CHARACTERIZATION OF A PROTEIN INVOLVED IN CELL MORPHOLOGY AND PYOMELANIN PRODUCTION IN *LEGIONELLA PNEUMOPHILA*

by

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A Dissertation

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy

Department of Biological Sciences

West Lafayette, Indiana

December 2020

THE PURDUE UNIVERSITY GRADUATE SCHOOL
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To my mother, Angela Cruz, my wife, Noemi Roman and my lovely daughters, Noemily, Ariana and Daisy for all the love and support, you are my all!

ACKNOWLEDGMENTS

Firstly, I would like to express my most sincere gratitude to my advisor, Dr. Zhao-Qing Luo for his mentorship. I have learned a tremendous amount through his guidance. Dr. Luo has taught me how to correctly set up experiments, the importance of controls and reading literature, how to think critically and work efficiently. In addition, Dr. Luo encouraged me to be the best communicator I could be, through accurate and concise reporting of Science. He has been enormously generous and patient throughout all these years. Thank you, Dr. Luo!

Secondly, I would like to thank past members of the Luo Lab. Dr. Wenhan Zhu, Dr. Jiazhang Qiu, Dr. Yao Liu, Dr. Ninghai Gan and Dr. Alix McCloskey. You were all so kind, friendly and helpful. Thanks to you guys, our lab environment was always welcoming and friendly to everybody. I will always appreciate all your technical help but also all the laughter we had in the daily conversations we shared. I am also very thankful of all the past members of Zhou lab. Through many years Dr. Zhou and his group shared their experiences and technical skills during our weekly joined lab meetings, and I learned a vast amount of these exchanges.

A special thanks to Dr. Peter Hollenbeck, a member of my thesis committee and my teaching supervisor during many years. I will always remember how passionate and cool you were in teaching all these young undergraduate students. You inspired and helped me realize that there is wisdom in sharing and helping others learn about Science. I now accept that is a wonderful feeling being able to help a very lost and frustrated undergraduate student, to understand concepts related to Cell Biology. I must confess that teaching was not something I considered enjoyable. But my experience with BIOL 231 and Dr. Hollenbeck truly changed this perspective for me forever! I would also like to thank the rest of my thesis committee, Dr. Daogou Zhou and Dr. James Samuel for all the help, scientific discussions and time invested in my research project.

Finally, I am eternally grateful to my wife, Noemi Roman, for all the unconditional support these years. This has been a long and stressful journey and you were always there for me after a mentally and physically-draining day in the lab. Thank you for taking care of our family while I spent all the extra hours in the lab and for all the sacrifices you have made so I could complete my doctoral degree.

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ABSTRACT

Legionella pneumophila is an intracellular pathogen and the etiological agent of Legionnaires' disease, a severe atypical pneumonia. This bacterium is ubiquitous to freshwater ecosystems where it spreads in the planktonic form but is primarily found associated with protozoa. Protozoa serve as a niche for its replication because the extracellular environment often does not offer sufficient nutrients to support the growth of this bacterium. *L. pneumophila* is an opportunistic pathogen in humans and utilizes an arsenal of virulence factors to colonize hosts and cause Legionnaires' disease. The transition between extracellular and intracellular milieus triggers a series of metabolic, morphological and genetic changes that define two developmental stages in this bacterium: replicative and transmissive. Relatively high concentration of nutrients triggers the replicative stage of growth, where the bacterium has the appearance of a thin, elongated rod without the presence of flagella. In addition, is characterized by active metabolism and expression of genes required for productive replication. In contrast, once nutrient levels are relatively low, *L. pneumophila* switches to its transmissive form. In this form, the bacterium activates a genetic program that includes the expression of many traits associated with the transmissive stage, such as coccoid cell shape, motility, pigmentation and important virulence factors. These multifaceted changes in gene expression leading the differentiation from replicative to the transmissive form, are controlled by two-component regulatory systems. Specifically, the LetAS two-component system plays a key role in the regulation of cell morphology and in the production of the pigment pyomelanin. Here we report the identification of a LetAS-regulated protein, Lpg0586 (designated as Larp1), capable of inducing changes in cell morphology and pigment production. We found that Larp1 expression was accompanied by accumulation of the RecA protein, but evaluation of *recA* deletion mutants indicated that RecA is not involved in cell morphology changes in *L. pneumophila*. The specific reason as to why RecA accumulates upon Larp1 expression remains to be elucidated. However, we show that upon synthetic HGA treatment, *L. pneumophila* cultures display cell elongation and increased RecA levels. Lastly, Larp1 expression restored pyomelanin production in an un-pigmented mutant and increased the transcription of important genes involved in the pyomelanin production pathway. Based on these findings, Larp1 is the first LetAS-regulated protein reported to be involved in pyomelanin production.

CHAPTER I. GENERAL INTRODUCTION

Legionella pneumophila and its protozoan host

Legionella pneumophila was first identified as a causative agent of disease following an outbreak of severe pneumonia during a 1976 American Legion Convention in Philadelphia (Brenner et al., 1979; Fraser et al., 1977; McDade et al., 1977). After the identification of the pathogen responsible for the outbreak, the disease was eventually designated as Legionnaires' disease. Later studies revealed that *L. pneumophila* is an intracellular pathogen ubiquitous in aquatic environments where it preferentially replicates in amoebae (Rowbotham, 1980). Specifically, this bacterium is able to replicate within soil and freshwater amoeba of the genera *Acanthamoeba, Naegleria* and *Dictyostelium* (Fliermans et al., 1981; Solomon et al., 2000). Once internalized, the phagosome containing the bacterium forms a unique membrane-bound compartment called the *Legionella*-containing vacuole (LCV). This compartment, evades the endocytic network by blocking fusion of the LCV with lysosomes in the first few hours, however the LCV eventually develops into a vacuole with the characteristics of lysosomes (Isberg et al., 2009; Sturgill-Koszycki & Swanson, 2000). *L. pneumophila* replicates within the LCV until nutrients in infected cells are depleted which is concomitant with the lysis of the host cells and the release of the bacteria to start the second infection cycle to restart the cycle (Molofsky & Swanson, 2004).

Legionella pneumophila: From aquatic environments to the host cell

L. pneumophila is a fastidious bacterium and nutrients in the environment often are not sufficient to support its replication, thus it is believed that very little planktonic bacterial growth occurs in natural settings. Instead, *L. pneumophila* preferentially parasitizes protozoa, such as amoeba, in the environment which provides nutrients to the bacteria as well as serving as a protective shelter from harsh environmental conditions (Cunha et al., 2016). Protozoa are eukaryotic unicellular microorganisms, many of which prey on bacteria. These microorganisms share similar cellular and molecular characteristics in phagosome biogenesis and maturation with mammalian macrophages. In fact, the development of the LCV is highly similar in these two hosts (Fields et al., 2002; Hilbi et al., 2007). Human exposure arises from contracting bacteria grown in

man-made aquatic environments, such as air circulation systems in hotels and assembly halls and other facilities equipped with large cooling systems. Bacteria originated from these man-made systems, are believed to be the major source of *L. pneumophila* in community-acquired infections. Other potable and non-potable water sources such as water fountains, taps, showers, air conditioners and evaporative condensers, have been reported to serve as source of infections with *L. pneumophila* (Cunha et al., 2016; Newton et al., 2010). However, human infection is considered accidental and a dead end for *L. pneumophila*, only one case of suspected human-human transmission has been reported to date (Correia et al., 2016). Whether this was an isolated event, or it represents a new mechanism of transmission, remains unclear and requires further investigation. Therefore, human infection by *L. pneumophila* occurs mostly because of compromised or poorly maintained man-made aquatic environments (Cunha et al., 2016; Hilbi et al., 2010). After inhalation of infected aerosols containing *L. pneumophila*, the bacterium reaches the lungs and is engulfed by alveolar macrophages wherein it establishes the LCV to support its intracellular replication, leading to tissue damage and development of Legionnaires' disease (Newton et al., 2010).

The populations with chronic lung diseases, such as the elderly or immune-compromised patients, are more susceptible to infections by *L. pneumophila* (CDC, 2018b; Gudiol et al., 2007; Newton et al., 2010; Oliva et al., 2018). Symptoms associated with Legionnaires' disease include cough, shortness of breath, fever, muscle aches and headaches (Daniels et al., 2000), which usually start 2 to 10 days after exposure but can take as long as 2 weeks. Legionnaires' disease can be treated with antibiotics and the majority of the patients make a full recovery (Gudiol et al., 2007). However, this disease can be fatal as one out of ten people that get infected will die (CDC, 2018b). The Centers for Disease Control and Prevention stated that in the United States alone, the number of reported cases since the 2000s have increased nearly 5 times but it is unclear whether this is a result of increased awareness and testing, higher persistence of *Legionella* in the environment or increased susceptibility of the population to the bacterium (CDC, 2018a). Due to lack of diagnostic and surveillance methods in many countries, worldwide occurrence is unknown. According to the World Health Organization, in Europe, Australia and the USA, there are about 10-15 cases reported per million population a year with 75-80% of the cases for patients over 50 years old and 60-70% being male (WHO, 2018).

Virulence factors of *Legionella pneumophila*

Legionella pneumophila encodes several well-characterized virulence factors, including LPS, some surface-associated proteins and type II and type IV secretion systems. For instance, the LPS O chain of *L. pneumophila* is unusually hydrophobic (Knirel et al., 1994; Zähringer et al., 1995), typical of synthetic particles that macrophages do not deliver to lysosomes effectively (de Chastellier et al., 1995; Zähringer et al., 1995). Importantly, type IV secretion-deficient mutants remain viable in mouse macrophages, probably because of the absence of key digestive factors (Joshi et al., 2001), even though these LCVs contain endosomal and LAMP-1 markers (Conover et al., 2003). This suggests that other factors besides type IV secretion (discussed below) are involved in the evasion of the lysosome with more digestive powers. Of note, *L. pneumophila* sheds outer membrane vesicles (OMVs) high in LPS into the supernatant of broth cultures (Fernandez-Moreira et al., 2006; Seeger et al., 2010). The role of LPS in the inhibition of phago-lysosomal fusion, was demonstrated by Swanson and colleagues by showing that purified OMVs attached to latex beads, displayed a delay in such fusion for up to 5 hours in macrophages (Fernandez-Moreira et al., 2006). By 10 hours, most of the beads (~70%) were delivered to lysosomes. However, the question remained as to whether the inhibition of phago-lysosomal fusion was specific to LPS or other factors present in the OMVs. Later it was shown that LPS not linked to OMVs was enough to inhibit phago-lysosomal fusion up to 5 hours, which establishes this surface molecule as an independent factor for the evasion of lysosomal degradation (Seeger et al., 2010).

L. pneumophila encodes a type II secretion system (T2SS) evolutionary related to type IV pili (Cianciotto, 2005; Johnson et al., 2006). Recently, through the application of electron cryotomography, the structure of the *L. pneumophila* T2SS has been observed *in vivo*, showing the similarities, but also some differences, with type IV pili (Ghosal et al., 2019). The T2SS of *L. pneumophila* secretes at least 25 substrates, however, recent *in silico* analysis predict that $\geq$60 proteins contain signal sequences and are predicted to be extracellular (Cianciotto, 2009; DebRoy et al., 2006). Some of the biochemical activities associated with these T2SS substrates include phosphatase, protease, lipolytic, surfactant secretion and low temperature growth (Cianciotto, 2013; Stewart et al., 2009; Söderberg et al., 2008). Notably, *L. pneumophila* T2SS mutants show significant intracellular growth defects in protozoa such as *Hartmannella vermiformis* and *Acanthamoeba castellanii* and to a lesser extent in murine models (Hales & Shuman, 1999a; Liles

et al., 1999; Rossier et al., 2004). Taken together, these observations indicated that the T2SS of *L. pneumophila* is another important virulence factor for this bacterium.

The macrophage infectivity potentiator (Mip) surface protein represents another long-known virulence factor of *L. pneumophila*. For instance, the Mip protein was shown to be required for productive infection of alveolar macrophages and protozoa such as *H. vermiformis* (Cianciotto et al., 1990; Cianciotto et al., 1989; Cianciotto & Fields, 1992). These studies described the first cloned *L. pneumophila* needed for optimal infectivity. Later, the Mip protein was defined as a peptidyl-proline *cis-trans*-isomerase (Fischer et al., 1992). The exact role of Mip in virulence is not clear, but it was later found to be required for the secretion of phospholipase C-activity into culture supernatants (DebRoy et al., 2006). Interestingly, the Mip-dependent phospholipase C activity observed in culture supernatants was dependent on type II secretion, which suggests that Mip is involved in the activation of a type II effector protein at the cell surface (DebRoy et al., 2006).

Among the virulence factors mentioned above, the Dot/Icm type IV protein translocation system (defect in organelle trafficking/intracellular multiplication), is considered the most essential virulence factor of this pathogen. The Dot/Icm system is a complex protein delivery system, distantly related to classical conjugation systems, composed of approximately 26 proteins that spans the inner and outer bacterial membranes (Kubori & Nagai, 2016), it injects over 330 protein substrates, also called effector proteins, into host cells (Ensminger, 2016). Simply put, the Dot/Icm system can be separated in 3 distinct regions, an inner membrane complex that interact with the soon-to be secreted substrates, a transmembrane complex connecting the inner and outer membranes and the outer membrane complex that forms the apparatus that “injects” proteins into the host cell to hijack important cell processes (Kubori & Nagai, 2016; Ninio et al., 2005; Vincent et al., 2006). The discovery of the essential role of the Dot/Icm system was achieved through the isolation of mutants that failed to replicate within macrophages and the identification of genomic regions capable of restoring this defect in intracellular multiplication (Berger & Isberg, 1993; Marra et al., 1992). The sheer amount of effector proteins shown to be translocated into host cells is significant and accounts for more than 10% of the predicted proteome, which is five times more than those predicted for *Salmonella enterica* and *Escherichia coli* O157:H7 (Ensminger, 2016). Recent genome sequencing reveals that more than 18,000 such effectors are present in 58

Legionella species (Burstein et al., 2016; Gomez-Valero et al., 2019), which represents a rich source of bacterial enzymes that target host cell biology by diverse biochemical mechanisms.

L. pneumophila Dot/Icm effector proteins are enormously diverse and capable of targeting a vast array of host cellular processes. (Burstein et al., 2016; Qiu & Luo, 2017). For instance, the first type IV effector ever characterized, called RalF, was found to colocalize with the small GTPase ARF1 and to be necessary for the recruitment of ARF1 to LCV (Nagai et al., 2002). It was also shown that RalF is capable of activating ARF1 through intrinsic guanine-exchange nucleotide (GEF) activity. Presumably activated ARF1, a small GTPase involved in membrane trafficking between Golgi and ER, aids in the biogenesis of the LCV through recycling of membrane coat proteins from the LCV back to the ER, which in turns promotes secretion of vesicles from the ER, an important feature in the expansion of the LCV (Kagan & Roy, 2002; Qiu & Luo, 2017). In addition to ARF1, *L. pneumophila* hijacks the activity of Rab1, another small GTPase important for vesicle trafficking (Hardiman et al., 2012) through other type IV effector proteins. Specifically, *L. pneumophila* injects the effectors SidM and AnkX into host cells to target Rab1. SidM (also DrrA) was shown to modify Rab1 through AMPylation, a process where the adenosine monophosphate group from ATP is transferred to the substrate, locking the small GTPase in its GTP-loaded activated form (Müller et al., 2010). In contrast, AnkX was shown to modify Rab1 through a process called phosphorylcholination, a reaction where the phosphorylcholine group from CDP-choline is transferred to the substrate Rab1, leading to the inhibition of its activity (Mukherjee et al., 2011; Tan et al., 2011). The modification of Rab1 by the effectors SidM and AnkX was shown to be regulated by proteins that reverse these modifications in a temporal manner such as the deAMPylator SidD (Neunuebel et al., 2011; Tan & Luo, 2011) and the dephosphorylcholinase Lem3 (Tan et al., 2011). Another significant finding came through the discovery that the effector SdeA transfers an ubiquitin variant to several Rab proteins, such as Rab33B, through unconventional ubiquitination (Qiu et al., 2016). In fact, this form of ubiquitination had not been seen before and defied the established ubiquitination mechanisms. The newly discovered ubiquitination reaction was independent of E1 and E2 enzymes, which typically mediate the activation of ubiquitin (Ub) through attachment of an AMP moiety to Ub. This enables the E3 ligase to transfer ubiquitin to a target substrate in the classical ubiquitination pathway. Strikingly, SdeA activates Ub through ADP-rybosylation, a reaction where ADP-ribose from nicotinamide adenine dinucleotide (NAD) is transferred to the target. This

biochemical activity originates from the presence of a mART motif in the center of the SdeA protein. The discovery of SdeA, a bacterial effector that carries out ubiquitination by non-canonical means, showed us that there is still much to learn and gain from studying bacterial effectors and their functions to potentially uncover new cellular signaling pathways. More recently, the effector MavC was shown to target NF-κB signaling pathway by ubiquitinating the E2 ligase UBE2N, in a non-canonical manner (Gan et al., 2020; Gan, Nakayasu, et al., 2019). Modification of UBE2N prevents formation of polyubiquitin chains, which ultimately prevents NF-κB activation early during infection. The characterization of MavC shed light on the unique ways that bacterial pathogens can exploit host immunity through a mechanism that involves ubiquitin signaling. The bacterial virulence factors described above represent only a small fraction of *Legionella's* repertoire and more recently in excess of 18,000 effectors spanning the whole *Legionella* genus and harboring eukaryotic-like motifs, were predicted through comparative genomics (Gomez-Valero et al., 2019). These unprecedented findings suggest that we still have much to learn of the *Legionella* life cycle and the interaction with eukaryotic hosts.

Morphological changes during *Legionella pneumophila* life cycle

Bacterial pathogens can alter their morphology in response to environmental cues that induce complex signal transduction pathways. This alteration is accompanied by physiological, metabolic and genetic changes in the bacterium and it was originally referred to as "microbial differentiation" in *L. pneumophila* (Molofsky & Swanson, 2004; Oliva et al., 2018). Simply put, *L. pneumophila* can alternate between two different forms, a replicative and a transmissive form. This differentiation is not unique to *Legionella* and it has been documented in other intracellular pathogens such as *Coxiella burnetii*, the causative agent of Q fever, and the sexually transmitted pathogen, *Chlamydia trachomatis* (Hammerschlag, 2002; van Schaik et al., 2013). In the replicative form, *Legionella* are metabolically active and appear as elongated and thin rods whereas, transmissive bacteria appear as short, flagellated rods **(Fig. 1-1)** (Faulkner & Garduño, 2002). In the replicative form, which is adopted when nutrient levels are high, *Legionella* constitutively express a repressor of transmission traits to inhibit traits such as motility and cytotoxicity to eukaryotic cells (Molofsky & Swanson, 2003). In contrast, bacteria in the transmissive phase appear when nutrients have been depleted after productive replication. Successful conversion into the infectious variant is achieved by activation of genes that govern

transmission traits required for lysosomal evasion, to escape from the host cell, survival in the extracellular milieu and invasion of a new host (Oliva et al., 2018). Two-component regulatory systems play an essential role in controlling the complex changes in gene expression that govern the differentiation from replicative to the transmissive form in *L. pneumophila* (Segal, 2013).

Two-component systems (TCSs) are a commonly used signal transduction machinery by which bacteria respond to stimuli from changes in their living environment. Pathogenic bacteria can utilize multiple TCS to regulate expression of virulence genes (Gotoh et al., 2010). TCSs sense environmental signals through the action of a membrane-bound sensor histidine kinase and a cytoplasmic transcriptional regulator which serves as a response regulator (Capra & Laub, 2012). Upon stimulation, the sensor histidine kinase autophosphorylates, which phosphorylates the response regulator, leading to its activation (Jung et al., 2012). Current knowledge suggests the involvement of at least four distinct TCSs (Cazalet et al., 2004) that regulate gene expression in *L. pneumophila*: the LqsRS, CpxRA, PmrAB, and LetAS (Gal-Mor & Segal, 2003a, 2003b; Rasis & Segal, 2009; Tiaden et al., 2007; Zusman et al., 2007).

The LqsRS two-component system

L. pneumophila synthetizes 3-hydroxypentadecane-4-one, which is a small, diffusible signaling molecule named *Legionella* auto-inducer 1 (LAI-1) (Tiaden, Spirig, & Hilbi, 2010). Response to this signaling molecule by *L. pneumophila* is mediated by the LqsRS TCS which is homologous to the prototype *V. cholerae* CqsAS TCS (Spirig et al., 2008). *Vibrio spp* produce three different classes of autoinducers: uranosyl boratediester, AI-2, N-acyl-L-homoserine lactones, AHLs, and hydroxyketones, AHKs, but the only known signaling compound utilized by *L. pneumophila* is AHK (Tiaden, Spirig, & Hilbi, 2010). Specifically, the autoinducer synthase, LqsA is responsible for the production of LAI-1 which is likely recognized by the sensor kinase, LqsS, which in turn activates the response regulator LqsR (Spirig et al., 2008; Tiaden, Spirig, Sahr, et al., 2010). Through DNA microarray experiments, Tiaden et al. discovered that the expression of 12 effector-encoding genes were affected by LqsR (Tiaden et al., 2007). Some cross-talk between the LetAS TCS (discussed below) and the LqsRS TCS occurs as the expression of LqsR requires the response regulator, LetA (Tiaden et al., 2007). In addition, Tiaden and colleagues found that deletion of the *lqsR* gene impaired the intracellular multiplication in macrophages and amoeba. Later, LqsS was found to upregulate the expression of at least 93 genes at least 2-fold,

many of these genes (Tiaden, Spirig, & Hilbi, 2010). Later, LqsS was found to upregulate the expression of at least 93 genes at least 2-fold, many of these are clustered in a 133 kb high plasticity genomic island, which is flanked by putative DNA-mobilizing genes and encodes multiple metal ion efflux pumps (Tiaden, Spirig, Sahr, et al., 2010). Surprisingly, the putative response regulator, LqsR, lacks a DNA binding motif so it is currently unknown how this protein affects gene expression (Segal, 2013). In sum, these studies establish the LqsRS TCS has an important role in the regulation of gene expression in the transmissive phase.

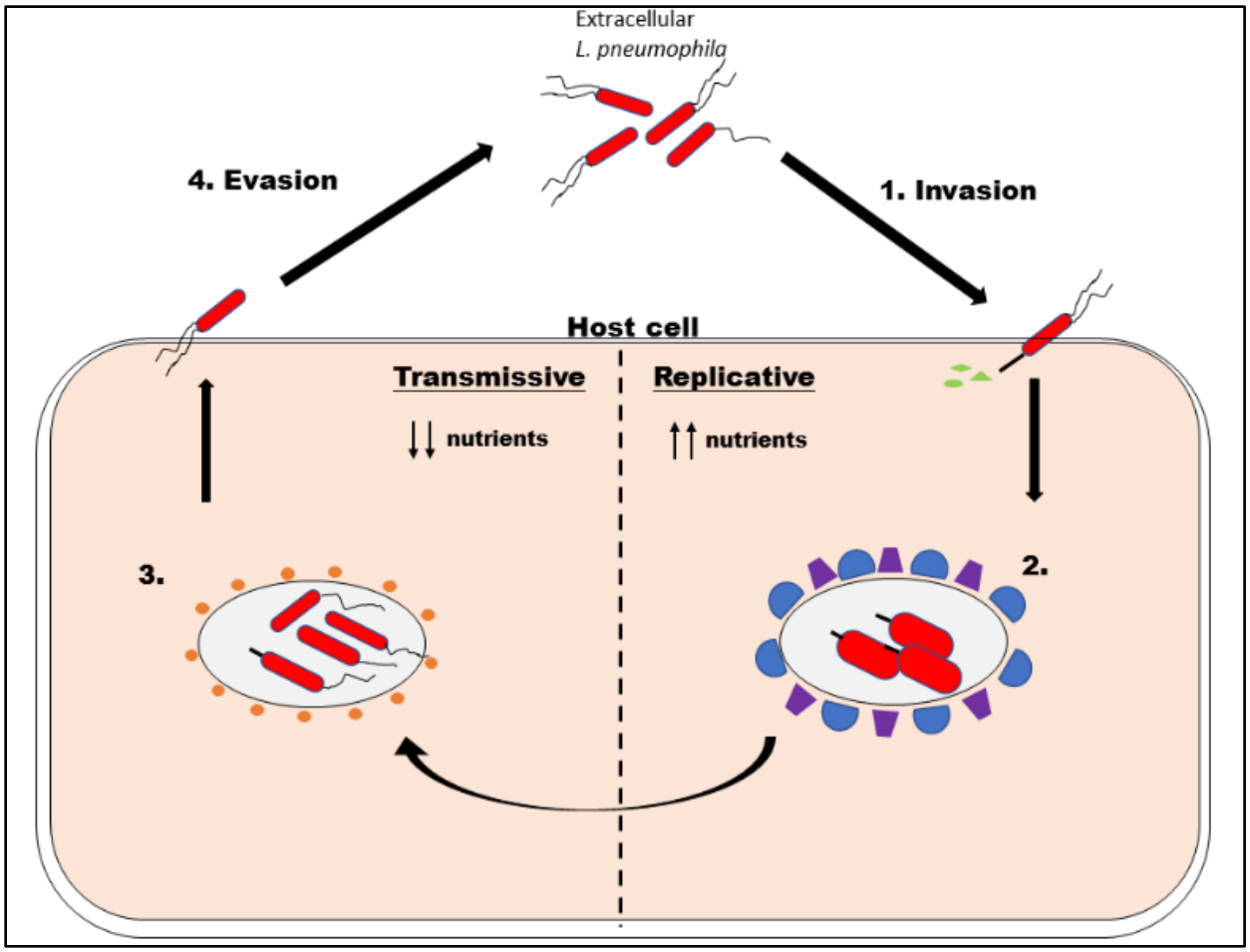

Figure 1-1. An overview of *L. pneumophila* morphological changes during its biphasic life cycle.
1.Uptake of virulent *L. pneumophila* by eukaryotic host cells (protozoa or macrophages) through phagocytosis. 2. After internalization, the bacteria evade fusion with the endosomal network and remodels the phagosomal membrane to form the LCV through interactions with the endoplasmic reticulum (ER), ER vesicles and mitochondria (blue and purple shapes). These interactions create an environment permissive for intracellular replication and survival. The Dot/Icm type IV secretion and its effectors (green shapes) allow for the modulation of host cell processes needed for the development of the LCV and intracellular multiplication of *L. pneumophila*. 3. Nutrient depletion triggers morphological changes and expression of transmissive traits such as flagella and cytotoxicity. 4. Transmissive bacteria lyse the host cell and are released into the extracellular milieu to start the cycle anew or survive as planktonic form.

The CpxRA two-component system

The CpxRA TCS has been studied in many bacteria where it has been designated as a stress response system since it can sense the disruption of many types of proteins present in the cell envelope, such as flagella, adhesins, secretion systems and others (Hunke et al., 2012). In these bacteria, the CpxRA TCS responds to protein misfolding by production of chaperones to deal with misfolded proteins. It also functions to downregulate the expression of these resident proteins of the cell envelope. Since many of these factors controlled by CpxRA are associated with virulence, this TCS is a significant contributor to virulence in many bacteria (Hunke et al., 2012). In *L. pneumophila*, the CpxRA TCS is composed of the CpxA sensor histidine kinase and the CpxR response regulator. The CpxRA TCS was originally discovered to be associated with virulence in *L. pneumophila* by Gal-Mor and Segal in 2003 (Gal-Mor & Segal, 2003a). By a genetic screening designed to search for an activator of the *icmR* gene, which serves as a chaperone to IcmQ of the Dot/Icm system of *L. pneumophila* (Duménil & Isberg, 2001), they found that CpxR controls the expression of *icmR* and to a lesser extent, the expression of the *icmV-dotA* and *icmW-icmX* operons. Additionally, a conserved CpxR binding site with the sequence GTAAA, was discovered in the regulatory region of *icmR*. Then in 2008, Altman and Segal found that another *icm/dot* gene, *lvgA*, and 11 effector-encoding genes are controlled by CpxR (Altman & Segal, 2008). More recently, the pool of *L. pneumophila* CpxR-controlled genes was extended to 38, which includes 27 effector-encoding genes (Feldheim et al., 2016). However, *cpxR* and *cpxA* deletion mutants did not show any defects for intracellular multiplication in the amoeba *A. castellanii* or HL-60 human-derived macrophages (Gal-Mor & Segal, 2003a). This might be to the high degree of functional redundancy observed in *L. pneumophila*, at least when referring to Dot/Icm effectors studies, which to date pose an experimental barrier and complicates research in the *Legionella* field (Isaac & Isberg, 2014).

The PmrAB two-component system

PmrAB is another TCS in *L. pneumophila* where PmrA and PmrB function as the response regulator and the sensor histidine kinase, respectively (Zusman et al., 2007). This system was identified in 2007, by Segal and colleagues through bioinformatic analysis of several effector-encoding genes, and the identification of a conserved regulatory sequence (cTTAATatT, less

conserved nucleotides are in lower case). Further analysis revealed that the PmrAB TCS activates the expression of several effector-encoding genes and that the PmrA response regulator is necessary for optimal intracellular growth in the amoeba host *A. castellanii*. Furthermore, the identification of this regulatory element led to the validation of many hypothetical proteins as effector-encoding genes in *L. pneumophila* as well as in *C. burnetii*, a close relative of *L. pneumophila* (Chen et al., 2010; Zusman et al., 2007). What exactly is the signal that activates the PmrAB TCS is not known but it might be related to pH levels of the bacterial phagosome (Segal, 2013). Perez and Groisman found that in *Salmonella enterica* the sensor PmrB detects acidic pH to activate PmrA (Perez & Groisman, 2007). Up to date, the expression of at least 43 effector-encoding genes are regulated by the PmrAB in *L. pneumophila*. This system also activates CsrA, an RNA-binding protein and global repressor regulated by the LetAS TCS (Al-Khodor et al., 2009; Rasis & Segal, 2009; Zusman et al., 2007).

The LetAS two-component system

The response regulator, LetA, and the sensor histidine kinase, LetS, constitute the LetAS TCS in *L. pneumophila* **(Fig. 1-2)**. The expression of genes associated with virulence in many pathogenic bacteria has been shown to be regulated by TCSs structurally similar to the LetAS system (Lapouge et al., 2008). Named GacAS in *Pseudomonas aeruginosa*, BarA-UvrY in *E.coli* and BarA-SirA in *S. enterica*, the LetAS TCS of *L. pneumophila* was first identified by Hammer et al., through a genetic screening utilizing the *mariner* transposon (Hammer et al., 2002) to search for mutants that negatively affected the expression of the flagellin gene via reporter gene fusion. They isolated 17 mutants that mapped to a gene predicted to be a GacA homologue and 5 mutants that affected a homologue of GacS. These researchers further showed that *letA* and *letS* mutants grown to post-exponential phase retained characteristics of exponential-phase bacteria, suggesting that this system regulates transition between these two phases. These mutants displayed phenotypes such as decreased levels of flagellin, low cytotoxicity, and the inability to prevent phago-lysosomal fusion during infection and defects in intracellular replication in macrophages. Further experiments showed that LetA is required to activate motility, cytotoxicity, promote macrophage infectivity and the capacity to evade phago-lysosomal fusion by the LCV (Hammer et al., 2002). Later, LetA was shown to regulate other traits such as pigment production and cell morphology. Specifically, *letA* mutants grown to post-exponential phase were observed to be

significantly elongated, contrary to the typical coccoid shape associated with similarly grown wild type strain. Furthermore, cultures of the *letA* mutant also accumulated less pyomelanin, the pigment produced by *L. pneumophila* at post-exponential growth phase (Molofsky & Swanson, 2003).

Accumulation of the alarmone ppGpp, due to nutrient depletion, is considered the activating signal of the LetAS TCS (Oliva et al., 2018). Upon activation, LetA induces the expression of small non-coding RNAs, RsmY and RsmZ which inhibit the global repressor CsrA, by means of sequestration, leading to de-repression of many virulence genes needed for transmission (Rasis & Segal, 2009; Sahr et al., 2009; Sahr et al., 2012). Moreover, CsrA has been shown to repress many traits associated with post-exponential phase bacteria such as coccoid cell shape, motility, pigmentation, stress resistance, sodium sensitivity, cytotoxicity and proficient macrophage infection (Fettes et al., 2001; Molofsky & Swanson, 2003). Later studies found that CsrA is essential for *L. pneumophila* growth in bacteriological media and only conditional mutants can be obtained (Molofsky & Swanson, 2003). Thus, the LetAS TCS together with CsrA are key regulatory machinery that control the cycling between replicative and transmissive forms of *L. pneumophila*.

Other factors involved in gene regulation in L. pneumophila

In addition to TCSs, other factors involved in the regulatory network in *L. pneumophila* include sigma factors and non-coding small RNAs (Oliva et al., 2018). For instance, starvation induces accumulation of the signaling alarmone (p)ppGpp. Accumulation of (p)ppGpp leads to increased levels of the alternative sigma factor RpoS ($\sigma^{S/38}$), which has been implicated in *E. coli*, in destabilizing the binding of sigma factor 70 with the RNA polymerase, promoting the integration of other alternative sigma factors (Jishage et al., 2002). RpoS accumulation in *L. pneumophila* was found to modulate traits associated with transmission such as motility, and expression of Dot/Icm effectors (Bachman & Swanson, 2004; Trigui et al., 2015). RpoS was also found to be necessary for intracellular growth in an amoeba host (Hales & Shuman, 1999b). Other sigma factors known to regulate virulence factors in *L. pneumophila* include the RpoN (σ^{54}) and the flagellar sigma factor FliA (σ^{28}), which work together in the regulation of proteins required for flagellum assembly (Oliva et al., 2018).

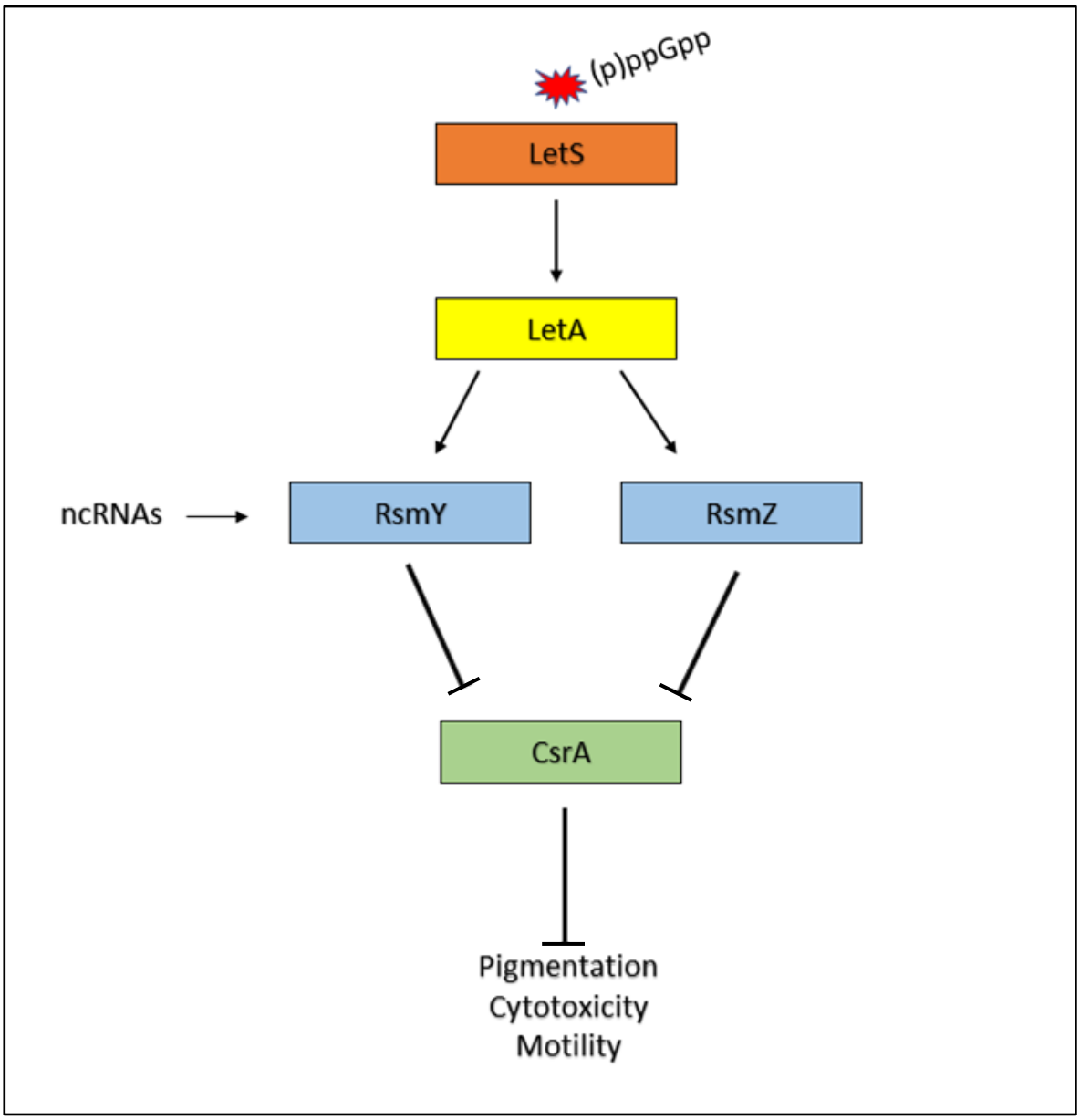

Figure 1-2. The LetAS TCS signaling pathway.
LetAS signaling is presumably activated by the alarmone (p)ppGpp upon nutrient starvation. LetA controls the expression of the small non-coding RNAs RsmY and RsmZ. RsmY and RsmZ relieve the repression exerted by the global regulator CsrA on virulence traits, thus inducing the expression of transmissive traits in *L. pneumophila* during post-exponential phase growth.

The role of small regulatory RNAs in the control of virulence factors and other cellular processes in bacterial pathogens has become an active area of research in recent years (González Plaza, 2020; Nitzan et al., 2017; Papenfort & Vogel, 2010). For example, the two sRNAs first identified to be implicated in the regulation of virulence traits in *L. pneumophila* , RsmY and

RsmZ, were found to be controlled by the LetAS TCS to ultimately inhibit the repressor CsrA by virtue of sequestration, preventing CsrA from binding their mRNA targets (Rasis & Segal, 2009; Sahr et al., 2009). Inhibition of CsrA leads to the de-repression of the expression of CsrA target mRNAs which include genes involved in virulence and other traits exhibited in post-exponential phase growth (Molofsky & Swanson, 2003; Rasis & Segal, 2009; Sahr et al., 2009). More recently, deep RNA sequencing revealed the presence of hundreds of sRNAs in *L. pneumophila*, including a subset that appears to be regulated by growth-phase, some sRNAs might influence the life cycle of this bacterium (Sahr et al., 2012). Recently Oliva et al characterized anti-hfq, one of these sRNAs that targets the *hfq* transcript to control the expression of the protein Hfq (Oliva et al., 2017). Hfq is an RNA binding protein known to facilitate the interaction between sRNAs and their mRNA targets, ultimately regulating pathways associated with metabolism, transport, energy production and membrane proteins (Boudry et al., 2014; Oliva et al., 2018).

In summary, *L. pneumophila* is armed with a sophisticated regulatory network, which includes TCSs, sRNAs and sigma factors to control the expression of many genes directly involved in cycling between replicative and transmissive forms and their corresponding traits.

Biosynthesis of pigments by microorganisms

Pigments are secondary metabolites produced by microorganisms which absorb light at a certain wavelength, as a result, the reflected light gives the pigment its color. These pigments have many useful biological properties such as anticancer, antioxidant and antimicrobial activities which makes understanding their production and chemical properties an attractive area for research (Narsing Rao et al., 2017). In addition, some pigments are of economic importance since they are used in the food and textile industries. Plants and microorganisms are the main source of natural pigments. However, plant-derived pigments present many drawbacks such as cultivation being restrained by seasonal growth, low pigment stability and solubility. Thus, microbial pigments from fungi or bacteria offer some advantages including the ability to produce at any season and the requirement of less space for similar quantity of products (Narsing Rao et al., 2017). Filamentous fungi produce a wide range of pigments such as carotenoids, melanin, flavins, violacein, among others (Dufossé et al., 2014). The use of *Monascus* for the production of “red rice” in oriental foods represents the oldest use of a fungal pigment on record (Narsing Rao et al., 2017). Similar

to fungi, bacteria produce many types of pigments such as carotenoids, melanin, violacein, prodigiosin and pyocyanin (Narsing Rao et al., 2017).

Types of pigments and their biological functions

Microbes produce many types of pigments such as pyocyanin, carotenoids, melanin, prodigiosin and violacein. For example, *P. aeruginosa* produces a blue pigment called pyocyanin first described in 1980 (Hassan & Fridovich, 1980). Pyocyanin **(Fig 1-3A)** is a pigment of clinical importance, it contributes to *P. aeruginosa* virulence and is considered a toxin that induces tissue damage and necrosis in mice infection models (Britigan et al., 1999; Lau et al., 2004). Significant amounts of pyocyanin have been recovered from the sputum of cystic fibrosis patients infected with *P. aeruginosa* (Lau et al., 2004). It causes imbalance of protease-antiprotease activity in airways of cystic fibrosis patients and secretion of Ig by lymphocytes (Lau et al., 2004). Pyocyanin have also been shown to contain antifungal activity *in vivo* in cystic fibrosis patients (Hughes & Kim, 1973). In cell infection models, pyocyanin has been shown to interfere with many mammalian cell pathways such as cell respiration, epidermal cell growth, ciliary beating, calcium homeostasis, apoptosis and the release of interleukin (Ran et al., 2003). Recently, pyocyanin was found to have antifungal activity against fungi of agricultural importance (DeBritto et al., 2020). These properties may contribute to the dominance of *P. aeruginosa* over other microbes in the lungs of cystic fibrosis patients.

Carotenoids are yellow, orange or red pigments mainly isolated from plants. However, many bacterial species such as, *Flavobacterium multivorum* and *Agrobacterium aurantiacum* also produce this type of pigment (Bhosale & Bernstein, 2004; Narsing Rao et al., 2017). In plants, carotenoids function to harvest energy from light to pass excitation energy onto chlorophyll to expand the range of the wavelength of light that can be harvested (Narsing Rao et al., 2017). Carotenoids also protect the chlorophyll from photodamage (Armstrong & Hearst, 1996). Commercially, carotenoids are used as vitamin supplements, feed supplements for animals and colorants (Kulandaisamy et al., 2013). β-carotene **(Fig 1-3B)** is the most common carotenoid found in plants and it is a precursor for vitamin A. Recently, studies have shown that managing β-carotene dietary intakes might decrease the risk of lung cancer in non-smokers patients (Goralczyk, 2009). Production of carotenoids can be associated in bacterial pathogens with agricultural importance. For example, the gram-negative bacterium *Pantoea stewartii*, the causative agent of a severe sweet

corn disease called Stewart's wilt (Mohammadi et al., 2012b), synthetizes a carotenoid-derived yellow pigment that protects the bacteria from UV damage and contributes to virulence *in planta* (Mohammadi et al., 2012a).

Prodigiosin **(Fig 1-3C)** is a red pigment first isolated from the bacteria *Serratia marcescens* in 1987 (Boger D, 1987). *Serratia marcescens* is a gram negative bacteria and the causative agent of nosocomial outbreaks as well as wound, respiratory and urinary tract infections (Hejazi & Falkiner, 1997). These bacteria were first referred to as *Serratia* in 1819 by an Italian pharmacist that studied the mode of transmission of a "red substance" that grew on damp surfaces and on polenta dishes (Nazzaro, 2019). Prodigiosin production is considered widespread and other bacteria such as actinomycetes, like *Streptomyces coelicolor* and marine bacteria, including *Hahella chejuensis* KCTC 2396 and *Pseudoalteromonas denitrificans*, have been shown to produce this pigment (Williamson et al., 2006). Prodigiosins are becoming an attractive area of research after evidence showing potential anticancer and immunosuppressive activities with little cytotoxicity emerged (Pérez-Tomás et al., 2003). Like other secondary metabolites, the physiological role of this pigment, if any, is still unclear. However, it has been proposed that this pigment might serve as a metabolic sink by consuming the overflow of NADPH from metabolic reactions and serve to promote bacterial adherence and dispersal (Williamson et al., 2006).

Violacein is a violet colored pigment produced by the bacterium *Chromobacterium violaceum* and first described in 1945 (Lichstein & Van De Sand, 1945) **(Fig 1-3D)**. In addition to *C. violaceum*, violacein pigmentation has been documented in other microorganisms such as *Collimonas sp*., *Duganella sp*., *Janthinobacterium lividum*, *Microbulbifer sp*., *Pseudoalteromonas luteoviolacea*, *Pseudoalteromonas tunicata*, and *Pseudoalteromonas ulvae* which can inhabit very different environments (Narsing Rao et al., 2017). Violacein has been shown to contain antibiotic activity against *Staphylococcus aureus* and *Staphylococcus albus* (Lichstein & Van De Sand, 1945). In their 1945 article, Lichstein and Van De Sand compared the effect of four *C. violaceum* strains on *S. aureus* and *S. albus* cultures. Of these four strains, three strains were isolated from fatal patient infections with robust pigment production and the other was a saprophytic ordinary lab strain with no evident pigment production. The authors found that the antibiotic activity correlated with violacein production as all the pigmented strains inhibited growth of *S. aureus* and *S. albus* while the non-pigmented strain had no effect (Lichstein & Van De Sand, 1945). Violacein also exhibits biological activities such as antifungal, antiviral, antioxidant and antiprotozoal

(Durán et al., 2016) which make this pigment an interesting area of research with a potential impact on healthcare and agricultural practices. In industrial applications, violacein has been utilized as a food and textile colorant, sunscreen and skin lotion additive and for plant pathogen control (Durán et al., 2012; Durán et al., 2016).

Melanin is a common black-brown pigment found in many living systems and formed by oxidative polymerization of phenolic or indolic compounds such as L-tyrosine **(Fig 1-3E)**. There are 3 types of melanins: Black-brown melanins or eumelanins, yellow or reddish are called pheomelanins and brownish melanins, which are derived from homogentisic acid (HGA) and are often referred to as pyomelanins (Nosanchuk & Casadevall, 2006). Melanin-producing microorganisms include fungi, bacteria and helminths. For some of these organisms, the capability to produce melanin pigments have been linked with virulence and pathogenicity (Nosanchuk & Casadevall, 2003). Melanin production and its contribution to virulence has been mostly studied in fungal pathogens. In fact, many human pathogenic fungi such as *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Asperigillus spp* and *Sporothrix schenckii*, synthetize melanin (Nosanchuk & Casadevall, 2003). For instance, a melanin-deficient strain of *Cryptococcus neoformans* was avirulent in murine infection models (Kwon-Chung et al., 1982; Rhodes et al., 1982). Intracerebral infections of mice with albino strains of *Cryptococcus neoformans* showed minimal tissue damage and induced a cytokine response, whereas, a revertant melanin-producing strain caused extensive damage and inhibited the cytokine response (Barluzzi et al., 2000). Melanin-producing fungi often synthetize large amounts of the pigment which points to the importance of this metabolic product. For example, 30% of the dry-weight of the fungi *Agaricus bisporus* accounts for melanin (Rast & Hollenstein, 1977). More recently, melanin produced and purified from a strain of *Streptomyces glaucescens* was found to contain antioxidant and anticancer activity against skin cancer cell lines (El-Naggar & El-Ewasy, 2017). In addition, melanin might contribute to survival in extreme environments since many black fungi, such as *A. alternata* and *Cladosporium sphaerospermum*, have prevailed in the contaminated soil surrounding the site of the Chernobyl nuclear reactor accident, where radiation levels are 10 thousand times higher than the human lethal dose (Zhdanova et al., 2000).

Melanin and L. pneumophila

The production of melanin in bacteria was discovered more than a century ago and the production was correlated with tyrosine metabolism. Skinner reported that actinomycetes growing in plates only produced dark coloration when tyrosine was present in the media (Skinner, 1938). Originally, biosynthesis of bacterial melanin was thought to occur exclusively through the DOPA pathway in which tyrosine is transformed by oxidation, while conserving the amino group, into L-3,4-dihydroxyphenylalanine, or L-DOPA. If the amino group is lost in this step, then homogentisate (HGA, **Fig 1-3E**) is generated. These molecules can then oxidize spontaneously to produce dopaquinones or benzoquinones which can then be polymerized to form melanin (Pavan et al., 2020). Melanins derived from DOPA are catalyzed by tyrosinases and laccases (Claus & Decker, 2006; Plonka & Grabacka, 2006).

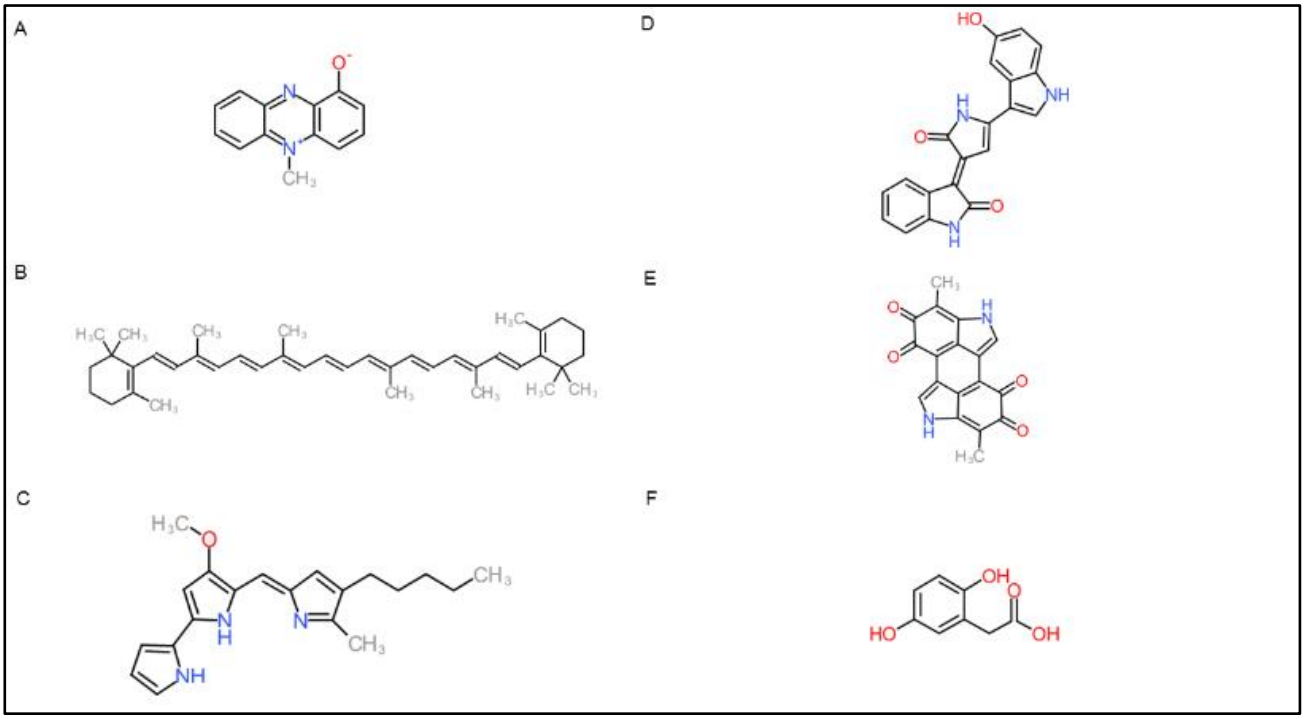

Figure 1-3: Chemical structure of pigments produced by microorganisms.
A. Pyocyanin, B, β-carotene, C, Prodigiosin, D, Violacein, E, Melanin, F, Pyomelanin precursor, HGA

In 1972, Yabuuchi and Ohyama discovered a brown pigment in *P. aeruginosa* that derived from homogentisate and was called pyomelanin (Yabuuchi & Ohyama, 1972). About 20 years later, Denoya et al. found that the enzyme 4-hydroxyphenylpyruvate dioxygenase, from

Streptomyces avermitilis, was responsible for the production of homogentisate (Denoya et al., 1994). Specifically, in the homogentisate pathway, tyrosine is metabolized in several steps **(Fig. 1-4)**. First, deamination of tyrosine is catalyzed by aromatic amino acid transferases to produce 4-hydroxyphenylpyruvate. Next, 4-hydroxyphenylpyruvate dioxygenase, acts on 4-hydroxyphenylpyruvate to produce homogentisate (Pavan et al., 2020). Once homogentisate is left to accumulate, autoxidation and polymerization occurs, producing pyomelanin. Otherwise, homogentisate can be degraded by homogentisate 1,2 dioxygenase, or *hmgA*, to produce 4-maleyl-acetoacetate. Generally, accumulation of pyomelanin can occur through increased activity of the enzyme 4-hydroxyphenylpyruvate dioxygenase or due to disruption or inactivation of homogentisate 1,2 dioxygenase. For example, strains of *Vibrio cholerae* HTX-3 and *Shewanella colwelliana* were found to synthetize elevated levels of homogentisate because of increased expression of 4-hydroxyphenylpyruvate dioxygenase (Kotob et al., 1995). On the other hand, transposon insertions in *hmgA* led to accumulation of homogentisate and increased pigmentation in clinical isolates of *P. aeruginosa* (Rodríguez-Rojas et al., 2009). Interestingly, hyperpigmentation in *P. aeruginosa* by inactivation of *hmgA* was associated with diminished killing ability in acute murine model of infection and decreased clearance and increased persistence in chronic lung infections (Rodríguez-Rojas et al., 2009). In *B. anthracis*, disruption of *hmgA* induced pigment accumulation and increased pigmentation levels were associated with UV damage protection but not antioxidant protection (Han et al., 2015). In contrast, pyomelanin produced by *Burkholderia cenocepacia* strain C5424 protected the bacterium from hydrogen peroxide treatment, suggesting that the pigment was capable of scavenging free radicals (Keith et al., 2007). *L. pneumophila* is another bacterium of clinical importance known to produce brown pigments derived from HGA.

Early work showed that *L. pneumophila* secretes a brown pigment into culture supernatants during stationary phase growth and this secretion is enhanced by the availability of L-tyrosine in the media (Baine & Rasheed, 1979; Baine et al., 1978; Vickers & Yu, 1984). Since then, substantial progress has been made in characterizing the pyomelanin production pathway in *L. pneumophila*. First, HGA is derived from tyrosine and phenylalanine metabolism. L-phenylalanine is first converted to L-tyrosine through phenylalanine hydroxylase encoded by *phhA* (gene lpg2647 in strain Philadelphia 1), L-tyrosine is then converted into 4-hydroxyphenylpyruvate by the amino acid transferase encoded by *hisC2* (lpg1998) and 4-hydroxyphenylpyruvate is then metabolized

by 4-hydroxyphenylpyruvate dioxygenase, encoded by *lly* (lpg2278), to produce HGA (Steinert et al., 2001; Wiater et al., 1994; Wintermeyer et al., 1994; Wintermeyer et al., 1991). In 1995, Steinert et al., showed that production of pyomelanin, also referred to as HGA-melanin, depends on the *lly* gene and that it conferred protection to ordinary light (Steinert et al., 1995). Then in 2007, while seeking for factors involved in the regulation of the *Legionella* siderophore, legiobactin, Chatfield et al., reported that the pyomelanin pigment produced by *L. pneumophila* confers the bacterium with ferric reductase activity (Chatfield & Cianciotto, 2007). The authors arrived at this conclusion after isolating a hyperpigmentation mutant (*hmgA* mutant) that was capable of inhibiting growth of a ferrous iron (Fe^{2+}) transport mutant (*feoB* mutant), in low-iron conditions. They proposed that pyomelanin had ferric reductase activity, thus reducing all the available ferric iron (Fe^{3+}) in the media into ferrous iron (Fe^{2+}), which arrested the growth of the *feoB* mutant as a result of ferric iron depletion. Generation of an *lly* mutant, which is impaired for pigment production and consequently, ferric reductase activity, validated their initial observations (Chatfield & Cianciotto, 2007). In 2013, Zheng et al. provided further evidence that pyomelanin is directly involved in ferric iron reduction and enhancement of iron uptake by *L. pneumophila* (Zheng et al., 2013). Together these observations propose that pyomelanin is a contributor in the iron acquisition pathway of *L. pneumophila*. As mentioned above, pyomelanin accumulation occurs during stationary phase growth. It is not clear how this pathway is regulated in *L. pneumophila*, but earlier work indicate that the LetAS TCS might be involved in inducing the production of pyomelanin, at least in part through the inhibition of the global repressor CsrA (Molofsky & Swanson, 2003, 2004).

To understand the signaling pathway that governs the development of filamentous cells in *L. pneumophila*, we identified a protein (lpg0586, hence forth Larp1, for LetAS-regulated protein 1) involved in the regulation of multiple phenotypes, ranging from cell morphology to pyomelanin production. Here we show that overexpression of this protein causes elongation in wild-type *L. pneumophila*. In addition, overexpression of Larp1 rescues the ability of the *letA* deletion mutant in pigment production. We found that a *larp1* deletion mutant showed decreased transcription of *phhA, lly* and *hisC2*. We also discovered that not only *larp1* is part of the LetAS regulon, but Larp1 regulates itself in an apparent negative feedback loop. These results suggest that Larp1 is a novel protein regulated by the LetAS TCS and involved in the pyomelanin production pathway of *L. pneumophila*.

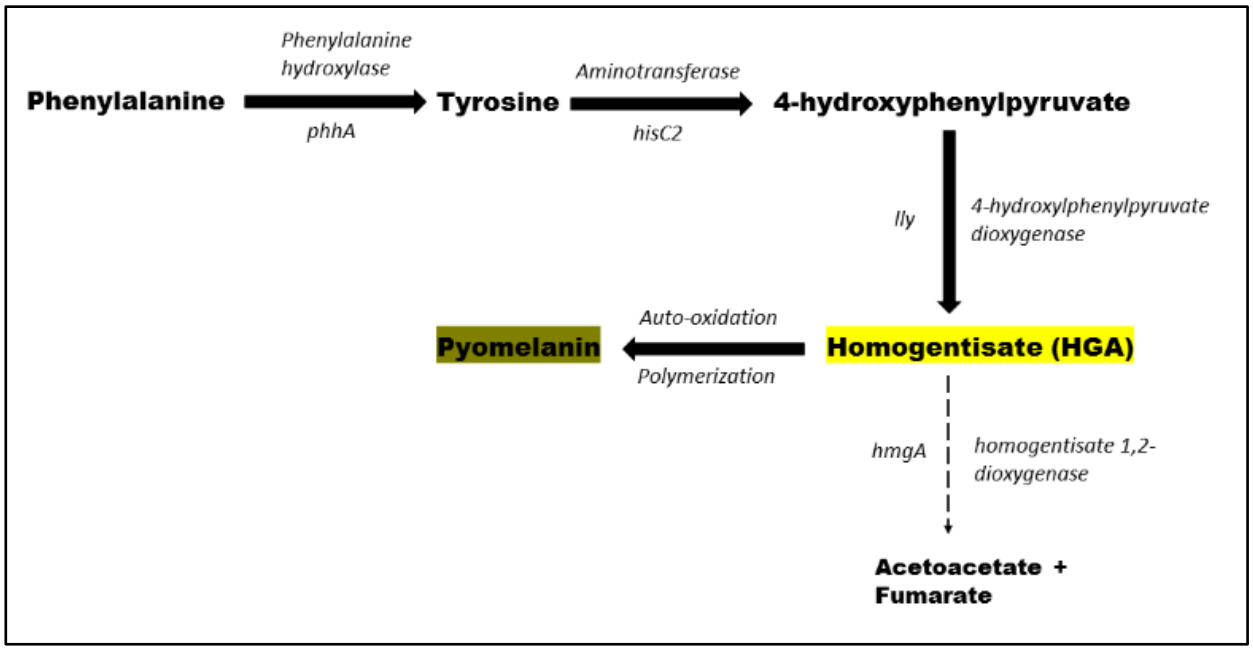

Figure 1-4. Pathway of phenylalanine/tyrosine-derived pyomelanin showing enzymatic steps to produce homogentisate.
Secreted HGA self-polymerizes and oxidizes to produce pyomelanin or degraded to acetoacetate and fumarate. Dashed line indicates degradation of HGA.

CHAPTER II. IDENTIFICATION OF A PROTEIN INVOLVED IN CELL MORPHOLOGY AND PYOMELANIN PIGMENT PRODUCTION IN *L. PNEUMOPHILA*

Abstract

Legionella pneumophila undergoes a transition in cell morphology in its life cycle. The replicative phase is characterized by elongated rods and active metabolism while in the transmissive phase, *L. pneumophila* cells are coccoid shaped, display flagella and produce the pigment, pyomelanin. Current knowledge points to the LetAS two-component system as one of the key regulators of cell morphology and pyomelanin production in *L. pneumophila*. However, which LetA-regulated proteins are directly involved in the regulation of cell morphology or pyomelanin production are currently unknown. Here, we initiated a screening to identify proteins potentially involved in cell morphology regulation. Our screening led to the discovery of a gene, *lpg0586* (designated as Larp1), that appears to participate in the regulation of multiple phenotypes such as cell elongation, RecA induction and increased accumulation of pigment in culture supernatants. In addition, we found that upon overexpression of Larp1, the observed cell elongation phenotype occurs in a *recA*-independent manner which contrasts with model organisms such as *E. coli*. Moreover, we found that overexpression of Larp1 induces accumulation of the pyomelanin pigment in culture supernatants and it is accompanied by increased transcription of key genes involved in the pyomelanin production pathway. Furthermore, we show that expression of Larp1 during stationary phase is dependent on the LetAS two-component system. These results provide further insights on the regulation of morphology and pigment production in *L. pneumophila* through the identification of a protein regulated by the LetAS two-component system.

Introduction

Within the LCV and in bacteriological media, *L. pneumophila* adopts a biphasic life cycle which includes morphological and gene expression changes which define two very distinct stages of its life cycle, the replicative and transmissive forms (Oliva et al., 2018). After internalization by eukaryotic hosts, this bacterium evades the endocytic network and lysosomal killing to form a

niche suitable for replication which include interactions with the ER vesicles and mitochondria (Isberg et al., 2009). *L. pneumophila* replicates effectively when nutrients are relatively high within the LCV and at this point in the life cycle, the bacterium is referred to as being in its replicative form (Faulkner & Garduño, 2002). The replicative form is characterized by metabolically active *Legionella* and the bacterial cells display a physical appearance of thin, elongated rods. Once nutrients are depleted after productive replication within the host, morphological and gene expression changes occurs which include expression of transmissive traits that help the bacteria escape its host to restart the cycle anew (Molofsky & Swanson, 2004). In addition, transmissive *Legionella* express a plethora of factors that promote motility, coccoid cell shape, pigmentation, cytotoxicity and other traits required for lysosomal evasion, escape from the host cell and survival in the environment (Molofsky & Swanson, 2003, 2004; Oliva et al., 2018). Some of these transmissive traits are, in part, controlled by two-component signal transduction pathways such as the LetAS two-component system (Segal, 2013). Current evidence suggests that the LetA TCS in *L. pneumophila* is involved in the regulation of pigment production, cell morphology, flagellin levels, among others transmissive traits required for successful infection (Hammer et al., 2002; Molofsky & Swanson, 2003). However, the specific mechanism on how cell morphology changes are regulated in *L. pneumophila* has not been described. Consequently, we set out to identify candidate genes with the potential to influence cell morphology and found that *lpg0586* (hence forth called *larp1*) overexpression induces two distinct phenotypes, cell elongation and pigment accumulation.

Results

Identification of a L. pneumophila protein involved in the induction of cell elongation

In order to identify proteins involved in regulating cell morphology we utilized a mutant derived from *L. pneumophila* JR32 strain that harbor a mutation in the thymidine production pathway (JR32*thyA*). This mutation induced cell elongation and filamentation upon thymidine starvation (Ahmad et al., 1998). Therefore, I inoculated cultures of JR32*thyA* and JR32 (Rao et al., 2013) in the absence of exogenous thymidine. After overnight growth, the presence of extensive cell filamentation in cultures of strain JR32*thyA* was confirmed and cells from both cultures were harvested to prepare total protein lysates, which were run in SDS-PAGE gels. Protein samples that

A

Accession	Description	MW [kDa]	avg fold
52627934	transcriptional regulator lpg 0586	34.75936	10.184
52628552	hypothetical protein lpg1209	29.7	5.591793
52629734	hypothetical protein lpg2414	11	3.925055
52627450	hypothetical protein lpg0084	56.6	3.693973
52628070	hypothetical protein lpg0722	15.5	3.472203
52628623	hypothetical protein lpg1281	14.6	2.852548
52630264	hypothetical protein lpg2959	57.4	2.642965
52628136	hypothetical protein lpg0788	45.2	2.538341
52627453	hypothetical protein lpg0087	23.5	2.413971
52628718	hypothetical protein lpg1377	12.7	2.141056

B

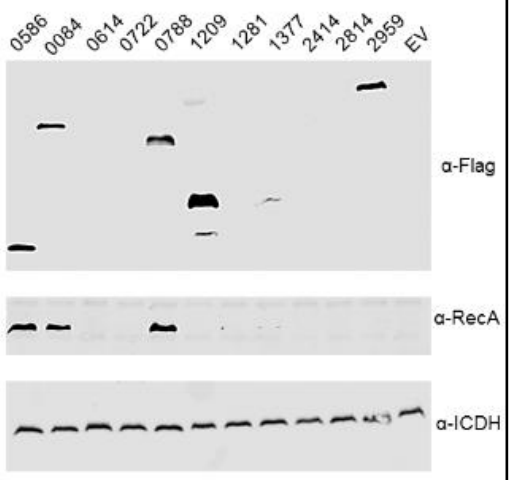

Figure 2-1. Hypothetical proteins that are upregulated in filamented cultures identified through proteomic analysis. A. Table of hypothetical proteins upregulated at least 2-fold in filamented cultures compared to non-filamented. Right column shows average fold increase of each protein obtained in 3 independent sets of samples analyzed by Mass spectrometry. B. Overexpression of indicated genes in *L. pneumophila* and the effect on RecA protein levels. EV, empty vector

ran into the gels for approximately 1 cm were stained with Coomassie brilliant blue and the bands containing proteins were excised and subjected to mass spectrometric analysis. We then compared identified proteins that specifically induced in cells of strain JR32*thyA*. Cultures were then harvested after overnight incubation and JR32thyA cultures were confirmed visually to contain extensive cell filamentation. These analyses led to the identification of 10 proteins of unknown function that upregulated for at least 2-fold in the JR32*thyA* when compared to the WT (**Fig. 2-1A**). To confirm the potential involvement of these proteins in inducing cell morphology changes, I cloned and overexpressed each of these genes in *L. pneumophila* utilizing the IPTG-inducible plasmid, pZLQKnflag (Gan, Zhen, et al., 2019). First, I found that overexpression of Lpg0586, Lpg0084 and Lpg0788 induced increased levels of the RecA protein when compared to the empty vector control samples **(Fig. 2-1B)**. Induction of RecA is typically associated with DNA damage and the induction of the SOS pathway in the model organism, *E. coli* (Cox, 1999, 2007b; Little, 1983). The "SOS response", is a genetic circuit that up regulates over 50 genes involved in DNA repair, DNA damage tolerance, inhibition of cell division and cell filamentation (Cox, 2007b; Little & Mount, 1982; Simmons et al., 2008). Very little is known about the response to DNA damage in *Legionella* and the existence of a canonical SOS pathway has not been confirmed in this bacterium (Charpentier et al., 2011). Importantly, among the 3 proteins identified that were

able to induce the accumulation of RecA, only Lpg0586 expression provoked both, cell elongation **(Fig 2-2)** and RecA induction.

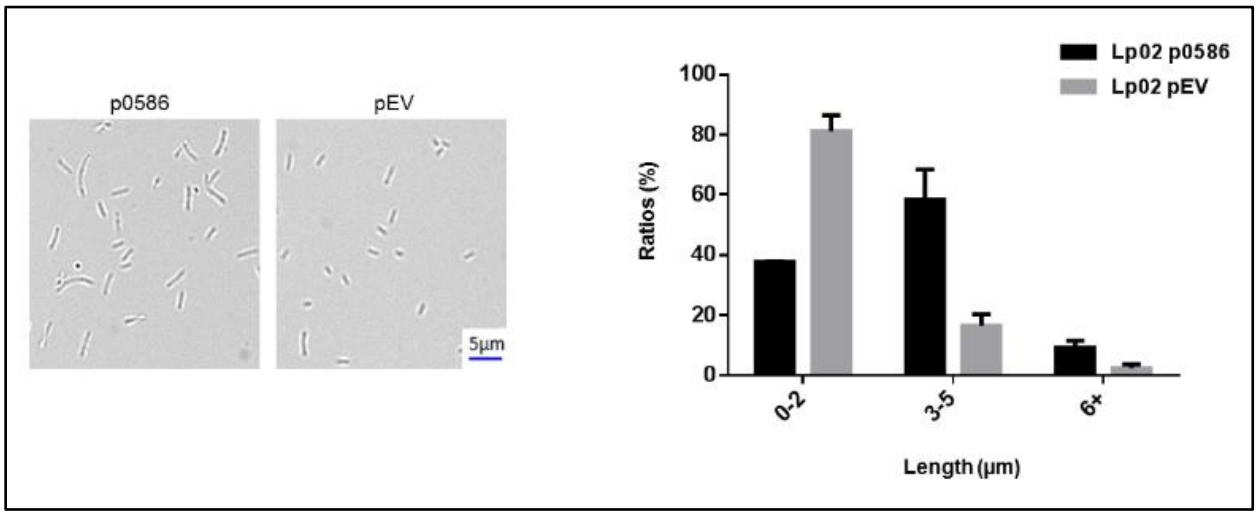

Figure 2-2. Overexpression of Lpg0586 induces cell elongation in *L. pneumophila*.
Left, Lp02 strain carrying a plasmid for 0586 overexpression or empty vector were inoculated in AYE and IPTG was added to 0.1mM for 16 hours. Samples were collected and bacteria were fixated using 4% paraformaldehyde before imaging. Labels: p0586, strain overexpressing Lpg0586, pEV, strain carrying empty vector. Right, Distribution of cell lengths from samples in left panel. Bacterial cells were fixated**,** and the distribution of cell lengths was plotted. The length of >300 cells was measured from 3 independent experiments utilizing the IPlab software package.

Lpg0586 is expressed during stationary phase and regulated by the LetAS two-component system

Given that overexpression of Lpg0586 induced cell elongation and increased RecA levels I decided to perform further experimentation to characterize this protein and possibly elucidate the mechanism by which overexpression of this protein induces these two phenotypes. First, I started by evaluating the expression profile of this protein in *L. pneumophila*. For this purpose, I first constructed a *L. pneumophila* strain by introducing a Flag tag into the carboxyl end of *lpg0586* to create strain, VR700, which allows the detection of Lpg0586 protein levels by immunoblotting with the Flag tag specific antibody. Next, overnight cultures grown in AYE were diluted to OD_{600nm} of 0.2. After dilution, cultures were maintained for 24 hours, at every 3 hours samples were withdrawn to monitor protein levels of Lpg0586 **(Fig. 2-3)**. We found that 3 hours after cells resume to grow in the new cultures, Lpg0586 levels started to decrease and became undetectable around 9 hours. At 3 hours post dilution, the bacterium is expected to be entering the growth and

replication phase. We also found that Lpg0586 levels started to rise again after 18hrs of incubation, which is corresponding to the stationary phase. These observations suggest that Lpg0586 is

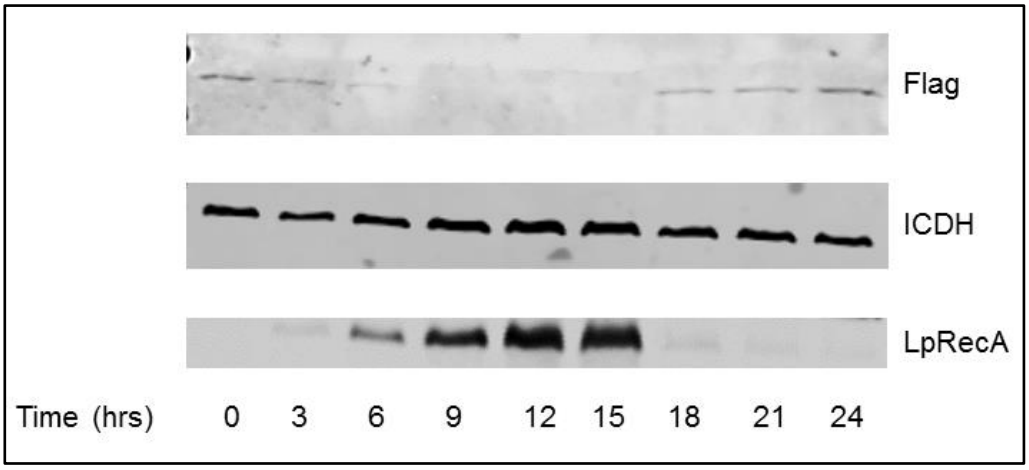

Figure 2-3. Expression profile of Lpg0586.
Overnight cultures of VR700 were diluted to OD_{600nm} of 0.2 and monitored for 24 hours. Samples were taken every 3 hours for immunoblotting with anti-Flag and anti-RecA antibodies.

primarily expressed during stationary phase and appears to be regulated by a cycle of degradation or low expression during replicative phase and accumulation or high expression during the stationary phase.

Our current understanding of the life cycle of *L. pneumophila* suggests that expression of transmissive traits is associated with the stationary phase in this bacterium (Hammer et al., 2002; Molofsky & Swanson, 2004; Oliva et al., 2018). Many genes involved in the expression of transmissive traits are regulated by the LetAS TCS, such as cell morphology and the display of coccoid shape upon reaching stationary phase (Molofsky & Swanson, 2003). Since LetAS activity and Lpg0586 expression pattern correlated with the stationary phase, I examined whether expression of Lpg0586 was under direct control of LetAS TCS. To this end, I constructed a *letA* deletion mutant (Lp02Δ*letA*). I also constructed pVR004, a plasmid that harbors *lpg0586*-Flag expressed by its cognate promoter. As shown in **Figure 2-4**, Lpg0586 was detected in the wild-type strain Lp02 (**Fig. 2-4**, left lane) when cultures reached the stationary phase. In contrast, expression of Lpg0586 was undetectable in the *ΔletA* strain (**Fig. 2-4, middle lane**). Importantly, although at considerably lower levels, introduction of a plasmid expressing LetA restored the expression of Lpg0586 (**Fig. 2-4, right lane**). These results suggest that Lpg0586 is under the regulatory control of the LetAS TCS. Because of this phenotype, we designated Lpg0586 as Larp1,

for LetA regulated protein 1, and this designation will be used throughout the remaining of the thesis.

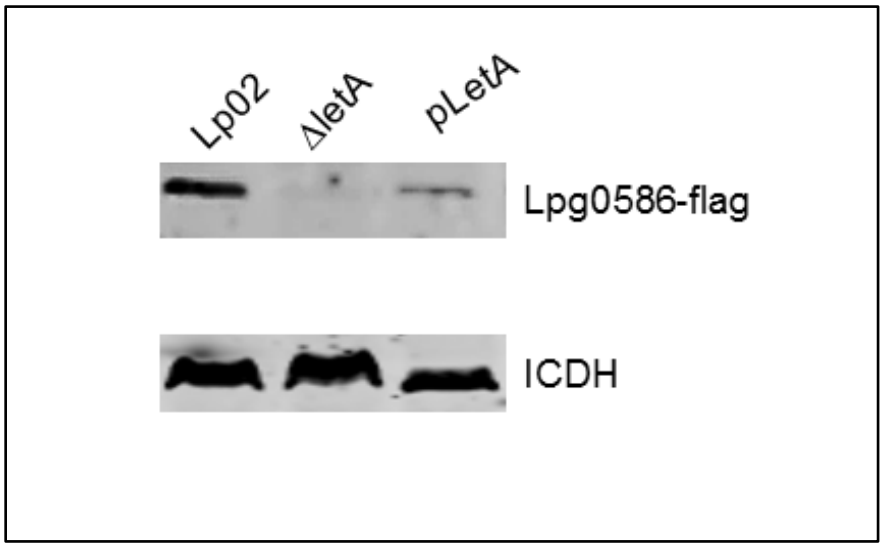

Figure 2-4. Lpg0586 is under the control of the LetAS two component system. Lp02, Lp02Δ*letA* or a complemented strain (pLetA) were assayed for the expression of Lpg0586. In addition, these strains were carrying a plasmid-borne copy of Lpg0586-flag with its native promoter. All samples were taken when cultures reach stationary phase. All the strains were grown in the presence of IPTG (0.025mM).

Imbalance in Larp1 expression during infection is detrimental to L. pneumophila growth

The results in **Figure 2-3**, indicated that Larp1 (Lpg0586) is primarily expressed in the stationary phase. To examine the role of this protein during infection, *Dictyostelium discoideum* cells were challenged with Lp02 WT, Lp02Δ*larp1* or strains from WT or Δ*larp1* backgrounds carrying plasmids for overexpression of Larp1. Intracellular replication was determined by obtaining total colony-forming units (CFUs) by plating lysates on CYE plates at the indicated time points post infection **(Fig. 2-5)**. Our results indicate that Larp1 is important for productive replication at 48hrs post-infection (hpi) as the Δ*larp1* (Lp02Δ*larp1* pEV) strain had reduced growth when compared to the WT strain (Lp02 pEV) or the complemented strain (Lp02Δ*larp1* pLarp1). The Δ*larp1* strain grew to comparable levels by 72 hpi. On the other hand, overexpression of Larp1 was detrimental to intracellular bacterial growth at 72 hpi since both, the WT and Δ*larp1* background strains carrying a plasmid for Larp1 overexpression (Lp02 pLarp1 and Lp02Δ*larp1*

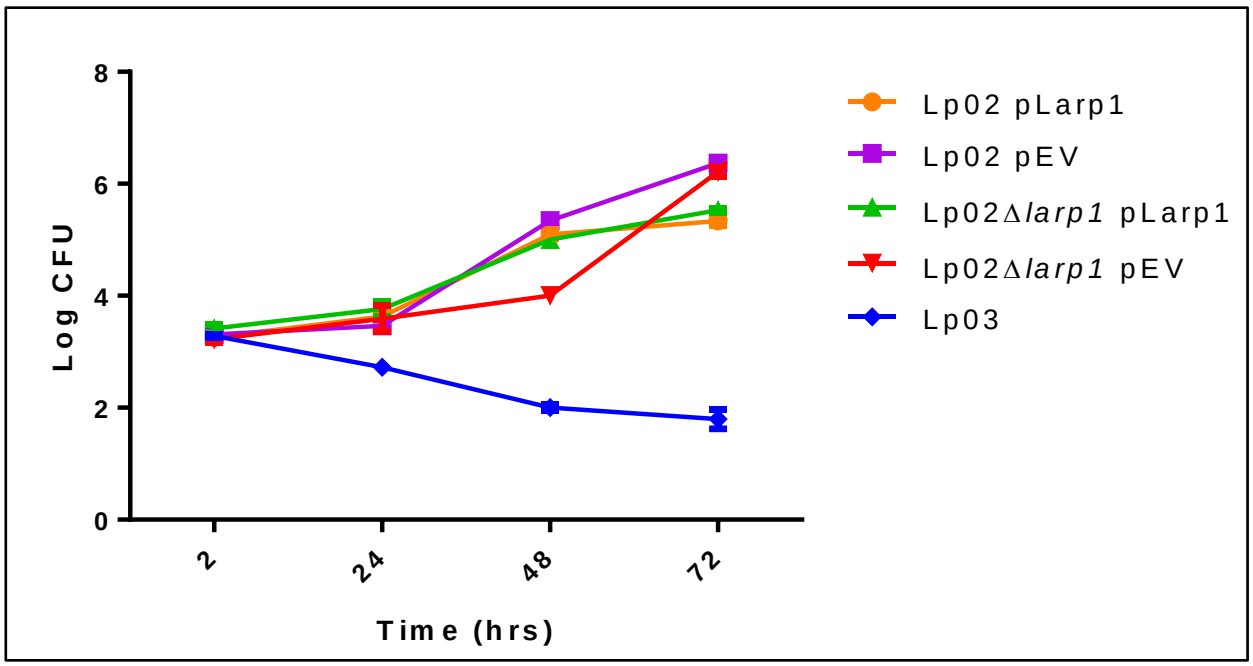

Figure 2-5. Larp1 expression levels affect *L. pneumophila* growth during infection.
Dictyostelium discoideum cells were infected with the indicated strains and bacterial counts were obtained by plating lysates on CYE plates and obtaining total bacterial counts at the indicated time points. IPTG was added to culture media of infected samples 2 hours post infection and maintained for the duration of the experiment.

pLarp1, respectively), exhibited reduced bacterial counts at this time point. Thus, deletion or overexpression of Larp1 impairs intracellular replication, suggesting that Larp1 expression is likely tightly controlled in the bacterium.

Larp1 autoregulates in a negative feedback loop

Larp1 (Lpg0586) is annotated as a putative transcriptional regulator in the KEGG (Kyoto Encyclopedia of Genes and Genomes, www.kegg.jp) database. This protein is a member of a family of proteins with unknown function (PF02622, domain of unknown function DUF179). However, there are no predicted DNA binding motifs in the protein sequence. RecA is a well-established example of proteins involved in the regulation of cell morphology in *E. coli* (discussed below) upon activation of the SOS response (Cole, 1983; Little, 1983; Mizusawa et al., 1983) and of self-regulating, at least indirectly. This self-regulation occurs because *lexA* and *recA* are both under the regulation of LexA by repression (Maslowska et al., 2019). Therefore, as the SOS response is activated, more RecA protein is produced but at the same time LexA levels start to increase gradually, eventually shutting down both the SOS response and *recA* induction (Simmons

et al., 2008). Since we found that disruption of Larp1 expression appears to be detrimental to *L. pneumophila* during infection, I asked whether this protein can regulate itself akin to LexA. For this purpose, I transformed Lp02 with two plasmids, pLarp1 and pVR0004, to create strain VR702. This strain harbors an inducible, plasmid-borne copy of a His-tagged Larp1 and a plasmid-borne copy of Flag-tagged Larp1, the expression of which is controlled by its native promoter. As shown in **Figure 2-6A**, Flag-tagged Larp1 levels were not detected upon induction of Larp1 from pLarp1. Similar results were obtained when I introduced the plasmid pLarp1 into the strain VR700 to create VR700 pLarp1 **(Fig. 2-6B)**. This strain allows for the monitoring of a chromosomally Flag-tagged Larp1 upon induction of a plasmid borne Larp1. Chromosomal Larp1 levels decreased in an IPTG dependent manner, suggesting that the endogenous Larp1 levels were directly affected by the levels of the plasmid-borne Larp1. This contrasts with the VR700 strain carrying an empty vector, VR700 (pEV), where the chromosomal Larp1 levels were unchanged in the presence of the inducer, IPTG. These results indicate that Larp1, overexpressed from a plasmid, can affect endogenous levels of Larp1 through a yet to be determined mechanism. These results also suggest that Larp1 is within a negative feedback loop. Likely this effect on endogenous Larp1 levels is through an indirect mechanism, since Larp1 does not encode any putative DNA binding motifs in its protein sequence.

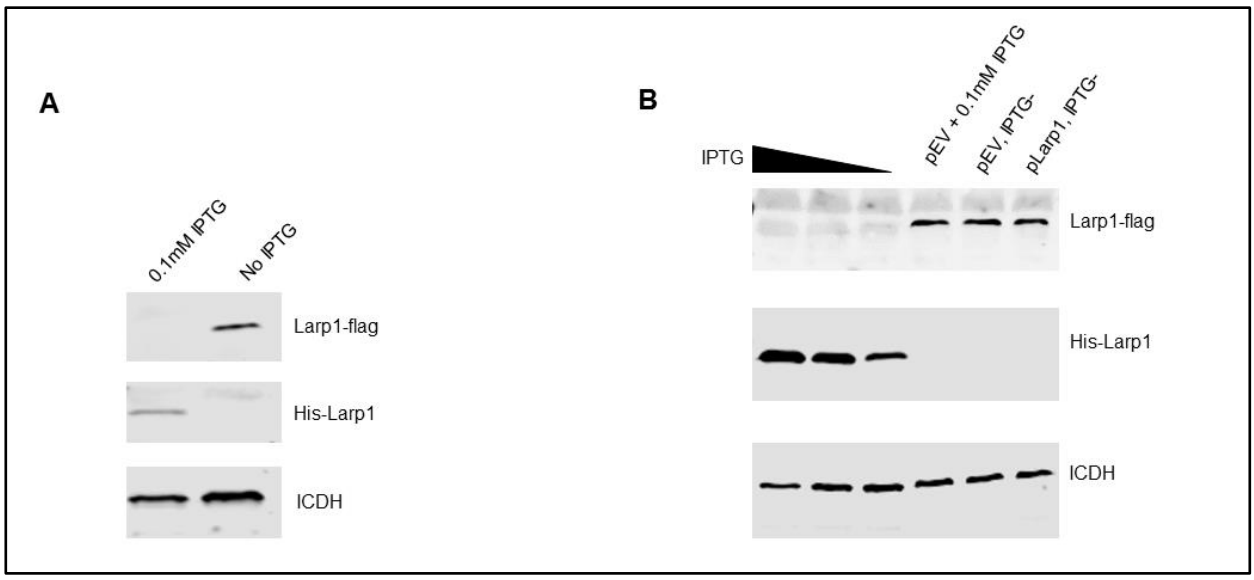

Figure 2-6. Larp1 self regulates in a negative feedback loop.
A. Strain VR702 was inoculated in AYE with or without IPTG. Plasmid-borne Larp1 was through immunoblotting with anti-His antibody and endogenous Larp1 with anti-flag antibody. B. Strain VR700 pLarp1 was inoculated in AYE with increasing amounts of IPTG and endogenous Larp1 levels were monitored using anti-flag antibody. Plasmid-borne Larp1 was detected by using anti-his antibody. A VR700 carrying an empty vector (pEV) was used as a negative control.

RecA expression affects cell morphology in E. coli but not in L. pneumophila

As shown above (**Fig. 2-1** and **Fig. 2-2**, respectively), overexpression of Larp1 induces accumulation of the RecA protein and causes cell elongation. In the model organism *E. coli*, activation of the RecA protein after DNA damage leads to SOS response induction via the expression of the *sulA* gene, which codes for a cell division inhibitor (Michel, 2005). Specifically, *sulA* expression is directly repressed by the SOS repressor LexA (Cole, 1983; Mizusawa et al., 1983). Therefore, upon DNA damage, RecA is activated and auto-cleavage of LexA follows, which allows the *sulA* promoter to initiate transcription, leading to the inhibition of cell division through binding of FtsZ by SulA, and extensive cell filamentation (Chen et al., 2012; Michel, 2005; Mound et al., 1973). In agreement, our findings suggest that overexpression of the *E. coli* RecA protein (EcRecA) is enough to induce cell elongation in the *E. col*i XL1-Blue strain (**Fig. 2-7A)**. Visual evaluation of XL1-Blue cultures carrying an inducible plasmid for overexpression of EcRecA, showed elongated cells upon induction with IPTG when compared to a strain carrying an empty vector (**Fig. 2-7A)**. Next, cell length in these cultures was visually evaluated and quantified using IPlab software. In bacterial cultures where EcRecA was overexpressed, around

20% of the cells were smaller than 2 µm in contrast to the cultures carrying the empty vector where the fraction of cells in this range was close to 80% (**Fig. 2-7A)**. In addition, EcRecA overexpression caused a significant increase in the population of bacterial cells with lengths between 3 and 5 microns. Similarly, cells overexpressing EcRecA had a larger population of cells longer that 6 microns when compared to cultures carrying an empty vector. These results indicated that overexpression of the RecA in *E. coli* is enough to provoke morphological changes in the form of elongated cells.

Since Larp1 overexpression induces cell elongation, I asked whether overexpression of RecA is enough to induce morphological changes in *L. pneumophila*. In contrast to *E. coli*, overexpression of the *L. pneumophila* RecA protein (LpRecA) did not induce changes in cell length in cultures of *L. pneumophila* when evaluated under a microscope (**Fig. 2-7B**). Cell length

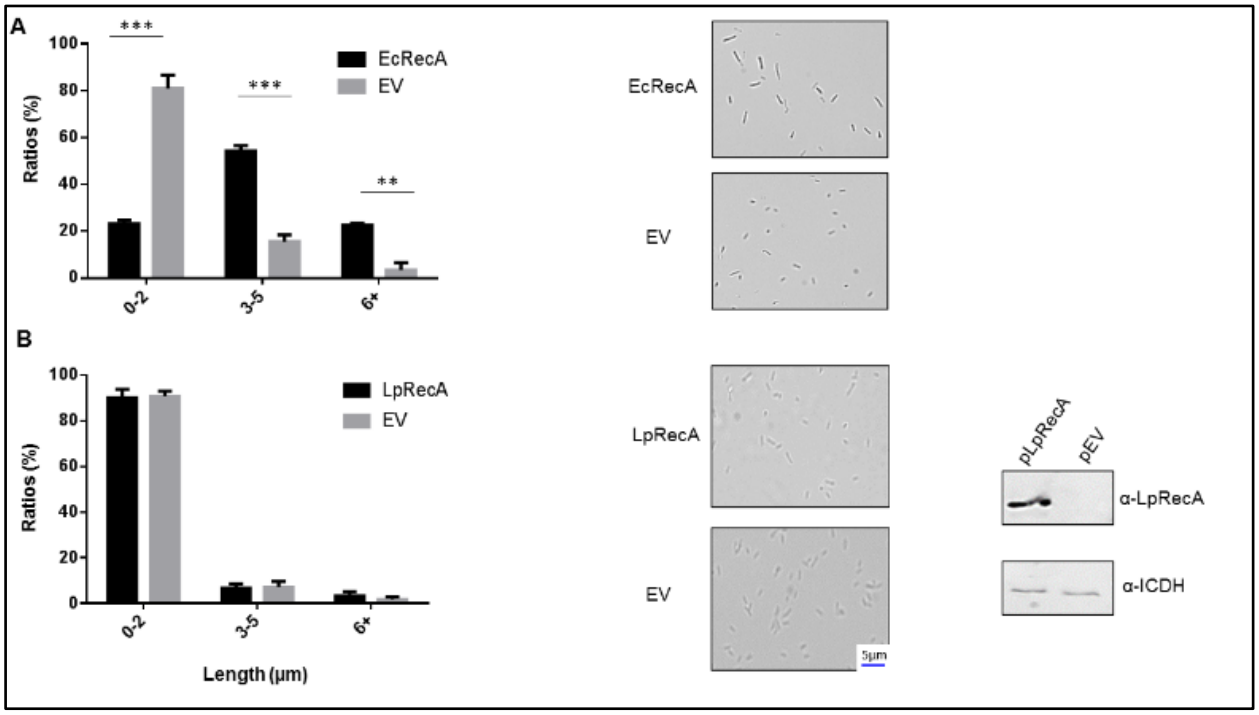

Figure 2-7. Effect of RecA overexpression on cell morphology in *E. coli* and *L. pneumophila*
A. Overexpression of RecA in *E. coli*. *E. coli* XL1-Blue cultures inoculated in LB supplemented with ampicillin and carrying pQE30::*EcrecA* or empty vector, were visually evaluated through microscopy after fixation and the distribution of cell lengths was plotted. The length of >300 cells was measured from 3 independent experiments utilizing the IPlab software package. P-value <0.01 (**); P-value <0.001 (***). B. RecA overexpression in *L. pneumophila*. Lp02 carrying p507::*LpRecA* (pRecA) or empty vector were inoculated in AYE with IPTG and incubated overnight. Bacterial cells were fixated and visually analyzed, and the distribution of cell lengths was plotted. The length of >300 cells was measured from 3 independent experiments utilizing the IPlab software package. Right panel shows immunoblotting of culture samples carrying p507::*LpRecA* or empty vector.

quantification indicated that the bacterial cells overexpressing LpRecA had almost identical lengths when compared to bacteria harboring an empty plasmid (**Fig. 2-7B**). These results indicate that overexpression of RecA is not enough to induce cell elongation in *L. pneumophila*.

Larp1-induced cell elongation is RecA independent

Next, I asked whether the cell elongation phenotype observed after Larp1 overexpression depends on RecA. For this purpose, Lp02 and Lp02 Δ*recA* were transformed with the plasmid pLarp1 or empty vector. After overnight incubation in AYE in the presence of IPTG, the cultures were visually analyzed by light microscopy followed by cell length quantification. We found that expression of Larp1 induced elongation in cells of Lp02 and Lp02 Δ*recA* backgrounds with no detectable differences (**Fig 2-8A-B**), which suggests that this is a RecA-independent event. Quantification of cell lengths indicated ~ 80% of cells were shorter than 2 µm in the Lp02 or Lp02 Δ*recA* cultures of the control strains harboring the empty vector. However, there was a significant shift in cell length in strains expressing Larp1 in both, the Lp02 and the Δ*recA* background. In these strains, only ~40% of the cells were shorter than 2 µm and more than 50% of the cells were between 3 and 5 µm **(Fig 2-8A)**. In addition, cells longer than 6 µm were readily detectable in the strains expressing Larp1 which was in contrast with those harboring an empty vector. Taken together, these results suggest that the cell elongation phenotype displayed by strains overexpressing Larp1 does not require the RecA protein. This was expected since our previous observations in *L. pneumophila* indicated that RecA overexpression (**Fig 2-7B**) does not affect cell morphology, but RecA overexpression in *E. coli* does influence cell morphology (**Fig 2-7A**). Furthermore, the RecA accumulation observed in strains overexpressing Larp1 **(Fig 2-8C)** might be an event that is downstream of Larp1 expression by an unrecognized mechanism. To test this idea, pRecA, a plasmid that directs the expression of RecA, was introduced into strain VR700, which harbored Flag-Larp1 expressed from its cognate promoter from the chromosome, and changes in Larp1 expression were monitored. No difference in Larp1 levels were detected upon RecA overexpression compared to the strain carrying an empty vector **(Fig 2-8C, right panel)**. These observations suggest that RecA accumulation occurs because of Larp1 expression and that RecA expression does not affect Larp1 levels.

Larp1 expression augments cell elongation phenotype of Lp02 ΔletA mutants

The LetA TCS of *L. pneumophila* has been shown to regulate several transmissive traits such as pigment production, cell morphology, and flagellin expression, among others (Hammer et

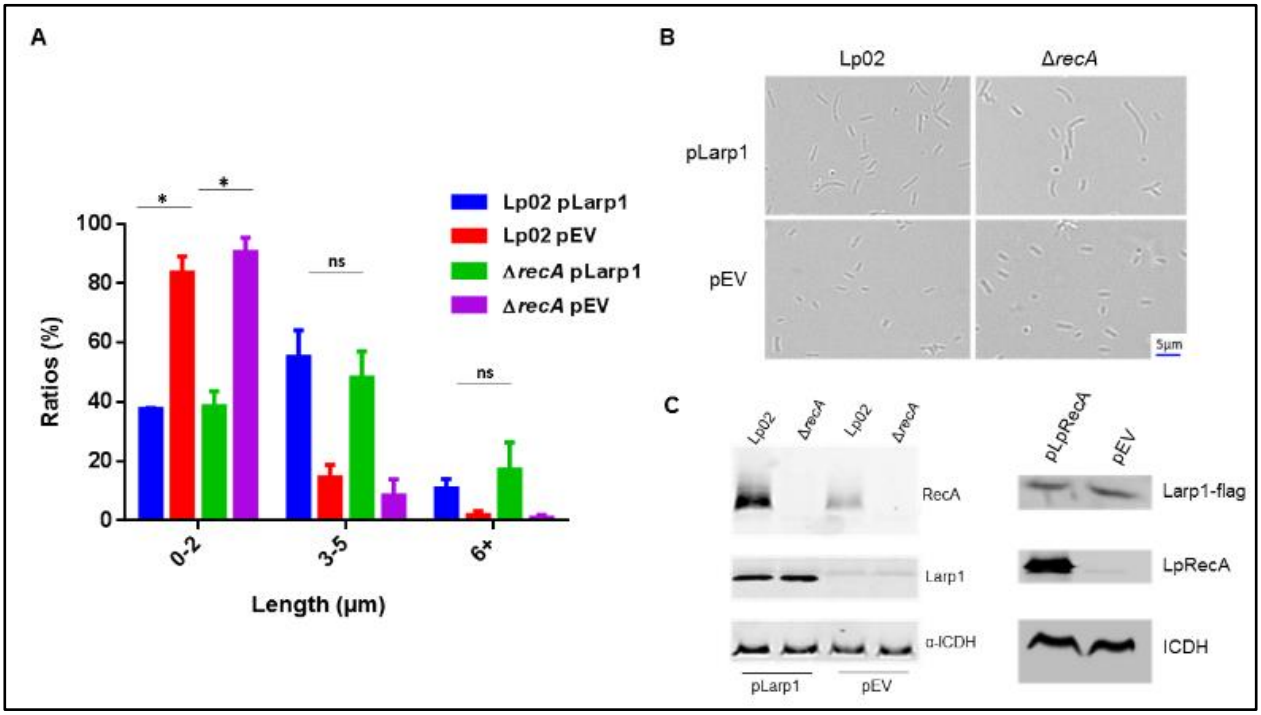

Figure 2-8. Larp1 causes cell elongation independent of RecA.
A. Distribution of cell length in Lp02 or Δ*recA* strains harboring a plasmid for Larp1 expression (pLarp1) or empty vector (pEV) inoculated in AYE with IPTG for 15hrs. P-value < 0.05 (*). The length of >300 cells was measured from 3 independent experiments utilizing the IPlab software package. B. Representative images of strains analyzed in panel A. C. Left, Immunoblotting showing expression of Larp1 in strains used in panel A. C, right panel, expression of RecA does not affect Larp1 levels, VR700 strain harboring pRecA or an empty vector was inoculated in AYE with IPTG and after overnight incubation samples were analyzed by immunoblotting.

al., 2002; Molofsky & Swanson, 2003; Oliva et al., 2018). Specifically, Δ*letA* mutant cells grow into filaments and the pigment pyomelanin does not accumulate in culture supernatants (Molofsky & Swanson, 2003). Thus, I tested the effect of Larp1 expression in Δ*letA* background strains. For this experiment, Δ*letA* mutant strain was transformed with pLarp1 or an empty vector. The resulting strains were then inoculated in AYE with IPTG overnight and bacterial cultures were visually evaluated for morphological changes. In agreement with observations by another group (Molofsky & Swanson, 2003), a larger proportion of cells in strain Lp02Δ*letA* (Δ*letA* pEV) are longer when compared to the WT strain (Lp02 pEV). Here, about 90% of the cells in the WT strain displayed lengths smaller than 2 µm in contrast to 60% in the Δ*letA* (**Fig 2-9**). Around 40% of the

cells in the Δ*letA* strain were between 3 and 5 µm compared to 8% in the WT strain, indicating that our Δ*letA* strain presented the expected filamentation phenotype observed by other research groups. Regarding the effect of Larp1 on the Δ*letA* strain, only about 10% of the cells in strain Δ*letA* (pLarp1) displayed lengths shorter than 2 µm whereas the ratio for the strain harboring the empty vector was around 60%. Furthermore, around 50% of the cells in the Δ*letA* (pLarp1) strain displayed lengths longer than 6 µm, compared to 10% in the Δ*letA* carrying an empty vector **(Fig 2-9)**. These results suggest that expression of Larp1 in the Δ*letA* background further enhances the cell elongation phenotype already presented by the *letA* deletion strain.

Larp1 expression restores pigmentation in the ΔletA mutants

Earlier studies have reported that deletion of the *letA* gene, which encodes the response regulator of the LetAS TCS in *L. pneumophila* (Gal-Mor & Segal, 2003b), causes changes in cell morphology and a decrease in the accumulation of the pyomelanin pigment in culture supernatants (Molofsky & Swanson, 2003; Sahr et al., 2009). Surprisingly, while examining the effect that Larp1 expression had on cell morphology in the Δ*letA* strain, I noticed that cultures of this strain

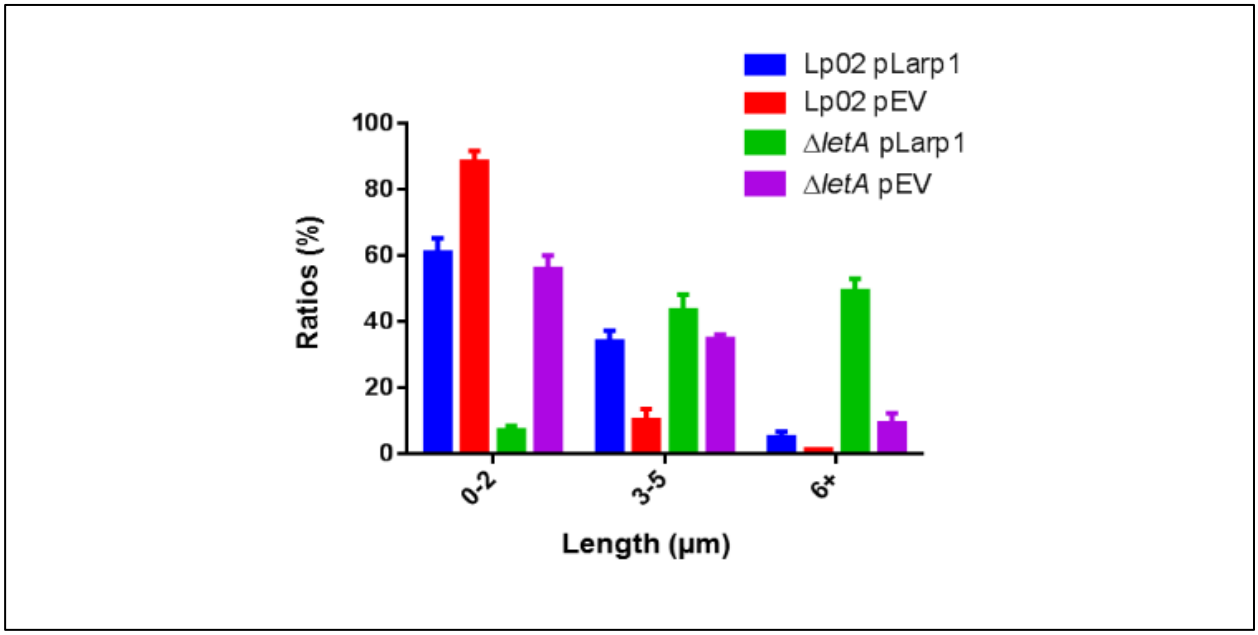

Figure 2-9. Cell morphology and the effect of Larp1 expression in Lp02 Δ*letA* background.
Distribution of cell length in Lp02 or Δ*letA* strains harboring a plasmid for Larp1 expression (pLarp1) or empty vector (pEV) inoculated in AYE with IPTG for 15hrs. The length of >300 cells was measured from 3 independent experiments utilizing the IPlab software package.

accumulated brown pigmentation in the culture supernatant, which was in contrast to the uninduced Δ*letA* (pLarp1) strain or Δ*letA* (pEV) both of which retained a light yellow color (**Fig 2-10A, left**). Determination of colony forming units (CFUs) indicated that the pigment accumulation in strain Δ*letA* (pLarp1) was not due to increased cell numbers since all the strains tested grew to similar numbers after overnight incubation, with the exception of Δ*letA* (pLarp1) grown in the presence of IPTG which had a slight decrease in cell numbers (**Fig 2-10A, right**). Next, I quantified the amount of pigment accumulated in culture supernatants by measuring optical density at 400nm (OD_{400nm}) of culture supernatants (Chatfield & Cianciotto, 2007; Zheng et al., 2013). These results showed that the Δ*letA* strain (pLarp1) exhibited increased pigment accumulation when compared to the uninduced strain or a strain carrying an empty vector (**Fig 2-10A, bottom**). Previous studies have reported that LetA is required for pigment accumulation in

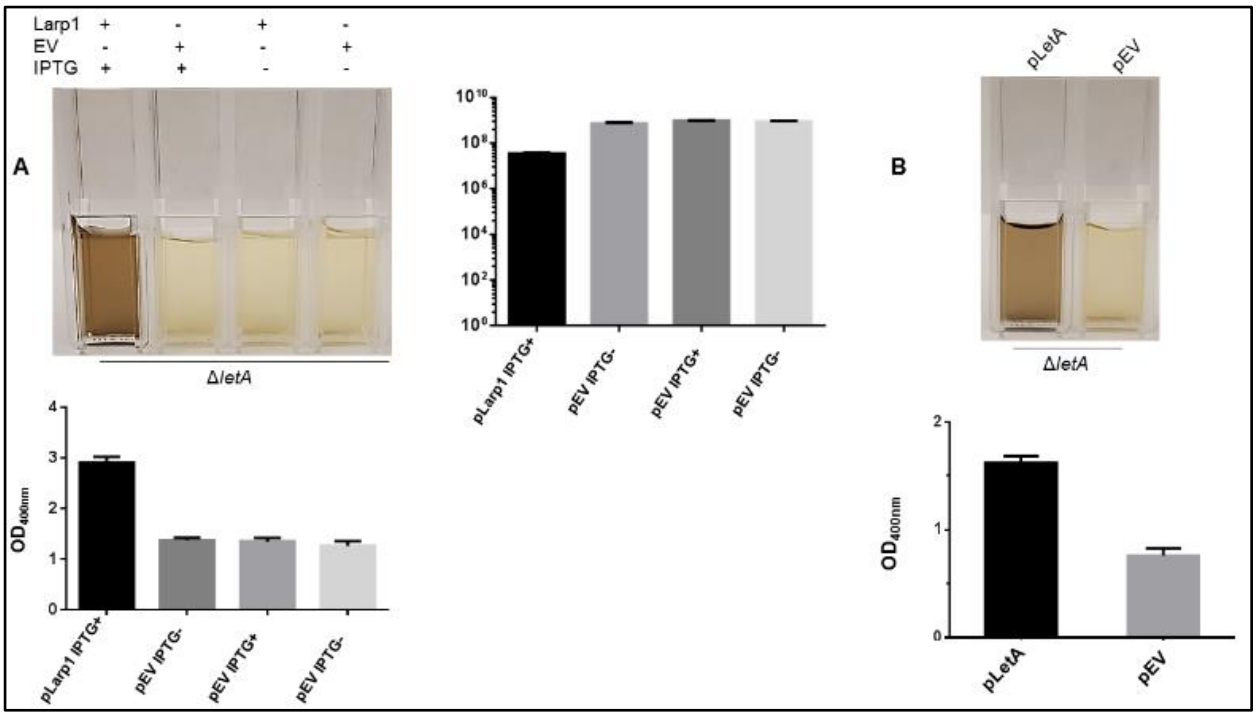

Figure 2-10. Larp1 expression restores pyomelanin accumulation in the Δ*letA* mutant strain. A. Left, Lp02 Δ*letA* was transformed with pLarp1 or an empty vector and strains were inoculated in AYE to ~5x10^8 CFU/ml in the absence or presence of IPTG for 15hrs. Bacterial cultures were then centrifuged, and supernatants are shown. Right, colony forming units were determined by plating serial dilutions in CYE plates for bacterial cultures in A (left panel). Bottom, OD_{400nm} measurements of culture supernatants shown in A (left panel). Overnight bacterial cultures used in A (left panel), were centrifuged and supernatants were collected for measurements of pyomelanin accumulation at OD_{400nm}. B. Lp02 Δ*letA* was transformed with pLetA or an empty vector and strains were inoculated in AYE in the presence of IPTG for 15hrs. Cultures were harvested, and supernatants are shown (top). Pyomelanin measurements taken at OD_{400nm} of culture supernatants are shown (bottom). Results shown are averages of 3 independent experiments.

L. pneumophila cultures (Molofsky & Swanson, 2003). In agreement with these reports, complementation of the Δ*letA* mutant strain with a plasmid expressing LetA, restored pigment accumulation (**Fig 2-10B top, bottom**). Taken together, these results suggest that expression of Larp1 in the Δ*letA* background restores pigment accumulation and propose a potential involvement of this protein in the induction of the pyomelanin production pathway.

Larp1 expression increases the transcription of genes involved in pyomelanin in ΔletA background

Our data suggest that expression of Larp1 in Δ*letA* background restores pigment accumulation in culture supernatants (**Fig 2-10A**), suggesting that Larp1 might have a role in the

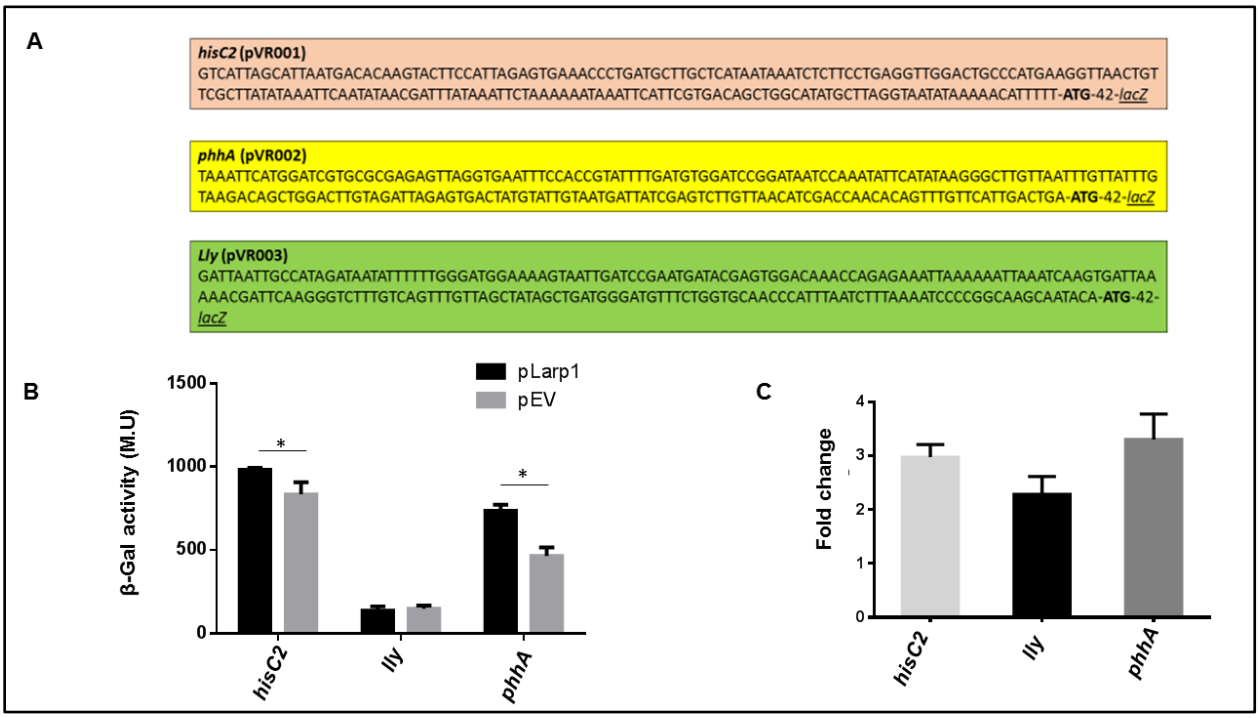

Figure 2-11. Larp1 expression in the Δ*letA* mutant affects the promoter activity of genes involved pyomelanin synthesis.
A. Diagram of LacZ fusions to promoter regions of genes involved in pyomelanin synthesis**.** The 200 bp PCR fragments corresponding to the upstream promoter region (UPR) of the genes *lly*, *phhA* or *hisc2* and the *lacZ* gene from *E. coli*, were ligated to the plasmid pBBR1MCS1 by three-way ligation and later introduced into the indicated *L. pneumophila* strains (see materials and methods for details). B. Expression of LacZ fusions of the promoter region of genes involved in pyomelanin synthesis (*hisc2*, *phhA* and *lly*) in Lp02 Δ*letA* harboring a plasmid for Larp1 expression (pLarp1) or an empty vector (pEV). β-galactosidase activity was measured as detailed in the materials and methods section. The results (Miller units, M.U) are averages of 3 independent experiments. P-value < 0.05 (*), P-value < 0.01 (**). C. Total RNA derived from Δ*letA* Larp1 or Δ*letA* pEV was used for qRT-PCR to assay mRNA levels. Relative mRNA levels in the Δ*letA* Larp1 and Δ*letA* pEV were compared using the deltaCT method ($2^{\Delta\Delta ct}$).

pyomelanin production pathway (also known as the HGA-melanin pathway) (Chatfield & Cianciotto, 2007; Levin et al., 2019). To gain insights into the mechanism of action of Larp1, I tested the effect of Larp1 expression on the promoter activity of three genes involved in pyomelanin synthesis. For this purpose, I constructed a series of plasmids where 200bp of the upstream promoter region of the genes, *hisc2, phhA, lly* and were fused to the *lacZ* gene from *E. coli* (Miller, 1972; Zusman et al., 2007) to create the plasmids pVR001, pVR002 and pVR003, respectively (**Fig 2-11A)**. These genes encode key enzymes in the pyomelanin production pathway (**see Fig. 1-4** for details) and correspond to the phenylalanine hydroxylase (*phhA*), aminotransferase (*hisC2*) and 4-hydroxylphenylpyruvate dioxygenase (*lly*) in *L. pneumophila* (Steinert et al., 2001; Wiater et al., 1994; Wintermeyer et al., 1994; Wintermeyer et al., 1991). The reporter plasmids were then introduced into strain Δ*letA* (pLarp1), for expression of Larp1 or a strain harboring an empty vector. The resulting strains were then inoculated in AYE with IPTG for 15hrs and β-galactosidase activity was measured. Fusion of the predicted promoter regions of the genes *hisC2* and *phhA* to LacZ, resulted in significant increase in β-galactosidase activity upon Larp1 expression vector (**Fig 2-11B**). In contrast, no significant difference was observed in β-galactosidase activity for the putative promoter corresponding to *lly*.

To further validate these observations, mRNA levels for the genes *hisc2*, *phhA* and *lly* were determined through qRT-PCR in the Δ*letA* pLarp1 strain and compared to a strain carrying an empty vector. By qRT-PCR and calculation of relative mRNA levels using the deltaCt method, we found that Larp1 expression in the Δ*letA* mutant strain increases the mRNA levels of all 3 genes involved in pyomelanin synthesis (**Fig 2-11C**). Specifically, the mRNA levels for *hisc2* and *phhA* increased 3-fold while a 2-fold increment was observed for *lly*. These results suggest that Larp1 increases accumulation of the pyomelanin pigment in bacterial cultures through the induction of the enzymes involved in the synthesis of this pigment.

Larp1 expression induces accumulation of pyomelanin in L. pneumophila.

To further assess the effect of Larp1 expression on pyomelanin accumulation in *L. pneumophila*, we measured the production of pigment in WT or Δ*larp1* strains expressing Larp1 or an empty vector. These strains were inoculated to ~5 x 10^8 CFU/ml in AYE with IPTG and pyomelanin accumulation was assayed at 24 hrs and 48 hrs by measuring the OD_{400nm} of culture supernatants. As shown in **Fig. 2-12**, the WT or Δ*larp1* expressing Larp1 did not display

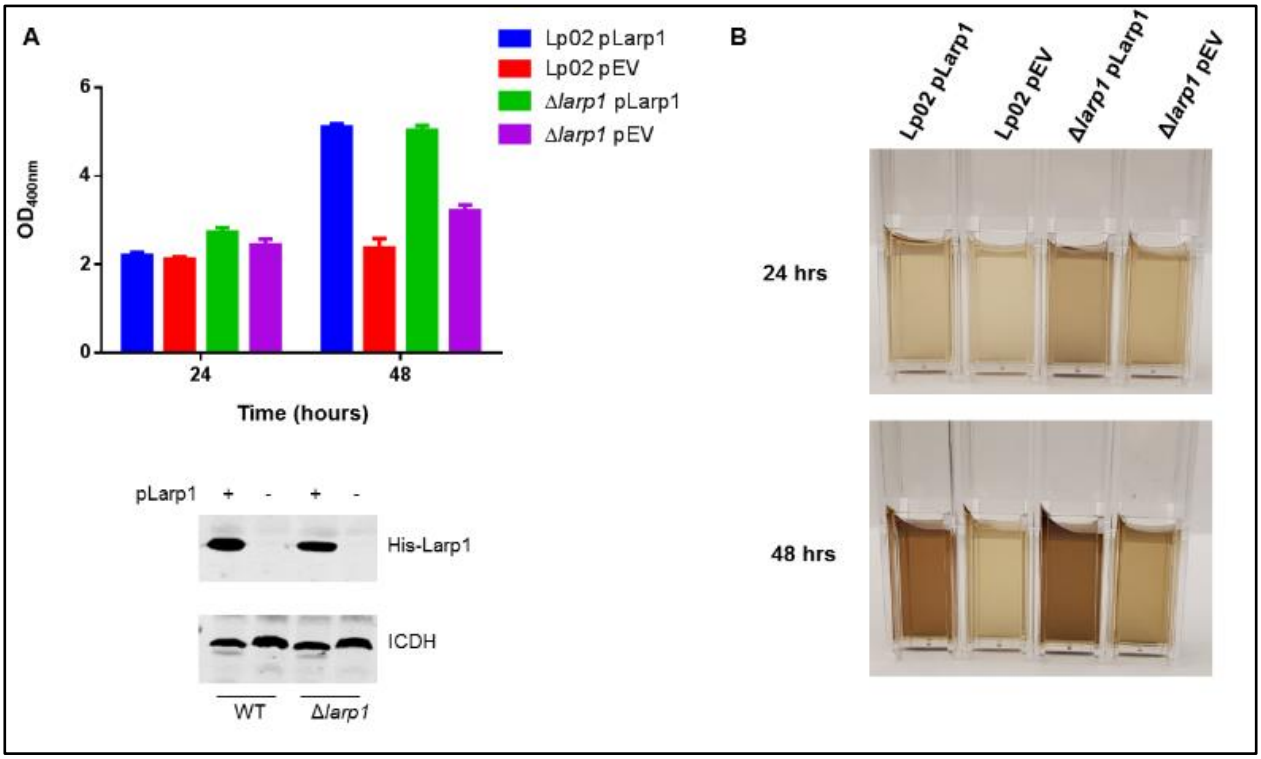

Figure 2-12. Pyomelanin accumulation in WT or Δ*larp1 Legionella* strains expressing Larp1.
A. Levels of pigment in culture supernatants of Lp02 WT or Δ*larp1* strains expressing Larp1 (pLarp1) or an empty vector (pEV) were determined by their OD_{400nm}. ~5 x 10^8 CFU/ml of the indicated strains were inoculated in AYE with IPTG at 37C in the presence of IPTG and at the indicated time points, culture supernatants were assayed. A, bottom panel, shows expression of Larp1 at 48hrs by immunoblotting. B. Image of culture supernatants of strains used in A, taken at 24 and 48 hrs.

significant pigment accumulation when compared with strains harboring an empty vector, at least during the first 24hrs. In addition, pigment in the WT strain (Lp02 pEV) accumulated to similar levels when compared to the Δ*larp1* strain (Δ*larp1* pEV). However, OD_{400nm} measurements at 48 hrs showed significant accumulation of the pigment in the strains expressing Larp1 when compared to control strains carrying an empty vector (**Fig. 2-12A**). The accumulation of the

pigment in culture supernatants was evident when the measurement was performed 24 and 48 hrs after the cultures were established (**Fig. 2-12B**). Taken together, these results suggest that Larp1 expression promotes pigment accumulation when cultures are evaluated at 48 hrs in the WT or *larp1* deletion background. On the other hand, a *larp1* deletion strain does not show decreased levels of pigment when compared to the WT, which suggests that this protein is not crucial for pigment production by *L. pneumophila*.

Synthetic HGA induces cell elongation and RecA

In *L. pneumophila*, oxidative polymerization of homogentisic acid (HGA) results in production of pyomelanin after being exported from the cell (Steinert et al., 2001). HGA is the first metabolite secreted of the pyomelanin synthesis pathway (Levin et al., 2019). Previous studies have shown that synthetic HGA contains antimicrobial activity and antagonizes the growth of *L. micdadei*, and to a lesser extent of *L. pneumophila* (Levin et al., 2019). Notable, HGA but not concentrated pyomelanin extracts (also called HGA-melanin) contained antimicrobial activity. To the contrary, pyomelanin have been implicated in providing improved iron scavenging capabilities and protection from light, thus being beneficial rather than detrimental to the bacterium (Chatfield & Cianciotto, 2007; Steinert et al., 1995). Thus, we proceeded to investigate whether there is a relationship between products of the pyomelanin synthesis pathway, cell elongation and RecA induction. For this purpose, we decided to utilize the commercially available pyomelanin precursor, HGA (Sigma Aldrich, Cat.# H0751), to treat bacterial cultures. Our results suggest that treatment of *L. pneumophila* cultures with synthetic HGA produced changes to cell morphology and RecA levels (**Fig. 2-13)**. We found *that L. pneumophila* cultures treated with HGA displayed elongated cells after ~19 hrs of incubation at 37C **(Fig. 2-13A, top)**. Measurements of cell lengths within these cultures showed that HGA treated samples contained a smaller fraction of cells with lengths < 2 µm when compared the control culture (not treated). Additionally, HGA treated cultures displayed increased cell lengths in the 3-5 µm range when compared to the non-treated control **(Fig. 2-13A, bottom)**. These observations suggest that exogenous HGA can induce cell elongation in *L. pneumophila* cultures. Next, we tested whether HGA treatment affects RecA levels. Indeed, RecA levels were affected upon HGA treatment **(Fig. 2-13B)**. We found that *L. pneumophila* cultures treated with 250µM HGA for 20hrs had increased levels of RecA. Furthermore, we show that this increase in RecA levels was concentration dependent. These results

propose that HGA alone is enough to cause changes to cell morphology and RecA levels. It is possible that the cell elongation and RecA induction phenotypes observed with Larp1 expression are due to overproduction of HGA, which ultimately leads to the increase levels of pyomelanin observed in Larp1-expressing cultures.

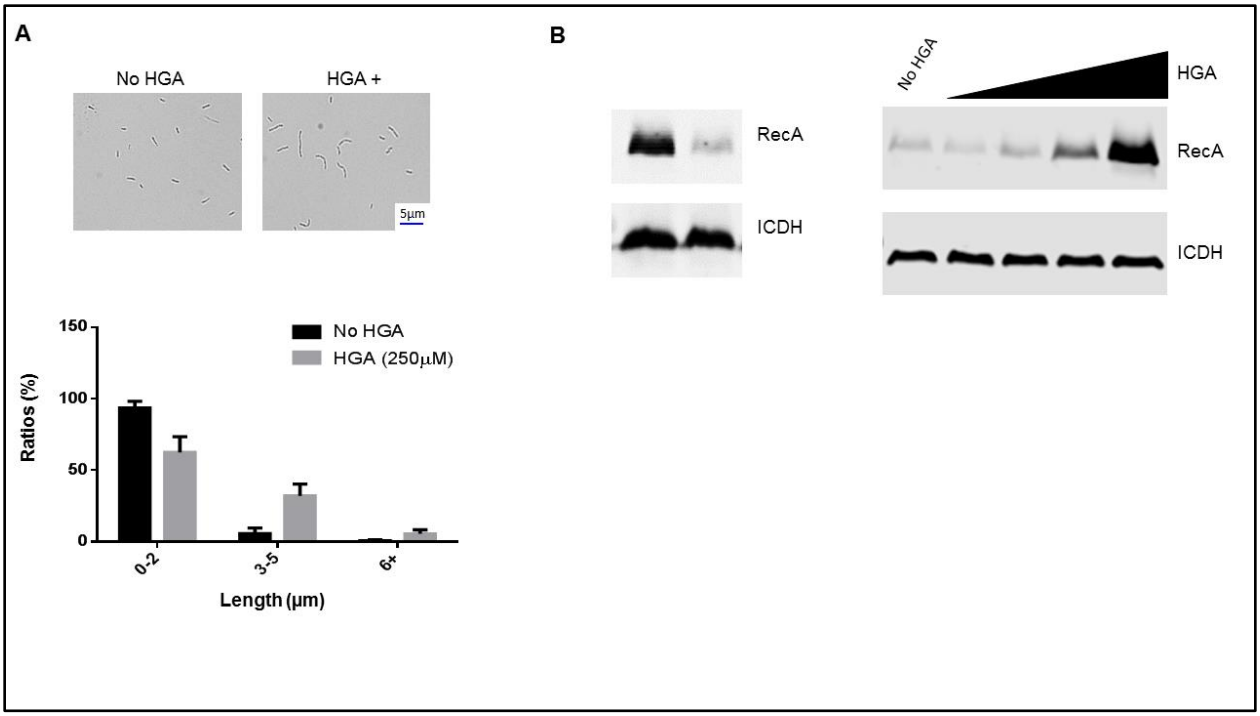

Figure 2-13. Synthetic HGA affects cell length and RecA levels.
A. Overnight cultures were diluted to OD_{600nm} of 0.3 and HGA was added to a final concentration of 250µM and incubated for 20hrs at 37C with shaking. Cultures were visually evaluated through microscopy after fixation (Top) and the distribution of cell lengths was plotted (bottom). The length of >200 cells was measured from 3 independent experiments utilizing the IPlab software package. B. Overnight cultures were diluted to OD_{600nm} of 0.3 and HGA was added to a final concentration of 250µM and incubated for 20hrs at 37C with shaking. RecA levels were determined by immunoblotting using an anti-RecA antibody. ICDH was used as loading control.

Larp1 affects the promoter activity of genes involved in pyomelanin synthesis

To provide further insights on how Larp1 might be affecting pyomelanin accumulation, I examined the effect of Larp1 expression on the promoter activity of *hisc2*, *phhA* and *lly* genes. For this purpose, the upstream promoter region of the genes, *hisc2*, *phhA*, *lly* were fused to *lacZ* and transformed into WT, Δ*larp1* or Δ*larp1* complemented strain, Δ*larp1* (pLarp1). The enzymes encoded by these genes have been reported to be essential for pigment production as deletion mutants of *lly* or *phhA* produce non-pigmented strains (Flydal et al., 2012; Steinert et al., 2001). Thus, affecting the internal balance of these metabolic enzymes might account for the increased pigment accumulation observed in Larp1 expressing strains. β-galactosidase activity assays performed 18 hrs after inoculation in AYE, showed a significant difference in activity of the *hisC2* promoter in the WT strain compared to Δ*larp1*, suggesting that endogenous Larp1 is sufficient to influence this promoter (**Fig 2-14A**). On the other hand, no significant differences in β-galactosidase activity were observed for *lly* or *phhA* promoters in the WT compared to Δ*larp1* mutant. qRT-PCR analysis followed by deltaCT calculations, showed decreased mRNA levels for *hisC2* and a slight decrease for *phhA*, in the WT strain when compared to the Δ*larp1* mutant (**Fig 2-14B**). Additionally, relative mRNA levels in WT *L. pneumophila* carrying pLarp1 or an empty vector, showed that Larp1 expression also increase expression of genes involved in pyomelanin synthesis in the WT background (**Fig. 2-14C).** However, a significant increase in β-galactosidase activity was observed for all 3 promoter regions in the Δ*larp1* complemented strain (Δ*larp1* pLarp1) compared to Δ*larp1* carrying an empty vector (red bars), suggesting that elevated levels of Larp1 lead to induction of transcription by these promoters. In sum, elevated levels of Larp1 induce the promoter activity of enzymes involved in pyomelanin synthesis which might account for the increased accumulation of pigmentation in culture supernatants.

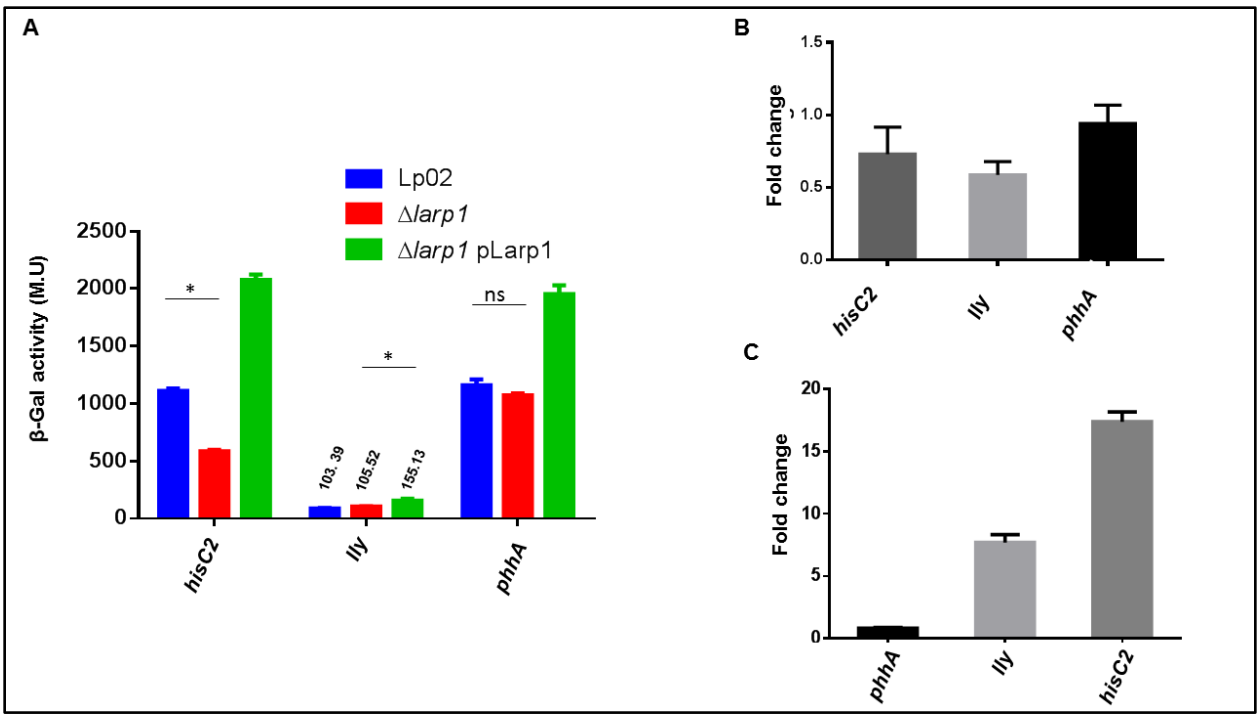

Figure 2-14. Effect of Larp1 expression on promoter activity of enzymes involved in pyomelanin synthesis. A. Expression of LacZ fusions of the promoter region of genes involved in pyomelanin synthesis (*hisc2*, *phhA* and *lly*) in Lp02 WT, Δ*larp1* or Δ*larp1* complemented strain (Δ*larp1* pLarp1) β-galactosidase activity was measured as detailed in the materials and methods section. The results (Miller units, M.U) are averages of 3 independent experiments. P-value < 0.05 (*). B. Total RNA derived from Lp02 WT or Δ*larp1* was isolated and used for qRT-PCR to assay mRNA levels. Relative mRNA levels in the Lp02 WT and Δ*larp1* were compared using the deltaCT method ($2^{\Delta\Delta ct}$). C. Total RNA derived from Lp02 expressing Larp1 or an empty vector was isolated and used for qRT-PCR to determine mRNA levels. Relative mRNA levels were compared using the deltaCT method ($2^{\Delta\Delta ct}$).

Discussion

In bacteriological media and during the establishment of a niche during infection of eukaryotic hosts, *L. pneumophila* displays two distinct developmental states: a replicative and a transmissive form (Oliva et al., 2018). When nutrients levels are adequate, the bacterium enters the replication stage characterized by active metabolism, cell appearance of thin, elongated rods (Faulkner & Garduño, 2002) and the induction of a genetic program for expression of other traits required for productive replication (Oliva et al., 2018). On the other hand, depletion of nutrients signals the bacterium to switch its genetic repertoire favoring the expression of traits associated with the transmissive form, which include coccoid cell shape, motility, pigmentation, among others, that aid the bacteria escape its host to start the cycle anew (Molofsky & Swanson, 2004; Oliva et al., 2018). Previous studies reported the involvement of the LetAS TCS in the regulation of cell morphology and pigmentation as well as several other transmissive traits (Hammer et al.,

2002; Molofsky & Swanson, 2003; Rasis & Segal, 2009; Sahr et al., 2009; Segal, 2013), which highlights the importance of the LetAS TCS in cycling between these developmental states. However, there is no knowledge of LetAS-regulated proteins involved in the regulation of cell shape or pigmentation. To gain more insights into the regulation of cell shape, I performed a screening to identify proteins involved in cell morphology changes in *L. pneumophila*.

Our screening led to the identification of 10 hypothetical proteins that were upregulated, at least 2-fold, upon thymidine starvation of JR32*thyA* strains when compared to WT JR32. After cloning and overexpressing each of these genes in *L. pneumophila*, Larp1 (Lpg0586) was found to induce cell elongation (**Fig. 2-2**) and increased RecA levels (**Fig 2-1**). RecA induction is associated with the activation of the SOS response in *E. coli* (Cox, 1999, 2007a; Little, 1983). This prompted us to test whether RecA expression alone in *E. coli* induce morphology changes. Indeed, our results showed that overexpression of RecA in *E. coli* led to cell elongation which suggests that cell morphology can be affected by the levels of RecA in this bacterium. However, similar experiments done in *L. pneumophila* showed that RecA expression does not trigger cell elongation (**Fig 2-7**). This suggests that cell morphology changes in *L. pneumophila* can occur independently of RecA, which is consistent with the observation that deletion of *recA* does not affect the induction of cell elongation provoked by cues such as Larp1 expression **(Fig. 2-8)**. Of note is that *L. pneumophila* does not encode the homologs of LexA or SulA, two proteins involved in triggering the SOS response in *E. coli* and it is predicted that the canonical SOS pathway it is not present in this bacterium (Charpentier et al., 2011). This is consistent with the observations that RecA overeexpression alone does not affect cell morphology. Thus, Larp1-induced cell elongation must occur through another mechanism that is RecA independent.

The LetAS TCS of *L. pneumophila* is known to regulate the expression of transmissive traits during the stationary phase, including cell morphology, pigmentation and a large cohort of Dot/Icm effectors (Gal-Mor & Segal, 2003b; Hammer et al., 2002; Molofsky & Swanson, 2003, 2004; Rasis & Segal, 2009). Larp1 expression seems to be the highest during stationary phase, which led us to investigate whether this protein is regulated by the LetAS TCS (**Fig. 2-3**). Western blot analysis of Δ*letA* deletion samples showed that Larp1 is under the regulatory control of the LetAS TCS (**Fig. 2-4**). Interestingly, expression of Larp1 in the *letA* deletion strain revealed that pyomelanin accumulation is restored in culture supernatants when compared to the strain carrying an empty vector, which suggests that Larp1 is involved in pigment production (**Fig 2-10**). These

observations propose that Larp1 functions downstream of LetA and that exogenous expression can bypass the requirement of LetA for pigmentation. Further analysis of Larp1-expressing strains showed that exogenous expression of Larp1 in WT, Δ*larp1* or Δ*letA* strains was enough to promote accumulation of pyomelanin in culture supernatants (**Fig 2-10 & 2-12**). Additionally, qRT-PCR analysis of *letA* deletion strains expressing Larp1 showed increased mRNA levels of *hisC2*, *phhA* and *lly*, the three enzymes involved in pyomelanin synthesis in *L. pneumophila* (**Fig 2-11**). Therefore, overexpression of these enzymes in the *letA* deletion background might be enough to drive accumulation of pyomelanin in culture supernatants. Exactly how Larp1 affects the transcription of these genes remains to be determined, but direct binding to the promoter regions is not expected since this protein does not encode any of the known DNA-binding domains. Intriguingly, reduced mRNA levels for *hisC2* and *lly* were detected in the *larp1* deletion strain when compared to the WT, however, this did not translate to reduced pigment accumulation (compare **Figs. 2-12 and 2-14**). In fact, pyomelanin accumulation in the Δ*larp1* and WT strains was similar at 24hrs, but the Δ*larp1* strain slightly accumulated more pigment at 48 hrs. Moreover, accumulation of pyomelanin in the *larp1* deletion strain to WT-like levels indicate that Larp1 is not crucial for pigment production.

Taken together, characterization of Larp1 revealed that (i) expression of this protein in *L. pneumophila* influences cell morphology and RecA levels through a yet to be determined mechanism, but potentially related to HGA production **(Fig. 2-13)**, and (ii) pyomelanin accumulation in culture supernatants is enhanced following Larp1 expression **(Fig. 2-15)**. These observations offer new insights regarding the pyomelanin precursor molecule, HGA and its biological activities. Previous studies have reported that HGA is a potentially detrimental molecule with antimicrobial activity against some *Legionella* species, including *L. pneumophila* (Levin et al., 2019). Perhaps, the Larp1-induced cell elongation and increased RecA levels are a consequence of overproduction of HGA, which ultimately leads to the increased pyomelanin levels observed in our experiments. Our data showing cell elongation and increased RecA levels after synthetic HGA treatment support this idea **(Fig 2-13)**. Malik and group reported that *L. pneumophila* produces HGA and is intrinsically more resistant to its antimicrobial activity during stationary phase of growth when the culture contains a relatively high cell density (Levin et al., 2019). Our findings that endogenous Larp1 expression peaks at stationary phase propose that Larp-1-induced pyomelanin production occurs when the bacterium can manage the stress associated

with increased production of HGA. Thus, Larp1 is produced only during stationary phase to prevent production of molecules, such as HGA, that could negatively impact the physiology of the bacterial cell through abnormal RecA levels and cell morphology changes.

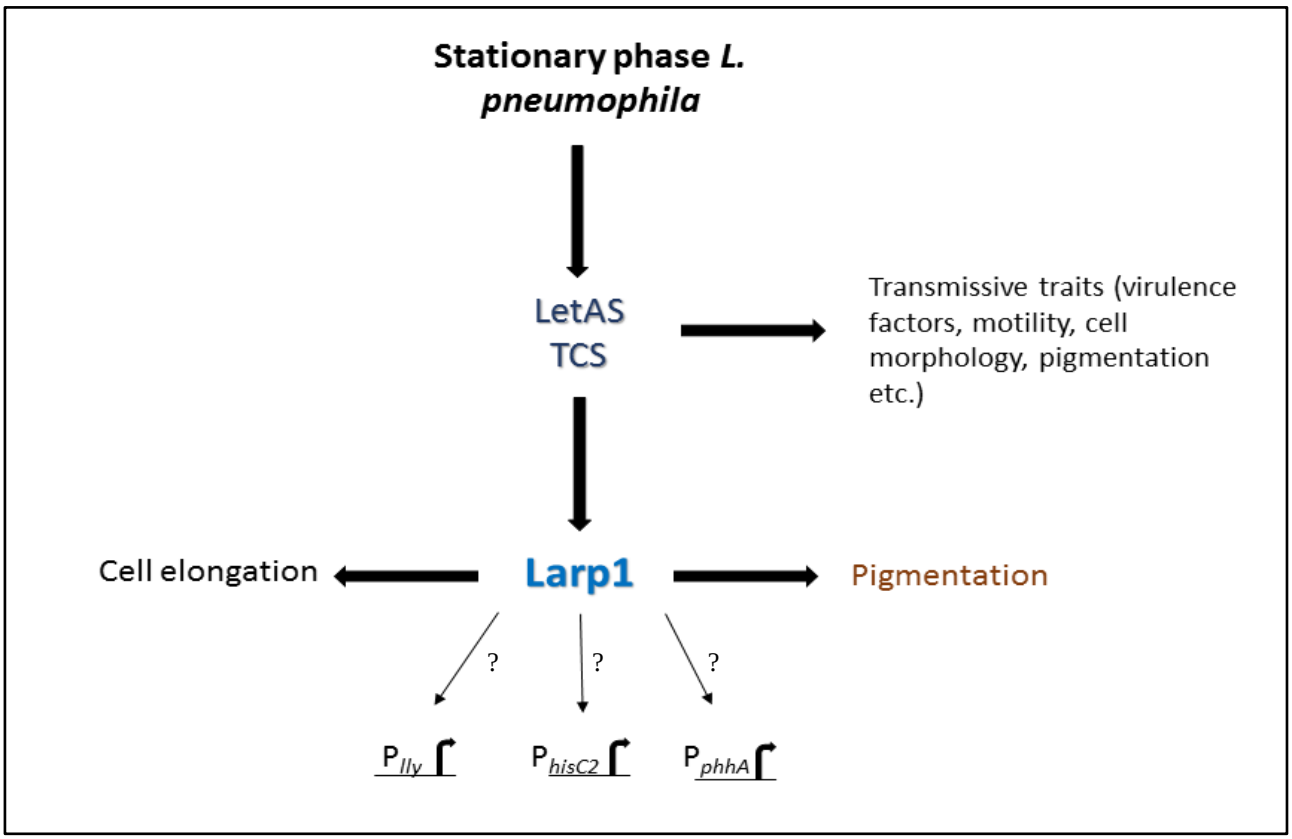

Figure 2-15. Larp1 affects cell morphology and pyomelanin accumulation in *L. pneumophila*.
During stationary phase, LetAS function to activate many traits associated with transmission. We show that LetA induced the expression of Larp1 during stationary phase. Larp1 was shown to affect cell morphology, through an unknown mechanism, and pigmentation of culture supernatants. Larp1-dependent pyomelanin accumulation correlated with increased transcription of genes known to be involved in the pyomelanin synthesis pathway.

CHAPTER III. MATERIALS AND METHODS

Media, bacterial strains and plasmid construction

Bacterial strains used in this study are listed in **Table A-1** and primers in **Table A-2**. All *L. pneumophila* strains generated in this study were derivates of the Philadelphia-1 Lp02 strain (Berger & Isberg, 1993). *L. pneumophila* strains were grown and maintained in CYE agar or AYE broth as previously described (Berger & Isberg, 1993). CYE or AYE was supplemented with thymidine when required (CYET or AYET). Antibiotics and IPTG were added to the media when needed at the following concentrations: kanamycin: 20µg/ml, chloramphenicol: 5µg/ml, streptomycin: 100µg/ml and IPTG: 0.1mM. *E. coli* strains were grown and maintained in LB agar with addition of antibiotics as necessary: kanamycin: 30µg/ml, chloramphenicol: 30µg/ml, ampicillin: 100µg/ml. The Lp02Δ*larp1*, Lp02Δ*letA* and Lp02Δ*recA* were constructed as described previously (Luo & Isberg, 2004). Briefly, the flanking regions of Δ*larp1* were amplified by PCR using the primer sets VRC1001-F/R and VRC1002-F/R. The fragments were then cloned into pSR47s by 3-way ligation. Next, the plasmids were introduced into Lp02 by tri-parental mating and clones were selected in CYET with kanamycin and streptomycin. Clones that no longer carried the vector backbone were selected in CYET with 5% sucrose. Lastly, mutants with the desired in-frame deletions were identified by PCR. Lp02Δ*letA* and Lp02Δ*recA* deletion mutants were constructed similarly. For Δ*letA* the primer sets used were: VRC1011-F/R and VRC1012-F/R and for Δ*lprecA*: VRC1013-F/R and VRC1014-F/R.

For construction of strain VR700, which expresses a Flag-tagged chromosomal *larp1* under the control of its native promoter, the *larp1* gene including ~400bp upstream of the ORF was amplified with the primer sets VRC1003-F/R and VRC1004-F/VRC1002-R. The fragments were cloned into pSR47s through 3-way ligation and knock-in mutants were isolated as described above. Mutants were identified by using the primers VRC1000-F and Flag tag-R.

For construction of plasmid pVR004, which expresses a copy of a Flag-tagged *larp1* gene under its native promoter, the Flag-tagged chromosomal *larp1* ORF was amplified from the strain VR700 using the primers VRC1003-F and VRC1004-R. The fragment was then ligated into pBBR1MCS1 (Kovach et al., 1994) to create the vector pVR004 and later introduced into Lp02 by electroporation.

For construction of *lacZ* fusions with the *L. pneumophila* genes involved in the pyomelanin production pathway, the *lacZ* gene (b0344) was amplified from *E. coli* MG1655 using primers lacZ-F and lacZ-R, which generates a fragment with restriction sites *XhoI* (5') and *HindIII* (3'). Then ~200bp upstream (from initiation codon) of the genes *hisC2, phhA* and *lly* were amplified using the appropriate primers listed in **Table A-2**. The upstream primer contains a KpnI restriction site and the downstream primer contains a *XhoI* site, which was designed to include the first 15 amino acids to generate an in-frame fusion with *lacZ*. The PCR products, after digestion with restriction enzymes, were ligated via 3-way ligation using the pBBR1MCS1 plasmid (Kovach et al., 1995; Kovach et al., 1994) cut with *KpnI* and *HindIII* restriction enzymes. Ligations were transformed into XL1-blue and clones were selected using LB agar supplemented with chloramphenicol (30µg/ml).

β-Galactosidase assays

β-galactosidase assays were performed as described previously (Miller, 1972; Zusman et al., 2007). *L. pneumophila* strains were inoculated in AYE broth with chloramphenicol and grown for ~18hrs at 37C with rotation. When needed, IPTG 0.1mM was added and 100µl of culture was used to assay β-galactosidase activity. ONPG was used as the substrate for β-galactosidase.

Measurement of secreted pyomelanin pigment

To measure pyomelanin in culture supernatants, bacteria were inoculated in 2-3 ml of AYE and induced with 0.1mM IPTG when needed. Cultures were calibrated to 1.5-2.0 OD_{600nm} before incubation. Cultures were then incubated at 37C with rotation and supernatants were collected at 24 or 48 hours and assayed for their absorbance at 400nm (Chatfield & Cianciotto, 2007) using a Beckman Coulter DU 650 spectrophotometer.

Quantitative RT-PCR

Total RNA was isolated from *L. pneumophila* using TRIzol Reagent and following manufacturer's instructions (Thermo-Fisher Scientific). Total RNA was digested with RNAse-free DNAse I (NEB) following manufacturer's protocol. GeneJET RNA Cleanup and Concentration Micro Kit (Thermo-Fisher Scientific) was used for RNA clean up and concentration after DNAse I reactions. Primers used for qRT-PCR are listed in **Table A-2**. qRT-PCR was

performed using the One-Step BrightGreen qRT-PCR-No Dye kit, following manufacturer's protocol (Applied Biological Materials, Canada), in a LightCycler 95 Real-Time PCR system (Roche Life Science).

Immunoblotting

Protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with 4% milk for 1 hour at room temperature. After blocking, membranes were incubated with the indicated primary antibodies for 1hr at room temperature with rocking: anti-his (Sigma), anti-ICDH, anti-LpRecA (polyclonal serum from Poccono Rabbit Farms) or anti-FLAG (Sigma). After incubation with primary antibody, membranes were washed 3 times with PBS-T followed by incubation with the appropriate IRDye infrared secondary antibody (Li-Cor Biosciences). Membranes were processed using the Odyssey infrared imaging system.

Intracellular growth assay

Dictyostelium discoideum strain AX4 was maintained in HL-5 medium as described previously (Li et al., 2005). *L. pneumophila* strains were grown in AYE to post-exponential phase as indicated by optical density (OD_{600nm}= 3.2-4.0) and bacterial motility observed under a light microscope. *D. discoideum* cells seeded in 24 well-plates at a density of 4.0 x 10^5 were infected with an MOI of 0.1 for determining the proficiency of intracellular growth of these strains. In all experiments, 2 hours after addition of bacteria to cultured cells, the infection was synchronized by washing the cells with room temperature PBS, 3 times. Colony forming units (CFUs) were determined by plating serially diluted saponin lysates in CYE agar plates. When indicated, IPTG was added to the culture media 2hrs post-infection and kept throughout the infection period.

Microscopy and cell morphology analysis

Morphology of bacterial cells from *L. pneumophila* or *E. coli* cultures were visually analyzed to determine cell length. Briefly, 60-80 µl of overnight culture was harvested and the pellet was resuspended in 350ul of 4% paraformaldehyde. Bacterial cells in 4% paraformaldehyde were incubated at room temperature for 20mins for fixation. Around 6-8ul of fixed sample was dropped into a microscope slide and covered with cover glass (22 x 22mm). Standard nail polish

was used to seal the cover glass around the edges. Finally, bacterial cells were imaged using the brightfield on an IX-81 Olympus fluorescence microscope. IPlab software (Scanalytics, Inc.) was used for processing images and for measuring of cell lengths of at least 250 bacterial cells.

Data presentation and statistical analysis

Student's t-test (two-sided) was implemented to compare the mean between two groups with at least 3 independent samples. GraphPad Prism software was used for data visualization.

APPENDIX

Table A-1. Bacterial strains and plasmids used in this study

Strains	Description	Reference/note
***L. pneumophila* strains**		
Lpo2		(Merriam et al., 1997)
Lp03		(Merriam et al., 1997)
Lp02(pZL507)		
Lpo2Δ*larp1*		
Lpo2Δ*larp1*(pLarp1)		
Lpo2Δ*larp1*(pLarp1)(pVR001)		
Lpo2Δ*larp1*(pLarp1)(pVR002)		
Lpo2Δ*larp1*(pLarp1)(pVR003)		
Lp02(pLarp1)		
Lp02(pLarp1)(pVR004)	VR702	
VR700	Lp02 carrying a flag-tagged chromosomal copy of *larp1*	
Lp02Δ*letA*		
Lp02Δ*letA*(pLetA)		
Lp02Δ*letA*(pLetA)(pVR004)	Designated VR701	
Lp02Δ*letA* (pLetA)(pVR001)		
Lp02Δ*letA*(pLetA)(pVR002)		
Lp02Δ*letA* (pLetA)(pVR003)		
Lp02(pRecA)		
Lp02Δ*lpRecA*		
Lp02Δ*lpRecA*(pLarp1)		
Lp02(pVR001)(pLarp1)		
Lp02(pVR002)(pLarp1)		
Lp02(pVR003)(pLarp1)		
E. coli		
XL1-Blue		Agilent
BL21-(DE3)		NEB
Plasmids		
pETSUMO	For expression of His6-SUMO-tagged proteins	Invitrogen, For expression of Larp1

Table A-1. continued

pZL507	**Plasmid for His6-tagged protein expression in *L. pneumophila***	**(Xu et al., 2010), expression of Larp1 and RecA**
pSR47s	R6K vector for gene knockout	(Xu et al., 2010)
pBBR1MCS1		(Kovach et al., 1994)
pZLQKn-flag	For expression of Flag tagged proteins in *L. pneumophila*	
pLarp1	Larp1 (*lpg0586*) cloned into pZL507	
pRecA	RecA (*lpg1801*) cloned into pZL507	
pVR001	pBBR1MCS1::P_{hisC2}::LacZ, for monitoring promoter activity of *hisC2*	
pVR002	pBBR1MCS1::P_{phhA}::LacZ, for monitoring promoter activity of *phhA*	
pVR003	pBBR1MCS1::P_{lly}::LacZ, for monitoring promoter activity of *lly*	
pVR004	pBBR1MCS1::*larp1*-flag, *larp1* ORF 3' flag-tagged and cloned with upstream promoter region	

Table A-2: Primers used in this study

Primers for plasmid construction	**Sequence (restriction sites are underlined)**	**note**
VRC1000-F	GCGGGATCCATGGCAATTATCAGTTCAC	For cloning *larp1* ORF
VRC1000-R	AGCGTCGACCTAGGCATGGCCAGCATC	For cloning *larp1* ORF
VRC1001-F	ATAGCGGCCGCGTGGGGAATCCGCTTTTGT	For *larp1* in-frame deletion
VRC1001-R	GCTGGATCCCATGGCAATCAATAGGTG	For *larp1* in-frame deletion
VRC1002-F	GCTGGATCCGGCATCAAAATGAATCAACTC	For *larp1* in-frame deletion
VRC1002-R	CGAGTCGACGCTTCGCCTTTAGTTTCTG	For *larp1* in-frame deletion
VRC1003-F	GCGCTCGAGCTTCAACTTCTTATACAGCTGG	For chromosomal flag tag knock-in of *larp1*-Promoter
VRC1003-R	GCGGGATCCCTACTTGTCGTCATCGTCTTTGTAGTCGGCATGGCCAGCATCAGAAGAGAGTTGATT	For chromosomal flag tag knock-in of *larp1*
VRC1004-F	GCGGGATCCAGGTGTGTATTTAGGTTTTGATT	For chromosomal flag tag knock-in of *larp1*
VR1004-R	GCGGGTACCCAGCGCATCTTCAAAATGAG	For PCR of *larp1* flag tagged ORF
VRC1005-F	GCGGGATCCTTGGTCAAGGAGCATTATTTG	For cloning l*etA* ORF
VRC1005-R	CGCGTCGACCTAATCATCTTGTGACAAATC	For cloning l*etA* ORF
VRC1006-F	GCAGGTACCGTCATTAGCATTAATGACACAAG	For cloning promoter region of *hisC2* (~200bp up)
VRC1006-R	GCGCTCGAGGGGTAATTGTTGAAAATCAATAG	For cloning promoter region of *hisC2* (~200bp up)
VRC1007-F	GCAGGTACCTTGGTATTTAGAGTATCGCCATG	For cloning promoter region of *phhA* (~500bp up)
VRC1007-R	GCGCTCGAGCTGAGCATCAGGGACATGTGCGAC	For cloning promoter region of *phhA* (~500bp up)

Table A-2: continued

VRC1008-F	**GCTGGTACCGATTAATTGCCATAGATAATA**	**For cloning promoter region of *lly* (~200bp up)**
VRC1008-R	GCGCTCGAGTGTATTGCTTGCCGGGGATT	For cloning promoter region of *lly* (~200bp up)
VRC1009-F	GCGCTCGAGATGACCATGATTACGGATTC	For cloning LacZ fusions with promoter regions of interest
VRC1009-R	GCGAAGCTTTTATTTTTGACACCAGACCA	For cloning LacZ fusions with promoter regions of interest
VRC1010-F	GCGGGTACCATGGAAGAAAATAAACAAAAAGC	For cloning of *LprecA* (lpg1801)
VRC1010-R	CGCGTCGACTTAGTCATCTATAGTCTCAAAAAG	For cloning of *LprecA* (lpg1801)
VRC1011-F	GTAGCGGCCGCGGCTGACCCTAAATACAAA	For in-frame deletion of *letA* (*lpg2646*)
VRC1011-R	GCGAGATCTGTCATCAACAATTAATACTTTA	For in-frame deletion of *letA* (*lpg2646*)
VRC1012-F	GCGAGATCTCACCGCATTATTGAGCACC	For in-frame deletion of *letA* (*lpg2646*)
VRC1012-R	GCAGTCGACGTAATTATCAAATCCGGAATTC	For in-frame deletion of *letA* (*lpg2646*)
VRC1013-F	GCGGCGGCCGCATGCAACTACCAGATATGTTGAA	For in-frame deletion of *lprecA* (*lpg1801*)
VRC1013-R	GCGAAGCTTTTGCGAAAGCGCCGCTGATAG	For in-frame deletion of *lprecA* (*lpg1801*)
VRC1014-F	GCGAAGCTTTTGGCTAGTTCATCAGAAGATC	For in-frame deletion of *lprecA* (*lpg1801*)
VRC1014-R	GCGGTCGACCAACATTTTCCAGATCATTATG	For in-frame deletion of *lprecA* (*lpg1801*)
***lacZ*-F**	GCGCTCGAGATGACCATGATTACGGATTC	For *lacZ* fusions
lacZ-R	GCGAAGCTTTTATTTTTGACACCAGACCA	For *lacZ* fusions

Table A-2: continued

Flag tag-R	**GTCGTCATCGTCTTTGTAGTC**	
Primers (qRT-PCR)	**Sequence**	**note**
hisC2-F	CCCATGCCGGAATACGTTCT	
hisC2-R	AAAGGGCTAATGGGCTGCAT	
lly-F	CAGCCATCCAGGCTATTGGT	
lly-R	TGGGTGAGATGGTCGATTGC	
phhA-F	CGCCTAAAGGGCTTAGAGCA	
phhA-R	GGCATTCGAAAAGCCACCAC	
16srRNA-F	AAGAAGCACCGGCTAACTCC	
16srRNA-R	AATCAACCACCTACGCACCC	

REFERENCES

Ahmad, S. I., Kirk, S. H., & Eisenstark, A. (1998). Thymine metabolism and thymineless death in prokaryotes and eukaryotes. *Annu Rev Microbiol*, *52*, 591-625. https://doi.org/10.1146/annurev.micro.52.1.591

Al-Khodor, S., Kalachikov, S., Morozova, I., Price, C. T., & Abu Kwaik, Y. (2009). The PmrA/PmrB two-component system of Legionella pneumophila is a global regulator required for intracellular replication within macrophages and protozoa. *Infect Immun*, *77*(1), 374-386. https://doi.org/10.1128/IAI.01081-08

Altman, E., & Segal, G. (2008). The response regulator CpxR directly regulates expression of several Legionella pneumophila icm/dot components as well as new translocated substrates. *J Bacteriol*, *190*(6), 1985-1996. https://doi.org/10.1128/JB.01493-07

Armstrong, G. A., & Hearst, J. E. (1996). Carotenoids 2: Genetics and molecular biology of carotenoid pigment biosynthesis. *FASEB J*, *10*(2), 228-237. https://doi.org/10.1096/fasebj.10.2.8641556

Bachman, M. A., & Swanson, M. S. (2004). Genetic evidence that Legionella pneumophila RpoS modulates expression of the transmission phenotype in both the exponential phase and the stationary phase. *Infect Immun*, *72*(5), 2468-2476. https://doi.org/10.1128/iai.72.5.2468-2476.2004

Baine, W. B., & Rasheed, J. K. (1979). Aromatic substrate specificity of browning by cultures of the Legionnaires' disease bacterium. *Ann Intern Med*, *90*(4), 619-620. https://doi.org/10.7326/0003-4819-90-4-619

Baine, W. B., Rasheed, J. K., Feeley, J. C., Gorman, G. W., & Casida, L. E. (1978). Effect of supplementall-tyrosine on pigment production in cultures of the Legionnaires' disease bacterium. *Current Microbiology*, *1*(2), 93-94. https://doi.org/10.1007/BF02605423

Barluzzi, R., Brozzetti, A., Mariucci, G., Tantucci, M., Neglia, R. G., Bistoni, F., & Blasi, E. (2000). Establishment of protective immunity against cerebral cryptococcosis by means of an avirulent, non melanogenic Cryptococcus neoformans strain. *J Neuroimmunol*, *109*(2), 75-86. https://doi.org/10.1016/s0165-5728(00)00319-2

Berger, K. H., & Isberg, R. R. (1993). Two distinct defects in intracellular growth complemented by a single genetic locus in Legionella pneumophila. *Mol Microbiol*, *7*(1), 7-19. https://doi.org/10.1111/j.1365-2958.1993.tb01092.x

Bhosale, P., & Bernstein, P. S. (2004). Beta-carotene production by Flavobacterium multivorum in the presence of inorganic salts and urea. *J Ind Microbiol Biotechnol*, *31*(12), 565-571. https://doi.org/10.1007/s10295-004-0187-9

Boger D, P. M. (1987). Total synthesis of prodigiosin. *Tetrahedron Letters*, *28*(22), 2499 - 2502. https://doi.org/https://doi.org/10.1016/S0040-4039(00)95451-0

Boudry, P., Gracia, C., Monot, M., Caillet, J., Saujet, L., Hajnsdorf, E., Dupuy, B., Martin-Verstraete, I., & Soutourina, O. (2014). Pleiotropic role of the RNA chaperone protein Hfq in the human pathogen Clostridium difficile. *J Bacteriol*, *196*(18), 3234-3248. https://doi.org/10.1128/JB.01923-14

Brenner, D. J., Steigerwalt, A. G., & McDade, J. E. (1979). Classification of the Legionnaires' disease bacterium: Legionella pneumophila, genus novum, species nova, of the family Legionellaceae, familia nova. *Ann Intern Med*, *90*(4), 656-658. https://doi.org/10.7326/0003-4819-90-4-656

Britigan, B. E., Railsback, M. A., & Cox, C. D. (1999). The Pseudomonas aeruginosa secretory product pyocyanin inactivates alpha1 protease inhibitor: implications for the pathogenesis of cystic fibrosis lung disease. *Infect Immun*, *67*(3), 1207-1212. https://doi.org/10.1128/IAI.67.3.1207-1212.1999

Burstein, D., Amaro, F., Zusman, T., Lifshitz, Z., Cohen, O., Gilbert, J. A., Pupko, T., Shuman, H. A., & Segal, G. (2016). Genomic analysis of 38 Legionella species identifies large and diverse effector repertoires. *Nat Genet*, *48*(2), 167-175. https://doi.org/10.1038/ng.3481

Capra, E. J., & Laub, M. T. (2012). Evolution of two-component signal transduction systems. *Annu Rev Microbiol*, *66*, 325-347. https://doi.org/10.1146/annurev-micro-092611-150039

Cazalet, C., Rusniok, C., Brüggemann, H., Zidane, N., Magnier, A., Ma, L., Tichit, M., Jarraud, S., Bouchier, C., Vandenesch, F., Kunst, F., Etienne, J., Glaser, P., & Buchrieser, C. (2004). Evidence in the Legionella pneumophila genome for exploitation of host cell functions and high genome plasticity. *Nat Genet*, *36*(11), 1165-1173. https://doi.org/10.1038/ng1447

CDC. (2018a). *Legionella: History, Burden, and Trends*. Centers for Disease Control and Prevention. https://www.cdc.gov/legionella/about/history.html

CDC. (2018b). *Legionnaires' Disease*. Centers for Disease Control and Prevention. https://www.cdc.gov/legionella/about/signs-symptoms.html

Charpentier, X., Kay, E., Schneider, D., & Shuman, H. A. (2011). Antibiotics and UV radiation induce competence for natural transformation in Legionella pneumophila. *J Bacteriol*, *193*(5), 1114-1121. https://doi.org/10.1128/JB.01146-10

Chatfield, C. H., & Cianciotto, N. P. (2007). The secreted pyomelanin pigment of Legionella pneumophila confers ferric reductase activity. *Infect Immun*, *75*(8), 4062-4070. https://doi.org/10.1128/IAI.00489-07

Chen, C., Banga, S., Mertensa, K., Webera, M. M., Gorbaslievaa, I., Tan, Y., Luo, Z.-Q., & Samuel, J. E. (2010). Large-scale identification and translocation of type IV secretion substrates by *Coxiella burnetii*. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(50), 21755-21760.

Chen, Y., Milam, S. L., & Erickson, H. P. (2012). SulA inhibits assembly of FtsZ by a simple sequestration mechanism. *Biochemistry*, *51*(14), 3100-3109. https://doi.org/10.1021/bi201669d

Cianciotto, N. P. (2005). Type II secretion: a protein secretion system for all seasons. *Trends Microbiol*, *13*(12), 581-588. https://doi.org/10.1016/j.tim.2005.09.005

Cianciotto, N. P. (2009). Many substrates and functions of type II secretion: lessons learned from Legionella pneumophila. *Future Microbiol*, *4*(7), 797-805. https://doi.org/10.2217/fmb.09.53

Cianciotto, N. P. (2013). Type II secretion and Legionella virulence. *Curr Top Microbiol Immunol*, *376*, 81-102. https://doi.org/10.1007/82_2013_339

Cianciotto, N. P., Eisenstein, B. I., Mody, C. H., & Engleberg, N. C. (1990). A mutation in the mip gene results in an attenuation of Legionella pneumophila virulence. *J Infect Dis*, *162*(1), 121-126. https://doi.org/10.1093/infdis/162.1.121

Cianciotto, N. P., Eisenstein, B. I., Mody, C. H., Toews, G. B., & Engleberg, N. C. (1989). A Legionella pneumophila gene encoding a species-specific surface protein potentiates initiation of intracellular infection. *Infect Immun*, *57*(4), 1255-1262. https://doi.org/10.1128/IAI.57.4.1255-1262.1989

Cianciotto, N. P., & Fields, B. S. (1992). Legionella pneumophila mip gene potentiates intracellular infection of protozoa and human macrophages. *Proc Natl Acad Sci U S A*, *89*(11), 5188-5191. https://doi.org/10.1073/pnas.89.11.5188

Claus, H., & Decker, H. (2006). Bacterial tyrosinases. *Syst Appl Microbiol*, *29*(1), 3-14. https://doi.org/10.1016/j.syapm.2005.07.012

Cole, S. T. (1983). Characterisation of the promoter for the LexA regulated sulA gene of Escherichia coli. *Mol Gen Genet*, *189*(3), 400-404. https://doi.org/10.1007/BF00325901

Conover, G. M., Derré, I., Vogel, J. P., & Isberg, R. R. (2003). The Legionella pneumophila LidA protein: a translocated substrate of the Dot/Icm system associated with maintenance of bacterial integrity. *Mol Microbiol*, *48*(2), 305-321. https://doi.org/10.1046/j.1365-2958.2003.03400.x

Correia, A. M., Ferreira, J. S., Borges, V., Nunes, A., Gomes, B., Capucho, R., Gonçalves, J., Antunes, D. M., Almeida, S., Mendes, A., Guerreiro, M., Sampaio, D. A., Vieira, L., Machado, J., Simões, M. J., Gonçalves, P., & Gomes, J. P. (2016). Probable Person-to-Person Transmission of Legionnaires' Disease. *N Engl J Med*, *374*(5), 497-498. https://doi.org/10.1056/NEJMc1505356

Cox, M. M. (1999). Recombinational DNA repair in bacteria and the RecA protein. *Prog Nucleic Acid Res Mol Biol*, *63*, 311-366.

Cox, M. M. (2007a). Motoring along with the bacterial RecA protein. *Nat Rev Mol Cell Biol*, *8*(2), 127-138. https://doi.org/10.1038/nrm2099

Cox, M. M. (2007b). Regulation of bacterial RecA protein function. *Crit Rev Biochem Mol Biol*, *42*(1), 41-63. https://doi.org/10.1080/10409230701260258

Cunha, B. A., Burillo, A., & Bouza, E. (2016). Legionnaires' disease. *Lancet*, *387*(10016), 376-385. https://doi.org/10.1016/S0140-6736(15)60078-2

Daniels, N. A., MacKinnon, L., Bishop, R., Altekruse, S., Ray, B., Hammond, R. M., Thompson, S., Wilson, S., Bean, N. H., Griffin, P. M., & Slutsker, L. (2000). Vibrio parahaemolyticus infections in the United States, 1973-1998. *J Infect Dis*, *181*(5), 1661-1666. https://doi.org/10.1086/315459

de Chastellier, C., Lang, T., & Thilo, L. (1995). Phagocytic processing of the macrophage endoparasite, Mycobacterium avium, in comparison to phagosomes which contain Bacillus subtilis or latex beads. *Eur J Cell Biol*, *68*(2), 167-182.

DeBritto, S., Gajbar, T. D., Satapute, P., Sundaram, L., Lakshmikantha, R. Y., Jogaiah, S., & Ito, S. I. (2020). Isolation and characterization of nutrient dependent pyocyanin from Pseudomonas aeruginosa and its dye and agrochemical properties. *Sci Rep*, *10*(1), 1542. https://doi.org/10.1038/s41598-020-58335-6

DebRoy, S., Dao, J., Söderberg, M., Rossier, O., & Cianciotto, N. P. (2006). Legionella pneumophila type II secretome reveals unique exoproteins and a chitinase that promotes bacterial persistence in the lung. *Proc Natl Acad Sci U S A*, *103*(50), 19146-19151. https://doi.org/10.1073/pnas.0608279103

Denoya, C. D., Skinner, D. D., & Morgenstern, M. R. (1994). A Streptomyces avermitilis gene encoding a 4-hydroxyphenylpyruvic acid dioxygenase-like protein that directs the production of homogentisic acid and an ochronotic pigment in Escherichia coli. *J Bacteriol*, *176*(17), 5312-5319. https://doi.org/10.1128/jb.176.17.5312-5319.1994

Dufossé, L., Fouillaud, M., Caro, Y., Mapari, S. A., & Sutthiwong, N. (2014). Filamentous fungi are large-scale producers of pigments and colorants for the food industry. *Curr Opin Biotechnol*, *26*, 56-61. https://doi.org/10.1016/j.copbio.2013.09.007

Duménil, G., & Isberg, R. R. (2001). The Legionella pneumophila IcmR protein exhibits chaperone activity for IcmQ by preventing its participation in high-molecular-weight complexes. *Mol Microbiol*, *40*(5), 1113-1127. https://doi.org/10.1046/j.1365-2958.2001.02454.x

Durán, M., Ponezi, A. N., Faljoni-Alario, A., Teixeira, M. F. S., Justo, G. Z., & Durán, N. (2012). Potential applications of violacein: a microbial pigment. *Medicinal Chemistry Research*, *21*(7), 1524-1532. https://doi.org/10.1007/s00044-011-9654-9

Durán, N., Justo, G. Z., Durán, M., Brocchi, M., Cordi, L., Tasic, L., Castro, G. R., & Nakazato, G. (2016). Advances in Chromobacterium violaceum and properties of violacein-Its main secondary metabolite: A review. *Biotechnol Adv*, *34*(5), 1030-1045. https://doi.org/10.1016/j.biotechadv.2016.06.003

El-Naggar, N. E., & El-Ewasy, S. M. (2017). Bioproduction, characterization, anticancer and antioxidant activities of extracellular melanin pigment produced by newly isolated microbial cell factories Streptomyces glaucescens NEAE-H. *Sci Rep*, *7*, 42129. https://doi.org/10.1038/srep42129

Ensminger, A. W. (2016). Legionella pneumophila, armed to the hilt: justifying the largest arsenal of effectors in the bacterial world. *Curr Opin Microbiol*, *29*, 74-80. https://doi.org/10.1016/j.mib.2015.11.002

Faulkner, G., & Garduño, R. A. (2002). Ultrastructural analysis of differentiation in Legionella pneumophila. *J Bacteriol*, *184*(24), 7025-7041. https://doi.org/10.1128/jb.184.24.7025-7041.2002

Feldheim, Y. S., Zusman, T., Speiser, Y., & Segal, G. (2016). The Legionella pneumophila CpxRA two-component regulatory system: new insights into CpxR's function as a dual regulator and its connection to the effectors regulatory network. *Mol Microbiol*, *99*(6), 1059-1079. https://doi.org/10.1111/mmi.13290

Fernandez-Moreira, E., Helbig, J. H., & Swanson, M. S. (2006). Membrane vesicles shed by Legionella pneumophila inhibit fusion of phagosomes with lysosomes. *Infect Immun*, *74*(6), 3285-3295. https://doi.org/10.1128/IAI.01382-05

Fettes, P. S., Forsbach-Birk, V., Lynch, D., & Marre, R. (2001). Overexpresssion of a Legionella pneumophila homologue of the E. coli regulator csrA affects cell size, flagellation, and pigmentation. *Int J Med Microbiol*, *291*(5), 353-360. https://doi.org/10.1078/1438-4221-00141

Fields, B. S., Benson, R. F., & Besser, R. E. (2002). Legionella and Legionnaires' disease: 25 years of investigation. *Clin Microbiol Rev*, *15*(3), 506-526. https://doi.org/10.1128/cmr.15.3.506-526.2002

Fischer, G., Bang, H., Ludwig, B., Mann, K., & Hacker, J. (1992). Mip protein of Legionella pneumophila exhibits peptidyl-prolyl-cis/trans isomerase (PPlase) activity. *Mol Microbiol*, *6*(10), 1375-1383. https://doi.org/10.1111/j.1365-2958.1992.tb00858.x

Fliermans, C. B., Cherry, W. B., Orrison, L. H., Smith, S. J., Tison, D. L., & Pope, D. H. (1981). Ecological distribution of Legionella pneumophila. *Appl Environ Microbiol*, *41*(1), 9-16.

Flydal, M. I., Chatfield, C. H., Zheng, H., Gunderson, F. F., Aubi, O., Cianciotto, N. P., & Martinez, A. (2012). Phenylalanine hydroxylase from Legionella pneumophila is a thermostable enzyme with a major functional role in pyomelanin synthesis. *PLoS One*, *7*(9), e46209. https://doi.org/10.1371/journal.pone.0046209

Fraser, D. W., Tsai, T. R., Orenstein, W., Parkin, W. E., Beecham, H. J., Sharrar, R. G., Harris, J., Mallison, G. F., Martin, S. M., McDade, J. E., Shepard, C. C., & Brachman, P. S. (1977). Legionnaires' disease: description of an epidemic of pneumonia. *N Engl J Med*, *297*(22), 1189-1197. https://doi.org/10.1056/NEJM197712012972201

Gal-Mor, O., & Segal, G. (2003a). Identification of CpxR as a positive regulator of icm and dot virulence genes of Legionella pneumophila. *J Bacteriol*, *185*(16), 4908-4919. https://doi.org/10.1128/jb.185.16.4908-4919.2003

Gal-Mor, O., & Segal, G. (2003b). The Legionella pneumophila GacA homolog (LetA) is involved in the regulation of icm virulence genes and is required for intracellular multiplication in Acanthamoeba castellanii. *Microb Pathog*, *34*(4), 187-194. https://doi.org/10.1016/s0882-4010(03)00027-5

Gan, N., Guan, H., Huang, Y., Yu, T., Fu, J., Nakayasu, E. S., Puvar, K., Das, C., Wang, D., Ouyang, S., & Luo, Z. Q. (2020). Legionella pneumophila regulates the activity of UBE2N by deamidase-mediated deubiquitination. *EMBO J*, *39*(4), e102806. https://doi.org/10.15252/embj.2019102806

Gan, N., Nakayasu, E. S., Hollenbeck, P. J., & Luo, Z. Q. (2019). Legionella pneumophila inhibits immune signalling via MavC-mediated transglutaminase-induced ubiquitination of UBE2N. *Nat Microbiol*, *4*(1), 134-143. https://doi.org/10.1038/s41564-018-0282-8

Gan, N., Zhen, X., Liu, Y., Xu, X., He, C., Qiu, J., Fujimoto, G. M., Nakayasu, E. S., Zhou, B., Zhao, L., Puvar, K., Das, C., Ouyang, S., & Luo, Z. Q. (2019). Regulation of phosphoribosyl ubiquitination by a calmodulin-dependent glutamylase. *Nature*, *572*(7769), 387-391. https://doi.org/10.1038/s41586-019-1439-1

Ghosal, D., Kim, K. W., Zheng, H., Kaplan, M., Truchan, H. K., Lopez, A. E., McIntire, I. E., Vogel, J. P., Cianciotto, N. P., & Jensen, G. J. (2019). In vivo structure of the Legionella type II secretion system by electron cryotomography. *Nat Microbiol*, *4*(12), 2101-2108. https://doi.org/10.1038/s41564-019-0603-6

Gomez-Valero, L., Rusniok, C., Carson, D., Mondino, S., Pérez-Cobas, A. E., Rolando, M., Pasricha, S., Reuter, S., Demirtas, J., Crumbach, J., Descorps-Declere, S., Hartland, E. L., Jarraud, S., Dougan, G., Schroeder, G. N., Frankel, G., & Buchrieser, C. (2019). More than 18,000 effectors in the. *Proc Natl Acad Sci U S A*, *116*(6), 2265-2273. https://doi.org/10.1073/pnas.1808016116

González Plaza, J. J. (2020). Small RNAs as Fundamental Players in the Transference of Information During Bacterial Infectious Diseases. *Front Mol Biosci*, *7*, 101. https://doi.org/10.3389/fmolb.2020.00101

Goralczyk, R. (2009). Beta-carotene and lung cancer in smokers: review of hypotheses and status of research. *Nutr Cancer*, *61*(6), 767-774. https://doi.org/10.1080/01635580903285155

Gotoh, Y., Eguchi, Y., Watanabe, T., Okamoto, S., Doi, A., & Utsumi, R. (2010). Two-component signal transduction as potential drug targets in pathogenic bacteria. *Curr Opin Microbiol*, *13*(2), 232-239. https://doi.org/10.1016/j.mib.2010.01.008

Gudiol, C., Verdaguer, R., Angeles Domínguez, M., Fernández-Sevilla, A., & Carratalà, J. (2007). Outbreak of Legionnaires' disease in immunosuppressed patients at a cancer centre: usefulness of universal urine antigen testing and early levofloxacin therapy. *Clin Microbiol Infect*, *13*(11), 1125-1128. https://doi.org/10.1111/j.1469-0691.2007.01805.x

Hales, L. M., & Shuman, H. A. (1999a). Legionella pneumophila contains a type II general secretion pathway required for growth in amoebae as well as for secretion of the Msp protease. *Infect Immun*, *67*(7), 3662-3666. https://doi.org/10.1128/IAI.67.7.3662-3666.1999

Hales, L. M., & Shuman, H. A. (1999b). The Legionella pneumophila rpoS gene is required for growth within Acanthamoeba castellanii. *J Bacteriol*, *181*(16), 4879-4889. https://doi.org/10.1128/JB.181.16.4879-4889.1999

Hammer, B. K., Tateda, E. S., & Swanson, M. S. (2002). A two-component regulator induces the transmission phenotype of stationary-phase Legionella pneumophila. *Mol Microbiol*, *44*(1), 107-118. https://doi.org/10.1046/j.1365-2958.2002.02884.x

Hammerschlag, M. R. (2002). The intracellular life of chlamydiae. *Semin Pediatr Infect Dis*, *13*(4), 239-248. https://doi.org/10.1053/spid.2002.127201

Han, H., Iakovenko, L., & Wilson, A. C. (2015). Loss of Homogentisate 1,2-Dioxygenase Activity in Bacillus anthracis Results in Accumulation of Protective Pigment. *PLoS One*, *10*(6), e0128967. https://doi.org/10.1371/journal.pone.0128967

Hardiman, C. A., McDonough, J. A., Newton, H. J., & Roy, C. R. (2012). The role of Rab GTPases in the transport of vacuoles containing Legionella pneumophila and Coxiella burnetii. *Biochem Soc Trans*, *40*(6), 1353-1359. https://doi.org/10.1042/BST20120167

Hassan, H. M., & Fridovich, I. (1980). Mechanism of the antibiotic action pyocyanine. *J Bacteriol*, *141*(1), 156-163. https://doi.org/10.1128/JB.141.1.156-163.1980

Hejazi, A., & Falkiner, F. R. (1997). Serratia marcescens. *J Med Microbiol*, *46*(11), 903-912. https://doi.org/10.1099/00222615-46-11-903

Hilbi, H., Jarraud, S., Hartland, E., & Buchrieser, C. (2010). Update on Legionnaires' disease: pathogenesis, epidemiology, detection and control. *Mol Microbiol*, *76*(1), 1-11. https://doi.org/10.1111/j.1365-2958.2010.07086.x

Hilbi, H., Weber, S. S., Ragaz, C., Nyfeler, Y., & Urwyler, S. (2007). Environmental predators as models for bacterial pathogenesis. *Environ Microbiol*, *9*(3), 563-575. https://doi.org/10.1111/j.1462-2920.2007.01238.x

Hughes, W. T., & Kim, H. K. (1973). Mycoflora in cystic fibrosis: some ecologic aspects of Pseudomonas aeruginosa and Candida albicans. *Mycopathol Mycol Appl*, *50*(3), 261-269. https://doi.org/10.1007/BF02053377

Hunke, S., Keller, R., & Müller, V. S. (2012). Signal integration by the Cpx-envelope stress system. *FEMS Microbiol Lett*, *326*(1), 12-22. https://doi.org/10.1111/j.1574-6968.2011.02436.x

Isaac, D. T., & Isberg, R. (2014). Master manipulators: an update on Legionella pneumophila Icm/Dot translocated substrates and their host targets. *Future Microbiol*, *9*(3), 343-359. https://doi.org/10.2217/fmb.13.162

Isberg, R. R., O'Connor, T. J., & Heidtman, M. (2009). The Legionella pneumophila replication vacuole: making a cosy niche inside host cells. *Nat Rev Microbiol*, *7*(1), 13-24. https://doi.org/10.1038/nrmicro1967

Jishage, M., Kvint, K., Shingler, V., & Nyström, T. (2002). Regulation of sigma factor competition by the alarmone ppGpp. *Genes Dev*, *16*(10), 1260-1270. https://doi.org/10.1101/gad.227902

Johnson, T. L., Abendroth, J., Hol, W. G., & Sandkvist, M. (2006). Type II secretion: from structure to function. *FEMS Microbiol Lett*, *255*(2), 175-186. https://doi.org/10.1111/j.1574-6968.2006.00102.x

Joshi, A. D., Sturgill-Koszycki, S., & Swanson, M. S. (2001). Evidence that Dot-dependent and -independent factors isolate the Legionella pneumophila phagosome from the endocytic network in mouse macrophages. *Cell Microbiol*, *3*(2), 99-114. https://doi.org/10.1046/j.1462-5822.2001.00093.x

Jung, K., Fried, L., Behr, S., & Heermann, R. (2012). Histidine kinases and response regulators in networks. *Curr Opin Microbiol*, *15*(2), 118-124. https://doi.org/10.1016/j.mib.2011.11.009

Kagan, J. C., & Roy, C. R. (2002). Legionella phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites. *Nat Cell Biol*, *4*(12), 945-954. https://doi.org/10.1038/ncb883

Keith, K. E., Killip, L., He, P., Moran, G. R., & Valvano, M. A. (2007). Burkholderia cenocepacia C5424 produces a pigment with antioxidant properties using a homogentisate intermediate. *J Bacteriol*, *189*(24), 9057-9065. https://doi.org/10.1128/JB.00436-07

Knirel, Y. A., Rietschel, E. T., Marre, R., & Zähringer, U. (1994). The structure of the O-specific chain of Legionella pneumophila serogroup 1 lipopolysaccharide. *Eur J Biochem*, *221*(1), 239-245. https://doi.org/10.1111/j.1432-1033.1994.tb18734.x

Kotob, S. I., Coon, S. L., Quintero, E. J., & Weiner, R. M. (1995). Homogentisic acid is the primary precursor of melanin synthesis in Vibrio cholerae, a Hyphomonas strain, and Shewanella colwelliana. *Appl Environ Microbiol*, *61*(4), 1620-1622. https://doi.org/10.1128/AEM.61.4.1620-1622.1995

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene*, *166*(1), 175-176. https://doi.org/10.1016/0378-1119(95)00584-1

Kovach, M. E., Phillips, R. W., Elzer, P. H., Roop, R. M., & Peterson, K. M. (1994). pBBR1MCS: a broad-host-range cloning vector. *Biotechniques*, *16*(5), 800-802.

Kubori, T., & Nagai, H. (2016). The Type IVB secretion system: an enigmatic chimera. *Curr Opin Microbiol*, *29*, 22-29. https://doi.org/10.1016/j.mib.2015.10.001

Kulandaisamy, V., Zakaria, Z. A., & Ahmad, W. A. (2013). Bacterial pigments and their applications. *Process Biochemistry*, *48*(7), 1065 - 1079. https://doi.org/https://doi.org/10.1016/j.procbio.2013.06.006

Kwon-Chung, K. J., Polacheck, I., & Popkin, T. J. (1982). Melanin-lacking mutants of Cryptococcus neoformans and their virulence for mice. *J Bacteriol*, *150*(3), 1414-1421. https://doi.org/10.1128/JB.150.3.1414-1421.1982

Lapouge, K., Schubert, M., Allain, F. H., & Haas, D. (2008). Gac/Rsm signal transduction pathway of gamma-proteobacteria: from RNA recognition to regulation of social behaviour. *Mol Microbiol*, *67*(2), 241-253. https://doi.org/10.1111/j.1365-2958.2007.06042.x

Lau, G. W., Hassett, D. J., Ran, H., & Kong, F. (2004). The role of pyocyanin in Pseudomonas aeruginosa infection. *Trends Mol Med*, *10*(12), 599-606. https://doi.org/10.1016/j.molmed.2004.10.002

Levin, T. C., Goldspiel, B. P., & Malik, H. S. (2019). Density-dependent resistance protects. *Elife*, *8*. https://doi.org/10.7554/eLife.46086

Li, Z., Solomon, J. M., & Isberg, R. R. (2005). Dictyostelium discoideum strains lacking the RtoA protein are defective for maturation of the Legionella pneumophila replication vacuole. *Cell Microbiol*, *7*(3), 431-442. https://doi.org/10.1111/j.1462-5822.2004.00472.x

Lichstein, H. C., & Van De Sand, V. F. (1945). Violacein, an Antibiotic Pigment Produced by Chromobacterium Violaceum. *The Journal of Infectious Diseases*, *76*(1), 47-51. https://doi.org/10.1093/infdis/76.1.47

Liles, M. R., Edelstein, P. H., & Cianciotto, N. P. (1999). The prepilin peptidase is required for protein secretion by and the virulence of the intracellular pathogen Legionella pneumophila. *Mol Microbiol*, *31*(3), 959-970. https://doi.org/10.1046/j.1365-2958.1999.01239.x

Little, J. W. (1983). The SOS regulatory system: control of its state by the level of RecA protease. *J Mol Biol*, *167*(4), 791-808. https://doi.org/10.1016/s0022-2836(83)80111-9

Little, J. W., & Mount, D. W. (1982). The SOS regulatory system of Escherichia coli. *Cell*, *29*(1), 11-22. https://doi.org/10.1016/0092-8674(82)90085-x

Luo, Z. Q., & Isberg, R. R. (2004). Multiple substrates of the Legionella pneumophila Dot/Icm system identified by interbacterial protein transfer. *Proc Natl Acad Sci U S A*, *101*(3), 841-846. https://doi.org/10.1073/pnas.0304916101

Marra, A., Blander, S. J., Horwitz, M. A., & Shuman, H. A. (1992). Identification of a Legionella pneumophila locus required for intracellular multiplication in human macrophages. *Proc Natl Acad Sci U S A*, *89*(20), 9607-9611. https://doi.org/10.1073/pnas.89.20.9607

Maslowska, K. H., Makiela-Dzbenska, K., & Fijalkowska, I. J. (2019). The SOS system: A complex and tightly regulated response to DNA damage. *Environ Mol Mutagen*, *60*(4), 368-384. https://doi.org/10.1002/em.22267

McDade, J. E., Shepard, C. C., Fraser, D. W., Tsai, T. R., Redus, M. A., & Dowdle, W. R. (1977). Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory disease. *N Engl J Med*, *297*(22), 1197-1203. https://doi.org/10.1056/NEJM197712012972202

Merriam, J. J., Mathur, R., Maxfield-Boumil, R., & Isberg, R. R. (1997). Analysis of the Legionella pneumophila fliI gene: intracellular growth of a defined mutant defective for flagellum biosynthesis. *Infect Immun*, *65*(6), 2497-2501. https://doi.org/10.1128/IAI.65.6.2497-2501.1997

Michel, B. (2005). After 30 years of study, the bacterial SOS response still surprises us. *PLoS Biol*, *3*(7), e255. https://doi.org/10.1371/journal.pbio.0030255

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Mizusawa, S., Court, D., & Gottesman, S. (1983). Transcription of the sulA gene and repression by LexA. *J Mol Biol*, *171*(3), 337-343. https://doi.org/10.1016/0022-2836(83)90097-9

Mohammadi, M., Burbank, L., & Roper, M. C. (2012a). Biological role of pigment production for the bacterial phytopathogen Pantoea stewartii subsp. stewartii. *Appl Environ Microbiol*, *78*(19), 6859-6865. https://doi.org/10.1128/AEM.01574-12

Mohammadi, M., Burbank, L., & Roper, M. C. (2012b). Pantoea stewartii subsp. stewartii produces an endoglucanase that is required for full virulence in sweet corn. *Mol Plant Microbe Interact*, *25*(4), 463-470. https://doi.org/10.1094/MPMI-09-11-0226

Molofsky, A. B., & Swanson, M. S. (2003). Legionella pneumophila CsrA is a pivotal repressor of transmission traits and activator of replication. *Mol Microbiol*, *50*(2), 445-461. https://doi.org/10.1046/j.1365-2958.2003.03706.x

Molofsky, A. B., & Swanson, M. S. (2004). Differentiate to thrive: lessons from the Legionella pneumophila life cycle. *Mol Microbiol*, *53*(1), 29-40. https://doi.org/10.1111/j.1365-2958.2004.04129.x

Mound, D. W., Walker, A. C., & Kosel, C. (1973). Suppression of lex mutations affecting deoxyribonucleic acid repair in Escherichia coli K-12 by closely linked thermosensitive mutations. *J Bacteriol*, *116*(2), 950-956. https://doi.org/10.1128/JB.116.2.950-956.1973

Mukherjee, S., Liu, X., Arasaki, K., McDonough, J., Galán, J. E., & Roy, C. R. (2011). Modulation of Rab GTPase function by a protein phosphocholine transferase. *Nature*, *477*(7362), 103-106. https://doi.org/10.1038/nature10335

Müller, M. P., Peters, H., Blümer, J., Blankenfeldt, W., Goody, R. S., & Itzen, A. (2010). The Legionella effector protein DrrA AMPylates the membrane traffic regulator Rab1b. *Science*, *329*(5994), 946-949. https://doi.org/10.1126/science.1192276

Nagai, H., Kagan, J. C., Zhu, X., Kahn, R. A., & Roy, C. R. (2002). A bacterial guanine nucleotide exchange factor activates ARF on Legionella phagosomes. *Science*, *295*(5555), 679-682. https://doi.org/10.1126/science.1067025

Narsing Rao, M. P., Xiao, M., & Li, W. J. (2017). Fungal and Bacterial Pigments: Secondary Metabolites with Wide Applications. *Front Microbiol*, *8*, 1113. https://doi.org/10.3389/fmicb.2017.01113

Nazzaro, G. (2019). *Etymologia: Serratia marcescens*. https://wwwnc.cdc.gov/eid/article/25/11/et-2511_article#:~:text=Serratia%20marcescens%2C%20which%20can%20cause,the%20pigment%20known%20as%20prodigiosin.

Neunuebel, M. R., Chen, Y., Gaspar, A. H., Backlund, P. S., Yergey, A., & Machner, M. P. (2011). De-AMPylation of the small GTPase Rab1 by the pathogen Legionella pneumophila. *Science*, *333*(6041), 453-456. https://doi.org/10.1126/science.1207193

Newton, H. J., Ang, D. K., van Driel, I. R., & Hartland, E. L. (2010). Molecular pathogenesis of infections caused by Legionella pneumophila. *Clin Microbiol Rev*, *23*(2), 274-298. https://doi.org/10.1128/CMR.00052-09

Ninio, S., Zuckman-Cholon, D. M., Cambronne, E. D., & Roy, C. R. (2005). The Legionella IcmS-IcmW protein complex is important for Dot/Icm-mediated protein translocation. *Mol Microbiol*, *55*(3), 912-926. https://doi.org/10.1111/j.1365-2958.2004.04435.x

Nitzan, M., Rehani, R., & Margalit, H. (2017). Integration of Bacterial Small RNAs in Regulatory Networks. *Annu Rev Biophys*, *46*, 131-148. https://doi.org/10.1146/annurev-biophys-070816-034058

Nosanchuk, J. D., & Casadevall, A. (2003). The contribution of melanin to microbial pathogenesis. *Cell Microbiol*, *5*(4), 203-223. https://doi.org/10.1046/j.1462-5814.2003.00268.x

Nosanchuk, J. D., & Casadevall, A. (2006). Impact of melanin on microbial virulence and clinical resistance to antimicrobial compounds. *Antimicrob Agents Chemother*, *50*(11), 3519-3528. https://doi.org/10.1128/AAC.00545-06

Oliva, G., Sahr, T., & Buchrieser, C. (2018). The Life Cycle of. *Front Cell Infect Microbiol*, *8*, 3. https://doi.org/10.3389/fcimb.2018.00003

Oliva, G., Sahr, T., Rolando, M., Knoth, M., & Buchrieser, C. (2017). A Unique cis-Encoded Small Noncoding RNA Is Regulating Legionella pneumophila Hfq Expression in a Life Cycle-Dependent Manner. *mBio*, *8*(1). https://doi.org/10.1128/mBio.02182-16

Papenfort, K., & Vogel, J. (2010). Regulatory RNA in bacterial pathogens. *Cell Host Microbe*, *8*(1), 116-127. https://doi.org/10.1016/j.chom.2010.06.008

Pavan, M. E., López, N. I., & Pettinari, M. J. (2020). Melanin biosynthesis in bacteria, regulation and production perspectives. *Appl Microbiol Biotechnol*, *104*(4), 1357-1370. https://doi.org/10.1007/s00253-019-10245-y

Perez, J. C., & Groisman, E. A. (2007). Acid pH activation of the PmrA/PmrB two-component regulatory system of Salmonella enterica. *Mol Microbiol*, *63*(1), 283-293. https://doi.org/10.1111/j.1365-2958.2006.05512.x

Plonka, P. M., & Grabacka, M. (2006). Melanin synthesis in microorganisms--biotechnological and medical aspects. *Acta Biochim Pol*, *53*(3), 429-443.

Pérez-Tomás, R., Montaner, B., Llagostera, E., & Soto-Cerrato, V. (2003). The prodigiosins, proapoptotic drugs with anticancer properties. *Biochem Pharmacol*, *66*(8), 1447-1452. https://doi.org/10.1016/s0006-2952(03)00496-9

Qiu, J., & Luo, Z. Q. (2017). Legionella and Coxiella effectors: strength in diversity and activity. *Nat Rev Microbiol*, *15*(10), 591-605. https://doi.org/10.1038/nrmicro.2017.67

Qiu, J., Sheedlo, M. J., Yu, K., Tan, Y., Nakayasu, E. S., Das, C., Liu, X., & Luo, Z. Q. (2016). Ubiquitination independent of E1 and E2 enzymes by bacterial effectors. *Nature*, *533*(7601), 120-124. https://doi.org/10.1038/nature17657

Ran, H., Hassett, D. J., & Lau, G. W. (2003). Human targets of Pseudomonas aeruginosa pyocyanin. *Proc Natl Acad Sci U S A*, *100*(24), 14315-14320. https://doi.org/10.1073/pnas.2332354100

Rao, C., Benhabib, H., & Ensminger, A. W. (2013). Phylogenetic reconstruction of the Legionella pneumophila Philadelphia-1 laboratory strains through comparative genomics. *PLoS One*, *8*(5), e64129. https://doi.org/10.1371/journal.pone.0064129

Rasis, M., & Segal, G. (2009). The LetA-RsmYZ-CsrA regulatory cascade, together with RpoS and PmrA, post-transcriptionally regulates stationary phase activation of Legionella pneumophila Icm/Dot effectors. *Mol Microbiol*, *72*(4), 995-1010. https://doi.org/10.1111/j.1365-2958.2009.06705.x

Rast, D. M., & Hollenstein, G. (1977). Architecture of the Agaricus bisporus spore wall. *Canadian Journal of Botany*, *55*, 2251-2262. https://doi.org/10.1139/b77-255

Rhodes, J. C., Polacheck, I., & Kwon-Chung, K. J. (1982). Phenoloxidase activity and virulence in isogenic strains of Cryptococcus neoformans. *Infect Immun*, *36*(3), 1175-1184. https://doi.org/10.1128/IAI.36.3.1175-1184.1982

Rodríguez-Rojas, A., Mena, A., Martín, S., Borrell, N., Oliver, A., & Blázquez, J. (2009). Inactivation of the hmgA gene of Pseudomonas aeruginosa leads to pyomelanin hyperproduction, stress resistance and increased persistence in chronic lung infection. *Microbiology*, *155*(Pt 4), 1050-1057. https://doi.org/10.1099/mic.0.024745-0

Rossier, O., Starkenburg, S. R., & Cianciotto, N. P. (2004). Legionella pneumophila type II protein secretion promotes virulence in the A/J mouse model of Legionnaires' disease pneumonia. *Infect Immun*, *72*(1), 310-321. https://doi.org/10.1128/iai.72.1.310-321.2004

Rowbotham, T. J. (1980). Preliminary report on the pathogenicity of Legionella pneumophila for freshwater and soil amoebae. *J Clin Pathol*, *33*(12), 1179-1183. https://doi.org/10.1136/jcp.33.12.1179

Sahr, T., Brüggemann, H., Jules, M., Lomma, M., Albert-Weissenberger, C., Cazalet, C., & Buchrieser, C. (2009). Two small ncRNAs jointly govern virulence and transmission in Legionella pneumophila. *Mol Microbiol*, *72*(3), 741-762. https://doi.org/10.1111/j.1365-2958.2009.06677.x

Sahr, T., Rusniok, C., Dervins-Ravault, D., Sismeiro, O., Coppee, J. Y., & Buchrieser, C. (2012). Deep sequencing defines the transcriptional map of L. pneumophila and identifies growth phase-dependent regulated ncRNAs implicated in virulence. *RNA Biol*, *9*(4), 503-519. https://doi.org/10.4161/rna.20270

Seeger, E. M., Thuma, M., Fernandez-Moreira, E., Jacobs, E., Schmitz, M., & Helbig, J. H. (2010). Lipopolysaccharide of Legionella pneumophila shed in a liquid culture as a nonvesicular fraction arrests phagosome maturation in amoeba and monocytic host cells. *FEMS Microbiol Lett*, *307*(2), 113-119. https://doi.org/10.1111/j.1574-6968.2010.01976.x

Segal, G. (2013). The Legionella pneumophila two-component regulatory systems that participate in the regulation of Icm/Dot effectors. *Curr Top Microbiol Immunol*, *376*, 35-52. https://doi.org/10.1007/82_2013_346

Simmons, L. A., Foti, J. J., Cohen, S. E., & Walker, G. C. (2008). The SOS Regulatory Network. *EcoSal Plus*, *3*(1). https://doi.org/10.1128/ecosalplus.5.4.3

Skinner, C. E. (1938). THE "TYROSINASE REACTION" OF THE ACTINOMYCETES. *J Bacteriol*, *35*(4), 415-424. https://doi.org/10.1128/JB.35.4.415-424.1938

Solomon, J. M., Rupper, A., Cardelli, J. A., & Isberg, R. R. (2000). Intracellular growth of Legionella pneumophila in Dictyostelium discoideum, a system for genetic analysis of host-pathogen interactions. *Infect Immun*, *68*(5), 2939-2947. https://doi.org/10.1128/iai.68.5.2939-2947.2000

Spirig, T., Tiaden, A., Kiefer, P., Buchrieser, C., Vorholt, J. A., & Hilbi, H. (2008). The Legionella autoinducer synthase LqsA produces an alpha-hydroxyketone signaling molecule. *J Biol Chem*, *283*(26), 18113-18123. https://doi.org/10.1074/jbc.M801929200

Steinert, M., Engelhard, H., Flügel, M., Wintermeyer, E., & Hacker, J. (1995). The Lly protein protects Legionella pneumophila from light but does not directly influence its intracellular survival in Hartmannella vermiformis. *Appl Environ Microbiol*, *61*(6), 2428-2430. https://doi.org/10.1128/AEM.61.6.2428-2430.1995

Steinert, M., Flügel, M., Schuppler, M., Helbig, J. H., Supriyono, A., Proksch, P., & Lück, P. C. (2001). The Lly protein is essential for p-hydroxyphenylpyruvate dioxygenase activity in Legionella pneumophila. *FEMS Microbiol Lett*, *203*(1), 41-47. https://doi.org/10.1111/j.1574-6968.2001.tb10818.x

Stewart, C. R., Rossier, O., & Cianciotto, N. P. (2009). Surface translocation by Legionella pneumophila: a form of sliding motility that is dependent upon type II protein secretion. *J Bacteriol*, *191*(5), 1537-1546. https://doi.org/10.1128/JB.01531-08

Sturgill-Koszycki, S., & Swanson, M. S. (2000). Legionella pneumophila replication vacuoles mature into acidic, endocytic organelles. *J Exp Med*, *192*(9), 1261-1272. https://doi.org/10.1084/jem.192.9.1261

Söderberg, M. A., Dao, J., Starkenburg, S. R., & Cianciotto, N. P. (2008). Importance of type II secretion for survival of Legionella pneumophila in tap water and in amoebae at low temperatures. *Appl Environ Microbiol*, *74*(17), 5583-5588. https://doi.org/10.1128/AEM.00067-08

Tan, Y., Arnold, R. J., & Luo, Z. Q. (2011). Legionella pneumophila regulates the small GTPase Rab1 activity by reversible phosphorylcholination. *Proc Natl Acad Sci U S A*, *108*(52), 21212-21217. https://doi.org/10.1073/pnas.1114023109

Tan, Y., & Luo, Z. Q. (2011). Legionella pneumophila SidD is a deAMPylase that modifies Rab1. *Nature*, *475*(7357), 506-509. https://doi.org/10.1038/nature10307

Tiaden, A., Spirig, T., & Hilbi, H. (2010). Bacterial gene regulation by alpha-hydroxyketone signaling. *Trends Microbiol*, *18*(7), 288-297. https://doi.org/10.1016/j.tim.2010.03.004

Tiaden, A., Spirig, T., Sahr, T., Wälti, M. A., Boucke, K., Buchrieser, C., & Hilbi, H. (2010). The autoinducer synthase LqsA and putative sensor kinase LqsS regulate phagocyte interactions, extracellular filaments and a genomic island of Legionella pneumophila. *Environ Microbiol*, *12*(5), 1243-1259. https://doi.org/10.1111/j.1462-2920.2010.02167.x

Tiaden, A., Spirig, T., Weber, S. S., Brüggemann, H., Bosshard, R., Buchrieser, C., & Hilbi, H. (2007). The Legionella pneumophila response regulator LqsR promotes host cell interactions as an element of the virulence regulatory network controlled by RpoS and LetA. *Cell Microbiol*, *9*(12), 2903-2920. https://doi.org/10.1111/j.1462-5822.2007.01005.x

Trigui, H., Dudyk, P., Oh, J., Hong, J. I., & Faucher, S. P. (2015). A regulatory feedback loop between RpoS and SpoT supports the survival of Legionella pneumophila in water. *Appl Environ Microbiol*, *81*(3), 918-928. https://doi.org/10.1128/AEM.03132-14

van Schaik, E. J., Chen, C., Mertens, K., Weber, M. M., & Samuel, J. E. (2013). Molecular pathogenesis of the obligate intracellular bacterium Coxiella burnetii. *Nat Rev Microbiol*, *11*(8), 561-573. https://doi.org/10.1038/nrmicro3049

Vickers, R. M., & Yu, V. L. (1984). Clinical laboratory differentiation of Legionellaceae family members with pigment production and fluorescence on media supplemented with aromatic substrates. *J Clin Microbiol*, *19*(5), 583-587. https://doi.org/10.1128/JCM.19.5.583-587.1984

Vincent, C. D., Friedman, J. R., Jeong, K. C., Buford, E. C., Miller, J. L., & Vogel, J. P. (2006). Identification of the core transmembrane complex of the Legionella Dot/Icm type IV secretion system. *Mol Microbiol*, *62*(5), 1278-1291. https://doi.org/10.1111/j.1365-2958.2006.05446.x

WHO. (2018). *Legionellosis*. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/legionellosis

Wiater, L. A., Sadosky, A. B., & Shuman, H. A. (1994). Mutagenesis of Legionella pneumophila using Tn903 dlllacZ: identification of a growth-phase-regulated pigmentation gene. *Mol Microbiol*, *11*(4), 641-653. https://doi.org/10.1111/j.1365-2958.1994.tb00343.x

Williamson, N. R., Fineran, P. C., Leeper, F. J., & Salmond, G. P. (2006). The biosynthesis and regulation of bacterial prodiginines. *Nat Rev Microbiol*, *4*(12), 887-899. https://doi.org/10.1038/nrmicro1531

Wintermeyer, E., Flügel, M., Ott, M., Steinert, M., Rdest, U., Mann, K. H., & Hacker, J. (1994). Sequence determination and mutational analysis of the lly locus of Legionella pneumophila. *Infect Immun*, *62*(3), 1109-1117. https://doi.org/10.1128/IAI.62.3.1109-1117.1994

Wintermeyer, E., Rdest, U., Ludwig, B., Debes, A., & Hacker, J. (1991). Characterization of legiolysin (lly), responsible for haemolytic activity, colour production and fluorescence of Legionella pneumophila. *Mol Microbiol*, *5*(5), 1135-1143. https://doi.org/10.1111/j.1365-2958.1991.tb01886.x

Xu, L., Shen, X., Bryan, A., Banga, S., Swanson, M. S., & Luo, Z. Q. (2010). Inhibition of host vacuolar H+-ATPase activity by a Legionella pneumophila effector. *PLoS Pathog*, *6*(3), e1000822. https://doi.org/10.1371/journal.ppat.1000822

Yabuuchi, E., & Ohyama, A. (1972). Characterization of "Pyomelanin"-Producing Strains of Pseudomonas aeruginosa. *International Journal of Systematic and Evolutionary Microbiology*, *22*(2), 53-64. https://doi.org/https://doi.org/10.1099/00207713-22-2-53

Zhdanova, N. N., Zakharchenko, V. A., Vember, V. V., & Nakonechnaya, L. T. (2000). Fungi from Chernobyl: mycobiota of the inner regions of the containment structures of the damaged nuclear reactor. *Mycological Research*, *104*(12), 1421-1426. https://doi.org/https://doi.org/10.1017/S0953756200002756

Zheng, H., Chatfield, C. H., Liles, M. R., & Cianciotto, N. P. (2013). Secreted pyomelanin of Legionella pneumophila promotes bacterial iron uptake and growth under iron-limiting conditions. *Infect Immun*, *81*(11), 4182-4191. https://doi.org/10.1128/IAI.00858-13

Zusman, T., Aloni, G., Halperin, E., Kotzer, H., Degtyar, E., Feldman, M., & Segal, G. (2007). The response regulator PmrA is a major regulator of the icm/dot type IV secretion system in Legionella pneumophila and Coxiella burnetii. *Mol Microbiol*, *63*(5), 1508-1523. https://doi.org/10.1111/j.1365-2958.2007.05604.x

Zähringer, U., Knirel, Y. A., Lindner, B., Helbig, J. H., Sonesson, A., Marre, R., & Rietschel, E. T. (1995). The lipopolysaccharide of Legionella pneumophila serogroup 1 (strain Philadelphia 1): chemical structure and biological significance. *Prog Clin Biol Res*, *392*, 113-139.

PUBLICATIONS

1. Weber, M. M.; Chen, C.; Rowin, K.; Mertens, K.; Galvan, G.; Zhi, H.; Dealing, C. M.; **Roman, V. A**.; Banga, S.; Tan, Y.; Luo, Z. Q.; Samuel, J. E. Identification of *Coxiella burnetii* type IV secretion substrates required for intracellular replication and Coxiella-containing vacuole formation. J Bacteriol. 2013 Sep: 195(17): 3914-3924.
2. Weber, M. M.; Faris, R.; van Schaik, E. J.; McLachlan, J. T.; Wright, W. U.; Tellez, A.; **Roman, V. A**.; Rowin, K.; Case, E. D.; Luo, Z. Q.; Samuel, J. E. The Type IV Secretion System Effector Protein CirA Stimulates the GTPase Activity of RhoA and Is Required for Virulence in a Mouse Model of *Coxiella burnetii* Infection. Infect Immun. 2016 Aug: 84(9): 2524-2533.
3. Hamada, N. A.; **Roman, V. A**.; Howell, S. M.; Wilker, J. J. Examining Potential Active Tempering of Adhesive Curing by Marine Mussels. Biomimetics. 2017 Aug: 2(3): 2-11.