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NEXT-GEN TRANSCRIPTOMICS REVEALS VARIABLE GENE EXPRESSION IN
STENOTROPHOMONAS MALTOPHILIA K279A WHEN GROWN IN CO-CULTURE
WITH *PSEUDOMONAS AERUGINOSA* 2192

By

David Jensen
B.S., Brigham Young University Hawaii

A Thesis
Submitted to the Faculty of the
College of Arts and Sciences of the University of Louisville
In Partial Fulfillment of the Requirements
for the Degree of

Master of Science in Biology

Department of Biology
University of Louisville
Louisville, Kentucky

May 2015

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David Jensen
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A Thesis Approved on

April 20, 2015

by the following Thesis Committee:

Dr. Deborah Yoder-Himes, Thesis Director

Dr. Michael Perlin, Thesis Committee Member

Dr. Donald Demuth, Thesis Committee Member

DEDICATION

To my loving parents and grandparents

Craig and Christine Jensen,

and

John and Marian Black.

“If I have seen further it is by standing on ye shoulders of Giants”

ACKNOWLEDGMENTS

I have been fortunate in life to be surrounded by so many inspiring and influential people. I would be remiss if I didn't thank those who have influenced my life for the better.

First I would like to thank my mentor, Dr. Deborah Yoder-Himes, without whom this work would have been nearly impossible. Her expectations and work ethic are only surpassed by her patience and support. She has taught me what it is like to think like a reasoning scientist and inspired me to become a higher functioning individual. For the guidance she has given I will be forever grateful.

I would also like to thank my graduate committee members Dr. Michael Perlin and Dr. Donald Demuth who have provided insight and expertise from their fields to make this research happen.

A thanks also goes to my family who has been so much of an influence on the person I have become. From my father, Craig Jensen's incessant guidance never to settle for something that is less than my best to my mother, Christine's example of selflessness and optimism, I have been fortunate to have their influence in my life. Gratitude is owed to my sisters Sara, Becky, Jessica and Elizabeth who have taught me the importance of being a brother, friend and ultimately a man. To my grandparents John and Marian Black, who have provided kind support and a roof over my head while attending school in Louisville. To my aunt Loraine and uncle Terry, my surrogate mother and father away

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Without my many influences I could not be where I am today.

ABSTRACT

NEXT-GEN TRANSCRIPTOMICS REVEALS VARIABLE GENE EXPRESSION IN *STENOTROPHOMONAS MALTOPHILIA* K279A WHEN GROWN IN CO-CULTURE WITH *PSEUDOMONAS AERUGINOSA* 2192

David Jensen

April 20, 2015

We are constantly surrounded by bacteria, many of which are found in cohabitation with other species in the same niches. These organisms can be particularly problematic when they infect the human body. Cystic fibrosis (CF) is of significant concern because of the frequency that those suffering from this autosomal recessive disease have in colonization by these infectious agents. Affecting more than 30,000 people in the U.S. alone. CF is due to a genetic mutation that causes a thick mucosal buildup in luminal surfaces. This viscous mucus creates a cultivation site for complex respiratory biofilms. Complications of CF include paralysis of respiratory cilia and an inability to absorb nutrients in the digestive tract. The study of these bacteria in mixed communities, which better represents their common modes of infection, is critical to understanding the mechanisms of virulence for these pathogens. Studying poly-microbial biofilm-forming organisms in monoculture has intrinsic inaccuracies. In this work, the phenotypes of opportunistic respiratory pathogens were observed in co-culture growth. *P. aeruginosa* 2192, *Pseudomonas aeruginosa* PAO1, *S. maltophilia* K279a, *Burkholderia*

dolosa AU0158, *B. cenocepacia* K56-2 and *Escherichia coli* pEBFP showed limited growth defects when spotted proximally in pairwise comparisons, but when testing effects of their growth on established bacterial lawns, inhibitions were more pronounced. The transcriptome of *P. aeruginosa* 2192 and *S. maltophilia* K279a were characterized in in mono- or co-cultures in three *in vitro* conditions using high-throughput Next-Gen Sequencing. This resulted in hundreds of significant changes in gene expression which indicate that these two CF pathogens can sense and respond to each other. Of the genes up-regulated in *S. maltophilia* K279a in response to growth with *P. aeruginosa* 2192, seven constructs were made for future complete gene deletion generation. This work provides a model where genes up-regulated in response to mixed communities can be identified and complete gene deletions can be made for future co-culture growth experiments.

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LITERATURE REVIEW

Cystic Fibrosis

Cystic fibrosis (CF) is the most common autosomal genetic disorder in the Caucasian population, (Davis, 1996) and it currently affects approximately about 30,000 people in the U.S. and over 70,000 worldwide (Cystic Fibrosis Foundation Patient Registry: Annual Data Report). CF is caused by a mutation in the CF transmembrane conductance regulator (*CFTR*) gene, which encodes an ATP-driven pump responsible for the flux of chloride ions across the apical membranes of mammalian cells (Sibley, 2006). Although there are over 800 different mutations that can be attributed to CFTR alteration, a single amino acid deletion ($\Delta F508$) is associated with 70% of the cases resulting in CF (Zielinski, 1995; Cebotaru, 2008). This mutation results in the malformation of the CFTR protein which prevents chloride ions from entering the lumen of lungs and digestive tract organs. Without this chloride ion flux, water is not drawn to the lumen, causing a thick mucosal build up in the respiratory tract (Harris, 2007). This viscous accumulation prevents mucociliary clearance that normally aids in keeping the lungs pathogen-free (Matsui, 1998). The CFTR protein also functions in the gastrointestinal tract and holds particular relevance to infants and young children, where absorption of necessary macromolecules is limited due to the mucosal coating on the inside of the intestinal lumen. But because symptoms in the CF lung present themselves as more acute, much of the research is focused on the respiratory system (Bodewes, 2015) which was attributed

to 68% of CF deaths in 2013 (Cystic Fibrosis Foundation Patient Registry: Annual Data Report). The other 32% consist of transplant-related complications, liver disease and suicide. Over time, those who suffer from this disorder will see their lungs deteriorate in function (Winnie, 1991) and may be subject to the effects of cystic fibrosis-related diabetes (CFRD) due to the need for high caloric intakes. Reports of CFRD have increased significantly since 1986 when data collection began. This has been attributed to increased detection methods in younger CF patients along with increased life expectancy, which has allowed more time for the patients to develop CFRD. Those who have CFRD have a lower average body-mass index (BMI) than those who have not acquired CFRD. Tropic effects include retinopathy, kidney disease, neuropathy and microalbuminuria, but are still less prevalent at less than 6% of occurrence (Cystic Fibrosis Foundation Patient Registry: Annual Data Report).

Even with these various complications, the prognosis of CF patients today is much different than it was just 30 years ago. In 1950 the median mortality age of those diagnosed with CF was 2 years old (Cystic Fibrosis Foundation Patient Registry: Annual Data Report, 2013). Since that time the average life expectancy has increased from 27 years in 1986 to 38 years old in 2013. Currently, over half of the CF population in the U.S. is over the age of 18, and that number has been rising continually (Cystic Fibrosis Foundation Patient Registry: Annual Data Report, 2013). With 66% of all patients being diagnosed in the first year of life, treatment and prevention of infection are improving. Detection is also improving; with sequencing advancements, 97% of all CF patients are identified through genetic screenings (Cystic Fibrosis Foundation Patient Registry: Annual Data Report, 2013). These advances follow increased awareness of the disease

and the pathogens that regularly accompany it. Even with increased exposure in mainstream research, scientists are still just starting to understand the many complexities of what it is like to live with CF.

The main causative agent of physical decline in CF patients does not come directly from the genetic mutation itself, but rather the resulting conditions of mucous accumulation in the lungs and the opportunistic bacteria that take advantage of that environment (Lipuma, 2010). Through genetic screenings of the 97% of those who are known to have CF, *Staphylococcus aureus* is the most common bacterial species isolated from younger patients while *Pseudomonas aeruginosa* is responsible for up to 80% of the total biomass in the lungs of adults (Razvi, 2009). This may be because, as life expectancies are increasing, the likelihood for *P. aeruginosa* infection will increase accordingly. Bacterial diversity is also manifested geographically due to variability in treatment practices globally. CF patients in the U.S. were shown to have less species diversity compared to United Kingdom CF patients (Stressmann, 2011). Treatment, diet, access to medical care and other lifestyle factors correlate with contracting a more diverse group of microbial colonizers. This diversity can increase the variables used in assessing treatment which makes effectively combating these pathogens much more difficult. It has also been shown that bacterial infections are not random, but that they are usually contracted in an age-specific fashion (Garber, 2008). *S. aureus* and *Haemophilus influenzae* tend to dominate as infectious agents in younger CF patients; as adults the common colonizers progress to other species like *Burkholderia cepacia*, *Stenotrophomonas maltophilia* and *P. aeruginosa* (Hutchison, 1999). Other bacteria associated with chronic CF infections that do not classify as primary or dominant

pathogens include *Streptococcus pneumoniae*, *Achromobacter xylosoxidans*, and *Klebsiella* species. Fungi isolated from CF patients include *Aspergillus*, *Candida*, *Chysonilia*, *Exophiala*, *Mucor* and *Penicillium* species. However, not only do free-living pathogens and fungi find themselves in those suffering from CF, but viruses like adenoviruses, influenza, picornaviruses, respiratory syncytial virus and parainfluenza viruses also take advantage of this environment (Sibley 2006). Despite the ability for viruses and fungi to proliferate in the CF community, they are not as detrimental to host health in comparison to CF-associated bacteria. When these bacteria infect the CF lung they are often cohabitate with other bacteria. This makes them difficult to study because the effects of one bacterium can easily be misappropriated to another if they have similar phenotypes (Rogers, 2003; Harris, 2007).

New fields of research with their own methods and materials have been created to better understand these mixed communities. Collection methods such as expectorated mucus collection (Sibley, 2008), bronchoalveolar lavage (Harris, 2007) and throat swabs (Gilligan, 1991) have been employed, but as pointed out by multiple researchers, these collections may be misrepresentative of the actual bacterial populations within the lung due to contamination of oropharyngeal-associated microbes during collection (Goddard, 2012; Gilligan, 1991; Thomassen, 1984). With advancements in identification of the oropharyngeal microflora, recommendations to adjust for the confounding strains are suggested. Researchers have used 16S rDNA sequencing techniques to establish phylogenetic diversity of microbes within CF patients which has shown promise, but new methods of collection are needed in order to produce a more representative model of the community ecology of the CF lung (Goddard, 2012).

Many bacteria grow in bacteria-rich, pathogenic powerhouses called biofilms. Biofilms (which will be elaborated upon later in this review) are difficult to study not only because of their physical makeup consisting of multiple bacteria that can communicate and secrete an extracellular matrix, but because many of the microbiological techniques used to examine bacteria in the past rely upon examination of a single species. Therefore, little is known about how bacterial interactions shape the pathogenesis of CF (Peters, 2012). Researchers have sufficient evidence suggesting that major bacterial players like *P. aeruginosa* can alter the growth of other bacteria (Collinson, 1996; Baldan, 2014; Bragonzi, 2012; Eberl, 2004; Korganokar, 2013). Within the past decade, advances in identifying and quantifying these biofilm-forming virulent bacteria have been on the rise (Markle, 2013). Significant advancements in bacterial sequencing are being used to understand more about the composition of these biofilms (Rogers, 2004), and medical treatments are being tailored to treat individuals suffering from this highly variable disease.

The Cystic Fibrosis Foundation Annual Report from 2013 outlines prescribed medications that are recommended for use. Some of these treatments focus on breaking up or decreasing the viscosity of the mucous buildup in the lungs with the introduction of recombinant human DNA (rhDNA) (Hodson, 1995) or inhaled hypertonic saline solutions (Robinson, 1997). However, newer methods for treatment are focusing on the pathogens and are mainly antibiotic; some of these include clarithromycin-tobramycin and azithromycin (Solleti, 2014). Multiple treatments are usually administered due to the range of symptoms that present themselves in CF patients. Unfortunately these antibiotics are becoming less effective every year (Hill, 2005). One such example of rising

resistance to antibiotics in the CF pathogens is *Staphylococcus aureus* , particularly the emergence of a methicillin-resistant strain that is one of the few bacteria that is increasing its occurrence of infection in CF.

S. aureus

The most commonly found isolate in young CF patients is *S. aureus* (Razvi, 2009). *S. aureus*, a Firmicute, are ubiquitous Gram-positive cocci carried by 37.2% of the human population as part of the commensal microflora (Kluytmans, 1997). As of 2013 it infected 56% of CF patients under the age of 2 years old, and by the age of 17, almost 80% of the CF population were carriers at one time (Cystic Fibrosis Foundation Patient Registry: Annual Data Report, 2013). Even though found in about half as many cases (23% compared to 51%) as methicillin-sensitive *S. aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA) has seen an increase among CF patients in their pre-teen years where loss of proper lung function (Goss, 2011), measured by forced expiratory volume (FEV₁), is the result of this particularly harmful infection (Dasenbrook, 2008). MRSA is also garnering increased attention because of its ability to acquire resistance to antibiotics other than methicillin (Gross, 2011). With the knowledge that this bacterium has been associated with much of the adolescent mortality, the scientific community has widely adopted the use of anti-staphylococcal drugs as methods of treatment. Although, there has been a decrease of MSSA due to these agents, there is concern that it allows colonization of other harmful airway infections to occur, specifically *P. aeruginosa* (Goss, 2011), the most prevalent bacteria in adult CF patients. Despite the decrease in MSSA, MRSA is of continued concern because of difficulties in administering treatment.

What makes *S. aureus* an organism of concern to researchers is its ability to not only opportunistically infect, but its ability to adapt to its environment. In most *S. aureus* nosocomial infections, the minority of transmission happens from patient-to-patient, so it is accepted that most of those infected with *S. aureus* obtain the infection from other non-human vectors (Price, 2014). *In vitro* and murine CF models both confirmed that in the early infection stages *S. aureus* are susceptible to bacterial competition, but during chronic infections by other organisms *S. aureus* has increased fitness and is better adapted to outcompete other colonizing bacteria (Baldan, 2014). This is attributed to the ability that it has to retain pathogenicity after generations of growth within the lung. However, *S. aureus* is not an obligate respiratory pathogen. It can be found in boils, impetigo, septic arthritis, osteomyelitis, endocarditis and meningitis to name a few (Gordon, 2008). Over time mammals have acquired specific defensins for such widespread pathogens, one of which is called type-II-secreted phospholipase A2 (sPLA2-IIA) (Pernet, 2014). This hydrolase has little effect on Gram-negative bacteria; it possesses significant bactericidal effects on Gram-positive bacteria and is the chief defensive molecule against Staphylococcal infections found in intestinal secretions, human tears and leukocytes (Qu, 1998).

S. aureus has been of interest to researchers since its discovery during a Scottish knee surgery in 1880 (Ogston, 1984). Over a century later, researchers are still exploring characterization methods that will give them insight into what makes *S. aureus* a dangerous nosocomial infection. One of the most comprehensive tools that researchers have to identify the genetic makeup of *S. aureus* is genetic sequencing. Currently there are 56 published complete genome sequences of *S. aureus* with hundreds more in the

draft phase (Genomes Online Database, <http://gold.jgi-psf.org>). Sequencing of the *S. aureus* genome has proven useful in modern research because it enables researchers the ability to examine gene expression in monocultures and it has also given insight into polymicrobial interactions and genes that play key roles in virulence (Zhu, 2009; Baba, 2008).

The virulence of *S. aureus* is directly connected with how it attaches to surfaces, invades host cells and causes harm using exotoxins. First, *S. aureus* uses adhesins to attach to host cells. The major class of these attachment proteins is anchored in the peptidoglycan of *S. aureus*. Attaching to extracellular matrix components of host cells, these are generally termed as microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) (Speziale, 2009). This primary attachment allows attachment to human fibrinogen, collagen and fibronectin (Flock, 1987). Characterized gene products of these MSCRAMMs are staphylococcal protein A (SpA), clumping factor (Clf), and fibronectin-binding protein A and B (FnbpA and FnbpB) (Lowy, 1998). Once the pathogen has attached to a host cell, it produces exoproteins, which are the main components of pathogenicity in *S. aureus* infections. Some of these exoproteins are nucleases, lipases, proteases, collagenase, hyaluronidase α - and β -hemolysins, leukocidin and Panton-Valentine leukocidin (PVL) (Kaneko, 2004; Dinges, 2000). These exoproteins can produce β -barrels in host plasma membranes and cause cellular leakage (Foster, 2005). Another common result of *S. aureus* infection is toxic shock syndrome (TSS). The exotoxins associated with this infection are termed pyrogenic toxin superantigens (PTSAs). Superantigenicity comes from the ability for this class of toxins to stimulate the extreme over-production of T-lymphocytes (Holtfreter, 2005). Evasion

of host defenses can come from staphylococcal complement inhibitor (SCIN) which prevents the formation of the complement protein C3b on the invader's surface and prevents opsonization and phagocytosis (Roosjakkers, 2005). Overall, *S. aureus* is considered a dangerous pathogen because of its multifactorial virulence factors. These factors make it difficult to prevent *S. aureus* attachment, obtaining nutrients by catalysis of host cells and evasion of host immune responses.

Pseudomonas aeruginosa

Shortly after the identification of *S. aureus*, *P. aeruginosa* was discovered in France and named after its blue-green coloration left on bandages (Wilson, 1998). In the year 2000, the first whole genome sequence of this organism was completed. Twenty six additional strains have been or are currently being sequenced since that time (Genomes Online Database, <http://gold.jgi-psf.org>). *P. aeruginosa* is aerobic and belongs to the 12-member family Pseudomonadaceae of the Gammaproteobacteria. This bacillus accounts for 10-15% of nosocomial infections, which are notoriously difficult to treat because of their natural antibiotic resistance and the ability to acquire gene encoding antimicrobial resistance (Blanc, 1998; Pechere, 1999). This acquisition is particularly worrisome for healthcare providers because of its ability to acquire multiple types of resistance simultaneously (McGowan, 2006), which makes treatment much more difficult to optimize. For the immunosuppressed like those with severe burns or malnutrition, *P. aeruginosa* is a severe threat to overall health. Once considered ubiquitous, this Gram-negative pathogen is the leading colonizer of CF patients over the age of 24 and is found in over 65% of cases reported (Cystic Fibrosis Foundation: Annual Data Report, 2013).

Not only is it the most prevalent in adult patients suffering from CF, but it also represents over 80% of total bacteria found in the lungs of each case reported (Folkesson, 2012). This gives insight into why it is generally accepted as one of the most dangerous and widely studied pathogens in the CF community.

Much of the research regarding *P. aeruginosa* concerns its virulence factors, how they affect host viability, what mechanisms are employed, and how they persist in the infection stages. Some of the virulence factors are: exotoxins, exoenzymes, proteases, lipopolysaccharides, pili, flagella, and mucoid exopolysaccharide (Passador, 1993). Flagella produced by *P. aeruginosa* are also significant virulence factors (Montie, 1987). Strains bearing mutations in the *fliC* gene (which encodes the monomer protein of the flagella) have shown to be significantly less infectious in an intranasal inoculation mouse infection model by a 30% decrease in mortality and greater than a 50% decrease in pneumonia. This is thought to be the case because the flagella can act as a tether to epithelial membranes (Feldman, 1998). Lipopolysaccharides and pili also aid in cellular attachment while exotoxins and enzymes are injected through Type III secretion system (T3SS) that provides a passage from the pathogen to healthy host tissues (Bleves, 2010). Two of these exotoxins secreted by the T3SS are ExoS and ExoT, both of which have GTPase-activating protein and ADP ribosyltransferase activity (Barbieri, 2004)). ExoS prevents actin production and ExoT discourages phagocytosis and in concert they work to promote apoptosis of healthy cells (Barbieri, 2004). Another exogenous effector is the phospholipase ExoU which can damage cellular membranes by folding into membrane binding, bridging and phospholipase domains while also modulating inflammatory response (Ballok, 2013). Results of inflamed tissues in the respiratory tract cause

coughing, pneumonia and, if spread to the bloodstream (septicemia), it can cause chills, disorientation, high fever and shock. Those who are immunosuppressed or suffering from CF are some of the most susceptible. Knowing these factors, a study showing *P. aeruginosa* isolated from CF sputum, inoculated in the infection models of *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Galleria mellonella* had decreased ability to infect these models over the time of chronic infection. However *P. aeruginosa* did not lose its ability to cause acute infection in CF models (Lorè, 2012), showing specific pathogenicity even when selective pressures are temporarily removed.

Unidentified environmental pressures within CF lungs have the ability to turn initial infections from non-mucoid isolates into mucoid strains through the production of the exopolysaccharide alginate (Speert, 1990). With this ability for *P. aeruginosa* to alter its phenotype it can be better suited to persist in its environment because of the physical protection against phagocytosis and recognition by the immune response that this thick alginate provides. One way this is done is by conjugation. Evidence of this exists in islands of genetic homology that have been found in *P. aeruginosa* that correlate with *Burkholderia cepacia*, another respiratory pathogen, suggesting that there is a regular exchange of genetic material between the two organisms (Eberl, 2004).

Persistence is also a significant issue that researchers are trying to understand. *P. aeruginosa* not only has the ability of effectively infect, but also to elude the host immune response. CF isolates are frequently anti-phagocytic because of their ability to produce alginate, which inhibits opsonization, thus making it invisible to defensive resident alveolar macrophages (Passador, 1993). These polymers can also be produced in concert with an extracellular polysaccharide composed of multiple compounds (discussed

further in the biofilm section of this review) making a thick matrix of diverse components (Rasamiravaka, 2015). This persistence is not only against host defenses, but also against other respiratory pathogens.

Gram-positive bacteria have shown to enhance the virulence of *P. aeruginosa* in co-culture. In the presence of N-acetyl-D-glucosamine (GlcNAc), a component of peptidoglycan, *P. aeruginosa* produces an excretory pyocyanin that has shown to inhibit surrounding Gram-positive growth, while simultaneously increasing GlcNAc catabolism genes during *in vitro* growth (Koraonkar, 2011 and 2013; Whiley, 2013). In summary, *P. aeruginosa* is well adapted to opportunistic growth, specifically within the CF lung because of its defenses, environmental sensing and persistence mechanisms that all have the ability to influence expression and phenotype based on lifestyle constraints.

Due to these many virulence factors, it has been difficult to find a treatment that will prevent *P. aeruginosa* infections from causing such havoc in the CF community. The most common treatments of *P. aeruginosa* are antibiotics. The main types of anti-pseudomonal antibiotics are fluoroquinolones that inhibit DNA gyrase, thus not allowing replication and transcription; penicillin derivatives, specifically those containing β -lactam rings, attributed to cell wall biosynthesis inhibition; and aminoglycosides, which inhibit bacterial translation by binding to and inhibiting the 30S subunit of the ribosome (Strateva, 2009). Even with the administration of these antibiotics, the lifestyle and adaptability of *P. aeruginosa* still make treatment difficult because of the ability to acquire multiple types antibiotic resistance from other organisms (Eberl, 2004). As antibiotics continue to be used for treatment of mutable bacteria, resistance is likely to increase such as has been seen with other antibiotics like penicillin over the past 50 years

(Aiello, 2006). As research techniques continue to advance and sequencing becomes more financially feasible, researchers strive to learn more about the pathogenicity of this nosocomial bacterium and how to effectively treat it.

Stenotrophomonas maltophilia

A traditionally non-clinical strain of bacteria that is garnering interest among the scientific community due to its pathogenicity is *S. maltophilia*. This Gram-negative bacillus is commonly found in aqueous environments both inside and outside of hospitals. Like *P. aeruginosa*, *S. maltophilia* is also a multiple drug-resistant pathogen; this is probably because of genetic homology that these two Gammaproteobacteria share (Brooke, 2012). In fact, *S. maltophilia* was originally classified as *Pseudomonas maltophilia* (or *Xanthomonas maltophilia*) until about 20 years ago. Thanks to increased research in phylogeny, it was classified as the first member of the genus *Stenotrophomonas* (Denton, 1998). Since that time at least five other strains have been added to the genus. *S. maltophilia* has even been called one of the “most worrisome” of the unusual Gram-negative non-fermenters in nosocomial infections (Fihman, 2012). This worry comes from its known colonization of up to 16 percent of adolescent and adult CF patients, most of which comes from healthcare provider facilities (Cystic Fibrosis Foundation Patient Registry: Annual Data Report, 2013), and while not commonly an initial colonizer of the CF lung, it follows closely after the initial colonizers *S. aureus* and *H. influenzae*. *S. maltophilia*, has only been completely sequenced four times, but with 16 genome projects in the draft phase there is still interest in identifying

the virulence factors associated with this CF pathogen (Genomes Online Database, <http://gold.jgi-psf.org>).

From this genome information have been able to better characterize *S. maltophilia* in respect to virulence factors. One such virulence factor is the gene *spgM*, which codes for a membrane-bound lipopolysaccharide. Its significance in virulence was elucidated when strains with *spgM* mutations showed a decrease of susceptibility to antimicrobial compounds (McKay, 2003). Another ability that has shown to have a significant effect on virulence is the production of diffusible signal factors (DSF's). One, identified as cis-11-methyl-2-dodecenoic acid (Wang, 2004), is dependent upon the two component sensing proteins encoded by the *rpfC* and *rpfG* genes, which are transcribed in an operon (Slater, 2000). *S. maltophilia* possesses the ability to make DSFs that control extracellular proteases and genes encoding proteins that control aggregative behaviors like RpfF (Huang, 2007; Fouhy, 2007). When mutations of the *rpfC* and *rpfG* genes were made, protease activity decreased, motility was attenuated, susceptibility to antibiotics increased, and lipopolysaccharide production was altered (Fouhy, 2007). These data show that DSF's play an important role in transcriptional control.

Despite current *S. maltophilia* research, its prevalence has steadily increased (Razvi, 2009) as measured by increased surveillance of transmission, widespread implementation of microbiological techniques that aid in the identification, and oversight by organizations like the Cystic Fibrosis Foundation (Saiman, 2003). It can also be attributed to the successful treatment practices of *P. aeruginosa*, leaving CF patients with lungs that can be more easily colonized by *S. maltophilia* (Samoins, 2012).

Most *S. maltophilia* infections are treated with antibiotics; but with its ability to obtain multi-drug resistance, it has been difficult to treat. Although initially considered non-virulent, *S. maltophilia* is a worrisome instigator of pulmonary deterioration (Waters, 2012; Dasenbrook, 2008). Antibiotics used are similar to those used to treat *P. aeruginosa* infection, with fluoroquinolones being the chief agent (Samonis, 2012; Cystic Fibrosis Foundation: Annual Data Report 2013). Biocides are another treatment option that has been used, but research conducted over a decade ago in blood isolates has already shown decreasing susceptibility to the current dosages (Higgins, 2001). Most of *S. maltophilia* infections are found within the respiratory system, but others include vascular, intra-abdominal, bloodstream, urinary tract and soft tissue infections (Fihman, 2012). Their variable nature in pathogenicity is attributed to the fact that they are motile, containing a few polar flagella, and can survive in poor nutrient environments (Brooke, 2012). With increased research, the virulence factors of *S. maltophilia* are beginning to come to light. With continued work on the effects of this bacteria researchers hope to decrease its persistence in the CF lung.

Biofilms

Bacteria frequently form structures called biofilms in environmental and host-associated niches. The formation of a biofilm can be divided into three stages: attachment, colonization and growth (Donlan, 2001). Biofilms are formed on solid, moist surfaces by the attachment of a planktonic (free floating) cell. This individual will then initiate growth of a microcolony. Microcolonies are small isogenic aggregates of the attached cell which are expressing adherence factors, allowing them to stick to a surface;

this is the attachment stage. Once the microcolony is established the colonization stage has begun. This stage is where the diversity of the biofilm is cultivated (Conibear, 2009). In complex biofilms, there are: primary colonizers, which attach to a solid surface (Donlan, 2001); secondary colonizers, which can attach to the surface or the established growth via ligands, pili or fimbriae (Ryan, 2003); bridging bacteria which allow other species that would not otherwise incorporate into the biofilm to attach, usually by ligand-surface protein interactions (Maestre-Reyna, 2013); and finally the species that attach to the bridging bacteria (Donlan, 2001; Maestre-Reyna, 2013). As these cells play differing rolls in the biofilm their numbers increase and the progression of the biofilm moves into the growth stage, where the maturation of the structure is enhanced by an extracellular matrix (Eberl, 2004). Some biofilms form from single species growth while others have dozens of different species arranged in highly organized arrangements called ultrastructures (Hung, 2013).

Biofilms are found in a variety of locations including abiotic and biotic surfaces (Kostakioti, 2013). The biofilm-forming bacteria that cohabitate in a niche are aided in survival by extracellular matrix production (Hall-Stoodley, 2004). This matrix is composed primarily of polysaccharides that can increase bacterial resistance to phagocytosis, antibiotic killing, and that can increase cell-to-cell communication (Lyczak, 2002; Passador, 1993). However, this matrix has many other structural elements including colonic acid, extracellular DNA (eDNA), cellulose and polyglucosamine, all of which allow for bacteria to persist in close proximity to one another (Kostakioti, 2013).

Despite the many advancements of drug delivery, biofilms have proven to be difficult targets for treatment. Because of the thick, viscous extracellular matrix produced

by these bacteria, it is difficult to get the intended drug in contact with the pathogens (Lyczak, 2002). The sharing of resistance among the bacteria that form biofilms exacerbates this treatment problem. A previous study demonstrated that under environmental constraints, *Escherichia cloacae* and *P. aeruginosa* could obtain resistance to multiple antibiotics through one transfer event even if the antibiotics were not chemically related (Sanders, 1984). Another transfer of genes that makes treatment difficult is the community acquisition of drug efflux pumps (Ma, 1994). Similar to the ABC family transporters, efflux pumps differ in the location of where binding proteins attach to comprise the holocomplex (Lyczak, 2002). Not all biofilm life is cohabitation; there is competition that exists and can shape the lifestyles of the species that reside therein. *P. aeruginosa* has an intrinsic ability to form biofilms, and because of its adhesion factors and rapid growth rate, it establishes primary colonization of the niche, and can outcompete other strains within the biofilm (Folsom, 2010). In the marine biofilm-forming bacterium *Pseudoalteromonas tunicata* extracellular inhibitory antibacterial toxins are released to inhibit other competitive organisms from incorporation into the biofilm (Rao, 2005).

Bacteria within biofilms also compete by sending, receiving, and intercepting signaling factors associated with quorum sensing systems (which will be discussed further later in this review). Conversely, not all biofilm-associated organisms compete; some bacteria can work together to form biofilms. *S. aureus* is often found in polymicrobial biofilms with *P. aeruginosa*. With the stages of infection that many CF patients follow it would be expected that *P. aeruginosa* would outcompete *S. aureus*. However, *S. aureus* was found to increase its rate of microcolony formation in the

presence of *P. aeruginosa* supernatant (Yang, 2011). This fact shows just how complex these interactions are, and that bacterial competition relies on many factors and functions on a variable scale. Despite the research striving to characterize these complex structures and how they work, investigators still do not fully understand the interactions of these biofilm-forming bacteria (Baldan, 2014) but an increased awareness of what biofilms mean to the scientific community concerning their ubiquity, resilience, persistence and competition warrants further research into these complex structures.

Bacterial communication

For a single celled organism (like the CF-associated bacteria discussed in this paper) to survive in a variety of environments, it needs to be able to sense its environment. Without sensory mechanisms these organisms would not be able to appropriately respond to potentially selective environments. For that reason bacteria have evolved multiple types of sensors, receptors, fimbriae, adhesins and membrane-associated proteins that allow them to take information from their surroundings and make transcriptional adjustments. Compensatory measures can be taken by sensing environmental variables such as temperature, pH, osmolality and pressure. Not only do bacteria understand the makeup of their abiotic environment but they can also sense biotic factors in the surrounding area like other bacterial products. For this reason systems have evolved whereby bacteria can communicate with one another.

The most common system associated with bacterial communication is quorum sensing (QS). First characterized in *Vibrio fischeri*, QS is a system in which bacteria can elicit a coordinated response to cellular density using concentrations of signaling

molecules (Ruby, 1996). In this seminal case, *V. fischeri* were found on the Hawaiian bobtail squid in light organs. It was found that these bacteria did not luminesce unless they reached a concentration threshold or “quorum”. Further investigation yielded that the autoinducer (AI) molecule, acyl homoserine lactone (AHL), was directly correlated with this luminescence, yielding characterization of the LuxI-R system. The LuxI-R system is composed of two main cellular components: LuxI- the synthase located in the cytoplasm, and LuxR- the receptor located on the cytoplasmic side of the plasma membrane (Kolibachuk, 1993). LuxI is constitutively synthesizing AHL, a freely diffusible signaling molecule that passes through the membrane into intercellular space. When another bacterium that is expressing the *lux* genes comes in contact with the signal AHL, it is taken up by the cell and the signal molecule binds allosterically to the LuxR active site. Once this is done the LuxR/AHL complex can bind to DNA, and can either activate or repress the transcription of target genes (Holcombe, 2011). One common outcome is inducing the transcription of the *luxICDABE* operon which produces proteins that function to give off light while inhibiting the *luxR* gene (Koch, 2005).

Another QS system that has been best described in *P. aeruginosa* involve alkyl quinolones (AQs). Similar to AHL signaling systems used in other bacteria, AQs are freely diffusible signaling molecules that promote the variable production of targeted genes. Much of the mechanisms employed to sense the AQ and the AHL signaling molecules are similar, which gives rise to the hypothesis that there is possible cross communication between the two systems (Dekimpe, 2009). *P. aeruginosa* is reliant upon QS signals for virulence in host systems. It is believed that over 10% of the encoded genome is expressed under the direction of QS signaling molecules (Wagner, 2003).

As previously discussed, a QS system using DSFs has been characterized in *S. maltophilia*. A study looking at the communication between *S. maltophilia* and *P. aeruginosa* showed that an *S. maltophilia* DSF not only influenced biofilm formation, but also increased resistance to antimicrobial peptides (Ryan, 2008). The same signal can also increase the antibiotic susceptibility of other bacteria like *Bacillus cereus* (Deng, 2014). This DSF, characterized as *cis*-11-methyl-2-dodecenoic acid, does not enter the cell and bind to a response regulator like AHL and AQ (Fouhy, 2007). Rather, this Gram-negative communication molecule binds to a membrane sensor that activates a response regulator involving the synthesis of the second messenger cyclic di-GMP mediating the response while keeping the DSF external (Ryan, 2011). These are not novel molecules though. Their derivatives have been found to be structurally homologous to other QS signaling molecules (Deng, 2014). For Gram-positive bacteria, the most researched QS systems are peptide-based. There are no Gram-positive bacteria that have been shown to produce AHLs (de Kievit, 2000), but bacteria like *S. aureus*, *Bacillus subtilis* and *Streptococcus pneumoniae* produce secreted autoinducing peptides (AIP). These peptides are received by a two-component system (TCS) sensor kinase membrane receptor (Yarwood, 2003; de Kievit, 2000). When the AIP binds, the sensor kinase is phosphorylated, which activates a cytoplasmic messenger AgrA that in turn complexes and phosphorylates a transcriptional response regulator (Novick, 2008). With the aid of sequencing data, there is increased understanding of the specificity that exists between many Gram-positive bacteria involving their species-specific AIP and their corresponding TCS (Rocha-Estrada, 2010; <http://www.ncbi.nlm.nih.gov>).

In addition, there is one type of QS system that is not specific to Gram-positive or Gram-negative bacteria. The autoinducer 2 (AI-2) class is an important biofilm formation signal for *Actinomyces oris*, *Streptococcus gordonii*, and *Porphyromonas gingivalis* in the oral cavity (Jakubovics, 2010). These AI-2s have siderophoric properties, specifically chelation of boron (Chen, 2002). There is still speculation on the importance of binding boron as even its potential uses within the cell has not been well understood (Loomis, 1992). But as a common QS molecule in both Gram-positive and negative bacteria, the AI-2 system is becoming a major target for researchers interested in QS systems because it is seen as a universal signal for interspecies communication. One point of interest is that boron is also found in some bacterial macrolides suggesting that it may be a good target for antibiotic therapy (Irschik, 1995).

With all the different forms of bacterial communication and the adaptability of pathogens found in biofilms containing QS signaling molecules, there are some strains that take advantage of signals not directly intended for them. This action has been coined bacterial “eavesdropping” (Ryan, 2008). One such biofilm bacterium that has shown such adaptability is *P. aeruginosa*. This Gram-negative CF pathogen does not have the AI-2 LuxS synthase. However it can detect the signaling molecule and differentially express specific genes (Duan, 2003). Similarly, *Klebsiella*, *Escherichia* and *Salmonella* do not have the cellular machinery to produce AHLs. They do have LuxR-like proteins that act as receptors for AHL though. In the case of *Salmonella*, an operon coding proteins involved in defense against the host is activated using the surrounding bacterial signals to act as warnings (Ahmer, 1998). Some bacteria have even devised methods to fight against eavesdropping. In a newly characterized method, *E. coli* uses nanotubes to share

cytoplasmic products and nutrients exclusively with other *E. coli* (Pande, 2015), thus eliminating the ability for other bacteria to eavesdrop. There are many cases of eavesdropping in vastly different bacteria. One thing is for certain though, bacteria have evolved signals to react in their environment in a coordinated way to increase their overall fitness, whether they were the intended recipient or not (Duan, 2003).

Next generation sequencing

Basically unchanged since the time of Robert Koch, the methods for determining bacterial identity have remained isolation on agar plates as well as a series of selective and/or differential growth tests (Zengler, 2009). Using advancements in technology, genetic sequencing has become a powerful tool that researchers are using to not only identify bacterial strains, but also gain a deeper understanding of how they function. In 1972 sequence of a single gene coding for a bacteriophage MS2 coat protein was completed in Gheringen, Germany (Min, 1972). It marked the beginning of the sequencing age and although it did not characterize an entire organism it provided a method of research that would revolutionize bacterial research. Five years later, the first genome fully sequenced, bacteriophage ϕ X174, was completed by Frederick Sanger (Sanger, 1977). It took seven years before a full genome, that of the Epstein-Barr virus, was sequenced in 1984 using the primer extension method developed by Sanger. Since that time advancements in genetic sequencing processes have included direct-blotting-electrophoresis which eliminated the need for radioactive isotopes for integration into immobilizing matrixes (Beck, 1984), and partial automation of the sequencing method (Smith, 1986). In 1995 the first full genome of a free-living entity, *Haemophilus*

influenzae, was sequenced (Fleischmann, 1995). Another significant event in genomics came in 2001, after decades of work and the cost of \$2.7 billion. A draft of the human genome was complete in its initial sequencing by the Center for Genome Research (Lander, 2001). Since that time, hundreds of whole genomes have been submitted for publication or are currently being researched (<http://www.ncbi.nlm.nih.gov>). This is due, in part, to the increased optimization of sequencing processes. With the implementation of methods such as pyrosequencing (454) and base-by-base (Illumina HiSeq) the cost has been decreasing drastically, and it is projected to become more economical with continual advancements in this expanding field (Norton, 2012).

In 2005, 454 introduced the first next-gen sequencing platform, using DNA fragments with attached adaptors on either end. Beads presenting primers complementary to the adaptors on the DNA fragments are introduced and after the DNA attach to the bead it is replicated so each bead is covered with fragment DNA. Each bead is separated into a well with a polymerase enzyme bead. The polymerase attaches to the DNA fragment and nucleotides are fed in waves. When one attach, light is emitted. When multiple of the same nucleotide is purportedly attached one after the other the light emitted is brighter. Based on when the light is emitted a sequence can be determined from the light emission profile. As of 2010, the cost of 454 sequencing is about \$0.02 per 1000 bases, which makes it a high output, relatively inexpensive sequencing method (Balzer, 2010; Liu 2012). This method has also allowed us to obtain the whole genome sequences of multiple bacteria. Having these genomes as a reference proves useful when looking at the transcriptome of those bacteria so we can understand what is being expressed and when.

Within two years of 454 sequencing's debut, Illumina sequencing was introduced to the scientific community. This is a base-by-base sequencing method that attaches DNA fragments to a slide that is covered with a lawn of primers. When the fragmented sample DNA, which has adapters on both ends that are complementary to the primers on the slide anneal, they will bend over and attach to the opposite side complementary primer. These are replicated multiple times leaving a cluster of copied fragment DNA attached to the slide. Primers are then added to the free side of the DNA and nucleotides bound to fluorescent moieties are washed over the slide. A laser that causes the emission of fluorescence detects the nucleotides attachment to the strand. The fluorescence pattern is then converted to a sequence for each cluster (Brown, 2012). Illumina sequencing reads are shorter fragments than 454 sequencing and therefore is not best suited for *de novo* sequencing, but can obtain the same number of reads within about half the time (Luo, 2012). Also the cost of Illumina sequencing is much less than 454 sequencing at about \$0.001 per 1000 bases (Liu, 2012). Due to these and other sequencing methods, much time and money is being put into sequencing different organisms. This is indicative of the direction that bacterial research is taking, and with the versatility of the different types of sequencing methods, the data generated by these technologies will vastly increase our understanding of these studied organisms (Quail, 2012).

Transcriptomics is also a segment of the genomics field that has become increasingly advanced. Next-gen sequencing offers the ability to identify the genes expressed by an organism under differing environmental conditions. Conditions that can cause a variable response or act as a selective pressure to bacteria are good scenarios for Next-Gen Sequencing. Transcriptomics is also valuable in understanding the

pathogenicity of microbes in different stages of infection by allowing researchers to see the variance of transcripts that bacteria express under specific conditions. This gives researchers useful insight into which targets antimicrobial therapies might be best suited. The transcriptomics studies are providing genetic information that were previously unattainable. Now researchers are able to look at mixed samples and identify the types of bacteria within the sample with a high degree of statistical significance (Goddard, 2012; Sibley, 2008). As we attempt to understand the microbiome of diverse habitats, genetic sequencing has drastically reduced the cost and time of characterization in these environments. Given these advancements, the next giant step forward, comparable to the first sequenced gene or the human genome, is for beginning-to-end sequencing including *in vitro/in vivo*, and *in silico* methods to be integrated into one process for whole genome sequencing (Frese, 2013). Although this leap may seem far off, the advancements in genomic sequencing are increasing exponentially, not only in possible reads and accuracy but also in affordable price per base and automation. This project sought to use what we know about CF associated pathogens, their lifestyles and communication mechanisms in order to understand more about how they can influence each other in co-culture.

INTRODUCTION

Bacteria have long been studied in order to understand how they infect hosts. As researchers have tried to address this question in the past, they would eliminate as many experimental variables as possible by studying virulent bacteria in monoculture. However, infectious bacteria are not always found in monoculture *in vivo*. During such infections, most bacteria are found in close association with other strains of differing genera. For this reason, poly-microbial studies have become increasingly important for revealing how bacteria influence each other, which may in turn hold clues to understanding how they affect the human population.

One such human polymicrobial disease which is exacerbated by polymicrobial infections is Cystic Fibrosis (CF). As the most common genetic disorder in the Caucasian population, CF currently affects approximately 30,000 people in the United States and over 70,000 worldwide (Cystic Fibrosis Foundation Patient Registry: Annual Data Report, 2013). This disorder results from alterations in a specific chloride channels in the lung, which results in the development of a thick mucus layer where bacteria colonize in biofilms. The most common infectious agents in CF lungs is *Pseudomonas aeruginosa*. In fact, over 95% of CF patients will be infected with *P. aeruginosa* at some point in their lives (Cystic Fibrosis Foundation Patient Registry: Annual Data Report, 2013). This is of great concern due to the virulent capabilities of *P. aeruginosa*. Another bacterium, *Stenotrophomonas maltophilia*, has been shown to be an alarming threat to CF patients

due to its increasing prevalence over the past decade (Stressmann, 2011; Fihman, 2012; Razvi, 2009). Infection with either species can lead to severe necrotizing pneumonia that can result in rapid pulmonary decline, sepsis and death.

Evidence has suggested that the bacterial composition found in one part of the airway is not indicative of the whole respiratory tract (Goddard, 2012). Regardless of fluctuations in the cast of accessory microbes, there are only a handful of bacteria routinely isolated from the lower respiratory tracts of CF patients. This finding suggests that there are major “players” in CF pathogenesis. The advancements made in genetic sequencing are playing a significant role in understanding the genes that contribute to these species’ robustness in co-culture as well as their role in virulence. These findings increase the scope of bacterial research to include the effects of bacterial communication and what genes are essential for their cohabitation in the CF lung. These genes may provide answers to questions regarding how communication is linked to bacterial virulence. The genes transcribed by one bacterium in co-culture may inhibit opportunistic growth of another species and subsequent infection by mechanisms not yet understood. The aim of this research was to better understand how two CF pathogens, *P. aeruginosa* and *S. maltophilia*, sense and respond to each other. Using morphological tests and genetic approaches, genes were identified that were up-regulated when these strains were grown in co-culture. Follow-up experiments to generate mutations in up-regulated genes are still underway. The results from this work have demonstrated that numerous genes are differentially regulated in response to co-culture of these CF pathogens. This suggests that *P. aeruginosa* and *S. maltophilia* have the ability to sense and respond to each other either directly or indirectly when grown in co-culture.

MATERIALS AND METHODS

Media and bacterial strains

Bacterial strains were maintained routinely in Luria Broth Lennox formulation (LB), which consisted of 1% tryptone, and 0.5% yeast extract and 0.5% NaCl (Lennox, 1955). For growth assays, three additional media were also used: Tryptic soy agar (TSA)[1], consisting of 0.05% soya peptone and NaCl, and 1.5% casein peptone with pH adjusted to 7.3 (MacFaddin, 1985); Soil extract medium (SEM)[2] supplemented with 30 mM glucose, containing 10% soil extract made by autoclaving 200 g of sieved UV-light sterilized corn root soil in 500 mL tap water for 20 min, then supplemented with 30 mM glucose, 0.05% CaCO_3 , pH adjusted to 6.8 and autoclaved for 45 minutes (Kilmer, 1982), approximately 50 mL of loose soil was obtained at 9:30 a.m. on May 23, 2013 from the roots of a corn stalk at the edge of a field (Latitude-Longitude: 38.326876,-85.460758) shaded by growing corn, with an air temperature of 13.9°C. Synthetic cystic fibrosis medium (SCFM)[3] was made by adding (per liter of medium) 100 mM amino acid stocks to a buffered base of 6.5 mL 0.2 M NaH_2PO_4 , 6.25 mL 0.2 M Na_2HPO_4 , 0.348 mL 1 M KNO_3 , 0.122 g NH_4Cl , 1.114g KCl , 3.03g NaCl , 10 mM MOPS and 779.6 mL MilliQ water. The amino acid stocks were added to the buffered base in the following volumes: L-aspartate, 8.27 mL; L-threonine, 10.72 mL; L-serine, 14.46 mL; L-glutamate-HCl, 15.49 mL; L-proline, 16.61 mL; L-glycine, 12.03 mL; L-alanine, 17.8

mL; L-cysteine·HCl, 1.6 mL; L-valine, 11.17 mL; L-methionine, 6.33 mL; L-isoleucine, 11.2 mL; L-leucine, 16.09 mL; L-tyrosine, 8.02 mL; L-phenylalanine, 5.3 mL; L-ornithine·HCl, 6.76 mL; L-lysine·HCl, 21.28 mL; L-histidine·HCl, 5.19 mL; L-tryptophan, 0.13 mL; and L-arginine·HCl, 3.06 mL (tyrosine, aspartate, and tryptophan were resuspended in 1.0 M, 0.5 M, and 0.2 M NaOH, respectively). This was then adjusted to a pH of 6.8 and filter sterilized through a sterile 500 mL Corning Bottle-Top Filter with a pore size of 0.2 μ m. After filtration the sterile components of: 1.754 mL 1 M CaCl₂, 0.606 mL 1 M MgCl₂, and 0.001g FeSO₄·7H₂O were added. Three milliliters 1 M D-glucose and 9.3 mL 1 M L-lactate (previously adjusted to a pH of 7.0) and the final volume was adjusted to 1 L with MilliQ water and stored in the dark at 4°C (Palmer, 2007). All agar was made by adding 12 g/L bacteriological agar to each medium and autoclaved for 30 minutes. In the case of SCFM, 500 mL of a 2X stock of filter-sterilized ingredients described above was added to 500 mL of autoclaved water containing 12 g bacteriological agar. All bacterial strains were maintained at 37°C with shaking at 300 rpm in liquid culture or on agar in a walk-in 37°C incubator as indicated.

P. aeruginosa PAO1 (a burn isolate), *P. aeruginosa* 2192 (a mucoid CF isolate), *B. dolosa* AU0158 (a CF isolate) and *Escherichia coli* pEBFP (a lab strain) were all obtained from the Stephen Lory lab collection at Harvard Medical School. *S. maltophilia* K279a (a blood isolate) was a gift from Nick Ciancotto at Northwestern University. *B. cenocepacia* K56-2 (a CF isolate) was obtained from John LiPuma (University of Michigan). *S. aureus* NRS77 was obtained through the Network of Antimicrobial Resistance in *Staphylococcus aureus* from BEI Resources (<http://www.narsa.net/>). *E.*

coli DH5 α was a gift from Michael Perlin (University of Louisville), and *E. coli* pRK2013 was obtained from Herbert Schweizer at Colorado State University.

Proximal growth test

To assess growth inhibition of differing bacterial strains, *P. aeruginosa* 2192, *P. aeruginosa* PAO1, *S. maltophilia* K279a, *B. dolosa* AU0158, *B. cenocepacia* K56-2, and *E. coli* pEBFP were grown in LB broth overnight at 37° C with shaking at 300 rpm. Ten microliter aliquots were spotted on LB agar plates with pairwise comparisons in three zones: isolated, proximal, and combined, in triplicate for each comparison. The plates were then incubated at 37°C overnight and colony morphology was observed after approximately 12 hours. Based on morphological observations, a designation of inhibition or no inhibition was made for each comparison and replicate.

Co-culture lawn inhibition test

After normalizing the concentration of each overnight liquid culture (using an Eppendorff BioPhotometer 6131) to an optical density at 600 nanometers (O.D.₆₀₀) of 0.02, 25 μ L of *P. aeruginosa* 2192, *P. aeruginosa* PAO1, *S. maltophilia* K279a, *B. dolosa* AU0158, *B. cenocepacia* K56-2 and *Escherichia coli* pEBFP were spread on LB and TSA plates making single species bacterial lawns. The plates were incubated at 37°C for three hours to allow a low-density lawn to establish. Ten microliters of each bacterial species were then spotted on top of the established lawns with the following dilutions O.D.₆₀₀ 1.0, 0.5 and 0.01. Broths matching the plate medium as well as sterile nutrient

broth were spotted as negative controls. Plates were dried and incubated overnight at 37°C. Three biological replicates were performed for each comparison and medium.

Growth curves

Triplicate samples of *P. aeruginosa* 2192 and *S. maltophilia* K279a were grown overnight in an SCFM at 37°C shaken at 300 rpm. Twenty milliliters of fresh SCFM was inoculated with 1 mL of the overnight culture and shaken at 300 rpm at 37°C. At one-hour time intervals, O.D.₆₀₀ measurements were taken of three replicates and the averages computed via Microsoft Excel.

Growth curves were then generated in SEM by overnight incubation with shaking at 300 rpm at 37°C of each bacterial strain to which 20 mL of fresh SEM broth was inoculated with 1 mL of overnight culture. At one-hour time intervals, O.D.₆₀₀ measurements were taken of three replicates and averages computed using Microsoft Excel.

Final growth curve generation was done by allowing each bacterial species (*P. aeruginosa* 2192, *S. maltophilia* K279a and *Staphylococcus aureus* NRS77) to grow overnight with shaking at 300 rpm at 37°C in LB broth. Fresh LB broth was brought to an O.D.₆₀₀ of 0.2 using an overnight culture in a total of 20 mL. At one-hour time intervals, O.D.₆₀₀ readings were taken of three replicates and averages computed via Microsoft Excel.

RNA purification

Pure cultures of *P. aeruginosa* 2192, *S. maltophilia* K279a and *S. aureus* NRS77 were grown in LB broth, diluted to an O.D.₆₀₀ of 0.1 in 20 mL of LB broth and incubated until the O.D.₆₀₀ reached ~2.0. Aliquots totaling 2 mL of each of the following combinations were collected: *P. aeruginosa* 2192 monoculture, *S. aureus* NRS77 monoculture, *S. maltophilia* K279a monoculture, *P. aeruginosa* 2192 + *S. aureus* NRS77 co-culture, *P. aeruginosa* 2192 + *S. maltophilia* K279a co-culture, *S. aureus* NRS77 + *S. maltophilia* K279a co-culture, *P. aeruginosa* 2192 + *S. maltophilia* K279a + *S. aureus* NRS77 tri-culture. Each of the co-culture and tri-culture mixtures was combined with equal colony forming units (CFU) of their respective bacteria based on growth curve data. Each combination was prepared for the “plated” condition by dispensing the prepared 2 mL inoculum on a LB agar plate and allowed to incubate with the petri plate upright for 2.5 hours at 37°C. Each plate was then scraped and suspended in an additional 1 mL of LB broth for hot phenol extraction. These samples were combined in their pairwise and tri-culture combinations with equal CFUs, diluted to an O.D.₆₀₀ of 0.2 in 20 mL of LB broth, and incubated at 37°C with shaking for the other two conditions. When the triplicate samples each reached mid-exponential phase (O.D.₆₀₀ ~ 0.9), the “liquid log” sample was collected for RNA extraction. The remainder of the liquid culture incubated an additional 12 hours (“stationary” sample). Hot phenol extraction (Masse, 2003) for each growth condition was performed by adding 700 µl of cell culture with 100 µL 8X lysis solution (20 mM sodium acetate, 0.5% sodium dodecyl sulfate, 1 mM EDTA) preheated to 65°C. Eight hundred microliters of 65°C acid phenol (pH = 4.5, Ambion) was added and the reactions were incubated for 5 minutes with vortexing every

20 seconds. The lysate was centrifuged at 15,000 rpm for 15 minutes at 4°C. The supernatant above the protein phase containing nucleic acids was collected after two additional rounds of phenol:chloroform:isoamyl alcohol (25:24:1) extraction. The top lysate layer was then mixed with an equal volume of chloroform:isoamyl alcohol (24:1) by vortexing then re-centrifuged. The supernatant was precipitated with 3 volumes of 95% ethanol at -80°C overnight with inversion of each tube after 4 hours. Samples were centrifuged at 15,000 rpm at 4°C for 10 minutes, the ethanol was poured off, the pellet rinsed with 500 µL of 75% ethanol, centrifuged for another 2 minutes at 15,000 rpm at 4°C, and allowed to air dry in a ventilated chemical fume hood. Each pellet was resuspended in 30 µL RNase-free water. DNA was removed from the samples by treating with 50 µL 10X DNase buffer and 5 µL DNaseI in 500 µL final volume. Reactions were incubated at 37°C for 1 hour and purified by phenol extraction and ethanol precipitation. The RNA quality was assessed using gel electrophoresis in a 1% agarose gel using SYBR Gold detection stain. RNA concentrations were then measured using a Thermo Scientific NanoDrop ND-2000c spectrophotometer.

Library preparation for Illumina sequencing

To enrich for mRNA from total RNA, each sample was purified using the MICROBExpress Bacterial mRNA Enrichment Kit (Life Technologies) that relies on the selective capture of bacteria rRNAs using magnetic beads bound to complementary oligonucleotides. To ensure specific 16S and 23S capture of each bacterial species in the sample custom multiplex oligonucleotides were constructed using DNA oligonucleotides (Integrated DNA Technologies) and used in place of the capture mix contained in the

MICROBExpress kit. Each oligonucleotide mix consisted of a poly adenosine sequence of 15 bases (for magnetic bead binding) followed by specific binding sequences that were found across the 16S and 23S rRNAs from *P. aeruginosa* 2192, *S. maltophilia* K279a and *S. aureus* NRS77: 16S rRNA binding oligonucleotides - (A)₁₅CACTGGAA, (A)₁₅GGATTAGA, (A)₁₅TCGTGAGA, (A)₁₅TTGTACAC; 23S rRNA binding oligonucleotides - (A)₁₅GTACCGTG, (A)₁₅AAAGGGAA, (A)₁₅CCGTAACT, (A)₁₅GCGAAATT, (A)₁₅GAACGTCG. Enriched mRNA samples were prepared for Illumina sequencing by adjusting each to a concentration of 20 ng/μL with a total volume of 5 μL each. For cDNA conversion and amplification, the NEBNext Ultra RNA Library Prep Kit for Illumina (New England BioLabs) was used following manufacturer's recommended protocols with two exceptions: the mRNA purification step was omitted, and Qiaquick PCR Purification Kit (Qiagen) was used for purification of the ligation in the place of AMPure XP Beads. New England BioLabs NEBNext Multiplex Oligos (Index Primer Set 1, New England BioLabs) were used for adding indices ("barcodes").

Sequencing and data analysis

High-throughput Illumina sequencing was carried out at the University of Wisconsin-Madison Biotechnology Center DNA Sequencing Facility on 3 lanes (7 barcoded samples/lane) of an Illumina HiSeq 2000 sequencer. Prior to sequencing, quality assessments of each library preparation were done for each sample using an Agilent Technologies 2100 Bioanalyzer. CLC Genomics Workbench (Qiagen) was used for sorting samples using the indices and alignment to each genome using the RNA-seq feature and standard default settings. Normalized output for each gene was expressed as

RPKM (reads per million reads per million reads). Libraries were assessed for the number of reads that aligned to single positions on each genome, gene coverage, and overall genome coverage to determine the quality of the data and identify comparisons that could be further investigated. *S. maltophilia* K279a mono-culture or *P. aeruginosa* 2192 mono-culture versus the *S. maltophilia* K279a + *P. aeruginosa* 2192 co-culture were selected for further analysis.

In silico experiments with CLC Genomics workbench were performed to compare the effect of culture conditions (whether community composition or environmental condition) using the “Set Up Experiment” function in the Transcriptomics Analysis menu. Each replicate compared co-culture growth to the monoculture sequence profiles in their respective condition. Statistical analysis for comparisons was performed using the Kal’s test for single samples and a Bonferroni multiple testing correction factor (for multiple comparison error rate), which provides a *p*-value for each gene comparison. PSORTb v3.0.2 (<http://www.psort.org/>) was used to predict protein localization for genes with significant fold changes (> 3-fold) and for all the predicted proteins in *S. maltophilia* K279a and chi-square tests were used to quantify significant differences between expected and observed frequencies in the predicted proteins. Likewise, clusters of orthologous gene (COG) functional classifications were assigned for each predicted protein, and comparisons between the genes with significant fold changes between mono- and co-culture and the entire predicted proteome of *S. maltophilia* K279a were statistically analyzed by chi-squared tests. The data used to select genes for further studies were 1) fold changes across differing growth conditions, 2) predicted PSORTb

subcellular locations and 3) predicted COG functional classifications. Fifteen genes were chosen for verification by qRT-PCR and mutant construction and analysis.

Quantitative Real-Time Polymerase Chain Reaction

Primers for qRT-PCR were optimized in concentration and used to assess the relative abundance of gene transcripts from three replicates of each *S. maltophilia* K279a mono-culture and *S. maltophilia* K279a + *P. aeruginosa* 2192 co-culture mRNA samples and growth condition (liquid log, stationary, plated), which were freshly prepared in triplicate. For optimization, the primer concentrations tested were 50, 300 and 900 ng/ μ L in pairwise combinations using 10^4 PCR amplicons per reaction for template. The forward and reverse primer concentrations were selected by identifying which concentration of each would yield a single 300-400 bp amplicon that have a melting temperature of 80-90°C. After optimization of primer concentrations, transcript levels in *S. maltophilia* K279a mono-culture and *S. maltophilia* K279a + *P. aeruginosa* 2192 co-culture mRNA samples were assessed using quantified PCR products as a standard curve (10^8 - 10^2 amplicons per tube in a 10-fold dilution) or 20 ng of purified mRNA samples as templates, primers surrounding a ~50-150 bp region internal to each gene (and nested inside the PCR amplicons used for standard curves), and Power SYBR Green RNA-to- C_T 1-Step Kit (Life Technologies) in 15 μ L final reaction volumes. Reactions contained 7.5 μ L Power SYBR Green RNA-to- C_T 1-Step Kit One-Step solution (Life Technologies), 0.12 μ L RT-PCR mix (Life Technologies), 3.33 μ L RNase-free water, 0.005 μ L glycogen (20 mg/mL), 1 μ L of template RNA/DNA and 0.36 μ L of each internal primer. Running conditions for qRT-PCR were: 48°C for 30 minutes, 95°C for 10 minutes, (95°C x 15

sec, 60°C for 75 seconds)_{40 cycles}, and 4°C hold. Each sample was run in triplicate and compared between conditions. Averages and standard deviations were calculated in Microsoft Excel.

Mutant construction

Overlap extension (SOE) (Horton, 1990) primers for differentially regulated genes in *S. maltophilia* K279a were created using sequences from the 500 base pairs upstream and downstream of each gene of interest retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) and the KEGG database (<http://www.genome.jp/kegg/>). For each gene, 4 primers were made: one upstream primer with an artificial *Bam*HI restriction enzyme recognition site embedded in the 5' end (Primer 1), one chimeric primer that flanked the 3' end of the upstream region fused directly to the 5' end of the downstream region (Primer 2), one primer that was the reverse complement of the second primer (Primer 3), and a downstream primer corresponding to the 3' end of the downstream region containing an artificial *Eco*RI restriction enzyme recognition site at its 5' end (Primer 4). Oligonucleotides corresponding to each primer sequence were generated by Integrated DNA Technologies at 25 nanomole scale and standard desalting purification. The primers were diluted to a working stock of 10 pm/μL. First round SOE PCR generated the flanking DNA regions for each gene and was performed using Hotstar High Fidelity Polymerase (Qiagen) with the manufacturer's recommended protocol. PCR amplicons generated were verified by 1% agarose gel electrophoresis and purified by Qiaquick PCR Purification Kit (Qiagen). Second round SOE PCR used 2 μL of the purified Round 1 SOE PCR products (upstream

and downstream regions) for templates and flanking primers and Primers 1 and 4 for each gene. Second round PCR products were verified by 1% agarose gel electrophoresis and purified by Qiaquick PCR Purification Kit. Restriction digests were performed using *Bam*HI-HF and *Eco*RI-HF (New England BioLabs) on the purified Round 2 PCR products and the pEX18Tc vector (Hoang, 1998; Zhang, 2001; Yang, 2009) according to the manufacturer's recommended protocols except reactions were incubated overnight at 37°C. Two microliters of Calf Intestinal Phosphatase (New England BioLabs) was added to the vector digest and an additional 3 µL of each enzyme was added to the inserts to "boost" each reaction. Inserts and the vector were incubated at 37°C for another hour and then at 65°C for 20 minutes to inactivate the restriction enzymes. Samples were purified using the Qiagen PCR Purification Kit and eluted into 50 µL DNase-free water. Ligation reactions containing 1 µL 10X T4 DNA ligase buffer (New England BioLabs), 1 µL T4 DNA ligase, 1 µL of the vector, and 3X or 10X molar excess of each insert in 10 µl final reaction volumes were incubated at 18°C overnight in a thermocycler. Negative controls for ligations were prepared in parallel that lacked insert (to check for single cut or uncut vectors) or lacked insert and ligase (to check for uncut vectors only). Reactions were first transformed into chemically competent *Escherichia coli* DH5α cells (Hanahan, 1985) by adding all 10 µL of each ligation reaction to 50 µL of thawed or fresh competent cells then incubated on ice for 30 minutes, heat shocked for 45 seconds at 42°C, then returned to ice. In parallel, transformations containing 1 µL of pEX18Tc uncut vector was used as a positive control or no DNA was added as a negative control. One milliliter of room temperature LB broth was added to each transformation, and the samples were shaken at 37°C for 1 hour at 300 rpm. Aliquots of 100 µL were plated on LB agar with 10 µg/mL

tetracycline (Tet 10) and 20 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Gold Biotechnology). The remaining 900 µl was centrifuged and resuspended in 100 µL of LB broth and plated on Tet 10 + X-gal LB agar. Colonies were assessed for white color (suggesting the *lacZα* fragment surrounding the multiple cloning site was disrupted) and verified by colony PCR with SOE primers 1 and 4. Freezer stocks were made from each successful ligated and transformed colony, by adding 1 mL of LB broth containing 40% glycerol to 1 mL overnight culture, and freezing in cryovials at -80°C. Plasmids were purified from each freezer stock using a QIAprep Spin Miniprep kit (Qiagen). Isolated plasmid samples were verified for purity and sent to Macrogen (<http://www.macrogenusa.net/>) for Sanger sequencing. Sequences of each transformed plasmid were compared to the expected sequences using BioEdit Biological Sequence Alignment Editor [<http://www.mbio.ncsu.edu/bioedit/bioedit.html>]. The percent correlation between Macrogen sequences and a synthesized theoretical sequence were calculated to assess the success of each insertion.

For transformation, electrocompetent *S. maltophilia* K279a cells were prepared following the method outlined by Ye *et al.* (2014) with a few modifications. To prepare competent cells, 100 µL of overnight cultured *S. maltophilia* K279a were inoculated into 5 mL of fresh LB medium and incubated at 37°C for 30 minutes. When O.D.₆₀₀ values reached 0.4, 4.5 mL of the cultures were placed on wet ice for 30 minutes. The cells were then pelleted by 15,000 rpm centrifugation at 4°C for 5 minutes. The supernatant was poured off and the pellet was washed three times with 3 mL of ice-cold 10% glycerol (v/v). Finally, pellets were resuspended in 300 µL fresh 10% glycerol and stored at -80°C. For transformations, 50 µL of electrocompetent cells were mixed with 2 µL of

plasmid DNA (200 ng/ μ L) and placed on ice for 5 minutes. The mixture was then transferred to a 0.1-cm cuvette and electroporated with a single pulse using a Bio-Rad Micropulser 2.0 and set to 1.8 kv (setting: Ec1). After the pulse, the mixture was immediately suspended in 950 μ L fresh LB medium and incubated at 37°C for one hour. Bacterial suspensions were then plated on antibiotic (Tet 10) plates in dilutions of LB at 1, 1:10, 1:100 and 1:1,000 (cell suspension to nutrient broth). After 24 hours plates were removed from incubation where colonies were counted and recorded.

For bacterial tri-parental conjugation three strains of bacteria were used. The recipient *S. maltophilia* K279a, donor strain of chemically competent *E. coli* DH5 α and the helper strain *E. coli* pRK2013. Transformed *E. coli* DH5 α containing pEX18Tc with gene deletion sequences and *E. coli* pRK2013 were grown under selection (Tet 10 and Kan 50 respectively) on LB agar overnight. *S. maltophilia* K279a was also grown overnight, but on LB agar with no selection. *S. maltophilia* K279a was heat shocked at 42°C for 2 hours in a standing tube while LB mating plates were dried at 37°C for 30 minutes. Two hundred microliters of both *E. coli* pRK2013 and *E. coli* DH5 α were mixed with 400 μ L *S. maltophilia* K279a and centrifuged at 15,000 rpm for 5 minutes. Mixture supernatant was then poured off and the pellet resuspended in 100 μ L fresh LB. The suspension was then plated on a dried LB agar plate and incubated overnight at 37°C. The mating mixture was scraped from the LB plate and resuspended in 2 mL LB. Aliquots of 400, 100 and 10 μ L were spread on selection plates (Tet 10, Carb 75) and incubated overnight at 37°C. Single colonies were counted after 24 hour incubation.

RESULTS

Proximal growth test

In order to study the effect of co-culture on *P. aeruginosa*, *S. maltophilia*, *Burkholderia cepacia* complex isolates, and *E. coli* (a lab strain control), we conducted experiments in which two strains were compared in isolated (Zone 1), proximal (Zone 2), or combined situations (Zone 3) to identify morphological changes that may occur when strains are grown near each other. These microbes can be found within similar niches and that was why they were chosen for these tests. For this, each strain was grown overnight in a nutrient rich medium and small aliquots were spotted on plates as shown in Figure 1A. All strains were tested in a pairwise fashion. For all pairwise comparisons after overnight incubation, Zones 1 and 2 showed no growth inhibition or changes in morphology for *P. aeruginosa* 2192 (a mucoid CF strain), *P. aeruginosa* PAO1 (a non-mucoid strain), *S. maltophilia* K279a, *B. dolosa* AU0158, *B. cenocepacia* K56-2 and *E. coli* pEBFP on any another strain (Table 1). As a note, *E. coli* pEBFP was chosen over the wild-type in this experiment for possible fluorescent identification in future assays. Observable growth inhibition was only seen between *P. aeruginosa* PAO1 and *P. aeruginosa* 2192 in which it appeared that *P. aeruginosa* PAO1 inhibited the growth of *P. aeruginosa* 2192 (Figure 1B). This was evident by the absence of *P. aeruginosa* 2192 when the combined sample was re-streaked and colony isolation only yielded non-mucoid colonies. This result suggests that *P. aeruginosa* PAO1 may be an inhibitor of

Figure 1. Proximal growth test. (A) Example layout for a proximal growth plate test. (B) Pairwise comparisons were performed for the bacterial strains *P. aeruginosa* 2192, *P. aeruginosa* PAO1, *S. maltophilia* K279a, *B. dolosa* AU0158, *B. cenocepacia* K56-2, and *E. coli* pEBFP. Zone 1- Isolated growth; Zone 2- Proximal growth; Zone 3- Combined growth. Example proximal growth test in which *P. aeruginosa* 2192 (left) was plated with *P. aeruginosa* PAO1 (right).

A.

B.

Table 1. Growth inhibition during proximal growth test.

Strain	<i>P. aeruginosa</i> 2192	<i>P. aeruginosa</i> PAO1	<i>S. maltophilia</i> K279a	<i>B. dolosa</i> AU0158	<i>B. cenocepacia</i> K56-2	<i>E. coli</i> pEBFP
<i>P. aeruginosa</i> 2192						
<i>P. aeruginosa</i> PAO1	^					
<i>S. maltophilia</i> K279a	x	x				
<i>B. dolosa</i> AU0158	x	x	x			
<i>B. cenocepacia</i> K56-2	x	x	x	x		
<i>E. coli</i> pEBFP	x	x	x	x	x	

^ - direction indicating the inhibited bacteria
x – No inhibition of growth

other *Pseudomonas* species but not of the other bacterial species tested; however, this bacterial inhibition may be due to the growth medium or concentration of the inoculations.

Co-culture lawn inhibition test

As a comparison to the proximal growth tests, strains were compared by inoculating high-density spots onto established lawns of bacteria to look for growth inhibition. This method is known as the spot-lawn assay and has been conducted in previous published research (Aguirre-von-Wobeser, 2014; Lo Giudice, 2007; Benkerroum, 1993).

Pairwise comparisons of all strains on LB medium were plated as shown in Figure 2A. All positive controls of bacterial lawns spotted with the same bacterium as on the lawn displayed growth. All six bacterial strains grew on established *S. maltophilia* K279a, *B. cenocepacia* K56-2 and *E. coli* pEBFP lawns (Table 2). Of those spotted bacteria that grew on established lawns, there was growth of the lawn directly up to the edge of the spotted region showing no discernable zones of inhibition. This suggests that there were no molecules produced by the spotted bacteria that were diffusible through the medium that could inhibit the growth of the established lawn bacteria. Conversely, none of the tested bacterial strains showed growth on the *P. aeruginosa* PAO1 lawn indicating that *P. aeruginosa* PAO1 actively suppressed the growth of the five other strains. Limited growth of *E. coli* pEBFP and *S. maltophilia* K279 was observed on *P. aeruginosa* 2192 and *B. dolosa* AU0158 lawns suggesting that *P. aeruginosa* 2192 and *B. dolosa* AU0158

Figure 2. Co-culture growth on single species lawn. **(A)** Layout of co-culture growth on bacterial lawns. Strains spotted on top of lawn are indicated. Approximate numbers of bacteria from each strain indicated on right for each spot. **(B)** Columns of individual bacterial species decreasing in concentration plated on an established lawn of single bacteria. Example plate shown here in which *P. aeruginosa* PAO1 was used for lawn on TSA medium.

A.

B.

Table 2. Morphological growth observations in spotted series on established bacterial lawn (LB).

Spotted Strain	Lawn Strain						
	Strain	<i>P. aeruginosa</i>	<i>P. aeruginosa</i>	<i>S. maltophilia</i>	<i>B. dolosa</i>	<i>B. cenocepacia</i>	<i>E. coli</i>
		2192	PAO1	K279a	AU0158	K56-2	pEBFP
<i>P. aeruginosa</i>	2192	O*	N [#]	O	O	O	O
<i>P. aeruginosa</i>	PAO1	O	O	O	O	O	O
<i>S. maltophilia</i>	K279a	N	N	O	N	O	O
<i>B. dolosa</i>	AU0158	O	N	O	O	O	O
<i>B. cenocepacia</i>	K56-2	O	N	O	O	O	O
<i>E. coli</i>	pEBFP	N	N	O	N	O	O

* O - Observed growth

- N - No observed growth

may slightly inhibit the growth of *E. coli* and *S. maltophilia* strains. The spot lawn assays were also replicated on an additional nutrient-rich medium, tryptic soy agar (TSA) to verify the results. The results were the same for growth on LB agar with two exceptions: *E. coli* pEBFP and *S. maltophilia* K279a showed no growth on *B. cenocepacia* K56-2 lawns; and *B. dolosa* AU0158 exhibited growth on the *P. aeruginosa* PAO1 lawn (Table 3). These results suggest that the medium on which the strains are plated may play a slight role in growth inhibition, as growth inhibition was different between TSA and LB media.

We conclude from these experiments that when grown in low-density lawns, *P. aeruginosa* PAO1 is an inhibitor of the tested strains and likewise is capable of growing on lawns of all the tested strains. *P. aeruginosa* PAO1 is known to produce a variety of toxins, siderophores and other compounds that may play a role in inhibiting growth of other strains; however, the mechanism for the growth inhibition in these studies remains to be explored. In both assays, *E. coli* pEBFP and *S. maltophilia* K279a do not inhibit the growth of any other strain. This was expected for *E. coli* pEBFP which is a lab strain that perhaps has lost the ability to compete with other bacteria. However, *S. maltophilia* K279a is a recent human isolate from the blood stream and *S. maltophilia* is commonly found in soils; thus it is surprising that it does not inhibit the growth of other strains.

P. aeruginosa, and *S. maltophilia* are simultaneously isolated from CF patients in many clinics. In addition, *S. aureus* is another CF pathogen that commonly exists in co-infections in CF patients; yet how it interacts with *P. aeruginosa* and *S. maltophilia* has not yet been studied. To address this, the transcriptomes of these species were analyzed to identify those genes whose expression changes in co-culture with other pathogens.

Table 3. Morphological growth observations in spotted series on established bacterial lawn (TSA).

Spotted Strain	Lawn Strain						
	Strain	<i>P. aeruginosa</i>	<i>P. aeruginosa</i>	<i>S. maltophilia</i>	<i>B. dolosa</i>	<i>B. cenocepacia</i>	<i>E. coli</i>
		2192	PAO1	K279a	AU0158	K56-2	pEBFP
	<i>P. aeruginosa</i> 2192	O*	N [#]	O	O	O	O
	<i>P. aeruginosa</i> PAO1	O	O	O	O	O	O
	<i>S. maltophilia</i> K279a	N	N	O	N	N	O
	<i>B. dolosa</i> AU0158	O	O	O	O	O	O
	<i>B. cenocepacia</i> K56-2	O	N	O	O	O	O
	<i>E. coli</i> pEBFP	N	N	O	N	N	O

* O - Observed growth

- N - No observed growth

Growth curves

To further understand the growth dynamics of pathogens that are common in the CF model we chose to include *S. aureus* in our research. To identify conditions under which CF pathogens could be grown, growth curves were performed with *P. aeruginosa*, *S. aureus* and *S. maltophilia*. This was done in order to identify which medium would be best suited for polymicrobial analysis as our previous experiments had differing results depending on the growth medium (Tables 2 and 3). The strains chosen from the proximal growth, and co-culture lawn tests were *P. aeruginosa* 2192 (chosen because it is a CF isolate rather than PAO1 which is a burn/lab isolate and because of the global inhibitory effects that *P. aeruginosa* PAO1 on the other tested strains) and *S. maltophilia* K279a, a blood isolate from a septic cancer patient (Crossman, 2008). *S. aureus* NRS77 is a blood isolate from a septic patient (Sassi, 2014). All three of these isolates were chosen because their genome sequences were publicly available; however no genomes are available for CF isolates of *S. aureus* or *S. maltophilia*.

Over 24 hours, *S. aureus* NRS77, *P. aeruginosa* 2192 and *S. maltophilia* K279a were grown in an artificial CF sputum medium (SCFM) that nutritionally mimics CF sputum. The *P. aeruginosa* growth in this medium yields a similar transcriptomic response as cells grown in actual CF sputum (Palmer, 2005; Palmer, 2007). The growth rate of *S. maltophilia* K279a was significantly lower than the *P. aeruginosa* 2192 growth rate (Table 4). The generation time during exponential growth of *P. aeruginosa* 2192 was significantly faster than that of *S. maltophilia* K279a. *S. maltophilia* K279a also had a lower growth yield after 24 hours compared to *P. aeruginosa* 2192 (Supplementary Table 1, Figure 3). *S. aureus* NRS77 on the other hand showed a slight decrease in

optical density from its inoculum O.D.₆₀₀ value (Figure 3). From this we conclude that *S. aureus* NRS77 would not grow in SCFM media and suggests that the SCFM medium could not be used to study co-culture growth of *S. aureus* NRS77. The lack of growth in this medium could be due to its sterile, limited nutrient composition. Each component added was filter sterilized and a defined amount of amino acids were included. Whereas in other nutrient rich media like TSA and LB there are compounds like yeast extract that contain partially digested proteins, vitamins, etc. that may allow for better growth of *S. aureus* NRS77.

To test to see if there was a growth medium that would provide similar growth rates and/or stationary O.D.₆₀₀ measurements better than SCFM medium for all three tested strains, a SEM growth curve was constructed for each bacterial species. The SEM media mimics soil conditions, where *P. aeruginosa* and *S. maltophilia* are naturally found. Despite *S. aureus* NRS77 not commonly being found in the soil, we decided to see if it would grow in this medium with the supplementation of 30 mM of glucose. In this medium, *S. maltophilia* K279a was the fastest growing strain, where *P. aeruginosa* 2192 had slower growth (Figure 4). *S. aureus* NRS77 showed no statistical evidence of growth in this medium (Supplementary Table 2 and Table 4). These data lead us to believe that the best growth medium to be used in co-culture experiments would have to be a previously defined medium in which *S. aureus* was known to grow.

The nutrient medium LB allows the growth of many bacterial strains. In this nutrient broth, each bacterial sample grew in logarithmic fashion (Figure 5). *S. aureus* NRS77 showed exponential growth and, after 24 hours obtained final O.D.₆₀₀ values that

Figure 3. Growth curves of *P. aeruginosa* 2192 (mucoid strain), *S. aureus* NRS77 and *S. maltophilia* K279a grown in artificial CF sputum medium. Optical density at 600 nm (O.D.₆₀₀) was measured for three biological replicates over the course of 24 hours. Averages were calculated and plotted for each strain. Error bars represent one standard deviation of the data. Dashed lines representative of time-points where data was not collected.

Figure 4. Growth curves of *P. aeruginosa* 2192 (mucoid strain), *S. maltophilia* K279a, and *S. aureus* NRS77 grown in soil extract medium supplemented with 30 mM glucose. Optical density at 600 nm (O.D.₆₀₀) was measured for two biological replicates over the course of 24 hours. Averages were calculated and plotted for each strain. Error bars represent one standard deviation of the data. Dashed lines representative of time-points where data was not collected.

were more similar to that observed in *P. aeruginosa* 2192 and *S. maltophilia* K279a than they were in both SEM and SCFM media (Supplementary Table 3). *P. aeruginosa* PAO1 typically grows in LB at a high rate within doubling times of 20-30 minutes. In our experiment, the mucoid strain exhibited a much slower growth rate and doubling time, perhaps due to the energy costs of producing high levels of alginate. *S. maltophilia* K279a also exhibited exponential growth but was significantly lower than both *P. aeruginosa* 2192 and *S. aureus* NRS77 in final O.D.₆₀₀ value (Figure 5, Table 4). A reason why *S. maltophilia* K279a may have had a lower growth than the others in a nutrient rich broth could be due to the optimal growth temperature for *S. maltophilia* (35°C), and we grew these bacteria at 37°C, the optimal temperatures for *P. aeruginosa* and *S. aureus* (Denton, 1998; Barbier, 2014; Rajkovic, 2006). Despite the stationary phases and generation times being different from each other, each bacterial strain in this tested medium grew at an exponential rate and achieved significant levels of growth in comparison with the SCFM and SEM growth curves and have thereby shown to be more useful than either media in further experiments.

Transcriptomic analysis

In order to obtain transcriptome information from *P. aeruginosa* 2192, *S. maltophilia* K279a and *S. aureus* NRS77, mono-, co- and tri-culture samples were prepared in an LB medium. The three conditions tested were: logarithmic phase in LB broth (“liquid log”), stationary phase in LB broth (“stationary”), or grown on LB agar for 2.5 hours (“plated”). Cells were harvested and the total RNA was isolated as described in

Figure 5. Growth curves of *P. aeruginosa* 2192, *S. maltophilia* K279a and *S. aureus* NRS77 grown in LB medium. Optical density at 600 nm (O.D.₆₀₀) was measured for two biological replicates over the course of 24 hours. Averages were calculated and plotted for each strain. Error bars represent one standard deviation of the data. Dashed lines representative of time-points where data was not collected.

Table 4. Growth rate, generation time and final O.D.₆₀₀ of *P. aeruginosa* 2192, *S. maltophilia* K279a, and *S. aureus* NRS77 in SCFM, SEM and LB medium.

Media	Strain	Growth rate (/min)	Generation time (min)	Stationary O.D. ₆₀₀
SCFM*	<i>P. aeruginosa</i> 2192	0.00783 ± 0.000161	89.1 ± 1.83	2.426 ± 0.08
	<i>S. maltophilia</i> K279a	0.00700 ± 0.000264	99.77 ± 3.69	2.194 ± 0.01
	<i>S. aureus</i> NRS77	NG ⁺	NG	NG
SEM [#]	<i>P. aeruginosa</i> 2192	0.001428 ± 0.000041	180.24 ± 10.41	1.107 ± .001
	<i>S. maltophilia</i> K279a	0.002729 ± 0.00018	144.5 ± 2.98	.632 ± .103
	<i>S. aureus</i> NRS77	NG	NG	NG
LB [✧]	<i>P. aeruginosa</i> 2192	0.003878 ± 0.000224	180.24 ± 10.41	5.15 ± .056
	<i>S. maltophilia</i> K279a	0.00483 ± 0.001	144.49 ± 2.979	2.83 ± 1.0
	<i>S. aureus</i> NRS77	0.00616 ± 0.000105	113.2 ± 1.93	5.83 ± .007

* - Synthetic Cystic Fibrosis Medium

- Soil Extract Medium

✧ - Luria Broth

⁺ - No growth

the Materials and Methods section. Total RNA was examined for its quality after DNaseI treatment by quantifying it on a spectrophotometer (Supplementary Table 4) and by agarose gel electrophoresis (Supplementary Figure 1). Gel electrophoresis yielded bands approximately corresponding to the expected 16S and 23S rRNA sizes of 1.5 and 2.9 kb respectively for most samples. *S. aureus* NRS77 also had bands at the expected sizes that were subtly discernable to the naked eye, but not reproducible using the Versadoc Gel Imaging apparatus (Supplementary Figure 1). Even though this detection was minimal, the concentration obtained was still suitable for continuation in purification and sequencing preparation. Each sample was then fragmented, converted to double-stranded DNA and ligated to Illumina-compatible multiplex oligonucleotides. These oligonucleotides incorporated a six base pair barcode on each adaptor-ligated cDNA (Supplementary Table 5). Each combination of strains was given a unique barcode. For example, all fragmented cDNAs from *P. aeruginosa* 2192 grown in monoculture were labeled with a CGTGAT barcode. In combination with another strain, the barcode would be different. Final purified cDNAs were assessed for quality using a spectrophotometer prior to sequencing to establish whether there was sufficient quantity and quality for submission (Supplementary Table 6). *S. aureus* NRS77 values in the plated and stationary conditions were not as high as *P. aeruginosa* 2192 or *S. maltophilia* K279a but did meet or exceed the 10 ng/μL threshold set for submission. Each sample was then submitted to the University of Wisconsin-Madison's DNA Sequencing Facility for sequencing on an Illumina Hi-Seq 2000 machine following quality assessments via an Agilent Bioanalyzer performed at the facility (Supplementary Figure 2).

The resultant reads were then aligned to the *P. aeruginosa* 2192, *S. maltophilia* K279a and *S. aureus* NRS77 genome sequences to verify that each sample contained RNAs corresponding to the appropriate strains (Table 5). Corresponding with a ten-to-twenty-fold coverage of genes within each genome, a 50,000 read lower threshold was established as the minimum number of reads in order for any combination and condition to be considered for further analysis. Sequenced samples aligned to genomes showed that despite an adequate number of reads in the plated and stationary liquid monoculture samples, *S. aureus* NRS77 was not represented by more than 50,000 reads in any of the co- or tri-culture experiments, thereby excluding *S. aureus* NRS77 sequencing data from further analysis. *P. aeruginosa* 2192 and *S. maltophilia* K279a on the other hand, were found in both plated and log liquid conditions to have reads exceeding the set minimum for statistical interpretation in both their monoculture and co-culture sequence data. The assessment of the libraries based on aligned reads to each genome yielded some overlap between monocultures of *P. aeruginosa* 2192 and *S. maltophilia* K279a, even when there was theoretically no *S. maltophilia* K279a in the sample. This however, may have been an aberration in the sequence alignment software. In the computation of reads, if there were paralogous genes in the genome to which the read could be aligned successfully, the read would be seen as redundant and automatically discarded (as the program can not discern which gene was expressed using this read thus it is removed from further analysis). This would artificially decrease the reads attributed to *P. aeruginosa* 2192 to these genes. *P. aeruginosa* 2192 is a pathogen that has been shown to possess such redundancies (Recinos, 2012).

Table 5. Number of reads aligned to each genome and assessment of each library based on reads by Sanger Sequencing according to indicated reference genomes.

	Culture used to prepare RNA/ cDNA*	Genome used for alignment		
		<i>P. aeruginosa</i> 2192	<i>S. aureus</i> NRS77	<i>S. maltophilia</i> K279a
Plated	PA	105,045	2,099	157,597
	SA	2,783	67,569	19,126
	SM	1,832	363	210,377
	PA+SA	3,358	300	9,462
	PA+SM	55,709	1,468	176,646
	SA+SM	446	94	26,022
	PA+SA+SM	87,953	3,164	296,878
Stationary Liquid	PA	112,310	2,077	111,885
	SA	1,704	252,123	15,555
	SM	8,497	856	156,214
	PA+SA	23,213	1,049	16,470
	PA+SM	35,245	463	171,985
	SA+SM	1,046	176	120,279
	PA+SA+SM	115,253	1,794	367,663
Log Liquid	PA	67,310	1,958	122,784
	SA	1,456	15,471	15,080
	SM	2,532	525	249,767
	PA+SA	3,060	135	83,186
	PA+SM	55,552	912	132,702
	SA+SM	4,050	1,373	50,139
	PA+SA+SM	99,333	2,588	215,998

* PA- *P. aeruginosa* 2192; SM- *S. maltophilia* K279a; SA- *S. aureus* NRS77

Table 6 displays the number of genes with at least a single aligned read. This was done to examine the percent of the genes in the genome that are represented in each sample. In each monoculture sample of *P. aeruginosa* 2192, genes with at least one read were near 90%, which is strong support for the sequenced sample correctly representing the supposed species in that sample. In the case of *S. maltophilia* K279a however, while the reads aligned in the stationary phase are high at 96%, the percent of more than one read in the plated and liquid log conditions are low at 6 and 44 percent, respectively. Despite these values being low, the genes aligned to the genome in both of these conditions for *S. maltophilia* K279a are high (Table 5). This can once again be attributed to paralogs being credited with the read and may be an effect of the sequencing software. The tri-culture sample had one or more reads for 89% and above in the *S. maltophilia* K279a genome, and 91% for *P. aeruginosa* 2192 in all three conditions. In tri-culture, *S. aureus* NRS77 had at least one read corresponding with each gene for 47% of the genes in the plated, 33% of the genes in the stationary and 40% for the genes in the log liquid conditions. This was less than half of the other two bacteria in the sample. This may have been the case due to possible growth inhibition by *S. maltophilia* K279a and/or *P. aeruginosa* 2192. This is confirmed also by the data in Table 5 which shows a low number of aligned reads to the *S. aureus* NRS77 genome from the tri-culture sample.

The fold coverage of each sample for the respective genomes showed that there was fold coverage exceeding 10X for *P. aeruginosa* 2192, and *S. maltophilia* K279a in all growth conditions (Table 7). In *S. aureus* NRS77 monoculture there was high fold coverage in the stationary phase of 87.79X and a low in the log liquid condition of 5.39X, with a 23.35X fold coverage in the plated condition. However, in tri- and co-culture

Table 6. Number and percent of genes in each reference genome with >0 reads aligned

	Culture used to prepare RNA/ cDNA*	Reference genome		
		<i>P. aeruginosa</i> 2192	<i>S. aureus</i> NRS77	<i>S. maltophilia</i> K279a
Plated	PA	5,533 (89%)	1,003 (35%)	3,163 (71%)
	SA	1,596 (26%)	2,623 (91%)	1,144 (26%)
	SM	1,128 (18%)	1,566 (55%)	253 (6%)
	PA+SA	1,039 (17%)	197 (7%)	469 (11%)
	PA+SM	5,180 (83%)	788 (27%)	3,781 (85%)
	SA+SM	330 (5%)	66 (2%)	579 (13%)
Stationary Liquid	PA+SA+SM	5,667 (91%)	1,357 (47%)	4,073 (92%)
	PA	5,833 (94%)	1,052 (37%)	2,867 (65%)
	SA	727 (12%)	2,799 (97%)	733 (17%)
	SM	2,722 (44%)	488 (17%)	4,249 (96%)
	PA+SA	2,172 (35%)	414 (14%)	1,005 (23%)
	PA+SM	4,544 (73%)	345 (12%)	4,158 (94%)
Log Liquid	SA+SM	666 (11%)	129 (4%)	2,392 (54%)
	PA+SA+SM	5,671 (91%)	946 (33%)	4,343 (98%)
	PA	5,180 (83%)	929 (32%)	2,932 (66%)
	SA	927 (15%)	1,952 (68%)	728 (16%)
	SM	1,269 (20%)	321 (11%)	1,930 (44%)
	PA+SA	1,118 (18%)	98 (3%)	397 (9%)
	PA+SM	5,106 (82%)	538 (19%)	3,605 (81%)
	SA+SM	2,034 (33%)	680 (24%)	3,486 (79%)
	PA+SA+SM	5,700 (91%)	1,155 (40%)	3,922 (89%)
	Total # of genes in genome	6,233	2,872	4,430

* PA- *P. aeruginosa* 2192; SM- *S. maltophilia* K279a; SA- *S. aureus* NRS77

Table 7. Fold coverage of reference genomes by Illumina reads.

	Culture used to prepare RNA/ cDNA*	Reference genome		
		<i>P. aeruginosa</i> 2192	<i>S. aureus</i> NRS77	<i>S. maltophilia</i> K279a
Plated	PA	16.85	0.73	35.57
	SA	0.45	23.53	4.32
	SM	0.29	0.13	47.49
	PA+SA	0.54	0.1	2.14
	PA+SM	8.94	0.51	39.87
	SA+SM	0.07	0.03	5.87
	PA+SA+SM	14.11	1.1	67.02
Stationary Liquid	PA	18.02	0.72	25.26
	SA	0.27	87.79	3.51
	SM	1.36	0.3	35.26
	PA+SA	3.72	0.37	3.72
	PA+SM	5.65	0.16	38.82
	SA+SM	0.17	0.06	27.15
	PA+SA+SM	18.49	0.62	82.99
Log Liquid	PA	10.8	0.68	27.72
	SA	0.23	5.39	3.4
	SM	0.41	0.18	56.38
	PA+SA	0.49	0.05	18.78
	PA+SM	8.91	0.32	29.96
	SA+SM	0.65	0.48	11.32
	PA+SA+SM	15.94	0.9	48.76

* PA- *P. aeruginosa* 2192; SM- *S. maltophilia* K279a; SA- *S. aureus* NRS77

experiments, *S. aureus* NRS77 showed a relatively smaller increase in fold coverage as all values for combination growth were at or below 1.10X in comparison to the other bacteria in the combination. Co-culture of *P. aeruginosa* 2192 and *S. maltophilia* K279a in all conditions had fold coverage of 5X or higher. Despite the fold coverage not being highly convincing of *S. aureus* NRS77 mRNA presence in combination samples, the data for *P. aeruginosa* 2192 and *S. maltophilia* K279a are indicative that the harvested mRNA from the mono- and co-culture samples indicative of their respective mRNA being found in the sample. This, taken with the data from Tables 4 and 5, indicate that genes in the combination samples of *P. aeruginosa* 2192 and *S. maltophilia* K279a are the most suitable to interpret in comparison to their monoculture growth in the plated and liquid log conditions.

Genes up-regulated in *S. maltophilia* K279a in response to co-culture with *P. aeruginosa* 2192

In order to identify the genes that were differentially regulated in *S. maltophilia* K279a when grown in the presence of *P. aeruginosa* 2192, the CLC Genomics Workbench analysis program was used. The *in silico* experiment of the co-culture *P. aeruginosa* 2192 and *S. maltophilia* K279a yielded *S. maltophilia* K279a genes that were up-regulated when compared to their monoculture growth, many of which displayed *p*-values of <0.05. An empirical limit was placed on the generated data to identify the genes with a fold change of 25 or higher. These data consisted of 140 up-regulated genes of *S. maltophilia* K279a in co-culture growth with *P. aeruginosa* 2192 compared to monoculture growth in the plated condition (Table 8). The same combination in the liquid log condition resulted

in 170 upregulated genes with the same fold increase (Table 9). Between the two data sets there were 84 common genes that were up-regulated in *S. maltophilia* K279a (Table 10). Many of these genes encode membrane-associated proteins, which was expected in co-culture growth. In both conditions some genes were found to come from the same operon. The genes *smeF* and *smeE*, for example, are located in the *smeDEF* operon (Zhang, 2001). This illustrates the fact that not only individual genes, but also operons can be up-regulated for a suite of coordinated responses (Alonso, 2001). In this case the *smeDEF* operon contains multidrug resistance genes that encode for a membrane protein (SmeF), an acriflavin resistance protein (SmeE) and a multidrug efflux pump (SmeD) (Zhang, 2001).

Genes down-regulated in *S. maltophilia* K279a in response to co-culture with *P. aeruginosa* 2192

The same experiment as above was conducted to identify the down-regulated genes of *S. maltophilia* K279a when grown in combination with *P. aeruginosa* 2192. Because there were no genes down-regulated to an equal degree as those up-regulated, the empirical limit of fold change was adjusted a five-fold decrease with a statistical significance of a *p*-value <0.05. In either the plated or liquid log conditions there were no genes that were down-regulated more than this value in *S. maltophilia* K279a when grown in combination with *P. aeruginosa* 2192. This leads us to believe that decreasing gene transcription from relative rates in monoculture growth is non-advantageous for *S. maltophilia* K279a when in co-culture with *P. aeruginosa* 2192 regardless of condition.

Table 8. Up-regulated *S. maltophilia* K279a genes when grown in the presence of *P. aeruginosa* 2192 in plated conditions compared to monoculture (>25-fold increase).

Gene	Fold change	SM means	SM+PA means	Annotation
<i>rplK</i>	119.1	11.08	1,319.59	50S ribosomal protein L11
<i>rplS</i>	98.85	11.82	1,168.82	50S ribosomal protein L19
<i>gyrA</i>	88.13	1.75	154.3	DNA gyrase subunit A
<i>acpP_1</i>	78.6	19.81	1,556.79	hypothetical protein
<i>smeF</i>	76.22	3.39	258.61	multidrug resistance outer membrane protein
<i>purL</i>	71.46	1.22	87.43	phosphoribosylformylglycinamide synthase
<i>sodA_2</i>	71.46	7.77	555	manganese superoxide dismutase
<i>accC</i>	70.27	3.47	244.15	biotin carboxylase
Smlt0184	69.08	16.5	1,140.07	conserved hypothetical protein
<i>tig</i>	61.93	3.67	227.14	trigger factor
<i>lon</i>	60.74	1.94	117.94	ATP-dependent protease
<i>rplL</i>	59.55	25.76	1,534.16	50S ribosomal subunit protein L7/L12
Smlt1498	57.17	1.95	111.27	outer membrane protein
<i>parC</i>	55.97	2.12	118.57	DNA topoisomerase IV subunit A
<i>lysS</i>	52.4	3.14	164.74	lysyl-tRNA synthetase
<i>alaS</i>	51.21	1.79	91.89	alanyl-tRNA synthetase
<i>fliC</i>	51.21	3.89	199.37	flagellin
<i>hupB</i>	51.21	17.41	891.67	DNA-binding protein HU-beta
<i>pnp</i>	51.21	9.02	461.69	polyribonucleotide phosphorylase
<i>carB</i>	50.62	2.93	148.38	carbamoyl-phosphate synthase large chain
<i>pheT</i>	50.02	2	99.82	phenylalanyl-tRNA synthetase beta chain
<i>qoxA</i>	47.64	4.57	217.52	transmembrane ubiquinol oxidase subunit 2 precursor
<i>rpsG</i>	47.64	20.06	955.45	30S ribosomal protein S7
<i>nuoB</i>	46.45	8.56	397.8	NADH dehydrogenase I chain B
<i>gltX</i>	45.26	3.39	153.22	glutamyl-tRNA synthetase
<i>dnaJ</i>	45.26	4.23	191.22	chaperone DnaJ protein
<i>eno</i>	45.26	3.68	166.37	enolase
<i>glyA</i>	45.26	3.79	171.55	serine hydroxymethyltransferase
<i>nuoJ</i>	44.07	7.23	318.81	NADH-ubiquinone oxidoreductase, chain J
Smlt3703	43.67	27.48	1,199.83	peptidoglycan-associated lipoprotein
<i>pyrG</i>	42.87	2.85	122.4	CTP synthase
Smlt0716	42.87	2.85	122.4	ABC transporter component protein
<i>pps</i>	42.87	4.14	177.6	7 phosphoenolpyruvate synthase
<i>sucD</i>	42.87	16.28	697.94	succinyl-CoA synthetase alpha chain
<i>fusA</i>	41.85	31.07	1,300.29	elongation factor G
<i>dnaX</i>	41.68	2.34	97.41	DNA polymerase III subunit Tau
<i>smeE</i>	40.69	9.13	371.6	acriflavin resistance protein B
Smlt2059	40.49	3.92	158.81	PQQ containing lipoprotein
Smlt0982	40.49	4.73	191.52	isocitrate/isopropylmalate dehydrogenase
<i>rpsT</i>	40.1	52.82	2,117.65	30S ribosomal protein S20

Table 8 ctd. Up-regulated *S. maltophilia* K279a genes when grown in the presence of *P. aeruginosa* 2192 in plated conditions compared to monoculture (>25-fold increase).

Gene	Fold change	SM means	SM+PA means	Annotation
<i>pilY1</i>	39.3	1.25	49.3	PilY1 protein
<i>etfS</i>	39.3	6.36	250.09	electron transfer flavoprotein subunit beta
<i>speA</i>	39.3	2.52	98.84	biosynthetic arginine decarboxylase
<i>rplJ</i>	38.41	35.41	1,359.91	50S ribosomal subunit protein L10
<i>rpoC</i>	38.31	13.51	517.69	DNA-directed RNA polymerase beta' chain
<i>atpF</i>	38.11	10.09	384.61	ATP synthase B chain
<i>rluB</i>	38.11	2.92	111.21	ribosomal large subunit pseudouridine synthase B
<i>rpsN</i>	38.11	15.53	592	30S ribosomal protein S14
<i>guaB</i>	37.52	6.52	244.61	inosine-5'-monophosphate dehydrogenase
<i>cspG</i>	36.92	22.64	835.68	cold shock protein
<i>smeP</i>	36.92	1.5	55.5	drug-resistance cell envelope-related protein
<i>Smlt1368</i>	36.92	2.06	76.07	conserved hypothetical protein
<i>lipA</i>	35.73	4.7	167.98	lipoic acid synthetase
<i>metG</i>	35.73	2.29	81.69	methionyl-tRNA synthetase
<i>nuoM</i>	35.73	3.15	112.55	NADH dehydrogenase I chain M
<i>smeH</i>	35.73	3	107.01	multidrug resistance efflux pump
<i>sppA</i>	35.73	2.47	88.32	protease IV
<i>topA</i>	35.73	1.91	68.12	DNA topoisomerase I
<i>atpD</i>	35.13	27.03	949.54	ATP synthase beta chain
<i>cyoA</i>	34.54	5.26	181.81	cytochrome O ubiquinol oxidase subunit II
<i>nusG</i>	34.54	8.47	292.64	transcription antitermination protein
<i>pcm_2</i>	34.54	7.34	253.35	hypothetical protein
<i>Smlt0083</i>	34.54	2.09	72.29	TonB dependent receptor protein
<i>Smlt2132</i>	34.54	3.1	107.09	aldehyde dehydrogenase
<i>lpdA</i>	33.94	5.26	178.37	dihydrolipoamide dehydrogenase
<i>icd</i>	33.74	6.41	216.46	isocitrate dehydrogenase
<i>leuS</i>	33.74	5.4	182.06	leucyl-tRNA synthetase
<i>accB</i>	33.35	9.9	330.23	biotin carboxyl carrier protein of acetyl-CoA carboxylase
<i>ahpF</i>	33.35	2.98	99.5	alkyl hydroperoxide reductase subunit F
<i>carA</i>	33.35	4.02	134.1	carbamoyl-phosphate synthase small chain
<i>prfC</i>	33.35	2.96	98.76	peptide chain release factor
<i>Smlt3526</i>	32.58	194.58	6,339.70	outer membrane lipoprotein
<i>accD</i>	32.16	5.35	172.13	acetyl-coenzymeA carboxylase carboxyl transferase subunit beta
<i>glmU</i>	32.16	3.47	111.73	UDP-N-acetylglucosamine synthesis bifunctional protein
<i>rnE</i>	32.16	4.33	139.21	ribonuclease E
<i>appB</i>	32.16	8.25	265.36	transmembrane cytochrome bd-II oxidase subunit II
<i>rplQ</i>	32.16	37.14	1,194.13	50S ribosomal protein L17
<i>Smlt3330</i>	32.16	3.04	97.79	N-acetylmuramoyl-L-alanine amidase
<i>rplO</i>	31.86	42.82	1,364.26	50S ribosomal protein L15

Table 8 ctd. Up-regulated *S. maltophilia* K279a genes when grown in the presence of *P. aeruginosa* 2192 in plated conditions compared to monoculture (>25-fold increase).

Gene	Fold change SM means		SM+PA means	Annotation
<i>exbD1</i>	30.96	11.16	345.51	biopolymer transport ExbD1 protein
<i>narK</i>	30.96	3.35	103.73	Major Facilitator Superfamily transmembrane nitrite extrusion protein
<i>metK</i>	30.96	7.84	242.88	S-adenosylmethionine synthetase
<i>recG</i>	30.96	2.25	69.69	ATP-dependent DNA helicase
<i>rpsR</i>	30.96	20.58	637.17	30s ribosomal subunit protein S18
Smlt3796	30.96	4.15	128.44	conserved hypothetical exported protein
<i>moaA</i>	30.96	4.85	150.04	molybdenum cofactor biosynthesis protein A
<i>betB</i>	29.77	3.23	96.08	betaine aldehyde dehydrogenase
<i>comL</i>	29.77	5.35	159.38	competence lipoprotein precursor
Smlt2039	29.77	4.58	136.34	deiminase
<i>frr</i>	29.77	8.56	255	ribosome recycling factor
<i>ftsI</i>	29.77	2.58	76.71	penicillin-binding protein 3 precursor
<i>secF</i>	29.77	4.85	144.27	protein-export membrane protein SecF
Smlt3472	29.77	1.23	36.71	conserved hypothetical exported protein
<i>rpsS</i>	29.18	35.21	1,027.37	30S ribosomal protein S19
<i>rplT</i>	29.18	26.41	770.53	50S ribosomal protein L20
<i>rpsM</i>	29.18	26.63	777.01	30S ribosomal protein S13
<i>rpsA</i>	28.81	59.21	1,705.70	30S ribosomal protein S1
<i>cysS</i>	28.58	3.45	98.67	cysteinyl-tRNA synthetase
Smlt4628	28.58	2.21	63.25	conserved hypothetical exported protein
<i>fdnG</i>	28.58	1.55	44.27	formate dehydrogenase-o, major subunit
<i>hflX</i>	28.58	3.63	103.63	GTP-binding phage-related protein
<i>serS</i>	28.58	3.71	106.06	seryl-tRNA synthetase
Smlt4339	28.58	4.45	127.21	branched-chain alpha keto acid dehydrogenase E1 beta subunit
Smlt4575	28.58	3.4	97.19	cytochrome oxidase subunit I
<i>tufB</i>	28.29	15.96	451.55	elongation factor Tu (Ef-Tu)
<i>rplN</i>	28.19	77.29	2,178.50	50S ribosomal protein L14
<i>rplB</i>	27.89	68.89	1,921.20	50S ribosomal protein L2
<i>atpC_2</i>	27.39	31.07	851.01	hypothetical protein
<i>gcvT</i>	27.39	4.27	116.98	aminomethyltransferase
<i>minD</i>	27.39	11.74	321.49	septum site-determining protein
Smlt2781	27.39	4.92	134.79	molybdenum cofactor biosynthesis protein (secreted protein)
Smlt3210	27.39	11.28	308.91	outer membrane antigen protein
Smlt4670	27.39	6.02	165.02	ABC transporter ATP-binding protein
<i>rmlB</i>	27.39	4.5	123.3	dTDP-glucose 4,6-dehydratase
Smlt3652	27.39	3.39	92.94	beta-lactamase protein
<i>rplE</i>	26.44	43.77	1,157.23	50S ribosomal protein L5
<i>dapF</i>	26.2	5.58	146.18	diaminopimelate epimerase

Table 8 ctd. Up-regulated *S. maltophilia* K279a genes when grown in the presence of *P. aeruginosa* 2192 in plated conditions compared to monoculture (>25-fold increase).

Gene	Fold change	SM means	SM+PA means	Annotation
<i>smeG</i>	26.2	3.7	97	RND family acriflavine resistance protein A precursor
<i>adhB</i>	26.2	3.53	92.46	alcohol dehydrogenase cytochrome c subunit
<i>argS</i>	26.2	5.63	147.48	arginyl-tRNA synthetase
<i>gcvH</i>	26.2	12	314.5	glycine cleavage system H protein
<i>rpmH</i>	26.2	67.42	1,766.57	50S ribosomal protein L34
<i>Smlt0777</i>	26.2	1.89	49.48	conserved hypothetical protein
<i>Smlt2831</i>	26.2	4.14	108.39	transmembrane LINOLEOYL-CoA DESATURASE (DELTA(6)-DESATURASE)
<i>qoxB</i>	25.9	9.49	245.76	quinol oxidase subunit 1
<i>surA</i>	25.61	7.07	181.12	survival protein SurA precursor
<i>groES</i>	25.61	33.01	845.22	10 kDa chaperonin
<i>dxs</i>	25.01	2.49	62.31	1-deoxy-d-xylulose-5-phosphate synthase
<i>mtcC</i>	25.01	4.01	100.32	cystathionine beta/gamma-lyase
<i>Smlt0277</i>	25.01	6.95	173.8	two-component transcriptional regulator response regulatory protein
<i>Smlt1133</i>	25.01	3.18	79.57	peptidase
<i>Smlt1704</i>	25.01	3.62	90.47	transmembrane CorC/HlyC family transporter
<i>Smlt3195</i>	25.01	3.36	83.96	conserved hypothetical protein
<i>Smlt3481</i>	25.01	11.57	289.25	conserved hypothetical protein
<i>Smlt3854</i>	25.01	5.52	138.07	YicC family protein
<i>Smlt4263</i>	25.01	5.51	137.83	ATP-dependent RNA helicase
<i>tpiA</i>	25.01	6.29	157.25	triosephosphate isomerase
<i>aroC</i>	25.01	4.31	107.68	chorismate synthase
<i>folE</i>	25.01	7.77	194.25	GTP cyclohydrolase I
<i>rho</i>	25.01	7.42	185.61	transcription termination factor

Table 9. Up-regulated *S. maltophilia* K279a genes when grown in the presence of *P. aeruginosa* 2192 in liquid log conditions compared to monoculture (>25-fold increase).

Gene	Fold change	SM means	SM+PA means	Annotation
<i>rplV</i>	114.57	11.71	1,341.30	50S ribosomal protein L22
<i>betA</i>	94.65	2.38	225.16	choline dehydrogenase
<i>Smlt0685</i>	84.69	0.55	46.92	repetitive surface protein
<i>metG</i>	79.7	1.93	153.49	methionyl-tRNA synthetase
<i>lpdA</i>	74.72	2.21	165.38	dihydrolipoamide dehydrogenase
<i>sucB</i>	74.72	3.33	248.69	dihydrolipoamide succinyltransferase E2 component
<i>gyrB</i>	69.74	1.63	113.51	DNA gyrase subunit B
<i>rpsH</i>	64.76	10.03	649.82	30S ribosomal protein S8
<i>rplX</i>	64.76	25.18	1,630.69	50S ribosomal protein L24
<i>Smlt0184</i>	64.76	13.9	900.28	conserved hypothetical protein
<i>Smlt0383</i>	64.76	4.48	290.02	histone H1-like protein
<i>mgtA</i>	64.76	1.45	93.94	transmembrane Mg(2+) transport ATPase
<i>putA</i>	59.78	1.24	74.35	bifunctional PutA protein
<i>gltA</i>	59.78	6.27	374.55	citrate synthase
<i>clpB</i>	59.78	1.55	92.55	heat shock chaperone ClpB
<i>rpsM</i>	54.8	11.21	614.54	30S ribosomal protein S13
<i>cirA</i>	54.8	1.87	102.57	colicin I receptor precursor
<i>Smlt3905</i>	54.8	2.71	148.34	TonB dependent receptor
<i>groEL</i>	53.97	14.56	785.69	GroEL protein
<i>metH1</i>	49.81	1.46	72.74	5-methyltetrahydrofolate--homocysteine methyltransferase
<i>atpH</i>	49.81	7.58	377.74	ATP synthase delta subunit protein
<i>Smlt1001</i>	49.81	1.29	64.42	autotransporter
<i>exbB1</i>	49.81	10.51	523.48	biopolymer transport exbB protein
<i>Smlt0025</i>	49.81	1.93	96.21	conserved hypothetical protein
<i>phaAB</i>	49.81	1.41	70.43	K(+)/H(+) antiporter subunit A/B (pH adaptation potassium efflux system protein A/B)
<i>Smlt4151</i>	49.81	1.48	73.54	TonB dependent receptor
<i>pdhB</i>	47.32	4.66	220.45	dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex
<i>groES</i>	44.83	13.9	623.27	10 kDa chaperonin
<i>asnS</i>	44.83	2.87	128.67	2 asparaginyl-tRNA synthetase
<i>Smlt2834</i>	44.83	1.7	76.32	autotransporter protein
<i>Smlt4687</i>	44.83	3.42	153.42	conserved hypothetical protein
<i>Smlt4045</i>	44.83	1.84	82.42	exported tail-specific protease precursor
<i>Smlt0136</i>	44.83	1.08	48.33	ferredoxin oxidoreductase
<i>Smlt0654</i>	44.83	1.21	54.1	helicase
<i>mdh</i>	44.83	4.06	181.87	malate dehydrogenase
<i>Smlt3703</i>	44.83	15.43	691.72	peptidoglycan-associated lipoprotein
<i>Smlt0187</i>	44.83	4.11	184.1	peroxidase
<i>phaD</i>	44.83	2.61	116.86	pH adaptation potassium efflux protein

Table 9 ctd. Up-regulated *S. maltophilia* K279a genes when grown in the presence of *P. aeruginosa* 2192 in liquid log conditions compared to monoculture (>25-fold increase).

Gene	Fold change	SM means	SM+PA means	Annotation
<i>GpT</i>	44.83	1.4	62.65	phage tail protein
<i>frr</i>	44.83	7.21	323.43	ribosome recycling factor
<i>Smlt4678</i>	44.83	2.57	115.06	RmuC family protein
<i>Smlt0882</i>	44.83	1.24	55.61	sensor histidine kinase/response regulator fusion protein
<i>acpP_1</i>	44.83	16.68	747.92	hypothetical protein
<i>nusA</i>	42.34	5.3	224.24	N utilization substance protein A
<i>Smlt1515</i>	39.85	3.77	150.24	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase
<i>Smlt4518</i>	39.85	4.13	164.66	ABC transporter ATP-binding protein
<i>lon</i>	39.85	1.64	65.18	ATP-dependent protease
<i>Smlt4656</i>	39.85	1.18	47.19	conserved hypothetical protein
<i>serA</i>	39.85	3.03	120.6	D-3-phosphoglycerate dehydrogenase
<i>Smlt2530</i>	39.85	1.57	62.57	DNA ligase family protein
<i>Smlt0198</i>	39.85	2.37	94.64	electron transfer flavoprotein-ubiquinone oxidoreductase
<i>recB</i>	39.85	1.09	43.35	exodeoxyribonuclease V beta chain
<i>Smlt3167</i>	39.85	3.24	129.01	NAD-dependent glutamate dehydrogenase
<i>nuoN</i>	39.85	2.74	109.21	NADH dehydrogenase I chain N
<i>Smlt3181</i>	39.85	2.97	118.19	nucleotide sugar dehydrogenase
<i>bglX</i>	39.85	1.84	73.36	periplasmic beta-glucosidase precursor
<i>RRM1</i>	39.85	3.44	137.08	ribonucleoside-diphosphate reductase large subunit
<i>sucD</i>	39.85	9.14	364.28	succinyl-CoA synthetase alpha chain
<i>Smlt0891</i>	39.85	9.67	385.4	transmembrane preprotein translocase subunit
<i>Smlt1331</i>	39.85	2.38	94.8	transmembrane protein
<i>Smlt0196</i>	39.85	1.09	43.52	two-component sensor/response regulator protein
<i>Smlt1542</i>	39.85	1.32	52.71	two-component system sensor kinase/response regulator fusion protein with GGDEF signalling domain
<i>maeB</i>	37.36	3.49	130.53	NADP-dependent malic enzyme
<i>Smlt1976</i>	36.53	1.51	55.32	conserved hypothetical protein
<i>dxs</i>	34.87	2.1	73.17	1-deoxy-d-xylulose-5-phosphate synthase
<i>Smlt4670</i>	34.87	5.07	176.95	ABC transporter ATP-binding protein
<i>acsA</i>	34.87	4.12	143.63	acetyl-coenzyme A synthetase
<i>hrpB</i>	34.87	1.6	55.73	ATP-dependent helicase
<i>ctpA</i>	34.87	2.76	96.35	carboxy-terminal processing protease precursor
<i>Smlt1978</i>	34.87	4.29	149.64	conserved hypothetical protein
<i>Smlt3859</i>	34.87	1.76	61.23	conserved hypothetical protein
<i>Smlt1246</i>	34.87	1.86	64.91	exported peptidase
<i>Smlt2728</i>	34.87	2.46	85.86	exported surface antigen protein
<i>mdoD</i>	34.87	2.49	86.99	glucan biosynthesis protein d precursor
<i>msbA</i>	34.87	2.29	79.82	lipidAexport ATP-binding/permease

Table 9 ctd. Up-regulated *S. maltophilia* K279a genes when grown in the presence of *P. aeruginosa* 2192 in liquid log conditions compared to monoculture (>25-fold increase).

Gene	Fold change	SM means	SM+PA means	Annotation
<i>ispB</i>	34.87	4.01	139.75	octaprenyl-diphosphate synthase
Smlt3229	34.87	1.87	65.27	peptidyl dipeptidase/oligopeptidase
Smlt1866	34.87	3.98	138.92	phage-related protein
Smlt1906	34.87	3.52	122.79	phage-related protein
<i>pilY1</i>	34.87	1.06	36.85	PilY1 protein
<i>pdhA</i>	34.87	3.7	128.91	pyruvate dehydrogenase E1 component, alpha subunit
Smlt4430	34.87	1.76	61.48	Rhs-family exported protein
<i>rne</i>	34.87	2.43	84.77	ribonuclease E
<i>rnr</i>	34.87	1.56	54.43	ribonuclease R (RNase R)
Smlt2364	34.87	5.3	184.67	SapC-related protein
Smlt0107	34.87	1.66	57.74	sensor histidine kinase transcriptional regulatory protein two-component regulator
Smlt4410	34.87	1.67	58.1	TonB dependent ferric enterobactin receptor
Smlt0626	34.87	2.05	71.6	transmembrane protein
Smlt1462	34.87	1.65	57.6	transmembrane protein
<i>pilF</i>	34.87	2.31	80.51	type IV pilus assembly protein
Smlt3626	34.87	1.13	39.46	hypothetical
<i>rpsC</i>	33.45	38.13	1,275.37	30S ribosomal protein S3
<i>rplY</i>	33.21	19.44	645.45	50S ribosomal protein L25
<i>pilL</i>	33.21	1.8	59.73	gliding motility sensor histidine kinase response regulator fusion transcriptional regulatory protein
<i>smeH</i>	33.21	3.78	125.67	multidrug resistance efflux pump
<i>acnB</i>	32.38	3.09	100.03	aconitate hydratase 2
<i>nuoD</i>	32.38	6.12	198.23	NADH dehydrogenase I chain D
Smlt0490 A	32.38	1.87	60.64	hypothetical
<i>rpsQ</i>	31.13	59.31	1,846.72	30S ribosomal protein S17
Smlt0387	30.89	69.87	2,158.05	conserved hypothetical protein
<i>rpmJ2</i>	29.89	31.78	949.74	50S ribosomal protein L36
<i>bioF</i>	29.89	3.27	97.77	8-amino-7-oxononanoate synthase
<i>adhC</i>	29.89	3.61	107.81	alcohol dehydrogenase class-III
<i>gcvT</i>	29.89	3.6	107.52	aminomethyltransferase
Smlt4577	29.89	3.09	92.34	ATP-binding ABC transporter protein
Smlt3443	29.89	4.63	138.5	ATP-binding protein
Smlt4339	29.89	3.75	112.05	branched-chain alpha keto acid dehydrogenase E1 beta subunit
<i>smmF</i>	29.89	1.27	38.1	cation efflux system protein
<i>traA</i>	29.89	1.44	43.03	conjugal transfer protein TraA
Smlt3472	29.89	1.04	31.04	conserved hypothetical exported protein
Smlt0332	29.89	3.56	106.37	conserved hypothetical protein

Table 9 ctd. Up-regulated *S. maltophilia* K279a genes when grown in the presence of *P. aeruginosa* 2192 in liquid log conditions compared to monoculture (>25-fold increase).

Gene	Fold change	SM means	SM+PA means	Annotation
Smlt2198	29.89	2.23	66.59	conserved hypothetical protein
Smlt4090	29.89	8.29	247.76	conserved hypothetical protein
Smlt4243	29.89	4.36	130.36	conserved hypothetical protein
Smlt4383	29.89	1.48	44.32	conserved hypothetical protein
<i>cbsB</i>	29.89	2.92	87.28	cystathionine beta-synthase
Smlt0567	29.89	3.07	91.7	D-amino acid dehydrogenase small subunit
Smlt4341	29.89	2.86	85.6	dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex
<i>uvrD</i>	29.89	3.65	109.14	DNA helicase II
<i>smeP</i>	29.89	1.27	37.85	drug-resistance cell envelope-related protein
<i>wbil</i>	29.89	2.09	62.52	epimerase/dehydratase polysaccharide-related biosynthesis protein
Smlt3450	29.89	3.98	119.07	exported endopeptidase
Smlt2308	29.89	2.13	63.62	flagellar hook-associated protein
<i>fdnG</i>	29.89	1.3	38.99	formate dehydrogenase-o, major subunit
<i>fumC</i>	29.89	2.8	83.8	fumarate hydratase C
Smlt0995	29.89	5.23	156.43	glutathione hydrolase
Smlt3742	29.89	2.25	67.15	glycoside hydrolase
Smlt4037	29.89	2.65	79.15	histidine sensor kinase/response regulator fusion protein
Smlt0076	29.89	8.5	254.07	hypothetical protein
Smlt1148	29.89	1.64	49.06	iron transport receptor protein
Smlt0373	29.89	2.93	87.48	leucine aminopeptidase
<i>hel</i>	29.89	4.33	129.51	lipoprotein E precursor (outer membrane protein p4)
Smlt1078	29.89	2.66	79.62	Major Facilitator Superfamily transmembrane transporter protein
Smlt0130	29.89	2.53	75.69	modulator of DNA gyrase
<i>smeB</i>	29.89	1.27	37.99	multidrug efflux protein
Smlt4280	29.89	1.24	37	multidrug efflux system transmembrane protein
<i>glnG</i>	29.89	2.76	82.59	nitrogen regulation protein nr(i)
Smlt1560	29.89	5.25	157.04	NUDIX hydrolase protein
Smlt1189	29.89	2.37	70.98	oxidoreductase
<i>ppi</i>	29.89	8.14	243.23	peptidyl-prolyl cis-trans isomerase
<i>cdsA</i>	29.89	4.78	142.97	phosphatidate cytidylyltransferase
Smlt0142	29.89	1.95	58.4	phospholipase
Smlt4470	29.89	4.16	124.27	pyridoxal-phosphate dependent enzyme
Smlt0044	29.89	1.97	58.75	RHS-repeat protein
<i>nrpE</i>	29.89	1.61	48.12	ribonucleoside-diphosphate reductase 2 alpha chain
Smlt2055	29.89	3.32	99.23	SAM methylase protein
Smlt3525	29.89	3.4	101.76	thiolase
Smlt2845	29.89	1.52	45.54	TonB dependent receptor
Smlt1175	29.89	1.39	41.46	TonB dependent receptor protein

Table 9 ctd. Up-regulated *S. maltophilia* K279a genes when grown in the presence of *P. aeruginosa* 2192 in liquid log conditions compared to monoculture (>25-fold increase).

Gene	Fold change	SM means	SM+PA means	Annotation
Smlt3446	29.89	1.42	42.39	TonB dependent receptor protein
<i>nusG</i>	29.89	7.14	213.31	transcription antitermination protein
Smlt4572	29.89	5.02	149.96	transmembrane acyltransferase
Smlt0538	29.89	3.64	108.69	transmembrane anchor protein
Smlt0526	29.89	2.91	87.09	transmembrane D-serine/D-alanine/glycine transporter
Smlt2251	29.89	1.76	52.69	transmembrane methyl-accepting chemotaxis protein
Smlt0555	29.89	2.43	72.53	transmembrane Na ⁺ /H ⁺ antiport transporter
Smlt2804	29.89	2.21	66.04	transmembrane protein
Smlt2137	29.89	4.82	144	universal stress family protein
<i>map_2</i>	29.89	5.15	154.01	Hypothetical protein
<i>prfB</i>	29.89	3.56	106.28	Hypothetical protein
<i>dat</i>	28.23	8.57	242.01	diaminobutyrate--2-oxoglutarate aminotransferase
Smlt1498	28.23	4.92	138.84	outer membrane protein
<i>pps</i>	27.4	3.49	95.59	7 phosphoenolpyruvate synthase
Smlt4632	27.4	2.08	56.87	conserved hypothetical exported protein
Smlt1009	27.4	1.47	40.24	glycine-rich autotransporter protein
<i>guaB</i>	27.4	5.49	150.47	inosine-5'-monophosphate dehydrogenase
Smlt1168	27.4	3.09	84.54	lysine-sensitive aspartokinase III
<i>pheT</i>	27.4	3.36	92.1	phenylalanyl-tRNA synthetase beta chain
<i>secA</i>	27.4	2.93	80.27	preprotein translocase SecA subunit
Smlt1741	27.4	7.71	211.36	Recombinase A
Smlt3805	25.74	38.13	981.4	outer membrane Omp family protein

Table 10. Averages of up-regulated *S. maltophilia* K279a genes when grown in the presence of *P. aeruginosa* 2192 in liquid log and plated conditions compared to monoculture (>25-fold increase).

Feature ID	Fold Change (plated)	Fold Change (log liquid)	Feature ID	Plated*log	Average (plated/log)
<i>rplK</i>	119.1	22.91	50S ribosomal protein L11	2728.58	71.005
<i>Smlt0184</i>	69.08	64.76	conserved hypothetical protein	4473.62	66.92
<i>rplV</i>	10.12	114.57	50S ribosomal protein L22	1159.45	62.345
<i>acpP_1</i>	78.6	44.83	acyl-carrier protein	3523.64	61.715
<i>metG</i>	35.73	79.7	methionyl-tRNA synthetase	2847.68	57.715
<i>rplS</i>	98.85	11.21	50S ribosomal protein L19	1108.11	55.03
<i>lpdA</i>	33.94	74.72	dihydrolipoamide dehydrogenase	2536.00	54.33
<i>gyrA</i>	88.13	19.1	DNA gyrase subunit A	1683.28	53.615
<i>smeF</i>	76.22	24.91	multidrug resistance outer membrane protein	1898.64	50.565
<i>lon</i>	60.74	39.85	ATP-dependent protease	2420.49	50.295
<i>purL</i>	71.46	24.91	phosphoribosylformylglycinamide synthase	1780.07	48.185
<i>sucB</i>	15.96	74.72	dihydrolipoamide succinyltransferase E2 component	1192.53	45.34
<i>Smlt3703</i>	43.67	44.83	peptidoglycan-associated lipoprotein	1957.73	44.25
<i>Smlt1498</i>	57.17	28.23	outer membrane protein	1613.91	42.7
<i>clpB</i>	24.22	59.78	heat shock chaperone ClpB	1447.87	42
<i>rpsM</i>	29.18	54.8	30S ribosomal protein S13	1599.06	41.99
<i>gyrB</i>	13.7	69.74	DNA gyrase subunit B	955.44	41.72
<i>Smlt0383</i>	18.46	64.76	histone H1-like protein	1195.47	41.61
<i>accC</i>	70.27	12.45	biotin carboxylase	874.86	41.36
<i>sucD</i>	42.87	39.85	succinyl-CoA synthetase alpha chain	1708.37	41.36
<i>rplX</i>	16.87	64.76	50S ribosomal protein L24	1092.50	40.815
<i>rplL</i>	59.55	20.64	50S ribosomal subunit protein L7/L12	1229.11	40.095
<i>pheT</i>	50.02	27.4	phenylalanyl-tRNA synthetase beta chain	1370.55	38.71
<i>frr</i>	29.77	44.83	ribosome recycling factor	1334.59	37.3
<i>pilY1</i>	39.3	34.87	PilY1 protein	1370.39	37.085
<i>groEL</i>	18.96	53.97	GroEL protein	1023.27	36.465
<i>pnp</i>	51.21	20.48	polyribonucleotide phosphorylase	1048.78	35.845
<i>gltA</i>	11.31	59.78	citrate synthase	676.11	35.545
<i>groES</i>	25.61	44.83	10 kDa chaperonin	1148.10	35.22
<i>pps</i>	42.87	27.4	7 phosphoenolpyruvate synthase	1174.64	35.135
<i>phaAB</i>	20.25	49.81	K(+)/H(+) antiporter subunit A/B (pH adaptation potassium efflux system protein A/B)	1008.65	35.03
<i>smeH</i>	35.73	33.21	multidrug resistance efflux pump	1186.59	34.47
<i>parC</i>	55.97	11.96	DNA topoisomerase IV subunit A	669.40	33.965
<i>exbB1</i>	18.03	49.81	biopolymer transport exbB protein	898.07	33.92
<i>Smlt0716</i>	42.87	24.91	ABC transporter component protein	1067.89	33.89
<i>rpsG</i>	47.64	19.93	30S ribosomal protein S7	949.47	33.785
<i>rnE</i>	32.16	34.87	ribonuclease E	1121.42	33.515
<i>smeP</i>	36.92	29.89	drug-resistance cell envelope-related protein	1103.54	33.405

Table 10 ctd. Averages of up-regulated *S. maltophilia* K279a genes when grown in the presence of *P. aeruginosa* 2192 in liquid log and plated conditions compared to monoculture (>25-fold increase).

Feature ID	Fold Change (plated)	Fold Change (log liquid)	Feature ID	Plated*log	Average (plated/log)
<i>dnaX</i>	41.68	24.91	DNA polymerase III subunit Tau	1038.25	33.295
<i>nusA</i>	24.22	42.34	N utilization substance protein A	1025.47	33.28
Smlt3905	10.89	54.8	TonB dependent receptor	596.77	32.845
<i>pdhB</i>	17.86	47.32	dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	845.14	32.59
<i>guaB</i>	37.52	27.4	inosine-5'-monophosphate dehydrogenase	1028.05	32.46
<i>alaS</i>	51.21	13.7	alanyl-tRNA synthetase	701.58	32.455
<i>nusG</i>	34.54	29.89	transcription antitermination protein	1032.40	32.215
<i>nuoN</i>	24.41	39.85	NADH dehydrogenase I chain N	972.74	32.13
<i>etfS</i>	39.3	24.91	electron transfer flavoprotein subunit beta	978.96	32.105
Smlt0198	22.63	39.85	electron transfer flavoprotein-ubiquinone oxidoreductase	901.81	31.24
Smlt4670	27.39	34.87	ABC transporter ATP-binding protein	955.09	31.13
Smlt4687	15.48	44.83	conserved hypothetical protein	693.97	30.155
Smlt0654	15.48	44.83	helicase	693.97	30.155
<i>dnaJ</i>	45.26	14.94	chaperone DnaJ protein	676.18	30.1
<i>glyA</i>	45.26	14.94	serine hydroxymethyltransferase	676.18	30.1
<i>dxs</i>	25.01	34.87	1-deoxy-d-xylulose-5-phosphate synthase	872.10	29.94
Smlt3472	29.77	29.89	conserved hypothetical exported protein	889.83	29.83
<i>qoxA</i>	47.64	11.62	transmembrane ubiquinol oxidase subunit 2 precursor	553.58	29.63
Smlt3859	23.82	34.87	conserved hypothetical protein	830.60	29.345
<i>icd</i>	33.74	24.91	isocitrate dehydrogenase	840.46	29.325
Smlt4339	28.58	29.89	branched-chain alpha keto acid dehydrogenase E1 beta subunit	854.26	29.235
<i>fdnG</i>	28.58	29.89	formate dehydrogenase-o, major subunit	854.26	29.235
<i>rpsN</i>	38.11	19.93	30S ribosomal protein S14	759.53	29.02
<i>atpF</i>	38.11	19.93	ATP synthase B chain	759.53	29.02
Smlt3167	17.59	39.85	NAD-dependent glutamate dehydrogenase	700.96	28.72
<i>gcvT</i>	27.39	29.89	aminomethyltransferase	818.69	28.64
<i>leuS</i>	33.74	23.25	leucyl-tRNA synthetase	784.46	28.495
<i>mdh</i>	11.91	44.83	malate dehydrogenase	533.93	28.37
<i>rplJ</i>	38.41	18.01	50S ribosomal subunit protein L10	691.76	28.21
<i>moaA</i>	30.96	24.91	molybdenum cofactor biosynthesis protein A	771.21	27.935
Smlt0982	40.49	14.94	isocitrate/isopropylmalate dehydrogenase	604.92	27.715
<i>RRM1</i>	15.48	39.85	ribonucleoside-diphosphate reductase large subunit	616.88	27.665
Smlt0891	15.48	39.85	transmembrane preprotein translocase subunit	616.88	27.665
<i>fusA</i>	41.85	13.43	elongation factor G	562.05	27.64
<i>maeB</i>	16.08	37.36	NADP-dependent malic enzyme	600.75	26.72

Table 10 ctd. Averages of up-regulated *S. maltophilia* K279a genes when grown in the presence of *P. aeruginosa* 2192 in liquid log and plated conditions compared to monoculture (>25-fold increase).

Feature ID	Fold Change (plated)	Fold Change (log liquid)	Feature ID	Plated*log	Average (plated/log)
Smlt3796	30.96	22.42	conserved hypothetical exported protein	694.12	26.69
<i>smeE</i>	40.69	12.57	acriflavin resistance protein B	511.47	26.63
Smlt3210	27.39	24.91	outer membrane antigen protein	682.28	26.15
<i>accD</i>	32.16	19.93	acetyl-coenzymeAcarboxylase carboxyl transferase subunit beta	640.95	26.045
Smlt1368	36.92	14.94	conserved hypothetical protein	551.58	25.93
<i>acnB</i>	19.4	32.38	aconitate hydratase 2	628.17	25.89
<i>atpD</i>	35.13	16.6	ATP synthase beta chain	583.16	25.865
<i>rpoC</i>	38.31	13.11	DNA-directed RNA polymerase beta chain	502.24	25.71
Smlt4656	11.51	39.85	conserved hypothetical protein	458.67	25.68
<i>secA</i>	23.82	27.4	preprotein translocase SecA subunit	652.67	25.61
Smlt0387	19.65	30.89	conserved hypothetical protein	606.99	25.27

This could be the case because *S. maltophilia* K279a may need additional protein products to aid in persistence when grown in co-culture.

Genes up-regulated in *P. aeruginosa* 2192 in response to co-culture with *S. maltophilia* K279a

Genes that were differentially regulated in *P. aeruginosa* 2192 when grown in the presence of *S. maltophilia* K279a were also identified using CLC Genomics Workbench. In this comparison the co-culture of *P. aeruginosa* 2192 and *S. maltophilia* K279a also yielded genes in *P. aeruginosa* 2192 that were up-regulated when compared to monoculture conditions. The same threshold of a fold change of 25 or higher was not used in this *in silico* experiment because there were no genes that exceeded that limit. Instead the fold-change threshold was set at a ten-fold increase but the significance value threshold remained the same with *p*-values of <0.05. There were 20 up-regulated genes of *P. aeruginosa* 2192 in co-culture growth with *S. maltophilia* K279a compared to monoculture growth in the liquid log condition (Table 11). That there were only 20 genes up-regulated at less than half of the fold change cutoff speaks to how much less *P. aeruginosa* 2192 changes its transcriptional profile when grown in co-culture with *S. maltophilia* K279a. This may be because *P. aeruginosa* 2192 is a CF isolate and is used to growing in co-culture therefore not needing to up-regulate many genes. Another reason may be because of the production of alginate by *P. aeruginosa* 2192 that could cause shrouding of sensory mechanisms. Or, another possibility for the comparatively low increase in comparison to *S. maltophilia* K279a could be that it is naturally a more virulent bacteria and expresses co-culture virulence genes at all times. The few annotated

genes that were up-regulated mostly encoded membrane proteins. It would be expected that membrane protein associated genes would be up-regulated when grown in these co-culture experiments. The same combination experiment in the plated condition also yielded 20 upregulated genes with a threshold often-fold increase (Table 12). These however were not the same 20 genes as in the liquid log condition, and only PA2G_01660 (a paralog of the gene encoding the TerC family integral membrane protein in *P. aeruginosa* PAO1) was up-regulated in both plated and liquid log samples as highlighted in Tables 11 and 12. We can conclude from these data that *P. aeruginosa* 2192 does not have as large of a response in genetic up-regulation in both plated and log liquid conditions as does *S. maltophilia* K279a when grown in combination. The caveat to this is that there was only one read for each gene in co-culture that was upregulated.

Genes down-regulated in *P. aeruginosa* 2192 in response to co-culture with *S. maltophilia* K279a

In contrast with *S. maltophilia* K279a, *P. aeruginosa* 2192 grown in combination with *S. maltophilia* K279a did have genes that were significantly down-regulated in both plated and liquid log conditions with *p*-values of <0.05. A five-fold decrease was once again used as the down-regulation threshold and 27 genes were found to be down-regulated in the liquid log condition which included genes encoding proteins linked to dehydrogenases, proteases and carboxylase, but most were hypothetical proteins (Table 13). In the plated condition, 26 genes had more than a five-fold decrease in transcription which included genes encoding multiple types of transferases, membrane-associated proteins and other enzymes (Table 14).

Table 11. Up-regulated *P. aeruginosa* 2192 genes when grown in the presence of *S. maltophilia* K279a in liquid log conditions compared to monoculture (>10-fold increase).

Gene	Fold change	#SM means	SM+*PA means	Annotation
PA2G_01388	23.02	22.41	515.87	hypothetical protein
PA2G_03990	19.39	11	213.35	hypothetical protein
PA2G_02538	19.39	18.48	358.23	hypothetical protein
PA2G_01660	18.17	14.23	258.64	integral membrane protein, TerC family
PA2G_04621	15.75	12.29	193.56	Phosphopantothenoylecysteine synthase/(R)-4'-phospho-N-pantothenoylecysteine decarboxylase
PA2G_05742	14.54	9.45	137.41	hypothetical protein
PA2G_03684	13.33	10.69	142.46	hypothetical protein
PA2G_02756	12.12	12.7	153.86	hypothetical protein
PA2G_04292	12.12	24.52	297.05	hypothetical protein
PA2G_02034	12.12	16.29	197.38	hypothetical protein
PA2G_03328	12.12	17.88	216.62	short-chain dehydrogenase
PA2G_03871	12.12	14.4	174.43	TonB protein
PA2G_05634	12.12	36.41	441.2	stringent starvation protein B
PA2G_00167	10.9	11.33	123.58	C4-dicarboxylate transport protein
PA2G_02898	10.9	18.52	202.01	hypothetical protein
PA2G_05080	10.9	17.2	187.51	hypothetical protein
PA2G_00206	10.9	12.83	139.91	membrane-bound lytic murein transglycolase A
PA2G_03715	10.9	17.69	192.87	permease of ABC transporter
PA2G_04001	10.9	14.19	154.74	hypothetical protein
PA2G_05173	10.9	26.2	285.73	hypothetical protein

* PA- *P. aeruginosa* 2192; #SM- *S. maltophilia* K279a;

Table 12. Upregulated *P. aeruginosa* 2192 genes when grown in the presence of *S. maltophilia* K279a in plated conditions compared to monoculture (>10-fold increase).

Gene	Fold change	SM [#] means	SM+PA* means	Annotation
PA2G_01387	30.17	14.17	427.39	hypothetical protein
PA2G_05590	28.28	15.56	439.96	superoxide dismutase
PA2G_01385	24.51	22.19	543.95	hypothetical protein
PA2G_01660	18.86	27.36	515.82	integral membrane protein, TerC family
PA2G_08034	17.91	253.86	4,547.44	hypothetical protein
PA2G_01939	15.08	15.95	240.54	hypothetical protein
PA2G_01389	14.61	24.65	360.17	hypothetical protein
PA2G_02251	13.2	5.28	69.69	hypothetical protein
PA2G_03181	13.2	22.35	294.96	hypothetical protein
PA2G_04461	11.31	9.82	111.15	hypothetical protein
PA2G_05322	11.31	20.08	227.22	hypothetical protein
PA2G_00275	11.31	12.9	145.94	cobalamin (5'-phosphate) synthase
PA2G_00414	11.31	6.58	74.48	two-component sensor
PA2G_02321	11.31	6.82	77.21	conjugal transfer protein TrbL
PA2G_02339	11.31	4.75	53.74	hypothetical protein
PA2G_03732	11.31	6.46	73.12	cardiolipin synthase
PA2G_05769	11.31	3.43	38.85	two-component sensor
PA2G_00773	10.37	14.29	148.24	hypothetical protein
PA2G_05417	10.37	14.23	147.57	two-component response regulator PilR
PA2G_01386	10.37	26.12	270.86	hypothetical protein

* PA- *P. aeruginosa* 2192; #SM- *S. maltophilia* K279a;

Of these down-regulated genes in *P. aeruginosa* 2192 when grown in co-culture with *S. maltophilia* K279a, there were no common genes that existed between the plated and liquid log conditions. These data suggest that although there are genes that are down-regulated when grown in the presence of *S. maltophilia* K279a, *P. aeruginosa* 2192 does not have a specific group of down-regulated genes in co-culture growth. The combination of all up and down-regulation data generated here leads us to focus our analysis on the common genes that were up-regulated in *S. maltophilia* K279a. When grown in co-culture with *P. aeruginosa* 2192, *S. maltophilia* K279a in the plated and liquid log conditions displayed a global response. Having no highly down-regulated genes indicates that in co-culture growth *S. maltophilia* K279a predominantly up-regulates gene transcripts. With only little up-regulation by *P. aeruginosa* 2192 the genetic response comes mainly from *S. maltophilia* K279a. For this reason as well as the sheer total number of up-regulated genes *S. maltophilia* K279a genes will be investigated for future mutations.

Table 13. Down-regulated *P. aeruginosa* 2192 genes when grown in the presence of *S. maltophilia* K279a in liquid log conditions compared to monoculture (>5-fold decrease).

Gene	Fold change	SM means	SM+PA means	Annotation
PA2G_02948	-5.36	92.37	17.22	prophage protease subunits of ATP-dependent proteases
PA2G_01749	-5.78	51.51	8.92	hypothetical protein
PA2G_01805	-5.78	86.23	14.93	hypothetical protein
PA2G_02355	-5.78	50.39	8.72	hypothetical protein
PA2G_02458	-5.78	46.72	8.09	two-component sensor
PA2G_02557	-5.78	107.66	18.63	NAD dependent epimerase/dehydratase-like protein
PA2G_02820	-5.78	56.21	9.73	hypothetical protein
PA2G_04034	-5.78	253.03	43.8	hypothetical protein
PA2G_04782	-5.78	104.41	18.07	similar to c4-dicarboxylate-binding protein
PA2G_05883	-5.78	68.87	11.92	hypothetical protein
PA2G_00970	-5.78	22.72	3.93	hypothetical protein
PA2G_02185	-5.78	134.36	23.26	hypothetical protein
PA2G_01612	-6.6	707.46	107.15	hypothetical protein
PA2G_03864	-6.6	151.79	22.99	short-chain dehydrogenase
PA2G_04024	-6.6	110.66	16.76	hypothetical protein
PA2G_00864	-6.6	377.31	57.15	hypothetical protein
PA2G_02927	-6.6	363.47	55.05	hypothetical protein
PA2G_04425	-6.6	80.2	12.15	c-type cytochrome
PA2G_06158	-6.6	47.16	7.14	usher CupC3
PA2G_02057	-7.43	68.57	9.23	acetyl-CoA carboxylase, biotin carboxylase
PA2G_06183	-8.25	69.16	8.38	pimeloyl-CoA synthetase
PA2G_01264	-8.25	70.75	8.57	hypothetical protein
PA2G_02563	-9.08	279.36	30.77	bacterial transferase hexapeptide-like protein
PA2G_02642	-9.08	90.79	10	hypothetical protein
PA2G_03559	-9.08	131.26	14.46	hypothetical protein
PA2G_00639	-9.08	110.72	12.2	hypothetical protein
PA2G_05449	-9.35	455.07	48.65	hypothetical protein

Table 14. Down-regulated *P. aeruginosa* 2192 genes when grown in the presence of *S. maltophilia* K279a in plated conditions compared to monoculture (>5-fold decrease).

Gene	Fold change	SM means	SM+PA means	Annotation
PA2G_03283	-5.3	94.16	17.76	hypothetical protein
PA2G_01889	-5.3	661.09	124.66	hypothetical protein
PA2G_01923	-5.3	83.43	15.73	hypothetical protein
PA2G_02055	-5.3	57.49	10.84	propionyl-CoA carboxylase
PA2G_02562	-5.3	89.05	16.79	hypothetical protein
PA2G_03849	-5.3	142.09	26.79	hypothetical protein
PA2G_04766	-5.3	233.33	44	hypothetical protein
PA2G_02028	-5.3	38.09	7.18	transporter
PA2G_04727	-5.83	127.39	21.84	hypothetical protein
PA2G_01258	-5.83	97.23	16.67	hypothetical protein
PA2G_02511	-5.83	201.77	34.59	General secretion pathway outer membrane protein H precursor
PA2G_04827	-5.83	73.18	12.54	two-component response regulator NtrC
PA2G_06034	-5.83	58.47	10.02	ATP-binding/permease fusion ABC transporter
PA2G_03660	-6.36	113.67	17.86	phenazine-specific methyltransferase
PA2G_03825	-6.36	146.46	23.01	hypothetical protein
PA2G_04440	-6.63	170.24	25.68	nitric-oxide reductase subunit B
PA2G_02894	-6.89	278.73	40.43	rhamnosyltransferase chain A
PA2G_03246	-6.89	163.05	23.65	type 4 fimbrial biogenesis protein PilF
PA2G_00339	-7.42	167.64	22.58	short-chain dehydrogenase
PA2G_04592	-7.96	123.63	15.54	rubredoxin reductase
PA2G_03464	-8.49	237.25	27.96	hypothetical protein
PA2G_03362	-9.02	290.03	32.17	molybdopterin biosynthetic protein B1
PA2G_03822	-9.02	998.98	110.81	hypothetical protein
PA2G_03376	-10.61	188.88	17.81	cyanide insensitive terminal oxidase
PA2G_03592	-15.91	951.97	59.83	hypothetical protein
PA2G_01154	-19.09	198.67	10.41	carbamoyl transferase

Gene selection

To refine our list of candidate *S. maltophilia* K279a genes for further experiments, the predicted subcellular localization of all the proteins within the *S. maltophilia* K279a genome was generated via PSORTb (Figure 6A). This software uses twelve different algorithms to predict whether proteins will be located in the cytoplasm, cytoplasmic membrane, periplasm, outer membrane or extracellular space, or it will return an unknown value. It has a limited value for proteins located in multiple locations in the cells and lipoproteins (Yu, 2010). These data allowed us to identify the genes that may potentially aid in persistence of *S. maltophilia* K279a based on where their corresponding proteins are used in the cell. The PSORTb data show that when grown in co-culture with *P. aeruginosa* 2192 in plated and liquid log conditions, *S. maltophilia* K279a transcribed a significant increase of genes coding for proteins associated with the cytoplasm and outer membrane. This was compared to all predicted proteins in the reference genome of *S. maltophilia* K279a. Under these same conditions, there was a decrease in cytoplasmic membrane associated proteins. This may be as a result of up-regulation of other defense and sensing mechanisms possessed by *S. maltophilia* K279a. It may also be necessary for *S. maltophilia* K279a to express genes encoding outer membrane and cytoplasmic proteins in order to compete (Cystic Fibrosis Foundation Patient Registry: Annual Data Report, 2013). Inversely, when *P. aeruginosa* 2192 was compared to co-culture with *S. maltophilia* K279a, expression of genes encoding cytoplasmic and outer membrane-associated proteins decreased while genes encoding membrane proteins significantly increased (Figure 6B). This, once again suggests a high level of

Figure 6. Subcellular localization predictions for proteins up-regulated in co-culture for (A) *S. maltophilia* K279a in response to co-culture with *P. aeruginosa* 2192 or (B) in *P. aeruginosa* 2192 in response to co-culture with *S. maltophilia* K279a. The genes predicted to encode proteins localized in the cytoplasm, cytoplasmic membrane, periplasm, outer membrane, extracellular, or unknown were tabulated in PSORTb software and compared between this data set versus all the proteins predicted from the entire genome. ***-*p*-value < 1e-10, *-*p*-value = 0.001

transcription for membrane-associated defense and sensing mechanisms when these are grown in co-culture.

Cluster of Orthologous Gene (COG) functional classifications were made from gene annotations in *S. maltophilia* K279a when it was grown in co-culture with *P. aeruginosa* 2192. In *S. maltophilia* K279a these classifications showed a global increase in genes encoding metabolic factors, specifically amino acid, nucleotide, lipid and inorganic ion transport and metabolism proteins (Figure 7A). This may have been the case because of the increased competition for metabolites in a co-culture environment and the need for *S. maltophilia* K279a to increase its ability to obtain them. There was a mixed response in cellular processes and signaling associated proteins with increases in signal transduction mechanisms and defense mechanisms, and decreases in cell motility and intracellular trafficking proteins. The increase of transcripts for these proteins suggest that the response to co-culture growth increases sensing and defense as well as modification of intercellular trafficking while possibly conserving energy by decreasing the abundance of proteins used in motility (Figure 7A). When *P. aeruginosa* 2192 was grown in co-culture with *S. maltophilia* K279a, there was no trend in functional up-regulation of *P. aeruginosa* 2192 genes with exceptions in the cellular processing and signaling categories. These included genes encoding proteins involved in motility and signal transduction (Figure 7B). The increase of metabolism-associated genes points to *S. maltophilia* K279a actively transcribing genes that change the catabolism of substrate into usable cellular products that may heighten the ability for *S. maltophilia* K279a to survive when grown in co-culture with *P. aeruginosa* 2192.

Figure 7. Cluster of orthologous gene distribution of proteins encoded by genes induced in (A) *S. maltophilia* K279a in response to co-culture with *P. aeruginosa* 2192 or (B) *P. aeruginosa* 2192 in response to co-culture with *S. maltophilia* K279a. COG designations are as follows: RNA processing and modification (A); Chromatin structure and dynamics (B); Translation, ribosomal structure and biogenesis (J); Transcription (K); Replication, recombination and repair (L); Cell cycle control, cell division, chromosome partitioning (D); Cell wall/membrane /envelope biogenesis (M); Cell motility (N); Post-translational modification, protein turnover, and chaperones (O); Signal transduction mechanisms (T); Intracellular trafficking, secretion, and vesicular transport (U); Defense mechanisms (V); Extracellular structures (W); Nuclear structure (Y); Cytoskeleton (Z); Energy production and conversion (C); Amino acid transport and metabolism (E); Nucleotide transport and metabolism (F); Carbohydrate transport and metabolism (G); Coenzyme transport and metabolism (H); Lipid transport and metabolism (I); Inorganic ion transport and metabolism (P); Secondary metabolites biosynthesis, transport, and catabolism (Q); General function prediction only (R); Function unknown (S). * - Chi squared distribution p -value <0.05.

These data provide not only predicted locations, but also cellular processes that we can use to identify the importance of each gene response to co-culture growth. For further study, genes associated with up-regulation in *S. maltophilia* K279a were chosen as there were 84 genes that were up-regulated in both liquid log and plated conditions. *P. aeruginosa* 2192 only had one gene in common in up-regulation between the two conditions which indicates that there is a higher transcriptional response in *S. maltophilia* K279a when grown in co-culture compared to *P. aeruginosa* 2192. From these data, selections of genes were made by identifying genes that might be necessary for *S. maltophilia* K279a to persist in co-culture growth with *P. aeruginosa* 2192.

Quantitative Real-Time Polymerase Chain Reaction

Before mutants were constructed, the accuracy of the sequencing data was confirmed by quantitative real-time polymerase chain reaction (qRT-PCR). Standard curves were made for each gene using optimized concentrations of forward and reverse primers to which mRNA samples would then be compared to obtain absolute values (rather than relative abundance to another transcript). The expected outcome for all up-regulated genes in *S. maltophilia* K279a was a positive fold change verified by qRT-PCR.

Based on PSORTb and COG classifications of protein function of differentially regulated gene products, sequences for qRT-PCR primers (Supplementary Table 7) were constructed based on the selected gene targets (Table 15). Eight of the genes verified the sequencing data. These were genes primarily encoding membrane proteins, as is the case for *smlt0278*, *Smlt3905*, *exbB1*, *pilY1*, *cyoA*, *wzt* and *Smlt1471*, or possible cellular

Table 15. *S. maltophilia* K279a genes selected for further study.

Gene ID	Protein ID	COG* designation	RNA-seq Average Fold Increase (plated/log)
Smlt0278	two component regulator sensor histidine kinase transmembrane transcriptional regulatory protein	T	18.3
Smlt3905	TonB dependent receptor	P	32.845
<i>exbB1</i>	biopolymer transport exbB protein	U	33.92
Smlt0184	conserved hypothetical protein (QS)	S	66.92
Smlt1471	ABC transporter	V	18.895
<i>pilY1</i>	PilY1 protein	NU	37.085
<i>wzt</i>	ABC transporter component, polysaccharide related	GM	20.09
<i>cyoA</i>	cytochrome O ubiquinol oxidase subunit II	C	24.74
Smlt4670	ABC transporter ATP-binding protein	Q	31.13
Smlt0716	ABC transporter component protein	R	33.89
Smlt3703	peptidoglycan-associated lipoprotein	M	44.25
<i>lon</i>	ATP-dependent protease	O	50.295
<i>smeH</i>	multidrug resistance efflux pump	N/A	34.47
<i>smeF</i>	multidrug resistance outer membrane protein	MU	50.565
<i>cydA</i>	cytochrome D ubiquinol oxidase subunit I	C	14.13

* - Clusters of Orthologous Groups (COG)

communication in the case of Smlt0184. Primer optimizations were performed before each gene was quantified by testing each primer at three differing concentrations. These optimized concentrations provided the best concentration of each primer for optimal melting temperature of a standard concentration of template (10^4 copies). Quantitative assessment in tandem with standard curves for each gene provided a copy number value for each gene transcribed in co-culture samples which were each repeated to generate transcript mRNA. These values were compared to transcripts from monoculture growth in the same condition. While the fold changes were different between the RNA-seq and qRT-PCR for any given gene, the trend of up-regulation under co-culture conditions was positive for all genes which is consistent with other research that verifies that trend increases of transcripts is sufficient to verify sequencing fold increase of target genes. Differences of magnitude seen in the data are specific to the increased sensitivity of RNA-seq methods in comparison to the less sensitive qRT-PCR method. This was shown by the values that were > 1 for the average fold-change increase of co-culture transcripts when compared to monoculture transcripts (Figure 8, Supplementary Table 8). With data consistent with RNA-seq analysis we can conclude from the qRT-PCR analysis that *S. maltophilia* K279a up-regulates our selected genes in response to co-culture with *P. aeruginosa* 2192. To test the hypothesis that these genes play some role in co-culture viability for *S. maltophilia* K279a, mutants with deletions of these genes will be constructed and tested for ability to persist in our co-culture model.

Figure 8. Up-regulation of select *S. maltophilia* K279a genes. Quantitative real-time PCR was used to assess the absolute quantities of Smlt0278, Smlt3905, *exbB1*, *pilY1*, *cyoA*, *wzt*, Smlt0184 and Smlt1471 in comparison to the fold change of RNA-seq experiments. Error bars indicate one standard deviation.

Construction of mutant plasmids

In order for genetic mutants to be constructed, primers that flank the genes *exbB1*, *cyoA*, *wzt*, *Smlt4670*, *lon*, *pilY1*, and *Smlt3905* needed to be generated by overlap extension techniques (Horton, 1990) which results in a complete and markerless gene deletion. The construction of these primers (Supplementary Table 9) was done by selecting sequences that were 500 base pairs (bp) upstream of the coding sequence, 500 bp downstream from the stop codon of the gene, and identifying primer binding sites with melting temperatures between 60 and 65°C that flank these regions. Primers that lie on the interface with the gene sequence were concatenated from chimeric primers that could hybridize to each other. The first round of PCR generated two fragments of DNA corresponding to the 500 bp upstream and 500 bp downstream of each gene. These products were purified and used as templates for the second round of PCR which yielded a 1000 bp DNA fragment that was introduced into the plasmid pEX18Tc (Supplementary Figure 3) at the *EcoR1* and *BamH1* restriction sites. To verify that this ligation was successful, each plasmid containing the 1000 bp insert was sequenced via Sanger sequencing yielding individual nucleotide sequences of submitted samples. Using BioEdit Biological Sequence Alignment Editor, the sequences were compared to the theoretical nucleotide sequence of what the plasmid vector construct should contain (a 500 bp upstream sequence followed by a 500 bp downstream sequence of the gene of interest inserted into the multiple cloning site of pEX18Tc). A percent correlation between the sequenced and theoretical nucleotide showed each construct that was successfully introduced into pEX18Tc (Table 16), all of which were over a 97% match. The reason why each gene did not have a 100% match was from some deletions in the

Table 16. Percent match of 500 bp flanking sequences used for gene deletion constructs.

Gene	Number of bases matching reference	Total bases in reference	Percent match for knockout insertion sequence
<i>exbB1</i>	1049	1080	97.13%
<i>cyoA</i>	1198	1198	100%
<i>wzt</i>	1150	1160	99.14%
<i>smlt4670</i>	1083	1110	97.57%
<i>lon</i>	1133	1160	97.67%
<i>pilY1</i>	1160	1161	99.91%
<i>smlt3905</i>	1127	1146	98.34%

connection between the 500 bp regions. This should have little effect in future experiments as the intent is to delete the gene, and these inserts still have high enough homology to their intended genomic targets to allow recombination to occur. Then only genes that had mutations in their 500 bp flanking sequences were *exbB1* and *smlt4679* which will be excluded for further gene deletion experiments.

Introduction of the pEX18Tc was attempted by tri-parental bacterial conjugations (Ditt, 1980) using the lower efficiency construct spot mating method, as well as transformation using electrocompetent *S. maltophilia* K279a (Ye, 2014). Both of these methods used to introduce the plasmids into *S. maltophilia* K279a were repeated at least three times for each gene using tetracycline as selection for successful integration. Neither of the methods was provided significant evidence that introduction of pEX18Tc with our insertion sequences into *S. maltophilia* K279a occurred. This was because obtaining pure monoculture of the recipient bacteria on selective media plates containing the conjugation mixture was not possible due to background growth. For this reason we could not be sure that the colonies that grew were the donor, helper or recipient strains.

DISCUSSION

Identifying growth inhibition is fundamental to how the co-culture growth model is assessed. Proximal growth tests have been one way in which this was done. In those tests, our data showed no growth inhibition when strains were grown in the proximal growth zone. However, this may not indicate that there are not diffusible inhibitory effects between strains. There has been work showing that *P. aeruginosa* PAO1 facilitates the colonization of *S. aureus* (Yang, 2011), whereas other research contends that *S. aureus* is outcompeted by established *P. aeruginosa* growth (Baldan, 2014). Further research showed that there were distinct differences in biofilm growth when *S. maltophilia* K279a and *P. aeruginosa* PAO1 were grown together because of a diffusible signaling factor (DSF) produced by *S. maltophilia* K279a (Ryan, 2008). This supports the premise that growth inhibition can be a key indicator to polymicrobial growth research of *S. maltophilia* K279a. This makes sense because the detection of biofilm morphology in *P. aeruginosa* PAO1, as previously referenced, was done via confocal laser microscopy. Our observations were made from colony formation and growth inhibition that was macroscopic in nature and did not detect such differences at that scale. In fact, our data indicating that *P. aeruginosa* PAO1 inhibited the growth of *P. aeruginosa* 2192 in the combined test only corroborates the notion that bacterial proximity may be a significant conditional factor influencing growth inhibition. This is because of the ability that *P. aeruginosa* 2192 had to grow in proximal growth zone, but not in combination with *P. aeruginosa* PAO1. The co-culture lawn inhibition tests showed no zones of inhibition

detected surrounding growing bacteria on low density bacterial lawns (Figure 2); indicating that even if DSFs were produced, they had little to no effect on growth inhibition of surrounding bacteria under our conditions or at least did not travel through a nutrient agar medium. On the other hand, if DSFs were produced they may have not diffused through the medium, but still may have had an effect on the bacterial lawn that they were in direct contact with. For this purpose, we conducted further co-culture experiments in liquid media where all bacteria and exogenous molecules would have direct contact to eliminate some of these variables. The focus of our research then shifted from identifying DSFs such as antibiotics, quorum sensing molecules, toxin or colicin-like production, to extracellular sensing involving proteins such as outer membrane proteins, transporters and receptors. To identify the effect of these proteins on co-culture growth we chose to use the most sensitive and informative method available – RNA-seq. We chose to focus our co-culture experiments on three bacteria, *S. aureus* NRS77, *S. maltophilia* K279a and *P. aeruginosa* 2192. The reason for including *S. aureus* NRS77 after not researching them in proximal tests and excluding *Burkholderia* species is because *S. aureus* is more often found in the CF lung than the *B. cepacia* complex members. Not only that, but most CF patients are initially colonized by *Staphylococcus* species at a young age (Pernet, 2014). Recent research has also shown that *P. aeruginosa* and *S. aureus* are often associated in co-culture when isolated from CF patients which makes *S. aureus* a good addition to our co-culture experiments (Baldan, 2014; Korgankar, 2013).

With the increasing level of sophistication in genomic data quantification and multiple comparison software capabilities, the most statistically powerful type of

transcriptomics is next-generation sequencing (Liu, 2012). In order to understand which genes were up-regulated in each bacterial strain during co-culture, transcripts isolated from monocultures within the same condition were used as the reference for each gene's expression. In the past, when looking at the levels of transcription of specific genes, microarrays were used (Malone, 2011; Yoder-Himes, 2010; Nookaew, 2012), but with the advent of high-throughput sequencing and the increased capacity for its computation, obtaining millions of reads corresponding to transcripts is not only useful, but is becoming increasingly practical (Liu, 2012; Zhao, 2014). For this reason we used Illumina HiSeq, which is a synthesis sequencing method that costs about seven cents per million bases read (Ross, 2013). RNA-seq data that comes from purified nascent mRNA allows researchers to identify which genes are being transcribed in real time for each bacterial strains, and is being done in much of the current co-culture research (Goddard, 2012; Duan, 2003; Harris, 2007; Liu, 2012; Ross, 2013). This method has limitations though. Despite the cost being relatively low, it still is not inexpensive and isolating enough quality mRNA can be difficult. RNA is also difficult to manipulate. RNA is a relatively fragile molecule because it is readily degraded by cellular or extracellular RNases that are ubiquitous. Additionally special treatment of all instruments, reagents, and working surfaces must be used such that they are meticulously sterilized to prevent degradation of samples. Despite these limitations, RNA-seq is the most sensitive technique available for examining whole cell responses to changes in conditions and thus we feel it was the best approach for our research questions. The co-culture methods we employed result in a comparison between two conditions. With so much data being generated, there needs to be an analysis tool that can handle these multiple comparisons.

For this reason we used the bioinformatic analysis program CLC Genomics Workbench, which can take millions of pieces of data, in this case gene reads, and compare them with other samples.

The results of our sequencing data varied depending upon condition and culture combination (Table 4). *S. aureus* NRS77 proved difficult to obtain sufficient reads aligned to its reference monoculture by being least represented in all combinations and conditions. Because this is a Gram-positive bacterium, the lysis of *S. aureus* NRS77 is more difficult than *P. aeruginosa* 2192 and *S. maltophilia* K279a. Isolation of *S. aureus* NRS77 mRNA was attempted multiple times. Even after these attempts, the highest yield samples did not produce enough quality mRNA to provide sufficient sequencing reads. Indeed this has been seen in other studies as well (Zhao, 2012). There could have been the possibility also that *P. aeruginosa* 2192 and *S. maltophilia* K279a were inhibiting its growth in co- and tri-culture and there was not a sufficient amount of cells to harvest mRNA from. This possibility is strengthened by the data showing sufficient reads aligned to the *S. aureus* NRS77 genome in mono-culture in two of the three conditions.

Reads of *P. aeruginosa* 2192 monoculture growth were aligned to *S. maltophilia* K279a when *P. aeruginosa* 2192 was grown in monoculture. It is not certain why this was the case. In most cases, the reads from a mono-culture of *P. aeruginosa* 2192 would not have been aligned to the genome of any other organism. We did this because of the low number of reads in samples containing *S. aureus* NRS77 and as a verification step as to the validity of each sample. We can only ascertain that because of the close phylogenetic relationship between *S. maltophilia* and *P. aeruginosa* (both Gram-negative

bacteria in the Gammaproteobacteria class), there may be some genes that are evolutionary conserved between the two species and that a bias in how we process the data in the genomics software leads to a false positive result in these cases. However, when we generated a list of the unmapped reads we BLASTed the sequences against the NCBI genome databases and found that that overwhelming majority were associated with *P. aeruginosa* species. Furthermore, there were sufficient reads that matched the expected transcriptome for each condition of *P. aeruginosa* 2192 monocultures to use for comparison in *in silico* experiments. There is also variability in reads across condition, particularly for *S. aureus* NRS77. For instance, when compared between the plated and liquid log conditions of growth in the *S. maltophilia* K279a and *S. aureus* NRS77 there was a 10 fold difference in reads aligned to the *S. aureus* NRS77 genome. This can be attributed to either sequencing shortfalls or a lack of quality mRNA harvested from each sample. Whatever the case there were some data that met our criteria for analysis. The data that exceeded our standards came from a single co-culture mixture and monocultures - *P. aeruginosa* 2192 and *S. maltophilia* K279a under liquid log and plated conditions. As mentioned in the Results section of this work, only *S. maltophilia* K279a genes were chosen for mutant construction. Mixtures containing *S. aureus* NRS77 were not analyzed further because of the underrepresentation of *S. aureus*-associated reads in the co-culture sequencing data (Tables 4, 5 and 6).

Based on the sequencing data, PSORTb sub-cellular location and COG functional analyses (Figures 6 and 7), 15 genes were chosen for mutant construction. Although empirical, the selection of genes that were responsible for membrane associated defense, sensing and transport proteins as well as those associated with drug resistance were of

primary interest. We chose genes that we thought might be playing a role in *S. maltophilia* K279a persistence in co-culture growth in either defensive or offensive capacities. The gene *smlt0184* is a hypothetical gene that is associated with QS systems in other bacteria. It was chosen because of the importance that QS plays in co-culture growth. A previous study examined its importance in *S. maltophilia* for the secretion of outer membrane vesicles (Devos, 2015), which shows that these DSF's are important signaling molecules in QS (Ryan, 2008). Also chosen for this reason *Smlt0278*, a predicted sensor kinase/response regulator chimera, was another possible quorum sensing-based choice, but also chosen because of the critical roles that these proteins play during extracellular environmental sensing which can change transcription accordingly. Transporters, receptors and pumps are the selective barriers of the cell, which is the reason why *Smlt3905*, *exbB1*, *Smlt1471*, *wzt*, *Smlt4670*, *Smlt0716*, and *smeH* were chosen for total gene deletions. Variable control of these genes can produce more or less of the proteins that regulate what goes into and comes out of the cell, which can be of interest when pathogens are growing in co-culture. The genes *cyoA* and *cydA*, which encode cytochrome *bo* terminal oxidase subunit II and cytochrome *bd*-I terminal oxidase subunit I respectively, were chosen because their encoded components of ubiquinol oxidase possesses the ability to protect the bacterial cell membranes from peroxidation (reference). Finally, *pilY1*, *Smlt3703*, and *smeF* were selected because of the potential roles that they could play in intercellular communication via membrane-bound protein interaction (Table 14).

To verify RNA-seq data, transcript levels were assessed via qRT-PCR. In all selected genes, the averages of liquid log and plated transcripts in the RNA-seq data were

confirmed by the positive increase of transcription values by qRT-PCR (Figure 8). Frequently the fold changes observed by qRT-PCR are muted compared to those observed by RNA-seq though the fold change trends are the same (Yoder-Himes, 2009; Harris, 2007; Grasso, 2015). For CF pathogens it has been shown that qRT-PCR values of fold change are consistently less than half when compared to molecular quantification techniques like microarrays and RNA-seq (Harris, 2007; Oh, 2010). Such has also been the case when compared to microarrays, although not to the same degree (Yoder-Himes, 2010; Malone, 2011; Nookaew, 2012). The gene *exbB1* in qRT-PCR fold change was shown to be up-regulated about 3 times more than the sequencing data. This was probably due to one replicate value being significantly larger than the other values. Had that sample been excluded, the other values would be more consistent with data from other measured genes (Table 15). In each case however, there was a positive fold change value in the qRT-PCR data, which was consistent with the RNA-seq data.

Not all genes selected for complete gene deletions were successfully inserted into pEX18Tc. Successful mutant construction required ligating whole gene deletion sequences into the vector and uptake of the resultant plasmid by *S. maltophilia* K297a through conjugation or transformation. Only seven of the selected gene deletion sequences could be successfully integrated into pEX18Tc (Table 17). The reasons for not being able to successfully integrate the other eight sequences could be attributed to PCR failure of primers self annealing or being nonspecific, degradation of the DNA, or primer design errors. To mitigate the possibility of improper lab technique each integration protocol was repeated at least three times before a construct was excluded.

S. maltophilia K279a did not take up pEX18Tc by transformation of electrocompetent cells or through tri-parental conjugation. Despite the failure to transform *S. maltophilia* K279a into pEX18Tc bearing the constructed gene deletion sequences, the work completed here will be of use in the future. With further optimization of a conjugation protocol to get *S. maltophilia* K279a to take up the plasmid vectors, there will be opportunity to see how *S. maltophilia* K279a interacts with *P. aeruginosa* 2192. This can be done by using the methods outlined previously in this work to grow mutants in co-culture with *P. aeruginosa* 2192 and assess *S. maltophilia* K279a mutant growth via isolation on selective media. With the methods described in this work, the *S. maltophilia* K279a mutants can be grown, and observations made according to their fitness, growth, morphology and potential virulence in the co-culture model with *P. aeruginosa* 2192. This research also provides a potential model for future work to be done using next generation sequencing methods to further characterize polymicrobial interactions between various bacterial species which is an upcoming field in microbiology due in large part to the knowledge gained in the Human Microbiome Project studies.

FUTURE DIRECTIONS

The work outlined in this thesis can be used for other research in the future. First, it can act as a model for co-culture experiments, specifically transcriptomics studies. Due to the optimization of the bacterial culturing conditions, co-culture experiments with other bacteria can be performed to assess the transcript changes when grown in co-culture. This work can also be used as a basis for growth models involving more than two bacteria grow together. In the case of sequencing and analysis of raw data, we have developed a pipeline that can be used for other organisms, not only of interest for CF researchers, but for those looking into polymicrobial interactions with respect to transcriptional changes induced by other bacteria.

Second, the data generated from this work has also allowed other work to be initiated with *P. aeruginosa* 2192. Currently, our lab is looking at a few selected genes that were upregulated in *P. aeruginosa* 2192 using these data as reference. As was done with *S. maltophilia* K279a, complete gene deletions can be made in these bacteria and observations made when re-introduced into the co-culture growth model.

Furthermore, from the genes that were up-regulated in our data sets, future work can look into the specificity of their up-regulation in co-culture growth of other bacterial strains not tested here. This would allow us to understand if the up-regulated genes are generic or co-culture specific. Furthermore, if there are *S. maltophilia* K279a genes that are necessary for bacterial viability in co-culture, future work can look at paralogs of that

gene and orthologs in other bacteria. With those identifications this method can be used to identify their contribution to virulence that they play when grown in co-culture with *P. aeruginosa* 2192. Personally I would like to continue using this research as a model to identify possible virulence linked genes within the oral microbiome while attending dental school at the University of Kentucky. Overall, this work can contribute to the field of polymicrobial communities by displaying a model system by which interactions can be quantified based on the genes expressed in each strain. It will also allow researchers to identify bacteria that are more persistent by trends in their transcription profiles being differentially regulated.

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APPENDIX

LIST OF ABBREVIATIONS

°C	degrees Celsius
AHL	acyl homoserine lactone
AI	autoinducer
AIP	autoinducing peptides
AQ	alkyl quinolones
ATP	adenosine triphosphate
BMI	body mass index
bp	base pair
Carb	carbenicillin
cDNA	complementary deoxyribonucleic acid
CF	cystic fibrosis
CFRD	cystic fibrosis-related diabetes
CFTR	cystic fibrosis transmembrane conductance regulator
COG	clusters of orthologous gene
DNA	deoxyribonucleic acid
DSF	diffusible signal factors
FEV ₁	forced expiratory volume
g	gram
GlcNAc	N-acetyl-D-glucosamine

Kan	kanamycin
L	liter
LB	Luria broth Lennox formulation
M	molar
mg	milligram
mL	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
MRSA	methicillin-resistant <i>S. aureus</i>
MSCRAMM	microbial surface component recognizing adhesive matrix molecules
MSSA	methicillin-sensitive <i>S. aureus</i>
ng	nanogram
nm	nanometers
O.D. ₆₀₀	optical density @ 600 nm
Oligo	oligonucleotide
PCR	polymerase chain reaction
PTSA	pyrogenic toxin superantigens
PVL	Panton-Valentine leukocidin
qRT-PCR	quantitative real-time polymerase chain reaction
QS	quorum sensing
rDNA	ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
RNA-seq	ribonucleic acid sequencing

rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SCFM	synthetic cystic fibrosis medium
SCIN	staphylococcal complement inhibitor
SEM	soil extract medium
SOE (PCR)	overlap extension polymerase chain reaction
sPLA2-IIA	type-II-secreted phospholipase A2
TCS	two-component system
Tet	tetracycline
TSA	tryptic soy agar
TSS	toxic shock syndrome
μL	microliter

SUPPLEMENTARY FIGURES AND TABLES

Supplementary Table 1. Optical densities at 600 nm indicating growth for *P. aeruginosa* 2192, *S. aureus* NRS77 and *S. maltophilia* K279a in artificial CF sputum medium.

	Strain														
	<i>P. aeruginosa</i> 2192					<i>S. maltophilia</i> K279a					<i>S. aureus</i> NRS77				
	Rep. 1	Rep. 2	Rep. 3	Avg.	St. dev.	Rep. 1	Rep. 2	Rep. 3	Avg.	St. dev.	Rep. 1	Rep. 2	Rep. 3	Avg.	St. dev.
	Time (hours)														
0	0.171	0.171	0.171	0.171	0.000	0.098	0.098	0.098	0.098	0.000	0.025	0.026	0.02	0.023	0.014
1	0.171	0.151	0.156	0.159	0.010	0.182	0.182	0.186	0.183	0.002	0.012	0.014	0.01	0.012	0.002
2	0.394	0.366	0.380	0.380	0.014	0.262	0.272	0.276	0.270	0.007	0.018	0.018	0.011	0.016	0.004
3	0.534	0.541	0.573	0.549	0.021	0.387	0.380	0.371	0.379	0.008	0.018	0.014	0.01	0.014	0.004
4	1.264	1.371	1.369	1.335	0.061	0.611	0.632	0.641	0.628	0.015	0.015	0.012	0.013	0.013	0.002
5	1.534	1.654	1.637	1.608	0.065	0.838	0.808	0.796	0.814	0.022	0.011	0.018	0.015	0.015	0.004
6	2.127	2.283	2.338	2.249	0.109	1.337	1.295	1.381	1.338	0.043	0.006	0.017	0.014	0.012	0.006
7	2.165	2.360	2.408	2.311	0.129	1.738	1.763	1.764	1.755	0.015	0.009	0.016	0.015	0.013	0.004
24	2.333	2.472	2.472	2.426	0.080	2.134	2.147	2.300	2.194	0.092	0.01	0.019	0.015	0.015	0.005

Supplementary Table 2. Growth curve for *P. aeruginosa* 2192, *S. maltophilia* K279a and *S. aureus* NRS77 in soil extract medium supplement with 30 mM glucose.

Time (hours)	Strain											
	<i>P. aeruginosa</i> 2192				<i>S. aureus</i> NRS77				<i>S. maltophilia</i> K279a			
	Replicate 1	Replicate 2	Average	St dev	Replicate 1	Replicate 2	Average	St dev	Replicate 1	Replicate 2	Average	St dev
0	0.226	0.223	0.225	0.002	0.305	0.295	0.300	0.007	0.266	0.209	0.238	0.040
1	0.390	0.378	0.384	0.008	0.270	0.287	0.279	0.012	0.306	0.233	0.270	0.052
2	0.519	0.542	0.531	0.016	0.232	0.271	0.252	0.028	0.342	0.251	0.297	0.064
3	0.664	0.674	0.669	0.007	0.219	0.300	0.260	0.057	0.382	0.293	0.338	0.063
4	0.791	0.786	0.789	0.004	0.248	0.321	0.285	0.052	0.467	0.336	0.402	0.093
5	0.813	0.813	0.813	0.000	0.264	0.281	0.273	0.012	0.529	0.410	0.470	0.084
6	0.863	0.876	0.870	0.009	0.265	0.299	0.282	0.024	0.610	0.490	0.550	0.085
7	0.902	0.898	0.900	0.003	0.308	0.314	0.311	0.004	0.670	0.498	0.584	0.122
24	1.017	1.016	1.017	0.001	0.276	0.301	0.289	0.018	0.705	0.559	0.632	0.103

Supplementary Table 3. Optical densities at 600 nm indicating growth for *P. aeruginosa* 2192, *S. aureus* NRS77 and *S. maltophilia* K279a in LB medium.

Time (hours)	Strain											
	<i>P. aeruginosa</i> 2192				<i>S. aureus</i> NRS77				<i>S. maltophilia</i> K279a			
	Replicate 1	Replicate 2	Average	St dev.	Replicate 1	Replicate 2	Average	St dev.	Replicate 1	Replicate 2	Average	St dev.
0	0.210	0.213	0.212	0.002	0.200	0.200	0.200	0.000	0.213	0.213	0.213	0.000
1	0.231	0.230	0.231	0.001	0.220	0.210	0.215	0.007	0.309	0.308	0.309	0.001
2	0.958	0.955	0.957	0.002	0.300	0.302	0.301	0.001	1.024	0.991	1.008	0.023
3	2.360	2.350	2.355	0.007	0.568	0.566	0.567	0.001	2.076	2.053	2.065	0.016
4	2.990	3.000	2.995	0.007	1.010	0.998	1.004	0.008	2.668	2.645	2.657	0.016
5	4.550	4.510	4.530	0.028	1.400	1.430	1.415	0.021	4.360	4.260	4.310	0.071
6	4.880	4.590	4.735	0.205	1.700	1.740	1.720	0.028	4.890	4.960	4.925	0.049
24	5.11	5.19	5.15	0.056	5.84	5.83	5.835	0.007	2.88	2.79	2.835	1

Supplementary Table 4. Total RNA concentration and quality data from cells harvested under mono-, co-, and tri-culture samples in plated, liquid log and stationary liquid growth conditions.

	Sample ID*	Date Prepared	Concentration (ng/ μ L)	A260	A280	260/280	260/230
Plated	PA	11/18/2013	197.7	4.942	2.855	1.73	1.82
	SA	11/18/2013	192.4	4.809	2.431	1.98	1.98
	SM	11/18/2013	117	2.924	1.419	2.06	2.27
	PA+SA	11/18/2013	802.3	20.058	9.668	2.07	2.19
	PA+SM	11/18/2013	218.4	5.46	2.708	2.02	2.12
	SM+SA	11/18/2013	1615.3	40.383	20.414	1.98	1.98
	PA+SM+SA	11/18/2013	1340.2	33.506	16.153	2.07	2.17
Log liquid	SA	11/18/2013	30.8	0.769	0.374	2.06	2.17
	SA	11/18/2013	162.1	4.051	1.942	2.09	2.23
	PA	11/18/2013	858.1	21.452	10.285	2.09	2.22
	PA	11/18/2013	820.5	20.512	9.919	2.07	2.24
	SM	11/18/2013	248.6	6.215	2.984	2.08	2.18
	SM	11/18/2013	322.2	8.055	3.895	2.07	2.16
	PA+SA	11/18/2013	627.2	15.68	7.55	2.08	2.24
	PA+SA	11/18/2013	554.7	13.867	6.717	2.06	2.14
	PA+SM	11/18/2013	589.2	14.729	7.068	2.08	2.26
	PA+SM	11/18/2013	723.3	18.083	8.689	2.08	2.24
	SM+SA	11/18/2013	140.7	3.518	1.714	2.05	2.2
	SM+SA	11/18/2013	238.8	5.971	2.87	2.08	2.23
	PA+SM+SA	11/18/2013	326.9	8.173	3.986	2.05	2.17
	PA+SM+SA	11/18/2013	636.4	15.91	8.212	1.94	2.09
	PA	11/18/2013	598.1	14.953	7.657	1.95	2.06
	PA	11/18/2013	835.3	20.883	10.434	2	2.17
	SA	11/18/2013	27.3	0.684	0.352	1.94	2.33
Log stationary	SA	11/18/2013	26.6	0.665	0.37	1.8	2.24
	SM	11/18/2013	1186.2	29.656	14.776	2.01	2.15
	SM	11/18/2013	1222.3	30.557	15.249	2	2.14
	PA+SA	11/18/2013	569.7	14.243	7.391	1.93	2.12
	PA+SA	11/18/2013	1390.3	34.758	17.378	2	2.18
	PA+SM	11/18/2013	880.8	22.019	10.889	2.02	2.13
	PA+SM	11/18/2013	835.5	20.889	10.323	2.02	2.15
	SM+SA	11/18/2013	239.3	5.983	3.015	1.98	2.09
	SM+SA	11/18/2013	321	8.026	4.047	1.98	2.06
	PA+SM+SA	11/18/2013	642.8	16.069	8.043	2	2.1
	PA+SM+SA	11/18/2013	564.7	14.117	7.044	2	2.13

* PA- *P. aeruginosa* 2192; SM- *S. maltophilia* K279a; SA- *S. aureus* NRS77.

Supplementary Figure 1. Electrophoresis of DNase-treated RNA samples. RNA gel electrophoresis on a 1% agarose gel using SYBR Gold stain to indicate presence of total RNA in samples from the plated condition. Lanes: (1) 1 kilobase (kb) ladder, (2) *P. aeruginosa* 2192 monoculture, (3) *S. aureus* NRS77 monoculture, (4) *S. maltophilia* K279a monoculture, (5) *P. aeruginosa* 2192 and *S. aureus* NRS77 co-culture, (6) *P. aeruginosa* 2192 and *S. maltophilia* K279a co-culture, (7) *S. maltophilia* K279a and *S. aureus* NRS77 co-culture, (8) *P. aeruginosa* 2192, *S. maltophilia* K279a, and *S. aureus* NRS77 tri-culture.

Supplementary Table 5. NEBNext Multiplex Oligos (Index Primer Set 1) with corresponding condition and combination designations.

Primer	Barcode Sequence	Name*	Condition	Lane for Illumina
1	CAAGCAGAAGACGGCATACGAGAT <u>CGTGAT</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	PA	Plated	1
1	CAAGCAGAAGACGGCATACGAGAT <u>CGTGAT</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	PA	Liquid Log	2
1	CAAGCAGAAGACGGCATACGAGAT <u>CGTGAT</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	PA	Liquid Stationary	3
2	CAAGCAGAAGACGGCATACGAGAT <u>ACATCGG</u> TGACT GGAGTTCAGACGTGTGCTCTTCCGATC	SM	Plated	1
2	CAAGCAGAAGACGGCATACGAGAT <u>ACATCGG</u> TGACT GGAGTTCAGACGTGTGCTCTTCCGATC	SM	Liquid Log	2
2	CAAGCAGAAGACGGCATACGAGAT <u>ACATCGG</u> TGACT GGAGTTCAGACGTGTGCTCTTCCGATC	SM	Liquid Stationary	3
3	CAAGCAGAAGACGGCATACGAGAT <u>GCCTAA</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	SA	Plated	1
3	CAAGCAGAAGACGGCATACGAGAT <u>GCCTAA</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	SA	Liquid Log	2
3	CAAGCAGAAGACGGCATACGAGAT <u>GCCTAA</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	SA	Liquid Stationary	3
4	CAAGCAGAAGACGGCATACGAGAT <u>TGGTCA</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	PA+SA	Plated	1
4	CAAGCAGAAGACGGCATACGAGAT <u>TGGTCA</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	PA+SA	Liquid Log	2
4	CAAGCAGAAGACGGCATACGAGAT <u>TGGTCA</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	PA+SA	Liquid Stationary	3
5	CAAGCAGAAGACGGCATACGAGAT <u>CACTGT</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	PA+SM	Plated	1
5	CAAGCAGAAGACGGCATACGAGAT <u>CACTGT</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	PA+SM	Liquid Log	2
5	CAAGCAGAAGACGGCATACGAGAT <u>CACTGT</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	PA+SM	Liquid Stationary	3
6	CAAGCAGAAGACGGCATACGAGAT <u>ATTGGC</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	SA+SM	Plated	1
6	CAAGCAGAAGACGGCATACGAGAT <u>ATTGGC</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	SA+SM	Liquid Log	2
6	CAAGCAGAAGACGGCATACGAGAT <u>ATTGGC</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	SA+SM	Liquid Stationary	3
7	CAAGCAGAAGACGGCATACGAGAT <u>GATCTG</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	PA+SA +SM	Plated	1
7	CAAGCAGAAGACGGCATACGAGAT <u>GATCTG</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	PA+SA +SM	Liquid Log	2
7	CAAGCAGAAGACGGCATACGAGAT <u>GATCTG</u> GTGACT GGAGTTCAGACGTGTGCTCTTCCGATC	PA+SA +SM	Liquid Stationary	3

* PA- *P. aeruginosa* 2192; SM- *S. maltophilia* K279a; SA- *S. aureus* NRS77. Underlined sequence- Illumina barcode.

Supplementary Table 6. cDNA quality assessment for submission for Illumina sequencing.

ID	Conditions	Concentration (ng/μl)	A260	A280	260/280	260/230	Lane for Illumina sequencing
<i>P. aeruginosa</i> 2192	plated	24.9	0.499	0.285	1.75	-5.08	1
<i>S. aureus</i> NRS77	plated	13.2	0.265	0.16	1.65	-1.49	1
<i>S. maltophilia</i> K279a	plated	15.4	0.307	0.176	1.75	-2.83	1
<i>P. aeruginosa</i> 2192 + <i>S. aureus</i> NRS77	plated	69.2	1.385	0.778	1.78	2.89	1
<i>P. aeruginosa</i> 2192 + <i>S. maltophilia</i> K279a	plated	37	0.739	0.414	1.79	14.31	1
<i>S. maltophilia</i> K279a + <i>S. aureus</i> NRS77	plated	27.5	0.55	0.355	1.55	1.89	1
<i>P. aeruginosa</i> 2192 + <i>S. maltophilia</i> K279a + <i>S. aureus</i> NRS77	plated	37.8	0.756	0.412	1.84	14.09	1
<i>P. aeruginosa</i> 2192	liquid-log	39.6	0.792	0.47	1.68	2.27	2
<i>S. aureus</i> NRS77	liquid-log	64.7	1.294	0.892	1.45	0.99	2
<i>S. maltophilia</i> K279a	liquid-log	17.3	0.346	0.189	1.83	1.4	2
<i>P. aeruginosa</i> 2192 + <i>S. aureus</i> NRS77	liquid-log	11.5	0.229	0.135	1.7	-1.12	2
<i>P. aeruginosa</i> 2192 + <i>S. maltophilia</i> K279a	liquid-log	23.2	0.463	0.256	1.81	-4.1	2
<i>S. maltophilia</i> K279a + <i>S. aureus</i> NRS77	liquid-log	12.4	0.247	0.158	1.56	5.37	2
<i>P. aeruginosa</i> 2192 + <i>S. maltophilia</i> K279a + <i>S. aureus</i> NRS77	liquid-log	33.9	0.678	0.377	1.8	7.32	2
<i>P. aeruginosa</i> 2192	stationary	38.7	0.774	0.437	1.77	9.62	3
<i>S. aureus</i> NRS77	stationary	10.7	0.213	0.114	1.87	-0.98	3
<i>S. maltophilia</i> K279a	stationary	31.6	0.632	0.412	1.53	1.39	3
<i>P. aeruginosa</i> 2192 + <i>S. aureus</i> NRS77	stationary	22.9	0.458	0.252	1.82	1.77	3
<i>P. aeruginosa</i> 2192 + <i>S. maltophilia</i> K279a	stationary	32.9	0.657	0.419	1.57	1.57	3
<i>S. maltophilia</i> K279a + <i>S. aureus</i> NRS77	stationary	11	0.22	0.129	1.71	-1.28	3
<i>P. aeruginosa</i> 2192 + <i>S. maltophilia</i> K279a + <i>S. aureus</i> NRS77	stationary	40.6	0.811	0.42	1.93	14.06	3

Supplementary Figure 2. Bioanalyzer results of submitted libraries for Illumina sequencing. Ten microliters of each cDNA library was assayed on the Agilent 2100 Bioanalyzer to assess the quality of the cDNA submitted for Illumina sequencing. DNA ladder sizes shown on the left in both panels. **(A)** Sample ID designations: (2) *P. aeruginosa* 2192 plated, (5) *S. aureus* NRS77 plated, (8) *S. maltophilia* K279a plated, (11) *P. aeruginosa* 2192 + *S. aureus* NRS77 plated, (17) *S. maltophilia* K279a + *S. aureus* NRS77 plated, (14) *P. aeruginosa* 2192 + *S. maltophilia* K279a plated, (20) *P. aeruginosa* 2192 + *S. maltophilia* K279a + *S. aureus* NRS77 plated, (23) *S. aureus* NRS77 log liquid, (24) *P. aeruginosa* 2192 log liquid, (27) *S. maltophilia* K279a log liquid, (28) *P. aeruginosa* 2192 + *S. aureus* NRS77 log liquid. **(B)** Sample ID designations: (31) *P. aeruginosa* 2192 + *S. maltophilia* K279a log liquid, (33) *S. maltophilia* K279a + *S. aureus* NRS77 log liquid, (35) *P. aeruginosa* 2192 + *S. maltophilia* K279a + *S. aureus* NRS77 log liquid, (37) *P. aeruginosa* 2192 stationary liquid, (38) *S. aureus* NRS77 stationary liquid, (41) *S. maltophilia* K279a stationary liquid, (43) *P. aeruginosa* 2192 + *S. aureus* NRS77 stationary liquid, (44) *P. aeruginosa* 2192 + *S. maltophilia* K279a stationary liquid, (47) *S. maltophilia* K279a + *S. aureus* NRS77 stationary liquid, (48) *P. aeruginosa* 2192 + *S. maltophilia* K279a + *S. aureus* NRS77 stationary liquid. Material at ~ 2000 bp possibly indicating remnant rRNA.

Supplementary Table 7. Primers used for qRT-PCR of *S. maltophilia* K279a in co-culture growth.

Primer name	Sequence
Smlt0278_SC_up	TGATGTCCGGCCGTCTGTGGTT
Smlt0278_SC_down	AGCGGTCGCGGGTGAGTCG
Smlt0278_INT_up	CCGCGTCCGTGGGTCTGG
Smlt0278_INT_down	GGTTCGCCTTCGCCGTATCG
Smlt3905_SC_up	CCGC TC GGTGGATGTGGA
Smlt3905_SC_down	GAAGTTCTTGCGCAGGATGATGTTGA
Smlt3905_INT_up	GGCGGGCAGCCAGACCTTCA
Smlt3905_INT_down	GACGTACTGGCCGCCCTGTTC
exbB1_SC_up	CCGGCGGCGTGGGTGAG
exbB1_SC_down	GGTCGCGCTGTTGATCTTGCTGAA
exbB1_INT_up	CCGTACCCGCGAAAGCAACA
exbB1_INT_down	GCCCCACACGGTACCCAGCAGA
PilY1_SC_up	GGGCGCCCGGATCAACACTA
PilY1_SC_down	GCCGGTACCGTCATCCCTTCCTG
PilY1_INT_up	GGCGCCCGGATCAACAACACTACAA C
PilY1_INT_down	CGTGCGAACCCGAAGACATCCA
cyoA_SC_up	A CGCCACCCCGCAGGAAATCTC
cyoA_SC_down	GAAGCCGGCCAGGGTCAG
cyoA_INT_up	GCAGGCGGCGATGGACACG
cyoA_INT_down	CAGGGCGAAGAAGGCGATGAGGTA
wzt_SC_up	GCCAGCCCGGATCGAGAAGGAC
wzt_SC_dpwn	GGGCAGCAGCGGCAGAGTGAAC
wzt_INT_up	CGCGCATGATCCCCTGACGAGT
wzt_INT_down	GGCGGCTGGATATAGGTGTAGGTGTG
Smlt0184_SC_up	CCTGGGTCTTCTGGCTGCTC
Smlt0184_SC_down	CATTCTTGAACCTGTCGGTCTCCTG
Smlt0184_INT_up	CCGACGGCTGGAA GGTGAAGG
Smlt0184_INT_down	GCTGTAGTCGCCGAAGATGTGGAAGT
Smlt1471_SC_up	TGCTGCTGGCCACGCTCAT
Smlt1471_SC_down	GACCATCTGCACCGCCGACTC
Smlt1471_INT_up	CAGCCCGGTGCCGACTTCTT
Smlt1471_INT_down	GCACGATGGCCTGGTTGAC

Supplementary Table 8. Average up-regulation of *S. maltophilia* K279a genes measured by qRT-PCR in liquid log and plated conditions.

Gene name	RNA seq fold increase plated	RNA seq fold increase liquid	qRT-PCR fold increase plated	qRT-PCR fold increase liquid	AVG qRT-PCR fold increase in both conditions
Smlt0278	16.67	16.94	-	9.18958684	4.414272075
			0.466516496	1.414213562	
			-	2.639015822	
Smlt3905	10.89	8.47	3.732131966	7.464263932	4.414620965
			2	2.29739671	
			0.535886731	3.482202253	
<i>exbB1</i>	18.3	14.12	0.757858283	2.29739671	53.96129387
			0.933032992	157.5864849	
			-	2	
<i>pilY1</i>	39.3	20.82	0.870550563	6.964404506	3.500150665
			0.933032992	N/A	
			0.870550563	0.035896824	
<i>cyoA</i>	34.54	1.88	0.406126198	13.92880901	6.518186064
			2.639015822	2.143546925	
			2.143546925	3.482202253	
<i>wzt</i>	20.25	6.59	2.462288827	5.656854249	2.926299011
			1.741101127	0.659753955	
			0.076946526	2.462288827	
Smlt0184	39.08	31.06	-	1.319507911	1.419115785
			0.707106781	1.071773463	
			-	1.866065983	
Smlt1471	17.86	15.06	0.267943366	1.148698355	1.652592282
			1.148698355	0.076946526	
			1.231144413	3.732131966	

Supplementary Table 9. Primers used for mutant construction for *S. maltophilia* K279a.

Primer name	Sequence	Description
<i>cyoA</i> _1	ATATAT <u>GGATCCC</u> GCTGCAGGTAGGCCGG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>cyoA</i>
<i>cyoA</i> _2	CCCTCGCAATTGGATCGACCGACATGTTGG GAAAACTCTCTCTTGAGTCGATC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>cyoA</i>
<i>cyoA</i> _3	GATCGACTCAAGAGAGAGTTTTCCCAACAT GTCGGTCGATCCAATTGCGAGGG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>cyoA</i>
<i>cyoA</i> _4	ATATAT <u>GAATT</u> CGGCAGCGAACTCACCGATC CAC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>cyoA</i>
<i>exbB1</i> _1	ATATAT <u>GGATCC</u> GTCGCCGCCGAAGCCGT	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>exbB1</i>
<i>exbB1</i> _2	ATCACCACACACAACAAAGGTAAGCGTCTT GCGGCGCGCGTC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>exbB1</i>
<i>exbB1</i> _3	GACGCGCGCCGAAGACGCTTACCTTTGTTG TGTGTGGTGAT	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>exbB1</i>
<i>exbB1</i> _4	ATATAT <u>GAATT</u> CGAATGCCATGGCTTATTGC CCCTTTTC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>exbB1</i>
<i>pilY1</i> _1	ATATAT <u>GGATCC</u> GTACGCAGCAGCGCGG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>pilY1</i>
<i>pilY1</i> _2	GTTTTCAAGCTTTGAGGAACCCGGTCCGAA GACATGCCCATGAGCCTG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>pilY1</i>
<i>pilY1</i> _3	CAGGCTCATGGGCATGTCTCGGACCGGGT TCCTCAAAGCTTGAAAAC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>pilY1</i>
<i>pilY1</i> _4	ATATAT <u>GAATT</u> CTGAGCTATTCGGGCGGGG AG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>pilY1</i>
<i>wzt</i> _1	ATATAT <u>GGATCC</u> CGCAATGCCAGCTACGTC AAA	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>wzt</i>
<i>wzt</i> _2	CTATGTCTGCTTCCACAAAATGCGCCGGGCG CATCGCAGGGACCG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>wzt</i>
<i>wzt</i> _3	CGGTCCTGCGATGCGCCCGCGCATTTTGT GGAAGCAGACATAG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>wzt</i>
<i>wzt</i> _4	ATATAT <u>GAATT</u> CCAGCTCCACCAGGCAATC G	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>wzt</i>
<i>Smlt1471</i> _1	ATATAT <u>GGATCC</u> GCTGGCCGGCTACACAGG C	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>Smlt 1471</i>
<i>Smlt1471</i> _2	GCTAAAGATGGCGAGGGAATGCGTCGCTTC GGGGTCGGATCCC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>Smlt 1471</i>
<i>Smlt1471</i> _3	GGGATCCGACCCGAAGCGACGCATTCCCT CGCCATCTTTAGC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>Smlt 1471</i>
<i>Smlt1471</i> _4	ATATAT <u>GAATT</u> CCAGCGGCATGCGCTGTTC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>Smlt 1471</i>

Underlined regions- Restriction site sequence

Supplementary Table 9 ctd. Primers used for mutant construction for *S. maltophilia* K279a.

Primer name	Sequence	Description
Smlt3905_1	ATATAT <u>GGATCC</u> GCAGCGTGACCTGGCCGA	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of Smlt3905
Smlt3905_2	CGCTACTAACACGTACCTTGGAGAGATTGTC CATCGAAGCCTGAAGGCAAG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of Smlt3905
Smlt3905_3	CTTGCCCTCAGGCTTCGATGGACAATCTCTC CAAGGTACGTGTTAGTAGCG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of Smlt3905
Smlt3905_4	ATATAT <u>GAATT</u> CCCGCAGTGGCGCGAACAT G	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of Smlt3905
Smlt4670_1	ATATAT <u>GGATCC</u> GCCTGGTACAGCTTCCAGT GC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of Smlt4670
Smlt4670_2	CGATGTCTAAAAAACGTTAATGGTGCCTGC CGTTCGTCCAAGCCAC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of Smlt4670
Smlt4670_3	GTGGCTTGGACGAACGGCAGGGCACCATT ACGTTTTTTAGACATCG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of Smlt4670
Smlt4670_4	ATATAT <u>GAATT</u> CCACGTGCACGGCCTCGAAG TAG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of Smlt4670
Smlt0716_1	ATATAT <u>GGATCC</u> GGCGGTGCCGGGCG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of Smlt0716
Smlt0716_2	GCGCCTACGGAGACCGCGGCAACCGGAAA GGCCGC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of Smlt0716
Smlt0716_3	GCGGCCTTTCCGGTTGCCGGTCTCCGTAG GCGC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of Smlt0716
Smlt0716_4	ATATAT <u>GAATT</u> CCAGCGATGAGGGAATACC CGATACC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of Smlt0716
Smlt3703_1	ATATAT <u>GGATCC</u> CGAGCCGACCTGGGCGC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of Smlt3703
Smlt3703_2	CTAGGAGCCACAAAGGTATCGCTCCATGC GCATTGGCATCAAATGATGC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of Smlt3703
Smlt3703_3	GCATCAGTTTGATGCCAATGCGCATGGAGG CGATACCTTTGTGGCTCCTAG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of Smlt3703
Smlt3703_4	ATATAT <u>GAATT</u> CTGCGCCGAATCATCGTACT TGCC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of Smlt3703
lon_Sm_1	ATATAT <u>GGATCC</u> GTGGTGCGACCCTGGAG G	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of lon_Sm
lon_Sm_2	GATGCCCTCCGGAGGCGGCGCGCTGTC CAATGGC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of lon_Sm
lon_Sm_3	GCCATTGGACAGCGCGCCGCTCCGGGA GGGCATC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of lon_Sm
lon_Sm_4	ATATAT <u>GAATT</u> CAGCGAGCCGCTTAGTTTAC AGCATCCTT	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of lon_Sm

Underlined regions- Restriction site sequence

Supplementary Table 9 ctd. Primers used for mutant construction for *S. maltophilia* K279a.

Primer name	Sequence	Description
<i>smeF</i> _1	ATATAT <u>GGATCC</u> GCGGCCCGCTGGG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>smeF</i>
<i>smeF</i> _2	CCTGCCATCCGGCAGTACCGAGGACAGCCG GCCAGCG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>smeF</i>
<i>smeF</i> _3	CGCTGGCCGGCTGTCCTCGGTACTGCCGGA TGGCAGG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>smeF</i>
<i>smeF</i> _4	ATATAT <u>GAATTC</u> GTACGAAACCTGTTCAATA CGATCGCCAAGAAG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>smeF</i>
<i>smeH</i> _1	ATATAT <u>GGATCC</u> GTGCGGTGTCGTTGCGC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>smeH</i>
<i>smeH</i> _2	CAAGCAGTAACGGGAACCTTCCGTCTCCAG CGGTGAACTGAAACGAGAAAAG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>smeH</i>
<i>smeH</i> _3	CTTTCTCGTTTCAGTTCACCGCTGGAGACGG AAGGTTCCCGTTACTGCTTG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>smeH</i>
<i>smeH</i> _4	ATATAT <u>GAATTC</u> GGCATTCCCGCTCCTTGGT CG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>smeH</i>
<i>cydA</i> _1	ATATAT <u>GGATCC</u> GCGGCCCGCTGGG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>cydA</i>
<i>cydA</i> _2	CCTGCCATCCGGCAGTACCGAGGACAGCCG GCCAGCG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>cydA</i>
<i>cydA</i> _3	CGCTGGCCGGCTGTCCTCGGTACTGCCGGA TGGCAGG	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>cydA</i>
<i>cydA</i> _4	ATATAT <u>GAATTC</u> GTACGAAACCTGTTCAATA CGATCGCC	for making knockout in <i>Stenotrophomonas maltophilia</i> K279a of <i>cydA</i>

Underlined regions- Restriction site sequence

Supplementary Figure 3. Plasmid map for pEX18Tc. Sewing by Overlap Extension (SOE) fragments for *S. maltophilia* K279a genes of interest have been cloned into the *Bam*HI/*Eco*RI sites of the multiple cloning site and sequenced using Sanger sequence to ensure no mutations have occurred.

CURRICULUM VITAE

NAME: David Craig Jensen

ADDRESS: University of Louisville Department of Biology
Shumaker Research Building Room 351
2210 S. Brook St.
Louisville, Kentucky 40208

DOB: Dayton, Ohio – July 21, 1984

EDUCATION
&TRAINING: B.S., Biology
Brigham Young University Hawaii
2006-2010

Orientation Characteristics of *Bacillus megaterium*,
Bacillus cereus, *Escherichia coli*, and *Klebsiella*
pneumonia in Magnetic and Electrically Charged
Environments
Mentor- Dr. Shane Gold

AWARDS: Biology Department Academic Award
2007

Student Leadership Award
2009
2010

Brigham Young University Hawaii Outstanding Presenters Award
2010

GSC Conference Travel Award
2014

PROFESSIONAL
SOCIETIES: American Society for Microbiology (2013 – Present)

NATIONAL
MEETING

PRESENTATIONS: American Society for Microbiology 114th General Meeting

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