtJ*	polyamine efflux pump subunit
4.44	KPN_03277	*mrkB*	type 3 fimbrial usher protein
4.41	KPN_01316		hypothetical protein
4.00	KPN_03008	*proV*	glycine betaine ABC transporter
3.99	KPN_02723	*fadJ*	enoyl-CoA hydratase
3.88	KPN_pKPN5p08207		hypothetical protein
3.82	KPN_03510	*fadH*	2,4-dieonyl-coa reductase
3.65	KPN_01727		hypothetical protein
3.49	KPN_01676		hypothetical protein

Table 3.S1. *K. pneumoniae* MGH 78578 transcriptional response to lung surfactant.

Fold Change (survanta over lactate)	Gene Title	Locus Tag	Target Description
19.16	*dhCA*	KPN_02053	putative acetyl-CoA:acetoacetyl-CoA transferase alpha subunit
12.25	*fadB*	KPN_04340	4-enzyme protein: 3-hydroxyacyl-CoA dehydrogenase
12.06	*fadE*	KPN_00235	acyl-CoA dehydrogenase
11.69	*dhCB*	KPN_02054	putative acetyl-CoA:acetoacetyl-CoA transferase beta subunit
8.68	*KPN_02058*	KPN_02058	putative transcriptional regulator (LysR family)
8.41	*atoB*	KPN_02055	beta-ketothiolase
7.41	*nemA*	KPN_01989	N-ethylmaleimide reductase
6.67	*mrkC*	KPN_03278	putative bacterial pili assembly chaperone
6.16	*Kp52D*	KPN_02505	Possible glycosyltransferase
5.96	*fadA*	KPN_04339	acetyl-CoA acetyltransferase
5.94	*KPN_02057*	KPN_02057	short chain dehydrogenase
5.58	*yneI*	KPN_01635	putative aldehyde dehydrogenase
5.4	*KPN_01159*	KPN_01159	hypothetical protein
5.36	*queC*	KPN_00406	putative (aluminum) resistance protein
5.04	*bdhB*	KPN_02056	3-hydroxybutyryl-CoA dehydrogenase
4.93	*fadI*	KPN_02724	acetyl-CoA acetyltransferase
4.68	*mdtJ*	KPN_01565	multidrug transport protein (SMR superfamily)
4.44	*mrkB*	KPN_03277	putative fimbrial usher protein
4.41	*KPN_01316*	KPN_01316	hypothetical protein
4	*proV*	KPN_03008	ATP-binding component of transport system for glycine
3.99	*fadJ*	KPN_02723	bifunctional fatty acid oxidation complex protein
3.88	*KPN_pKPN5 p08207*	KPN_pKPN5 p08207	hypothetical protein
3.82	*fadH*	KPN_03510	2,4-dienoyl-CoA reductase
3.65	*KPN_01727*	KPN_01727	hypothetical protein
3.49	*KPN_01676*	KPN_01676	hypothetical protein
3.47	*evgA*	KPN_03480	putative bacterial regulatory protein
3.44	*KPN_04108*	KPN_04108	putative inner membrane protein
3.37	*yjcB*	KPN_04460	hypothetical protein
3.36	*KPN_02822*	KPN_02822	putative glycoside hydrolase

3.34	*yehS*	KPN_02561	hypothetical protein
3.32	*mrkD*	KPN_03279	putative fimbrial-like protein
3.22	*KPN_pKPN3 p05887*	KPN_pKPN3 p05887	transposase
3.19	*yfeH*	KPN_04411	putative cytochrome oxidase
3.14	*arnA*	KPN_03845	hypothetical protein
3.14	*thiL*	KPN_00375	thiamine biosynthesis protein ThiI
3.11	*glnp-like*	KPN_pKPN5 p08192	hypothetical protein
3.09	*pheS*	KPN_02176	phenylalanyl-tRNA synthetase alpha subunit
3.07	*yjbD*	KPN_04410	hypothetical protein
3.06	*KPN_03102*	KPN_03102	putative cytoplasmic protein
3.03	*zupT*	KPN_03453	zinc transporter ZupT
2.92	*leuB*	KPN_00079	3-isopropylmalate dehydrogenase
2.91	*KPN_01393*	KPN_01393	hypothetical protein
2.91	*lamB*	KPN_04425	maltoporin precursor
2.9	*pdhR*	KPN_00117	transcriptional regulator of pyruvate dehydrogenase
2.89	*ybjM*	KPN_00879	hypothetical protein
2.85	*KPN_01370*	KPN_01370	hypothetical protein
2.83	*gidB*	KPN_04145	glucose-inhibited division protein B
2.83	*KPN_02483*	KPN_02483	Possible glycosylhydrolase
2.79	*nemR*	KPN_01988	hypothetical transcriptional regulator
2.78	*ychJ*	KPN_02205	hypothetical protein
2.74	*glnK*	KPN_00413	nitrogen regulatory protein P-II 2
2.74	*KPN_01160*	KPN_01160	hypothetical protein
2.73	*KPN_02440*	KPN_02440	putative bacterial regulatory protein
2.73	*mdtI*	KPN_01566	multidrug transport protein (SMR superfamily)
2.72	*mqo*	KPN_01554	malate:quinone oxidoreductase
2.71	KPN_pKPN3 p05873	KPN_pKPN3 p05873	putative alginate lyase
2.7	*KPN_00759*	KPN_00759	putative IS1 transposase
2.68	*ybjN*	KPN_00884	putative sensory transduction regulator
2.67	*strB*	KPN_pKPN5 p08180	streptomycin resistance protein B
2.66	*KPN_04560*	KPN_04560	putative arginine-binding periplasmic protein
2.65	*fic*	KPN_03553	hypothetical protein
2.64	*gltd*	KPN_03625	glutamate synthase

2.61	*KPN_01878*	KPN_01878	putative transcriptional regulator
2.61	*KPN_01729*	KPN_01729	carbohydrate kinase
2.61	*KPN_00524*	KPN_00524	putative bacterial extracellular solute-binding protein
2.6	*yfgB*	KPN_02847	putative pyruvate formate lyase activating enzyme 2
2.59	*stbE*	KPN_00262	putative fimbriae
2.59	*KPN_02063*	KPN_02063	putative acetyl transferase
2.59	KPN_pKPN5 p08193	KPN_pKPN5 p08193	hypothetical protein
2.58	*avtA*	KPN_03936	valine--pyruvate transaminase
2.57	*ygjP*	KPN_03514	hypothetical protein
2.57	*ugd*	KPN_02493	UDP-glucose dehydrogenase
2.56	*catC*	KPN_01874	putative muconolactone delta-isomerase
2.55	*mrkA*	KPN_03276	putative fimbrial-like protein
2.55	*secM*	KPN_00101	SecA regulator SecM
2.54	*nrdI*	KPN_03005	hypothetical protein
2.53	*fhuA*	KPN_00165	outer membrane pore protein
2.53	*secA*	KPN_00102	translocase
2.52	*KPN_00213*	KPN_00213	D- and L-methionine transport protein (ABC superfamily
2.51	*ybbL*	KPN_00468	putative ATP-binding component of a transport system
-3.04	*hutI*	KPN_00792	Imidazolonepropionase
-3.17	*hutH*	KPN_00796	histidine ammonia lyase
-3.55	*paaK*	KPN_01479	phenylacetate-CoA ligase
-5.06	*paaF*	KPN_01474	probable enoyl-CoA hydratase
-5.08	*hutU*	KPN_00795	putative urocanase
-5.23	*paaC*	KPN_01471	phenylacetic acid degradation protein
-5.35	*paaI*	KPN_01477	phenylacetic acid degradation protein
-5.86	*paaE*	KPN_01473	probable phenylacetic acid degradation NADH oxidoreductase
-6.65	*paaD*	KPN_01472	phenylacetic acid degradation protein

Table 3.S2. Primers used in this study.

Primer Name	Sequence	Function
Kpprl_leuABCD_KO_F1_PvuI	aatcgatcgAGACGCACAGATGGAACACG	*leuABCD* gene deletion construct
Kpprl_leuABCD_KO_SOE_R1	gccacgcatcaacggtcCTACTGGCTCATGGTTTGGATCCTT	*leuABCD* gene deletion construct
Kpprl_leuABCD_KO_SOE_F2	taggaccgttgatgcgtggcGCAAGCTGCCGGCGTTTATG	*leuABCD* gene deletion construct
Kpprl_leuABCD_KO_R2_PvuI	taacgatcgTCCTGGCAGAGCTCAAGAATAC	*leuABCD* gene deletion construct
Kpprl_proV_R2_KpnI	aataggtaccAAGGGCGAGTGAgAAGCCCC	*proV* gene deletion construct
Kpprl_proV_KO_SOE_F2	gTGActgTAGGTGTGAGTCaGCGAGGGGGTGAATCATGGCTGA	*proV* gene deletion construct
Kpprl_proV_SOE_R1	tGACTCACACCTAcagTCAcGCCATGCAATAGAGAAGTTCCTG	*proV* gene deletion construct
Kpprl_proV_KO_F1_SphI	atagcatgcTTCGCGTCTTTACCGGATTGA	*proV* gene deletion construct
Kpprl_mrk_KO_F1_bamHI	ataggatccGATGGCGCTGATGGGATTGA	*mrkABC* gene deletion construct
Kpprl_mrk_KO_SOE_R1	gctctagcgtcacactcacgtCATTGCCATTTCCTTGTCAGAG	*mrkABC* gene deletion construct
Kpprl_mrk_KO_SOE_F2	acgtgagtgtgacgctagagcTAAGTGACGTTAAAAGGCCGGG	*mrkABC* gene deletion construct
Kpprl_mrk_KO_R2_kpnI	taaggtaccGGTTCATAGCCACCGCATCCA	*mrkABC* gene deletion construct
Kpprl_mdtJI_KO_F1_xmaI	taTcccgGGATGGCGCCCATACCTTCTAC	*mdtJI* gene deletion construct
Kpprl_mdtJI_KO-SOER1	gctgcgcagTCAtcgTTAactCATGATCCCTTCTCCTGCTTGAGA	*mdtJI* gene deletion construct
kpprl_mdtJI_KO_SOEF2	agtTAAcgaTGActgcgcagcTGACGTCTCGCCGCCTGCAAAG	*mdtJI* gene deletion construct
Kpprl_mdtJI_KO_R2_xmai	AAATcccgggAGCACAAGCAGAACACTGGT	*mdtJI* gene deletion construct
KpprL_leu_KO_scrn_F	CCGCCTGGATTACTTCAATGTC	PCR screen for gene deletion
Kpprl_leu_KO_scrn_R	CGATATCCGTTGCCAGACCAA	PCR screen for gene deletion
VK055_413-5_KO_scrnR	GTTCACGGGTCAGCATCTCA	PCR screen for gene deletion
VK055_413-5_KO_scrnF	CTGGTCAAAGGCTGGAAAGC	PCR screen for gene deletion
kppr1_proV_KO_scrnR	TCAATCGAGACAATCCCGACA	PCR screen for gene deletion
Kpprl_proV_KO_scrnF	CAGGCGAAGCATCAACGCA	PCR screen for gene deletion
Kpprl_mrkC_KO_scrnF	GTAGGCGGGTCGGATAACAG	PCR screen for gene deletion
Kpprl_mrkC_KO_scrnR	CGCTTCTTTCACCGAGACCT	PCR screen for gene deletion
Kpprl_mdtJI_KO_scrnR	CCAGGGACAGAATGCCGTA	PCR screen for gene deletion
Kpprl_mdtJI_KO_scrn_F	CTATGCGTTGTGGGAAGGG	PCR screen for gene deletion
Kpn_mdtJ_qRT_R	TTTTTCTGGGTGCCGGACTT	qRT-PCR
Kpn_leuB_qRT_F	ACCAGCCAGTATGACGTTGG	qRT-PCR
Kpn_leuB_qRT_R	AGCCCCTGATACAGTTTGGC	qRT-PCR
Kpn_04184_qRT_F	CGC TGG TTG AGG CAT TTA TT	qRT-PCR
Kpn_04184_qRT_R	GCT GAA CAT GGC TAA CTG AC	qRT-PCR
Kpn_proV_qRT_F	CAT GGT TCG CCT TCT CAA TC	qRT-PCR
Kpn_proV_qRT_R	AAG GAC TGA AAA ACC ATC GC	qRT-PCR
Kpn_02789_qRT_F	CCA TCC GTT TGC TGT TAC TG	qRT-PCR
Kpn_02789_qRT_R	CCG TTT TTG ACG AAG ATG CT	qRT-PCR
Kpn_mrkB_qRT_F	TAA AGA GAC GCT GTG GTG GC	qRT-PCR
Kpn_mrkB_qRT_R	TTG ATG GCG AGA CTA CTG CC	qRT-PCR
Kpn_fadB_qRT_F	AAG ATG TCG AGA CGC CGA AG	qRT-PCR
Kpn_fadB_qRT_R	GGT TTC CGC TAG TAC GGC TT	qRT-PCR
Kpn_02053_qRT_F	CCATTTGTTCACGGGTCAGC	qRT-PCR
Kpn_02053_qRT_R	CGGCCGACACTACATCCTT	qRT-PCR
Kpn_03845_qRT_R	ATG CTC AAC GTC CTG GTA GC	qRT-PCR
Kpn_03845_qRT_F	CTG CGA CGG GCA GAT CAT TA	qRT-PCR

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CHAPTER 4:

Identification of genes associated with *Stenotrophomonas maltophilia* growth in Cystic Fibrosis sputum.

Graham G. Willsey[1,2], Korin M. Eckstrom[1], Lauren A. Hinkel[1], Annette E. LaBauve[3], Kristin C. Schutz[1], John J. LiPuma[4], and Matthew J. Wargo[1,2*]

4.1 Abstract

Stenotrophomonas maltophilia is an MDR respiratory pathogen of environmental origin that has more than tripled in prevalence in patients with Cystic Fibrosis (CF) over the last decade. Sputum serves as the primary nutrient source for microbes colonizing the CF lung and has been shown to contain many of the host-derived signals that drive the virulence-associated phenotypes of several CF pathogens. Here, we characterized the transcriptional responses of three *S. maltophilia* strains during growth in synthetic CF sputum media (SCFM2) to gain insight into how this organism interreacts with the host in the CF lung. These efforts led to the identification of 238 transcripts expressed by all three strains that reflect nutrient utilization by *S. maltophilia* during CF lung infection. We also compared the SCFM2 transcriptomes of two *S. maltophilia* CF isolates with the SCFM2 transcriptome of the acute infection model strain, *S. maltophilia* K279A. In doing so, we identified CF isolate-specific signatures in gene expression that are suggestive of adaptation to the CF lung, including the repression of genes involved in cell motility and biofilm formation, increased expression of oxidative stress-related genes, and increased expression of alternative cytochromes associated with growth in low oxygen environments. Many of these transcriptional changes correlated with phenotypes observed *in vitro*, as the CF isolates failed to form surface-adhered biofilms in SCFM2 and were inherently more resistant to oxidative stress than K279A. Collectively, this work provides novel insight into the interactions occurring between *S. maltophilia* and the CF lung environment and revealed potential virulence factors and metabolism-related genes that will be the subject of future research efforts.

4.2 Introduction

Cystic Fibrosis (CF) is the most common lethal genetic condition affecting the Caucasian population and is caused by mutations within the cystic fibrosis transmembrane conductance regulator (CFTR) that result in impaired chloride and bicarbonate transport across the epithelial cells lining several organ systems. Lack of functional CFTR within the respiratory tract leads to the accumulation of thick, viscous mucus in the airways that impairs mucociliary clearance and dampens the innate immune response. These conditions promote chronic respiratory tract infections that lead to progressive lung function decline, and eventually death (1, 2). While *Pseudomonas aeruginosa* and *Staphylococcus aureus* remain the most common bacterial pathogens in adults with CF, improvements in clinical care practices and antipseudomonal therapeutic strategies have led to the emergence of other multi-drug resistant (MDR) respiratory pathogens in individuals with CF (3-5).

Stenotrophomonas maltophilia is an MDR respiratory pathogen of environmental origin that has more than tripled in prevalence in patients with CF over the last decade (6, 7). Current estimates indicate that more than 13 % of the worldwide CF population is infected with *S. maltophilia,* a figure that is expected to climb in coming years (4, 8). Despite this alarming increase in respiratory tract colonization frequency, there is still debate over the role of this organism in CF pathogenesis (9, 10). Recent reports, however, have concluded that infection with *S. maltophilia* is correlated with an elevated risk of acute exacerbation requiring hospitalization, acceleration in lung function decline, and respiratory failure requiring lung transplant (11, 12).

Several studies have examined the virulence-associated phenotypes of *S. maltophilia* CF isolates and the genotypic evolution of this organism over the course of chronic infection (13-16). While these studies have provided valuable insight into *S. maltophilia* adaptation to the CF lung, the molecular mechanisms permitting lung colonization and virulence have remained largely unknown. The low virulence of *S. maltophilia* in mammalian models (17, 18) has hindered research efforts into pathogenic mechanisms as a lung infection model that demonstrates bacterial growth does not exist. Nevertheless, invertebrate infection models, bioinformatics, and tissue culture-based approaches have been useful in identifying virulence-associated genes including a variety of secreted proteases and hydrolytic enzymes, adhesins, and siderophores (19-25).

However, it is important to note that many of these studies were performed under *in vitro* conditions that fail to reflect the host environment at the site of infection. The molecular interactions between *S. maltophilia* and the host are largely unknown. Environmental conditions such as the composition and availability of nutrients, osmotic conditions, oxygen tension, and pH significantly influence gene expression and therefore impinge upon the virulence of pathogenic bacteria. Characterizing *S. maltophilia* interactions with the host environment is thus critical to our understanding of the mechanisms permitting colonization and infection of the CF lung.

The thick, viscous mucus that characterizes the CF lung is expectorated as sputum, and is comprised of mucin, high molecular weight DNA, serum components, and cell debris resulting from chronic infection. This complex milieu serves as the primary nutrient source for microbes colonizing the CF lung and has been shown to contain many of the host-derived signals that promote the virulence-associated phenotypes of CF

pathogens such as *P. aeruginosa* and *Burkholderia cenocepacia* (26-29). In this study, we sought to examine how *S. maltophilia* interacts with and utilizes this important aspect of the CF lung environment. To accomplish this, we characterized the transcriptional responses of *S. maltophilia* K279A and two *S. maltophilia* CF isolates during growth in synthetic cystic fibrosis sputum media (SCFM2), a defined media developed to closely resemble the composition of native CF sputum (30, 31). These efforts led to the identification of potential virulence genes and metabolic pathways that likely contribute to *S. maltophilia* fitness within sputum. Furthermore, we compared the gene expression profiles of both *S. maltophilia* CF isolates with the acute infection model strain *S. maltophilia* K279A. In doing so, we identified CF isolate-specific signatures in gene expression that are suggestive of adaptation to the CF lung, including the repression of genes involved in cell motility/biofilm formation, increased expression of oxidative stress-related genes in addition to a cytochrome associated with growth in low oxygen environments. Many of these transcriptional changes correlated with phenotypes observed *in vitro*, as the CF isolates failed to form surface-adhered biofilms in SCFM2 and were inherently more resistant to oxidative stress than K279A. Collectively, this work provides novel insight into the interactions occurring between *S. maltophilia* and the CF lung environment and identified potential virulence factors and metabolism-related genes that will be the subject of future research efforts.

4.3 Materials and Methods

4.3.1 Bacterial strains and media

Stenotrophomonas maltophilia K279A (ATCC BAA-2423), a clinical bacteremia isolate and model strain, and two *S. maltophilia* cystic fibrosis isolates, AU30115 (GW273) & AU32848 (GW275) were included in this study. AU30115 (GGW273) and AU32848 (GW275) were obtained from the library of John LiPuma (University of Michigan), and were selected from a small set of CF isolates for their genetic tractability, antibiotic susceptibility profiles, and capacity to grow in minimal media. *S. maltophilia* strains were maintained in LB (Luria Broth), and modified MOPS minimal media supplemented with 40 mM sodium lactate, 5 mM glucose, and 250 µM L-methionine. Gene induction and metabolomic experiments were performed using synthetic CF sputum media (SCFM2), prepared as described by Turner *et. al* (31). As a control condition for these experiments, *S. maltophilia* strains were cultured in modified MOPS minimal media containing 4 mM sodium lactate and 250 µM L-methionine.

4.3.2 Genomic DNA extraction from *Stenotrophomonas maltophilia* CF clinical isolates and Illumina sequencing library preparation

Genomic DNA was purified from GW273 and GW275 cultures that were grown in LB for 18 h on a roller drum at 37˚C through CTAB extraction. Co-purified RNA was removed from the genomic DNA samples by treatment with RNaseA/T1 (NEB), and the samples were then assessed for integrity through Bioanalyzer and quantified using a Qubit fluorometer as we have done previously (32).

4.3.3 Growth Conditions and RNA purification for RNA-Seq

S. maltophilia K279A, GW273, and GW275 were grown overnight at 37 ˚C in MOPS minimal media supplemented with 40 mM sodium lactate, 5 mM D-glucose, and 500 µM L-methionine. The following day, the cultures were collected through centrifugation, washed twice with 1 ml of MOPS media, and resuspended in freshly prepared SCFM2 (31) and MOPS media supplemented with 4 mM lactate and 250 µM L-methionine at a final concentration of 0.3 OD_{600} units. Each strain was cultured in technical duplicate per condition for 4 h at 37 ˚C while shaking at 170 RPM. Following the induction period, cells were collected via centrifugation and immediately resuspended in 800 µl of ~80 ˚C RNAzol. Total RNA was then extracted from these samples using Zymo Research's RNA Miniprep Kit. Residual genomic DNA was removed from each sample through DNaseI treatment (NEB), and the RNA was re-purified using Qiagen's RNeasy kit as we have done previously (32). The quality of each resulting RNA sample was then assessed via Agilent Bioanalyzer and quantified through Qubit fluorometer.

4.3.4 RNA-seq library preparation

The purified RNA was treated with Ribo-Zero Gold Epidemiology kit (Illumina) following manufacturer's instructions. The RNA was fragmented using the NEBNext Magnesium RNA Fragmentation Module (33) with a 3 min incubation at 94°C followed by a cleanup step using the RNA Clean & Concentrator-5 (Zymo Research). Double-stranded, tagged cDNA was generated from 50ng of fragmented RNA as previously described (34, 35).

A qPCR assay was performed on the cDNA samples to determine the correct number of cycles required to generate the final sequencing library as well as to assess the efficacy of normalization and enrichment (34). The final library was created by PCR amplifying the samples with the full-length sequencing adapters and 6-mer ScriptSeq (Epicentre) barcodes. Following PCR, the samples were cleaned, and size selected in a two-step cleanup using 0.75 volumes of AMPure XP beads followed by 0.15 volumes to achieve a final library with an average size of ~330bp. Libraries were combined with 12 samples each, in equal amounts and concentrated using the DNA Clean & Concentrator-5 (Zymo Research). Prior to sequencing the final libraries were quantified using qPCR. Combined libraries were sequenced on an Illumina NextSeq using 75 cycle kits. Samples were loaded at 1.8 pM and 75 base single-end reads were obtained. RNA-seq was performed in biological duplicate for each strain and each condition, using RNA collected from two independent SCFM2 induction experiments that were performed on separate days.

4.3.5 RNA-Seq data processing & analysis

Quality assessment of raw sequencing data was assessed using FastQC (v. 0.11.6) (36). Adapters and low-quality sequences were removed using Trimmomatic (v. 0.38) removing TruSeq SE adapters, clipping 3 bp from the leading and trailing ends, and a sliding window of 4:15 to a minimum length of 36 bp (37). Post-trimming quality was again assessed via FastQC prior to mapping and assembly. Transcriptome assembly and quantification was performed using Rockhopper2, a program targeted at bacterial transcriptome analysis and using the default parameters with verbose output (38, 39).

Reads from each sample were mapped to the reference genome of *Stenotrophomonas maltophilia* strain K279A in order to improve annotation and utility of results (40). Differential gene expression was calculated using the raw counts from Rockhopper and normalized to library size using edgeR (38, 41)**.** Only genes with at least 2 counts per million (cpm) and occurred in at least 2 samples were included in analysis in order to reduce noise. Expression was calculated as SCFM2 over lactate treatments, both within isolates and globally in order to establish responses specific to clinical isolates in addition to those in specific to media type. Significance was established at *P*-adjusted value of <0.05 after Benjamini & Hochberg correction (FDR <0.05).

4.3.6 Assessment of *S. maltophilia* resistance to oxidative stress

S. maltophilia K279A, GW273, and GW275 were cultured in LB overnight on a roller drum at 37 °C. The following day, dilutions of these cultures were grown in LB until cells achieved log phase of growth. Cells from each were then collected by centrifugation and washed with DPBS, and then adjusted to an OD_{600} of 0.5 in DPBS. Fifty microliters of cells from each strain were then added in technical duplicate to 50 µl of DPBS supplemented with hydrogen peroxide to achieve final concentrations of 0, 2, 4, and 8 mM. These cultures were then incubated at 37°C for 2 hours without shaking, and surviving CFU counts were determined through serial dilution. Percent survival was reported as a percent of the survival observed in the 0 mM hydrogen peroxide condition for each strain. Statistical significance was determined using 2-way ANOVA with Tukey's post-test for multiple comparisons.

4.3.7 Assessment of *S. maltophilia* biofilm formation in SCFM2

S. maltophilia K279A, GW273, and GW275 were cultured overnight at 37 °C on a roller drum in MOPS minimal media supplemented with 25 mM lactate, 5 mM glucose, and 500 µM L-methionine. The following day, cells from each culture were collected via centrifugation, washed in MOPS media, and then adjusted to an OD_{600} of 0.5 units. Each OD_{600}-adjusted culture was then added 1:10 to fresh SCFM2 media prepared as described above. These cultures were then incubated for 18 h at 37 °C and agitated at 170 RPM to reflect the partial aeration and mixing that occurs within the lung. Following incubation, the suspended cells were removed from the culture dish, the wells washed, and the biofilm material remaining adhered to the wells was stained with 0.1% crystal violet, incubated for 15 minutes, and then thoroughly rinsed with water (42). This experiment was performed three times.

4.4 Results

4.4.1 *S. maltophilia* response to synthetic cystic fibrosis sputum media

Our primary goal in the study was to characterize the conserved transcriptional response of *S. maltophilia* during growth in media closely resembling the nutrient and ion content found in the lungs of individuals with cystic fibrosis. To accomplish this, we used RNA-Seq to measure the transcriptional responses of two *S. maltophilia* clinical CF isolates and the model strain *S. maltophilia* K279A during growth in SCFM2 compared to MOPS lactate minimal media as a control condition. The CF isolates, GW273 and GW275, were chosen for inclusion in this study for their ability to grow in minimal

media as well as their antibiotic resistance profiles and genetic tractability. *S. maltophilia* K279A, an acute bacteremia isolate, was included in this study because it is the standard lab strain, and it represents a recent colonizing strain that has not undergone selection pressure to adapt to the CF lung environment.

Comparing the aggregate responses of each *S. maltophilia* strain to SCFM2 relative to the aggregate responses to MOPS lactate media, we identified 238 shared genes that exhibited greater than a 2.5-fold increase in transcript abundance in response to SCFM2 ($p<0.05$). These changes represent the "conserved" *S. maltophilia* response to SCFM2 and are described in further detail below. A summary of the conserved transcriptional changes is found in **Figure 4.1**, with transcripts categorized into groups reflecting their known, or bioinformatically predicted function (43-46). The 50 most highly induced transcripts within the conserved *S. maltophilia* response to SCFM2 can be found in **Table 4.1.** Since the control condition for our analysis was MOPS minimal media supplemented with lactate and ammonia as sole carbon and nitrogen sources, the genes repressed during growth in SCFM2 were not addressed in our analysis as they largely reflect housekeeping genes and general metabolism involved in lactate and ammonia utilization. Nevertheless, a complete listing of the *S. maltophilia* conserved transcriptional response to SCFM2 can be found in **Supplementary Table 4.1**, while the individual transcriptional responses of each strain are summarized in **Supplementary Figure 4.1.**

4.4.1.1 SCFM2 alters *S. maltophilia* metabolism

Growth under oxygen limiting conditions

Nearly thirty transcripts were expressed by *S. maltophilia* that reflect changes in cellular metabolism to support survival and growth under microaerophilic conditions. Nineteen genes within the membrane-associated nitrate reductase locus (*Smlt2764-82)* exhibited between 4.68 and 21.64-fold increases in transcript abundance, while transcription of the sulfite reductase operon, *cysJ-Sm2763*, increased 1064.48 and 777.72-fold, suggesting that nitrate and sulfite are likely utilized by *S. maltophilia* as electron sinks to regenerate NAD^+ and $NADP^+$ under these conditions (47). Also expressed was the formate dehydrogenase, *fdnHI,* that permits the use of formate as an electron donor when nitrate is used as an electron acceptor under oxygen-limiting conditions [4.47 & 4.94-fold increase] (48). In addition, SCFM2 also stimulated transcription of the cytochrome bd-II oxidase operon, *(cydC cydD, Smlt3284, appD)*, whose products permit the generation of a proton motive force in low oxygen environments to facilitate oxidative phosphorylation [3.10 to 3.80-fold increase] (49).

SCFM2 induces the expression of numerous hypothetical nutrient transporters

Fourteen uncharacterized genes related to nutrient uptake were expressed by all three *S. maltophilia* strains during growth in SCFM2. Nine genes encoding hypothetical TonB-dependent receptor proteins exhibited between 2.58 and 7.01-fold increases in transcript abundance. Also expressed within the conserved response were five genes encoding uncharacterized porins and ABC transporter-related proteins [2.48 to 5.67-fold increases in abundance].

Amino acid metabolism

Seventeen transcripts related to the metabolism of amino acids were induced by all three *S. maltophilia* strains during growth in SCFM2. These findings mirror similar observations in *Pseudomonas aeruginosa* (30) and suggest that amino acids also serve as preferred carbon and nitrogen sources for *S. maltophilia* during growth in the CF lung. Eight of the transcripts expressed by *S. maltophilia* under these conditions function in the metabolism of branched chain amino acids, including the *Smlt0237-9* and *Smlt4338-41* operons [8.10 to 19.85-fold increase], and leucine responsive regulatory protein, *lrp* [2.87- fold increase]. Also expressed were genes related to the metabolism of tyrosine and phenylalanine, with *hmgA, hppD, maiA,* and *fahA* exhibiting between 3.89 to 13.53-fold increases in transcript abundance. Other transcripts related to the metabolism of amino acids expressed during growth in SCFM2 reflect the catabolism of alanine and serine, *(dadAX),* proline, (*putA),* methionine *(metH2),* and glycine *(gcvP)* [2.49 to 13.73-fold increase].

Phosphatidylcholine catabolism

Seven transcripts related to fatty acid metabolism (*psrA, fadBA, fadH, bpoA, fadE, Smlt3352)* were also expressed, indicating that *S. maltophilia* actively degrades phosphatidylcholine during growth in SCFM2 [3.20 to 9.04-fold increase in transcript abundance]. Other SCFM2-induced transcripts suggest that the liberated phosphocholine head group is also metabolized by *S. maltophilia* to obtain the osmoprotectant, glycine betaine. Transcription of the glycine betaine/proline transporter, *proP,* increased 6.64-

fold while genes within the choline oxidase operon, *betI* and *betB*, increased 3.54 & 2.0-fold respectively.

DNA metabolism

Exposure to SCFM2 also stimulated transcription of several genes related to the metabolism of purines and pyrimidines, suggesting that *S. maltophilia* utilizes extracellular DNA as a nutrient source during growth in CF sputum. Specifically, transcription of five NrdR regulon members was observed, including multiple ribonucleotide reductase genes (*nrdEF, nrdB1)* and associated thioredoxin (*trxA*) and flavodoxin (*Smlt2840*) genes [2.94 to 5.26-fold increase in expression]. Other nucleotide metabolism-related genes expressed by *S. maltophilia* under these conditions include the glutamine amidotransferase, *guaA*, nucleoside hydrolase, *Smlt1222*, and nucleotide pyrophosphatase, *Smlt1449* [3.18 to 3.86-fold increase].

Mucin degradation

Previous studies have indicated that *S. maltophilia* is capable of degrading mucin (50). Mucin is the most abundant host-derived protein in CF sputum, and the only protein present in SCFM2. Our gene expression data support these earlier observations and suggest that this glycoprotein is actively metabolized by *S. maltophilia* during growth in SCFM2. Four uncharacterized proteases were expressed under these conditions, including a secreted M14-like carboxypeptidase encoded by *Smlt0603* [2.88-fold increase], and three type V secretion system autotransporters with serine protease

(*Smlt4145 & Smlt1350)* and clpP/crotonase-like (*Smlt1001)* N-terminal domains [5.27 to 9.09-fold increases in transcript abundance] (19).

N-acetylglucosamine & sugar metabolism

SCFM2 also induced the expression of six genes involved in the uptake and metabolism of N-acetylglucosamine, including *nagPRB2AX* and *nagK2* (2.58 to 5.25-fold increase in expression) (51). Genes related to the metabolism of sugars absent in SCFM2 were also expressed, including the alpha-1,2-mannosidase, *mnnA*, mannoside-specific TonB receptor, *Smlt2179*, and maltooligosyltrehalose trehalohydrolase, *Smlt2759* [2.53 to 4.07-fold increase]. The role of these genes during growth in SCFM2 is unclear but could relate to the degradation of glycan linkages present on mucin.

Cation transport

Two cation transport systems were expressed by all three *S. maltophilia* strains during growth in SCFM2, including the high affinity ATP-driven potassium transporter, *kdpABC* [5.28 to 6.89-fold increase], and low affinity phosphate transporter system encoded by *Sm1705 & pitA* [2.68 and 3.00-fold increase].

4.4.1.2 Stress response

A significant fraction of the transcripts expressed by *S. maltophilia* during growth in SCFM2 reflect several distinct stress responses. Six transcripts involved in the heat shock/unfolded protein response were expressed by all three *S. maltophilia* strains during

growth in SCFM2. Specifically, the *dnaJ/dnaK* and *groEL/groES* chaperonin systems exhibited between 2.6 and 13.46-fold increases in transcript abundance. In addition, transcription of the heat shock proteins, *hslU* and *htpG* displayed 2.60 and 5.94-fold increases in expression under these conditions.

Seven transcripts within the conserved *S. maltophilia* response to SCFM2 reflect metabolic stress related to the accumulation of excess nitrogen. Most notably, the SMR-family polyamine efflux pump encoded by *mdtJI* exhibited nearly a 300-fold increase in transcript abundance. Similarly, transcription of two hypothetical LysE-family amino acid efflux pumps, *Smt1238* and *Smlt3365*, increased 99.97 and 2.57-fold during growth in SCFM2. Also expressed under these conditions, were the *adiC* arginine/agmatine antiporter, *adiA* arginine decarboxylase, and associated cation efflux pump encoded by *Smlt2941* that have also been implicated in acid resistance [5.37 to 8.83-fold increases] (52).

Other transcriptional changes indicate that growth in SCFM2 invokes oxidative stress in *S. maltophilia*. Most notably, the glutathione-dependent formaldehyde detoxification genes encoded by *adhC, Smlt3977,* and *fghA* exhibited between 2.93 and 3.35-fold increases transcript abundance. Transcription of the reactive oxygen species neutralizing manganese superoxide dismutase, *sodA,* also increased 3.5-fold under these conditions(53, 54).

Several genes associated with antibiotic resistance were also expressed by all three *S. maltophilia* isolates during growth in SCFM2. Included within this group are the aminoglycoside resistance gene, *Smlt3615,* and *sugE,* encoding an SMR-family efflux pump conferring resistance to quaternary ammonium compounds [3.09 and 2.76-fold

increases in transcript abundance] (55, 56). In addition, transcription of an uncharacterized fusaric acid-like drug efflux system encoded by *Smlt4660-3,* was also induced during growth in SCFM2 [3.00 to 4.36-fold increase] (56).

4.4.1.3 SCFM2 alters *S. maltophilia* motility/biofilm gene expression

Elements of the *S. maltophilia* conserved transcriptional response to SCFM2 also reflect changes in cell motility. Transcription of the type IV twitching pili assembly genes encoded within the *pilMNOPQ, pilHIJ,* and *ppdD* operons exhibited between 2.83 and 3.98-fold increases in transcript abundance under these conditions. The functional consequences of these transcriptional changes are unclear, as type IV pili mediate twitching motility, surface attachment during the initiation of biofilm formation, and cell to cell adhesion during biofilm maturation.

4.4.2 *S. maltophilia* isolates exhibit distinct gene expression profiles during growth in SCFM2

The second major goal of this study was to identify CF-isolate specific transcripts expressed during growth in SCFM2 that could reflect adaptation to the host lung environment. To address this question, we compared the SCFM2 transcriptional responses of *S. maltophilia* GW273 and GW275 with that of the model strain K279A. These efforts led to the identification of 85 transcripts that exhibited similar expression patterns in the CF isolates that were not shared with K279A. More specifically, twenty-nine transcripts within the CF isolates exhibited more than a 2.5-fold increase in

expression relative to K279A, while the remaining 56 transcripts exhibited at least a 2.5-fold decrease in expression relative to K279A ($P<0.05$). A summary of these transcriptional changes can be found in **Figure 4.2**, with genes categorized into groups reflecting their known, or bioinformatically predicted function(43-46). The top 25 transcripts exhibiting the greatest changes in expression relative to *S. maltophilia* K279A can be found in **Tables 4.2 & 4.3,** while a complete listing can be found in **Supplementary table 4.2.**

4.4.2.1 *S. maltophilia* CF isolates exhibit altered expression of metabolism-related transcripts

One of the most striking differences between the CF isolates and K279A's responses to SCFM2 involve the expression of transcripts related to nutrient acquisition. Three uncharacterized OMP family genes exhibited between 22.07 and 4.04-fold increases in expression in the CF isolates relative to K279A during growth in SCFM2, including *Smlt2944*, *Smlt3805*, and *Smlt4119*. Similarly, six transcripts including an uncharacterized ABC transporter system (*Smlt4100, Smlt4102, Smlt4103)* and three TonB-dependent receptor genes *(Smlt0461, Smlt3478, Smlt4387)* exhibited significantly lower levels of expression in the CF isolates relative to K279A under these conditions. Most strikingly, the transcript abundance of TonB-dependent receptor gene encoded by *Smlt4387* was more than 190-fold higher in *K279A* relative to GW273 and GW275 during growth in SCFM2.

Several metabolism-related transcripts expressed by the CF isolates during growth in SCFM2 support adaptation to the host lung environment. Expression of *prpB,* encoding a 2-methylisocitrate lyase that functions in the metabolism of the mucin catabolite, proprionic acid, increased nearly ten-fold in the CF isolates relative to K279A (50). It was previously determined that the ability of *P. aeruginosa* to synthesize riboflavin was required for this organism's fitness during growth in SCFM2 (31). Surprisingly, growth in SCFM2 induced the expression of two transcripts involved in the biosynthesis of riboflavin, *ribE & ribB,* in the *S. maltophilia* CF isolates [6.49 & 5.94-fold more transcripts relative to K279A].

The CF isolates also exhibited distinct expression patterns in genes related to cation transport. In particular, the CF isolates likely maintain larger manganese pools during growth in SCFM2 than K279A, as evidenced by the more than 47-fold decrease in expression of the manganese efflux pump, *yebN,* and 2.52-fold increase in the manganese uptake protein, *mntH,* relative to K279A. The CF isolates also expressed a predicted low affinity iron permease gene, *Smlt4069,* that was not induced by SCFM2 in K279A [3.20-fold difference]. Finally, the CF isolates expressed significantly lower levels of genes associated with phosphate uptake compared to K279A, including: *pstS, pstC, pstB, phoU,* and *oprP* [4.84 to 12.80-fold lower transcript abundance].

4.4.2.2 *S. maltophilia* CF isolates express fewer transcripts related to cell motility and biofilm formation

S. maltophilia K279A expressed significantly higher levels of thirty transcripts related to flagellar synthesis and type IV pili assembly relative to the CF isolates during growth in SCFM2. More specifically, 26 flagellar synthesis genes in K279A exhibited between 2.51 and 17.86-fold higher transcript levels during growth in SCFM2 compared to GW273 and GW275 under these conditions (*flgABCDEFGHIJK, fliEFGHIJKLMN, flhFAB, fliA, fleQ,* and *Smlt2258)*. Similarly, the abundance of four transcripts related to type IV pili assembly (*pilV, pilW, pilY1, pilH)* were between 2.71 and 2.90-fold higher in K279A relative to both CF isolates. In contrast, the CF isolates expressed 3.84-fold more transcripts of a predicted oar-family adhesin, *Smlt1619,* compared to K279A. Collectively, these differences in flagellar and type IV pili expression indicate that *S. maltophilia* CF isolates are less motile relative to K279A. Furthermore, flagella and type IV twitching pili have previously been shown to be critical for *S. maltophilia* biofilm development on cultured airway cells, mouse tracheal mucus, and abiotic surfaces (14, 57, 58).

4.4.2.3 *S. maltophilia* CF isolates express higher levels of alternative cytochromes

Other SCFM2-induced transcriptional changes specific to the *S. maltophilia* CF isolates reflect adaptation to growth in microaerophilic environments. Transcript levels of the cytochrome bd-II oxidase genes, *(cydA cydB, and Smlt3284)* whose products facilitate oxidative phosphorylation in low oxygen environments (49), were induced between 9.11

and 11.79-fold higher in the CF isolates compared to K279A. Similarly, the CF isolates also expressed significantly higher levels of the *Smlt1756-8* operon that encodes a predicted alternative cytochrome C of unknown function [5.04 to 9.7-fold increase in transcript abundance relative to K279A].

4.4.2.4 *S. maltophilia* CF isolates express more transcripts related to heat shock and oxidative stress resistance

Interestingly, both *S. maltophilia* CF isolates expressed more transcripts related to heat shock and oxidative stress than K279A during growth in SCFM2. Transcripts for the universal stress protein, *usp*, and heat shock chaperone, *clpB*, were 3.60 and 4.02-fold higher in abundance in the CF isolates relative to K279A during growth in SCFM2. Similarly, transcript levels of the hydrogen peroxide neutralizing, alkyl hydroperoxide reductase, *ahpC*, were 2.48-fold higher in the CF isolates than in K279A under these conditions.

4.4.3 *S. maltophilia* CF isolates generate less surface-attached biofilm mass during growth in SCFM2

Comparison of the three *S. maltophilia* strains transcriptional responses to SCFM2 revealed that the CF isolates expressed significantly less flagellar synthesis transcripts relative to the bacteremia isolate, *S. maltophilia* K279A. Since flagella have previously been implicated in *S. maltophilia* biofilm formation on abiotic surfaces and human CF-derived bronchial epithelial cells (14, 57), we were curious to determine if

their expressed influenced biofilm formation during growth in SCFM2. To test this, we cultured *S. maltophilia* K279A, GW273, and GW275 in SCFM2 and MOPS minimal media, and then used crystal violet staining to compare the resultant biofilm material adhered to the culture dish the following day. As shown in **Figure 4.3,** *S. maltophilia* K279A generated a robust biofilm at the air liquid interface during growth in SCFM2, while the CF isolates failed to generate substantial biofilm under these conditions.

4.4.4 *S. maltophilia* CF isolates are more resistant to oxidative stress

Our expression data suggested that the *S. maltophilia* CF isolates expressed significantly higher levels of two cytochromes and an alkyl-hydroperoxide reductase during growth in SCFM2 compared to the acute bacteremia isolate, K279A. Moreover, the CF isolates expressed higher levels of the *mntH* manganese uptake protein and repressed the *yebN* manganese efflux pump, indicating that these strains likely hold larger manganese pools in their cytosol than K279A. These observations suggested that GW273 and GW275 could be more resistant to oxidative stress than *S. maltophilia* K279A. To test this hypothesis, we examined the ability of each strain to survive treatment with various concentrations of hydrogen peroxide during logarithmic growth in LB. As shown in **Figure 4.4,** both CF isolates exhibited increased resistance to hydrogen peroxide relative to K279A.

4.5 Discussion

The occurrence of *Stenotrophomonas maltophilia* within the CF community has more than tripled over the last decade, with recent estimates indicating that nearly 15 % of the world CF population is currently infected with this bacterium. Despite this growing prevalence, our understanding of *S. maltophilia* virulence and the genes required for survival in the CF lung lags far behind other CF pathogens. The viscous mucus found within the CF lung consists of heavily glycosylated mucin, high molecular weight DNA, serum components, and cell debris resulting from chronic infection. This substance, which is expectorated as sputum, serves as the primary nutrient source for pathogens growing in the CF respiratory tract and is known to influence the virulence of *P. aeruginosa* and *B. cenocepacia* (26, 30).

Here, we characterized the transcriptional responses of three *S. maltophilia* strains during growth in synthetic cystic fibrosis sputum media (SCFM2), with the goal of identifying conserved genes and metabolic pathways contributing to *S. maltophilia* fitness in the CF lung (31). Our efforts revealed 238 shared transcripts that were expressed by two *S. maltophilia* CF isolates (GW273 and GW275) and the acute infection model strain K279A, which we included to represent a recent, non-adapted colonizer of the CF lung **(Figure 4.1 & Table 4.1).** Our second goal was to identify *S. maltophilia* CF isolate-specific transcripts expressed during growth in SCFM2 that could reflect adaptation to the CF lung environment. To address this question, we compared the SCFM2 transcriptional responses of the CF isolates with K279A. These efforts led to the identification of 85 transcripts that exhibited similar expression patterns in the CF isolates

that were not shared with K279A **(Figure 4.2, Tables 4.2 & 4.3).** The major findings of this work are discussed below.

4.5.1 Characterization of the conserved *S. maltophilia* response to SCFM2

CF sputum is a nutrient rich milieu that can facilitate exponential bacterial growth *in vitro* and support bacterial cell densities as high as 10^9 CFU/ml *in vivo* (59, 60). Surprisingly, elements of the conserved *S. maltophilia* transcriptional response to SCFM2 suggest that growth in this media invokes a significant degree of stress in *S. maltophilia,* as evidenced by the numerous heat shock proteins, drug efflux pumps, and oxidative stress resistance genes expressed under these conditions **(Figure 4.1).** Furthermore, the expression of numerous amino acid and polyamine efflux pumps was shared between all three *S. maltophilia* strains, suggesting that growth in SCFM2 results in metabolic stress that leads to the accumulation of excess nitrogen **(Figure 4.1)**.

Nearly twenty transcripts related to the catabolism of amino acids were expressed by all three *S. maltophilia* strains during growth in SCFM2 **(Figure 4.1 & Table 4.1)**. These findings reflect similar observations in *P. aeruginosa* and suggest that amino acids also serve as preferred carbon and nitrogen sources for *S. maltophilia* during growth in the CF lung (30). Moreover, many of the amino acid catabolism-related transcripts expressed by *S. maltophilia* under these conditions are orthologues of genes expressed by *P. aeruginosa* during growth in SCFM2 *(putA, dadAX, hmgA, hppD, Smlt4339-41, fahA,* and *maiA*), indicating that these organisms likely have similar nutritional preferences within the CF lung (30).

All three *S. maltophilia* strains expressed genes that indicated they metabolized N-acetylglucosamine (GlcNAC) (51) and phosphatidylcholine under these conditions **(Figure 4.1)**. Included within the conserved SCFM2 response was a two-gene operon, encoding a BpoA-like peroxidase and a FadE-like acyl-CoA dehydrogenase that we have previously determined to be expressed by *S. maltophilia* K279A upon exposure to the phosphatidylcholine-rich, pulmonary surfactant (Willsey & Wargo, unpublished data). These data suggest that the ability to metabolize the fatty acid tails of phosphatidylcholine could be important for *S. maltophilia* fitness within two very different respiratory tract infection niches.

Nearly thirty genes within the *S. maltophilia* SCFM2 transcriptome reflect alterations in electron transport to support growth in low oxygen environments, such as those found in the CF lung **(Figure 4.1 & Table 4.1)**. Many of the transcriptional changes we observed suggest that *S. maltophilia* utilizes nitrate and sulfite as alternative electron acceptors during growth in SCFM2 **(Table 4.1)**. It was previously suggested that *S. maltophilia* is inactive within CF sputum due to this organism's inability to utilize nitrate for respiration under anaerobic conditions (61). These findings are not unexpected, as *S. maltophilia* is an obligate aerobe and lacks the machinery required to undergo assimilatory denitrification (7, 47). Instead, *S. maltophilia* likely utilizes nitrate and sulfite as electron sinks to facilitate the regeneration of NAD^+ and $NADP^+$ under conditions of low oxygen availability (47). The significance of these findings is unclear; although we suspect that the use of nitrate and sulfite as alternative electron acceptors likely contribute to *S. maltophilia* growth and survival in the microaerophilic regions of the CF lung.

Several aspects of the conserved *S. maltophilia* response to SCFM2 could contribute to virulence within the CF lung **(Figure 4.1)**. Three uncharacterized T5SS autotransporters with predicted protease effector domains (*Smlt1001*, *Smlt1350*, *Smlt4145*), and a predicted M14-family carboxypeptidase (*Smlt0603*) were expressed during growth in SCFM2. While the function of these proteases is unknown, we suspect that they could be involved in the metabolism of mucin, which is the only protein present within SCFM2 (31). Future research efforts will focus on characterizing these enzymes and examining their potential roles in the degradation of this glycoprotein. Furthermore, transcription of the polyamine efflux pump, *mdtJI*, increased nearly three hundred-fold under these conditions **(Figure 4.1 & Table 4.1)**. The expression of *mdtJI* was recently shown to be necessary for *Klebsiella pneumoniae* fitness in a mouse model of acute pneumonia (32). It is therefore plausible that the expression of pump could similarly influence *S. maltophilia* fitness in the context of the CF lung.

4.5.2 *S. maltophilia* CF isolates response to SCFM2 reflect adaptation to the host lung

Previous studies in *S. maltophilia* demonstrated the importance of flagella and type IV pili in biofilm development on cultured airway epithelial cells and abiotic surfaces (14, 57). Interestingly, the most striking differences between the SCFM2 transcriptomes of the *S. maltophilia* CF isolates and *S. maltophilia* K279A centered on the expression of these structures, as the CF isolates expressed significantly lower levels of nearly thirty transcripts related to flagella and type IV pili synthesis **(Figure 4.2 &**

Table 4.3). Using a crystal violet biofilm staining assay, we were further able to show that the *S. maltophilia* CF isolates failed to generate surface attached biofilms in SCFM2, while the acute infection strain, K279A, developed robust biofilms in this media **(Figure 4.3).** These data correlate with similar observations in *P. aeruginosa* and *S. maltophilia* clinical CF isolates, where the expression of flagella and the ability to generate biofilm diminishes over the course of chronic infection (14, 62, 63). In line with these observations, the *S maltophilia* CF isolates also expressed nearly twenty-fold lower levels of the PstSACB phosphate importer relative to K279A **(Table 4.3),** as disruption of these genes has been shown to inhibit biofilm formation in *Pseudomonas* species (64). To our knowledge, this study provides the first insight into the biofilm forming capacity of *S. maltophilia* under conditions reflective of the CF lung environment.

The *S. maltophilia* CF isolates also exhibited distinct expression profiles for genes relating to nutrient transport **(Figure 4.2, Tables 4.2 & 4.3).** Most notably, the CF isolates expressed three uncharacterized outer membrane porins (OMPs) that exhibited between 4 and 22-fold higher transcript levels relative to K279A *(Smlt2944, Smlt3805, and Smlt4119)*. SCFM2 also repressed the transcription of numerous uncharacterized TonB-dependent receptor and ABC transporter transcripts in the CF isolates. In particular, the TonB-dependent receptor encoded by *Smlt4387* exhibited nearly a 200-fold decrease in transcript abundance relative to K279A **(Table 4.3)**. These data suggest that CF lung-adapted *S. maltophilia* strains could have unique metabolic requirements. Alternatively, the different porin and TonB-dependent receptor expression patterns could reflect selection pressures resulting from previous antibiotic stress or detection by the host immune system.

S. maltophilia was recently shown to be capable of metabolizing mucin *in vitro* (50). While the genes involved in this process have not been identified, our transcriptome analysis revealed that the *S. maltophilia* CF strains expressed nearly ten-fold more transcripts of the 2-methylisocitrate lyase, *prpB* during growth in SCFM2 relative to *S. maltophilia* K279A **(Figure 4.2 & Table 4.3).** This enzyme functions in the breakdown of the short chain fatty acid, propanoic acid that is generated during the catabolism of mucin by several CF pathogens and oral microbes (50, 65). Flynn *et. al* demonstrated that propanoic acid is relatively abundant in CF sputum (~15 mM), and can serve as nutrient source for *P. aeruginosa*, which lacks the ability to degrade mucin (50). These observations lead us to believe that the increased expression of *prpB* by *S. maltophilia* CF isolates could contribute to fitness during infection.

Our data also revealed the clinical CF isolates expressed more than ten-fold higher levels of cytochrome bd-II that is involved in growth under microaerophilic conditions (49). Similarly, the CF isolates also expressed an operon encoding a diheme cytochrome c-553 of unknown function (*Smlt1756-8)* **(Table 4.3).** We suspect that the increased expression of these cytochromes could support accelerated growth of *S. maltophilia* in low oxygen environments, such as those found in the mucus filled terminal bronchioles within the CF lung.

S. maltophilia CF isolates also expressed higher levels of the manganese importer *mntH* and nearly 50-fold lower levels of the manganese efflux pump *yebN* compared to K279A **(Tables 4.2 & 4.3).** The accumulation of manganese is known to protect against oxidative stress in several pathogenic bacteria, and deletion of *yebN* was shown to increase resistance against superoxide and hydrogen peroxide-mediated killing in the

Stenotrophomonas-related plant pathogen, *Xanthomonas oryzae pv. Oryzae* (53, 54, 66-68). The *S. maltophilia* CF isolates also expressed more transcripts associated with resistance against environmental stressors, and our hydrogen peroxide challenge experiments revealed that these strains were inherently more resistant to oxidative stress than K279A **(Figure 4.4).** These data strongly suggest that the *S. maltophilia* CF strains have undergone selection pressure for increased resistance against reactive oxygen species. Interestingly, our data contrast with observations made by *Pompilio et. al*, who demonstrated that *S. maltophilia* CF isolates were often more susceptible to oxidative stress than isolates of environmental origin (14). However, the discrepancies in our observations could result from differing culture conditions, or our small sample size, which is an inherent limitation of this study.

4.5.3 Conclusions

In summary, we characterized the transcriptional responses of three *S. maltophilia* strains during growth in synthetic CF sputum media (SCFM2) to gain insight into how this organism interreacts with the host at the site of infection. Using this approach, we identified numerous conserved transcripts expressed by all three strains that likely reflect nutrient utilization by *S. maltophilia* during CF lung infection. This work identified nitrate and sulfite as potentially important alternative electron acceptors under these growth conditions and also provides evidence suggesting that *S. maltophilia* preferentially utilizes amino acids for carbon and nitrogen sources within the CF lung. We also compared the SCFM2 transcriptional responses of two *S. maltophilia* CF isolates

with the *S. maltophilia* model strain, K279A, to identify CF isolate-specific signatures in gene expression associated with adaptation to the host lung environment. This work revealed that CF isolates express different genes related to nutrient acquisition, fewer transcripts related to biofilm formation, and increased levels of transcripts associated with respiration in low oxygen environments. Many of these transcriptional changes correlated with phenotypes observed *in vitro*, as the CF isolates failed to generate surface-adhered biofilms in SCFM2 and exhibited increased resistance to oxidative stress. Collectively, this work provides novel insight into the interactions occurring between *S. maltophilia* and the CF lung environment and identified potential virulence and metabolism-related genes that will be the subject of future research efforts.

4.6 Chapter 4 figures

Figure 4.1 Conserved *S. maltophilia* transcriptome changes in response to synthetic cystic fibrosis sputum media (SCFM2). (**A**) Volcano plot of transcripts detected through RNA-seq as exhibiting at least a 2.5-fold change in expression ($P < 0.05$) in all three *S. maltophilia* strains following exposure to SCFM2 (**B**) Conserved SCFM2-induced transcripts were categorized into groups reflecting their known, or bioinformatically-predicted functions.

Figure 4.2 *S. maltophilia* CF-isolate specific transcriptome changes induced by synthetic cystic fibrosis sputum media (SCFM2). (**A**) Volcano plot of transcripts detected through RNA-seq as exhibiting at least a 2.5-fold change in expression ($P < 0.05$) in GW273 and GW275 following exposure to SCFM2 (**B**) SCFM2-altered transcripts were categorized into groups reflecting their known, or bioinformatically-predicted functions

Figure 4.3 *S. maltophilia* CF isolates fail to generate surface-attached biofilms during growth in synthetic cystic fibrosis sputum. *S. maltophilia* K279A, GW273, and GW275 were cultured in SCFM2. After 18 h, the liquid cultures were removed and the extracellular material remaining adhered to the culture dish were stained with 0.1 % crystal violet.

Figure 4.4 *S. maltophilia* CF isolates are more resistant to oxidative stress than *S. maltophilia* K279A. *S. maltophilia* K279A, GW273, and GW275 were cultured in LB until mid-log phase of growth, at which point each strain was challenged with 0, 2, 4, and

8 mM of hydrogen peroxide. After 2 h, the remaining cells surviving was determined through serial dilution and CFU counting. The percent survival was then determined for each strain through comparison against the untreated cultures. The data shown summarizes three independent experiments that were performed in biological duplicate. Statistical significance was determined using 2-way ANOVA with Tukey's post-test for multiple comparisons.

Figure 4.S1 Volcano plots depicting SCFM2-induced transcripts in individual *S. maltophilia* strains. **(A-C)** Volcano plots of transcripts detected through RNA-Seq as exhibiting at least a 2.5-fold change in expression ($P < 0.05$) in each *S. maltophilia* strain following exposure to SCFM2. The 25 most highly induced transcripts are labeled for each strain.

4.7 Chapter 4 tables

Table 4.1

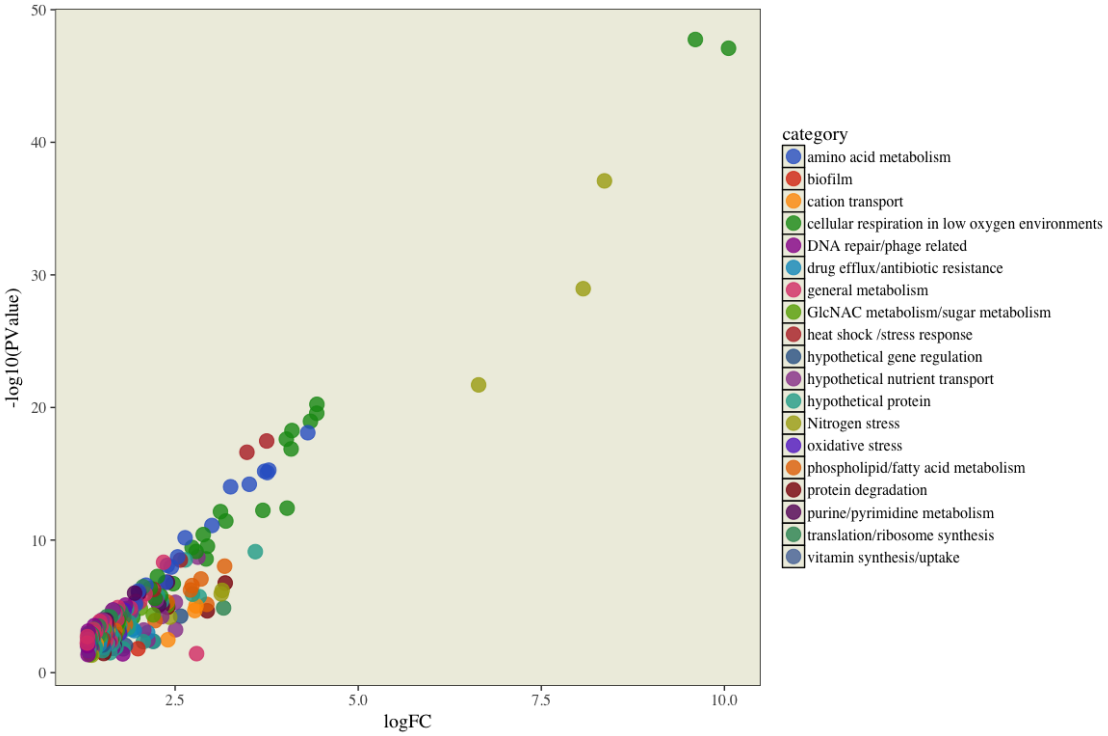
Table 4.2

Table 4.3

Table 4.S1

Table 4.S2

A.



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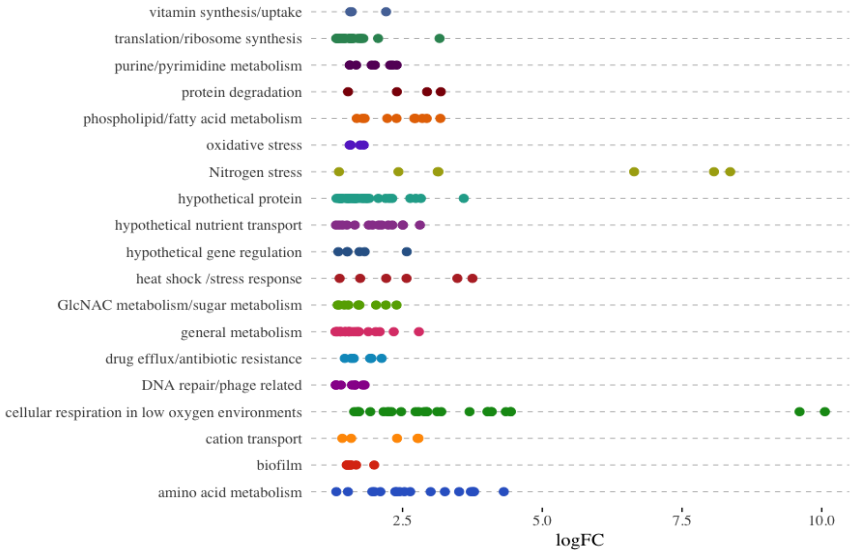
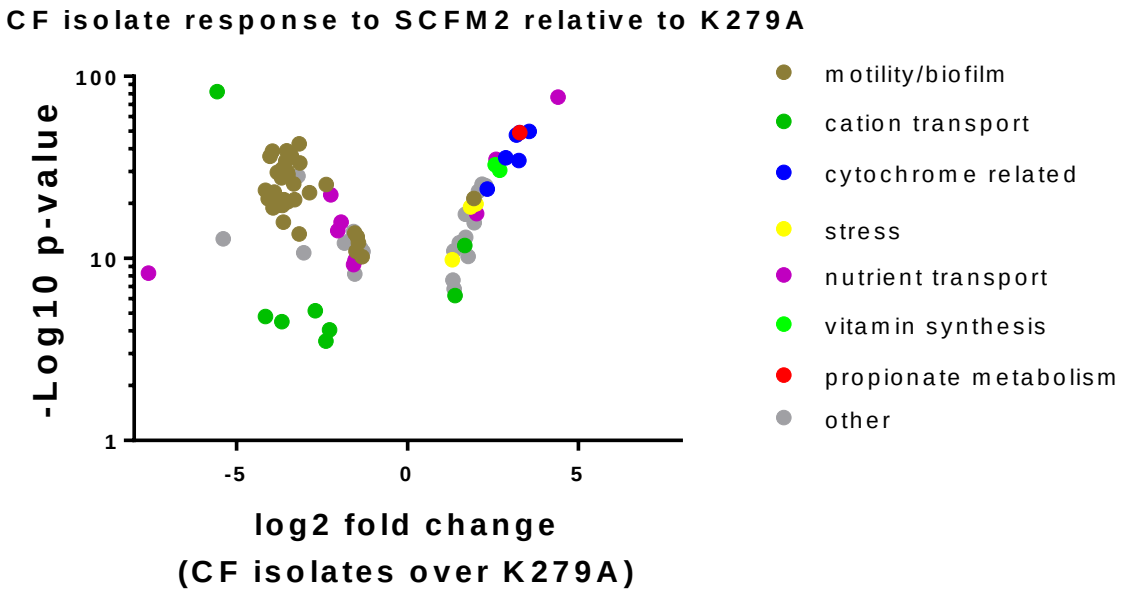


Figure 4.1 Conserved *S. maltophilia* transcriptome changes in response to synthetic cystic fibrosis sputum media (SCFM2).

A.



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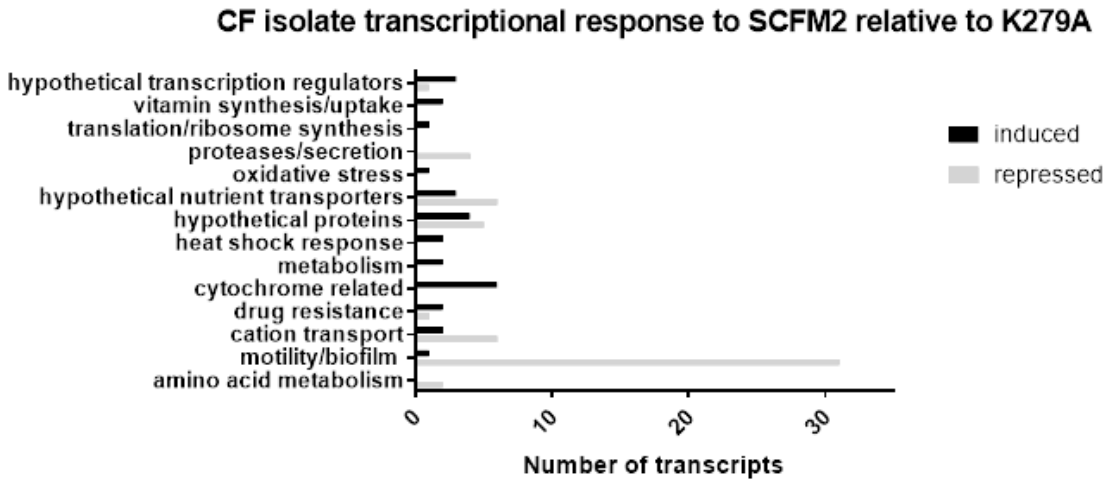


Figure 4.2 *S. maltophilia* CF-isolate specific transcriptome changes induced by synthetic cystic fibrosis sputum media (SCFM2).

Figure 4.3 *S. maltophilia* CF isolates fail to generate surface-attached biofilms during growth in synthetic cystic fibrosis sputum.

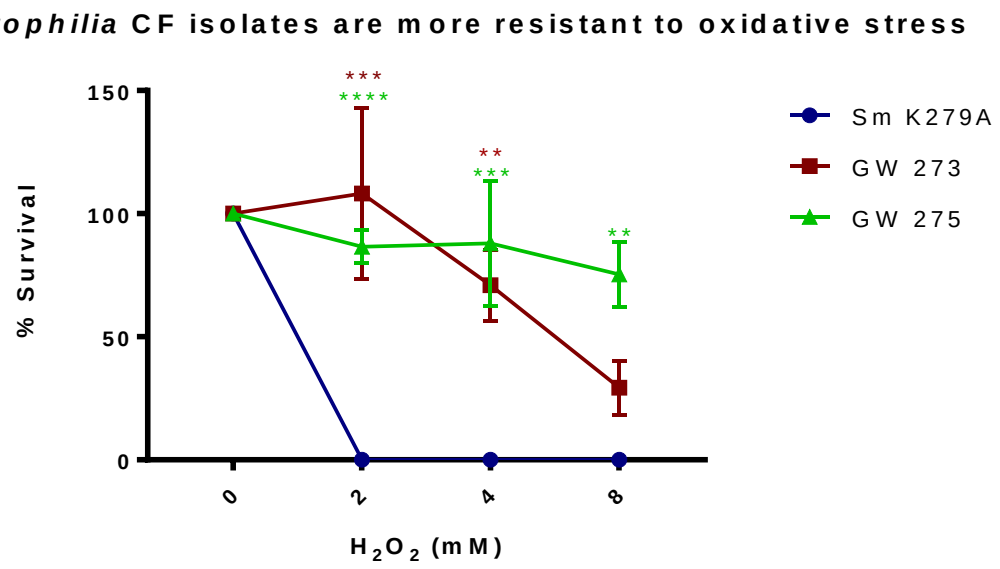
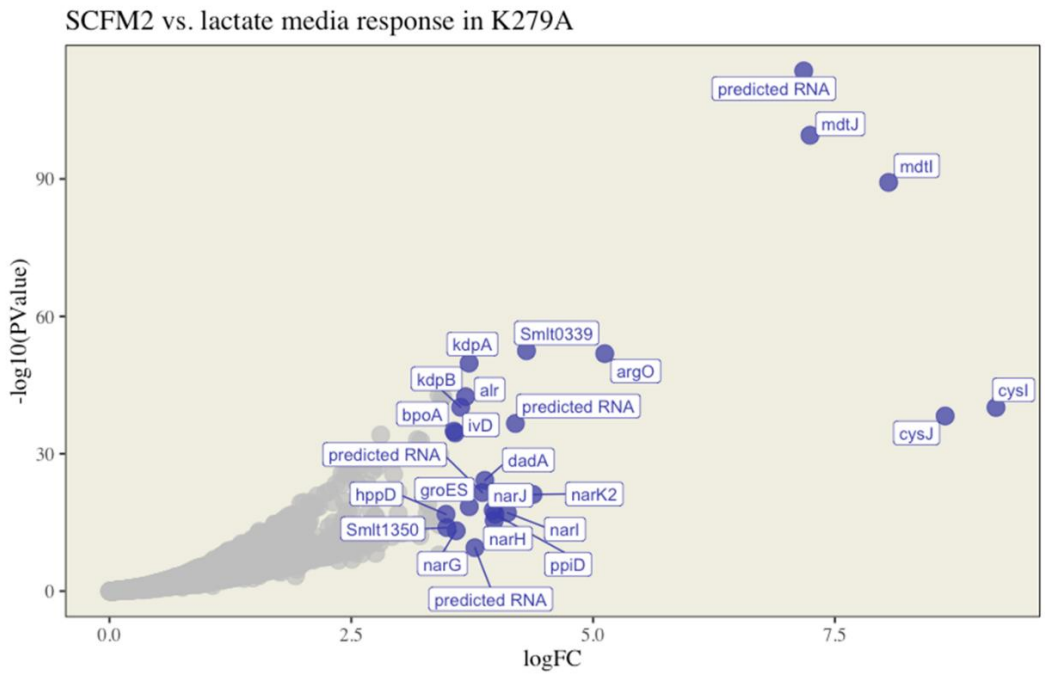
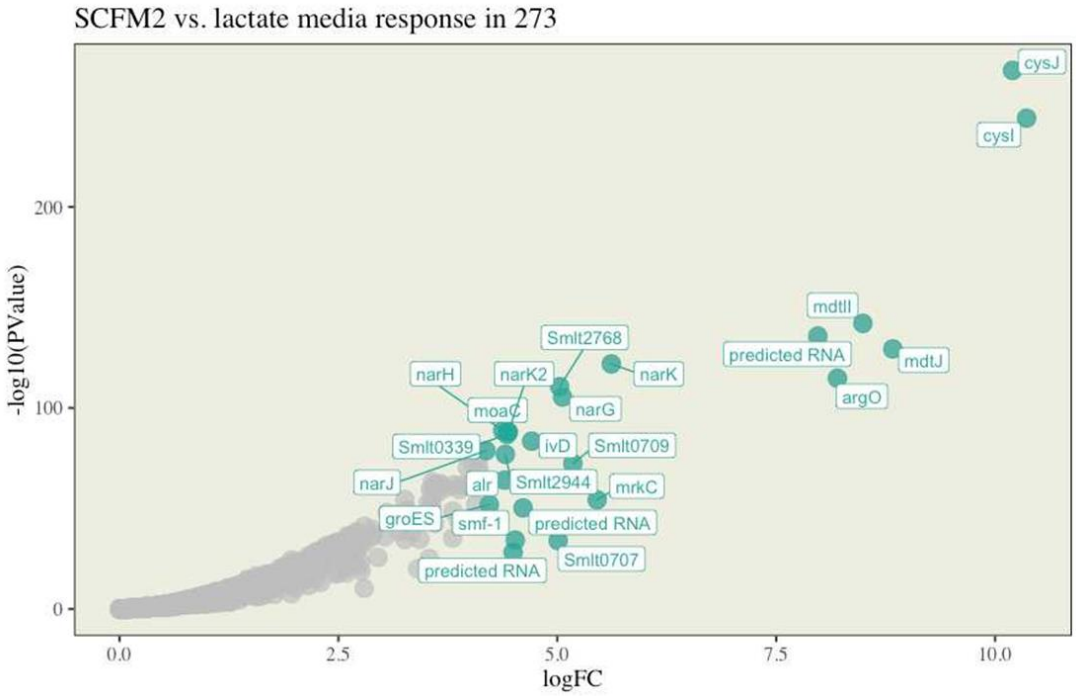


Figure 4.4 *S. maltophilia* CF isolates are more resistant to oxidative stress than *S. maltophilia* K279A.

A.


SCFM2 vs. lactate media response in K279A
predicted RNA
mdtJ
mdtI
cysI
cysJ
kdpA
Smlt0339
argO
kdpB
alr
predicted RNA
bpoA
ivD
predicted RNA
dadA
hppD
groES
narJ
narK2
Smlt1350
narI
narH
narG
ppiD
predicted RNA
-log10(PValue)
0
30
60
90
0.0
2.5
5.0
7.5
logFC

B.


SCFM2 vs. lactate media response in 273
cysJ
cysI
mdtII
predicted RNA
mdtJ
argO
Smlt2768
narK
narH
narK2
moaC
narG
Smlt0339
ivD
Smlt0709
narJ
alr
Smlt2944
mrkC
groES
smf-1
predicted RNA
predicted RNA
Smlt0707
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0
100
200
0.0
2.5
5.0
7.5
10.0
logFC

C.

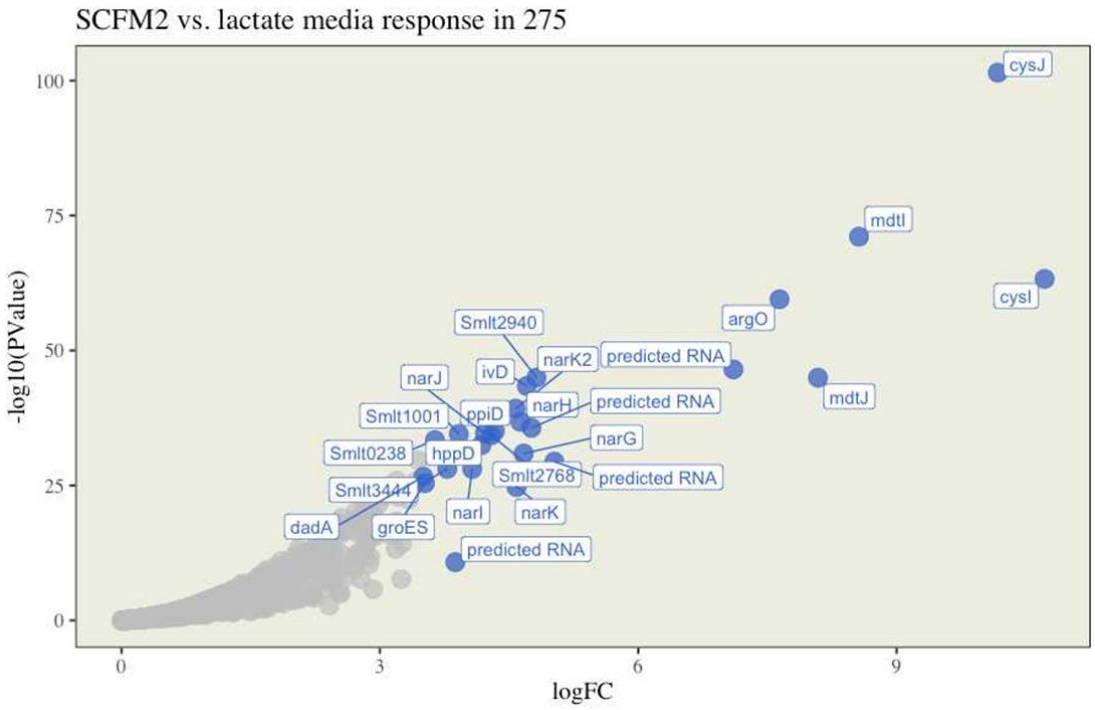

Figure 4.S1 Volcano plots depicting SCFM2-induced transcripts in individual *S. maltophilia* strains.

Table 4.1 Summary of the 50 most highly induced transcripts expressed by all three *S. maltophilia* strains in response to synthetic cystic fibrosis sputum media (SCFM2).

Gene ID	Name	Fold Change	Product Description	P-Value
Smlt2762	-	1064.48	methyl-accepting chemotaxis protein	8.08E-48
Smlt2763	*cysJ*	777.72	sulfite reductase flavodoxin containing subunit	1.76E-48
Smlt2852	*mdtI*	329.00	polyamine efflux protein	8.11E-38
Smlt2851	*mdtJ*	269.03	polyamine efflux protein	1.10E-29
Smlt1238	-	99.98	lysE-family amino acid efflux protein	1.97E-22
Smlt2774	*narG*	21.64	respiratory nitrate reductase subunit alpha	5.75E-21
Smlt2769	*narK2*	21.61	MFS transmembrane nitrite extrusion transporter	2.72E-20
Smlt2773	*narH*	20.36	respiratory nitrate reductase subunit	1.10E-19
Smlt0239	*ivD*	19.85	acyl CoA dehydrogenase	7.98E-19
Smlt2772	*narJ*	17.09	respiratory nitrate reductase subunit delta	5.47E-19
Smlt2770	*ppiD*	16.96	rotamase/peptidyl-prolyl cis-trans isomerase	1.34E-17
Smlt2775	*narK*	16.33	MFS transmembrane nitrite extrusion transporter	3.90E-13
Smlt2771	*narI*	16.21	respiratory nitrate reductase subunit gamma	2.44E-18
Smlt0568	*alr*	13.73	alanine racemase	5.34E-16
Smlt4330	*hppD*	13.53	4-hydroxyphenylpyruvate dioxygenase	8.07E-16
Smlt4215	*groES*	13.46	co-chaperonin GroES	3.36E-18
Smlt0567	*dadA*	13.20	D-amino acid dehydrogenase small subunit	6.47E-16
Smlt2768		12.98	oxygen-independent coproporphyrinogen III oxidase	5.73E-13
Smlt0339	-	12.08	hypothetical protein	7.47E-10
Smlt0238	-	11.41	acyl CoA carboxyltransferase	6.17E-15
Smlt4214	*groEL*	11.16	chaperonin GroEL	2.38E-17
Smlt0426	*putA*	9.56	proline dehydrogenase	9.53E-15
Smlt2781	*moaC*	9.14	molybdenum cofactor biosynthesis	3.66E-12
Smlt1001	-	9.09	autotransporter, crotonase clP domain	1.73E-07
Smlt3647	*bpoA*	9.04	peroxidase BpoA	9.26E-09
Smlt2940	-	8.95	translational inhibitor protein	1.3073E-05
Smlt2941	-	8.83	cation efflux transmembrane protein	6.1914E-07
Smlt2942	*adiA*	8.75	orn/arg/lys decarboxylase	1.1392E-06
Smlt2780	*moeA*	8.69	molybdopterin biosynthesis protein	7.15E-13
Smlt0237	-	8.01	biotin carboxylase	7.9225E-12
Smlt2777	*mobA*	7.68	molybdopterin-guanine dinucleotide biosynthesis	2.9042E-10
Smlt1350	-	7.66	outer membrane autotransporter	2.1619E-05
Smlt3649	-	7.63	hypothetical protein	7.1065E-06
Smlt2779	*moaD*	7.59	molybdopterin converting factor subunit 1	2.6479E-09

Smlt2767	*fnr2*	7.38	fumarate and nitrate reduction transcriptional regulator	3.8355E-11
Smlt2051	*fadA*	7.22	acetyl-CoA acetyltransferase	8.5939E-08
Smlt4368	-	7.10	hypothetical protein	1.8384E-06
Smlt0359	-	7.02	TonB dependent receptor protein	1.9373E-09
Smlt2418	-	6.93	monooxygenase	0.03672886
Smlt2778	*moaE*	6.90	molydopterin converting factor subunit 2 protein	7.1775E-10
Smlt0408	*kdpA*	6.89	potassium-transporting ATPase subunit A	9.0027E-06
Smlt0407	*kdpB*	6.81	potassium-transporting ATPase subunit B	2.0564E-05
Smlt3122	-	6.67	hypothetical protein	1.2031E-06
Smlt2782	*moaA*	6.64	molybdenum cofactor biosynthesis protein A	3.6016E-10
Smlt2706	*proP*	6.64	proline/betaine transporter	2.7114E-07
Smlt2052	*fadB*	6.54	3-hydroxyacyl-CoA dehydrogenase oxidoreductase	5.7945E-07
Smlt1569	-	6.22	hypothetical protein	3.1279E-09
Smlt4341	-	6.22	branched-chain alpha-keto acid dehydrogenase subunit	6.7076E-11
Smlt2243	-	5.96	SpoVT/AbrB domain transcriptional regulator	5.4353E-05
Smlt1809	*htpG*	5.94	heat shock protein 90	3.2153E-09

Table 4.2 Summary of the 25 most highly induced transcripts expressed by *S. maltophilia* CF isolates during growth in synthetic cystic fibrosis sputum media (SCFM2).

Gene ID	Name	Fold Change	Product Description	P-Value
Smlt2944	-	21.22	OMP-family porin	1.13E-77
Smlt3283	*cydB*	11.79	transmembrane cytochrome bd-II oxidase subunit II	1.40E-50
Smlt3610	*prpB*	9.75	2-methylisocitrate lyase	1.01E-49
Smlt1757	-	9.70	cytochrome c family protein	7.98E-50
Smlt3284	-	9.59	transmembrane cyd operon protein	3.02E-35
Smlt3282	*cydA*	9.11	cytochrome D ubiquinol oxidase subunit I	2.46E-48
Smlt1756	-	7.35	TonB dependent receptor protein	1.91E-36
Smlt0727	*ribE*	6.49	riboflavin synthase subunit alpha	3.69E-31
Smlt3805	-	6.02	OMP-family protein	9.67E-36
Smlt0728	*ribB*	5.92	3,4-dehydroxy-2-butanone 4-phosphate synthetase	2.07E-33
Smlt1758	*adhB*	5.04	alcohol dehydrogenase cytochrome c subunit	9.79E-25
Smlt1691	-	4.90	FAD sensors of blue light domain-containing protein	9.49E-26
Smlt1459	-	4.53	hypothetical protein	3.02E-26
Smlt0232	*aceA*	4.23	isocitrate lyase	3.66E-24
Smlt4119	-	4.07	OMP-like channel protein	2.37E-18
Smlt3732	*clpB*	4.02	heat shock chaperone ClpB	1.18E-20
Smlt1598	-	3.87	ABC transporter ATP-binding protein	2.09E-16
Smlt1619	-	3.84	predicted oar family adhesin	4.24E-22
Smlt4591	*usP*	3.59	universal stress protein	6.88E-20
Smlt0773	-	3.41	hypothetical protein	5.55E-11
Smlt2112	-	3.26	TetR family transcriptional regulator	8.32E-14
Smlt1597	-	3.24	HlyD family secretion protein	3.10E-18
Smlt4069	-	3.20	iron-permease family protein	1.74E-12
Smlt1737	*hflX*	2.86	GTP-binding phage-like protein	6.30E-13
Smlt2838	*mntH*	2.62	manganese ion transporter protein	5.48E-07

Table 4.3 Summary of the 25 most repressed transcripts in *S. maltophilia* CF isolates during growth in synthetic cystic fibrosis sputum media (SCFM2).

Gene ID	Name	Fold Change	Product Description	P-Value
Smlt4387	-	-190.82	TonB dependent receptor protein	5.12E-09
Smlt4626	*mntP*	-47.45	transmembrane protein	5.86E-83
Smlt4386	-	-42.07	hypothetical protein	1.60E-13
Smlt2315	*flgD*	-17.86	flagellar basal body rod modification protein	1.97E-24
Smlt1551	*pstC*	-17.84	phosphate transport system permease	1.60E-05
Smlt2314	*flgE*	-16.81	flagellar hook protein FlgE	5.91E-22
Smlt2316	*flgC*	-16.21	flagellar basal body rod protein FlgC	4.46E-37
Smlt2273	*flhA*	-15.45	flagellar biosynthesis protein FlhA	1.37E-39
Smlt2313	*flgF*	-15.30	flagellar basal body rod protein FlgF	1.14E-19
Smlt2272	*flhF*	-14.94	flagellar biosynthesis regulator FlhF	6.87E-24
Smlt2317	*flgB*	-13.99	flagellar basal body rod protein FlgB	2.20E-30
Smlt2311	*flgH*	-13.95	flagellar basal body L-ring protein	3.12E-20
Smlt2312	*flgG*	-12.85	flagellar basal body rod protein FlgG	1.95E-28
Smlt1552	*pstS*	-12.80	phosphate transport system substrate-binding	3.19E-05
Smlt2274	*flhB*	-12.63	flagellar biosynthesis protein FlhB	8.87E-32
Smlt2309	*flgJ*	-12.59	flagellar rod assembly protein/muramidase	3.14E-20
Smlt2308	*flgK*	-12.42	flagellar hook-associated protein FlgK	1.57E-16
Smlt2310	*flgI*	-12.27	flagellar basal body P-ring protein	8.86E-22
Smlt2286	*fliI*	-11.77	flagellum-specific ATP synthase	4.93E-35
Smlt2282	*fliM*	-11.56	flagellar motor switch protein FliM	9.12E-40
Smlt2289	*fliF*	-11.43	flagellar MS-ring protein	1.12E-30
Smlt2288	*fliG*	-11.39	flagellar motor switch protein	3.69E-21
Smlt2290	*fliE*	-11.27	flagellar hook-basal body complex protein	1.39E-29
Smlt2281	*fliN*	-10.57	flagellar motor switch protein	2.13E-37
Smlt2287	*fliH*	-10.08	flagellar assembly protein, FliH	2.39E-26

Table 4.S1 List of transcripts expressed by all three *S. maltophilia* strains in response to synthetic cystic fibrosis sputum media (SCFM2)

Gene ID	Name	Fold Change	Product Description	P-Value
Smlt2762	-	1064.48	methyl-accepting chemotaxis protein	8.08E-48
Smlt2763	*cysJ*	777.72	sulfite reductase flavodoxin containing subunit alpha	1.76E-48
Smlt2852	*mdtI*	329.00	transmembrane efflux protein	8.11E-38
Smlt2851	*mdtJ*	269.03	transmembrane efflux protein	1.10E-29
Smlt1238	*argO*	99.98	arginine efflux	1.97E-22
Smlt2774	*narG*	21.64	respiratory nitrate reductase subunit alpha	5.75E-21
Smlt2769	*narK2*	21.61	MFS transmembrane nitrite extrusion transporter protein	2.72E-20
Smlt2773	*narH*	20.36	respiratory nitrate reductase subunit	1.10E-19
Smlt0239	*ivD*	19.85	acyl CoA dehydrogenase	7.98E-19
Smlt2772	*narJ*	17.09	respiratory nitrate reductase subunit delta	5.47E-19
Smlt2770	-	16.96	rotamase/peptidyl-prolyl cis-trans isomerase family protein	1.34E-17
Smlt2775	*narK*	16.33	major facilitator superfamily transmembrane nitrite extrusion protein	3.90E-13
Smlt2771	*narI*	16.21	respiratory nitrate reductase subunit gamma	2.44E-18
Smlt0568	*alr*	13.73	alanine racemase	5.34E-16
Smlt4330	-	13.53	4-hydroxyphenylpyruvate dioxygenase	8.07E-16
Smlt4215	*groES*	13.46	co-chaperonin GroES	3.36E-18
Smlt0567	*dadA*	13.20	D-amino acid dehydrogenase small subunit	6.47E-16
Smlt2768	-	12.98	oxygen-independent coproporphyrinogen III oxidase	5.73E-13
Smlt0339	-	12.08	hypothetical protein	7.47E-10
Smlt0238	-	11.41	acyl CoA carboxyltransferase	6.17E-15
Smlt4214	*groEL*	11.16	chaperonin GroEL	2.38E-17
Smlt0426	*putA*	9.56	bifunctional proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase	9.53E-15
Smlt2781	*moaC*	9.14	bifunctional molybdenum cofactor biosynthesis protein MoaC/MogA	3.66E-12
Smlt1001	-	9.09	autotransporter	1.73E-07
Smlt3647	*bpoA*	9.04	peroxidase BpoA	9.26E-09
Smlt2940	-	8.95	translational inhibitor protein	1.31E-05
Smlt2941	-	8.83	cation efflux transmembrane protein	6.19E-07
Smlt2942	*adiA*	8.75	orn/arg/lys decarboxylase	1.14E-06
Smlt2780	*moeA*	8.69	molybdopterin biosynthesis protein	7.15E-13
Smlt0237	-	8.01	biotin carboxylase	7.92E-12
Smlt2777	*mobA*	7.68	molybdopterin-guanine dinucleotide biosynthesis protein A	2.90E-10
Smlt1350	-	7.66	outer membrane autotransporter	2.16E-05
Smlt3649	-	7.63	hypothetical protein	7.11E-06

Smlt2779	*moaD*	7.59	molybdopterin converting factor subunit 1	2.65E-09
Smlt2767	*fnr2*	7.38	fumarate and nitrate reduction transcriptional regulator	3.84E-11
Smlt2051	*fadA*	7.22	acetyl-CoA acetyltransferase	8.59E-08
Smlt4368	-	7.10	hypothetical protein	1.84E-06
Smlt0359	-	7.02	TonB dependent receptor protein	1.94E-09
Smlt2418	-	6.93	monooxygenase	3.67E-02
Smlt2778	*moaE*	6.90	molydopterin converting factor subunit 2 protein	7.18E-10
Smlt0408	*kdpA*	6.89	potassium-transporting ATPase subunit A	9.00E-06
Smlt0407	*kdpB*	6.81	potassium-transporting ATPase subunit B	2.06E-05
Smlt3122	-	6.67	hypothetical protein	1.20E-06
Smlt2782	*moaA*	6.64	molybdenum cofactor biosynthesis protein A	3.60E-10
Smlt2706	*proP*	6.64	proline/betaine transporter	2.71E-07
Smlt2052	*fadB*	6.54	3-hydroxyacyl-CoA dehydrogenase oxidoreductase	5.79E-07
Smlt1569	-	6.22	hypothetical protein	3.13E-09
Smlt4341	-	6.22	branched-chain alpha-keto acid dehydrogenase E2 subunit	6.71E-11
Smlt2243	-	5.96	SpoVT/AbrB domain transcriptional regulator	5.44E-05
Smlt1809	*htpG*	5.94	heat shock protein 90	3.22E-09
Smlt4340	-	5.79	hypothetical protein	1.86E-09
Smlt3789	-	5.68	TonB dependent receptor protein	5.77E-04
Smlt1095	-	5.67	transmembrane YitT family protein (ABC transporter)	4.93E-06
Smlt2765	-	5.56	ABC molybdenum transport-related membrane protein	1.94E-07
Smlt4329	*hmgA*	5.46	homogentisate 1,2-dioxygenase	1.08E-08
Smlt2943	*adiC*	5.37	arginine:agmatin antiporter	6.50E-05
Smlt0406	*kdpC*	5.28	potassium-transporting ATPase subunit C	3.29E-03
Smlt4145	-	5.27	extracellular serine protease	1.14E-05
Smlt2841	*nrdF*	5.26	ribonucleotide-diphosphate reductase subunit beta	1.49E-07
Smlt4338	*pdhA*	5.25	pyruvate dehydrogenase E1 component subunit alpha	8.07E-09
Smlt4023	*nagP*	5.25	major facilitator superfamily transmembrane sugar transporter	8.45E-06
Smlt2053	*psrA*	5.25	TetR family regulatory protein	4.69E-06
Smlt3579	*gcvP*	5.17	glycine dehydrogenase	1.53E-07
Smlt2245	*acnB*	5.07	bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	4.71E-09
Smlt2839	*trxA*	5.01	thioredoxin	9.08E-06
Smlt3740	-	4.98	TonB dependent receptor protein	5.84E-05
Smlt2244	-	4.97	hypothetical protein	4.22E-06
Smlt3846	*fdnI*	4.94	formate dehydrogenase, cytochrome b556 (FDN) subunit	1.65E-06
Smlt2840	-	4.85	flavodoxin	7.97E-06
Smlt0960	-	4.79	hypothetical protein	1.57E-06
Smlt2766	-	4.78	molybdenum transport-related, substrate-binding	5.57E-08

			protein	
Smlt3444	-	4.74	TonB dependent receptor protein	3.50E-06
Smlt2764	-	4.68	molybdenum transport-related, ATP-binding protein	2.50E-06
Smlt3648	*fadE*	4.68	acyl-CoA dehydrogenase	1.19E-04
Smlt1993	*dnaJ*	4.62	chaperone protein DnaJ	5.30E-07
Smlt1067	*btuB*	4.61	TonB-dependent receptor	4.31E-03
Smlt4025	*nagk2*	4.60	glucokinase	4.37E-05
Smlt2082	-	4.58	transmembrane anchor protein	4.30E-03
Smlt3845	*fdnH*	4.47	formate dehydrogenase, iron-sulfur subunit	4.32E-07
Smlt0602	-	4.37	TonB dependent receptor protein	3.47E-03
Smlt4661	-	4.36	transmembrane protein	9.58E-04
Smlt4339	-	4.30	branched-chain alpha keto acid dehydrogenase E1 subunit beta	2.52E-07
Smlt3222	-	4.26	fumarate hydratase	1.12E-06
Smlt2944	-	4.21	porin	6.10E-04
Smlt0538	-	4.19	transmembrane anchor protein	4.57E-03
Smlt1506	*tsf*	4.18	elongation factor Ts	3.31E-07
Smlt2180	*mnnA*	4.07	Alpha-1,2-mannosidase	1.38E-05
Smlt3902	*ppa*	4.05	inorganic pyrophosphatase	4.31E-06
Smlt2842	*nrdE*	4.01	ribonucleotide-diphosphate reductase subunit alpha	9.87E-07
Smlt0608	*faa*	3.99	fumarylacetoacetate (FAA) hydrolase family protein	6.94E-07
Smlt3757	*ppdD*	3.98	prepilin peptidase dependent protein D	1.55E-02
Smlt3340	-	3.90	TonB dependent receptor protein	4.28E-06
Smlt0609	*maiA*	3.89	maleylacetoacetate isomerase	7.43E-06
Smlt1449	-	3.86	phosphodiesterase-nucleotide pyrophosphatase	1.00E-06
Smlt4663	-	3.84	outer membrane efflux protein	6.75E-04
Smlt3279	*cydC*	3.79	transmembrane ABC transporter ATP-binding protein, cytochrome related	6.72E-05
Smlt4662	-	3.78	HlyD family secretion protein	5.15E-04
Smlt2783	-	3.74	hypothetical protein	3.24E-05
Smlt3905	-	3.72	TonB dependent receptor protein	2.60E-05
Smlt4539	-	3.70	aldo-keto reductase/oxidase	1.35E-05
Smlt4453	-	3.66	two-partner secretion system protein	1.58E-04
Smlt2806	-	3.62	hypothetical protein	4.25E-04
Smlt0560	-	3.57	hypothetical protein	1.45E-05
Smlt3021	-	3.54	TetR family transcriptional regulator	8.79E-03
Smlt2239	*betI*	3.54	TetR family regulatory protein	2.30E-04
Smlt3689	*recQ*	3.53	ATP-dependent DNA helicase	8.00E-06
Smlt3238	*sodA*	3.50	superoxide dismutase	1.97E-05
Smlt3150	*rpsF*	3.48	30S ribosomal protein S6	6.57E-05
Smlt2018	-	3.47	hypothetical protein	6.62E-04

Smlt0539	-	3.46	hypothetical protein	1.59E-02
Smlt1053	-	3.45	hypothetical protein	3.82E-02
Smlt3352	-	3.43	acyl-CoA dehydrogenase oxidoreductase	3.76E-04
Smlt0876	*rplY*	3.39	50S ribosomal protein L25/general stress protein Ctc	2.35E-05
Smlt2017	-	3.35	hypothetical protein	2.75E-04
Smlt3977	-	3.35	hypothetical protein	1.07E-03
Smlt1992	*dnaK*	3.34	molecular chaperone DnaK	6.02E-05
Smlt1464	-	3.34	pseudouridine synthase	5.78E-05
Smlt3175	-	3.31	ArsR family transcriptional regulator	4.81E-04
Smlt4022	*nagR*	3.31	LacI family transcriptional regulator	1.77E-04
Smlt2776	-	3.30	hypothetical protein	1.04E-04
Smlt0982	-	3.29	isocitrate dehydrogenase	1.22E-05
Smlt4021	*nagB2*	3.27	phosphosugar-binding protein	6.86E-04
Smlt3280	*cydD*	3.26	ABC transporter ATP-binding protein, cytochrome related	2.12E-04
Smlt4116a	*atpC*	3.26	F0F1 ATP synthase subunit C	9.84E-05
Smlt3637	*prfC*	3.24	peptide chain release factor 3	2.60E-05
Smlt0540	-	3.23	hypothetical protein	1.24E-02
Smlt0952	*fadH*	3.20	2,4-dienoyl-CoA reductase [NADPH]	6.43E-04
Smlt3407	*tpiA*	3.20	triosephosphate isomerase	3.36E-05
Smlt3284	-	3.20	transmembrane cyd operon protein	2.12E-03
Smlt2932	-	3.18	glutamine amidotransferase class-I	8.34E-03
Smlt3672	*pilI*	3.18	pilus biogenesis protein	1.80E-03
Smlt2933	-	3.17	hypothetical protein	1.48E-02
Smlt1432	-	3.16	HD domain signalling protein	2.53E-03
Smlt2054	*ndk*	3.15	nucleoside diphosphate kinase	5.03E-05
Smlt4687	-	3.15	hypothetical protein	3.82E-05
Smlt4595	*uvrD*	3.14	DNA-dependent helicase II	1.87E-05
Smlt1048	-	3.14	bacteriophage tail protein I	1.43E-02
Smlt1801	-	3.13	hypothetical protein	6.73E-03
Smlt2030	*uup*	3.13	ABC transporter ATP-binding protein	2.00E-05
Smlt3283	*appB*	3.10	transmembrane cytochrome bd-II oxidase subunit II	2.78E-03
Smlt3615	-	3.09	aminoglycoside 6'-N-acetyltransferase	1.50E-03
Smlt0559	-	3.08	hypothetical protein	1.20E-03
Smlt2931	-	3.07	racemase	4.24E-03
Smlt0877	*pth*	3.07	peptidyl-tRNA hydrolase	6.19E-05
Smlt1375	*rimM*	3.06	16S rRNA-processing protein RimM	7.03E-05
Smlt3594	-	3.05	transmembrane protein	3.14E-02
Smlt1061	-	3.02	phage integrase	5.29E-03
Smlt2808	-	3.02	hypothetical protein	9.86E-04

Smlt3616	*pdxH*	3.01	pyridoxamine 5'-phosphate oxidase	3.70E-04
Smlt3148	*rplI*	3.00	50S ribosomal protein L9	6.56E-04
Smlt1706	*pitA*	2.99	low-affinity inorganic phosphate transporter integral membrane protein PITA	7.25E-04
Smlt4660	-	2.99	MarR family transcriptional regulator	6.95E-03
Smlt3825	*pilM*	2.99	type 4 fimbrial biogenesis protein PilM	2.42E-04
Smlt0767	*metF*	2.98	5,10-methylenetetrahydrofolate reductase	6.00E-03
Smlt3821	*pilQ*	2.98	type II/III pilus secretin	1.49E-03
Smlt3490	-	2.98	hypothetical protein	5.76E-03
Smlt3976	-	2.97	esterase	1.19E-03
Smlt0878	-	2.97	GTP-dependent nucleic acid-binding protein EngD	5.89E-05
Smlt0731	-	2.96	thiamine monophosphate kinase	9.46E-04
Smlt1374	*rpsP*	2.95	30S ribosomal protein S16	4.47E-04
Smlt3149	*rpsR*	2.95	30S ribosomal protein S18	7.22E-04
Smlt1222	-	2.94	inosine-uridine preferring nucleoside hydrolase	1.08E-04
Smlt0444	*rpsU*	2.94	30S ribosomal protein S21	4.04E-04
Smlt0248	-	2.94	ribonucleotide diphosphate reductase small subunit	2.06E-04
Smlt1700	-	2.94	monooxygenase family protein	3.83E-03
Smlt3978	*adhC*	2.93	alcohol dehydrogenase class-III	6.25E-04
Smlt3824	*pilN*	2.93	type 4 fimbrial biogenesis protein PilN	1.01E-03
Smlt2807	-	2.93	transmembrane protein	6.37E-04
Smlt0206	*dhaA*	2.91	haloalkane dehalogenase	9.51E-04
Smlt1800	-	2.91	TPR repeat-containing protein	3.36E-04
Smlt3321	*suhB*	2.91	inositol-1-monophosphatase	1.37E-04
Smlt0047	-	2.90	fructose-1,6-bisphosphatase	3.80E-04
Smlt4110	*atpC*	2.89	F0F1 ATP synthase subunit epsilon	2.06E-04
Smlt2179	-	2.89	Mannosides-regulated TonB-dependent outer membrane receptor	5.46E-04
Smlt3822	*pilP*	2.88	type 4 fimbrial biogenesis protein PilP	1.84E-03
Smlt0603	-	2.88	peptidase m14, carboxypeptidase A	3.55E-02
Smlt4137	-	2.87	transmembrane protein	1.93E-03
Smlt0566	*lrp*	2.87	leucine-responsive regulatory protein	1.25E-02
Smlt3673	*pilH*	2.86	two-component response regulator transcriptional regulator	4.16E-03
Smlt3588	-	2.85	methyl-accepting chemotaxis protein	2.13E-02
Smlt3906	-	2.85	hth transcriptional regulator	2.45E-03
Smlt2853	-	2.85	LysR family transcriptional regulator	8.16E-03
Smlt4658	-	2.84	hypothetical protein	5.50E-04
Smlt3823	*pilO*	2.84	type 4 fimbrial biogenesis protein PilO	2.57E-03
Smlt3724	-	2.84	ABC transporter ATP-binding protein	1.05E-04
Smlt3671	*pilJ*	2.83	pilus biogenesis protein PilJ/methyl accepting chemotaxis protein	4.36E-03

Smlt3480	-	2.80	rRNA large subunit methyltransferase	2.54E-03
Smlt4111	*atpD*	2.80	F0F1 ATP synthase subunit beta	3.16E-04
Smlt4117	-	2.78	F0F1 ATP synthase subunit A	3.51E-04
Smlt0097	-	2.78	hypothetical protein	4.20E-03
Smlt3886	*adk*	2.77	adenylate kinase	1.36E-04
Smlt0944	*mdh*	2.77	malate dehydrogenase	3.26E-04
Smlt4304	*sugE*	2.76	chaperone protein	7.63E-03
Smlt4020	*nagA*	2.75	N-acetylglucosamine-6-phosphate deacetylase	2.12E-03
Smlt1507	*rpsB*	2.75	30S ribosomal protein S2	3.10E-04
Smlt0398	*argS*	2.73	arginyl-tRNA synthetase	1.54E-03
Smlt0083	-	2.68	TonB dependent receptor protein	5.25E-04
Smlt1705	-	2.68	pit accessory protein	6.44E-03
Smlt1280	*obgE*	2.67	GTPase ObgE	8.20E-04
Smlt4115	*atpF*	2.67	F0F1 ATP synthase subunit B	8.80E-04
Smlt0726	-	2.66	hypothetical protein	1.05E-02
Smlt3878	-	2.65	lipoprotein	1.59E-02
Smlt4585	-	2.65	hypothetical protein	1.71E-02
Smlt3614	-	2.64	hypothetical protein	1.24E-02
Smlt0104	*rdgC*	2.63	recombination associated protein	2.82E-04
Smlt3316	*efp*	2.63	elongation factor P	5.33E-04
Smlt4114	*atpH*	2.62	F0F1 ATP synthase subunit delta	1.20E-03
Smlt3406	*secG*	2.62	preprotein translocase subunit SecG	5.57E-04
Smlt2229	-	2.62	hypothetical protein	2.92E-03
Smlt3508	*phnA*	2.62	alkylphosphonate uptake protein	1.03E-03
Smlt1124	-	2.60	transmembrane protein	4.71E-03
Smlt4315	-	2.60	acetyltransferase	2.46E-03
Smlt2805	-	2.60	hypothetical protein	3.02E-03
Smlt4075	*hslU*	2.59	ATP-dependent protease ATP-binding subunit HslU	2.06E-03
Smlt3374	*pheS*	2.58	phenylalanyl-tRNA synthetase subunit alpha	1.28E-03
Smlt4019	*nagX*	2.58	transmembrane protein	1.24E-02
Smlt3115	-	2.58	TonB-dependent receptor	1.69E-02
Smlt3365	-	2.57	LysE family amino acid efflux	4.74E-02
Smlt1459	-	2.56	hypothetical protein	2.91E-03
Smlt0922	*rplR*	2.55	50S ribosomal protein L18	1.13E-03
Smlt2677	-	2.55	TetR family regulatory protein	9.95E-03
Smlt3213		2.54	ThiF domain-containing protein	1.74E-03
Smlt4113	*atpA*	2.54	F0F1 ATP synthase subunit alpha	1.28E-03
Smlt0920	*rpsH*	2.53	30S ribosomal protein S8	2.23E-03
Smlt2759	-	2.53	Maltooligosyltrehalose trehalohydrolase protein	4.35E-02
Smlt3387	*truB*	2.53	tRNA pseudouridine synthase B	4.00E-03

Smlt3873A	-	2.50	azurin	1.33E-02
Smlt3779	-	2.50	ferredoxin	2.33E-02
Smlt0945	*rluA*	2.50	ribosomal large subunit pseudouridine synthase A	3.08E-03
Smlt0774	-	2.49	hypothetical protein	2.17E-02
Smlt3176	*metH2*	2.49	5-methyltetrahydrofolate--homocysteine methyltransferase	6.36E-03
Smlt0942	*typA*	2.49	GTP-binding protein	1.10E-03
Smlt3137	*gyrA*	2.48	DNA gyrase subunit A	7.39E-04
Smlt1049	-	2.48	phage baseplate assembly protein	4.31E-02
Smlt1241	-	2.48	major facilitator superfamily transmembrane transporter	3.08E-03
Smlt1044	-	2.47	major tail tube protein	9.14E-03
Smlt4112	*atpG*	2.47	F0F1 ATP synthase subunit gamma	1.91E-03
Smlt0645	*etfA*	2.46	electron transfer flavoprotein subunit alpha	5.88E-03

Table 4.S2 *S. maltophilia* CF isolate-specific transcriptional changes occurring during growth in synthetic cystic fibrosis sputum media (SCFM2).

Gene ID	Name	Fold Change	Product Description	P-Value
Smlt4387	-	-190.82	TonB dependent receptor protein	5.12E-09
Smlt4626	*mntP*	-47.45	transmembrane protein	5.86E-83
Smlt4386	-	-42.07	hypothetical protein	1.60E-13
Smlt2315	*flgD*	-17.86	flagellar basal body rod modification protein	1.97E-24
Smlt1551	*pstC*	-17.84	phosphate transport system permease	1.60E-05
Smlt2314	*flgE*	-16.81	flagellar hook protein FlgE	5.91E-22
Smlt2316	*flgC*	-16.21	flagellar basal body rod protein FlgC	4.46E-37
Smlt2273	*flhA*	-15.45	flagellar biosynthesis protein FlhA	1.37E-39
Smlt2313	*flgF*	-15.30	flagellar basal body rod protein FlgF	1.14E-19
Smlt2272	*flhF*	-14.94	flagellar biosynthesis regulator FlhF	6.87E-24
Smlt2317	*flgB*	-13.99	flagellar basal body rod protein FlgB	2.20E-30
Smlt2311	*flgH*	-13.95	flagellar basal body L-ring protein	3.12E-20
Smlt2312	*flgG*	-12.85	flagellar basal body rod protein FlgG	1.95E-28
Smlt1552	*pstS*	-12.80	phosphate transport system substrate-binding exported periplasmic protein	3.19E-05
Smlt2274	*flhB*	-12.63	flagellar biosynthesis protein FlhB	8.87E-32
Smlt2309	*flgJ*	-12.59	flagellar rod assembly protein/muramidase FlgJ	3.14E-20
Smlt2308	*flgK*	-12.42	flagellar hook-associated protein FlgK	1.57E-16
Smlt2310	*flgI*	-12.27	flagellar basal body P-ring protein	8.86E-22
Smlt2286	*fliI*	-11.77	flagellum-specific ATP synthase	4.93E-35
Smlt2282	*fliM*	-11.56	flagellar motor switch protein FliM	9.12E-40
Smlt2289	*fliF*	-11.43	flagellar MS-ring protein	1.12E-30
Smlt2288	*fliG*	-11.39	flagellar motor switch protein	3.69E-21
Smlt2290	*fliE*	-11.27	flagellar hook-basal body complex protein	1.39E-29
Smlt2281	*fliN*	-10.57	flagellar motor switch protein	2.13E-37
Smlt2287	*fliH*	-10.08	flagellar assembly protein, FliH	2.39E-26
Smlt2285	*fliJ*	-9.87	flagellar fliJ protein	1.02E-21
Smlt4272	-	-9.21	hypothetical protein	4.99E-29
Smlt2284	*fliK*	-9.01	flagellar hook-length control protein	2.49E-14
Smlt2319	*flgA*	-9.00	flagellar basal body P-ring biosynthesis protein FlgA	2.96E-43
Smlt2283	*fliL*	-8.90	flagellar basal body-associated protein FliL	3.62E-34
Smlt2888	-	-8.21	AraC family transcriptional regulator	1.92E-11
Smlt2270	*fliA*	-7.28	RNA polymerase sigma factor for flagellar regulon FliA	1.19E-23
Smlt1555	*oprP*	-6.49	polyphosphate-selective porin O	6.94E-06
Smlt1549	*pstB*	-5.24	phosphate transporter ATP-binding protein	3.02E-04
Smlt2258	-	-5.19	methyl-accepting chemotaxis receptor	3.33E-26

Smlt1548	*phoU*	-4.85	phosphate transport system-like protein	9.00E-05
Smlt0461	-	-4.73	TonB outer membrane protein oar family	4.46E-23
Smlt4100	-	-4.11	HlyD family transporter protein	6.04E-15
Smlt3478	-	-3.85	TonB dependent receptor protein	1.56E-16
Smlt2874	-	-3.59	type II secretion/pilus assembly transmembrane protein	7.09E-13
Smlt2871	-	-3.12	exported fimbriae assembly protein	1.66E-13
Smlt0526	*cycA*	-2.99	D-alanine/D-serine/glycine permease	9.14E-15
Smlt4102	-	-2.98	ABC transporter permease	5.76E-10
Smlt2201	*smeY*	-2.91	secretion protein-HlyD family	6.05E-09
Smlt3598	*pilH*	-2.91	two-component response regulator	1.73E-14
Smlt4103	-	-2.90	transmembrane permease	1.63E-10
Smlt1622	*pilV*	-2.84	type 4 fimbrial biogenesis protein PilV	1.13E-11
Smlt1625	*pilY1*	-2.78	PilY1 protein	6.57E-14
Smlt4371	-	-2.78	hypothetical protein	2.54E-13
Smlt4641	*tatA*	-2.76	twin arginine translocase A	3.66E-12
Smlt1623	*pilW*	-2.71	type 4 fimbrial biogenesis protein PilW	6.56E-13
Smlt0956	-	-2.71	hypothetical protein	3.66E-11
Smlt3293	*proA*	-2.66	gamma-glutamyl phosphate reductase	8.34E-12
Smlt4084	-	-2.57	exported oligopeptidase	4.64E-12
Smlt2295	-	-2.51	nitrogen regulation protein NR(I)	6.19E-11
Smlt0014	-	-2.47	hypothetical protein	1.26E-11
Smlt0841	*ahpC*	2.48	alkyl hydroperoxide reductase subunit c	1.48E-10
Smlt0882	-	2.52	sensor histidine kinase	2.54E-08
Smlt3005	-	2.55	VirB9	1.05E-11
Smlt1075	-	2.57	GntR family transcriptional regulator	1.57E-07
Smlt2838	*mntH*	2.62	natural resistance associated macrophage protein	5.48E-07
Smlt1737	*hflX*	2.86	GTP-binding phage-like protein	6.30E-13
Smlt4069	*Fet4-like*	3.20	iron-permease family protein	1.74E-12
Smlt1597	-	3.24	HlyD family secretion protein	3.10E-18
Smlt2112	-	3.26	TetR family transcriptional regulator	8.32E-14
Smlt0773	-	3.41	hypothetical protein	5.55E-11
Smlt4591	*usP*	3.59	universal stress protein	6.88E-20
Smlt1619	-	3.84	predicted oar family adhesin	4.24E-22
Smlt1598	-	3.87	ABC transporter ATP-binding protein	2.09E-16
Smlt3732	*clpB*	4.02	heat shock chaperone ClpB	1.18E-20
Smlt4119		4.07	OMP-like channel protein	2.37E-18
Smlt0232	*aceA*	4.23	isocitrate lyase	3.66E-24
Smlt1459	-	4.53	hypothetical protein	3.02E-26
Smlt1691	-	4.90	FAD sensors of blue light domain-containing	9.49E-26

			protein	
Smlt1758	*adhB*	5.04	alcohol dehydrogenase cytochrome c subunit	9.79E-25
Smlt0728	*ribB*	5.92	3,4-dehydroxy-2-butanone 4-phosphate synthetase	2.07E-33
Smlt3805		6.02	OMP-family protein	9.67E-36
Smlt0727	*ribE*	6.49	riboflavin synthase subunit alpha	3.69E-31
Smlt1756	-	7.35	TonB dependent receptor protein	1.91E-36
Smlt3282	*cydA*	9.11	cytochrome D ubiquinol oxidase subunit I	2.46E-48
Smlt3284	-	9.59	transmembrane cyd operon protein	3.02E-35
Smlt1757	-	9.70	cytochrome c family protein	7.98E-50
Smlt3610	*prpB*	9.75	2-methylisocitrate lyase	1.01E-49
Smlt3283	*cydB*	11.79	transmembrane cytochrome bd-II oxidase subunit II	1.40E-50
Smlt2944	-	21.22	OMP-family porin	1.13E-77

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CHAPTER 5:

Conclusions & future directions

5.1 Dissertation summary

Nosocomial lung infections are a growing concern in the United States, with more than 300,000 cases reported annually. More than 30 % of these opportunistic infections are caused by the Gram-negative bacteria, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (1, 2). Similarly, Gram-negative bacteria establish chronic infections in individuals with cystic fibrosis (CF) that are closely linked with the progressive decline in lung function associated with disease advancement and are difficult or impossible to eradicate (3, 4). *P. aeruginosa* has historically established chronic infections in the vast majority of CF individuals by their third decade of life (4, 5). Unfortunately, as antipseudomonal therapy has improved, more antibiotic resistant species have taken hold, including *Stenotrophomonas maltophilia*, which now colonizes more than 10 % of individuals with CF (6, 7).

Regardless of the source or specifics of the infection, Gram-negative respiratory infections are becoming increasingly difficult to treat due to the rising incidence of multiple drug resistance among these organisms (8-10). Pan-antibiotic Gram-negative species, including *P. aeruginosa* and *K. pneumoniae* have recently been isolated in the clinical setting, making it clear that new therapeutics will soon be needed to combat these infections (11-13). To aide in the development of new therapeutics, a greater understanding of how these organisms transition from the environment to the host lung is

needed. The goal of the research outlined in this dissertation was to expand our knowledge of how opportunistic Gram-negative respiratory pathogens recognize, exploit, and adapt to the host lung environment. The major findings of this work are briefly summarized below, with remaining questions and future directions discussed in the following sections.

5.2 Chapter 2 summary

Sarcosine is present in many eukaryote-associated environments inhabited by *Pseudomonas aeruginosa* and is most often encountered as an intermediate in the metabolism of choline. Phosphatidylcholine constitutes an estimated 70 % of the dry weight of human pulmonary surfactant and serves as an important source of choline for *P. aeruginosa* during infection (14, 15). We previously determined that the sarcosine catabolic operon (*sox* operon) of *P. aeruginosa* is induced by the glutamine amidotransferase1-like AraC-family regulator (GATR) GbdR in response to the choline catabolites, glycine betaine and dimethylglycine (16). However, transcription of the *sox* operon was still observed in response to sarcosine in a *gbdR* deletion mutant, indicating that an independent sarcosine-responsive transcription factor also acted at this locus (17).

As described in chapter two, we used a transposon-based genetic screen to identify PA4184, or SouR (Sarcosine oxidation & utilization Regulator), as a second GATR that controls the expression of the *sox* operon in response to sarcosine. Through growth assays with *souR* and *gdbR* single and double deletion strains, we demonstrated that SouR is required for growth when sarcosine is utilized as a sole carbon and nitrogen

source. We also examined the inducing ligand specificity of SouR through β-galactosidase assays and determined that the activity of this regulator is specific to sarcosine. Additionally, we characterized the transcriptome response to sarcosine governed by SouR using microarrays and performed electrophoretic mobility shift assays (EMSAs) to identify promoters directly regulated by this transcription factor. Finally, we characterized PA3630, or GfnR (Glutathione-dependent formaldehyde neutralization Regulator), as the regulator of the glutathione-dependent formaldehyde detoxification system in *P. aeruginosa* that is expressed in response to formaldehyde released during the catabolism of sarcosine. This study expands our understanding of sarcosine metabolic regulation in bacteria through the identification and characterization of the first known sarcosine-responsive transcriptional regulator. The identification of SouR and GfnR also further clarified the regulatory mechanisms governing choline catabolism by *P. aeruginosa* within the respiratory tract, leading to the revised regulatory model depicted in **Figure 5.1.**

5.2.1 Remaining questions regarding the transcriptional regulation of sarcosine catabolism in *P. aeruginosa*

In Chapter two, we identified and characterized SouR as the first known sarcosine responsive transcription regulator. We determined that this regulator was required for *P. aeruginosa* growth when sarcosine is utilized as a sole carbon and nitrogen source, and characterized SouR's regulon using microarrays and EMSAs. Through this work we identified at least two additional promoter regions that are directly controlled by SouR,

and characterized a second formaldehyde-responsive regulator controlling the expression of the glutathione-dependent formaldehyde detoxification system that is induced during growth on sarcosine. Despite these findings, several unanswered questions remain regarding the transcriptional regulation of sarcosine catabolism.

5.2.1.1 How do SouR and GbdR contribute to the regulation of the *sox* operon during glycine betaine catabolism?

Glycine betaine catabolism in *P. aeruginosa* and many other proteobacteria proceeds through a series of oxidative demethylation events that generate sarcosine as an intermediate. Catabolism of glycine betaine in *P. aeruginosa* is regulated by two transcription factors, GbdR and SouR (18, 19). GbdR induces genes encoding all steps in the pathway in response to GB and dimethylglycine, while SouR only induces the genes for sarcosine catabolism in response to sarcosine (18). Our promoter mapping and EMSA experiments revealed that SouR and GbdR directly stimulate transcription from the same region of the *glyA1* promoter. Interestingly, promoter mapping with *souR* and *gbdR* single & double deletion strains revealed that SouR significantly contributed to P_{glyA1} expression during the catabolism of glycine betaine. **(Supplementary Figure 2.1).** These observations suggest that SouR and GbdR either bind this promoter simultaneously during the catabolism of glycine betaine, or that SouR could have a higher binding affinity for the *glyA1* promoter than GbdR.

The SouR and GbdR regulons overlap at two other promoters (P_{sdaB} & P_{PA2762}), making the latter hypothesis more likely. DNAseI footprinting with MBP-tagged GbdR

was previously used to identify this regulator's binding site within the *plcH* promoter (19). A similar approach with MBP-tagged SouR and GbdR could be used to elucidate the respective binding sites of each regulator within the *glyA1* promoter. Alternatively, ChIP-seq could be used to map the half sites of each regulator throughout the genome, and would identify similar interactions occurring in the promoter regions of other shared regulon members (20).

5.2.1.2 What is the role of SouR within the *adhC* promoter?

In Chapter 2 we identified GfnR as the formaldehyde-responsive LysR-family transcription regulator that is divergently transcribed from the glutathione-dependent formaldehyde detoxification system it regulates (*adhC & PA3628)* (18, 21, 22). We were therefore surprised that our electrophoretic mobility shift assays (EMSAs) revealed MBP-tagged SouR bound within the *adhC* promoter with specificity, suggesting that SouR also impinges on control of this operon's expression during growth on sarcosine **(Figure 2.6).** However, we demonstrated through quantitative RT-PCR that GfnR, and not SouR, stimulated transcription of the glutathione-dependent formaldehyde detoxification genes during growth on sarcosine **(Table 2.2).**

Several hypotheses could explain these contradictory findings. First, the binding of MBP-SouR to the *adhC* promoter that we observed through EMSA could be a technical artifact. Due to the inherently low solubility of AraC-family regulators (23), we were not able to purify GbdR and SouR without the addition of an N-terminal tag. MBP-GbdR and MBP-SouR are not capable of responding to their respective inducing ligands

in vitro, indicating that the addition of these tags might compromise some portion of their function. Furthermore, EMSAs are performed under artificial buffering conditions with extreme excesses of protein that do not reflect the cytosolic environment in bacteria (24).

Alternatively, the SouR binding site within P_{adhC} we detected through EMSA could belong to GbdR. The serial demethylation of glycine betaine in *P. aeruginosa* generates between two and three moles of formaldehyde per mole of glycine betaine consumed, depending on the availability of tetrahydrofolate (25, 26). In contrast, the oxidative demethylation of sarcosine only generates formaldehyde in the absence of this cofactor (27, 28). Since glycine betaine catabolism generates significantly more formaldehyde than sarcosine catabolism, selection pressure would be more likely to lead to the acquisition of GbdR as a secondary regulator of these genes. The role of GbdR in the regulation of the glutathione-dependent formaldehyde detoxification operon could be tested through quantitative RT-PCR using RNA collected from *P. aeruginosa* WT, *ΔgbdR, ΔgfnR,* and *ΔgbdR ΔgfnR* strains following exposure to glycine betaine and formaldehyde.

5.2.1.3 Does the glutathione-dependent formaldehyde detoxification system contribute to *P. aeruginosa* fitness during growth on glycine betaine?

Oxidative demethylation of glycine betaine in *P. aeruginosa* results in the generation of two to three moles of formaldehyde per mole of glycine betaine consumed, depending on the availability of tetrahydrofolate (25, 26). In contrast, the N-methyl group of sarcosine is only lost as formaldehyde in the absence of this cofactor (27, 28). Since

our sarcosine growth assays with *gfnR* and *adhC* transposon-disruption mutants revealed slight (but significant) defects growth relative to our control strain **(Figure 2.6),** it is likely that more drastic growth defects would be observed in these mutants during growth on glycine betaine. Similar experiments should be performed with *P. aeruginosa* wild type and *gfnR* deletion strains using glycine betaine as the sole carbon and nitrogen source to determine the role of this formaldehyde detoxification system in the catabolism of glycine betaine.

5.2.2 SouR and GbdR as models for examining GATR function

GbdR and SouR are members of the glutamine amidotransferase1-like subfamily of AraC transcription regulators (GATRs), and their sequence homology and phylogenetic distribution suggest common ancestry. Despite nearly 30,000 entries cataloged in Genbank's non-redundant database (29), very little is known about these regulators aside from their structural organization. GATRs exhibit the same two domain architecture as other AraC's, with an N terminal ligand/effector binding domain and a C terminal DNA-binding domain separated by a flexible linker sequence (30, 31). However, their ligand binding domain is distinct from canonical AraC-family regulators in that they share close structural homology with type-1 glutamine amidotransferase (GATase1) family of enzymes (32). These domains are commonly found in enzymes involved in the metabolism of nitrogen, and similarly, all characterized GATase1 containing AraC's respond to amine containing compounds. The widespread distribution of GATRs attests to their importance and necessitates a closer examination of their mechanism of action.

The close phylogenetic similarities between SouR and GbdR and their partially overlapping regulons provide us with a powerful system to understand the molecular basis of the ligand selectivity and promoter specifies for the GATR subfamily.

5.2.2.1 Determining the DNA sequence requirements specifying SouR vs GbdR recruitment to the *glyA1* promoter

The SouR and GbdR regulons overlap at three promoters. However, GbdR also regulates the expression of at least eight other operons that are not recognized by SouR (18, 19). The DNA sequences within these shared promoters that dictate SouR and GbdR binding specificity are largely unknown, and should be examined in greater detail. We have devised a genetic screen to identify the DNA binding requirements within the *glyA1* promoter specifying SouR vs. GbdR-mediated recruitment **(Figure 5.2).** Although this screen is ongoing, preliminary results suggest that C102T eliminates SouR-dependent, but not GbdR-dependent expression from P_{glyA1}.

Future research efforts should confirm this phenotype by engineering this point mutation (C102T) into the *glyA1* promoter of the sarcosine oxidase transcriptional reporter strain (GGW009*)* via site directed mutagenesis and allelic exchange. A gain-of-function approach should also be considered, where a GbdR-regulon specific promoter such as P_{gbcA} can be randomly mutagenized, inserted into a promoterless *lacZYA* reporter vector, transformed into PA14 *ΔgbdR ΔgbcAB* and then blue-white screened for sarcosine-specific induction (mediated by SouR).

5.2.2.2 Uncovering structural determinants of SouR inducing ligand specificity

Our investigations into the inducing ligand specificity of SouR revealed that dimetheylglycine and glycine failed to stimulate SouR-mediated transcriptional activity, while the synthetic molecule, ethylglycine, could induce with ten-fold lower activity than sarcosine **(Figure 2.2).** These findings suggest that the secondary amine moiety of sarcosine is involved in ligand recognition by SouR. However, it is still not known how GATRs sense and bind their inducing ligand.

A crystal structure has been solved of the GATase1-like domain of the SouR orthologue in *Pseudomonas putida*, 3GRA (33, 34). The GATase1-like domain has been proposed to be the inducing ligand-binding region of GATRs, and 3GRA therefore represents the only GATR crystal structure with a known inducing ligand. Recrystallizing this domain in the presence of sarcosine and ethylglycine should be performed to identify the GATR ligand binding pocket, and potentially uncover ligand-induced structural changes associated with GATR dimerization and transcriptional activation.

5.2.2.3 Examining the GATR activation mechanism

The AraC activation mechanism is highly conserved, where monomers undergo structural rearrangement within their N-terminal arm upon ligand binding that allow for dimerization and an increased affinity for DNA binding. Although several variations exist, including the "light switch" mechanism of AraC, the binding of ligand is associated with structural rearrangements that facilitate dimerization and DNA binding that stimulate transcription through recruiting RNA polymerase (35).

We devised a gain-of-function genetic screen to identify residues in SouR that confer constitutively active and sarcosine hyper-responsive phenotypes to examine the activation mechanism of GATRs **(Figure 5.3)**. Using this approach, we identified five mutations within *souR* that resulted in sarcosine hyper-responsive and constitutively active phenotypes **(Figure 5.4A)**. Threading these residue substitutions onto the N-terminal domain crystal structure of the *P. putida* SouR ortholog (3GRA) revealed that these mutations all mapped within the predicted dimerization interface **(Figure 5.4B)** (33, 36, 37)**.** Interestingly, these residue substitutions are believed to disrupt dimerization, suggesting that the GATR activation mechanism is distinct from canonical AraC-family regulators, as dimerization, at least via this interface, appears to inhibit activation.

Data from this screen was recently used by our group to generate constitutively active GATRs in *P. aeruginosa* and *Burkholderia thailandensis* (38), demonstrating that dimerization-mediated inhibition is likely conserved among these regulators. Despite this progress, the GATR activation mechanism remains poorly understood. This screen should be repeated with a new pool of *souR* mutants to ensure that mutational saturation of *souR* is reached. Further insight into the structural rearrangements associated with GATR activation could also be collected through chymotrypsin protection experiments with MBP-tagged SouR and GbdR (39). To accomplish this, each regulator would be incubated in the presence and absence of their respective inducing ligands and DNA fragments spanning the *glyA1* promoter. These samples would then be briefly treated with chymotrypsin, and then electrophoresed on native and denaturing polyacrylamide gels. Evidence for conformational changes associated with ligand and DNA binding could then

be identified through comparing the differential migration patterns of protein fragments between conditions and peptide fingerprinting.

5.2.3 Methods

5.2.3.1 Genetic screen to identify bases within the *glyA1* promoter conferring binding specificity to SouR and GbdR

The *glyA1* promoter region of *P. aeruginosa* PA14 was mutagenized through error-prone PCR using Agilent Technologies GeneMorph II Random Mutagenesis Kit and the PglyA1F-500 and P-glyA1-R1-KpnI primer set. Mutated promoter fragments were purified via Thermo's GeneJet kit and ligated into the *lacZYA* reporter vector, pMW5, and transformed into DH5α *E. coli*. The resulting transformants were scraped, pooled, and plasmid DNA was then harvested using Qiagen's Miniprep kit. The pooled mutant plasmids were then transformed into PA14 *ΔgbcAB* via electroporation and plated on *Pseudomonas* Isolation Agar (PIA) supplemented with 50 μg/ml of gentamicin.

The resulting *P. aeruginosa* colonies that arose were scraped, pooled, and dilutions were then plated on MOPS agar supplemented with 25 mM pyruvate, 20 μg/ml gentamicin, 140 μg/mL X-gal and either 1mM sarcosine, 1mM glycine betaine, or no added inducer as a control. Colonies that appeared as nonresponsive on glycine betaine or sarcosine plates were then picked and grown with shaking overnight in MOPS with 20 mM pyruvate, 5 mM glucose, and 10 μg/mL gentamicin at 37 ºC. These cultures were then plated in triplicate on all three of the MOPS condition plates by dipping a pipette tip into the overnight growth and then slightly penetrating the surface of the agar with the

tip. Plasmid DNA was then harvested from strains that appeared to respond to one inducer but not the other, and re-transformed via electroporation into PA14 *ΔgbcAB* to confirm that the observed differences in regulation were plasmid-based. The *glyA1* promoter regions were then sequenced using the PglyA1F-500 and P-glyA1-R1-KpnI primer set.

5.2.3.2 Genetic screen to identify constitutive and sarcosine hyper-responsive SouR mutants

A plasmid carrying *souR* under the control of the gene's native promoter (pGW006)(18) was mutagenized through passage in XL-1 Red *E. coli* cells using the manufacturer's recommendations. Mutated plasmid was then harvested using Qiagen's MiniPrep kit, and transformed through electroporation into the GGW038 (PA14 Δ*gbdR ΔsouR* $P_{glyA1::lacZYA}$) transcriptional reporter strain (18). Transformants were selected for on Pseudomonas Isolation Agar (PIA) supplemented with 50 μg/ml of gentamicin and 140 μg/ml X-gal. *P. aeruginosa* colonies exhibiting β-galactosidase activity under these conditions were then picked and subjected to a secondary screen to characterize their gain-of-function phenotype via growth on MOPS agar supplemented with 25 mM pyruvate, 20 μg/mL gentamicin, 140 μg/mL X-gal in the presence and absence of 1mM sarcosine.

Plasmid DNA was then harvested from mutants exhibiting constitutive or sarcosine hyper-responsive phenotypes, and sequenced with the PA14_9770 Rescue Construct F & R primer set. Residue substitutions that were identified were then mapped

to a Phyre2-threaded *Pseudomonas putida* SouR ortholog, 3GRA (57 % identity) (33, 36). The corresponding mutations were then modeled and visualized using Chimera software package (37).

5.3 Chapter 3 Summary

The interactions that occur between *Klebsiella pneumoniae* and the host lung are poorly understood. Pulmonary surfactant serves as an initial point of contact for inhaled bacteria entering the lung and is thought to contain molecular cues that facilitate colonization and pathogenesis. In chapter three we characterized the transcriptional response of *K. pneumoniae* MGH 78578 to purified pulmonary surfactant to examine this important ecological transition. These efforts revealed numerous alterations within the *K. pneumoniae* transcriptome that are associated with host colonization, adaptation, and virulence *in vivo*. Notable transcripts expressed under these conditions include genes involved in capsule synthesis, LPS modification, antibiotic resistance, biofilm formation, and metabolism.

We also examined the contributions of surfactant-induced transcripts to *K. pneumoniae* survival using engineered isogenic KPPR1 deletion strains in a murine model of acute pneumonia. Through these infection studies, we identified the MdtJI polyamine efflux pump and ProU glycine betaine ABC transporter as significant mediators of *K. pneumoniae* survival within the lung, and also confirmed the importance of endogenous branched chain amino acid biosynthesis to bacterial survival during infection. Finally, we determined that pulmonary surfactant promotes type 3 fimbriae-

mediated biofilm formation in *K. pneumoniae* and identified two surfactant constituents that drive this response (phosphatidylcholine and cholesterol). Collectively, this study provides novel insight into the interactions occurring between *K. pneumoniae* and the host at an important infection site and demonstrates the utility of purified lung surfactant preparations for dissecting host-lung pathogen interactions *in vitro*.

5.3.1 Purified pulmonary surfactant as a tool for examining host-lung pathogen interactions

Our work with *K. pneumoniae* described in chapter 3 together with our previous studies in *P. aeruginosa* provide compelling evidence supporting the efficacy of Survanta for examining host-lung pathogen interactions (16, 40). However, since concentrated human pulmonary surfactant is not readily obtainable for such studies, it is uncertain how closely Survanta represents this critical aspect of the host lung environment. Nevertheless, porcine lungs share many important physiological features with human lungs including similar respiration rates, which is reflected in the phospholipid composition of their pulmonary surfactant. In order to validate the use of purified surfactant preparations in future transcriptional profiling studies, the transcriptional responses of *K. pneumoniae* and *P. aeruginosa* to porcine lung surfactant should be measured via microarray and compared against their previously determined responses to Survanta. Several protocols have been devised to purify pulmonary surfactant from porcine and other animal sources that would facilitate this process (41-43).

Survanta (Beractant) is a bovine pulmonary surfactant preparation that is standardized with added triglycerides and other fatty acids (44), which could influence bacterial gene expression in ways that are not biologically relevant. Several other pulmonary surfactant preparations are commercially available including Curosurf & Alveofact that differ from Survanta in their phospholipid composition, protein content, animal source, and methods used for purification (44, 45). The transcriptional responses of *K. pneumoniae* to these alternative surfactant preparations should be measured through quantitative RT-PCR and then compared with the Survanta and porcine surfactant microarray data. These efforts would identify the commercial surfactant preparation best fit for future host-lung pathogen interaction studies.

In chapter 3, we also identified surfactant-induced transcripts in *K. pneumoniae* that influence virulence and bacterial fitness in the mouse lung. Despite these findings, it is uncertain if surfactant-induced transcripts of *K. pneumoniae* are expressed during lung infection. To address this, quantitative RT-PCR should be performed on select genes of interest using RNA isolated from *K. pneumoniae* cells collected from the lungs of mice at different time points following oropharyngeal aspiration.

5.3.2 Identifying the diguanylate cyclase(s) influencing type 3 fimbriae expression in pulmonary surfactant

Transcription of type 3 fimbriae (Mrk fimbriae) is dependent on the intracellular accumulation of the secondary messenger, cyclic-di-GMP that is controlled by multiple integrated regulatory networks in *K. pneumoniae*. (46-52). Research efforts by multiple

groups have determined that the cyclic-di-GMP responsive LuxR-family transcription regulator, MrkI, directly interacts with the PilZ-domain containing MrkH to stimulate type 3 fimbriae expression, and that the MrkJ phosphodiesterase degrades cyclic-di-GMP to repress expression from the *mrk* promoter (46, 49, 51, 52). Surprisingly however, the environmental signals and diguanylate cyclases acting upstream of MrkH/I and MrkJ to drive type 3 fimbriae expression have remained largely unknown. As described in chapter 3, we revealed that two components of pulmonary surfactant, phosphatidylcholine and cholesterol, stimulate type 3 fimbriae transcription and biofilm formation *in vitro* **(Figures 3.7 and 3.8)**. These findings are a significant contribution towards our understanding of the signals within the host environment that influence virulence-associated phenotypes during infection. Future research efforts should focus on identifying the diguanylate cyclases that stimulate type 3 fimbriae transcription in response to these newly identified inducing signals.

Several experimental approaches could be used to identify the genes encoding these diguanylate cyclases. Microarrays could be performed using RNA collected from *K. pneumoniae* cells that were grown in MOPS lactate minimal media as control, or the same media independently supplemented with either 1 mM cholesterol or 1 mM phosphatidylcholine. Diguanylate cyclases expressed under these conditions could then be studied in further detail using engineered gene deletion strains. Alternatively, a high throughput genetic screen could be employed, where 96 well plates of *K. pneumoniae* KPPR1 transposon mutants can be examined for their ability to generate biofilm during growth in the presence of phosphatidylcholine and cholesterol through crystal violet staining. A brute-force approach could also be taken, where the fifteen conserved

diguanylate cyclases encoded within the core *K. pneumoniae* genome (53) could be individually deleted from the KPPR1 chromosome through allelic exchange, and then screened for their capacity to generate biofilm mass in MOPS lactate media supplemented with either phosphatidylcholine or cholesterol.

5.3.3 Determine how *mdtJI* expression promotes *K. pneumoniae* fitness during acute pneumonia

Our mouse infection experiments revealed that deletion of *mdtJI,* encoding a polyamine efflux pump resulted in more than a ten-fold decrease in bacterial lung burden relative to the *K. pneumoniae* KPPR1 WT strain **(Figure 3.4)**. These findings represent the first evidence that polyamines contribute to *K. pneumoniae* virulence. Nevertheless, it remains to be determined how secreted polyamines influence *K. pneumoniae* fitness during infection.

Numerous studies have demonstrated that pathogen-secreted polyamines can disrupt the host immune response during infection. Bacterial-derived putrescine and spermidine are abundant within the gingival fluid of inflamed periodontal pockets and have been shown to disrupt oral crevicular polymorphonuclear leukocyte (PMN) function through impairing chemotaxis, stimulating degranulation/respiratory bursts, and promoting apoptosis in vitro (54-57). While the impact of bacterial-derived polyamines on respiratory tract pathogenesis is less clear, spermidine secretion by the fungal pathogen *Pneumocystis jiroveci* has similarly been demonstrated to promote alveolar macrophage apoptosis in mouse and rat models of pneumonia (58).

Enumeration of infiltrating leukocytes in the broncho-alveolar lavage fluid (BALF) collected from mice infected with the *ΔmdtJI* strain revealed significantly lower PMN and macrophage counts relative to mice infected with the WT strain **(Figure 3.4).** These data suggest that polyamine secretion by *K. pneumoniae* disrupts the host immune response during acute pneumonia. To test this hypothesis, *K. pneumoniae* WT and the *ΔmdtJI* strain could be cultured in MOPS lactate media in the presence and absence of Survanta, and then incubated with cultured macrophages and neutrophils. Flow cytometry and cytokine panels could then be used to compare the innate immune responses resulting from exposure to each *K. pneumoniae* strain/culturing condition. In addition, the same culturing conditions could also be used for *in vitro* phagocytosis experiments to determine if the expression of *mdtJI* influences uptake or survival within these leukocytes.

Alternatively, the expression of *mdtJI* could directly influence *K. pneumoniae* survival against the effects of the innate immune system. Secreted polyamines have been shown to alter the charge, shape, and permeability of outer membrane porins of *E. coli,* resulting in increased resistance against cationic antimicrobial peptides and several classes of antibiotics (59, 60). Similarly, surface-localized spermidine has been reported to promote *P. aeruginosa* resistance against reactive oxygen species mediated killing (61). The ability of MOPS-Survanta treated *K. pneumoniae* WT and *mdtJI* deletion strains to survive hydrogen peroxide challenge could be measured to test this hypothesis.

5.4 Chapter 4 summary

Stenotrophomonas maltophilia is an MDR respiratory pathogen of environmental origin that has more than tripled in prevalence in patients with cystic fibrosis (CF) over the last decade (6, 7, 62). Despite its prevalence and correlations with poor clinical outcomes (6, 7, 63, 64), our understanding of *S. maltophilia* virulence and the genes required for survival in the lung lags far behind other CF pathogens. The thick, viscous mucus that characterizes the CF lung (sputum) serves as the primary nutrient source for colonizing microbes and has been shown to contain many of the host-derived signals that drive the virulence-associated phenotypes of several CF pathogens (65-68). In chapter four, we characterized the transcriptional responses of three *S. maltophilia* strains during growth in synthetic CF sputum media (SCFM2) to examine how this organism interreacts with the host at the site of infection (69).

These efforts led to the identification of 238 transcripts that were expressed by all three strains that largely reflect nutrient utilization by *S. maltophilia* during CF lung infection. We also compared the SCFM2 transcriptomes of two *S. maltophilia* CF isolates with the SCFM2 transcriptome of the acute infection model strain, *S. maltophilia* K279A. This allowed us to uncover CF isolate-specific signatures in gene expression that are suggestive of adaptation to host lung, including the repression of genes involved in cell motility and surface-adhered biofilm formation, increased expression of oxidative stress-related genes, and the induction of alternative cytochromes associated with growth in low oxygen environments. Many of these transcriptional changes correlated with phenotypes observed *in vitro*, as the CF isolates failed to form surface-adhered biofilms in SCFM2 and were inherently more resistant to oxidative stress than K279A. Collectively, this

work provides novel insight into the interactions occurring between *S. maltophilia* and the CF lung environment and identified potential virulence factors and metabolism-related genes that will be the subject of future research efforts.

5.4.1 Identifying genes required for *S. maltophilia* fitness during growth in SCFM2

By characterizing the transcriptomes of multiple *S. maltophilia* strains during growth in SCFM2, we generated some of the first data illustrating how this organism interacts with and utilizes the host lung environment during infection. Nevertheless, it remains to be determined which genes in *S. maltophilia* promote fitness and growth within this important infection niche. Turner and colleagues recently utilized a high-throughput Tn-Seq based growth competition to uncover genes within two model *P. aeruginosa* strains that were essential for growth in SCFM2 (69, 70). This work represents an important contribution to the field, and ultimately concluded that genes conferring fitness during growth in SCFM2 were largely associated with anabolic processes and restricted to the core *P. aeruginosa* genome.

Research efforts in our lab are currently focused on adapting this experimental approach to *S. maltophilia*. We have successfully generated pooled transposon mutant libraries of *S. maltophilia* K279A, GW273, and GW275 **(Figure 5.5.)**, and performed growth competitions with each in SCFM2 using similar methodology as described by Turner *et. al* (69). However, we selected for *S. maltophilia* transposon mutants using MOPS minimal media rather than Luria Broth (LB) to exclude amino acid and cofactor auxotrophs from our pooled transposon mutant libraries that we believe could mask less

dramatic, but more pathogenesis-relevant defects in catabolism-related genes in our SCFM2 growth competition experiments. Future work will focus on sequencing the input and outgrowth populations from these competition experiments to identify genes associated with *S. maltophilia* fitness in acute and CF-adapted strains during growth in synthetic CF sputum media.

5.4.2 Examining the role nitrate reduction to *S. maltophilia* fitness during growth in SCFM2 under low oxygen conditions

Mucus within airways of the CF lung contain hypoxic microenvironments that result from continuous reactive oxygen and nitrogen species production by polymorphonuclear leukocytes (PMNs) (71, 72). Interestingly, the ability of *P. aeruginosa* and several other CF pathogens to survive within the CF lung has been suggested to result from their capacity to grow anaerobically under these conditions (73). Chronic respiratory bursts mediated by PMNs in the CF lung generate nitrate as a biproduct, which is found in CF sputum at physiologically sufficient levels (~350 µM) to support significant anaerobic growth in *P. aeruginosa* (74, 75). Moreover, the membrane-bound nitrate reductase has been demonstrated to be required for anaerobic growth by *P. aeruginosa* in synthetic CF sputum (76), and antibodies against this enzyme have been detected in sera collected from CF patients, indicating that nitrate is likely utilized for respiration during infection (77).

Interestingly, although *S. maltophilia* is an obligate aerobe (62), our SCFM2 transcriptomics data suggest that this organism actively expresses nitrate reductase under

these conditions **(Table 4.1).** Because *S. maltophilia* is incapable of fully respiring nitrate as a terminal electron accepter in the electron transport chain, we suspect that the use of nitrate as an electron sink permits *S. maltophilia* growth and survival within the microaerophilic regions of the CF lung by facilitating the regeneration of NAD^+ and $NADP^+$ (78). These findings could have important clinical implications for treating *S. maltophilia* CF infections, as oxygen levels influence many physiological features in pathogenic bacteria, including susceptibility to antibiotics (79-81).

Ongoing research efforts in the Wargo lab are focused on testing this hypothesis. A nitrate reductase gene deletion strain has been engineered into the K279A genetic background that will be used to compare growth kinetics relative to the WT strain in SCFM2 under atmospheric and oxygen limiting conditions. In addition, the susceptibility of these strains to clinically relevant antibiotics will also be investigated during growth under these conditions.

5.4.3 Are *S. maltophilia* CF-isolate specific genes expressed during growth in SCFM2 reflective of adaption to the CF lung environment?

In chapter 4, we compared the gene expression profiles of two *S. maltophilia* CF isolates with *S. maltophilia* K279A to identify transcripts associated with adaptation to the host lung environment. These efforts revealed numerous CF isolate-specific signatures in gene expression that are suggestive of adaptation to the CF lung, including the expression of oxidative stress resistance genes and cytochromes associated with growth in low oxygen environments, as well as the repression of genes involved in cell

motility and biofilm formation **(Table 4.2)**. We were further able to demonstrate that many of these transcriptional changes correlated with phenotypes observed *in vitro,* as the CF isolates failed to form surface-adhered biofilms in SCFM2 and were inherently more resistant to oxidative stress than K279A **(Figures 4.3 & 4.4)**.

Although our observations with *S. maltophilia* CF isolates mirror similar findings in CF-adapted *P. aeruginosa,* our conclusions are drawn from an extremely limited sample size. Quantitative RT-PCR should be used to address this concern by examining the expression of these genes in additional *S. maltophilia* CF, acute infection, and environmental isolates during growth in SCFM2. Similarly, the capacity of these additional strains to survive oxidative stress and generate biofilms during growth in SCFM2 could also be investigated using the methodology described in chapter 4.

5.4.4 Identifying genes within *S. maltophilia* associated with mucin degradation

The capacity to degrade mucin is not conserved among CF pathogens, as *P. aeruginosa* cannot metabolize this glycoprotein *in vitro* without the aid of other microbes (82). Presumably, the inability of *P. aeruginosa* to metabolize mucin is due to the lack of a sufficient extracellular protease or glycan degrading activity by this organism. *S. maltophilia* has previously been shown to be able to utilize mucin as a nutrient source (82), which could contribute to this organism's ability to colonize the CF lung. Therefore, identifying and characterizing the genes involved in mucin metabolism is warranted.

Three T5SS autotransporters with predicted protease effector domains *(Smlt1001, Smlt1350, Smlt4145),* and an uncharacterized secreted protease (*Smlt0603*) were

expressed by all three *S. maltophilia* strains during growth in SCFM2. Since mucin is the only protein found within this media (69), examining the potential contribution of these proteases should be tested first. To accomplish this, gene deletion strains could be engineered into *S. maltophilia* K279A through allelic exchange, and then studied for their ability to grow in MOPS minimal media supplemented with mucin as the sole carbon and nitrogen source. A similar high-throughput approach could also be taken using the ordered *S. maltophilia* K279A transposon mutant library that we recently created. Alternatively, microarrays could be used to identify genes associated with mucin degradation.

5.4.5 Examining biofilm formation by *S. maltophilia* CF and acute infection isolates

In chapter 4 we showed that the *S. maltophilia* acute infection strain, K279A, formed a robust, surface-attached biofilm during growth in SCFM2, while neither *S. maltophilia* CF isolate generated robust biofilms under these conditions **(Figure 4.2).** Several previous studies have indicated that flagella and type IV pili influence biofilm formation in *S. maltophilia* through facilitating substrate adhesion and providing stability (83-85). Our SCFM2 transcriptomics data support the roles of these structures in sputum-mediated biofilm production, as *S. maltophilia* K279A expressed significantly more transcripts related to the assembly of these structures compared to the CF isolates **(Figure 4.2)**. Nevertheless, the core genome of *S. maltophilia* encodes multiple cellular adhesins, including the temperature sensitive Smf-1 fimbriae and various predicted adhesins (62, 86, 87). Therefore, the contributions of flagella and type IV fimbriae to SCFM2-mediated

biofilm formation should be confirmed using crystal violet staining assays with gene deletion strains engineered into *S. maltophilia* K279A background. In addition, GW273 and GW275 (CF isolates) should be subjected to flagellar staining and tested on swimming and twitching SCFM2 agar plates to determine if these strains generate functional flagella and type IV pili. Finally, additional *S. maltophilia* CF and non-CF isolates should be examined for their capacity to generate biofilms during growth in SCFM2 to examine the conservation of these phenotypes.

Although both CF isolates failed to generate robust surface-attached biofilms during growth in SCFM2, the ability of these strains to generate suspended cell aggregates (type 2 biofilms) was not investigated. A growing body of evidence suggests that *P. aeruginosa* and other CF pathogens do not adhere to alveolar epithelial cells during chronic infection, but often grow in visible cell aggregates (macrocolonies) adhered to mucus within the intraluminal spaces of the lungs (88-91). In the future, SCFM2 cultures of *S. maltophilia* K279A and each CF isolate should be examined for type 2 biofilm production through fluorescence in situ hybridization (FISH) or confocal laser scanning microscopy with strains harboring a plasmid constitutively expressing mCherry (pGW72).

5.4.6 Methods

5.4.6.1 Bacterial strains and growth conditions

Stenotrophomonas maltophilia K279A (ATCC BAA-2423), AU30115 (GW273) & (GW275) were maintained in LB (Luria Broth), unless otherwise noted. *Escherichia*

coli SM10 harboring plasmid pBT20 (MJW500) was maintained in LB supplemented with 10 µg/mL of gentamicin(18). Synthetic CF sputum media 2 (SCFM2) was freshly prepared before each transposon mutant competition assays as previously described(69). A control minimal media was formulated to support the growth of all three *S. maltophilia* strains comprising a 3:1 mixture of M63 and MOPS minimal medias supplemented with 50 mM sodium pyruvate, 10 mM glucose, 10 mM glutamate, and 10 µg/ml of gentamicin.

5.4.6.2 Transposon mutant library construction

S. maltophilia K279A, GW273, and GW275 were transposon mutagenized through conjugation-mediated mating with an *Escherichia coli* SM10 strain carrying a mobilizable Mariner-based transposable element on plasmid pBT20 (MJW500) as we have done previously (18). Briefly, cultures of *S. maltophilia* K279A, GW273, and GW275 were prepared in LB, and MJW500 was confluently plated on LB agar supplemented with 10 µg/mL of gentamicin the night prior. The following day, dilutions of the *S. maltophilia* overnight cultures were inoculated into 50 ml of LB and grown to mid-log phase. Cells were then collected through centrifugation and resuspended in 500 µl of LB at an OD_{600} of 40 units. MJW500, harboring plasmid pBT20, was scraped from the LB gentamicin plates, and gently resuspended in LB to obtain and OD_{600} of 20 units. The OD-adjusted *S. maltophilia* strains were then gently mixed 1:1 with MJW500, and the mating mixtures were spotted on LB agar in 50 µl replicates. After an hour incubation at 30 ˚C, ten mating spots from each strain were scraped and resuspended in 10 ml LB,

mixed 2:1 with 50% filter sterilized glycerol, and frozen in 1 ml aliquots at 80 ˚C until needed.

To select for *S. maltophilia* transposon integrants, glycerol stocks from each mating were thawed, mixed with 9 ml of MOPS media, and spread on 100 MOPS agar plates supplemented with 100 mM sodium pyruvate, 5 mM D-glucose, 1 mM methionine, and 100 μg/ml of gentamicin. After 48 h of growth at 37 ˚C, between 100,000 and 125,000 unique transposon mutants from each *S. maltophilia* strain were scraped and resuspended in 60 ml of MOPS media at an OD_{600} of 2.0 units. Forty milliliters of filter-sterilized 50 % glycerol was then added to each OD-adjusted culture, mixed via vortexer, aliquoted into 1.5 ml freezer tubes, and then frozen at -80 ˚C.

Transposon integration into *S. maltophilia* K279A, GW 273, and GW 275 was confirmed through PCR. Briefly, genomic DNA was isolated through CTAB extraction from eight randomly selected colonies per strain that arose following gentamicin selection. Genomic DNA was also extracted from the parental *S. maltophilia* strains to serve as controls. PCR was then performed on the purified genomic DNA using Q5 2x MM (NEB) and primers specific to the gentamicin resistant cassette mobilized from plasmid pBT20. Transposon insertion sites were then identified for each sample using a PCR-based strategy (92, 93) to ensure incorporation into the genome occurred randomly. To accomplish this, gDNA was first amplified with a transposon-specific forward primer (Rnd-TnM20) and one of two arbitrary primers (Rnd1-PA-Arb-2 or Rnd1-PA-Arb-3) originally designed for *P. aeruginosa* (93). A second round of amplification was then performed using the Rnd2-TnM20 and Rnd2-Arb-primer primer set, as previously described (92). The amplified products were then purified via Thermo-Fisher's GeneJet kit and sequenced using the

transposon specific primer BT20TnMSeq as previously described (93). Sequencing reads were then mapped to the genome of *S. maltophilia* K279A through NCBI BLAST.

5.4.6.3 Construction of an ordered *S. maltophilia* K279A transposon mutant library

To generate an ordered *S. maltophilia* K279A transposon mutant library, two glycerol stocks from mating with SM10 were thawed, mixed, diluted 1:10 in MOPS media, and then plated on MOPS agar plates supplemented with 100 mM sodium pyruvate, 5 mM D-glucose, 1 mM L-methionine, and 100 μg/ml of gentamicin to select for transposon disruption mutants. After 48 h of growth at 37 ˚C, 15,000 colonies were manually plated on LB agar supplemented with 100 μg/ml of gentamicin via toothpick and regrown overnight at 37 ˚C. The following day, a 43 pin replicator tool was used to transfer the gentamicin-resistant colonies that arose to 96 well plates containing LB supplemented with 100 μg/ml of gentamicin. These plates were then grown overnight shaking at 37 ˚C. The following day filter sterilized glycerol was added to each well to achieve a 20 % final concentration, and the 96 well plates of transposon mutants were then frozen at -80 ˚C.

5.4.6.4 Transposon mutant growth competitions in SCFM2 and M63-MOPS minimal media

Transposon mutant growth competitions were performed as previously described (69). Freezer stocks of *S. maltophilia* K279A, GW273, and GW275 transposon mutants were thawed at room temperature and inoculated into 12 ml of freshly prepared SCFM2 and 12 ml of M63-Mops media at a starting concentration of ~2 E 6 CFU/ml. The cultures were

then grown at 37 ˚C and 170 RPM for roughly nine generations to a cell density of ~2 E 9 CFU/ml, at which point the cells were collected through centrifugation, and frozen at -80 ˚C.

5.5 Chapter 5 figures

Figure 5.1 Transcriptional regulation of choline catabolism in *P. aeruginosa*. Transcription regulators influencing the catabolism of choline sources found within pulmonary surfactant. Each transcription regulator is color-coordinated to match the

choline catabolite(s) that influence their activity. Colored arrows radiating from each regulator signify genes under their control.

Figure 5.2 Schematic of genetic screen to identify DNA sequence determinants of SouR & GbdR binding within P_{glyA1}.

Figure 5.3 Schematic of genetic screen to identify constitutive and sarcosine hyper-responsive SouR mutants.

Figure 5.4 Mutations within the GATase1-like domain of SouR result in constitutive and sarcosine hyper-responsive phenotypes. (A) Residue substitutions that resulted in constitutive and hyper-responsive phenotypes are shown aligned within the primary sequence of SouR. **(B)** Mutations that resulted in gain of function phenotypes were threaded onto 3GRA crystal structure using Phyre2 and then modeled using Chimera. Substitutions depicted in part A are color-matched to their corresponding location within the model.

Figure 5.5 Construction of *S. maltophilia* K279A, GW273, and GW275 transposon mutant libraries. *S. maltophilia* K279A, GW273, and GW275 were transposon mutagenized through conjugation-mediated mating with an *Escherichia coli* SM10 strain carrying a mobilizable Mariner-based transposable element on plasmid pBT20. To select for *S. maltophilia* transposon integrants, each mating mixture was resuspended in MOPS, diluted 1:10, and then plated on 100 MOPS agar plates supplemented with 100 mM

sodium pyruvate, 5 mM D-glucose, 1 mM methionine, and 100 µg/ml of gentamicin. Between 100,000 and 125,000 unique transposon mutants from each *S. maltophilia* strain were then scraped, pooled and resuspended in LB before being mixed with glycerol and frozen at -80 ˚C. In the above panel, (-) depicts the lack of growth of non-mutagenized *S. maltophilia* strains plated on MOPS in the presence of 100 µg/ml of gentamicin, while (+) depicts the growth of transposon mutants from each *S. maltophilia* strain.

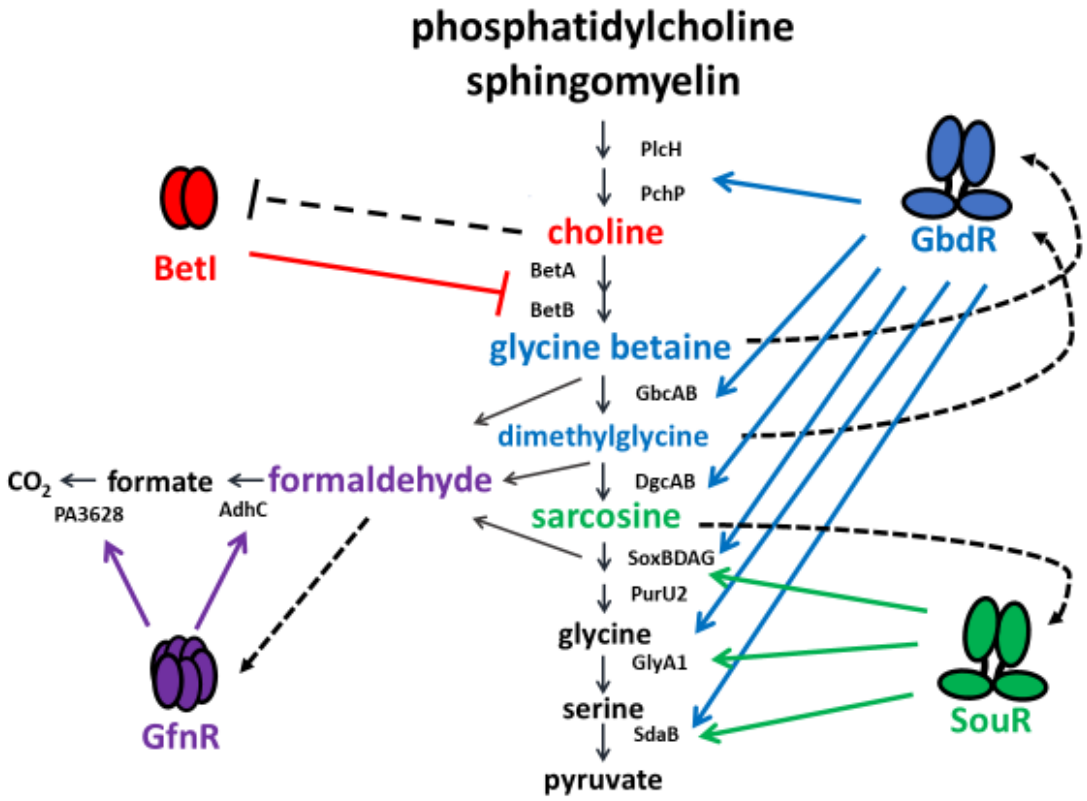


Figure 5.1 Transcriptional regulation of choline catabolism in *P. aeruginosa.*

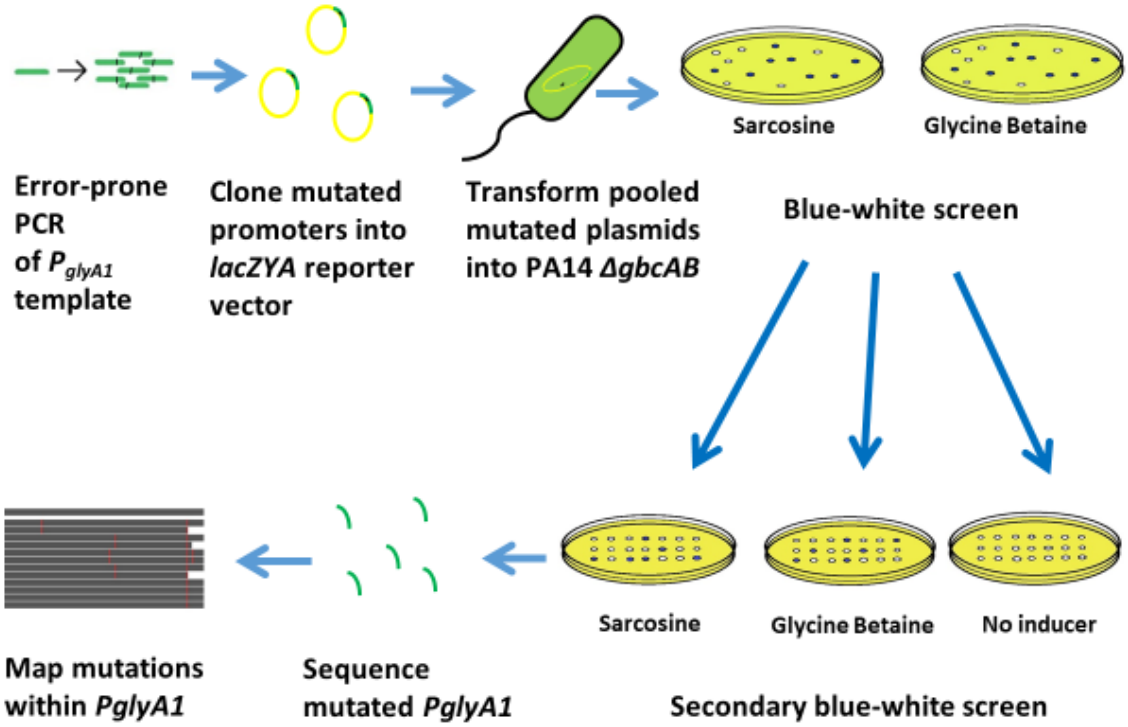


Figure 5.2 Schematic of genetic screen to identify DNA sequence determinants of SouR & GbdR binding within P_{glyA1}.

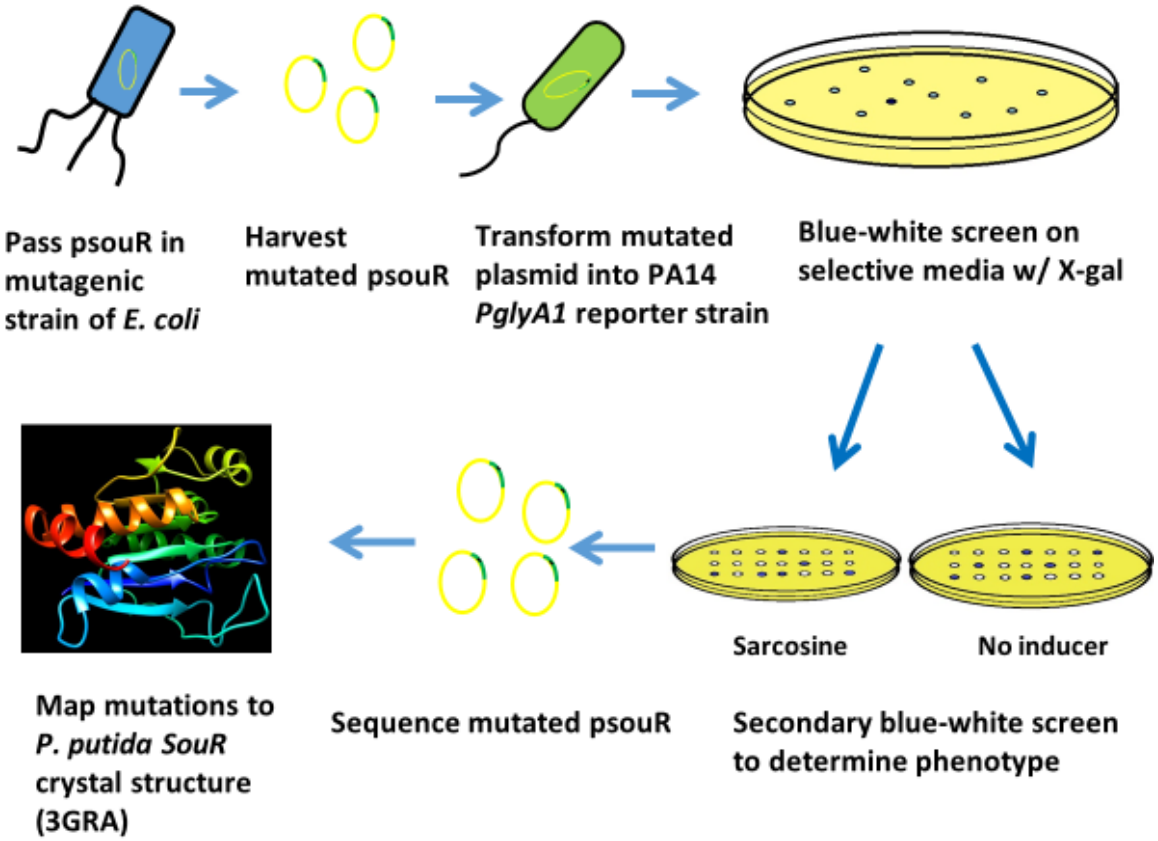
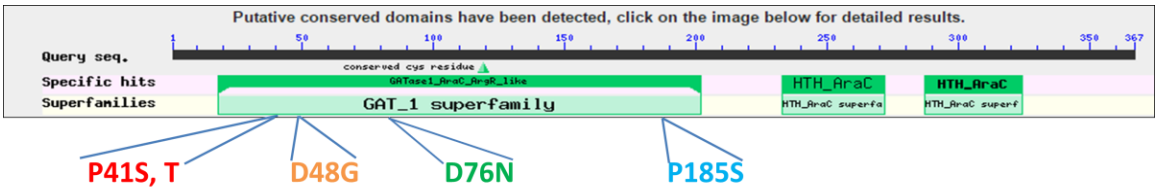


Figure 5.3 Schematic of genetic screen to identify constitutive and sarcosine hyper-responsive SouR mutants.

A.



B.

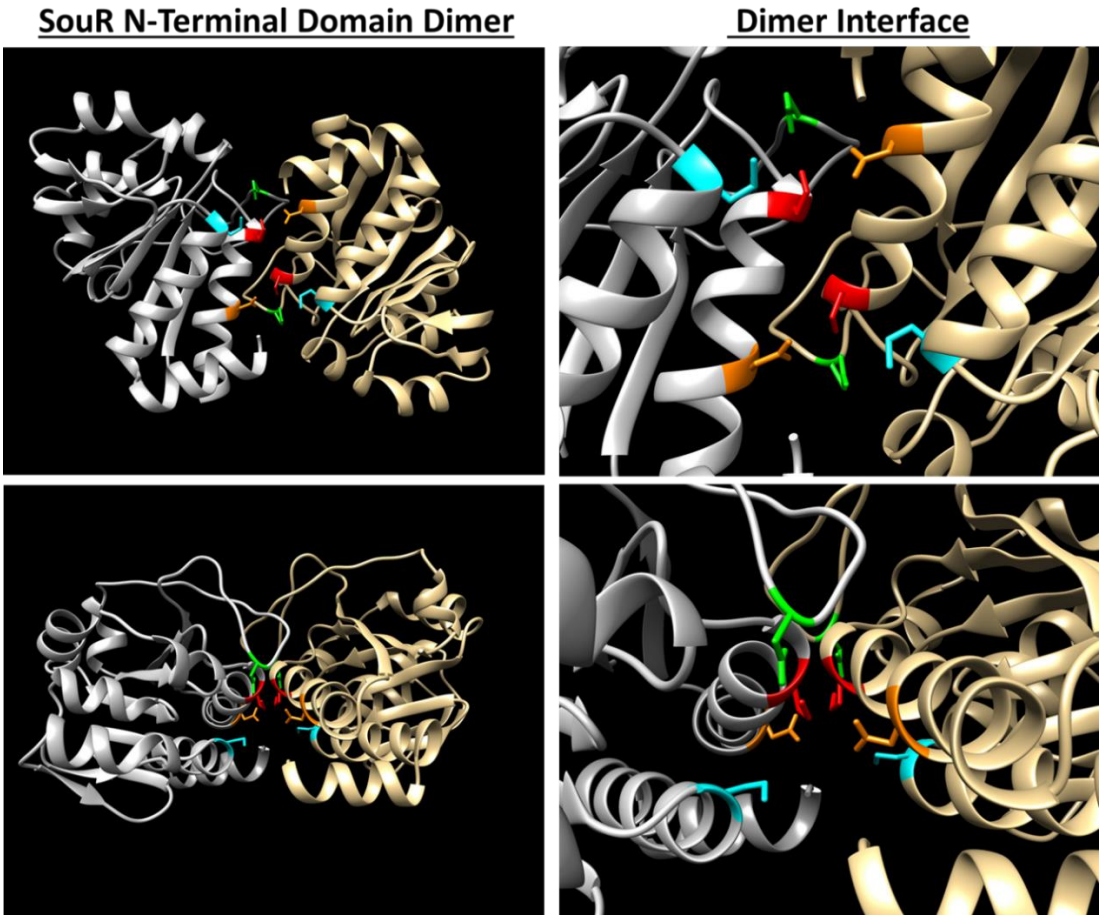


Figure 5.4 Mutations within the GATase1-like domain of SouR result in constitutive and sarcosine hyper-responsive phenotypes**.**

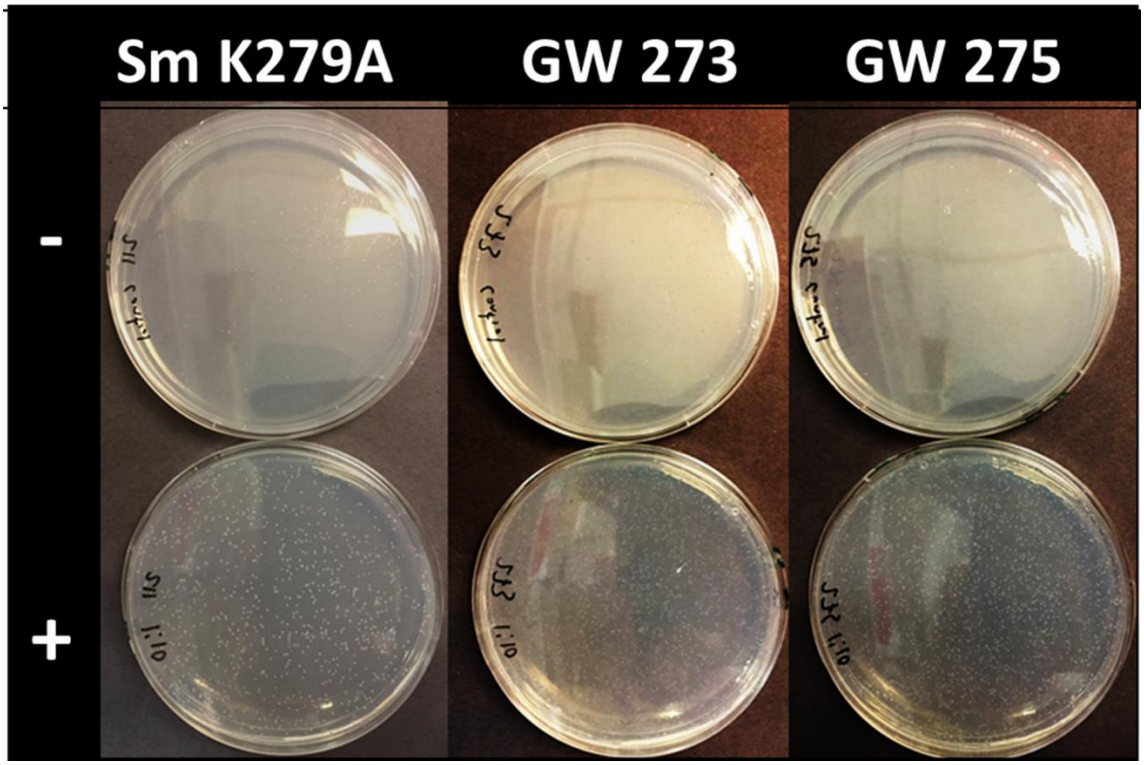


Figure 5.5 Construction of *S. maltophilia* K279A, GW273, and GW275 transposon mutant libraries.

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