EVALUATION OF THE p45 MOBILE INTEGRATIVE ELEMENT AND ITS ROLE IN *Legionella pneumophila* VIRULENCE

A Dissertation

by

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ABSTRACT

Legionella pneumophila are aqueous environmental bacilli that live within protozoal species and cause a potentially fatal form of pneumonia called Legionnaires' disease. Not all *L. pneumophila* strains have the same capacity to cause disease in humans. The majority of strains that cause clinically relevant Legionnaires' disease harbor the p45 mobile integrative genomic element. Contribution of the p45 element to *L. pneumophila* virulence and ability to withstand environmental stress were addressed in this study.

The *L. pneumophila* Philadelphia-1 (Phil-1) mobile integrative element, p45, was transferred into the attenuated strain Lp01 via conjugation, designating p45 an integrative conjugative element (ICE). The resulting trans-conjugate, $Lp01^{+p45}$, was compared with strains Phil-1 and Lp01 to assess p45 in virulence using a guinea pig model infected via aerosol. The p45 element partially recovered the loss of virulence in Lp01 compared to that of Phil-1 evident in morbidity, mortality, and bacterial burden in the lungs at the time of death. This phenotype was accompanied by enhanced expression of type II interferon in the lungs and spleens 48 hours after infection, independent of bacterial burden.

The p45 ICE was also evaluated for its contribution(s) to *L. pneumophila's* capacity to withstand adverse environmental conditions. Results from these *in vitro* experiments revealed that p45 impacts sensitivity to sodium and ability of the bacteria to enter host cells. The ability to enter hosts and live in the presence of sodium contribute to *L. pneumophila's* environmental fitness, and both are also associated with virulence. Sensitivity to sodium is utilized by researchers in the *Legionella* field as an *in vitro* proxy for virulence, where more sensitive bacteria tend to be more virulent. Thus, these results support that p45 ICE has a great impact on *L. pneumophila* virulence.

DEDICATION

To David Charles Schmitt.

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INTRODUCTION

Legionella pneumophila: the aqueously-ubiquitous, environmental, amoeba-dwelling bacteria with a dynamic versatile genome that inadvertently generated a human pathogen.

I.1 *Legionella pneumophila*

The environmentally ubiquitous bacteria, *Legionella pneumophila*, are Gram-negative pleomorphic bacilli that cause a potentially fatal form of pneumonia called Legionnaires' disease [1-3]. They are aerobic non-spore forming gammaproteobacteria with a single polar flagellum that produce catalase, oxidase, and beta-lactamase [4]. *L. pneumophila* are facultative intracellular organisms that often live as constituents of biofilm communities or within protozoal hosts [2, 5, 6].

L. pneumophila pathogenesis initiates when the bacteria locate and adhere to a suitable host cell such as amoeba or human alveolar cell [6-8]. They subsequently enter the host cell by phagocytosis, often via coiling phagocytosis, an uncommon distinct mechanism [5, 9, 10]. Following internalization, the bacteria subvert the host's natural endocytic trafficking pathways that lead to acidification and lysosomal degradation in order to form a replicative niche inside the arrested vacuole [11]. This vacuole, termed the *Legionella* containing vacuole (LCV) is often closely associated to endoplasmic reticulum, ribosomes, and mitochondria [12]. The LCV is where the bacteria survive and replicate until the host's nutrients have been depleted. The bacteria will then escape by lysing the host cell, free to infect adjacent host cells [13]. *L. pneumophila* display

a biphasic lifecycle with transmissive and replicative forms having distinct gene expression profiles [14].

Legionella pneumophila relies on its type IVb secretion system (T4bSS) Dot/Icm (Defect in organelle trafficking/Intracellular replication mutant) for the ability to form the LCV and live within host cells [15, 16]. The Dot/Icm facilitates translocation of more than 300 effector molecules across its cell wall, into the cytoplasm of its host [17]. With such a large arsenal of effector proteins, it has been suggested that many share functional redundancies [17]. This is evident both through genetic sequence analysis and because single effector knockout studies rarely produce phenotypes [17]. Thus, the vast majority of Dot/Icm effectors have unknown functions. Collectively, these effectors modulate and subvert the host's normal defenses against intracellular pathogens such as apoptosis, cytokine production, endosome acidification, and fusion with lysosomes in order to create and maintain a favorable environment for the bacteria.

Natural hosts of *Legionella spp.* encompass a large range of protozoal species including members of *Acanthamoeba*, *Dictyostelium*, *Vermamoeba* (*Hartmanella*), *Naegleria*, and *Tetrahymena* genera [12]. Co-evolution of *L. pneumophila* with its many various single-celled eukaryotic hosts, is likely a contributing factor in the reason they encode such a copious and redundant group of effectors [6]. In addition, *L. pneumophila* encodes the largest number of molecules with eukaryotic-like domains of any known bacterial species [18]. The majority of these eukaryotic-like domains are located on secreted effector proteins, having a large degree of interaction with molecules of their eukaryotic hosts [18, 19]. This co-evolutionary process between *L. pneumophila* and their eukaryotic hosts is also presumably responsible for the bacteria's ability to infect and live within human alveolar cells [6]. Humans are incidental hosts of *L. pneumophila*,

which only became at risk for disease upon the development and widespread utilization of aerosol-producing technologies.

I.2 Transmission

Legionnaires' disease, a form of legionellosis, is transmitted via inhalation of contaminated aerosol droplets. Aerosol droplets created by systems that utilize water reservoirs such as air conditioners, fountains, spas, humidifiers, and even showers. *Legionella pneumophila* was identified as the etiologic agent of Legionnaires' disease following the 1976 outbreak in Philadelphia, PA at an American Legion Conference at the Bellevue-Stratford Hotel [20, 21]. As a result of that outbreak more than 200 people were hospitalized and 48 died. Later it was determined that the source of the epidemic was a cooling tower contaminated with the bacteria subsequently named *Legionella pneumophila* strain Philadelphia-1 [20, 21]. Unlike most pneumonia-causing bacteria, *Legionella pneumophila* does not pass from one human to another. Due to this, legionellosis cases occur predominately as nosocomial infections and point-source epidemics. A large number of outbreaks have been associated with hotels, cruise ships, and nursing homes [22].

Legionella pneumophila are capable of withstanding vast ranges and varieties of environmental conditions. These organisms are found in plumbing and industrial water systems treated with harsh biocides including chlorine and bromine. They can live in temperatures ranging from 15 to 43°C, pH ranging from 3 to 8, and can survive for months in various types of water including tap, purified, deionized, sterilized, and environmentally acquired [23, 24]. They live and form biofilm communities on various materials commonly used for water handling, and benefit from metal corrosion [24]. They encode a large amount of heavy metal and toxin efflux systems

[25]. These survival abilities under adverse conditions are partially due to mechanisms *L. pneumophila* have developed or acquired, and partially due to protection provided by their protozoal hosts.

I.3 Legionellosis

Humans can acquire two versions of respiratory disease following infection with *Legionella pneumophila*, Pontiac fever or Legionnaires' disease, collectively referred to as legionellosis. Pontiac fever is an acute self-limiting illness that presents as flu-like symptoms and clears within 2-5 days. Because of its acute flu-like nature, Pontiac fever is rarely clinically diagnosed. Legionnaires' disease is a form of pneumonia that can become fatal if not properly treated. Legionnaires' disease most commonly affects immunocompromised individuals including cigarette smokers, those with chronic lung diseases, and people greater than 50 years of age [11]. Symptomatic presentation of Legionnaires' disease generally includes high fever, headache, muscle aches, shortness of breath, and an unproductive cough following a 2-14 day incubation period. An unproductive, non-sputum producing, cough is a distinctive symptom shared between the two forms of legionellosis. It is also, probably a contributing factor as to why legionellosis is not transmitted from human to human.

Since the signs and symptoms of Legionnaires' disease are mostly non-distinct and vary among patients a second form of diagnosis is often applied. There are a number of clinically accepted and utilized methods for the diagnosis of Legionnaires' disease, none of them without shortcomings [26]. Currently the most common diagnostic for legionellosis is the urine antigen test, which detects the presence of *L. pneumophila* serogroup 1 *Legionella* antigen (La), in the patient's urine [27]. This is a quick, simple, and cost-effective diagnostic tool, however, a

significant caveat is that the test is specific to serogroup 1 strains of *Legionella*. The majority of Legionnaires' disease cases are caused by serogroup 1 strains, however up to 20% of cases are not caused by serogroup 1 strains and therefore would be misdiagnosed using this method. Once diagnosed, Legionnaires' disease and can be treated using macrolides and fluoroquinolones.

I.4 Clinically Relevant *Legionella pneumophila*

There are over sixty recognized species part of the *Legionella* genus and over seventy serogroups. However, around 90% of clinical legionellosis cases are caused by *Legionella pneumophila* [22, 28]. Of the cases caused by *L. pneumophila*, approximately 80-90 % are caused by serogroup 1 strains [22, 29, 30]. This disparity is not simply due to environmental prevalence of *L. pneumophila* serogroup 1 strains [31]. Rather, these strains possess mechanistic attributes which gain them the ability to transmit, infect, and live within the conditions of alveolar tissue of a human host, ultimately eliciting a disease response. So, there exist both pathogenic disease-causing *Legionella* and innocuous non-pathogenic *Legionella* [32]. What makes this distinction however, has not yet been determined [30, 32]. All *Legionella*, pathogenic and innocuous, infect and inhabit protozoa, the presumed evolutionary stepping stool from water to human lungs. All *Legionella* also encode and utilize the Dot/Icm secretion system [30, 33-35], although effector substrates vary between strains [34].

While *L. pneumophila* serogroup 1 strains have the greatest incidence in causing clinical legionellosis, there are a handful of other *Legionella* species and serogroups that contribute to clinical disease [22, 36]. Other species associated with causing disease in humans include *Legionella anisa*, *Legionella bozemanii*, *Legionella fallonii*, *Legionella longbeachea*, *Legionella micdadei* and *Legionella wadsworthii*. Among these, the most notable is *Legionella longbeachea*

which accounts for nearly 30% of clinical legionellosis cases throughout Australia and New Zealand [22]. Although the species listed above, along with others, have been determined to cause a sizeable number of clinical legionellosis cases, *L. pneumophila* and *L. longbeachea* seem to be more virulent, not only based upon predominance in clinical cases, but also because members of these two species are more frequently associated with fatalities due to Legionnaires' disease [36].

Within the species of *Legionella pneumophila* there are over thirty-five serogroups. The vast majority of clinically relevant legionellosis cases are caused by members of *L. pneumophila* serogroup 1, however not exclusively. Clinical legionellosis cases have been reportedly caused by members of at least fifteen *L. pneumophila* serogroups [36]. *L. pneumophila* Thunder Bay and other members of serogroups 6 have not only been associated with human disease, but have been the causes of major epidemic outbreaks [36, 37]. Therefore, despite that there exists an undeniable skew towards *Legionella pneumophila* serogroup 1 strains' ability to infect and cause disease in humans, pathogenicity of these organisms is not exclusively linked to species nor serogroup [29, 30, 36, 38]. This phenomenon is comprehensible considering that these are accidental pathogens which did not mechanistically evolve with direct influence from human host conditions.

Since it became evident that neither species nor serogroups could adequately decipher between pathogenic and innocuous *Legionella*, attempts have been made to develop a new grouping or typing scheme that would do just that. Predominately, these attempts have been genomic sequence-based and hinge on phylogenetic relatedness [29, 34]. Results from such studies enhance understanding of these organisms, and have identified genetic factors distinguishing pathogenic *Legionella* from innocuous. However, the majority of these factors do not phenotypically correlate with pathogenesis or virulence.

I.5 Genome

The genome of *Legionella pneumophila* strain Philadelphia-1, an isolate from the original 1976 epidemic in Pennsylvania, was sequenced by Chien *et al.* in 2004 [25]. The genome consists of a single circular chromosome made up of 3,397,754 base pairs with approximately 38% G+C content. It revealed amino acid antiporters, large numbers of heavy metal and toxin efflux systems, diverse metabolic and synthetic pathways, many eukaryotic-like protein domains, and evidence of extensive horizontal transfer. This was the first of a multitude of *L. pneumophila* strains and isolates to be sequenced which continues to grow and enhance our understanding of these dynamic bacteria.

Through the ever-growing body of *Legionella* genomic information, something that continually becomes more evident is its diversity and versatility [19, 28, 32, 38]. Congruency among general features of the *Legionella* genome are largely maintained, however each species has distinctive genomic content with around 60% of genes being species specific [30]. Even within single species, *Legionella* genomes display substantial plasticity. Evident in a study comparing two similar serogroup 1 strains of *Legionella pneumophila*, Lens and Paris, where 10 and 14% respectively of encoded genes were strain specific [19]. In a broader study, the genome sequences from 53 isolates of *L. pneumophila* representing 12 serogroups were compared, and revealed *L. pneumophila* strains containing up to 199 strain specific genes [34]. This immense display of genetic variability contributes to *Legionella*'s abilities to inhabit such a diverse collection of environmental conditions.

I.6 Mobile Genomic Elements

The depth of genetic miscellany of *Legionella spp.* centers around their accommodation of mobile genetic elements and propensity for horizontal transfer [28]. *Legionella spp.* play host to numerous types mobile elements, both inter- and intra-genomic [39]. This includes transposons, mobile integrative elements, and integrative conjugative elements. Integrative genetic elements can exist as circular extra-chromosomal (episomal) plasmid-like structures or linearly integrated within the chromosome in a site-specific manner [39]. Mobile elements such as these share commonalities yet are all distinct [40, 41]. They tend to have enriched G+C content as compared to the chromosomal DNA, encode phage-like products, and encode tRNA flanking the insertion site. Some such elements also have the ability to horizontally transfer via conjugation, called integrative conjugative elements [42, 43]. These contribute greatly to the diversity and plasticity of the *Legionella* genome. Integrative conjugative elements often encode secretion systems, most often type IVa secretion systems, capable of facilitating their own translocation [44].

An example of a mobile integrative element, identified in *Legionella pneumophila* Philadelphia-1 (Phil-1), is the p45 element [25]. This element, p45, is 45.5 Kbp with approximately 42 % G+C content, site-specifically integrates within the chromosome which is flanked by $tRNA^{Arg}$, encodes phage-related genes, conjugation machinery, and a type IVa secretion system called *Legionella* Vir homolog (Lvh). Lvh was named for its similarity to the first described type IVa secretion system, VirB/VirD of the bacterial plant pathogen, *Agrobacterium tumefaciens* [45]. Notably, this element is more commonly contained by clinically relevant pathogenic *Legionella spp.* as compared with innocuous strains, spanning both species and serogroups [30, 46]. A number of studies have evaluated components of the p45 element such as the Lvh T4aSS [4, 40, 47] and

the $tRNA^{Arg}$ [48] as they contribute to pathogenesis. Although, concrete evidence regarding *L. pneumophila* virulence associated with this or other mobile integrative elements is lacking in the field.

This study evaluates the p45 mobile integrative element as it contributes to *L. pneumophila* virulence. This was accomplished by the introduction of p45 into an attenuated *L. pneumophila* strain, Lp01 [46, 49]. Conjugation was utilized for the transfer of p45, which successfully generated a p45-containing Lp01 ($Lp01^{+p45}$) strain, in addition to earning p45 the designation of integrative conjugative element. The newly constructed $Lp01^{+p45}$ strain, Lp01, and Philadelphia-1, the pathogenic stain from which p45 was transferred, were compared for their pathogenic qualities and virulence *in vivo*. These studies revealed that the p45 integrative conjugative element contributes to virulence of *L. pneumophila* in the guinea pig model infected via aerosol. In addition, *L. pneumophila* that contain p45 were more susceptible to sodium-induced fatality and enter host cells better than those without, both traits having implications to virulence. Although this study established an important role of p45 in *L. pneumophila* virulence, continued evaluation of the element is required for a more comprehensive understanding of the mechanisms involved.

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CHAPTER II

LEGIONNAIRES' DISEASE MORTALITY IN GUINEA PIGS DEPENDS ON THE PRESENCE OF P45 MOBILE GENOMIC ELEMENT

Legionella pneumophila are the cause of Legionnaires' disease, a potentially fatal form of pneumonia. These gram negative facultative intracellular bacilli live ubiquitously in water reservoirs, often as constituents of biofilm communities and/or intracellularly within protozoal hosts and cause human disease when inhaled in aerosol droplets. Not all strains of *L. pneumophila* display the same level of virulence when it comes to their human incidental hosts. As environmental organisms, *L. pneumophila* have highly plastic genomes which harbor numerous inter- and intra-genomic mobile elements, enhancing their abilities to live in diverse conditions. One such element, p45 mobile integrative element, is contained by *L. pneumophila* strains predominant in causing clinical disease. This element was evaluated for its contribution to *L. pneumophila* virulence by comparing clinically isolated strain Philadelphia-1 which contains p45, strain Lp01 which was derived from Philadelphia-1 and lacks p45, and a Lp01 strain in which the p45 element was reintroduced, $Lp01^{+p45}$. Through the process of p45 introduction into Lp01, the p45 element was found to be capable of horizontal transfer via conjugation, categorizing p45 as an integrative conjugative element. Virulence associated with the p45 integrative conjugative element was assessed by comparison of the three *L. pneumophila* strains in a guinea pig model infected via aerosol. Virulence of the Lp01 strain was drastically attenuated as compared with Philadelphia-1 in both morbidity and mortality of the guinea pigs. $Lp01^{+p45}$-infected animals displayed intermediate morbidity and mortality as compared with the other *L. pneumophila* strains,

suggesting an important role of p45 in the virulence associated with certain strains of *L. pneumophila*.

II.1 Introduction

Legionnaires' disease (LD) is a potentially fatal form of pneumonia particularly in individuals over the age of 55 years and those with compromised respiratory immune function such as smokers or those who suffer from cystic fibrosis [1, 2]. LD was originally identified to be caused by *Legionella pneumophila* Philadelphia-1 (Phil-1) via inhalation of aerosol droplets produced by a contaminated air cooling tower at the Belleview-Stratford Hotel in Philadelphia, PA in 1976 [2, 3]. Members of the Legionella genus are Gram negative facultative intracellular bacilli that ubiquitously inhabit water reservoirs often as constituents of biofilm communities and/or within protozoal hosts. Presumably *L. pneumophila* gained the functions and mechanisms necessary to utilize human alveolar cells as viable hosts through a co-evolutionary process with environmental eukaryotic single celled hosts [4, 5]. *L. pneumophila* is one of over sixty recognized species of *Legionella*, approximately 80% of which have never been associated with clinical LD [6, 7]. Thus, *Legionella spp.* are predominately environmental inhabitants and humans became incidental hosts upon the emergence of aerosol-producing technologies.

Despite there being more than 35 serogroups and 60 recognized species, over 85% of clinical cases have been caused by serogroup 1 (SG1) strains of *L. pneumophila* [7]. Surveys conducted comparing Legionella species isolated clinically or environmentally concluded that this is not simply because disease-causing *L. pneumophila* are more prevalent in the environment [6-8]. Rather, they have acquired through mutation or horizontal genetic transfer the abilities to infect and persist within human hosts, occasionally provoking disease. While it is true that serogroup 1

strains of *L. pneumophila* are associated with incidence of LD [6, 7, 9], the exceptions do exist. Members of *L. pneumophila* SG6 and SG12 have been the cause clinical LD epidemics [7, 59], in addition to non-*L. pneumophila* species such as *L. longbeachae* which is responsible for around 30 % of clinical LD in Australia and New Zealand [7]. This study focuses on virulence linked to genomic variations which distinguish pathogenic from innocuous *Legionella spp.*

As environmental organisms *Legionella* have the abilities to withstand environmental stressors including temperature, osmotic pressure, pH, nutrient deprivation, and presence of a number of antibiotics. These capabilities are enhanced in *Legionella spp.* due to both intricate regulatory systems [10, 11], and genomic plasticity rich with inter- and intra-genomic mobile elements [12-16]. Two classes of mobile genomic elements (MGE) prevalent within *Legionella spp.* are mobile integrative elements (MIE) and integrative conjugative elements (ICE). Both MIEs and ICEs can exist either as circular extra-chromosomal (episomal) entities or as segments linearly integrated within the chromosome in a site-specific manner [16]. Such elements often have higher G+C content than the chromosomal DNA, encode phage related genes, and are flanked by tRNA regions [14, 15]. As the name suggests, ICEs can be passed horizontally via conjugation most often facilitated by a self-encoded secretion apparatus [13-16].

Strains within *L. pneumophila* contain many versions of MIEs and ICEs, although inconsistently among them [12-14]. One such element, p45 is contained within *L. pneumophila* strain Phil-1 (illustrated in Figure 1A), shares commonalities with standard MIEs such as higher G+C content compared to surrounding DNA, flanking tRNA [17], and an encoded integrase [18-20], while also including conjugation machinery, phage-related genes, and a type IVa secretion system which suggests p45 may actually be an ICE [15, 19, 20]. The type IVa secretion system

called Lvh, for Legionella Vir homolog, was named for its relation to the originally identified type IV secretion system (Vir) of the bacterial plant pathogen, *Agrobacterium tumefaciens* [21].

Lvh was identified as a potential mediator of pathogenesis for *L. pneumophila* prior to the knowledge that it was encoded within p45 MIE [22]. Subsequent to its discovery, Lvh received a lot of attention due to similarities between it and *Legionella's* Dot/Icm type IVb secretion system (T4bSS), essential for intracellular replication and host transmission [19, 23]. After years of conflicting results, it now appears that Lvh plays a role *L. pneumophila* pathogenesis under certain conditions, however does not completely compensate for loss of Dot/Icm functionality [22, 24]. Regardless of whether or not the Lvh T4aSS contributes to *Legionella*'s pathogenesis or virulence in mammalian hosts, the p45 MIE on which it is encoded is not included in the genome of all *Legionella* species. Furthermore, p45 has been found present with greater prevalence in disease-causing strains [25-27]. Together this information prompted the question: is the presence of p45 not only genetically associated with *Legionella* virulence, but also phenotypically?

To examine this question, we compared four similar *L. pneumophila* SG1 strains Phil-1, AA100, JR32, and Lp01 for their abilities to cause disease in a guinea pig aerosol infection model. Lp01, the only strain tested to lack the p45 mobile genomic element, was attenuated compared to the other *L. pneumophila* strains. Contribution of p45 to *L. pneumophila* virulence was further evaluated by the reintroduction of p45 into Lp01 from Phil-1. The resulting strain, $Lp01^{+p45}$, was then compared with Lp01 and Phil-1 for its ability to cause disease in guinea pigs. The p45 element restored the ability of Lp01 to cause morbidity and mortality in infected guinea pigs mediated by a type II interferon pro-inflammatory immune response.

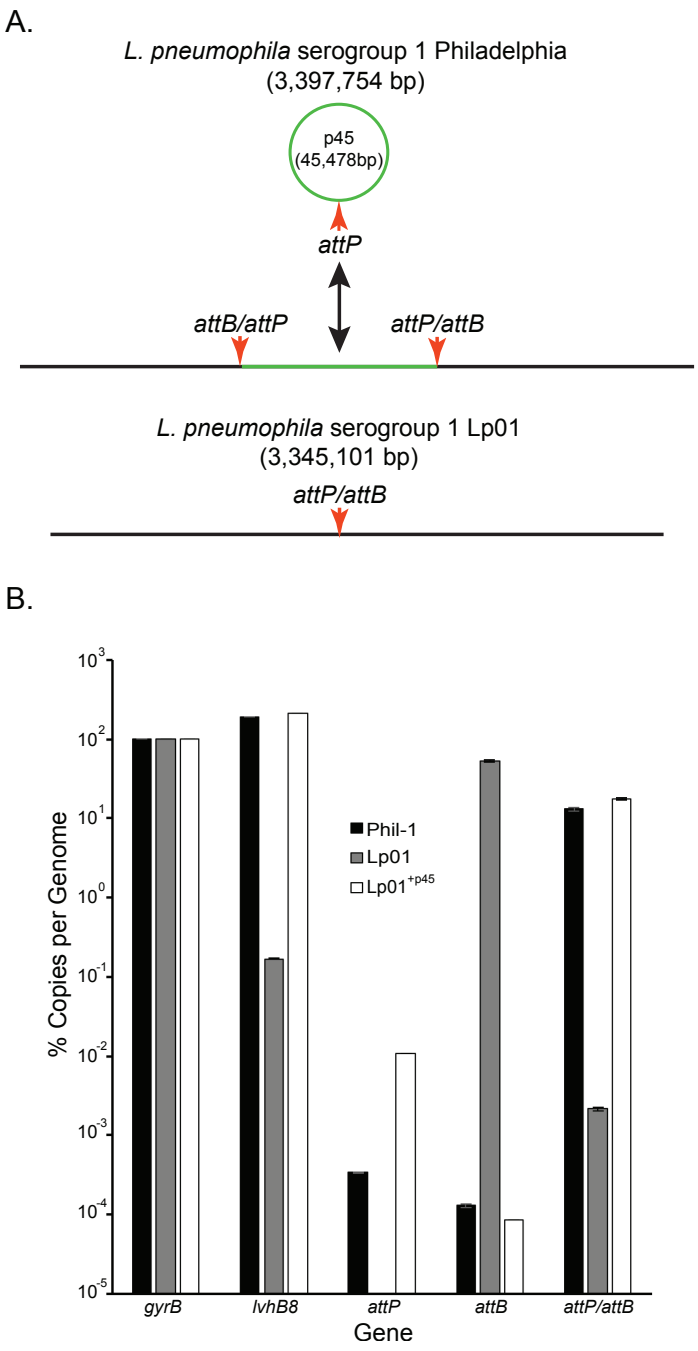

Figure 1. ***Legionella pneumophila*** **Phil-1 mobile integrative element, p45.** (A) Illustration of p45 MIE (green line) both episomal and integrated linearly into the *L. pneumophila* chromosome (black line), where red arrow markers depict site-specific integration sites *attP* and *attB* located on p45 and the chromosome respectively. (B) Quantitative PCR of genomic DNA from *L. pneumophila* Phil-1, Lp01, or Lp01^{+p45} used to measure total p45 (*lvhB8*), episomal p45 (*attP*), closed chromosomal integration site (*attB*), and chromosomally integrated p45 (*attP/attB*), relative to that of the chromosome (*gyrB)* which was set to equal 100% expression. Data represent results of three independent experiments executed in triplicate and are arbitrarily set so that the total p45 (*lvhB8*) group equals 100%. Data points and error bars represent group means and standard deviations respectively.

II.2 Results

II.2.1 p45 is a mobile integrative element

In order to confirm p45 as a mobile integrative element (illustrated in Figure 1A) genomic DNA from *L. pneumophila* strain Philadelphia-1 was compared with that of Lp01, a strain known to lack p45 MIE [28]. Results from quantitative PCR analysis shown in Fig 1B, verify the presence of the p45 element in *L. pneumophila* strain Phil-1 by targeting *lvhB8*. In addition, both episomal and chromosomally integrated forms of p45 were detected, by a closed p45 *attP* site and the *attP/attB* integration junction, respectively (Figure 1B). The episomal form of p45 attributed around 20-30% of total p45 while the remainder was site-specifically integrated within the chromosome (Figure 1B). These results confirm the presence of p45 along with its mobile and integrative properties within *L. pneumophila* Phil-1.

II.2.2 Virulence attenuation in *Legionella pneumophila* correlates with p45 loss

L. pneumophila serogroup 1 strains Phil-1, Lp01, AA10, and JR32 were used to infect guinea pigs via aerosol transmission in order to compare the strains' virulence *in vivo*. *L. pneumophila* Phil-1 and AA100 were clinically isolated, whereas JR32 and Lp01 were separately derived from Phil-1 as restriction minus and streptomycin resistant strains [28]. Genomic sequencing and analysis of Lp01 was discovered to have been cured of p45 MIE [28]. Few other genetic differences exist between Phil-1 and Lp01 [28], providing a nearly clean test between the two strains of the influence of p45 MIE on virulence. In order to compare the virulence of Lp01 to that of Phil-1, AA100, and JR32 we used the most physiologically relevant transmission and infection model available, that being, a guinea pig infection model by the aerosol route. Aerosol transmission was used considering that inhalation of contaminated aerosol droplets is the only natural route of *L. pneumophila* transmission to humans. Guinea pigs were chosen as the host

model based on the immunologic similarities shared with humans and lacking in mice. Furthermore, there is only one susceptible mouse model used for *L. pneumophila*, A strain, commonly called A/J mice [29]. However, A/J mice harbor hypo-functional NAIP5 in macrophages, which is an important component of the NLRC4 inflammasome particularly for protection against bacteria that express flagellin and/or type three secretion system components, such as *L. pneumophila* [29-31].

Guinea pigs infected with Phil-1, AA100, and JR32 displayed disease signs in as little as one day, and all succumbed to mortality within three days subsequent infection (Figure 2A). This was not true of the animals infected with Lp01, which displayed disease symptoms such as decreased energy and increased respiratory rate, however ultimately recovered and returned to baseline health after seven days post infection (data not included). In addition, bacterial burden at the time of death (ToD) in the lungs of animals infected with Lp01 was significantly lower, having more than three logs less CFU than those infected with strains containing p45, Phil-1, AA100, and JR32 (Figure 2B). These data clearly depict a virulence attenuation of Lp01 in the guinea pig model relative to similar strains of the same species and serogroup.

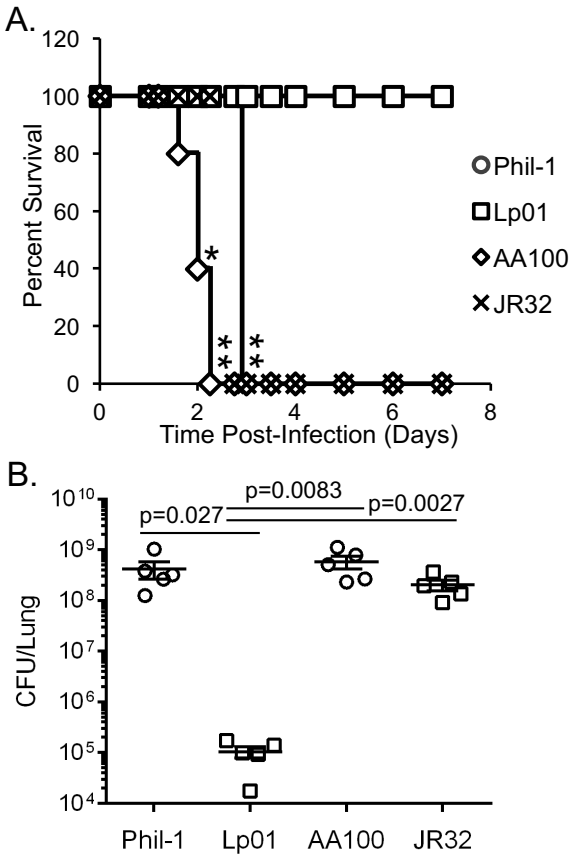

Figure 2. Variation of virulence within *Legionella pneumophila* serogroup 1 strains. Percent survival (A) and bacterial burden in lungs at the time of death (B) of guinea pigs infected with *L. pneumophila* Phil-1, Lp01, AA100, or JR32 via Madison aerosol chamber. Data from survival experiment are from a single independent experiment representative of two, with five animals per infection group. Error bars represent group standard deviations. *p<0.05 **p<0.01

A separate guinea pig infection experiment evaluating lung pathology was performed with *L. pneumophila* Lp01 and the parent strain from which it originated, Phil-1. Following aerosol infection animals were euthanized after 24 or 48 hours for evaluation of lung pathology, described in A-2 Table. Lp01-infected animals exhibited less pathological damage collectively compared to those infected with Phil-1 at 24 and 48 hours post infection, by analysis of H&E stained lung histopathology (A-2 Table). Guinea pigs infected with Lp01 displayed mild to moderate bronchiolitis with moderate alveolar pneumocyte accumulations after 24 hours of infection.

Pathology progressed to acute/subacute stage bronchiolar and alveolar pneumonia 48 hours after infection. Lung tissue of guinea pigs infected with Phil-1 were similar yet more severe, having acquired acute to subacute stage multifocal alveolitis & bronchiolitis with moderate pneumocyte accumulations at a frequency too numerous to count by 24 hours post infection. After 48 hours Phil-1 infected animals' lung pathology progressed to subacute and chronic stages with progressive, organizing alveolar fibrosis and massive neutrophil infiltration. These histopathology results also included in more detail in A-2 Table in Appendix A. Collectively, *L. pneumophila* strain Lp01 displayed a profound virulence attenuation *in vivo* compared to similar SG1 strains including its parent strain, Phil-1.

II.2.3 The p45 mobile integrative element is conjugative

In order to determine if the virulence attenuation displayed by Lp01 compared with similar *L. pneumophila* Serogroup 1 strains was caused by its lack of p45 MIE, we sought to reintroduce the element into Lp01 from Phil-1. Standard methods of pDNA isolation and transformation were unsuccessful, presumably due to the element's size. Therefore, conjugation was used despite the fact that p45 itself had not previously been documented to undergo conjugation. p45 MIE had, however, been shown to encode conjugation machinery [32] and may have been acquired originally through horizontal transfer [19].

A kanamycin resistance cassette (*aph*) was introduced within the p45 MIE of *L. pneumophila* Phil-1 in order to produce a donor strain for conjugation, Phil-262. Kanamycin resistance was used to select for bacteria containing p45 after conjugation, while streptomycin resistance selected for the recipient strain, Lp01, and against the Phil-262 donor strain background. Because streptomycin resistance arises naturally with substantial frequency in *L. pnuemophila* Phil-1 strains, conjugation and subsequent kanamycin and streptomycin selection was performed

with individual donor (Phil-262) and recipient (Lp01) cultures as controls. While the donor-only culture of Phil-262 did indeed accrue streptomycin resistance following the conjugation assay at an efficiency of approximately 1 x 10^{-8}, this was significantly less than the Phil-1-Lp01 (donor-recipient) efficiency of kanamycin and streptomycin resistance acquisition of approximately 6 x 10^{-7} (Figure 3 and B-2 Figure), suggesting that p45 conjugation into Lp01 was successful. To confirm that these were true p45 trans-conjugates, colonies from two of the conjugation experiments were screened for the presence of p45 and the Lp01 background via PCR (representative PCR gel images of one such trans-conjugate shown in Figure 4A). While all of the isolates chosen contained the p45 element, only 50 % were of the Lp01 genomic background. One isolate found to be of the Lp01 genomic background and to contain p45, Lp01^{+p45}, was chosen to use for subsequent experiments throughout this study.

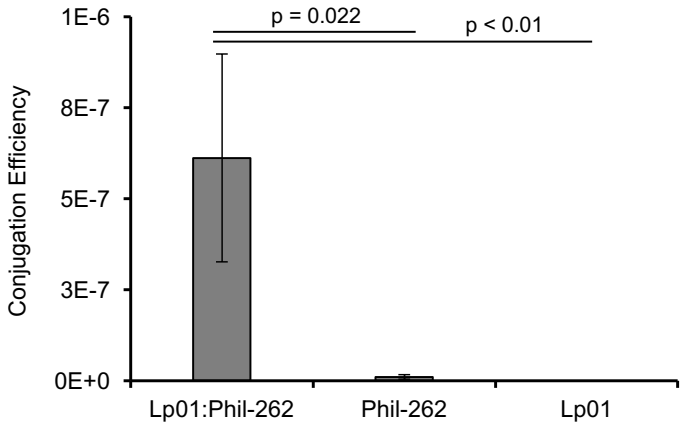

Figure 3. Conjugation of p45 mobile integrative element. Efficiency of p45 conjugation was determined by comparing CFU grown on BCYE + kanamycin (25 μg/ml) + streptomycin (200 μg/ml) with those grown on BCYE alone following conjugation. These data are from three independent experiments conducted in triplicate. The data points and error bars represent group averages and standard deviations respectively.

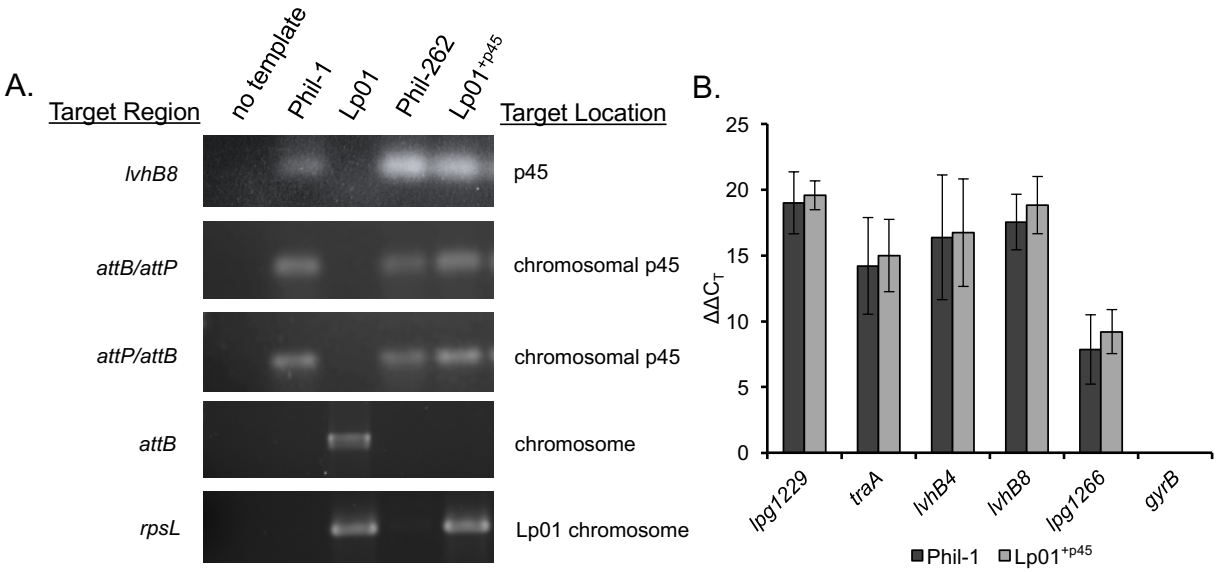

Figure 4. Confirmation of p45 conjugation from Phil-262 to Lp01. (A) Representative images of PCR products visualized by gel electrophoresis and EtBr staining. PCR target gene regions and genomic locations listed on the left and right respectively of the gel images. (B) mRNA expression of various p45-encoded genes by *L. pneumophila* Phil-1 or Lp01^{+p45} relative to Lp01 and *gyrB*. Data points and error bars represent group averages and standard deviations, respectively.

Presence and chromosomal integration of p45 in Lp01^{+p45} were quantified by qPCR, shown in Figure 1B, and had no significant differences as compared to the p45 of *L. pneumophila* Phil-1. Lp01^{+p45} was further confirmed to include a transcriptionally functional p45 by RT-qPCR of target genes dispersed throughout the element (Figure 4B). In addition, it was confirmed that Lp01^{+p45} was of the Lp01 genomic background by sequencing regions of genomic DNA previously described to have small nucleotide polymorphisms with respect to Phil-1 [28]. There were seven regions which differed between Lp01 and Phil-1 genome sequences reported by Rao *et al.* other than its lack of the p45 MIE. Here we compared six of these reported variations, listed in Table 1, excluding the difference within an intergenic region. In addition to confirmation of the Lp01 genomic background of trans-conjugate Lp01^{+p45}, it was also determined that the Lp01 strain used in this study included only three of these six polymorphisms previously reported (Table 1) [28].

Table 1. Confirmation of Lp01 genomic background by sequencing regions with nucleotide polymorphisms relative to Phil-1.

Annotated ID[a]	Gene	Nucleotide Present				
		Phil-1[a]	Phil-1[b]	Lp01[c]	Lp01[b]	Lp01^{+p45}
lpg0324	*rpsL*	A	A	G	G	G
lpg0671	*ndh*	GGCCGAAAT	GGCCGAAAT	9 bp Del.	9 bp Del.	9 bp Del.
lpg0716	h. p.	C	C	T	C	C
lpg0718	proton symporter	C	C	T	C	C
lpg2506	*luxN*	C	C	A	A	A
lpg2669	*ftsE*	T	T	C	T	T

[a] *Legionella pneumophila* strain Phil-1 sequenced in a previous report, GeneBank accession number: AE017354.1 [32].
[b] *Legionella pneumophila* strains used in this study.
[c] *Legionella pneumophila* strain Lp01 sequenced in a previous report [28].

Transfer of p45 mobile integrative element via conjugation successfully resulted in Lp01^{+p45} trans-conjugates, albeit at a relatively low frequency. Importantly the success of this mechanism for p45 horizontal transfer classifies the element as an integrative conjugative element (ICE). Furthermore, a valuable clone for this study was produced and shown to include fewer polymorphisms within the remaining genetic material than previously reported [28].

II.2.4 Introduction of p45 into *Legionella pneumophila* Lp01 partially restores virulence

The virulence of *L. pneumophila* strains Phil-1, Lp01, and Lp01^{+p45} were compared *in vivo* by assessing morbidity and mortality of guinea pigs infected via aerosol with each. Animals infected with Phil-1 all died within 4 days post infection (Figure 5A) and exhibited severe disease within 2 days (Figure 5B). Lp01-infected animals displayed less severe disease throughout the course of infection, ultimately clearing disease symptoms by day 7 post infection (Figures 5A-B). Guinea pigs infected with Lp01^{+p45} displayed an intermediate survival trend compared to those of Phil-1 and Lp01-infected animals, three out of five animals died between 5 and 6 days post

infection (Figure 5A). The Lp01^{+p45} infection group as a whole displayed more severe disease, than those infected with Lp01, specifically at 2 days, 3 through 5 days, and again at 7 and 7.5 days post infection (Figure 5B). Severity of disease as depicted in Figure 5B as total 'Disease Scores' were compiled using three categories of morbidity assessment which included respiratory rate increase, body weight decrease, and general behaviors (A-1 Table). Disease scores for individual animals over the course of infection are included in B-4 Figure.

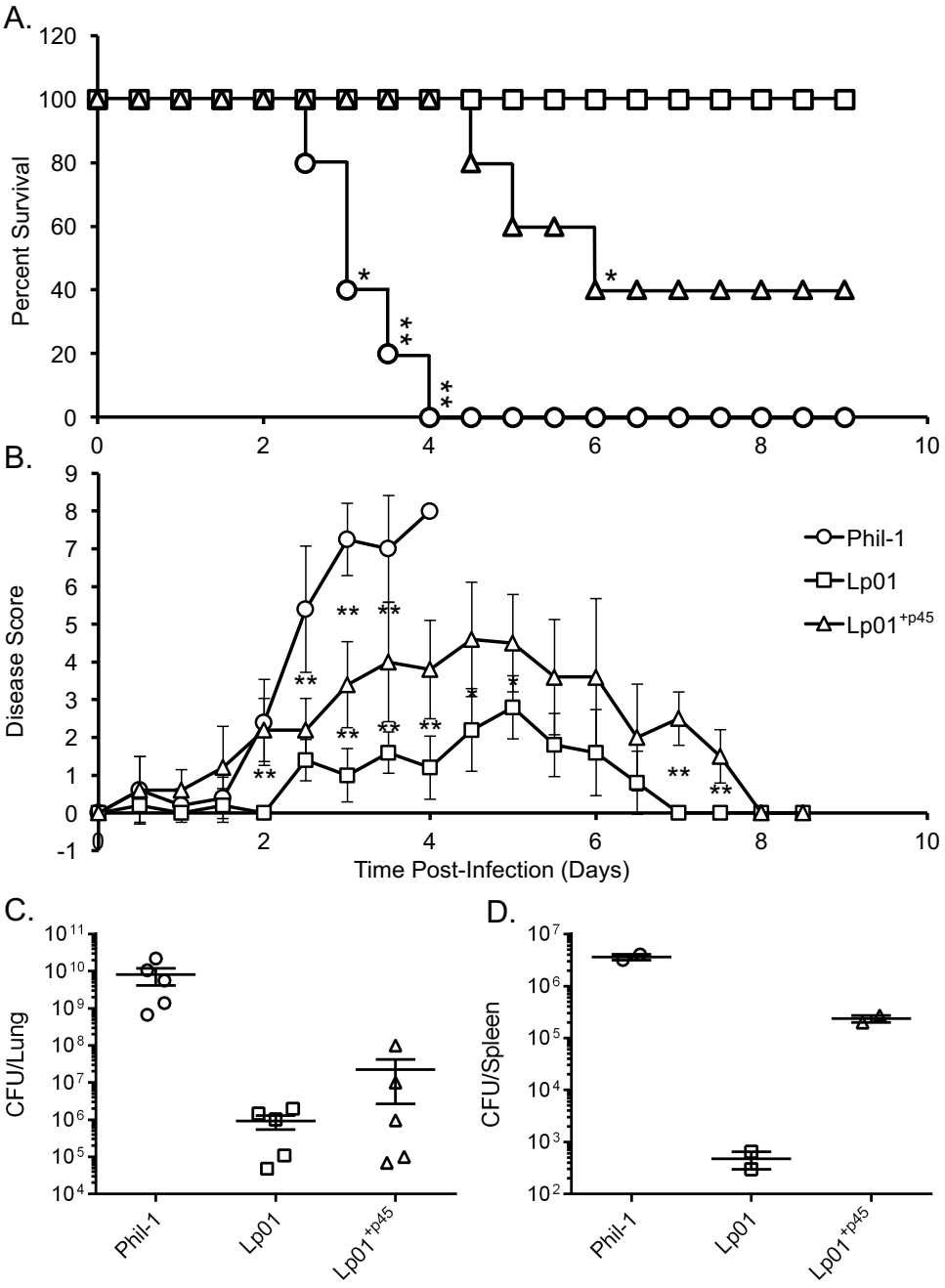

Figure 5. Contribution of p45 to *Legionella pneumophila* virulence *in vivo*. Percent survival (A), disease scores (B), and bacterial burden in lungs (C) and spleens (D) at time of death of guinea pigs infected via aerosol with *L. pneumophila* Phil-1, Lp01, or Lp01^{+p45}. Disease scores represent combined behavior, respiratory rate, and weight loss scores averaged for each group (B). Data are from a single independent experiment representative of two, and executed with five animals per infection group. Error bars represent standard deviation. *$p<0.05$ **$p<0.01$

Bacterial burden in the lungs at the time of death for each animal displayed a consistent trend (Figure 5C), Phil-1-infected animals having more than three logs more CFU per lung than those infected with Lp01. Bacterial burden in the lungs of guinea pigs infected with Lp01^{+p45} had an intermediate phenotype between the Phil-1 and Lp01, consistent with the intermediate morbidity and mortality phenotypes. Bacterial burden in the lungs of Lp01^{+p45}-infected animals had approximately one log more and two logs less than those infected Lp01 and Phil-1 respectively (Figure 5C). This trend was exaggerated among the three infection groups with respect to bacterial burden in the guinea pigs' spleens, where animals infected with Lp01^{+p45} were burdened with greater that two logs more CFU at time of death than those infected by Lp01 (Figure 5D). Together these data support involvement of p45 ICE in *L. pneumophila* virulence in the guinea pig model.

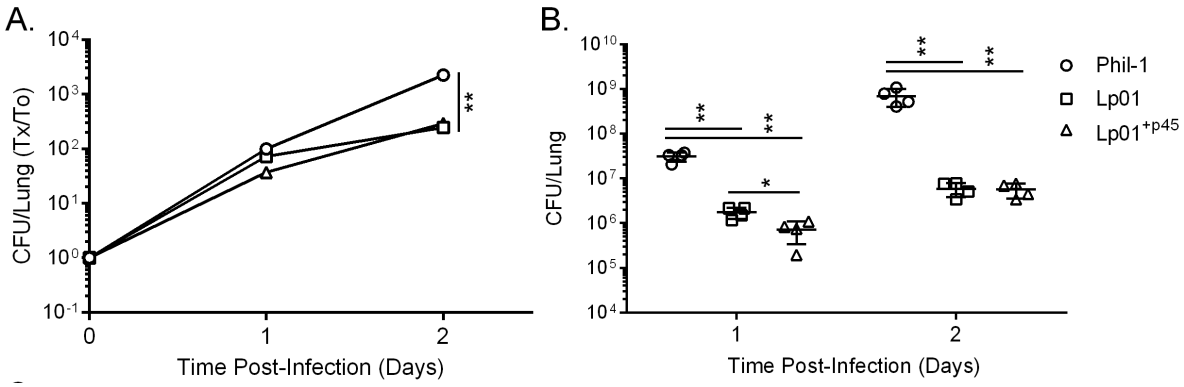

Figure 6. Bacterial burden in guinea pig lungs following *Legionella pneumophila* infection. (A) Bacterial growth in lungs of guinea pigs infected with *L. pneumophila* Phil-1, Lp01, or Lp01^{+p45}. Data points represent CFU/Lung 24 and 48 hours following infection normalized relative to CFU/Lung of the seeding animals. (B) Bacterial burden in individual animals measured by CFU/Lung, 24 and 48 hours following aerosol infection. Error bars represent standard deviation, and data represent a single independent experiment executed with four animals per group. *p<0.05 **p<0.01

II.2.5 *L. pneumophila* that contain p45 induce greater IFNγ expression in guinea pigs

To elucidate the pathological mechanisms underlying the decreased morbidity and mortality of guinea pigs infected with Lp01 relative to those infected with *L. pneumophila* containing p45, animals infected by aerosol were sacrificed 24 or 48 hours after infection. At these time points, lung and spleen tissues were utilized for bacterial burden (Figures 6A-B), cytokine expression (Figures 7A-C), and histopathological analysis (Figures 8A-H). Like previously, *L. pneumophila* Phil-1 had grown significantly more than Lp01 in the lungs after 24 and 48 hours post infection (Figures 6A-B). This increase was not complemented by the presence of p45 in animals infected with Lp01^{+p45}. Similarly, bacterial burden in the guinea pigs' spleens were also significantly higher in the animals infected with Phil-1 as compared to Lp01 and Lp01^{+p45} (B-5B Figure). There was, however, a significant seeding difference between the Phil-1 group and the other two (B-5A Figure), which could be a contributing factor in the differences in bacterial growth burden in the lungs and spleens 48 hours after infection. Despite this seeding disparity, sound comparisons can still be drawn concerning guinea pigs' pathological response to *L. pneumophila* infection and between Lp01 and Lp01^{+p45} infected groups. Disease scores followed a similar trend to previous infections, where Phil-1-infected animals displayed the most severe morbidity and Lp01^{+p45}-infected animals were intermediate between Phil-1 and Lp01 (B-5C Figure).

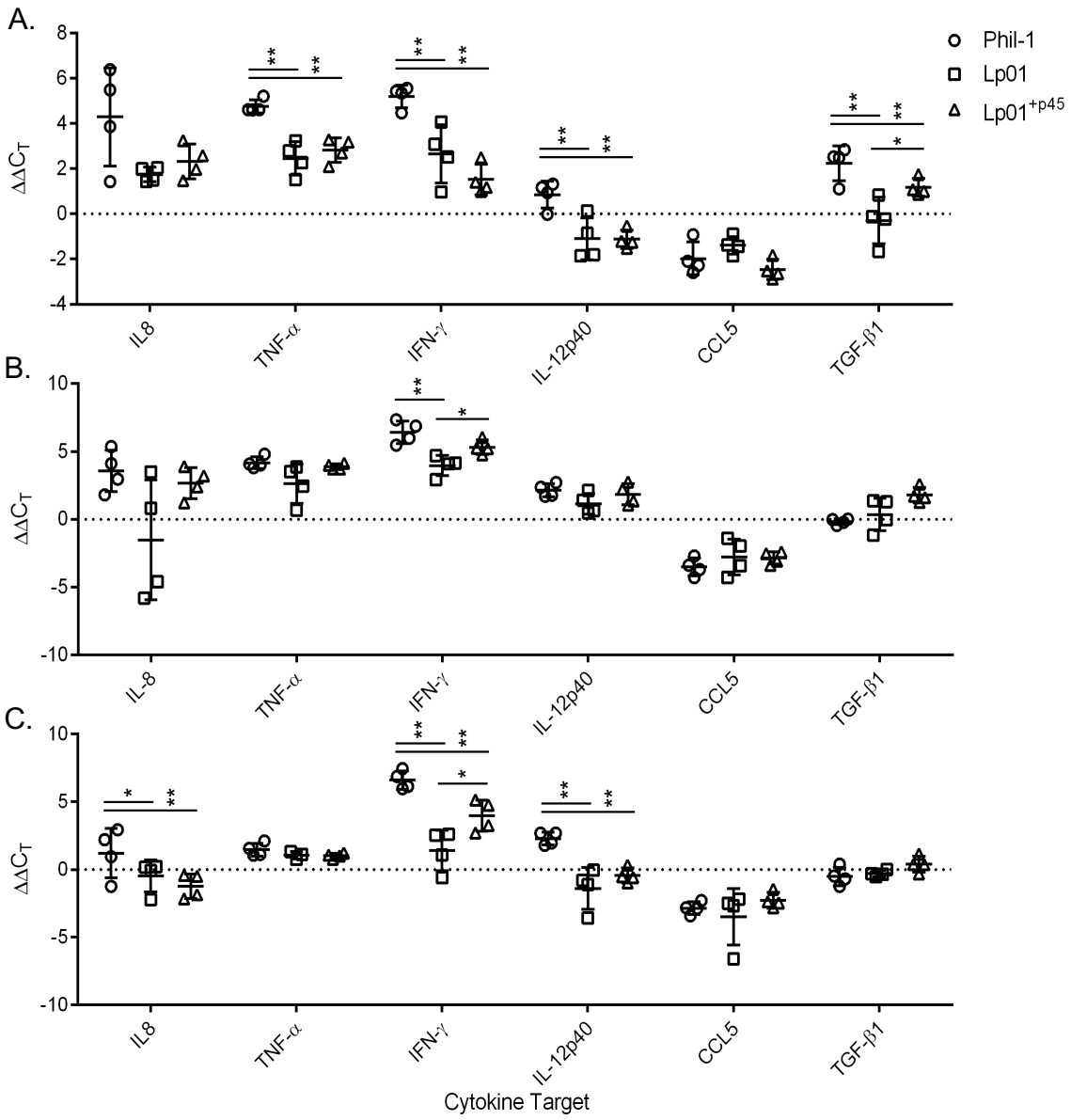

Figure 7. Guinea pig cytokine response to *Legionella pneumophila* infection. Cytokine expression measured by RT-qPCR in the lungs (A-B) and spleens (C) of guinea pigs 24 (A) and 48 hours (B-C) after infection, expression was calculated relative to HPRT and uninfected tissue. Error bars represent standard deviation, and data represent a single independent experiment executed with four animals per group. *p<0.05 **p<0.01

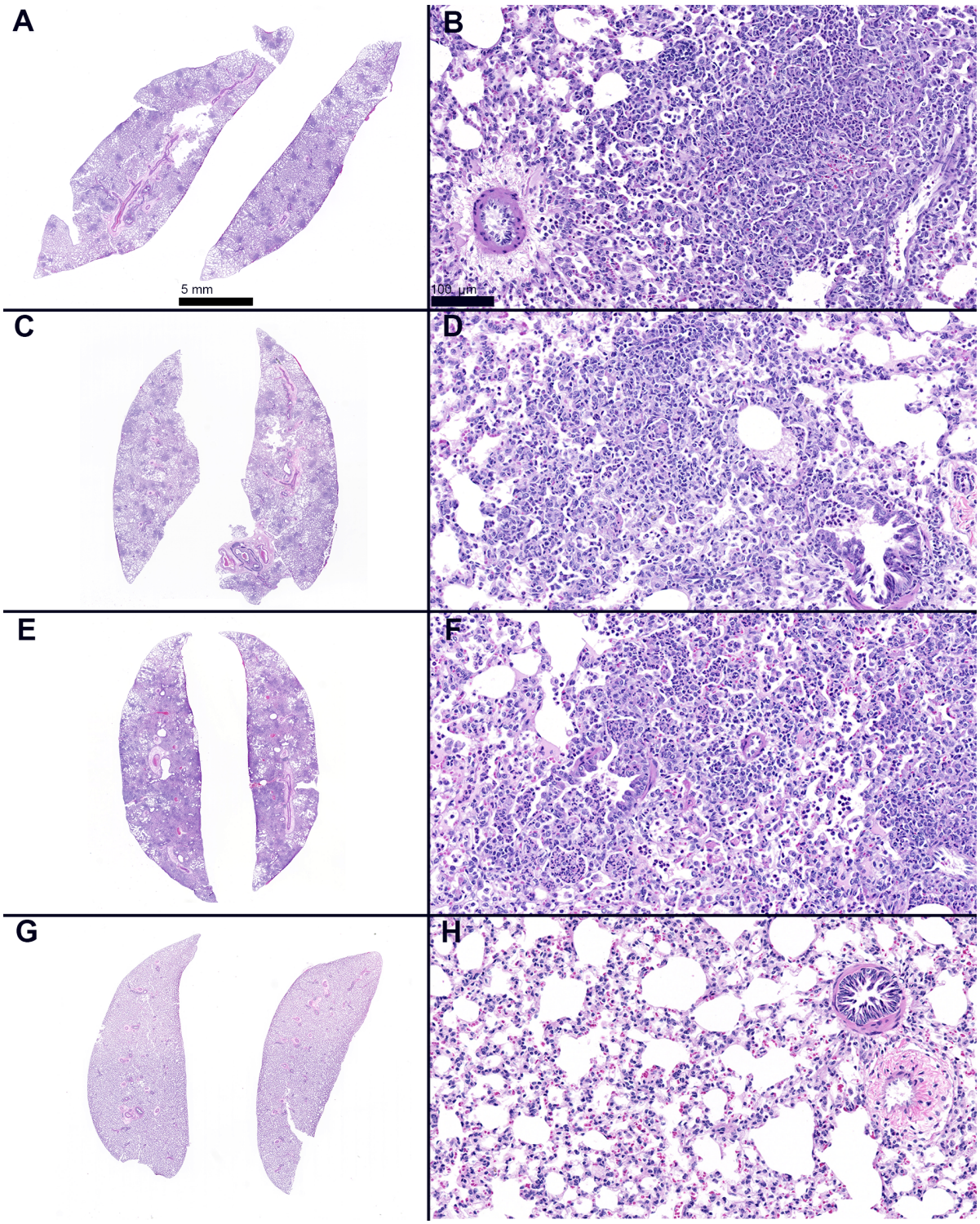

Figure 8. Lung histopathology of guinea pigs infected with *Legionella pneumophila*. Representative images of H&E stained lung tissue from guinea pigs 48 hours after infection with *L. pneumophila* Lp01^{+p45} (A-B), Lp01 (C-D), or Phil-1 (E-F) and lung tissue from an uninfected animal (G-H). Images in the left (A, C, E, G) column were taken at 5 mm magnification while those in the right (B, D, F, H) were high magnification at 100 µm. Images are representative of the condition of lungs in four animals per infection group.

Expression of IL-8, IL-12p40, TGF-β1, IFN-γ, CCL5, and TNF-α mRNA were measured in the lungs 24 and 48 hours after infection. Guinea pigs infected with Phil-1 displayed significantly higher expression of TNF-α, IFN-γ, IL-12p40, and TGF-β1 mRNA after 24 hours as compared with those infected with Lp01 and Lp01^{+p45} (Figure 7A). TGF-β1 expression was significantly higher in animals infected with Lp01^{+p45} at this time as compared with those infected with Lp01 (Figure 7A). The profile of TGF-β1 mRNA expression in the lungs shifted from 24 (Figure 7A) to 48 (Figure 7B) hours post infection, where at 48 hours post infection there were no longer significant differences among the three groups (Figure 7B). Similarly, the differences between TNF-α and IL-12p40 mRNA expression by animals infected with Phil-1 and those infected with Lp01 and Lp01^{+p45} after 24 hours diminished by 48 hours (Figure 7B). IFN-γ mRNA expression in the lungs of Phil-1 infected animals remained significantly higher than those infected with Lp01 48 hours post infection, yet were not significantly higher than those infected with Lp01^{+p45} (Figure 7B). In addition, expression of IFN-γ mRNA in the lungs of guinea pigs infected with Lp01^{+p45} was significantly greater than those infected with Lp01 after 48 hours of infection (Figure 7B). Spleens of guinea pigs infected with Phil-1 48 hours following infection displayed greater expression levels of IL-8, IL-12p40, and IFN-γ mRNA as compared to those infected by Lp01 and Lp01^{+p45} (Figure 7C). Similar to the lungs at the same time point, the spleens of animals infected with Lp01^{+p45} expressed higher levels of IFN-γ mRNA relative to Lp01-infected animals (Figure 7C).

To gain better insight into guinea pig's pathological response to *L. pneumophila* infection a histopathological analysis of H&E stained lungs were compared, along with that of an uninfected animal (Figures 8A-H and A-3 Table). Macroscopically the lungs from Phil-1-infected animals were markedly firm, wet, mottled tan to red and consolidated with numerous pinpoint, firm foci.

Animals infected by Lp01 and Lp01^{+p45} had minimally to mildly firm and consolidated lungs with fewer, variably apparent, pinpoint, firm foci. Microscopically, animals infected with Phil-1 (Figures 8E-F) had moderate to severe coalescing bronchopneumonia characterized by numerous coalescing foci of infiltrating heterophils, macrophages, and lymphocytes filling alveoli. Admixed with the inflammatory infiltrates was proteinaceous edema fluid, fibrin, karyorrhectic debris, and hemorrhage. Within these foci, often alveolar septa were atelectic and expanded by similar infiltrating inflammatory cells. Heterophils were occasionally within bronchioles and bronchi, however, the bronchiolar epithelium was largely spared. Rare bronchioles were variably lined by necrotic epithelial cells or contained sloughed epithelial cells admixed with the inflammatory infiltrate. Diffusely, the lungs of Phil-1 infected animals had evidence of moderate to severe diffuse alveolar damage as well. The alveolar damage was characterized by proteinaceous edema fluid and variable heterophils and foamy macrophages admixed with fibrin strands within the alveolar spaces, and congestion of the alveolar septa. Lymphatics and perivascular/peribronchial interstitium were expanded by edema fluid (Figures 8E-F).

Guinea pigs infected with Lp01^{+p45} (Figures 8A-B) or Lp01 (Figures 8C-D) had mild to moderate multifocal bronchopneumonia characterized by alveoli filled with numerous, distinct foci of infiltrating heterophils, macrophages and lymphocytes admixed with fibrin exudation, proteinaceous edema fluid, karyorrhectic debris and rare erythrocytes microscopically observed in the lungs. These animals had fewer foci and often lacked the coalescing nature of the inflammation observed in animals infected with Phil-1 (Figure 8E). Similarly, bronchioles were occasionally affected with inflammatory infiltrates within the lumen and rare bronchiolar epithelial cells undergoing necrosis. Diffuse alveolar damage was limited to mild and occasionally moderate within Lp01^{+p45} (Figures 8A-B) and Lp01-infected (Figures 8C-D) animals and less prominent

proteinaceous exudation and foamy macrophages within the alveoli and variable alveolar capillary congestion. Lymphatics and perivascular/peribronchial interstitium were expanded by edema fluid (Figures 8A-D). Taken together, Phil-1 infection caused robust pro-inflammatory bronchopneumonia in the lungs of infected guinea pigs, while infection with Lp01 or Lp01^{+p45} provoked mild to moderate lung pathology. Descriptions of lung pathology for each individual guinea pig are contained in A-3 Table. The severity of pathological damage in lungs of animals infected by Lp01 were not discernably different from those infected with Lp01^{+p45}.

L. pneumophila that contain the p45 element instigated a more robust IFN-γ transcriptional response in both the lungs and spleens of infected guinea pigs, not accompanied by increased bacterial burden. The increased expression of type II interferon in the lungs of Lp01^{+p45}-infected animals compared with those infected with Lp01 was not distinguished on a histopathological level.

II.3 Discussion

The vastness of genetic variation and plasticity within the species of *L. pneumophila* SG1 have been extensively documented, noting the prevalence of mobile elements [18, 26, 35, 36]. Such variations with respect to mobile elements have on occasion been experimentally correlated with phenotypic variations such as host cell entry, biofilm formation, and nitrogen fixation, however mostly in non-*Legionella* species [16, 19, 25, 37]. Yet, even fewer have actually experimentally evaluated the implications of these genetic variations on virulence *in vivo*. This study addresses this gap with the use of a physiologically relevant route of infection (aerosol route) and the guinea pig model.

Method and model organism are of utmost importance in the study of microbial pathogenesis and virulence. Inhalation of *L. pneumophila*-contaminated water droplets is the natural mode of LD transmission in humans and therefore was chosen as the route of infection for its physiological relevance. The guinea pig was selected as the *in vivo* model for this study for many reasons in addition to their historical relationship with LD [3]. Perhaps the most important reason being that they exhibit susceptibility to *L. pneumophila*, whereas the majority of mouse models do not. Furthermore, guinea pigs bear closer physiological resemblance to the human immune system as compared to mice and other common research animals with respect to IL-8, IL-12p40, and the complement system among others [30]. A drawback of the guinea pig model, and benefit of mice, is the lack of experimental reagents and underlying literature available involving guinea pig immunology [30]. Through the use of this infection model system, it was discernable that presence of p45 ICE enhanced virulence of *L. pneumophila*.

Although the importance of p45 ICE in *L. pneumophila* virulence has been demonstrated in this study, the precise genes or gene products responsible have yet to be determined. From what is known of the 42 proposed genes encoded on p45, the most alluring prospects for involvement in *L. pneumophila* virulence are the genes that make up the Lvh T4aSS. As mentioned previously, evidence suggests both that Lvh does not contribute to pathogenesis and/or virulence [22], and that it does contribute [24, 25]. Notably, the majority of studies in support, emphasize the importance of mimicking environmental conditions as closely as possible [24]. By using lower growth temperature of 30°C, water treatment, and passage in amoeba prior to experimental use, defects in Dot/Icm effector translocation and intracellular survival have been partially rescued [24] by Lvh subunit VirD4 [38]. Although the "water-stress treatment" described by Bandyopadhyay *et al.* 2007 was not strictly adhered to in this study, the methods of bacterial suspension in water rather

than BYE, and amoebae passage of *L. pneumophila* strains prior to experimental use were followed, and results support the notion of importance in mimicking environmental conditions for research of *Legionella* pathogenesis and virulence. Although this study does not directly address involvement of Lvh, the results from this study together with previous evidence [24, 25, 38, 39] advocate for continued studies into the function(s) of *L. pneumophila* Type IVa and P-type secretion systems, particularly Lvh. Mechanistic details concerning the function of p45 in virulence could also be pursued by mutagenesis of individual genes encoded on p45.

This study was the first that assessed cytokine and chemokine expression during the host response in a guinea pig model of *L. pneumophila* infection. This is particularly important because of the immune similarities guinea pigs share with humans, not shared with mice or rats [30]. Previous studies have evaluated cytokine expression in response to *L. pneumophila* infection using cell culture, mice, and sera from human clinical cases [40-42]. By these methods, it has been established that a pro-inflammatory TH1 type response develops in response to *L. pneumophila* infection based on increase expression of TNF-α, IL-1β, IL-12, and from human tissues, IL-8 [40-42]. Our results align with previous reports while also illustrating a dramatic induction of IFN-γ from both the lungs and spleen of Phil-1-infected guinea pigs (Figures 7D-F). Furthermore, this increase of type II interferon expression is likely a contributing cause of guinea pig mortality, since the increase was associated with p45-containing *L. pneumophila* and thus also associated with causing mortality (Figures 2A and 5A).

Because the increase of IFN-γ did not correlate well with bacterial burden (Figure 6) increase in the animals' lungs or spleens (Figure 7), indicating the involvement of a *L. pneumophila* secreted factor is involved. This could occur by a plethora of conceivable methods, one being increased secretion mediated by Lvh of a molecule or molecules traditionally exported

by another system. Another potential explanation could be secretion of a molecule encoded on p45 mediated by Lvh T4aSS or one of *L. pneumophila's* numerous other secretion systems such as Lsp T2SS, Lss T1SS, or dot/icm T4bSS. Other possibilities such as induction of type II interferon through immune signaling (not tested in this study), or the manipulation of the host immune response via effector function or dysfunction. The non-coding RNA of p45, *lpr0035*, may also be involved, having been implicated in *L. pneumophila* pathogenesis in a previous report [17]. There are countless imaginable possibilities for how p45 may be involved in L. pneumophila virulence, however likely mechanism of IFNγ-mediated mortality associated with the presence of p45 is included in greater detail in Chapter IV.

Guinea pig morbidity, mortality, and increased IFN-γ expression are associated with p45 presence within *L. pneumophila*. Collectively, the information gained from this study reveals an important role for the newly described ICE, p45, in mammalian virulence of *L. pneumophila* Phil-1 as shown in the guinea pig model and supported by genomic prevalence in clinically relevant strains [26]. Importantly, this study highlights the significance of IFN-γ in the host response to *L. pneumophila* infection, having implications on patient treatment possibilities.

Evidence compiled here, together with previous findings, suggest that the traits which enhance virulence in mammalian hosts, like p45, were acquired by *L. pneumophila* and subsequently passed via horizontal transfer to other *L. pneumophila* strains, including those belonging to other serogroups. *L. pneumophila* Thunder Bay and HL06041035, members of SG6 and SG12 respectively were both isolated clinically and have been implicated in causing human disease epidemics and both contain predicted mobile integrative elements which encode the *lvh/lvr* region [26]. Future directions from this study could address involvement of p45 on *Legionella*

virulence in a broader sense by comparing *L. longbeachae* strain D-4968 which includes a mobile element with the *lvh/lvr* region, with strain NSW-150 which lacks these [26].

The virulence attenuation of Lp01 per the lack of p45 ICE as described by this study, can be argued as both negative and positive situationally, with respect to laboratory research. In the endeavor of insight pertaining to *L. pnuemophila* virulence or pathogenicity, a strain genetically and functionally less removed from clinical relevance would serve better. However, in the pursuit of information relating to the Dot/Icm T4bSS and/or its effector molecules, the absence of unknown contributions made by Lvh and other p45 components may likely serve as advantageous.

The p45 ICE of *L. pneumophila* Phil-1 enhances virulence as shown by increased morbidity and mortality of infected guinea pigs accompanied by increased IFN-γ expression. Although a direct pathogenic mechanism to explain this phenomenon has not been distinguished, these results illustrate the importance of p45 ICE in the severity of host response to *L. pneumophila* infection. A better understanding of host response to LD could be pursued using this model system along with the emerging depth of reagents available for it.

II.4 Methods

II.4.1 Bacterial strains and growth conditions

Legionella pneumophila Philadelphia-1 (Phil-1) and its derivative strain, Lp01, were both provided graciously by Ralph Isberg and Michele Swanson. Lp01 is a streptomycin-resistant variant and restriction deficient mutant of the clinical strain Phil-1 [43]. JR32, a gift from Howard Shuman, is also derived from the parent strain Phil-1, and is similarly streptomycin resistant and restriction deficient [44]. AA100 was isolated as a streptomycin resistant mutant of 130b [45], a clinical isolate from the Wadsworth Veterans Administration Hospital in Los Angeles, CA [46].

The *Legionella* strains were grown as described previously [47], on buffered charcoal yeast extract (BCYE) agar plates or shaken in buffered yeast extract (BYE) broth, and kept at 30 or 37°C and 5% CO_2 for 3-5 days. *L. pneumophila* strains were passed through *Acanthamoeba castellanii* amoebae prior to pathogenic and virulence related experiments [24, 48, 49]. *L. pneumophila* used for experiments were grown from 4°C stock lawns, which were grown from -80°C glycerol stocks. Stock lawns were kept and used at 4°C for no longer than 5 days and passaged no more than twice [39]. *Escherichia coli* XL1-Blue strains were used for propagation of plasmids, grown in Luria-Bertani (LB) liquid media or agar plates at 37°C and 5% CO_2 overnight.

II.4.2 Protozoal strains and growth conditions

Acanthamoeba castellanii (ATCC 30234), an environmental host for *L. pneumophila*, was maintained in M712 media at 22°C in 75-cm^2 tissue culture flasks in the dark. Amoebae viability and enumeration were determined using Trypan Blue stain and a hemocytometer [49, 50].

II.4.3 Chromosomal integration and excision of p45

Genomic DNA from *L. pneumophila* Phil-1 and Lp01 cultures was isolated by standard phenol/chloroform extraction as previously described [51]. Quantitative PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems), ~50 ng/µl of genomic DNA, and oligonucleotides targeting *attB, attP*, the *attP/attB* junction, *lpg1249*, and *gyrB/lpg0004* described in A-4 Table. Quantities were determined by comparing target C_T values from *L. pneumophila* Phil-1 with those of *gyrB* and then with the same calculated values from Lp01 DNA, which lacks the p45 MIE. Samples were run in triplicate and each primer set was accompanied by negative control wells with no template.

II.4.4 Vector construction

The plasmid pMV262 containing the *aph* kanamycin resistance cassette was isolated from XL1-Blue *E. coli* strain Ψec485 [52] using standard phenol/chloroform midi-prep extraction as previously described [51], and digested by restriction enzymes, *AfeI* and *BamHI*-HF. The digested pMV262 was then ligated with a ~200 bp fragment amplified by PCR (primers listed in A-4 Table) from the p45 MIE of *L. pneumophila* Phil-1, also digested by *BamHI*-HF and *AfeI*. The ligated product was transformed by electroporation into *E. coli* XL1-Blue as described previously [53] and selected for on BCYE with kanamycin (25 μg/ml). The resulting plasmid, pJDC305, in *E. coli* Ψec445 was confirmed to contain the *aph* cassette by PCR (primers listed in A-4 Table) and restriction enzyme digestion, and later transformed into *L. pneumophila* Phil-1 by electroporation [51]. Bacteria successfully transformed by pJDC305 were selected using BCYE with kanamycin (25 μg/ml). Colonies chosen were verified to contain pJDC305 and its integration within the p45 element by homologous recombination, using PCR (primers listed in A-4 Table). The resulting strain, Ψlp76 (Phil-262), with a kanamycin resistance tagged p45 element (pJDC321), was subsequently used as the donor strain for conjugation of p45.

II.4.5 Conjugation of p45

Legionella cultures grown as lawns on BCYE agar plates at 30°C and 5% CO_2 for 2-3 days, were used to inoculate BYE liquid broth, and grown to post-exponential phase (OD_{600} 1.2-1.4) with shaking at 30°C. The bacteria were then diluted to an OD_{600} of ~1. The donor strain, Phil-262, has the vector pJDC321 which is the p45 element containing *aph* kanamycin resistance. The recipient strain, Lp01, was selected against the Phil-1 background donor strains by streptomycin resistance. Cultures of the donor alone, the recipient alone, and a 1:1 ratio of both were mixed and placed on sterile filter discs with 0.45 μm pores using a vacuum filtration system [20, 54]. The

discs were then placed with bacteria facing up on BCYE plates containing DNase at 30°C in the presence of 5% CO_2 for 4 hours. The bacteria were then resuspended in BYE, diluted, and plated to select for Lp01 trans-conjugates containing p45 (Ψlp77 or Lp01^{+p45}) on BCYE containing kanamycin (25 μg/ml) and streptomycin (200 μg/ml), and for comparison BCYE alone. The efficiency of conjugation was determined by dividing the number of colony forming units (CFU) that grew on the selective BCYE containing streptomycin (200 μg/ml) and kanamycin (25 μg/ml), with the number that grew on non-selective BCYE plates.

II.4.6 Lp01^{+p45} trans-conjugate confirmation

II.4.6a PCR analysis of trans-conjugates. Oligonucleotides listed in A-4 Table were used to verify the presence of p45, its integration into the chromosome, and to distinguish the Lp01 background from that of Phil-1. The presence of p45 and its site-specific integration into the chromosome were determined by targeting p45-encoded *lvhB8*, disruption of the chromosomal *attB* site, and the *attP/attB* p45-chromosomal integration junctions. In order to screen for the Lp01 genetic background by PCR, oligonucleotides were designed to detect a single nucleotide polymorphism within *rpsL* (*lpg0234*) [28]. The 3' tip of the forward primer was designed to complement the guanine residue of *rpsL* in Lp01, rather than the adenine encoded by Phil-1. Genomic DNA (~80 μg) was isolated as described previously [51] from colonies selected on BCYE containing kanamycin (25 μg/ml) and streptomycin (200 μg/ml) resulting from Phil-262/Lp01 conjugation. PCR was executed with an Applied Biosystems Veriti thermocycler, products were separated by gel electrophoresis, and visualized using ethidium bromide stain and UV light.

II.4.6b RT-qPCR analysis of Lp01^{+p45}. RNA was isolated from *L. pneumophila* Phil-1, Lp01, and Lp01^{+p45} cultures suspended in TRIZOL reagent using the standard chloroform method as

described previously [51]. The Super Script III Reverse Transcription System from Invitrogen was used to convert RNA samples to cDNA following manufacturer's recommendations. Approximately 50 ng/µl cDNA per reaction was used for RT-qPCR with primers which target transcripts from *lpg1266, lpg1254, lpg1241, lpg1229, lpg1249*, and *gyrB/lpg0004* (A-4 Table), and SYBR Green PCR Master Mix by Applied Biosystems following manufacturer's recommendations. Experiments were done with a Thermofisher Scientific StepOnePlus Real Time PCR System, in 96-well plates. Each reaction target was accompanied by negative control wells with no template, and samples were run in triplicate. Expression values were calculated by comparing target C_T values to those of *gyrB*. Those values (ΔC_T) were then compared to the same from Lp01, resulting in $\Delta\Delta C_T$.

II.4.6c Sequencing of small nucleotide polymorphisms between Phil-1 and Lp01. Sequencing was performed by Eton Biosciences using genomic DNA (~80 µg) isolated from *L. pneumophila* Phil-1, Lp01, and Lp01^{+p45} of genetic regions which harbor small polymorphism differences between the published genomes of Phil-1 (AE017354.1) [32] and Lp01 [28] located in *lpg0324, lpg0671, lpg0716, lpg0718, lpg2506*, and *lpg2669* gene regions (Table 1). Oligonucleotides used for PCR amplification, RT-qPCR, and sequencing are included in A-4 Table.

II.4.7 Guinea pig infections

Female Hartley guinea pigs (250 - 300 g) from Charles River Laboratories were infected with *Legionella pneumophila* cultures grown on BCYE plates at 37°C and 5% CO_2 and suspended in sterile ddH_2O, as detailed in B-1 Figure, using a Madison aerosol chamber [55]. The bacterial cultures used for nebulization in the Madison Chamber were normalized using OD_{600} in order to achieve similar seeding values within the lungs between groups. These normalizations were determined by seeding dose experiments prior to virulence experiments. Animals infected with *L.*

pneumophila were monitored and scored for disease severity using three types of criteria: 1) weight loss, 2) respiratory rate, and 3) general behaviors, which include lethargy, skin coloration, and fur ruffles. These criteria were scored as described in A-1 Table. When necessary, euthanasia was accomplished by overdose of pentobarbital (FatalPlus) through intra-peritoneal injection and completed by cardiac exsanguination. Following euthanasia, the upper right lobe of the lungs and half the spleens were collected in Falcon tubes in 5 ml sterile ddH_2O, homogenized, diluted, and plated on BCYE containing 5 μg/ml polymixin B, and incubated in 37°C and 5% CO_2 for 3-6 days. Slices of the remaining lungs and spleens were either immediately added to, homogenized, and frozen at -80°C in TRIZOL reagent, or fixed in 10 ml buffered formalin at 22°C (RT) for later embedding and staining for histopathology.

Each experiment utilized two animals per infection group to determine the number of bacteria seeded in the lungs following aerosol infection. Seeding animals were euthanized 4 hours after infection, the upper lobes of both lungs were harvested, added to 5 ml ddH_2O, homogenized, plated on BCYE with polymixin B (5 μg/ml), and incubated 3 – 5 days for CFU. Survival experiments were conducted with 2 seeding and 5 experimental guinea pigs per infection group, while the immune response experiment utilized 2 seeding and 4 experimental guinea pigs per time point per infection group, with 1 uninfected control.

II.4.8 Cytokine expression analysis

RNA was isolated from tissue samples frozen in TRIZOL reagent using the standard chloroform method as described previously [51]. The Super Script III Reverse Transcription System from Invitrogen was used to convert RNA samples to cDNA following the manufacturer's recommendations. Approximately 50 ng/μl cDNA per reaction was used for RT-qPCR with primers that target transcripts for guinea pig IL-8 [56], IL-12p40, TNF-α, IFN-γ, TGF-β1, CCL5,

and HPRT [57, 58] (A-4 Table) and SYBR Green PCR Master Mix by Applied Biosystems following the manufacturer's recommendations. Experiments were done with a Thermofisher Scientific StepOnePlus Real Time PCR System in 96-well plates. Expression values were calculated by comparing sample target C_T values to those of hypoxanthine-guanine phosphoribosyltransferase (HPRT), which produced ΔC_T values. The ΔC_T values were then compared to those from the corresponding tissue of uninfected guinea pig, which produced the $\Delta\Delta C_T$ values. Samples were run in triplicate, and each primer set was accompanied by negative controls lacking cDNA template.

II.4.9 Histopathology of guinea pig lung tissue

All animals were submitted for necropsy after terminal euthanasia 24 or 48 hours following *L. pneumophila* infection. Necropsies were performed, and sections of lung, liver, spleen and kidney were collected for microscopic evaluation. Tissues required for microscopic evaluation were trimmed, processed routinely, embedded in paraffin, and stained with hematoxylin and eosin. Microscopic evaluation was conducted by board-certified veterinary pathologists Garry Adams (A-2 Table) or Quinci Plumlee (Figs 8A-H and A-3 Table) in a 'blinded' manner via light microscopy.

II.4.10 Statistical analysis

The significance of the results was determined by using Student's T-test or analysis of variance, as appropriate. P values less than 0.05 were considered significant. Microsoft Excel V15.26 and GraphPad Prism V5 software were utilized for statistical analysis and figure formation. * $p<0.05$ ** $p<0.01$

II.5 References

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CHAPTER III

THE P45 MOBILE INTEGRATIVE ELEMENT INFLUENCES *Legionella pneumophila* HOST CELL ENTRY AND SENSITIVITY TO SODIUM

Legionella pneumophila are environmental bacteria found ubiquitously in both natural and man-made water reservoirs, often as constituents of biofilm communities and/or intracellularly within protozoal hosts. In the event that these bacilli become aerosolized in water droplets then inhaled by humans, they can cause a potentially fatal form of pneumonia called Legionnaires' disease. Strains of *L. pneumophila* have highly plastic genomes which harbor numerous inter- and intra-genomic elements, enhancing their abilities to live in diverse environmental conditions. One such mobile genomic element, p45 encodes the Lvh (Legionella Vir homolog) type IVa secretion system. This element was evaluated for its contribution to *L. pneumophila* environmental resilience and pathogenicity to host cells by comparing clinically isolated strain Philadelphia-1 which contains p45, strain Lp01 which was derived from Philadelphia-1 and lacks p45, and a Lp01 strain in which the p45 element was reintroduced, Lp01^{+p45}. Sensitivity to sodium and entry into J774A.1 monocytic cells were both influenced by the presence of the p45 element.

III.1 Introduction

Legionella pneumophila are Gram negative facultative intracellular bacilli that ubiquitously inhabit water reservoirs [1-3]. These environmental bacteria often live as constituents of biofilm communities or intracellularly within protozoal hosts [1]. Coevolution with single celled eukaryotic hosts presumably equipped these bacteria with the abilities to also utilize human alveolar cells as hosts [4, 5]. Humans can become incidental hosts to *L. pneumophila* when the

bacteria are aspirated as contaminated aerosol droplets [6, 7]. Infected individuals can then develop a potentially fatal form of pneumonia called Legionnaires' disease, or an acute self-limiting flu-like illness called Pontiac fever [8]. Not all *L. pneumophila* strains exhibit the same ability to cause severe human disease [9, 10]. It has been suggested that inconsistencies in the ability of different strains to cause clinically relevant disease is not due to environmental prevalence, rather that virulence of *L. pneumophila* strains vary [11, 12]. Highly virulent *L. pneumophila* strains share commonalities among them including secretion of virulence factors [13] and certain genetic components such as the p45 integrative conjugative element .

As environmental organisms *L. pneumophila* are capable of withstanding vastly diverse conditions, aided by intricate regulatory systems [14, 15] and plastic genomes [16-18]. The *L. pneumophila* genome varies greatly among strains, partially due to the prevalence of inter and intra-genomic mobile elements [19]. One such class of genomic elements, called mobile integrative elements (MIE), are capable of existing as extra-chromosomal (episomal) circular plasmid-like structures, or linearly integrated site-specifically within the chromosome [20, 21]. Distinguishing features of mobile integrative elements include higher G+C content than the chromosomal DNA, flanked by tRNA, and encode phage-related genes and conjugation machinery [17, 22]. Often these elements have the capacity to transfer from one bacterium to another via conjugation adding to the dynamic plasticity of the Legionella genome, such elements are referred to as integrative conjugative elements (ICE).

An example of a MIE in *L. pneumophila* Philadelphia-1 (Phil-1) is the p45 element, which was recently found to also have the ability to conjugate, making it an ICE (shown previously). p45 includes features common to MIEs and ICEs, and encodes the Legionella Vir homolog (Lvh) type IVa secretion system (T4aSS) along with the *lvr* gene cluster which includes a homolog of the

CsrA global regulator, LvrC [23]. Recently, p45 has been shown to impact *L. pneumophila* virulence in the guinea pig model (shown previously). Although, it is probable that p45 has a role(s) in withstanding conditions in *Legionella*'s natural environment, yet these are not understood.

In the current study, the *L. pneumophila* p45 ICE was evaluated for its role in various environmental conditions and interactions with host cells by comparing the wild type strain, Phil-1, that naturally carries p45 [24] with strain Lp01 that does not carry p45 [25, 26], and a Lp01 strain with a reintroduced p45 ICE ($Lp01^{+p45}$). We found that although p45 does not appear to impact growth *in vitro*, it has an impact upon susceptibility to the environmental stress from NaCl. Acidic pH had a greater effect on viability of Lp01 than Phil-1, but this effect was not corrected by p45, suggesting that other differences between Phil-1 and Lp01 are responsible for greater susceptibility to acidic environments. Similarly, the effect of reactive oxygen species and high temperature on bacterial viability were not affected by p45 and pigmentation levels were similar. However, p45 does appear to play a role during entry into host cells, both the environmental amoeba *Acanthamoeba castellanii* and the murine macrophage cell line J774A.1. These observations suggest that the p45 ICE is important for survival of Legionella in high salt environments and efficient infection of host cells that serve as the replicative compartment both during disease and in the water environments.

III.2 Results

III.2.1 Involvement of p45 in environmental stress resistance

$Lp01^{+p45}$ was compared with Lp01 and Phil-1 for the ability to recover from stressful conditions. Conditions tested included heat (56 °C), presence of H_2O_2, acidic pH, and presence of

sodium. Sensitivity to sodium has been used for decades in the *Legionella* field as an *in vitro* proxy for virulence, where strains more sensitive to sodium tend to be more virulent [27, 28]. *L. pneumophila* Phil-1 and Lp01^{+p45} displayed similar levels of resistance to sodium, while Lp01 was significantly less susceptible (Figures 9 and 10). This phenotype was consistent in both resistance to sodium stress (Figure 9) and resistance to sodium in the growth environment (Figure 10). Resistance to heat and the presence of H_2O_2 did not differ between the three strains, while Phil-1 recovered better than the Lp01 and Lp01^{+p45} strains after acidic pH stress (Figure 9).

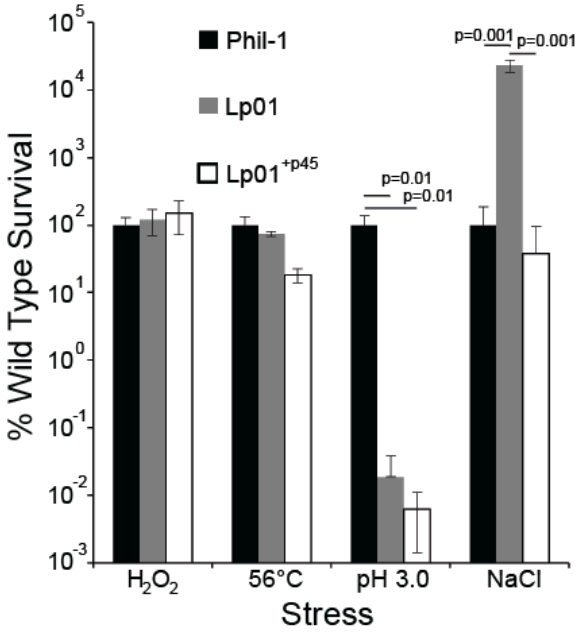

Figure 9. ***Legionella pneumophila*** **resistance to environmental stressors.** *L. pneumophila* Phil-1, Lp01, or Lp01^{+p45} culture resistance to heat (56 °C), H_2O_2, pH 3 citrate, and 1% sodium treatments. Data represent results of three independent experiments executed in triplicate, and error bars represent standard deviations.

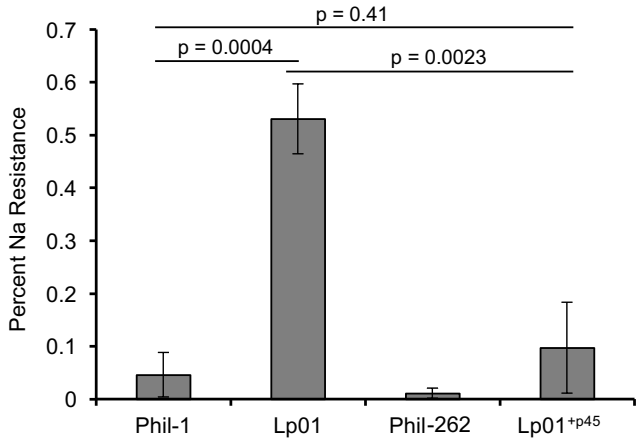

Figure 10. ***Legionella pneumophila*** **growth in the presence of sodium.** *L. pneumophila* Phil-1, Lp01, or Lp01^{+p45} culture resistance to sodium determined by CFU/ml grown on BCYE supplemented with 0.5 % NaCl compared to cultures grown on BCYE alone. Data are from three independent experiments conducted in triplicate. Error bars represent standard deviation.

III.2.2 Involvement of p45 in *Legionella pneumophila* pigmentation production

The three *L. pneumophila* strains were evaluated for their abilities to produce pigmentation, which provides an advantage for bacteria living in the environment [29]. Similar levels of pigmentation were detected in the supernatant of Lp01 and Lp01^{+p45} cultures (Figure 11). Pigment detected in the Phil-1 culture matched that of Lp01 and Lp01^{+p45}, until after 24 hours where Phil-1 supernatant pigmentation was significantly higher than the other two (Figure 11). Considering that there was no significant increase of Phil-1 bacterial growth at these time points (Figure 12), the increased detection of pigment in the Phil-1 culture was likely due to increased production.

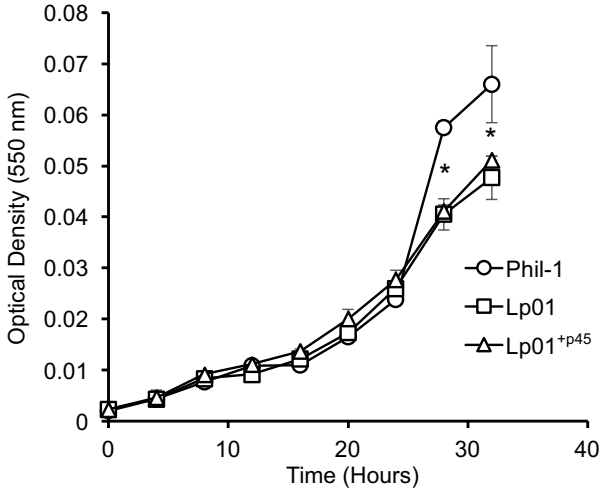

Figure 11. ***Legionella pneumophila*** **pigment production.** Pigment production was measured in the supernatant of *L. pneumophila* Phil-1, Lp01, or Lp01^{+p45} grown in BYE liquid media by optical density (OD_{550}). Data represent results of two independent experiments executed in triplicate, and error bars represent standard deviations. *p<0.05 **p<0.01

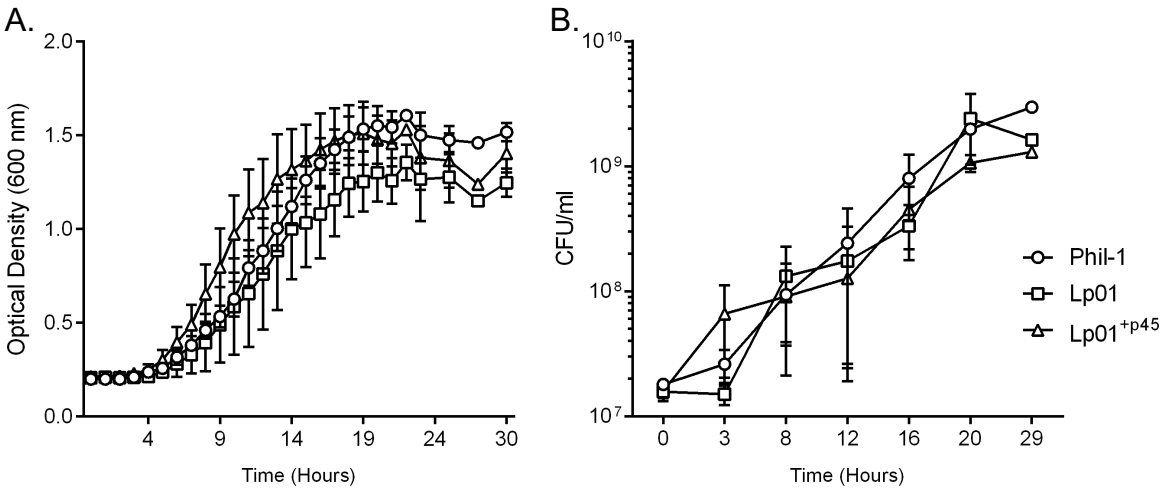

Figure 12. ***Legionella pneumophila*** **growth in liquid media.** *L. pneumophila* Phil-1, Lp01, and Lp01^{+p45} cultures grown in BYE liquid media measured by OD_{600} (A) and CFU (B). Markers and error bars represent group means and standard deviations respectively. Data represent results of three independent experiments executed in triplicate, and error bars represent standard deviations.

III.2.3 Involvement of p45 in *Legionella pneumophila* host cell entry

L. pneumophila strains Phil-1, Lp01, and Lp01^{+p45} were also assessed for their ability to enter various host cell types, environmental and mammalian. Infection of a mammalian monocytic cell line revealed that Lp01 was reduced in its entry ability as compared to Phil-1 (Figure 13A). Introduction of p45 in Lp01^{+p45}, rescued the attenuation of Lp01 in monocyte entry (Figure 13A). When used to infect *Acanthamoebae castellanii,* one of *Legionella*'s well studied environmental hosts, there were no significant differences in entry between any of the strains (Figure 13B), although all three strains maintained a trend consistent to that of monocytic cell entry (Figure 13A). Growth within *A. castellanii* amoebae hosts was similar among the three *L. pneumophila* strains (Figure 14). Together these data suggest involvement of p45 in *L. pneumophila* entry into host cells, particularly mammalian.

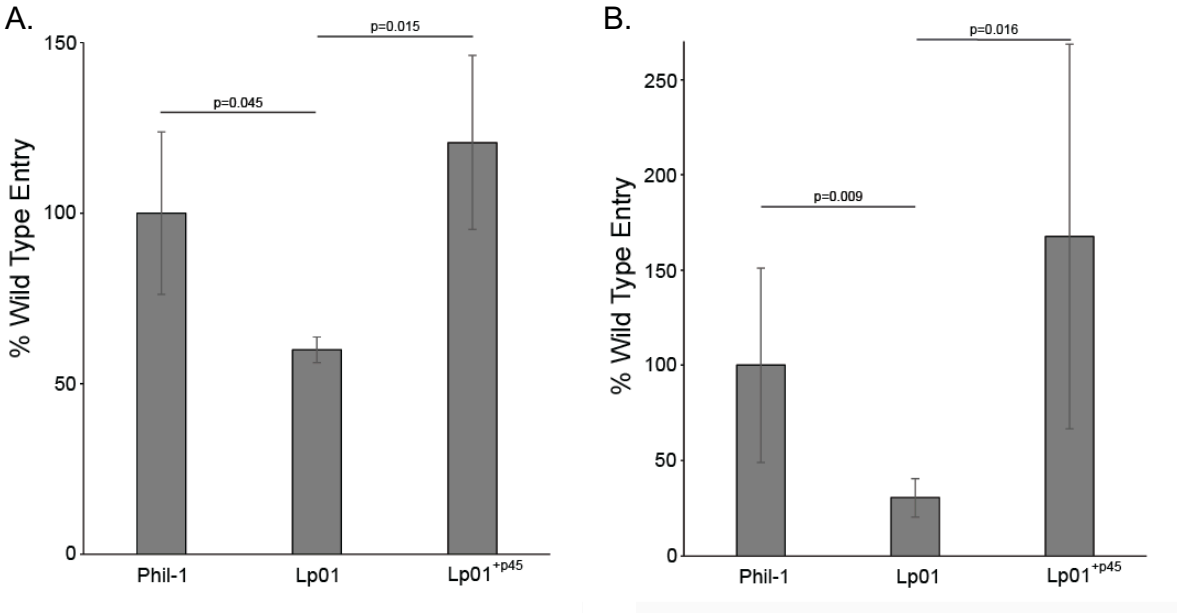

Figure 13. ***Legionella pneumophila*** **entry into host cells.** Host cell types J774A.1 macrophages (A) or *A. castellanii* amoeba (B) infected with *L. pneumophila* Phil-1, Lp01, or Lp01^{+p45} with MOI ~10 and ~50 respectively. Data represent results of three independent experiments executed in triplicate, and error bars represent standard error means.

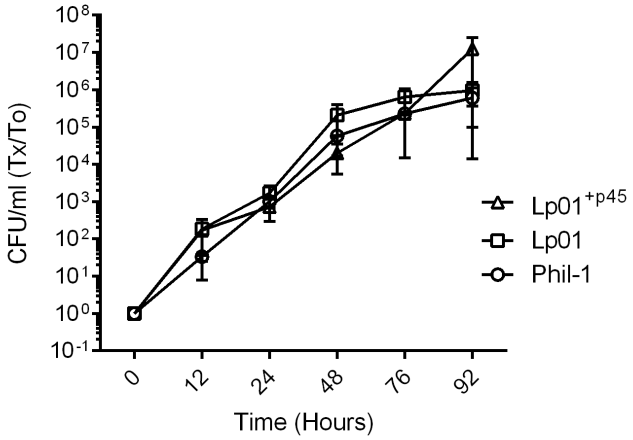

Figure 14. ***Legionella pneumophila*** **growth within amoebae.** Intracellular growth of *L. pneumophila* Phil-1, Lp01, or Lp01^{+p45} within *A. castellanii* infected with a MOI ~50, represented as CFU/ml normalized to entry CFU/ml (Tx/To). Data represent results of two independent experiments executed in triplicate, and error bars represent standard error means.

III.2.4 Reactive nitrogen species (RNS) response of amoebae to non-self rRNA

Production of reactive nitrogen species (RNS) is a common defense utilized by eukaryotic cells in response to infection or foreign debris. Here we determined whether *A. castellanii* produce RNS in response to the presence of 23S rRNA ORN, a sequence conserved among Gram negative bacterial species. Transfected *A. castellanii* responded to cytosolic *Orientia* 23S rRNA ORN in a dose-dependent fashion (Figure 15A), and responded similarly to the 23S rRNA ORN of other Gram negative bacterial genera at a common concentration (Figure 15B). Although, there was no difference in RNS production by amoeba stimulated with the mutant 23S rRNA ORN, this may suggest the importance of something other than the sequence alone in this detection mechanism. Regardless, amoeba induced RNS production in response to intracellular detection of a Gram negative 23S rRNA, which includes *L. pneumophila*. It's probable, however, that a robust RNS response to *L. pneumophila* infection would be dampened by functions of Dot/Icm effector molecules.

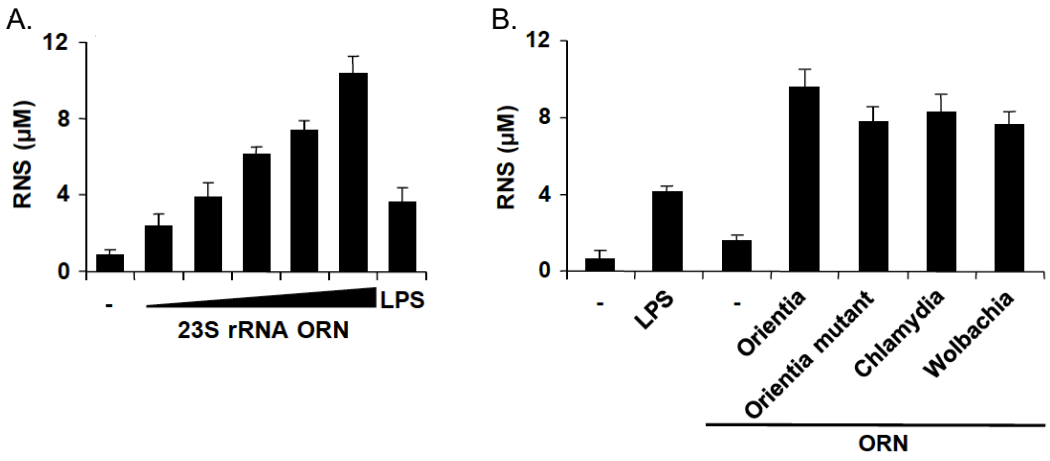

Figure 15. Reactive nitrogen species (RNS) production in response to bacterial rRNA. *Acanthamoeba castellanii* stimulated with *Orientia* 23S rRNA ORN at various concentrations (0, 0.1, 0.5, 1, 2, or 4 μg/ml) (A), with 23S rRNA ORN (4 μg/ml) from bacterial genera *Chlamydia*, *Wolbachia*, and *Orientia* natural sequence and a mutated sequence (B), or with 1 μg/ml LPS at 30°C for 10 hours. Data points represent combined data from three separate experiments conducted in triplicate, and error bars represent standard deviation.

III.3 Discussion

Phenotypic differences between *L. pneumophila* Phil-1 and Lp01 that were rescued by the presence of p45 in strain Lp01^{+p45} include entry into J774A.1 monocytic cells and sensitivity to sodium. Since sodium sensitivity represents a good proxy for virulence [27, 28], more virulent strains of *Legionella* are likely to exhibit greater sensitivity to sodium. Although a mechanistic explanation for this association is not fully understood, at least in some cases the defect has been localized to dotA, involved in type IV secretion [28, 30, 31]. Prior studies have also demonstrated a relationship between sodium sensitivity and the DotU and IcmF subunits of the Dot/Icm type IVb secretion system [32]. Interestingly, there are a number of Dot/Icm components that share similarities with those of the Lvh type IVa secretion system [33], presenting a likely explanation for the increased resistance to sodium displayed by Lp01 in comparison to strains with p45.

Regardless of mechanistic explanation, increased sodium sensitivity displayed by Phil-1 and Lp01^{+p45} suggests the presence of p45 is associated with *L. pneumophila* virulence.

Regulation of sodium resistance is impacted by the global regulator, CsrA [14]. *L. pneumophila* possess four homologs of CsrA and one of these is encoded on p45, lvrC [23, 33]. The lvrC (*lpg1257*) gene maintains 39% identity with csrA (*lpg1593*) [23], is located within the lvh/lvr region, and is believed to be involved in regulation of Lvh [23]. Phenotypes known to be regulated by *Legionella* csrA (*lpg1593*) include pigmentation, motility, and resistance to stressful conditions such as acidic pH, heat, osmotic pressure, sodium, and H2O2 [14]. When tested, phenotypes such as heat, sodium, H2O2, and acid resistance did not correlate with *L. pneumophila* strains that carry p45. Susceptibility to sodium stress was the only csrA-associated phenotype tested that correlated with the presence of the p45 ICE, suggesting that lvrC does not greatly impact all of the same phenotypic characteristics that the *Legionella* csrA gene does and it likely plays other roles during disease or in the environment.

The ability of *L. pneumophila* to enter both mammalian and protozoan host cells is impacted by the p45 ICE. Entry into the environmental host *A. castellanii* was influenced by the presence of p45 in similar manner to that observed in the murine macrophage cell line J774A1. These observations are consistent with our previous studies indicating that entry into both environmental and mammalian host cells can occur by similar mechanisms [26, 34-38]. This phenotype might suggest that this host cell infection advantage was selected for in the environment and that the ability to infect mammalian cells conferred by p45 ICE is a consequence of evolution to increase the efficiency of infecting protozoa. It should be taken into account that *A. castellanii* are not the only environmental hosts for *Legionella*. In fact, there are numerous protozoan species that can serve as hosts, including *Vermamoeba (Hartmanella)*, *Naegleria* and *Tetrahymena* as well

as most likely many others that have not yet been well characterized [7]. *Legionella* strains interact and parasitize various protozoal hosts with different efficiencies, but the extent of this variance is not known. Therefore, the effects on host cell infection observed in our studies may not necessarily be consistent among all protozoa capable of serving as replicative hosts for *Legionella* and these alternative hosts may have unknown and/or inconsistent impacts on interactions with mammalian hosts. Furthermore, we could not extensively test all environmental conditions and further work is needed to examine those that are likely to play a role in regulation of p45, including temperature [38]. The presence of additional regulatory elements that are known to impact virulence-related characteristics, including noncoding RNA [39], makes investigation of the global regulatory consequences of the p45 ICE important for understanding pathogenesis of *Legionella*.

Collectively, our observations suggest a role for the p45 ICE in *Legionella pneumophila* host cell infection and resistance to environmental salt conditions. These beneficial phenotypic characteristics suggest that the p45 ICE contributes to *L. pneumophila* survival in the environment and could impact the ability of these bacteria to cause disease. We are particularly interested in whether the distribution of the p45 correlates with strains of *Legionella* that are more likely to cause infections that lead to clinical symptoms and are responsible for larger epidemics. Examination of the distribution of p45 ICE in environmental and epidemic-associated isolates is warranted to better elucidate its role in the epidemiology of Legionnaires' disease.

III.4 Methods

III.4.1 Bacterial strains and growth conditions

Legionella pneumophila Philadelphia-1 (Phil-1) and its derivative strain, Lp01, were both provided graciously by Ralph Isberg and Michele Swanson. Lp01 is a streptomycin-resistant

variant and restriction deficient mutant of the clinical strain Phil-1 [40]. *Legionella* strains were grown as described previously [41], on buffered charcoal yeast extract (BCYE) agar plates or shaken in buffered yeast extract (BYE) broth, and kept at 30 or 37°C and 5% CO_2 for 3-5 days. *L. pneumophila* used for experiments were grown from 4°C stock lawns, which were grown from -80°C glycerol stocks. Stock lawns were kept and used at 4°C for no longer than 5 days. No strains were passed more than three times before experimental use.

III.4.2 Protozoal strains and growth conditions

Acanthamoeba castellanii (ATCC 30234), an environmental host for *L. pneumophila* [42], was maintained in M712 media at 22°C in 75-cm^2 tissue culture flasks in the dark as described before [34]. Prior to experimental use, *A. castellanii* were seeded in 24-well plates at 5 x 10^5 cells/well for 12 hours, the media was then washed and replaced with M712 high-salt media and incubated at 37°C and 5% CO_2 for 1 hour. Amoebae viability and enumeration were determined using Trypan Blue stain and a hemocytometer. The amoebae were passaged no more than sixteen times prior to being used in experiments.

III.4.3 Cell culture, strains and growth conditions

The mouse monocytic cell line, J774A.1 cells were maintained in 75-cm^2 untreated tissue culture flasks with RPMI 1640 with 2mM L-glutamine and 5% heat inactivated FBS at 37°C and 5% CO_2, as described previously [43]. Monocytic tissue culture cells were seeded in 24-well plates at 2.5 x 10^5 cells/well 18 hours prior to experimental use. Trypan Blue stain and a hemocytometer were used to count cell numbers and determine viability. Tissue culture cell lines were passaged no more than sixteen times prior to being utilized for experiments.

III.4.4 Stress resistance assay

Cultures of *L. pneumophila* Phil-1, Lp01, and Lp01^{+p45} grown on BCYE were suspended in BYE alone or BYE containing a stress treatment, at an OD_{600} of ~0.1. Stress treatments included 1% (w/v) NaCl, pH 3 citrate, 56°C, and 3mM H_2O_2 as previously described [44]. Cultures were incubated with 210 rpm shaking at 37°C for 30 minutes (with the exception of the heat stress samples, which were kept at 56°C in BYE) before diluting and plating the samples on BCYE which were then incubated at 37°C and 5% CO_2 for 3–5 days. CFU that grew from cultures kept in BYE alone at 37°C were compared with those from the stress treated samples and the results were calculated as percent resistance relative to wild type (Phil-1), with wild type resistance set to 100%.

III.4.5 Sodium sensitivity assay

Sodium sensitivity assays were performed as described previously [27, 28], by diluting *L. pneumophila* Phil-1, Lp01, and Lp01^{+p45} cultures to OD_{600} ~1 in BYE broth then plated on both BCYE containing 100mM NaCl and plain BCYE. After 3-4 days in 37°C with 5% CO_2, the number of CFU present on BCYE with 0.5% NaCl were compared with those that grew on BCYE alone and the results calculated as percent resistance.

III.4.6 Pigmentation assay

Pigment production was monitored by optical density (550 nm) of culture supernatant as described before [14, 29]. *L. pneumophila* Phil-1, Lp01, or Lp01^{+p45} grown on BCYE were used to inoculate BYE at an optical density of 0.2 (600 nm) shaken (210 rpm) at 37 °C for 32 hours. Every four hours the optical density (550 nm) of each culture's supernatant was measured, and results were calculated by normalizing the optical densities throughout the experiment to those at time 0.

III.4.7 Growth rate in liquid media

L. pneumophila Phil-1, Lp01, and Lp01^{+p45} cultures grown on BCYE were used to inoculate BYE broth and shaken (210 rpm) at 37°C for 32 hours. Optical density (600 nm) measurements were determined every half hour and the number of viable bacteria was quantified by CFU on BCYE plates every 3 hours. Results were calculated by normalizing the optical densities or CFU throughout the experiment to those at time 0.

III.4.8 Entry and intracellular growth assays

Entry assays were performed essentially as described previously [26]. Briefly, *L. pneumophila* cultures grown on BCYE plates were suspended and diluted using the appropriate medium for the host cells which were to be infected. Bacteria were added to 24-well plates containing either J774A.1 [43] cells or *A. castellanii* strain Neff [35] at a multiplicity of infection of approximately 10 or 50, respectively. Following 30 minutes of incubation, wells were washed twice and then incubated for 2 hours with the appropriate medium containing 100 μg/ml of gentamicin, then washed twice more. Host cells were lysed in sterile ddH_2O and the number of intracellular bacteria was determined by CFU on BCYE plates. Percent entry was then determined by dividing the CFU of intracellular bacteria by the CFU used for infection, then multiplied by 100. In order to determine intracellular growth, following treatment with gentamicin and washing fresh media was replaced in each well and incubated for the appropriate amount of time before the host cells were lysed and CFU were determined like previously.

III.4.9 Reactive nitrogen species (RNS) production assay

All reagents were prepared in RNase-free conditions to reduce risk of RNA degradation. The amoebae, *Acanthamoeba castellanii* (ATCC 30234), were grown to 90% confluency at 22°C in the dark in 75-cm^2 tissue culture flasks (Falcon) in PYG broth as described previously [34, 35].

Amoebae were then seeded in 48-well tissue culture plates at a concentration of 2 x 10^5 cells per well. The amoeba were allowed to adhere for 12 hours at 22°C in the dark before the media was replaced with 150 µl high salt (HS) buffer [42, 45] and allowed to equilibrate to 30°C for 1 hour. Various concentrations of purified 23S rRNA ORN were mixed with N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) at a 1:6 ORN:DOTAP ratio in DEPC ddH_2O and incubated at room temperature for 15 min. The ORN-DOTAP mixtures were then added to wells containing the amoebae. *Escherichia coli* O55:B5 purified LPS was used as a positive control at a concentration of 1µg/mL. The 48-well plates containing amoebae were swirled gently 6 times to evenly distribute the stimulants before returning to 30°C for 10 hours.

Following incubation, the concentration of RNS produced by each sample was measured by Griess assays based on a standard curve, as described previously [46]. In order to measure RNA present in supernatants, 50 µl of each supernatant was transferred into two wells of a 96-well flat bottom plate. Then 25 µl of 1% sulfanilamide in 5% H_3PO_4 was added to each well, the well gently mixed and left at room temperature in the dark for 5 min. Next 25 µl of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride was added to each well and again left for 5 min in the dark at room temperature. The absorbance for each well was read at 550 nm using a PerkinElmer Envision plate reader. Standard curves were constructed for each experiment using known concentrations of $NaNO_2$ diluted in HS buffer, in addition to a blank consisting of only HS buffer. The blank absorbance value was subtracted from all other values prior to construction of the standard curve and data analysis. Each experiment was repeated three times using triplicate wells of amoebae.

III.4.10 Statistical analysis

The significance of the results was determined by using Student's T-test or analysis of variance, as appropriate. P values less than 0.05 were considered significant. Microsoft Excel

V15.26 and GraphPad Prism V5 software were utilized for statistical analysis and figure formation.

* $p<0.05$ ** $p<0.01$

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CHAPTER IV

SUMMARY

Results from this study clearly depict that inclusion of the newly described integrative conjugative element, p45, contributes to *Legionella pneumophila* virulence. Enhanced virulence, represented by morbidity and mortality of guinea pigs infected via aerosol, was accompanied by increased IFNγ expression. Inclusion of the p45 element in *L. pneumophila* also has a positive impact on host cell entry, which may be a contributing factor to the overall virulence associated with p45. These results contribute a valuable perspective to the understanding of the potentially fatal pneumonia caused by *L. pneumophila*. Yet, questions remain, and require further evaluation to gain a more comprehensive understanding of *Legionella pneumophila* virulence.

IV.1 Mechanism by which p45 Contributes to IFNγ-Mediated Guinea Pig Mortality

The role of IFNγ during *Legionella pneumophila* infection has almost exclusively been regarded as protective [1-6]. Yet, an excess of IFNγ can have deleterious effects for the host due to uncontrolled inflammation [7]. During *L. pneumophila* infection, IFNγ is produced largely by T_H1 and NK cells activated in response to IL-1, IL-12, and IL-18 signaling [8]. *L. pneumophila*-infected cells express IL-1, IL-12, and IL-18 through MAP kinase and MyD88 mediated NFκB signaling, which then induce the production of TNFα and IFNγ by adjacent cells [9]. In this study guinea pig morbidity and mortality due to infection with p45-containing *L. pneumophila* were accompanied by enhanced IFNγ expression in the lungs and spleens. This suggests p45-associated mortality of guinea pigs is influenced by an inflammatory response mediated by type II interferon. An increase of IL-12p40 mRNA expression was not associated with the p45 element in this study,

which supports a model in which IL-12 production is modified post-transcriptionally. There are countless other possibilities as to why IL-12p40 expression differences at the transcriptional level would not be detected. Results from this study together with evidence from previous literature suggest a mechanism (illustrated in Figure 16) by which the presence of p45 influences IFNγ-mediated mortality.

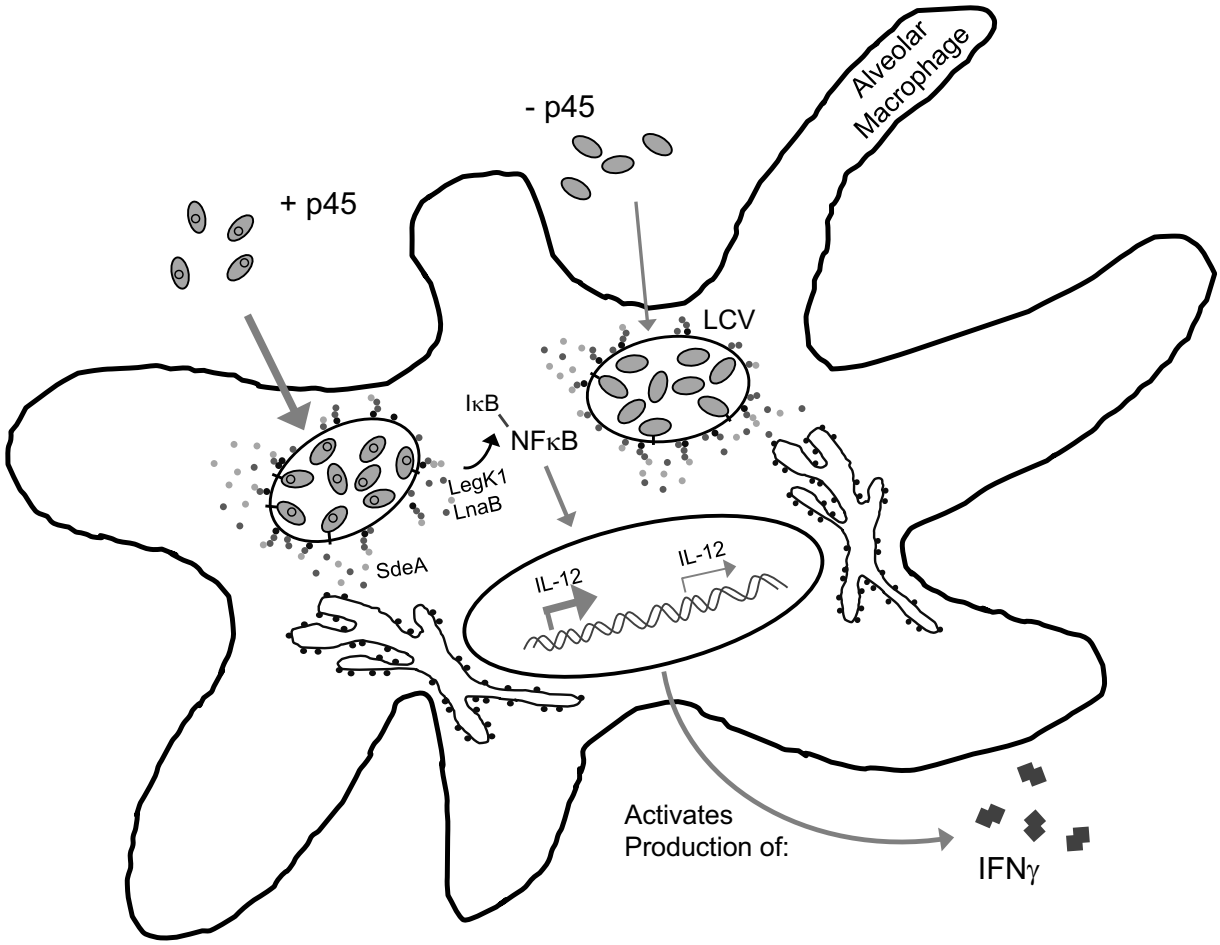

Figure 16. Mechanism by which the p45 element contributes to type II interferon-mediated *Legionella pneumophila* virulence. Upon inhalation *L. pneumophila* enter alveolar cells such as macrophages, those containing p45 (designated as '+ p45') more proficiently than those that do not (designated as '– p45'). Once inside the host cell, *L. pneumophila* secrete effector molecules (depicted as dots) that subvert the host signaling and defense pathways in order to create a replicative niche within the *Legionella*-containing vacuole (LCV). Translocation of these effector molecules occurs via the Dot/Icm type IVb secretion system, however in bacteria that contain p45 the Lvh type IVa secretion system may augment translocation of specific effector molecules. Effector molecules with functions that result in increased production of pro-inflammatory cytokines such as IL-12, then activate NK and T cells that then produce IFNγ. Effector molecules such as SdeA, which make post-translational modifications or LegK1 and LnaB, which destabilize the IκB-NFκB interaction allowing NFκB activated producing IL-12 and other pro-inflammatory mediators for prolonged periods. The subsequent enhanced IFNγ production by hosts infected with p45-containing *L. pneumophila* then leads to excessive inflammation and host death.

The most prominent feature of *L. pneumophila* intracellular pathogenesis is the Dot/Icm type IVb secretion system and the excess of 300 effector molecules it transfers into its hosts in order to subvert the natural signaling pathways and create a replicative niche. A number of these effectors modulate production of pro-inflammatory cytokines including INFγ. The p45 element encodes a type IVa secretion system named the *Legionella* Vir homolog (Lvh) for its similarity to the originally described VirB/VirD type IVa secretion system of the bacterial plant pathogen, *Agrobacterium tumefaciens* [10]. There have been a couple studies that have shown that the Lvh type IVa secretion system augments secretion of certain Dot/Icm effector molecules [11, 12]. One such study found that the Lvh coupling protein, VirD4, interacts with and influences translocation of Dot/Icm effectors including LepB, SdeA, and AnkQ [11]. Importantly, these effectors function in the disruption of host innate signaling pathways, specifically in ubiquitination pathways in the case of SdeA [13]. Therefore, the Lvh type IVa secretion system and effectors known to be translocated by it may be responsible for guinea pig mortality mediated by IFNγ production.

The distinct possibility remains that there may be Lvh effector substrates unidentified that are responsible or contributing to the virulence associated with p45-containing *L. pneumophila*. There are a handful of Dot/Icm effectors described to modulate cytokine and chemokine production both transcriptionally and translationally [14-16], that could be involved in the pro-inflammatory IFNγ response mediated by p45. Two that have been characterized, LegK1 and LnaB, interfere with IκB, which results in prolonged activation of NFκB [17, 18]. There are also effectors that modulate cytokine and chemokine production translationally such as SidI and SidL [9, 19]. A single or combination of effectors like these could then lead to increased production of IFNγ from uninfected bystander cells via production of cytokines such as IL-12, IL-18, and TNFα.

There are certainly also other Dot/Icm and/or Lvh effectors that influence IFNγ expression by similar or alternative mechanisms that are currently uncharacterized.

Aside from secretion systems and effector molecules as prospects for p45-mediated *L. pneumophila* virulence, there is also a non-coding $tRNA^{arg}$ (*lpr0035*) included on p45 that has a role in regulation of pathogenesis and virulence related traits [20]. This element influences RpoS, LepB, and PmrA regulation of numerous traits including host cell entry and Dot/Icm effector expression [20]. Although there has only been one detailed description of *lpr0035* function in *L. pneumophila* virulence, together with implications of the Lvh type IVa secretion system and its confirmed and hypothetical effector substrates provides a conscionable mechanism by which p45 influences IFNγ-mediated mortality. In order to assess the validity of the mechanism described above (Figure 16), additional studies will be necessary.

There are a number of different approaches that could be used to evaluate the role of NFκB signaling in p45-containing *L. pneumophila*-infected cells. Various host cell types such as *A. castellanii* amoeba, U937 monocytic cells, A451 epithelial cells, or primary cells could be used for *in vitro* infections with *L. pneumophila* containing or lacking p45. Initially transcriptional expression of cytokines known to be regulated by NFκB like IL-6 could be assessed by RT-qPCR. Similarly, translation of the same products could be detected by ELISA. To then target specific components of the NFκB signaling pathway, antibody-mediated immunofluorescence by confocal microscopy could be utilized. Nuclear localization of p65 would be an important target for such experiments. In addition to evaluation of the entire p45 element, these experiments could also be conducted with specific knockouts in individual components of p45, such as the non-coding *lpr0035* or Lvh genes.

Experiments to evaluate the proposed mechanism might include *in vitro* host cell infections with p45-containing or p45-lacking *L. pneumophila* with TEM1-effector fusions to assess translocation efficiency along with cytokine and chemokine production. Effectors known to influence cytokine and chemokine production such as LepB, SdeA, AnkQ, SidI, SidL, LegK1, and LnaB would be ideal to include in this type of experiment [11, 17, 18, 21]. Effectors more efficiently translocated by Lvh-encoding bacteria could then be knocked out, either as single or multiple gene mutations, and those strains then utilized for infection of guinea pigs or various pathogenesis experiments.

Contribution of Lvh specifically could be studied by knocking out an essential gene from Lvh in *L. pneumophila* Phil-1 and in trans-conjugate strain, $Lp01^{+p45}$. These strains could be used in the guinea pig aerosol infection model utilized in this study to determine Lvh contribution to *L. pneumophila* virulence. They could then be used for countless translocation and intracellular pathogenesis studies. Interestingly, a large portion of Dot/Icm and effector function studies have been conducted using strain Lp01, diminishing any potential contributions that Lvh may provide to effector translocation. This could either be considered positive or negative depending on the focus of the experiment. In the case of effector function or translocation experiments the lack of Lvh could be beneficial, in that it decreases variables. Although, with respect to natural pathogenesis and virulence experiments, lacking Lvh would be further from physiological relevance and therefore would detract from the study. Either way, results from this study provide important information for future researchers to make better informed decisions for experimental setup and methods.

IV.2 Translational Potential

The p45 integrative conjugative element has previously been associated with *Legionella* species and *L. pneumophila* strains that are pathogenic to humans as compared with innocuous strains [12, 22], which was supported by the results of the current study. To further assess the contribution of the p45 element in *L. pneumophila* virulence, p45 could be introduced via conjugation into *Legionella* species and *L. pneumophila* strains which have previously been considered innocuous. Strains such as *Legionella cherrii*, *Legionella shakespearei*, *Legionella lytica*, or *Legionella moravica* to list a few [23]. The innocuous *Legionella* strains could then be compared with their p45-containing counterpart in their ability to infect and cause disease in guinea pigs infected via aerosol.

There are a number of pathogenic *L. pneumophila* strains that do not include the p45 element such as Corby and Lorraine [24]. These strains do, however, contain mobile integrative elements that encode P-type secretion systems [24-26]. P-type secretion systems are a form of type IVa secretion systems, like Lvh [24, 27]. Under the assumptions of the proposed mechanism depicted in Figure 16 these P-type systems may function similarly to that of Lvh, providing *L. pneumophila* strains such as Corby and Lorainne virulence.

A translational possibility that resulted from this research could be by the development of a water test for detection of pathogenic *Legionella spp.* exclusively, and applied for aerosol-producing water systems. Such a test could target genetic or molecular patterns specific to pathogenic *Legionella spp.*, such as p45 and like elements or molecules produced by them. In addition, there have been a handful of genes identified through computational methods that correlate with disease causing *Legionella spp.* such as the hyp operon and Dot/Icm effectors CegC4 and Lem25 [24]. A combination of pathogen-specific targets used for a rapid detection water test

that could distinguish pathogenic *Legionella* strains from innocuous, in aerosol-producing water reservoirs would radically reduce incidence of LD point source epidemics.

The research completed in this report has ultimately helped elucidate the perplexing nature of virulence of an accidental pathogen. While further analysis will be necessary to gain a more comprehensive understanding of the mechanisms involved in p45-mediated virulence of *Legionella pneumophila*, these results provide an important platform for further inquiry. In addition, the results from this study provide valuable information about a commonly used *L. pneumophila* laboratory strain Lp01, previously thought to display similar phenotypes as its parent strain, Phil-1.

IV.3 References

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APPENDIX A

ADDITIONAL TABLES

A-1 Table. Disease scores used to assess guinea pig morbidity.

	Category (Score Range)[a]		
Score	**Weight Loss (0-4)**	**Respiratory Rate (0-4)**	**General Behavior (0-3)**
0	weight loss of <5%	<10% increase in mean baseline value with normal breathing pattern	normal appearance of fur and skin at nares and extremities; very active, alert, and excitable when observed
1	weight loss of <10%	<25% increase in mean baseline value with normal breathing pattern	ruffled appearance of fur with normal skin color at nares and extremities; moderate activity and alertness when observed
2	weight loss of <15%	<50% increase in mean baseline value with normal breathing pattern	ruffled appearance of fur with normal skin color at nares and extremities; little activity when observed (lethargic)
3	weight loss of >15% but <20%	<100% increase in mean baseline value with heavy/labored breathing pattern	ruffled appearance with of fur with cyanotic appearance of skin at the nares and the extremities; little visible activity (lethargic)
4	weight loss of >20%	>100% increase in mean baseline value with heavy/labored breathing pattern with visible gasping for breath	NA

[a] A maximum score in any individual category or a combined category score of 8 or more indicates the necessity for euthanasia.

A-2 Table. Guinea pig lung histopathological descriptions corresponding to section II.2.2 of Chapter II.

Time (Hours)	Strain	Stage	Frequency	Distribution	Description[a]
24	Lp01	Peracute-Acute	3-5	Focal Peribronchioloar-Random Alveolar	Very mild focal acute neutrophilic alveolitis with numerous Type II plump blue cytoplasm pneumocytes, alveolar macrophage accaccumulations
	Lp01	Peracute-Acute	4-6	Focal Peribronchioloar-Random Alveolar – perivascular	Mild to moderate multifocal acute neutrophilic alveolitis & bronchiolitis with moderate alveolar macrophage & Type II pneumocyte accumulations
	Phil-1	Acute-Subacute	Too numerous to count	Multifocal Bronchioloar, Peribronchiolar, Alveolar	Moderate multifocal acute neutrophilic alveolitis & bronchiolitis with moderate alveolar macrophage & Type II pneumocyte accumulations
	Phil-1	Acute-Subacute	Too numerous to count	Multifocal Bronchioloar, Peribronchiolar, Alveolar	Marked multifocal acute neutrophilic alveolitis & bronchiolitis with moderate alveolar macrophage & Type II pneumocyte accumulations
48	Lp01	Acute-Subacute	18	Multifocal Bronchioloar, Peribronchiolar, Alveolar	Severe acute multifocal purulent bronchiolar and alveolar pneumonia with massive PMNs, abundant alveolar macrophages & Type II pneumocytes
	Lp01	Acute-Subacute	30+	Diffuse Bronchial, Bronchioloar, Peribronchiolar, Alveolar	Profound acute multifocal purulent bronchial, bronchiolar and alveolar pneumonia with massive PMNs, massive alveolar macrophages & Type II pneumocytes
	Lp01	Subacute - Chronic?	30+	Diffuse Bronchial, Bronchioloar, Peribronchiolar, Alveolar	Profound acute multifocal purulent bronchial, bronchiolar and alveolar pneumonia with massive PMNs, massive alveolar macrophages & Type II pneumocytes, alveolar fibrosis

[a] Diagnoses were determined by a board-certified veterinarian from H&E stained lung sections from guinea pigs infected with *L. pneumophila* via aerosol transmission.

A-2 Table. (Continued) Guinea pig lung histopathological descriptions corresponding to section II.2.2 of Chapter II.

Time (Hours)	Strain	Stage	Frequency	Distribution	Description[a]
48	Phil-1	Subacute - Chronic?	Too numerous to count	Diffuse, Bronchioloar, Peribronchiolar, Alveolar	Profound acute multifocal purulent bronchiolar and alveolar pneumonia with massive PMNs, massive alveolar macrophages & Type II pneumocytes, with progressive alveolar fibrosis
	Phil-1	Chronic	Too numerous to count	Diffuse, Bronchial Bronchioloar, Peribronchiolar, Alveolar	Profound acute diffuse lobar purulent bronchial, bronchiolar and alveolar pneumonia with massive PMNs, massive alveolar macrophages & Type II pneumocytes, with advanced progressive alveolar fibrosis
	Not Infected	N/A	2	Alveolar	One small microgranuloma with birefringent material (foreign body/chitin?) & one very focal alveolar focus

[a] Diagnoses were determined by a board-certified veterinarian from H&E stained lung sections from guinea pigs infected with *L. pneumophila* via aerosol transmission.

A-3 Table. Guinea pig lung histopathology descriptions corresponding to section II.2.5 of Chapter II.

Infection Strain	Morphologic Diagnosis[a]
Phil-1	severe, coalescing bronchopneumonia with atelectasis; severe diffuse alveolar damage with pulmonary edema, and hemorrhage
Phil-1	moderate, multifocal bronchopneumonia with minimal atelectasis; moderate diffuse alveolar damage with pulmonary edema, and hemorrhage
Phil-1	severe, coalescing bronchopneumonia with atelectasis; moderate diffuse alveolar damage with pulmonary edema, and hemorrhage
Phil-1	severe, coalescing bronchopneumonia with atelectasis; moderate diffuse alveolar damage with pulmonary edema, and hemorrhage
Lp01	moderate, multifocal bronchopneumonia; mild, diffuse alveolar damage with pulmonary edema, and hemorrhage
Lp01	severe, coalescing bronchopneumonia with atelectasis; moderate, diffuse alveolar damage with pulmonary edema, and hemorrhage
Lp01	moderate, coalescing bronchopneumonia with atelectasis; moderate, diffuse alveolar damage with pulmonary edema and minimal hemorrhage
Lp01	mild, multifocal bronchopneumonia with atelectasis; mild, diffuse alveolar damage with pulmonary edema
$Lp01^{+p45}$	mild, multifocal bronchopneumonia with atelectasis; mild, diffuse alveolar damage with pulmonary edema
$Lp01^{+p45}$	mild, multifocal bronchopneumonia; minimal, diffuse alveolar damage with pulmonary edema
$Lp01^{+p45}$	mild, multifocal bronchopneumonia with minimal atelectasis; mild, diffuse alveolar damage with pulmonary edema
$Lp01^{+p45}$	mild, multifocal bronchopneumonia with atelectasis; mild, diffuse alveolar damage with pulmonary edema and minimal hemorrhage
Uninfected	normal

[a] Diagnoses were determined by a board-certified veterinarian from H&E stained lung sections from guinea pigs 48 hours post-infection with *L. pneumophila* via aerosol transmission.

A-4 Table. Oligonucleotides.

Primer Name (RE cut site)	Nucleotide Sequence 5'-3'	Target Gene ID[a]	Target Gene/Region	Target Organism
Cloning				
P45inst-F (*AfeI*)	ATATAGCGCTAGGAGCTAAATGTGCAGC	*lpg1237*	p45	L. p. Phil-1
P45inst-R (*BamHI*)	ATATGGATCCTTTGCTCCCGAAAGCCTG			
PCR				
P45t2RE-F1 (*BamHI*)	TATAGGATCCTCCAAGCCAGAAAAATG	*lpg1237*	p45	L. p. Phil-1
P45t2RE-R1 (*AatII*)	TATAGACGTCTGATGGAGTGAAGGGTTG			
P45-262F1	CGTCGACATCGATAAGCTTCG		pJDC305/p45	L. p. Phil-1/p45
P45-262R1	GGCAGGTGTGTTATTGCTATGC			
attPF[b]	AAGGCAATCCTGTTCGCATAC		*attP*/p45	L. p. Phil-1/p45
attPR	TCTCCAAGCCACCAATAAAGC			
LppF[c]	TATATTGATGGCGGTGCTGCC		*attB*	L. p.
LppR	ATCGAGATTGAGTTTACATCCC			
Lp01rpsL-F	GCACACCAGGTAAATCCC	*lpg0324*	*rpsL*	L. p. Lp01[d]
Lp01rpsL-R	AACGTGATGGTAGGTCGC			
qPCR				
qP45-F	TGATGCATCAAGCTATCGCG			L. p. Phil-1/p45
qP45-R	ATACCACTTCTGCCAATGCG			
qCRM-F	AAGGTATCGGAAACCGAACC			L. p.
qCRM-R	CTTCCTGGATAAAGAGGAGC			
Sequencing				
rpsLMut-F	TCCATAACAGACAGCGCACC	*lpg0324*	*rpsL*	L. p.
rpsLMut-R	AGACGCGGTGTATGTACTCG			
rpsL-N-F	CGTTGATGAACTTAGCAAGCAGTTC			
Phil-ndh-F3 (*AatII*)	ATATGACGTCAAGCACCGCCCTGTTTTGC	*lpg0671*	*ndh*	L. p.
Phil-ndh-R3 (*NheI*)	ATATGCTAGCTTTAGCCTTGATGGTTTGCC			
ndh-N-F	TCACACCATCGCTATCAATTTGC			
hpMut-F	GATACTGGCGGATGGTTTGG	*lpg0716*	*h.p.*	L. p.
hpMut-R	CAGTGGCAGTACCACTTTCG			
hp-N-F	CATCACTCTCAACTTCAGCAGAG			
gspMut-F	CTGCCAACTTAATGGCAACC	*lpg0718*	*gsp*	L. p.
gspMut-R	ACCATTGTCGCTATGGGATC			
gsp-N-F	ACCAAATCCATGCGCATCCC			
luxNMut-F	CTATCTCGTGCAAACAAGGC	*lpg2506*	*luxN*	L. p.
luxNMut-R	AACTGAAGATGCAAGCCACC			
luxN-N-F	GGCACCAATGATTATTGCTGC			
ftsEMut-F	CACCAATAGATGCTGAGGCG	*lpg2669*	*ftsE*	L. p.
ftsEMut-R	AAGTAACCCATGGCCAGCAC			
ftsE-N-F	GGAAGAGTCCCCAGAGGAGC			

[a] AE017354.1 [31].
[b] Used with LppR to detect *attP/attB* junction.
[c] Used with attPR to detect *attB/attP* junction.
[d] Detects G residue in Lp01 *rpsL* as compared with A residue in Phil-1 [27].

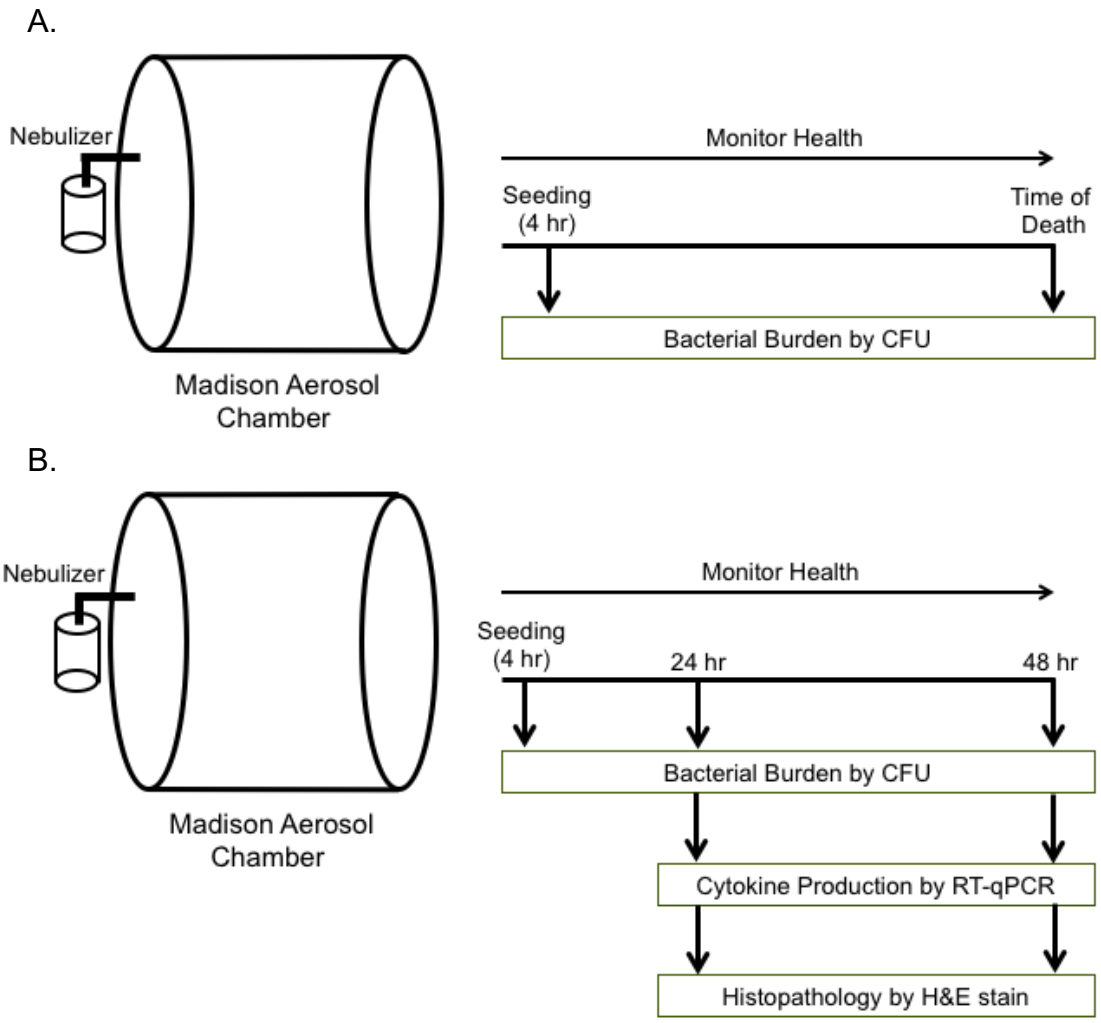
A-4 Table. (Continued) Oligonucleotides.

Primer Name (RE cut site)	Nucleotide Sequence 5'-3'	Target Gene ID[a]	Target Gene/Region	Target Organism
RT-qPCR				
qLvhB8-F	TGATGCATCAAGCTATCGCG	*lpg1249*	*lvhB8*	L. p. Phil-1/p45
qLvhB8-R	ATACCACTTCTGCCAATGCG			
qAttB-F	GCTATAAGGGTAGACTTGCG		*attB*	L. p.
qAttB-R	TAAAGCAAGGGTGCTTTAGG			
qAttP-F	TTGCATAGAGACACATGGAG		*attP*	L. p. Phil-1/p45
qAttP-R	TTTGCACCCTGAGTGCAC			
qGyrB-F	AGCATCATCACCTGTTGGAG	*lpg0004*	*gyrB*	L. p.
qGyrB-R	CCATGCAATGGAATGATGGC			
qMEase-F	AAGCGAATAGATGCCTACGC	*lpg1266*	methylase	L. p. Phil-1/p45
qMEase-R	AGGGAGTATGATGGCATTGC			
qLvhB4-F	TGGAATTGCCAGTGAAACCC	*lpg1254*	*lvhB4*	L. p. Phil-1/p45
qLvhB4-R	GAGGTAATCAGAGAGGCTCG			
qTraA-F	GCACCACTTTACTACCTGCC	*lpg1241*	*traA*	L. p. Phil-1/p45
qTraA-R	TATCCAAGCAGGGATGGAGG			
qSSR-F	ATGCCGTAAATCATGCCAGC	*lpg1229*	recombinase	L. p. Phil-1/p45
qSSR-R	AGCAGTTCGCGCTATATACC			
TGFb1-GP-F	CATCGATATGGAGCTGGTGAAG		TGF-β1	Guinea Pig
TGFb1-GP-R	GCCGTAATTTGGACAGGATCTG			
TNFa-GP-F	CCTACCTGCTTCTCACCCATACC		TNF-α	Guinea Pig
TNFa-GP-R	TTGATGGCAGAGAGAAGGTTGA			
IFNg-GP-F	ATTTCGGTCAATGACGAGCAT		IFN-$\boldsymbol{\gamma}$	Guinea Pig
IFNg-GP-R	GTTTCCTCTGGTTCGGTGACA			
CCL5-GP-F	CTGGCCCACTGCTTAGCAAT		CCL5	Guinea Pig
CCL5-GP-R	CCTTGCTTCTTTGCCTTGAAA			
IL12p40-GP-F	CCACAGTTTCATGCCACAAGA		IL-12p40	Guinea Pig
IL12p40-GP-R	CCATTCGCTCCACGATGAG			
IL8-GP-F2	TAGGGTGGCAGATTTAACTCA		IL-8	Guinea Pig
IL8-GP-R2	TCAGGAATTGGCTTGCTAC			
HPRT-GP-F	AGGTGTTTATCCCTCATGGACTAATT		HPRT	Guinea Pig
HPRT-GP-R	CCTCCCATCTCCTTCATCACAT			

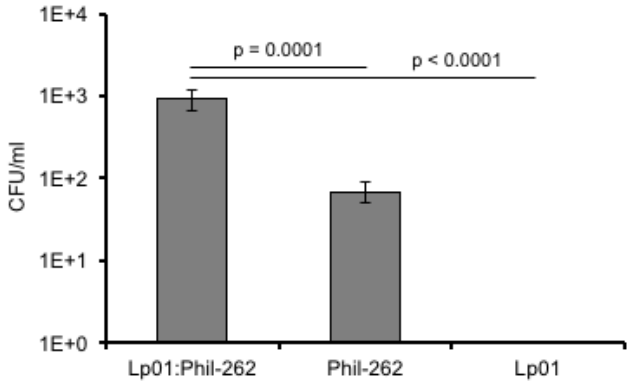
[a] AE017354.1 [31].
[b] Used with LppR to detect *attP/attB* junction.
[c] Used with attPR to detect *attB/attP* junction.
[d] Detects G residue in Lp01 *rpsL* as compared with A residue in Phil-1 [27].

APPENDIX B

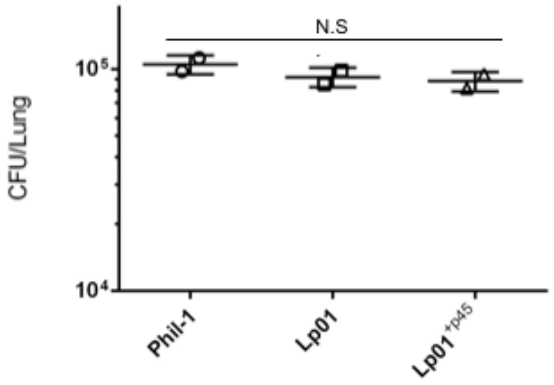
ADDITIONAL FIGURES



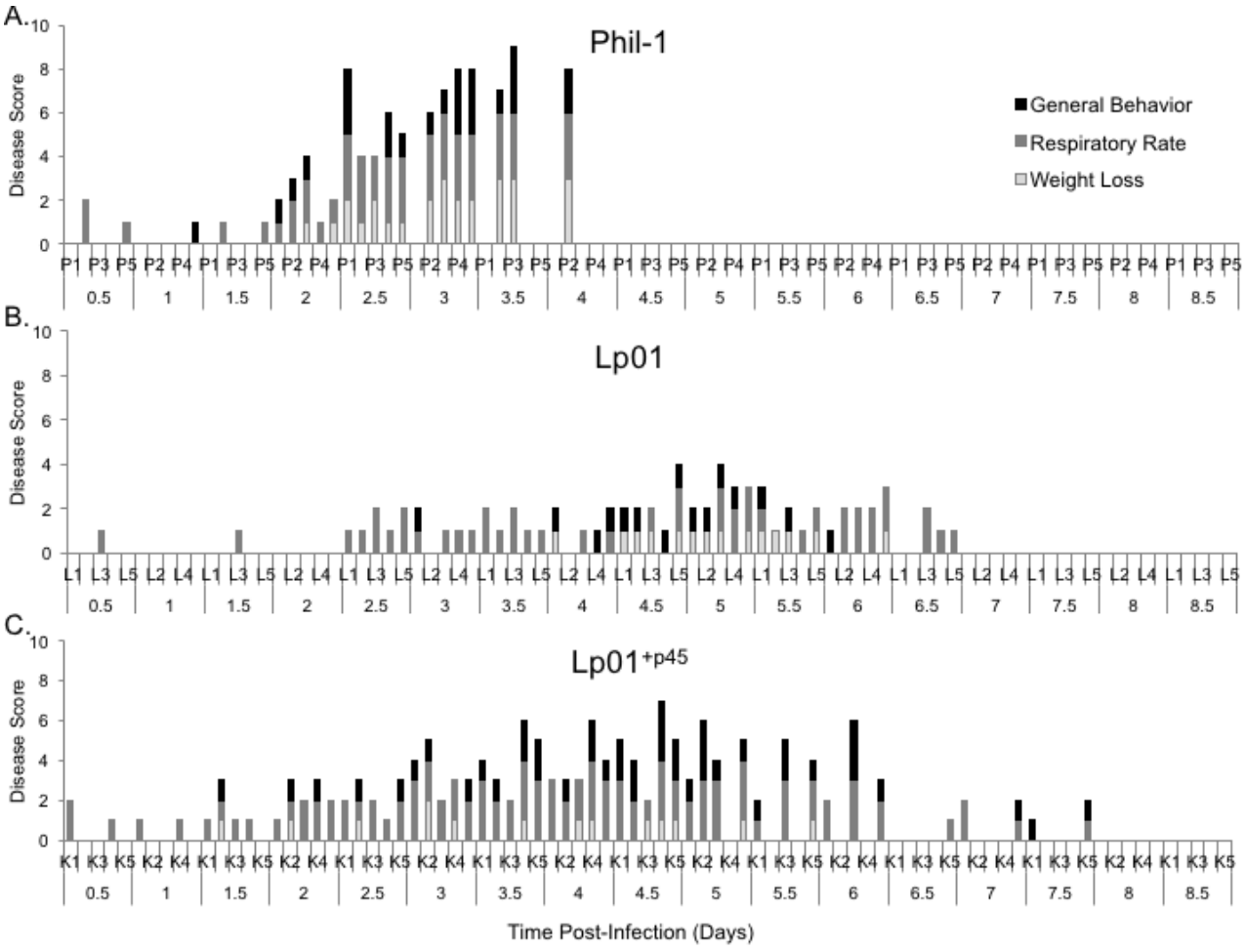
B-1 Figure. Experimental design of aerosolized infection of guinea pigs. *L. pneumophila* cultures suspended in sterile ddH_2O were delivered from the nebulizer to guinea pigs located within the Madison Chamber for 10 minutes of exposure. Two animals per infection group were euthanized 4 hours after infection and evaluated for seeding CFU in the lungs. Following infection animals' morbidity was monitored twice daily throughout the course of the experiment using the score system described in A-1 Table. In the case of survival experiments (A), bacterial burden in the lungs and spleens was determined by CFU at the time of death. In the case of pathological experiments (B), animals were euthanized 24 or 48 hours after infection, at which point the lungs and spleens were harvested and prepared for bacterial burden, cytokine expression, and histopathology.



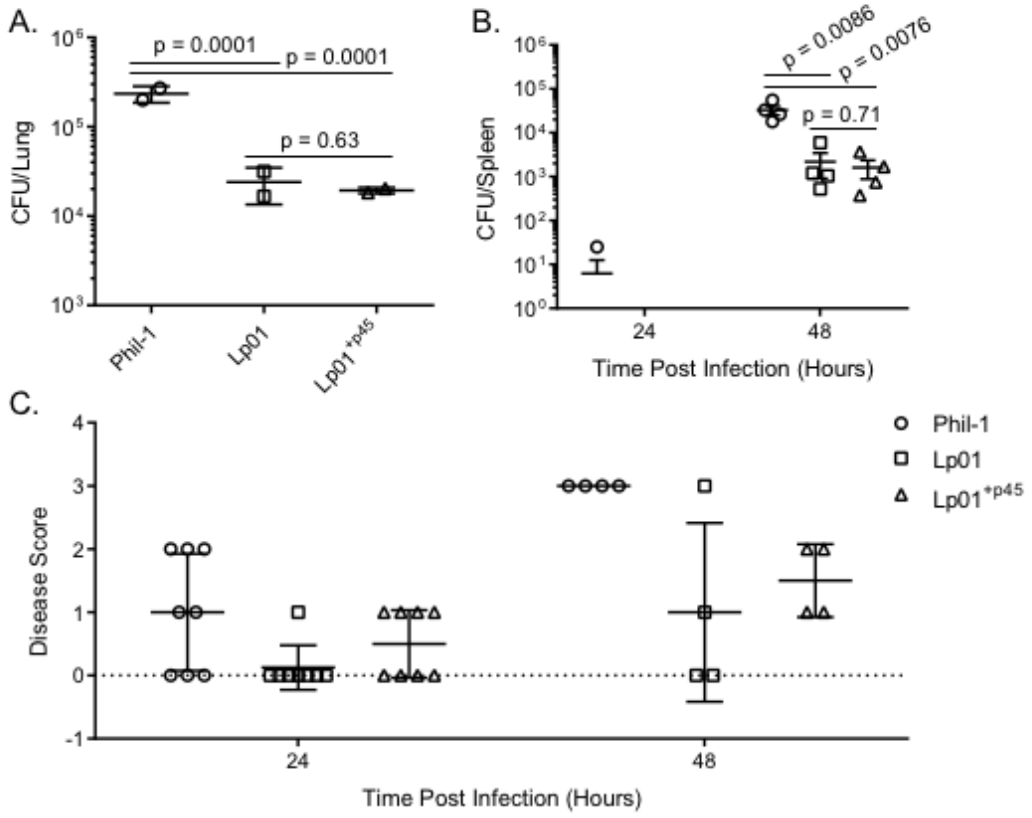
B-2 Figure. p45 conjugation from *Legionella pneumophila* Phil-262 to Lp01. The number of CFU/ml that grow on BCYE containing kanamycin (25 μg/ml) and streptomycin (200 μg/ml) following conjugation of *L. pneumophila* cultures containing 1:1 Phil-1:Lp01, Phil-1 alone, or Lp01 alone. This data corresponds to that of Chapter II, Figure 3. Data points and error bars represent group averages and standard deviation, resectively.



B-3 Figure. Seeding of *Legionella pneumophila* in guinea pig lungs. Seeding bacterial burden in lungs of guinea pigs 4 hours post aerosol infection with *L. pneumophila* Phil-1, Lp01, or Lp01^{+p45}. This data corresponds to that of Chapter II, Figure 5. Markers and error bars represent individual animals and group standard deviation respectively.



B-4 Figure. Disease scores of individual guinea pigs over the course of infection. Disease scores in each category as detailed in A-1 Table of individual animals infected via Madison aerosol chamber with *L. pneumophila* Phil-1 (A), Lp01 (B), or Lp01^{+p45} (C). This data corresponds to that of Chapter II, Figure 5.



B-5 Figure. Guinea pig immune response to *Legionella pneumophila* infection. (A) Seeding bacterial burden in lungs of guinea pigs 4 hours post aerosol infection with *L. pneumophila* Phil-1, Lp01, or Lp01^{+p45}. (B) Bacterial burden in guinea pig spleens at 24 and 48 hours post infection. (C) Disease score totals of guinea pigs infected with Phil-1, Lp01 or Lp01^{+p45}. This data corresponds to that of Chapter II, Figures 6-8. Markers and error bars represent individual animals and group standard deviation respectively.

APPENDIX C

NOMENCLATURE

ATCC	American Type Culture Collection
BCYE	Buffered Charcoal Yeast Extract
BYE	Buffered Yeast Extract
cDNA	chromosomal Deoxyribonucleic Acid
CFU	Colony Forming Units
C_T	Cycle Threshold
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
G+C	Guanine Cytosine
gDNA	genomic Deoxyribonucleic Acid
H&E	Hematoxylin and Eosin
HI	Heat Inactivated
IACUC	Institutional Animal Care and Use Committee
ICE	Integrative Conjugative Element
IFN	Interferon
IL	Interleukin
LCV	*Legionella*-Containing Vacuole
LD	Legionnaires' Disease
MGE	Mobile Genomic Element

MIE	Mobile Integrative Element
MOI	Multiplicity of Infection
mRNA	messenger Ribonucleic Acid
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	potential of Hydrogen
qPCR	quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
rRNA	ribosomal Ribonucleic Acid
RT	Room Temperature
RT-qPCR	Real Time quantitative Polymerase Chain Reaction
SS	Secretion System
tRNA	transfer Ribonucleic Acid
T2SS	Type II Secretion System
T4aSS	Type IVa Secretion System
T4bSS	Type IVb Secretion System
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor
UIC	Uninfected Control