7-25-2014

Phenotypic Analysis of the Regulatory Role of the Leucine-Responsive Regulatory Protein (LrpPA) in Pseudomonas aeruginosa

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Phenotypic Analysis of the Regulatory Role of the Leucine-Responsive Regulatory Protein (Lrp_{PA}) in *Pseudomonas aeruginosa*

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A thesis Submitted to the Graduate Faculty of Kennesaw State University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Integrative Biology in the Department of Biology and Physics

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ABSTRACT

Nutrient acquisition is critical to survival and infection by the opportunistic bacterium *Pseudomonas aeruginosa*. This pathogen expresses a number of virulence factors that are a part of the starvation response and are important in host-pathogen interactions. Additionally, *P. aeruginosa* is resistant to a large number of antibiotics and has become difficult to treat once it has colonized a tissue. New pharmaceutical treatments are sought while the metabolism of this organism must be fully understood to select new targets for therapy. The leucine-responsive regulatory protein (Lrp) could be a promising target for treatment. The ortholog in *Escherichia coli* is a global regulator of metabolism and regulates many genes related to amino acid degradation, transport and synthesis. There are structural and functional similarities that indicate that Lrp in both species plays a similar role. In this study, the role of Lrp in *P. aeruginosa* was investigated using a microbial and molecular approach to determine if Lrp regulates more genes than the published single operon, *dadRAX*. The results of this study suggest that Lrp plays a role in regulating important virulence factors and growth patterns in both nutrient-rich and nutrient-poor media, and thus may act as a global regulator in the metabolism of *P. aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa, a Gram-negative bacillus native to soil and aqueous environment, is capable of inhabiting a wide variety of environments. This bacteria has been shown to attach and grow in biofilms on PVC pipes, persist in distilled water, live on the surface of human skin or cause infection within many body tissues. It is an opportunistic pathogen of burn victims, immunocompromised individuals, and cystic fibrosis (CF) patients. *P. aeruginosa* is problematic in hospital settings, causing an estimated 51,000 healthcare-acquired infections (HAI) in the U. S. per year (www.cdc.gov). Since the treatment of *P. aeruginosa* infections with antibiotics became common practice, antibiotic resistant strains have developed and pose serious complications in successful treatment (1). About 13% of HAI are from multi-drug resistant strains, and 6% of these infections are fatal (www.cdc.gov). *P. aeruginosa* is particularly challenging for CF patients because it can inhabit the lungs (often as a co-infection with *Staphylococcus aureus*) and form a biofilm in the sticky mucus that cannot be penetrated by therapeutic antibiotics (2). Once their respiratory pathway is colonized, the biofilm is difficult to remove from these patients. The vast majority of CF patients will die from these infections.

Virulence Factors

Pseudomonads express a number of virulence factors during infection that cause damage to host tissues. For this study, the focus was on two particular virulence factors produced by *P. aeruginosa* that previous studies indicate are toxic in animal models: the siderophore pyoverdine and biofilm formation. Both of these virulence factors are under the control of the quorum-sensing network (3, 4, 5), a complex signaling system found in many

bacteria that coordinates cell-cell communication. Bacteria secrete signaling molecules called autoinducers that accumulate at high population densities. Bacteria have the ability to change the genetic expression of the entire population based on the concentration of these autoinducers (reviewed in 6, 7). *P. aeruginosa* has three quorum sensing circuits, two of which are homologs of the LuxR/LuxI system common to Gram-negative bacteria. The other system is called the *Pseudomonas* quinolone signal (PQS) system and is closely connected to the other two systems. All three systems are responsible for virulence factor production, including elastase, exotoxin A, pyocyanin, and rhamnolipids (reviewed in 6, 8). This information has made the quorum sensing network a popular target for pharmaceutical development, motivating many researchers to develop quorum sensing inhibitors.

Siderophores are fluorescent peptides that chelate iron from the environment for use in metabolic pathways and are under the control of quorum sensing under iron limited conditions. Siderophores are produced in response to low internal iron levels and are secreted into the environment where they bind iron. Receptors on the outer membrane of the bacterium attach and transport the iron-loaded siderophores. Once internal iron levels reach a specific concentration, siderophore secretion is halted in order to prevent toxic levels of iron accumulating inside the cell (9).

Siderophores such as pyoverdine are considered virulence factors because infection of a host is dependent on iron extracted from host tissues for bacterial growth. Naturally, mammals produce lactoferrins and transferrin to remove soluble iron from their tissues thereby making iron unavailable to invading pathogens (10). Siderophores are secreted in response to this

deprivation, allowing the bacterium to survive in an environment that would otherwise be inhospitable for the species.

Pyoverdine is secreted by an efflux pump composed of three genes, *pvdRT-ompQ* and is located close to the biosynthetic genes of pyoverdine (11). An outer membrane protein, FpvA, receives both iron-bound and metal-bound pyoverdine from the exterior of the cell and transports it to the periplasmic space. Iron is released from pyoverdine, but other metals remain bound, and both free and metal-bound pyoverdine are expelled back to the exterior by the efflux pump to extract more iron from the cell's surroundings.

Liquid-killing assays performed on the nematode animal model *Caenorhabditis elegans* have shown that pyoverdine leads to disruption of iron homeostasis and death (12). A study conducted in immunodeficient mice challenged with *P. aeruginosa* demonstrated that pyoverdine produced during infection contributes to overall ability to cause disease and lethality (13). A mutant strain deficient in *pvdA*, an L-ornithine N^5-oxygenase that is critical to the production of pyoverdine, showed reduced virulence and dissemination when inoculated intranasally, measured by relative CFUs in the blood (9). These studies demonstrated that a molecule that is utilized for nutrient acquisition acts as a virulence factor in a host-pathogen interaction.

Biofilm formation is another important virulence factor used by *P. aeruginosa*. The transition from a planktonic lifestyle (free swimming) to a biofilm community (attached) requires genetic changes that alter cellular morphology and metabolism. These transcriptional changes involve alternative metabolic pathways and resistance to chemical treatment, which

lead to an increase in virulence (14). As previously mentioned, bacteria including *P. aeruginosa* secrete signaling molecules called autoinducers that coordinate cell-cell communication. These autoinducers are primary signals in biofilm formation. Cyclic di-GMP serves as the second messenger in biofilm formation. High levels of internal cyclic di-GMP induce the changes needed to begin construction of the biofilm matrix, and low levels signal morphological changes to a motile, planktonic form (3). Biofilm formation, therefore, is linked to nutrient availability to the cell.

Since antibiotic treatment is problematic against *P. aeruginosa*, other methods are sought to inhibit or prevent infection. In order to discover new methods of treatment, the organism must first be fully understood. *P. aeruginosa* has the ability to survive in a large variety of environments, which desmonstrates a diversity of metabolic pathways enabling the utilization of a variety of carbon sources. The genetic regulation of these metabolic pathways may be a key target for drug therapy, thus investigation into the role of both the enzymes involved and their transcriptional regulators may be important.

Leucine-Responsive Regulatory Protein (Lrp)

One transcription factor that is conserved among many prokaryotes is the leucine-responsive regulatory protein, Lrp. These proteins belong to the Lrp/AsnC family of transcriptional regulators. Both bacterial and archaeal species have Lrp orthologs that demonstrate conservation of structure and function of the protein.

The DNA binding capability of Lrp from *Vibrio cholerae* and *Proteus mirabilis*, which share 92% and 98% identity with the *E. coli* Lrp protein (Lrp_{EC}), respectively, have been

extensively characterized. Microarray analysis revealed that the Lrp from these two bacteria significantly affected half of the 400 genes regulated by the *E. coli* Lrp when expressed in an *lrp*-deficient *E. coli* strain, demonstrating conservation of function (15). Although these proteins are very similar in amino acid sequence, the authors of this study also investigated the effects of minor changes within the sequence on the transcriptional efficacy. The sequence of the helix-turn-helix (HTH) motif of *E. coli* was altered to contain the sequence from either *V. cholerae* or *P. mirabilis*. The *V. cholerae* mutant showed reduced transcription of the *lrp* gene in *E. coli*, but the *P. mirabilis* mutant showed a 2- to 3-fold increase in transcription. This demonstrates that minor changes in amino acid sequence influence the level of transcription of the genes under the regulation of Lrp. However, these hybrid proteins were still able to bind to genes in *E. coli*, which shows some functional conservation.

Three other enteric bacteria also have amino acid sequence similarity (16). The *lrp* primers of *E. coli* also amplify sequences from *Salmonella typhimurium, Enterobacter aerogenes,* and *Klebsiella aerogenes*. Each strain produced a protein product of the same size that had been previously demonstrated to bind to the *ilvIH* promoter region, a characterized *lrp*-regulated operon of *E. coli*. This further demonstrates high sequence similarity and function in these three species.

Bacillus subtilis, a Gram-positive, spore-forming bacillus, uses an *lrp* homolog to regulate branched-chain amino acid transport, which has also been described for Lrp_{EC} (17). It was determined to be encoded as the first gene in an operon for the transport of these amino acids. It is identical to the *azlB* gene, which is the gene that allows resistance of 4-azaleucine (18).

Pyrococcus furiosis, a thermophilic archaean, possesses the protein LrpA, which has 28% sequence identity with Lrp_{EC} (19). This protein, like Lrp_{EC}, down regulates its own expression. In addition, *Sulfolobus sulfataricus*, another thermophilic archaean, uses a homolog of Lrp to negatively regulate its own promoter region (20), which shows conservation of this specific function across eubacteria and archaea.

As previously mentioned, the most widely studied Lrp is found in *E. coli.* In *E. coli*, a Gram-negative bacillus species, about 400 genes, or ten percent of the total genome, are regulated by Lrp (21). Because it regulates 10% of the genome of *E. coli*, Lrp_{EC} is considered a global regulator of metabolism. The *lrp* gene in *E. coli* is about 494 nucleotides long and codes for a 18.8 kDa protein product.

The DNA binding region of Lrp is found at the N-terminus, which contains a helix-turn-helix (HTH) motif (22). The leucine-binding domain is located at the C-terminus. X-ray crystallography has revealed that the protein first forms homodimers that can bend DNA 90^{o} and assemble to form an octameric ring, capable of bending DNA 360^{o} (23). The assembly of Lrp is shown in **Figure 1**. Some genes show several Lrp binding sites in the same promoter region, which would also support the findings that Lrp forms polymers when bound to DNA.

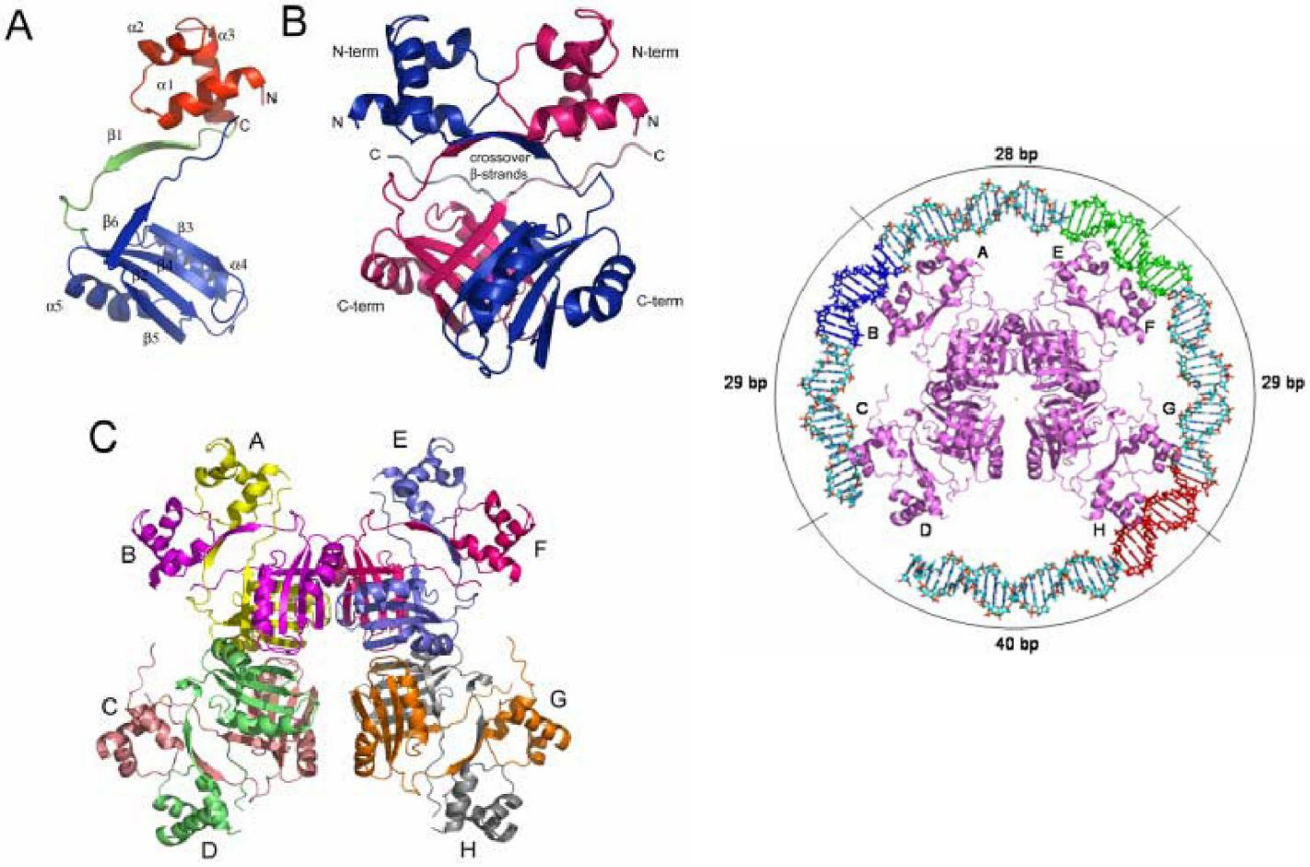

Figure 1. Lrp assembly in *E. coli.* Lrp monomers assemble into dimers then form homooctamers to bend DNA 360°. (A) An Lrp monomer forms (B) a homodimer with both HTH domains adjacent and both ligand-binding domains adjacent. (C) A complete octameric assembly of Lrp can bend DNA 360° to regulate DNA, which is shown in (D). Images were taken from (23).

Some of the genes up regulated by Lrp_{EC} are involved in amino acid biosynthesis, pilin synthesis, and ammonia assimilation, while Lrp_{EC} down regulates amino acid catabolism, peptide transport, and *lrp* itself (21, 22, 24). Lrp responds differentially to the presence of leucine, depending on the promoter to which it is bound. In genes negatively or positively

regulated by Lrp, the response to leucine binding can be positive, negative, or neutral (25). This flexibility in genetic regulation likely contributes to the adaptability of organisms with these transcription factors (26). Because of the role in metabolic gene regulation, Lrp_{EC} has been placed in the family of proteins known as feast/famine regulatory proteins (FFRP) (25).

The conservation of structure and function across these diverse groups of prokaryotes indicates that the role of Lrp may be conserved among all bacteria that possess this gene. The *P. aeruginosa* genome includes a leucine-responsive regulatory protein (Lrp_{PA}), but it has not been studied as extensively as Lrp_{EC}. It has only been demonstrated to bind to the promoter of the *dadRAX* operon (27). This operon is comprised of *dadX*, which is one of two alanine racemases in the *P. aeruginosa* chromosome, and *dadA*, which is an alanine dehydrogenase that has broad specificity. Lrp of *P.* aeruginosa, also published as *DadR,* will be referred to as Lrp_{PA} in this study. There are four binding regions for Lrp on the *dadRAX* promoter region, one of which is more divergent than the others (28). The explanation for the divergent regions was that binding affinities for Lrp would vary resulting in differing regulatory effects on gene expression. The presence of the four binding boxes also demonstrated that Lrp_{PA} may also form an assembly similar to that of Lrp_{EC} when bound to the promoter of *dadRAX* (28). Even more similarly, the homolog of *dadRAX* in *E. coli* is also regulated by Lrp_{EC}. Beyond these studies, however, the role of Lrp in *P. aeruginosa* is not well understood.

The conservation of structure and some function supports the possible role that Lrp can act as a global regulator of the *P. aeruginosa* genome. A side-by-side comparison of Lrp in *E. coli* and *P. aeruginosa* using 3-D modeling (**Figure 2**) shows that the N-terminus and the C-

terminus, which are the DNA-binding domain and the leucine-binding domain, respectively, are conserved in both proteins. The N-terminus shows the helix-turn-helix motif, which is the DNA-binding domain.

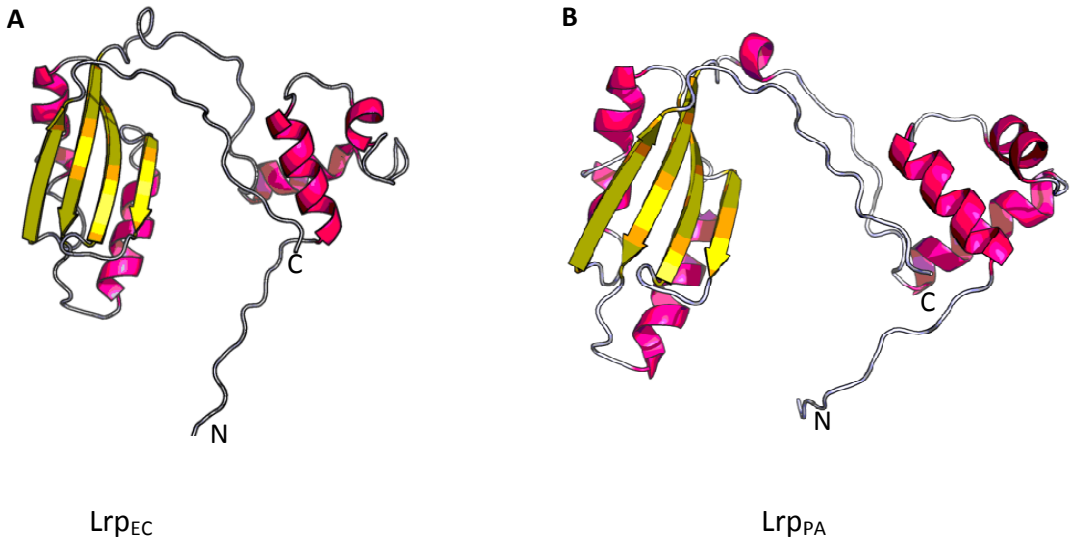

Figure 2. **Structural comparison of Lrp_{EC} and Lrp_{PA}.** A side-by-side comparison of the structure of an Lrp monomer from *E. coli* (A) and *P. aeruginosa* (B). Both proteins show a helix-turn-helix (HTH) motif at the N-terminus, a known DNA binding region. The C-terminus contains the amino acid binding region for both proteins as well. Images were obtained using the RaptorX Protein Structure Prediction Tool.

Parks and Griffin (2011) demonstrated that *in trans* complementation of Lrp_{PA} in a *lrp* knockout of *E. coli* is partially functional and can restore partial function of the native Lrp protein (29). Through expressing Lrp_{PA} in *E. coli*, the investigators were able to complement a

growth-deficient *lrp*$_{EC}$ mutant in minimal media. Lrp$_{PA}$ could not, however, activate expression of *gltB::lacZ,* another characterized Lrp$_{EC}$ – regulated gene. This demonstrated some conservation of structure-function between the two species, but it also suggested that some functions are likely species-specific. This emphasizes the need for careful study of individual Lrp proteins.

Due to the partial complementation of an *lrp*-deficient strain of *E. coli* by Lrp$_{PA}$ and the demonstration that Lrp$_{PA}$ regulates a metabolic operon in *P. aeruginosa*, it is plausible to expect that there may be other nutrient-acquisition genes that Lrp$_{PA}$ regulates. Conservation of the structure and function of this protein across both bacteria and archaea also demonstrates that Lrp$_{PA}$ may have a larger regulon than has been previously published. In this study, the Lrp$_{PA}$ regulon was examined by characterizing the phenotype of an *lrp* knockout of *P. aeruginosa*. The *P. aeruginosa lrp*- mutant was used to study virulence factor production and lethal effects on *C. elegans* and the results were compared to the wild-type strain of *P. aeruginosa*.

MATERIALS AND METHODS

Strains and Media

Pseudomonas aeruginosa strain PAO1 was originally isolated in Melbourne, Australia in the early 1950s from a wound infection. The whole genome of this strain was sequenced in 2000 and is maintained on a publically-accessible database, www.pseudomonas.com (30). A *Pseudomonas aeruginosa* PAO1 *lrp* knock-out mutant was obtained from the *P. aeruginosa* PAO1 transposon mutant library available through the Manoil Lab at the University of Washington Genome Sciences and is designated PW9942:*lrp*-C04::IS*lacZ*/hah (31). Within the transposon used to generate the *lrp*-knock-out mutant are genes providing resistance to tetracycline and β-lactam antibiotics. A graphic illustrating the insertion is shown in **Figure 3**.

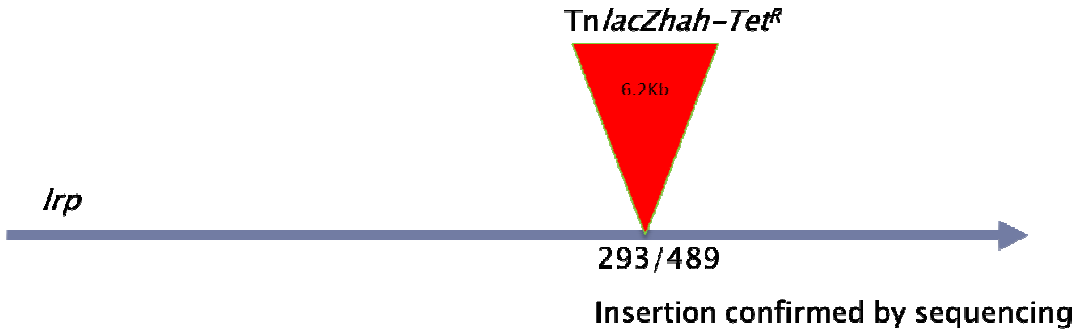

Figure 3. Transposon insertion into *P. aeruginosa* PAO1 *lrp* from a transposon-mediated mutant library. The *lrp* gene is 489 base pairs in *P. aeruginosa* strain PAO1. In PW9942:*lrp*-C04::IS*lacZ*/hah, the transposon insertion containing tetracycline resistance and a β-lactamase gene is inserted at 293 base pairs into the *lrp* gene, confirmed by sequencing.

The strain of *Caenorhabditis elegans* used in the killing assays was the wild-type N2 strain (*Caenorhabditis* Genome Center), isolated by Warwick L. Nicholas in Bristol, Great Britain in 1951 from mushroom compost (www.wormbase.org). *C. elegans* was used as a model for virulence in *P. aeruginosa* because of its suitability for studying host-pathogen interactions, its easy maintenance and short generation time, its similar innate immunity to humans, and its response to human pathogens (32). Worms were maintained at room temperature and grown on Nematode Growth Medium (NGM) Lite with *E. coli* strain OP50 as the food source.

P. aeruginosa PAO1 and PW9942:*lrp*-C04::IS*lacZ*/hah were grown in either Luria-Bertani broth (LB) (Difco, Franklin Lakes, NJ) or succinate broth (0.4% succinic acid, 0.02% $MgSO_4$ heptahydrate, 0.1% $(NH_4)_2SO_4$, 0.6% K_2HPO_4, 0.3% KH_2PO_4) under aeration on a rotating platform at 220 rpm within a 37^{o}C incubator. *E. coli* strains were grown at 37 °C in Luria-Bertani broth (LB) (Difco, Franklin Lakes, NJ) with shaking at 220 rpm.

PCR Confirmation of PW9942:lrp-C04::ISlacZ/hah

The strains of *P. aeruginosa* from University of Washington Genome Center were subjected to polymerase chain reaction to determine the presence of *lrp*. The *lrp* gene was amplified by polymerase chain reaction from *Pseudomonas aeruginosa* O1 (PAO1) chromosomal DNA with the forward primer 5'-GGAATTCCCGAGCCAGACGGGGAGCCTCCATCCATGCGTACC-3' and reverse primer 5'-GTGAATTCGGTCAATCCTAATCCGGAACCGGTAGGTCGAGCGA-3'. GoTaq G2 Hot Start MasterMix (Promega, Madison, WI) was used to amplify *lrp* using the following PCR cycle: 94^{o}C for 1

minute, then 32 cycles of 94^{o}C for 30 seconds, 55^{o}C for 1 minute, and 72^{o}C for 1 minute. The 500-base pair PCR product was run on a 1.5% agarose gel containing ethidium bromide with a 100 base pair ladder (Thermo Scientific, Waltham, MA) and visualized by UV 15luorescence using a Biorad Imaging System.

Growth Curve

24-hour growth curves were generated in both LB broth and succinate broth. Both *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::Is*lacZ*/hah were grown in 25 mL of broth with shaking at 220rpm at 37^{o}C. At 0, 8, 16, and 24 hours, 500 µL of the broth culture was collected, serially diluted and spread onto LB agar for the LB broth cultures and on Minimal A agar for the succinate broth cultures. Colonies were counted as an indication of viable cell count and colony forming units (CFUs) per mL were calculated at each time point. For each type of media, trials were run in duplicate and repeated 3 times. Statical relevance was determined using the student t-test.

Cell Size

In order to observe differences in cellular structure, cell length was compared in both LB and succinate broth at 16 hours for both *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::Is*lacZ*/hah. Sixteen hours was selected based on the results of the virulence factor assays, where a significant difference was measured between *P. aeruginosa* PAO1 and *P. aeruginosa*

PW9942:*lrp*-C04::Is*lacZ*/hah. Cultures of both *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::Is*lacZ*/hah were grown in 3 mL of either LB or succinate broth and a loopful of each was harvested at 16 hours and spread onto a glass microscope slide. The cells were heat-fixed to the slide surface, stained with 1% crystal violet for 1 min, and rinsed with distilled water. Slides were viewed under an inverted microscope at 1000X magnification. Between 50 and 100 cells were measured from each slide using the Image Processing and Analysis in Java (ImageJ), a program designed to edit and analyze images. This program was written by Wayne Rasband (Research Services Branch, National Institutes of Mental Health, Bethesda, MD) and inspired by NIH Image for Macintosh. This experiment was repeated 4 times in duplicate for LB and succinate broth. To determine a significant difference between cell sizes of *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::Is*lacZ*/hah, a *t*-test of the average cell length was determined for each type of media.

Siderophore Assay

Siderophores are pigmented iron-chelating agents that solubilize exogenous iron in order to support metabolism. Pyoverdine, a yellow-green siderophore, is a nutrient acquisition compound and is hypothesized to be regulated by Lrp. To determine if a correlation between pyoverdine production and Lrp could be observed, pyoverdine production was measured in both the wild-type and the mutant.

P. aeruginosa PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::Is*lacZ*/hah were streaked from a frozen stock onto MinA agar and incubated at 37^{o}C for 24 hours. Importantly, the use of fresh

strains is important for siderophore production (Griffin, unpublished data). An approximately equal amount of inoculum, confirmed by CFU collection, was added to 25 mL of succinate broth. At 0, 8, 16, and 24 hours, 500 µL of the culture was harvested and centrifuged at 13,200 rpm at 4^{O}C for 10 minutes. One hundred microliters of the supernatant was pipetted into a 96-well plate and a measurement of the OD was taken at a wavelength of 405 nm to measure pyoverdine production. CFUs were determined at each time point to demonstrate viable cell counts in each flask at each time. This experiment was done in duplicate and repeated three times.

For each time trial, a *t*-test was done to measure for significant differences in pyoverdine production between the wild-type and the mutant.

Biofilm Assay

Biofilm formation is influenced by nutrient availability for many bacterial species. Lrp is hypothesized to play a role in biofilm formation and was therefore measured in both the wild-type and the mutant.

P. aeruginosa PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::Is*lacZ*/hah were streaked onto Minimal A agar and allowed to grow at 37^{O}C for 24 hours. One colony from each species was selected and resuspended into 5 mL of either LB or succinate broth. One mL of each suspension was removed and added to 3 separate wells of a 24-well plate. Cultures were allowed to grow statically for 24 hours at 37^{O}C then the free cells were removed using a micropipette. One

percent crystal violet was added to each well and was allowed to incubate at room temperature for 5 minutes. The liquid supernatant containing free cells were removed. Wells were rinsed with distilled water three times and allowed to dry. OD measurements were taken at 540 nm. This experiment was done in triplicate and repeated 3 times. A *t*-test was used for the optical density of the crystal violet-stained biofilm for *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::Is*lacZ*/hah to determine a significant difference in biofilm formation.

Caenorhabditis elegans Killing Assay

C. elegans Maintenance and Growth

To determine if Lrp plays a role in overall virulence in an established animal model, a *C. elegans* killing assay was used as in previous publications (32, 33). Adult *C. elegans* N2 hermaphrodites were selected and killed with a bleach solution (500 mM NaOH and 20% household bleach) in order to harvest eggs and assure young adults for the assay. The larvae were allowed to develop to adulthood for four days before use in an experiment.

Preparation of *P. aeruginosa strains*

60mm plates with MinA agar were used for the growth of *P. aeruginosa* and for the killing assays. MinA agar was chosen because of higher expression of virulence factors under nutrient stress. *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::Is*lacZ*/hah were inoculated into 3 mLs of succinate broth and grown for 24 hours at 37^{o}C. Fifteen microliters of this broth was spread onto 60mm Petri dishes containing MinA and grown for 24 hours at 37^{o}C.

The plates were allowed to cool from 37^{o}C to room temperature for one hour before worms were added to prevent heat shock and death.

Killing Assay

Thirty adult hermaphrodite *C. elegans* were seeded onto a MinA agar plate containing either *P. aeruginosa* PAO1 or *P. aeruginosa* PW9942:*lrp*-C04::Is*lacZ*/hah and NGM-Lite plate with *E. coli* OP50 as a control, and worms analyzed every 24 hours for 96 hours. Every 24 hours, the worms were transferred to a new plate of the same bacteria. Worms were considered dead when they no longer responded to touch, and were removed from the plate. Worms that died for reasons other than bacterial consumption (ie. during plate transfer or from desiccation) were not included in the calculation of survival. Percentage of survival was calculated for each time point by dividing the sum of total worms surviving by total of worms that died. The results were subjected to a t-test to determine a difference in virulence between *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::Is*lacZ*/hah. This assay was repeated for three independent trials. A t-test was used at each time point to determine differences in survivability between the control strain *E. coli* OP50 and *P. aeruginosa* PAO1. A *t*-test was also used to determine a significant difference between OP50 and *P. aeruginosa* PW9942:*lrp*-C04::Is*lacZ*/hah and between *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::Is*lacZ*/hah.

Construction of pET300:lrp$_{PA}$

The *lrp* gene was amplified by polymerase chain reaction from *Pseudomonas aeruginosa* O1 (PAO1) using the same procedure as the confirmation of the *lrp* knockout (described above). The 500-base pair product was extracted from a 1.5% agarose gel (Qiagen, Germantown, MD) and inserted into the vector pCR2.0 using standard TA cloning (Invitrogen, Carlsbad, CA). The plasmid was then used in the pET300NT destination vector kit (Invitrogen, Carlsbad, CA) and was transformed into *E. coli* BL21(DE3), a strain of *E. coli* that is engineered to express high quantities of protein. The plasmid was purified with a MiniPrep Spin Kit (Qiagen, Germantown, MD) and sequenced to ensure the correct orientation (Functional Biosciences). *E. coli* BL21 + pET300NT:*lrp$_{PA}$* was maintained on media containing 50 μg/mL of carbenicillin.

Purification of Lrp

E. coli BL21(DE3) + pET300NT:*lrp$_{PA}$* was streaked on LB agar containing 50 μg/mL of carbenicillin and grown at 37^{o}C overnight. A 12 mL broth was inoculated using a single colony and allowed to grow overnight with shaking (220 rpm) in a 37^{o}C incubator. One liter of broth was inoculated with a 1:100 dilution of the overnight broth and was allowed to grow under the same shaking conditions for 2 hours and 15 minutes. At an OD 600 nm of 0.6, the culture was induced with 10 mLs of 100mM IPTG and allowed to continue to grow for 4 additional hours. The culture was centrifuged at 5,000 rpm for 15 minutes at 4^{o}C. The supernatant was removed and the resulting pellets were frozen overnight in a $^{-}$80^{o}C freezer to aid in the lysis of the cells. The cells were then resuspended in lysis buffer (10mM Tris pH 8.0, 500mM NaCl, 10mM

imidazole, 0.05% Tween 20) containing DNase I and lysozyme (10µg/ml) and allowed to sit on ice for 30 minutes. The suspension was homogenized briefly and passed through a French Press at 1280 Psi. The cell lysate was centrifuged at 10,000 rpm for 30 minutes at 4^{o}C. The supernatant was collected and mixed with HisPur cobalt resin beads (Thermo Scientific, Rockford, IL) and incubated on a rotating table at 4^{o}C for 2 hours. The lysate and beads were passed through a column equilibrated with 2 mLs of lysis buffer. The beads were washed 3 times with 10mLs of wash buffer (10mM Tris pH 8.0, 500mM NaCl, 25mM imidazole, 0.05% Tween 20). Five elution fractions of 1mL each were collected using elution buffer (10mM Tris pH 8.0, 500mM NaCl, 250mM imidazole, 0.05% Tween 20). 25µLs of each sample were mixed with 25 µLs of Laemmli Sample Buffer (Bio-Rad, Hercules, CA), boiled at 100^{o}C for 5 minutes and centrifuged at maximum speed for 5 minutes. 15 µLs of each sample were run through a 5-15% SDS-PAGE gradient gel for visual confirmation of Lrp production. The concentrations of protein in each elution was calculated by Bradford Assay then combined and dialyzed in 20mM Tris pH 7.6 overnight at 4˚C.

Electromobility Shift Assay

DNA regions were selected as targets for the activity of Lrp in an electromobility shift assay (EMSA). The targets selected for this assay were a positive and negative control segment amplified by PCR from PAO1. Positive control DNA was the *dadRAX* operon, selected based on the work of He *et al.* (2011) (28). The *dad* operon amplified contained three of the four published binding boxes and was amplified using the forward primer 5’ –

CATCGGCGCCGGCAACATGG – 3' and the reverse primer 5' – GCGCCAAGGCCTGGCACACGG – 3'. The following PCR program was used: 1 minute at 94^{o}C, then 94^{o}C 1 minute, 55 ^{o}C for 1 minute, 72 ^{o}C for 90 seconds repeated 30 times. GoTaq G2 Hot Start MasterMix (Promega, Madison, WI) with 10% DMSO (due to the high genomic GC content of *Pseudomonas* DNA) was used for the reaction. The negative control segment was the *proC* gene, a standard housekeeping gene that is constitutively expressed and is not known to be regulated by *lrp*. The *proC* gene was amplified using GoTaq G2 Hot Start MasterMix (Promega, Madison, WI) with 10% DMSO and the forward primer 5' – CCATGGCTTCTGCGACAGGAATTCCCG – 3' and reverse primer 5' – CCGATGACACCGCTGCCAAGG – 3'. The PCR cycle used was as follows: 94^{o}C for 1 minute, then 32 cycles of 94^{o}C for 30 seconds, 55^{o}C for 1 minute, and 72^{o}C for 1 minute. The product was gel-extracted using Qiagen DNA extraction kit (Qiagen, Germantown, MD) and spectrophotmetrically-quantified for use in this assay.

The EMSA protocol was adopted from a previous publication (28). A 4-15% Tris-Glycine electrophoresis gel (BioRad, Hercules, CA) was used with Tris-Glycine native running buffer and run at 100V. The target DNA and the protein were used at ten times the concentration due to no visibility of DNA in early trials, but the same buffer was used for the incubation for the binding.

BioLayer Interferometry

In addition to the EMSA, the DNA-binding capability of Lrp to target regions of the *P. aeruginosa* chromosome was also monitored using biolayer interferometry (BLI). BLI uses coated biosensors, which detect white light as it is reflected by two surface boundaries, the molecule coating the surface as well as the internal boundary of the sensor. Changes in interference occur as the number of molecules bound to the sensor changes during molecular interaction. This allows binding to be observed in real time and allow not only confirmation of interaction but the kinetics of both binding and release (www.ForteBio.com).

The same DNA targets from the EMSA were used in this assay to represent positive and negative controls for binding. BLI was monitored using the OctectQK System (ForteBio, Menlo Park, CA) equipped with Ni-NTA bound biosensors (ForteBio, Menlo Park, CA) that were used to bind to the His-tag on the N-terminus of the purified Lrp protein. Because previous trials of dipping the biosensor in eluted Lrp did not show activity with DNA, the sensor was instead dipped into cleared lysate to capture the protein in its native conformation before it was subjected to column purification. The sensors were dipped in cleared lysate from *E. coli* BL21(DE3) with or without the DNA construct containing lrp_{PA} to show background noise from nonspecific binding. The sensors were mixed in cleared lysate for 15 minutes then transferred to TBS-T for 5 minutes to wash nonspecific proteins from the sensor. This established a baseline of nonspecific binding. The sensor was placed in TBS-T and a saturating concentration of target DNA for five minutes then subjected to a wash step for five minutes to measure

dissociation of the DNA. Binding was measured at 25^{o}C. The nm shift in interference that occurs as the proteins bind was measured in real-time.

RESULTS

PCR Confirmation of Knockout

In order to demonstrate the disruption of the lrp gene by the presence of the transposon insert*ion*, PCR was utilized to amplify *lrp* from *P. aeruginosa* PAO1 and the mutant. The transposon in the mutant contains a tetracycline and *lacZ* fusion gene. This insertion lengthens the *lrp* gene to nearly 7Kb, which is not expected to amplify using *Taq* polymerase in a standard PCR cycle. The expected size of the product is 532 base pairs in the wild-type. The results of the PCR are shown in **Figure 4.** Amplification of *lrp* was achieved in PAO1 as expected and is shown in lane 2. The product is approximately 530 base pairs, which was the expected size of the amplicon. In the mutant, *lrp* did not amplify, which suggested that the transposon renders the amplicon too long for Taq amplification and is consisted with published results from the Manoil mutant library paper (31). This indicates that *lrp* is no longer a functional gene and that it is a true knockout. Any phenotypic differences observed between the wild-type and the mutant are due to a disruption of *lrp*. The amplification was attempted a minimum of three times on three independently isolated genomic samples for both mutant and wild-type.

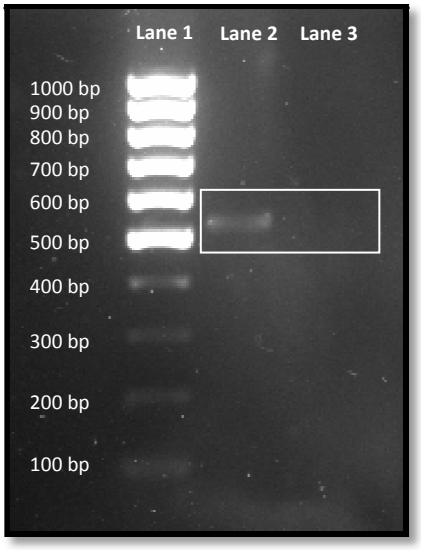

Figure 4. *lrp* amplification by PCR. 10µl of a 25µl reaction was loaded and electrophoresed through a 1.5% agarose gel. Lane 1: 100 base pair ladder. Lane 2: 500 base pair *lrp* amplicon for *P. aeruginosa* PAO1. Lane 3: PCR reaction from *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah. The absence of an amplicon is consistent with the presence of the transposon inserted into the *lrp* gene.

After PCR confirmation of the mutant, the phenotype of both *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah was characterized. The colony morphology and growth of both *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah on a minimal A agar plate is shown in **Figure 5.** At 24 hours, the colonies were reduced in size and the pigmentation from siderophores was also less prevalent in *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah. The changes in morphology were presumed to be mitigated by the expression of *lrp* as this is the

only genetic difference between both strains. The exact role of Lrp_{PA} in these morphological changes has yet to be determined.

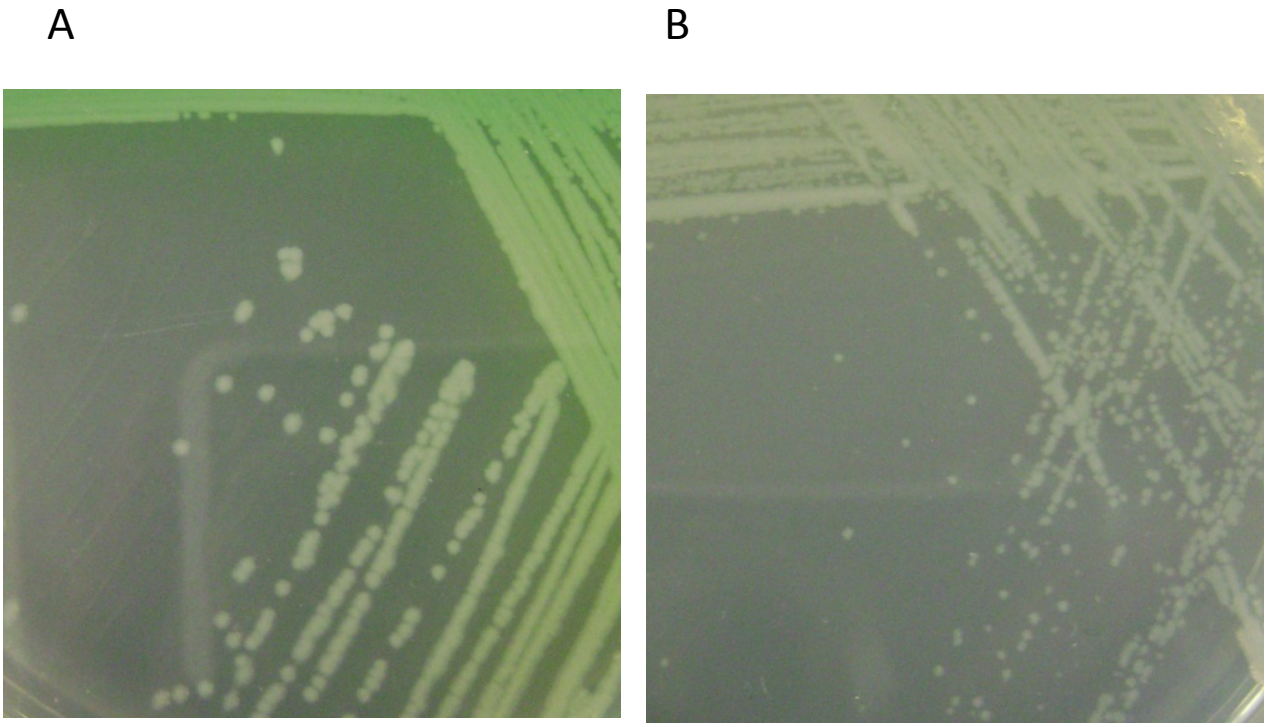

Figure 5. Phenotype of *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah on minimal A agar. (A) The wild-type strain *P. aeruginosa* PAO1 displayed cream colonies and green pigmentation of siderophores. (B) *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah displayed colonies that were reduced in size and the pigmentation from siderophores also appeared to be lower than that of *P. aeruginosa* PAO1.

Growth Curves

Wild-type and mutant *P. aeruginosa* were monitored for growth in nutrient-rich LB broth and nutrient-poor succinate broth for 24 hours. Growth was monitored every 8 hours by

colony forming units (CFU). *P. aeruginosa* PAO1 grew exponentially between 0 and 8 hours then began to decrease in cell count before 16 hours. The CFU count remained about the same from 16 to 24 hours. *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah, however, did not enter exponential growth until after 16 hours and appeared to grow more slowly than the wild-type strain PAO1. The trends in growth are shown in **Figure 6**.

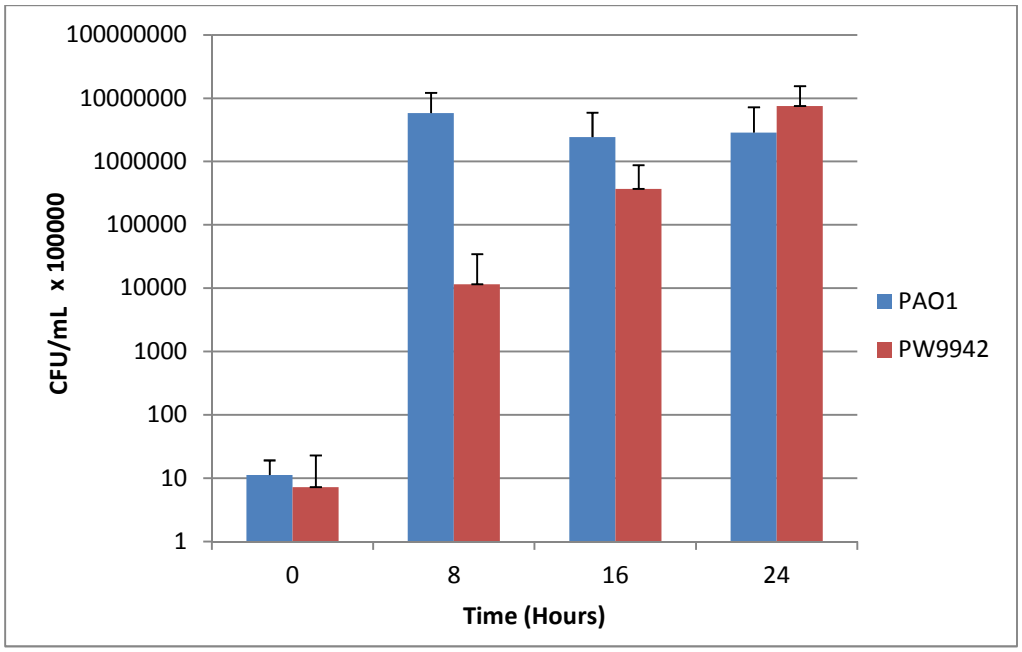

Figure 6. Growth of *Pseudomonas aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah (PW9942) in LB broth. The growth curves for *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah (PW9942) in LB broth over 24 hours were measured every 8 hours and expressed as a concentration of CFU/mL. The error bar above each point represents 1 standard deviation for three trials done in duplicate.

In contrast to nutrient-rich LB broth, a growth curve in minimal broth was also conducted for both *P. aeruginosa* strains as shown in **Figure 7.** The growth curves for *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah in succinate broth displayed comparable growth to those observed in LB broth. *P. aeruginosa* PAO1 showed exponential growth through 8 hours then stationary and decline phases between 8 and 24 hours. For *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah growth was exponential through 24 hours.

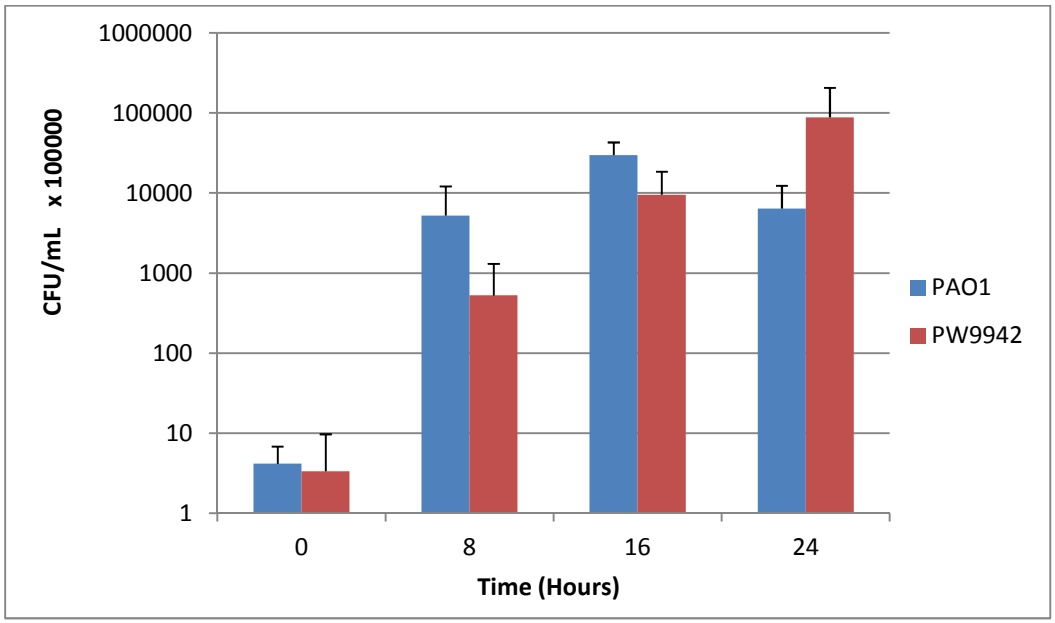

Figure 7. Growth of *Pseudomonas aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah (PW9942) in succinate broth. The growth curves for *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah (PW9942)in succinate broth over 24 hours, measured every 8 hours by CFUs. Colony forming units of *P. aeruginosa* PAO1 increase until 16 hours then begin to decrease. *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah CFUs continue to increase for the 24 hours and growth is exponential between 16 and 24 hours. The error bars represent 1 standard deviation for three trials done in duplicate.

A side-by-side comparison of the wild-type in both media is shown in Figure 8. Viable cell count decreased by approximately two orders of magnitude in succinate broth compared to the more nutritious LB broth. The limited nutrients in succinate broth were unable to support as high of a concentration of cells at each time point that was measured. The same media comparison is seen with mutant in Figure 9. However, all a reduction in growth is seen in succinate as compared to LB by the mutant, the mutant demonstrated a continuation in growth. It appears that it has not yet saturated nutrients that are available in the media. Whether this is due to a reduction in nutrient assimilation of transport or decreased metabolism as a result of lacking Lrp remains to be determined.

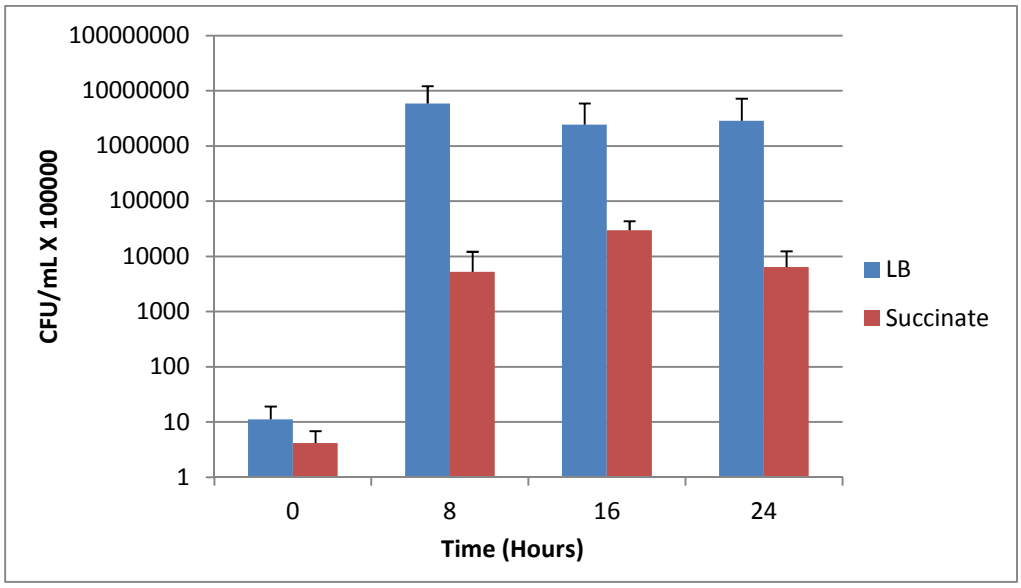

Figure 8. Growth rate of *P. aeruginosa* PAO1 in LB and succinate broth. Viable cell counts were higher in LB broth than in succinate broth for *P. aeruginosa* PAO1. The higher nutrient availability of LB allowed a higher concentration of cells at each time point.

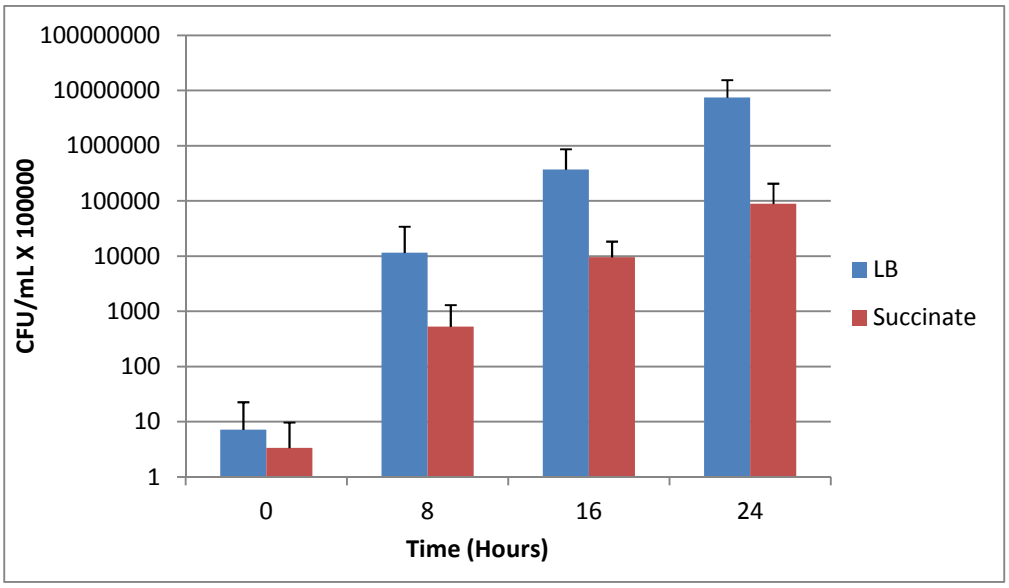

Figure 9. Growth rate of *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah in LB and succinate broth. *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah (PW9942) grows to a higher concentration of cells in LB broth than in succinate broth. The nutrients are more limiting in succinate broth and did not allow as much growth as LB broth.

Cell size

We hypothesized that cell size could be affected as a consequence of nutrient acquisition and loss of Lrp activity. Cell size was determined using an inverted light microscope and ImageJ imaging program. A sample cell from each slide that represents the average is shown in **Figure 10.** Cell size was determined for both *P. aeruginosa* strains grown in LB broth and in succinate broth. The results are shown in **Figure 11**. In LB broth (panel A and C), *P.*

aeruginosa PAO1 had a significantly larger size than *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah. However, the cell size of *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah was significantly larger than PAO1 when grown in succinate broth (panel B and D).

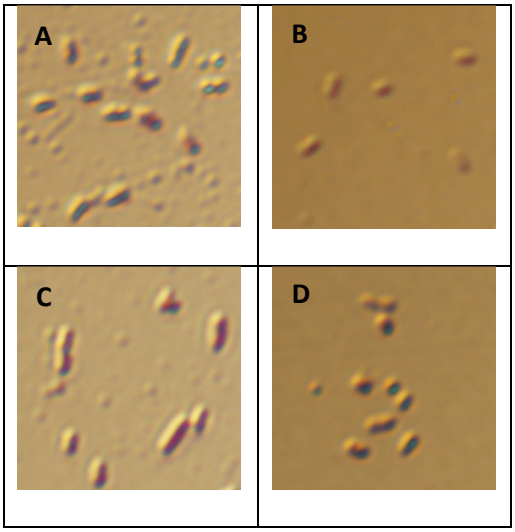

Figure 10. Micrographs comparing cell morphologies of *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah grown in two different broths. A sample of cells from each specimen that represent typical sizes are shown in each panel. (A) *P. aeruginosa* PAO1 grown in LB broth. (B) *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah grown in LB broth. (C) *P. aeruginosa* PAO1 grown in succinate broth. (D) *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah in succinate broth.

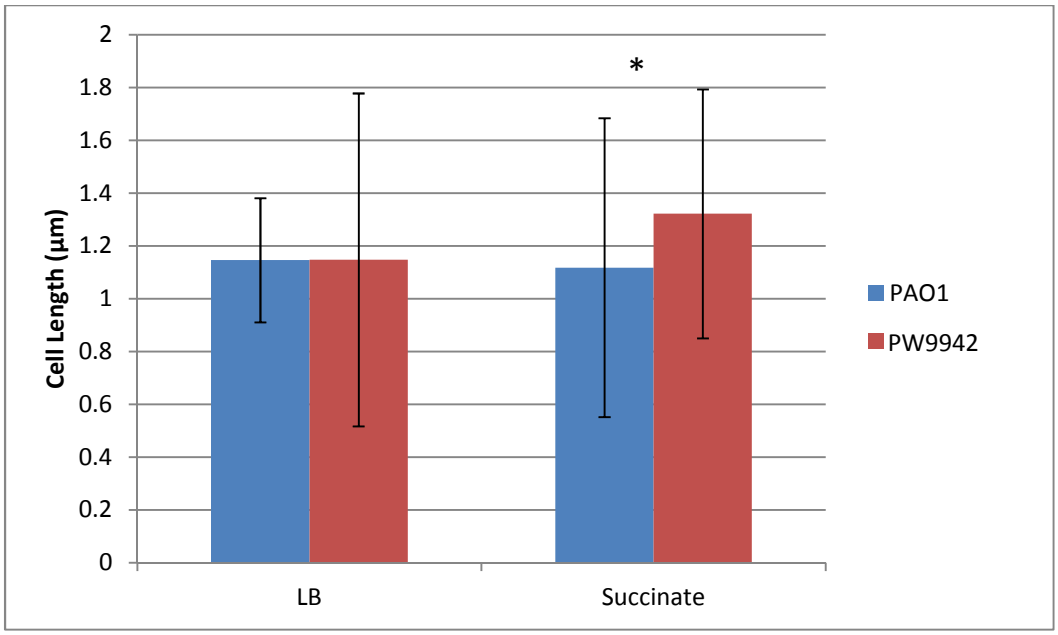

Figure 11. Cell size in LB and succinate broth. *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah (PW9942) were not significantly different sizes in LB broth. *P. aeruginosa* PAO1 was significantly smaller than *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah in succinate broth, shown in the second set of bars. Error bars represent 1 standard deviation of 4 trials in duplicate.

Siderophore Assay

Pyoverdine, a siderophore produced by *P. aeruginosa,* is considered a virulence factor and is important for iron acquisition from the environment. To determine if Lrp activity affected the production of this virulence factor, pyoverdine production was measured spectrophotometrically at 405 nm from a cell-free extract every 8 hours for 24 hours. Colony forming units (CFUs)/mL were calculated for each strain at all four time points to confirm

similarity in cell concentrations. The results of siderophore production for both *P. aeruginosa P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah in succinate broth are shown in **Figure 12**. The cell concentration appeared to be slightly lower for *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah than *P. aeruginosa* PAO1 at 8 hours, but had reached the same concentrations by 16 hours. Most notably, the mutant grew to a higher living cell density at 24 hours than the wild-type.

Pyoverdine production was significantly reduced for the PW9942:*lrp*-C04::IS*lacZ*/hah at later stages of growth. Pyoverdine production was first detected in the wild-type at 16 hours while *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah did not produce detectable pyoverdine until 24 hours. Pyoverdine levels were significantly different between *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah at both 16 and 24 hours.

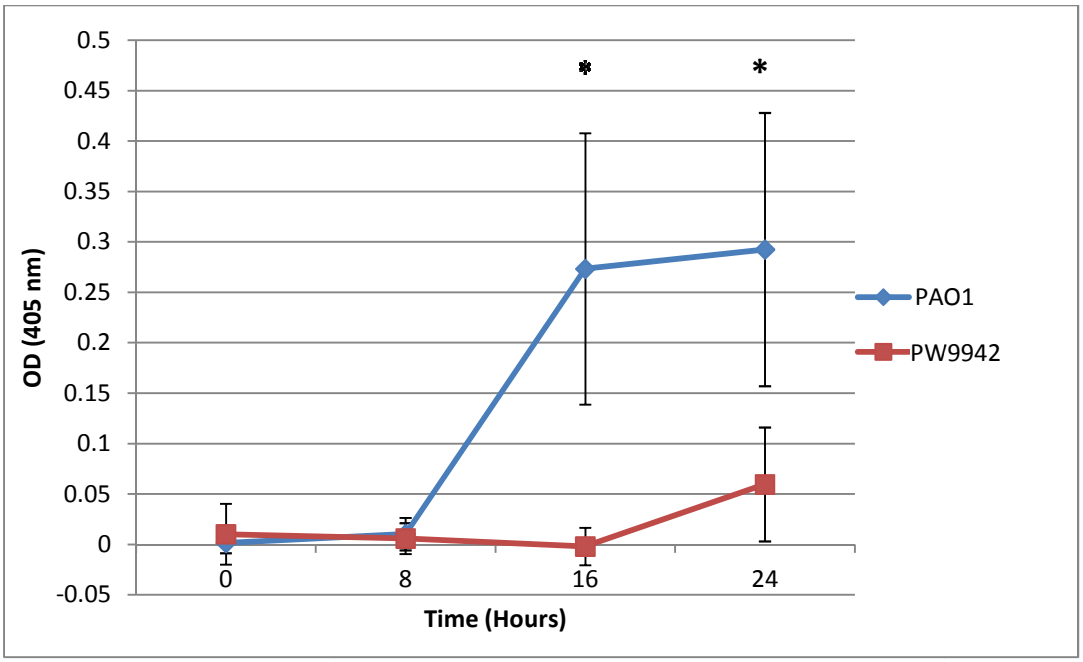

Figure 12. Spectrophotometry of pyoverdine production. Pyoverdine production from *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*C04::IS*lacZ*/hah was measured in cell-free extracts every 8 hours over a 24 hour period. The amount of pyoverdine is indicated as the absorbance of light (optical density= OD) at 405nm. A significant difference in pyoverdine production was measured at 16 and 24 hours for the wild-type and the mutant.

Biofilm Assay

Biofilm formation also promotes the virulence of pathogenic bacteria therefore biofilm formation was measured in a static culture for both *P. aeruginosa* strains in both LB broth and succinate broth. Cultures were grown for 24 hours in each broth, stained with crystal violet and measured spectrophotometrically at 540 nm. Absorbance is directly proportional to biofilm thickness; meaning, the higher the absorbance, the greater adherent cell population and

biofilm thickness. At 24 hours, biofilm production by *P. aeruginosa* PAO1 in LB broth was significantly greater than *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah ($p<0.05$) as shown in **Figure 13**. There was no significant difference found between the bacteria strains when grown in succinate broth, which suggested that the media is not nutritious enough to allow the dense biofilm layer to form for either the wild-type or the mutant.

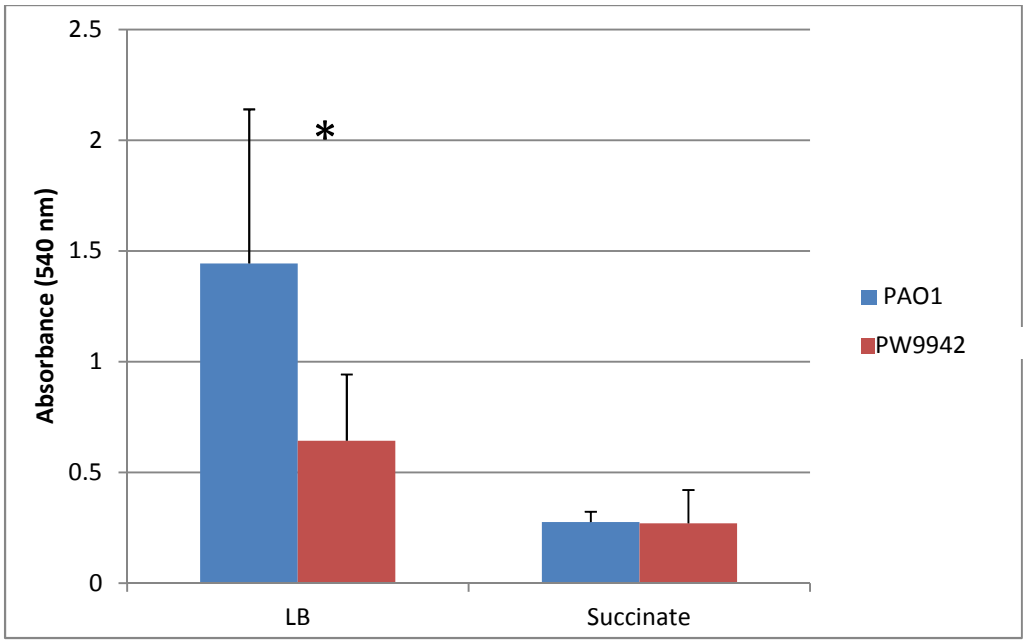

Figure 13. Twenty four hour biofilm formation in LB and succinate broth. Biofilm formation of static cultures of *P. aeruginosa* PAO1 and PW9942:*lrp*-C04::IS*lacZ*/hah (PW9942) at 24 hours in LB and succinate broths. Biofilm formation was indicated by absorbance of light at 540 nm. A significant difference was measured for LB broth, which is shown in the first set of bars. No significant difference was observed for biofilm formation when grown in succinate broth.

Because *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah showed no significant difference in biofilm formation in 24 hours in succinate broth, a 48 hour assay was performed. With more time, the cells might have been able to form a biofilm. The extended time did not result in significant differences as shown in **Figure 14**. These results suggested that the nutrients available to both strains were limiting and did not provide a suitable supply of energy to form a biofilm.

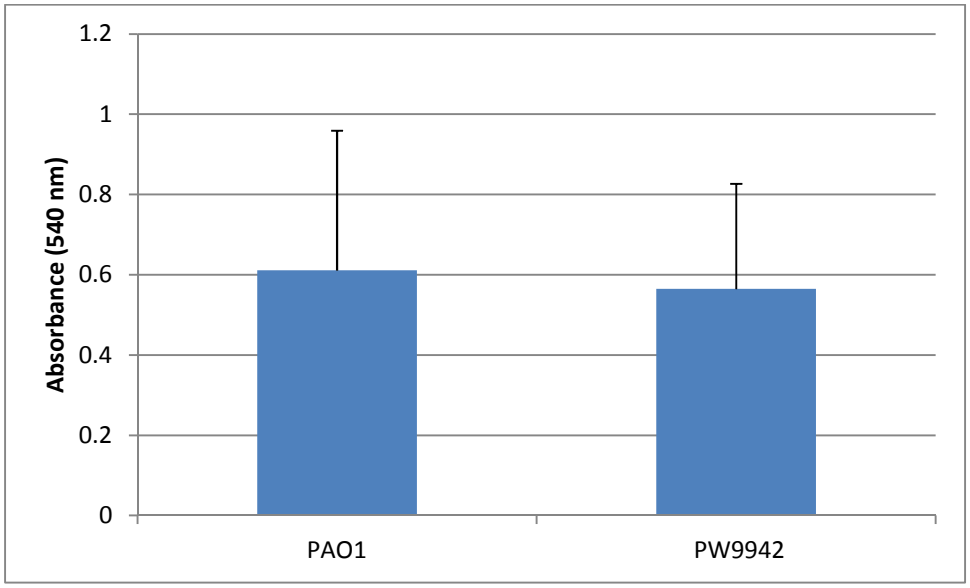

Figure 14. *P. aeruginosa* PAO1 and PW9942:*lrp*-C04::IS*lacZ*/hah biofilm formation in succinate broth. Biofilm formation in 48 hour static cultures of *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah (PW9942) in succinate broth shows no significant difference calculated between the wild-type and the mutant. Error bars represent 1 standard deviation of three trials in triplicate.

C. elegans Killing Assay

Siderophore production and biofilm formation vary significantly when comparing *P. aeruginosa* PAO1 to the *lrp* knock-out mutant. We wanted to determine if lethality would also differ between the wild-type and the mutant. *C. elegans* is an established model organism for infection by *P. aeruginosa* and was used in a 96-hour assay to determine differences in killing between *P. aeruginosa* PAO1 and PW9942:*lrp*-C04::IS*lacZ*/hah. Percentage survival was calculated every 24 hours for 96 hours as shown in **Figure 15.** Originally, all strains were grown on NGM-Lite however no death occurred over a four-day span with the wild-type PAO strain (data not shown). We attributed this as possibly being due to a lack of virulence factor induction due to the nutrient composition of the medium. *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah were grown on MinA plates for all subsequent assays to induce the expression of virulence factors in future experiments. Using a t-test, a significant difference in lethal effect occurred as compared to *E. coli* OP50 and *P. aeruginosa* PAO1 ($p=0.04$), as well as *E. coli* OP50 and *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah ($p=0.015$). Previous reports have shown that *P. aeruginosa* is toxic to *C. elegans*, which was supported by the data and analyses from this assay (32, 33). However, no significant difference (0.942) was determined between *P. aeruginosa* PAO1 and the *P. aeruginosa lrp-* mutant (PW9942:*lrp*-C04::IS*lacZ*/hah). These results are contradictory to the predicted outcomes of this assay in which *P. aeruginosa* PAO1 whould have caused greater fatalities in the challenged nematode population than *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah.

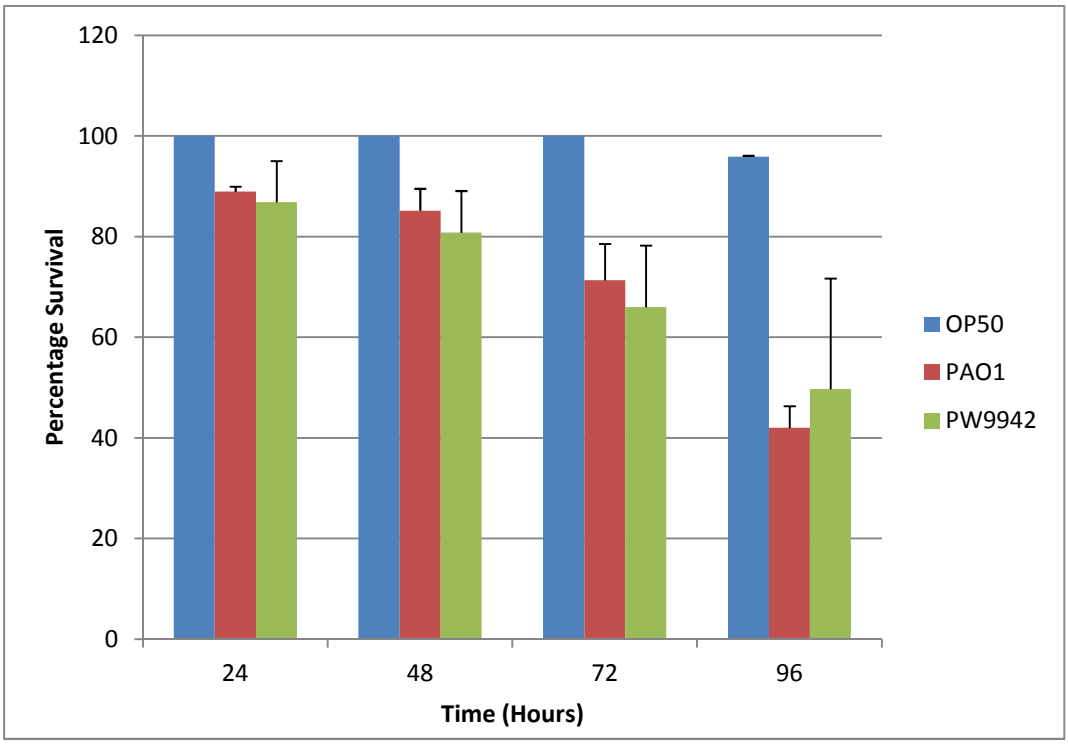

Figure 15. Pathogenicity of *P. aeruginosa* PAO1 and *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah. Percentage survival of *C. elegans* was calculated every 24 hours for 4 days. *C. elegans* were on a lawn of *E. coli* OP50, *P. aeruginosa* PAO1, and *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah (PW9942) and were transferred at the time of counting to a new culture of bacteria. A t-test reveals a significant difference between survival on *E. coli* OP50 and both *P. aeruginosa* strains, but no observable difference between PAO1 and PW9942:*lrp*-C04::IS*lacZ*/hah was seen.

Purification of Lrp

To test the binding of Lrp to specific DNA targets, the protein was purified using an affinity column. Lrp was over-expressed in *E. coli* BL21 DE3 + pET300:lrp_{PA} in LB supplemented with carbenicillin (50µg/ml) and purified with using a cobalt resin column. The large-scale purification resulted in successful isolation of His-tagged Lrp. (Of note, small scale protein purification using Qiagen his-tag columns was attempted many times and was unsucessful). SDS-PAGE analysis revealed a product of 22kDa, which is the predicted size of the monomer of Lrp with the 6X-histidine tag on the N-terminus. A Bradford assay was performed to calculate the amount of protein from each elution. **Table 1** shows typical readings of the elutions at 595 nm. Concentration of Lrp in the elutions was highest in the second elution therefore this fraction was dialyzed and used in all subsequent purifiction procedures.

	Elution 1	**Elution 2**	**Elution 3**	**Elution 4**	**Elution 5**
OD (595nm)	0.336	0.615	0.433	0.313	0.294
Concentration (µg/mL)	166.75	460.61	237.41	153.36	143.11

Table 1. Purification of Lrp_{PA} from an *E. coli* expression system. Protein concentrations from 5 column elutions were measured by Bradford assay at 595nm. A standard curve constructed from known concentrations of bovine serum albumin was used to calculate the concentration of protein in each elution.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed a purified protein of about 20 kDa, which is the predicted size of the monomer of Lrp with the 6X-histidine tag on the N-terminus. A SDS-PAGE of the dialyzed protein product is shown in lane 2 in **Figure 16**. All 5 elutions of protein showed a strong single band at the expected size of the Lrp product, with the highest amount of protein recovered in the second elution. This indicated that there was a high Lrp concentration in the column prep and was suitable for use in binding assays.

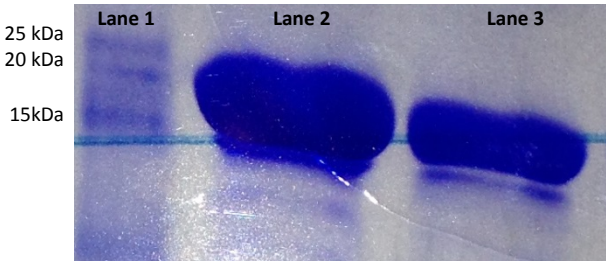

Figure 16. SDS-PAGE of Lrp_{PA} column purification. SDS-PAGE of the elution from a preparation of 6X his-tagged Lrp reveals a large single band of product at the expected size of 20 kDa. Lane 1 shows a standard protein ladder. Lane 2 contains the product from the second elution of Lrp. Lane 3 is the product of the third elution of Lrp.

Electromobility Shift Assay (EMSA)

Positive and negative control DNA probes were amplified by PCR for the EMSA to determine Lrp activity. The published binding site of the *dad* operon was amplified as the positive control. The DNA probe was successfully amplified by PCR to produce a single band,

which was gel-extracted from a 1.5% agarose gel. The *dad* operon amplicon is the expected 283 base pairs.

Lrp was purified and was subjected to electromobility shift assay (EMSA) testing attempt to demonstrate binding of the protein to DNA target sequences. The *dad* binding region was selected for this assay as a positive control for Lrp binding. The Tris-glycine native gel used in this the assay is shown in **Figure 17.** Lane 1 is a standard 100 base pair DNA ladder. Lanes 2 - 5 have 0.2nM *dad* binding site target DNA with varying concentrations of Lrp. Lane 2 has no Lrp to show baseline DNA movement. No discernable shift in DNA mobility was detected at any DNA concentration.

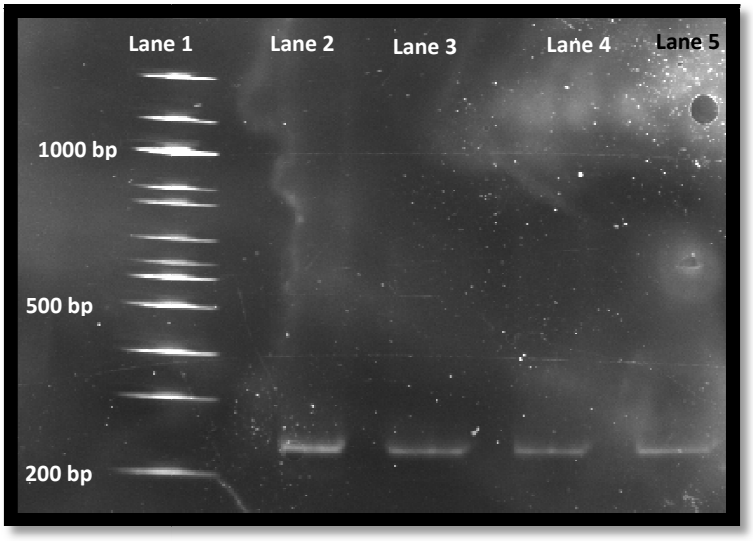

Figure 17. EMSA with positive control DNA probe. Negative and positive DNA controls were subjected to an EMSA with Lrp to detect activity of the purified protein. The gel shows a 100 base pair ladder in lane 1. Lane 2 has no Lrp to show the migration pattern of free probe. Lane 3 has 200 nM Lrp, lane 4 has 400 nM Lrp, and lane 5 has 800 nM. All samples were run with 5 mM L-alanine and EMSA buffer at 100V in a Tris-Glycine buffer.

BioLayer Interferometry

In addition to the EMSA, BioLayer Interferometry (BLI) was used to detect binding of Lrp to DNA targets. BLI also allows measurement of the kinetics of binding and release and makes it a useful tool for measuring molecular interactions. Cleared lysate from *E. coli* BL21 with and without the DNA construct was added to Ni-NTA sensors. Recombinant Lrp showed strong binding indicating that the 6X His-tag on the protein is present and exposed in the *E. coli* containing lrp_{PA} expression vector construct. The interferometry signal from the binding of Lrp to the probe is shown in **Figure 18**. The steep peak labeled "+ Lrp" demonstrates the change in refraction as Lrp from the cleared lysate bound to the biosensor. Some nonspecific binding occurs in the cleared lysate from the strain with no plasmid, but this binding is significantly less is less than that of the cleared lysate containing plasmid expressing Lrp.

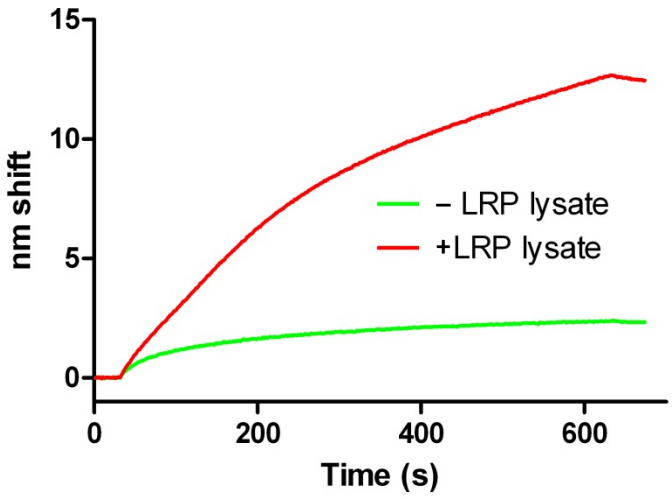

Figure 18. Cleared lysate interaction with Ni-NTA biosensors measured by BioLayer Interferometry (BLI). Refraction changes occur as Lrp from cleared lysate binds to Ni-NTA coated sensors in BLI. The shift in absorbance is shown on the y-axis as Lrp_{PA} binds, and the x-axis shows the time in seconds. *E. coli* BL21 cleared lysate is shown in green and lysate from *E. coli* BL21 with pET300:*lrp*$_{PA}$ is shown in red.

To test the ability of DNA-binding by the purified protein product, the positive control DNA sequence *dad* binding site was added to the sample. No binding was detected, which means that the purified product is inactive in the selected buffer, most likely due to misfolding.

DISCUSSION

Reports on *lrp* in *P. aeruginosa* suggest that it plays a role in nutrient acquisition and may regulate a larger spectrum of genes than has been previously published. The homology of Lrp_{PA} to the Lrp of other bacteria along with the findings of this study provide evidence that Lrp is part of a larger regulatory network and may serve as a potential drug target to reduce the virulence of *P. aeruginosa*.

P. aeruginosa lacking Lrp does not grow at the same rate as wild-type strain in both nutritious and minimal media, which suggests that *lrp* is influential in standard growth patterns. Growth is delayed because *lrp* may play a role in nutrient acquisition. If the ability to acquire nutrients for metabolism is affected by *lrp* as proposed, growth would be expected to be slower in comparison to the wild-type. Since Lrp may regulate metabolic genes, the response to changes in the media as nutrients are depleted may cause a delayed reaction to alterations in the metabolic pathway being utilized. This would lead to slower growth, which is what was observed in the lrp^- mutant *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah.

In addition to affecting the growth rate, the *lrp* gene was also influential in cell size. Because the growth of *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah was slower than PAO1 in nutritious broth, we expected that the cell size may be smaller. However, there was no difference in cell size in rich media. In minimal media, the wild-type was similar in size to both wild-type and mutant in nutritious broth. The mutant, however, was significantly larger than PAO1 in minimal media. Despite the fact that the mutant has a lower viable cell count at 16 hours, the cell size is significantly larger. The cells do not grow to large densities yet their cell

size is larger than that of wild-type. One suggestion for this observation involves the Krebs cycle, a metabolic pathway present in *P. aeruginosa*. One intermediate of this pathway involves succinate, which is the sole carbon source in the minimal media used in this assay. The finding that there is no difference in cell size in succinate broth indicates that the involvement of Lrp in this metabolic pathway is unlikely. Succinate is a sugar and may not be metabolized by enzymes that are regulated by Lrp. In contrast, the change in cell size in the more nutrient-rich LB broth suggests that there is a metabolic pathway used by *P. aeruginosa* in this medium that is affected by the presence of Lrp. Tryptone is one ingredient in the broth, which is a peptide resulting from the cleavage of casein by trypsin. If Lrp_{PA} plays a similar role as Lrp_{EC}, metabolism of proteins and amino acids may also be regulated by Lrp_{PA}. Individual metabolic pathways that are regulated by Lrp must be identified before a relationship between cell size and Lrp can be determined.

One observation of note in regards to the slides observed in the cell size assay was the presence of extracellular material (not quantified). Extracellular materials were visible on slides containing *P. aeruginosa* PAO1 (**Figure 10)**, but little to none of the same extracellular materials were observed on slides of *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah. The cells were the same size as the wild-type at the same time period, however no presence of extracellular substances when grown in either LB or succinate broths was observed. This could also support the previous suggestion that Lrp interacts with nutrient acquisition because extracellular materials may be metabolic and structural waste products and dependent on metabolic pathways within the bacterium. An example of this link between nutrient metabolism and extracellular material is biofilm formation, which is also limited in the lrp^- mutant.

Biofilm formation on untreated surfaces was also limited in statically-grown *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah when compared to *P. aeruginosa* PAO1 in nutritious broth. At 24 hours, *P. aeruginosa* PAO1 forms a significantly larger biofilm compared to that of PW9942:*lrp*-C04::IS*lacZ*/hah, indicating that *lrp* plays a role in biofilm formation. However, there is no difference between the strains in biofilm formation at 24 and 48 hours in succinate. This suggests that the media is not supportive enough to provide for both the metabolic needs of the bacterium in addition to the formation of a complex, polysaccharide biofilm. LB broth contains more organic carbon compounds, which allows the wild-type bacterium to maintain its metabolism in addition to forming a biofilm. In contrast, *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah is presumed not to be able to acquire its nutrients as efficiently and therefore does not form a biofilm as thick as the wild-type.

P. aeruginosa is a known pathogen of *C. elegans*. A killing assay was used to measure virulence in wild-type and mutant strains. *C. elegans* was chosen because of similarities of the innate immune system to humans making it an acceptable animal model of infection. The *C. elegans* killing assay was expected to demonstrate that overall virulence of *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah was less than that of PAO1 because the two strains had shown a significant difference in individual virulence factors in previous assays. However, the killing assay demonstrated that there was no significant difference in virulence between the two strains. Both strains were virulent in comparison to the avirulent control strain *E. coli* OP50, which confirmed previous reports using the same killing assay with *P. aeruginosa*. *C. elegans* has been determined to be affected by colonization of *P. aeruginosa* and consequent production of hydrogen cyanide in the gut (33). Although all factors that make *P. aeruginosa*

virulent in *C. elegans* are not known, it is highly likely that the *hcn* gene responsible for the manufacturing of HCN is not regulated by *lrp,* therefore the absence of the *lrp* gene does not affect virulence toward *C. elegans*.

In addition to characterizing the phenotype of an *lrp*-deficient mutant, protein-DNA interactions were attempted to detect binding of Lrp to select DNA targets. Large-scale purification of Lrp showed that a single product can be obtained using the pET300NT plasmid. In a small-scale experiment using the same construction in a 50 mL culture and a Qiagen Ni-NTA spin kit, no product was detected either by Coomassie stain in a SDS-PAGE gel or by a western blot. This may be because there was not enough protein from a 30 mL culture to be detected by either of these methods. The nickel in the spin column may also have a lower affinity for the His-tag in the 3-dimensional structure of the protein for attachment to the column surface. Large-scale protein preparations of 500 mls or higher with cobalt-coated resin beads were the best conditions for the preparation of the Lrp protein at concentrations that produced a visible band on a Coomassie-stained SDS-PAGE gel.

The EMSA and BLI assays did not demonstrate DNA binding with sequences published to be regulated by Lrp. The protein product from the elution step was present as a single band yet no interaction with DNA containing the Lrp binding sites of the *dad* operon could be demonstrated. The same publication demonstrated that dialysis into 20mM Tris pH 7.6 would be suitable for Lrp activity in an EMSA. We could not reproduce this result as no activity was detected by either method during our analysis. The EMSA showed a presence of the *dad* gene by SYBR green staining. A change in interference would have been detected if the protein was

in an active conformation and was able to bind to the target DNA. Lrp shows a sharp increase in refraction when binding to the biosensor, which indicates that the His-tag is exposed and has a high affinity for the Ni-NTA coated biosensors. However, no change in refraction occurs when the protein-bound biosensors are exposed to either negative or positive control DNA. BLI was also tested in the presence of 5 mM alanine for DNA targets, yet still no activity was detected.

Although the molecular interaction assays were unsuccessful in determining whether Lrp is capable of binding to DNA targets with specificity, there are other findings in these molecular assays that support the hypothesis that *lrp* regulates metabolic genes beyond the *dad* operon. Because there is a constitutively expressed alanine racemase in *P. aeruginosa*, there is still racemase activity without the use of *dadX*. That indicates that the differences observed between the wild-type and the mutant cannot be attributed to changes in the expression of *dadRAX*. For example, biofilm formation decreases significantly on non-treated surfaces. More metabolic genes must be affected by the absence of *lrp* than *dadRAX* to create a statistically significant difference in the phenotype. Also, the differences in growth rates cannot be fully dependent on an alanine racemase and dehydrogenase being expressed at different levels. The only carbon source available to *P. aeruginosa* was succinate in the minimal broth, which will not interact with DadA or DadX, but there was still an effect on the growth curve in the *lrp* deficient mutant.

There are several phenotypic effects observed in the mutant that appear to be beyond changes to the regulation of *dad*. Although the Lrp regulon seems to be larger than has been previously published, it may not regulate as many genes as Lrp does in *E. coli*. The overall

virulence of *P. aeruginosa* does not decrease significantly in a *C. elegans* killing assay even though two known virulence factors are significantly decreased in the mutant. This means that *P. aeruginosa* virulence in an animal model is not affected by *lrp*.

Biofilm formation is under the control of quorum sensing and this study shows that biofilm formation is limited in an *lrp* knock-out mutant strain of *P. aeruginosa*. This suggests that Lrp, as a transcription factor, interacts with or is a part of the quorum sensing network. Several of the effects observed in the mutant are similar to the findings of a previous study that used quorum sensing inhibitors (34). The authors demonstrated that there was a decrease in production of the virulence factor pyocyanin, formally classified as a siderophore, a reduction in biofilm formation and a reduction in virulence toward both *C. elegans* and human lung epithelial cells. Because the results of the quorum sensing study was similar to the findings of this study, it is reasonable to conclude that Lrp behaves similarly and may interact with the quorum sensing network in *P. aeruginosa*. O'Loughlin *et al.* (2014) concluded that the inhibitor partially prevented RhlR, a quorum sensing regulator, from allowing the expression of these virulence factors. It may be possible that Lrp is directly or indirectly regulated by this protein.

To continue this research and support these findings, a phenotypic characterization of an RhlR mutant using the same assays of this study could test the hypothesis that Lrp is regulated by RhlR. If the same effects in pyoverdine and biofilm formation and *C. elegans* killing assay are observed that may suggest that RhlR positively regulates Lrp. Because RhlR may play a larger role than Lrp, more extreme phenotypic changes may occur and virulence in *C. elegans* may decrease. A second project that will show the Lrp regulon is measuring RNA levels of DNA

targets in the *lrp* mutant. Quantitative PCR may be used to demonstrate changes in the levels of transcription of target DNA, such as genes that code for the changes observed in *P. aeruginosa* PW9942:*lrp*-C04::IS*lacZ*/hah measured in this study, or genes with homologs in *E. coli* that are regulated by Lrp_{EC}. If Lrp_{PA} demonstrates activity in the future by BLI or EMSA, it may also be used for a DNA footprinting assay to isolate target sequences that Lrp is bound to in *P. aeruginosa*. In combination with the bioinformatics output, this may provide the necessary information to identify the regulon of Lrp.

Lrp may play a larger role than previously thought and may serve as a target for pharmaceutical treatment in the future. Innate antibiotic resistance is on the rise in *P. aeruginosa* and Lrp may be a suitable alternative target to down regulate the virulence factors that are problematic in infection. The advantage of selecting a target that does not cause death in the bacteria but rather a suppression of virulence factors is that there is no selection for resistance to the treatment. This and previous studies indicate that Lrp may be a suitable long-term treatment for infection by *P. aeruginosa*.

ACKNOWLEDGEMENTS

This research was made possible by funding from the NSF-FACES grant, CSM Mentor-Protégé program, CETL Incentive Award, and OVPR grant. I would like to thank past Griffin lab members Lindsey Parks, Lisley Garcia, Leonard Amodio, III, Mary Girton, and Candice Caroline Bramlett for their assistance in data collection. Additional contribution was provided by Dr. Martin Hudson and undergraduate researchers in the *C. elegans* killing assay, Dr. Eric Albrecht in microscopy, and Dr. Jonathan McMurry and undergraduate researchers in the purification of Lrp and BLI. Finally, I would like to thank my thesis committee, Dr. Donald McGarey, Dr. Xueya Hauge, and Dr. Carol Chrestensen for all of their support throughout the program and for allowing me the use of equipment and materials. I would also like to thank my thesis advisor Dr. Melanie Griffin for her support and guidance in both graduate and undergraduate research. Finally, I would like to thank my parents, Nick and Linda Schaedle, for their support of my education since 1995, and my husband Nate, who kept me motivated and helped support me throughout the program.

REFERENCES

1. **Todar K.** 2010. Todar's Online Textbook of Bacteriology: Opportunistic Infections Caused by *Pseudomonas aeruginosa.*
2. **Hoiby N.** 1998. *Pseudomonas* in cystic fibrosis: Past, present, and future. Cystic Fibrosis Trust, London, United Kingdom.
3. **Masak J, Cejkova A, Schreiberova O, Rezanka T.** 2014. *Pseudomonas* biofilms: possibilities of their control. FEMS Microbiol. Ecol. **88**:1-14.
4. **Visca P, Imperi F, Lamont IL.** 2007. Pyoverdine siderophores: from biogenesis to biosignificance. Trends Microbiol. **15**:22-30.
5. **Whiteley M, Lee KM, Greenberg EP**. 1999. Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U. S. A. **96**:13904–13909.
6. **Rutherford ST, Bassler BL**. 2012. Bacterial quorum sensing: Its role in virulence and possibilities for its control. Cold Spring Harb. Perspect. Biol. 2:a012427.
7. **Ng W, Bassler BL.** 2009. Bacterial quorum-sensing network architectures. Ann. Rev. Gen. **43**:197-222.
8. **Tashiro Y, Yawata Y, Toyofuku M, Uchiyama H, Nomura N.** 2013. Interspecies interaction between *Pseudomonas aeruginosa* and other microorganisms. Microbes Environ. **28**:13-24.
9. **Visca P, Ciervo A, Orsi N.** 1994. Cloning and nucleotide sequence of the *pvdA* gene encoding the pyoverdine biosynthetic enzyme L-ornithine N^5-oxygenase in *Pseudomonas aeruginosa*. J. Bacteriol. **176**:1128–1140.

10. **Ratledge C, Dover LG.** 2000. Iron metabolism in pathogenic bacteria. Annu. Rev. Microbiol. **54**:881-941.
11. **Schalk IJ and Guillon L.** 2013. Pyoverdine biosynthesis and secretion in *Pseudomonas aeruginosa*: Implications for metal homeostasis. Environ. Microbiol. **15**:1661-1673.
12. **Kirienko NV, Kirienko DR, Larkins-Ford J, Wahlby C, Ruvkun G, Ausubel FM.** 2013. *Pseudomonas aeruginosa* disrupts *Caenorhabditis elegans* iron homeostasis, causing a hypoxic response and death. Cell Host Microbe. **13**:406-416.
13. **Takase H, Nitanai H, Kazuki H, Otani Y.** 2000. Impact of Siderophore production on *Pseudomonas aeruginosa* infections in immunosuppressed mice. Infection and Immunity. **68**:1834-1839.
14. **Watnick P, Kolter R.** 2000. Biofilm, City of Microbes. J. Bacteriol. **182**:2675-2679.
15. **Lintner RE, Mishra PK, Srivastava P, Martinez-Vaz BM, Khodursky AB, Blumenthal RM.** 2008. Limited functional conservation of a global regulator among related bacterial genera: Lrp in *Escherichia*, *Proteus* and *Vibrio*. BMC Microbiol. **8**:article 60.
16. **Friedberg D, Platko JV, Tyler B, Calvo JM**. 1995. The amino acid sequence of Lrp is highly conserved in four enteric microorganisms. J. Bacteriol. **177**:1624-1626.
17. **Belitsky BR, Gustafsson MC, Sonenshein AL, Von Wachenfeldt C**. 1997. An *lrp*-like gene of *Bacillus subtilis* involved in branched-chain amino acid transport. J. Bacteriol. **179**:5448-5457.
18. **Ward JB, Jr, Zahler SA.** 1973. Regulation of leucine biosynthesis in *Bacillus subtilis*. J. Bacteriol. **116**:727–735.

19. **Leonard PM, Smits SH, Sedelnikova SE, Brinkman AB, de Vos WM, van der Oost J, Rice DW, Rafferty JB**. 2001. Crystal structure of the Lrp-like transcriptional regulator from the archaeon Pyrococcus furiosus. EMBO J. **20**:990-997.

20. **Napoli A, van der Oost J, Sensen CW, Charlebois RL, Rossi M, Ciaramella M.** 1999. An Lrp-like protein of the hyperthermophilic archaeon Sulfolobus solfataricus which binds to its own promoter. J. Bacteriol. **181**: 1474-1480.

21. **Cho BK, Barrett CL, Knight EM, Park YS, Palsson BO**. 2008. Genome-scale reconstruction of the Lrp regulatory network in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **105**:19462-19467.

22. **Haney SA, Platko JV, Oxender DL, Calvo JM.** 1992**.** Lrp, a leucine-responsive protein, regulates branched-chain amino acid transport genes in *Escherichia coli.* J Bacteriol. **174**:108-15.

23. **de los Rios S, Perona JJ.** 2007. Structure of the *Escherichia coli* leucine-responsive regulatory protein Lrp reveals a novel octameric assembly. J. Mol. Biol. **366**: 1589-1602.

24. **Platko JV, Willins DA, Calvo JM**. 1990. The *ilvIH* operon of *Escherichia coli* is positively regulated. J. Bacteriol. **172**:4563 – 4570.

25. **Calvo JM, Matthews RG.** 1994. The leucine-responsive regulatory protein, a global regulator of metabolism in *Escherichia coli*. Am. Soc. Microbiol. **58**:466-490.

26. **Yokoyama K, Ishijima SA, Clowney L, Koike H, Aramaki H, Tanaka C, Makino K, Suzuki M.** 2005. Feast/famine regulatory proteins (FFRPs): *Escherichia coli* Lrp, AsnC and related archaeal transcription factors. FEMS Microbiol. Rev. **30**:89-108.

27. **Boulette ML, Baynham PJ, Jorth PA, Kukavica-Ibrulj I, Longoria A, Barrera K, Levesque RC, Whitely M.** 2009. Characterization of alanine catabolism in *Pseudomonas aeruginosa* and its importance for proliferation *in vivo*. J. Bacteriol. **191**:6329-6334.

28. **He W, Li C, Lu CD.** 2011. Regulation and characterization of the *dadRAX* locus for L-amino acid catabolism in *Pseudomonas aeruginosa*. J. Bacteriol. **193**:2107-2115.

29. **Parks LN, Griffin MC**. 2011. Preliminary characterization of the role of Lrp and leucine effects in *Pseudomonas aeruginosa*. Proceeding from 111th General Meeting of the American Society of Microbiology. May 2011. New Orleans, LA

30. **Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrock-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, Olson MV.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature. **406**: 959-964.

31. **Jacobs MA**, **Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S, Will O, Kaul R, Raymond C, Levy R, Chun-Rong L, Guenthner D, Bovee D, Olson MV, Manoil C.** 2003. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA. **100**:14339-14344.

32. **Kirienko NV, Cezairliyan BO, Ausubel FM, Powell JR.** 2014. *Pseudomonas aeruginosa* PA14 pathogenesis in *Caenorhabditis elegans*. Methods Mol. Biol. **1149**:653-669.

33. **Gallagher LA, Manoil C**. 2001. *Pseudomonas aerugionsa* PAO1 Kills *Caenorhabditis elegans* by Cyanide Poisoning. J. Bacteriol. **183**:6207 – 6214.

34. **O'Loughlin CT, Miller LC, Siryaporn A, Drescher K, Semmelhack MF, Bassler BL.** 2014. A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa* virulence and biofilm formation. P. Natl. Acad. Sci. USA. **110**:17981–17986.