Fall 2014

Host cell death in Legionella pneumophila pathogenesis and immunity

Wenhan Zhu
Purdue University

Graduate School Form 30
(Revised 08/14)

PURDUE UNIVERSITY
GRADUATE SCHOOL
Thesis/Dissertation Acceptance

This is to certify that the thesis/dissertation prepared

By Wenhan Zhu

Entitled
Host Cell Death in Legionella pneumophila Pathogenesis and Immunity

For the degree of Doctor of Philosophy

Is approved by the final examining committee:

Zhao-Qing Luo

Daoguo Zhou

Peter J. Hollenbeck

Mark C. Hall

To the best of my knowledge and as understood by the student in the Thesis/Dissertation Agreement, Publication Delay, and Certification/Disclaimer (Graduate School Form 32), this thesis/dissertation adheres to the provisions of Purdue University's "Policy on Integrity in Research" and the use of copyrighted material.

Zhao-Qing Luo

Approved by Major Professor(s): _____

_____

Approved by: Richard J. Kuhn 12/01/2014

Head of the Department Graduate Program Date

HOST CELL DEATH IN *LEGIONELLA PNEUMOPHILA* PATHOGENESIS AND IMMUNITY

A Dissertation
Submitted to the Faculty
of
Purdue University
by
Wenhan Zhu

In Partial Fulfillment of the
Requirements for the Degree
of
Doctor of Philosophy

December 2014
Purdue University
West Lafayette, Indiana

ACKNOWLEDGEMENTS

I would like to express my most sincere gratitude to my family, especially to my mother. My mother is the greatest mother in the world. She made countless sacrifices so I could pursue my career and have a better life. She has always been patient, loving and supportive in the past 29 years of my life. She set me admirable examples for never to give up when the times get tough, dust up and move on when the situation is less than perfect. She also taught me to be kind, understanding and considerate to everyone around me. She is my role model, without whom I will not be able to complete this thesis, let along to obtain the degree. She is the most important reason why I work hard for my future, so I can pay her back with the best I could. I would also thank my father, who constantly encourage me to do the best; my grandmother, who is always loving and nourished me ever since I was a kid.

I would like to thank my wonderful advisor, Dr. Zhao-qing Luo, who had been immensely supportive to my career at Purdue University. He is a passionate person when it comes to science. He could not sleep without knowing the result of the assay he set up and he will literally jump into the sky when he or we had exciting discoveries. His passion inspired many people including me to pursue scientific career with the burning passion. He sets up a hard-working and rewarding research environment in the lab, and always encourages lab members to explore more possibilities. Dr. Luo has high standards for his lab members in reading, understanding, connecting, and writing scientific literatures, and goes out of his way to help his lab members to achieve and surpass these standards. I

deeply appreciate his devotion of tremendous amount of time and patience out of his incredibly busy schedule into going through my thesis word by word. From grammatical errors to logical organization, Dr. Luo sat with me and patiently guide me through these problems. He set up a wonderful example for me to pursuit my career in academia.

I would also dedicate my gratitude to Dr. Yunhao Tan, who is a former labmate and good friend of mine. We studied at the same college and pursued our Ph.D degree in the same university under the same advisor. He is the most passionate and energetic scientist I have ever come across. He works more than 12 hours in lab and runs at least five miles every day. The amount of work he had done in lab is simply unbelievably large. He is simply a machine that loves science with a passion. He showed me the path of being a successful scientist is paved with hard work, and this notion continues to inspire me till today. I would also thank my former labmate Dr. Yancheng Liu, Dr. Li Xu, who are both very knowledgeable and kind. They taught me a lot when I started in Dr. Luo's lab. I also appreciate the companion of Chelsey Dankenbring, who is always funny and make everyone around her laugh. Dr. Lili Tao, who is also a brilliant physician, had brought much fun and laughter to lab. She is smart, funny, and simply fantastic at what she does. Dr. Tao is also very generous at sharing her knowledge and expertise. I can still remember her teaching me to dissect mouse to isolate femur bones. She finished the process with such incredible efficiency and precision that no tissue, blood vessels and what so ever was damaged at all throughout the whole process. Of course, also unforgettable is her laughing at me when I was performing the same procedure, which gave me "much confidence" in the days that followed. She also brought the project that became my main focus later, which I owed tons of thanks for.

Dr. Jiazhang Qiu is a fantastic labmate. He is a person that is easy to approach, lots of fun, and a great mentor. He is known to have a great pair of

hands, which produce the most beautiful experimental results on the first attempt. His immunostaining is so gorgeous that it reminds one the stars in the sky on a beautiful night. He is also a good friend who understands my frustration both in lab and life, gives me constant encouragement and extremely useful advices, and has the magical ability to turn my bad day down-side-up. For that, I would like to express my most sincere gratitude to Dr. Jiazhang Qiu.

I am truly grateful that I have three wonderful committee professors. Dr. Zhou is very passionate, kind yet critical when I present my research projects, which benefits me greatly over these years. Dr. Hollenbeck is extremely knowledgeable, always provides extremely insightful advices and always offers to help me in my projects. My project on VipD will not be completed without his generous help in quantitative microscope. Dr. Hall provided me with lots of helpful comments and great help in yeast genetics.

I would also like to thank Dr. Mattoo, the expert in hardcore biochemistry, and Dr. Deng, who knows everything in fish immunology, for their generosity in providing insightful advices and equipment. The members in Luo Lab, Zhou Lab, Mattoo Lab and Deng Lab are awesome, as they had gave me much help and joy over my six years in this department. I am truly grateful for know these wonderful people.

TABLE OF CONTENTS

Page

Page

Page

Page

NOTE

Chapter I, II, and III of the thesis contain contents of the following published units:

1. Zhu, W., et al., *Comprehensive identification of protein substrates of the Dot/Icm type IV transporter of Legionella pneumophila.* PLoS One, 2011. **6**(3): e17638
2. Zhu, W., et al., *Induction of caspase 3 activation by multiple Legionella pneumophila Dot/Icm substrates.* Cellular Microbiology, 2013. **15**(11):p. 1783-95.
3. Tao, L., et al., *Induction of rapid cell death by an environmental isolate of Legionella pneumophila in mouse macrophages.* Infect Immun, 2013. **81**(9): p. 3077-88.
4. Zhu, W., et al., *Sensing cytosolic RpsL by macrophages induces lysosomal cell death and termination of bacterial infection*. Manuscript under peer review.

ABSTRACT

Zhu, Wenhan Ph.D., Purdue University, December 2014. Interactions between Host Cell Death Pathways and *Legionella pneumophila.* Major Professor: Zhao-Qing Luo.

Legionella pneumophila is an intracellular pathogen that causes a severe, atypical pneumonia termed Legionnaires' disease. Upon entering the host cell, *L. pneumophila* resides in a membrane-bound vacuole, in which the bacterium evades lysosomal fusion and replicates. The establishment of the vacuole requires the Dot/Icm (Defect in organelle trafficking/intracellular multiplication) transport system, which translocates a large number of substrates into host cells to re-orchestrate various cellular processes, such as intracellular trafficking, protein synthesis and host cell death pathways. Therefore, a key step in understanding the biology of *Legionella* is to dissect the mechanisms of action of the Dot/Icm substrates. A large number of Dot/Icm substrates with diverse biochemical functions have been identified by various strategies, based on one or more characteristics associated with the candidate proteins. Given the high level of diversity exhibited by the identified substrates, it is possible that some substrates have been missed in these screenings. The "missing" substrates will certainly hinder further studies of *L. pneumophila* pathogenesis. To solve this problem, we took a systematic approach to test Dot/Icm-dependent translocation of *L. pneumophila* hypothetical proteins larger than 100 residues. By fusing the gene of interest with β-lactamase, we determined the transfer of the fusions into mammalian cells using the β-lactamase reporter substrate CCF4-AM. 164 Dot/Icm substrates were identified in this screening, which led to the expansion of the list of known Dot/Icm substrates by 70 effectors. These efforts have

facilitated future studies on the biology of *L. pneumophila* and its interaction with hosts.

As an important determinant of the outcome of infection, cell death pathways are the target of *L. pneumophila*. Surprisingly, permissive macrophages challenged by *L. pneumophila* are resistant to exogenous cell death stimuli despite harboring high levels of active caspase-3. How this bacterium achieves such a balance in these cells remains elusive. Several reports have described *L. pneumophila* effectors that contribute to pro-survival signaling cascades, however, the Dot/Icm substrates that are responsible for caspase-3 activation has not been documented, nor was the mechanisms underlying such activation. Taking advantage of the expanded list of *L. pneumophila* Dot/Icm effectors, we initiated a screening to survey all known effectors for the ability to activate caspase-3. Our screening led to the discovery that at least five Dot/Icm substrates, Lpg0716, Lpg0898, Lpg1831, Lpg1625 and Lpg2716, activate caspase-3 when ectopically expressed in mammalian cells. We further demonstrated that one of these effectors, VipD (lpg2831), is a phospholipase A2 that hydrolyzes the phosphatidylethanolamine (PE) and phosphatidylcholine (PC) on the mitochondrial membrane in a manner that appears to require host cofactor(s). The lipase activity is essential to its ability to activate caspase-3 via the production of free fatty acids and 2-lysophospholipids, which destabilize the mitochondrial membranes. Mitochondria membrane destabilization may contribute to cytochrome c release and subsequent activation of caspase-3. Moreover, whereas caspase-3 activation does not seem to play significant roles in limiting *L. pneumophila* replication in permissive macrophages, it is essential to restricting bacterial growth in dendritic cells. We further demonstrated that genetic ablation of all five caspase-3 activating effectors dampens apoptosis activation in dendritic cells. Our result demonstrate that the activation of host apoptosis pathway is a collective effects of multiple effectors with diverse biochemical activities.

Host cell death is an integrated component of the immune defense against infections. Although amazing strides have been made in how the innate immune system responds to *Legionella* at the interface of host cell death, most if not all of these knowledge was acquired using laboratory strains derived from a few clinical isolates. To investigate how primary macrophages respond to *Legionella* environmental isolates, which are responsible for most of the Legionnaires' disease outbreaks so far, we analyzed the intracellular growth of a few such isolates in A/J mouse macrophages, which is permissive to most, if not all, laboratory strains. None of the *Legionella* environmental isolates were able to replicate in these macrophages. Further investigation into one of the isolates, LPE509, revealed that such growth restriction is not due to the lack of important pathogenic determinants, as this strain is competent of replication in two protozoan hosts and the human macrophage U937 cell. Moreover, the inability of LPE509 to replicate intracellularly is accompanied by host cell death of unique features, as it is independent of most cell death signaling components known to control *Legionella* infection. In an attempt to screen for the factor(s) of LPE509 responsible for host cell death and growth restriction, we found that a spontaneous streptomycin resistant mutant had gained the ability to overcome the growth restriction in primary mouse macrophages. Analysis of the mutant strain showed a lysine to arginine substitution on position 88 of the 30S ribosome small subunit S12 (RpsL, lpg0324) severely attenuates the host cell death induction and enables the bacterium to replicate in macrophages. However, another spontaneous streptomycin-resistant mutation (Lys 43 to Asn) of *rpsL* did not allow efficient replication of LPE509 in A/J macrophages, suggesting that streptomycin per se is not the cause of replication restriction of LPE509. Further sequence analysis revealed that commonly used laboratory strains such as Lp02 and JR32 also harbor the K88R allele of *rpsL*. Replacing $rpsL_{K88R}$ allele with its wild type counterpart completely abolishes the ability of these strains to replicate in A/J mouse macrophage, suggesting that *rpsL* governs the intracellular fate of *L. pneumophila*. These results together with the conservation of *rpsL* gene across

diverse bacterial species prompted us to test whether the RpsL protein *per se* is responsible for the induction of host cell death. Indeed, delivery of RpsL into mouse macrophages elicits a noncanonical form of cell death involved in the permeabilization of lysosomal membranes. Upon the loss of lysosome membrane integrity, various hydrolases are released into the cytosol, where they process diverse substrates and cause cell death. Amongst these hydrolases, cathepsin B plays an important role in RpsL induced cell death, as genetic ablation of this gene dampens host cell death induced by recombinant $RpsL_{WT}$ or *Legionella* expressing wild type RpsL. Taken together, our results suggested that RpsL is a microbial associated molecular pattern (MAMP) that triggers a unique cell death pathway in mouse macrophages, which limits the spread of invading bacterial pathogens.

CHAPTER I: GENERAL INTRODUCTION

Legionella and Type IV Secretion System

In 1976, during the American Legion Convention, 221 American veterans were affected by a mysterious infectious disease, which claimed 34 lives. Three years later, the causative agent for this highly concerning outbreak was identified and designated as *Legionella pneumophila,* which is a gram-negative bacterium that belongs to the γ-proteobacteria [1]. Further studies revealed that *L. pneumophila* is a facultative intracellular pathogen that infects a wide range of hosts, from fresh water amoebae to mammalian alveolar macrophages [2]. Inhalation of *L. pneumophila* contaminated aerosols by immune-compromised individuals can result in an atypical acute pneumonia known as Legionnaires' disease [3]. Early studies of *L. pneumophila* pathogenesis using techniques such as electron microscopy revealed many interesting features associated with infection of this bacterium. For example, upon phagocytosis by macrophages, the phagosome containing *L. pneumophila* does not undergo the canonical pathway of lysosomal fusion for degradation; rather it forms an organelle, the *Legionella* containing vacuole (LCV), which recruits selected host proteins and lipids to construct a niche permissive for bacterial replication (**Fig. 1-1**) [4-6].

To better understand the pathogenesis of the *L. pneumophila*, genetic screenings were initiated to identify genes that are essential for this bacterium to replicate intracellularly. By passing *L. pneumophila* in rich medium, which potentially relives the bacterium from selection pressure to infect eukaryotic hosts, Horwitz obtained a mutant named 25D, which had lost the ability to replicate in human monocyte and escape phagosome-lysosome fusion [7]. The Shuman

group later attempted to complement the growth defect of the 25D mutants with genomic fragments of the wild type bacterium to identify the genetic elements that are important for the bacterial intracellular replication [7]. These efforts led to the identification of the *icm* (defect in intracellular multiplication) genes [7]. On the other hand, based on the observation that thymidine-auxotrophs of *L. pneumophila* showed decreased survival in human monocytes in the absence of exogenous thymidine, the Isberg group reasoned that mutants that are also defective in intracellular replication should survive better, because such strains would need less thymidine for replication [8]. Utilizing the “thymidineless death” strategy to enrich *L. pneumophila* mutants that are unable to replicate intracellularly, the Isberg group generated a Tn-*5* based transposon library of Lp02 [9], a thymidine auxothroph of *L. pneumophila*, and screened for mutants that survive thymidine starvation. Analysis of the genomic fragments that could complement the intracellular replication defects of these mutants revealed that the *dot* (defect in organelle trafficking) genes are important for the intracellular replication and subversion of host organelle trafficking [10]. Taking advantage of the phenotypic link between resistance to low amount of sodium and the inability to replicate in macrophages, the same group obtained a total of 26 *L. pneumophila* mutants that are incapable of multiplying intracellularly [11]. It is worth noticing that most of the *dot* and *icm* genes are identical, and the sum of these genes was proposed to assemble into an apparatus essential for *L. pneumophila* infection. Although genetic inactivation of many of these genes leads to impaired organelle recruitment and defects in intracellular multiplication, the functions of these genes mutated in the mutants were elusive for a few years.

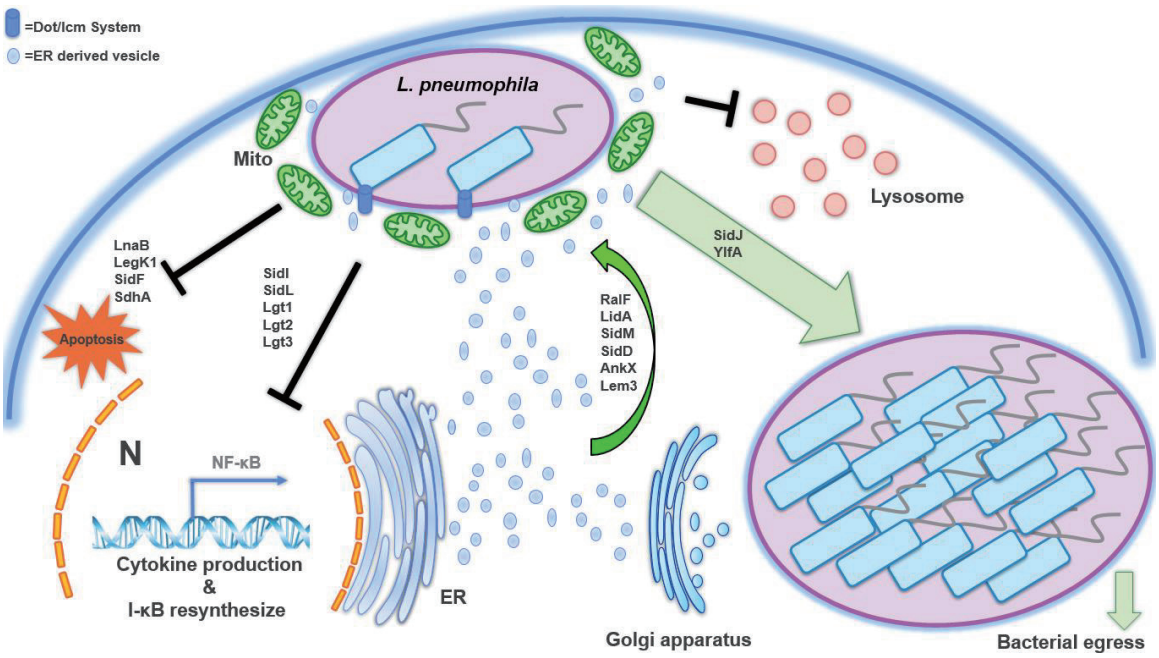


Fig. 1-1 Life cycle of *L. pneumophila* Upon phagocytosis, *L. pneumophila* injects a large number of Dot/Icm substrates to manipulate numerous host processes, such as evasion of lysosomal fusion and recruitment of ER-derived vesicles, to establish a replicative vacuole. The bacterium replicates in the vacuole to high numbers before lysing the host cells and egress.

Further analysis revealed that the Dot/Icm proteins are required for transferring DNA such as the IncQ plasmid RSF1010. Such capability, together with the association of virulence, and the limited homologies of some Dot/icm proteins with the structural components of the *Agrobacterium tumefaciens* T-DNA transfer system, prompted researchers to postulate that the Dot/Icm system proteins assemble into a complex with conjugation activity, which belongs to the Type IV Secretion System (T4SS) [12]. Shortly after Vogel *et al* proposed the possible origin of the Dot/Icm system, the sequence of the *Shigella* Collb-P9 IncI conjugative plasmid became available [13, 14]. The close resemblance at both gene sequences and organization level between the Dot/Icm genes and the *tra/trb* genes on the Collb-P9 plasmid prompted Segal and Shuman to propose that the Dot/Icm system should be defined as a Type IVb secretion system, while the machines that are similar to the *A. tumefaciens* VirB system being Type IVa [13, 15].

The molecular functions of the Dot/Icm components were mostly revealed through 1) analyzing the phenotypes or 2) the change of Dot/Icm macro-molecular structure associated with the genetic ablation of one or more of these components, or 3) the physical interactions between different components or substrates of the system. For example, deleting IcmF and IcmH decreases the protein level of DotH and DotG, suggesting that IcmF/H may function to regulate the homeostatic levels of DotH and DotG [16]. Further biochemical and genetic analysis suggested that DotG forms the central tunnel of the apparatus, with DotC and DotD being the outer membrane components [17]. Advances in electron microscopes have made visualization of multi-protein, macro-molecule machinery such the bacterial translocation system possible [18]. A recent transmission electron microscopy analysis of *Legionella* Dot/Icm system demonstrated the ring-shaped structure of the core complex. This report also showed the importance of DotC, D, H, G and DotF in Dot/Icm system, as the Dot/Icm could not form the ring-shaped structure in the absence of DotC/D and H,

and forms a premature and distinct structure in the absence of DotG or DotF [19]. Analysis of other Dot/Icm components such as IcmS and IcmW revealed that these two proteins interact with multiple substrates, and that the deletion of either one of these two proteins abolishes the translocation of some, but not all substrates [20]. Therefore, these two proteins are considered as the soluble chaperones of the Dot/Icm substrates [21]. Moreover, yet another Dot/Icm component, IcmT, has been linked with bacterial pore-formation mediated exist from infected cells, as ablating this gene causes the bacterium to be “trapped” inside of the infected cell [22].

The most important function of a specialized bacterial secretion system is to deliver into the host cells the effector proteins that can subvert host processes for the benefit of the bacterium. It is therefore not surprising that the nature of the substrates of the Dot/Icm secretion system had attracted extensive attention since the discovery of the system [15]. Although the Dot/Icm of *L. pneumophila* could deliver DNA into the host as seen in *Agrobacterium tumefaciens*, it is also possible that this system delivers protein substrates, as in the case of *Bordetella pertussis* [23, 24]. The requirement for this transport system within minutes of bacterial uptakes hints towards the latter possibility [25]. By examining the partially sequenced *L. pneumophila* genome, Sexton and Vogel noticed a bacterial ORF with extensive homology to Sec7 domain found in some eukaryotic proteins involved in vesicle trafficking, which functions as an GEF (guanine exchange factor) for the small GTPase Arf1[26]. The presence of the Sec7 domains hinted that this protein may potentially be a substrate of the Dot/Icm system, as other bacterial proteins with eukaryotic homology, such as Sptp and YopH from *S. typhimurium* and *Y. pestis* respectively, had been demonstrated to be secreted by bacterial translocation systems [27]. Experimental verification of the translocation of this protein, named RalF, lent support to the proteinaceous nature of the Dot/Icm substrates [28]. Since the revelation of RalF as the first substrate of the Dot/Icm system, extensive efforts have been devoted to the

mining of new substrates of the system as well as their molecular functions, which had and will continue to shed light on the understanding of the biology of *L. pneumophila*.

Translocation Assay System and Dot/Icm Substrate Identification

Intensive efforts devoted into the mining of Dot/Icm substrates and the dissection of their biochemical functions have made exciting progress in the past decade. Effector proteins with novel enzymatic function and intriguing virulence strategies have greatly enhanced our knowledge not only on the biology of *L. pneumophila* but also of bacterial pathogenesis in general. However, our understanding of *L. pneumophila* virulence factors is far from complete. This is partly due to the incomplete knowledge of protein substrates translocated by Dot/Icm system. Several methods have been developed to measure Dot/Icm mediated translocation of protein substrates:1) detection of the presence of the protein candidates using immunostaining of infected cells or/and isolated LCV [28]; 2) interbacterial transfer of a fusion protein containing the candidate gene and the Cre recombinase, which removes the marker gene from the genome of the recipient cell (an antibiotic resistant marker, for example). The transfer is then measured by the deletion of this marker in the recipient cells [29]; 3) restoration of a transfer-deficient mutant of SidC by fusing this mutant to the gene of interest (GOI) [30, 31]; 4) bacteria-to-mammalian cell transfer and activation of a fusion protein containing GOI and CyaA, the calmodulin-dependent adenylate cyclase from *Bordetella pertussis* [32, 33]; 5) the transfer of a beta-lactamase fusion protein into the mammalian cell. As the fusion protein could cleave the pre-loaded substrate CCF-4AM to make it emit different wavelength of fluorescence than its intact form, the protein transfer is then measured by the ratio of florescence intensity by the cleaved/intact substrates [34]. Most of the Dot/Icm substrates were discovered by studying the candidate genes gathered by the following approaches: 1) bioinformatics analysis to retrieve candidate genes with structural features or functional domains which are only found in eukaryotic

proteins [35]; physical interaction with components of the Dot/Icm apparatus [29, 36]; screening of their ability of disrupting cellular processes in *Saccharomyces cerevisiae* [37-39]; expression regulation profiling which is similar to known effectors [40, 41]; or self-learning computing strategies to search for protein harboring one or more features mentioned above [42]. The combination of these search methods and translocation assays have led to the identification of 204 protein substrates translocated by Dot/Icm apparatus by 2010. As all previous screenings relied on the aforementioned criteria to select candidate genes, it is likely that these screens will miss some substrates with undefined features, especially considering the sheer number and the diverse function of the Dot/Icm substrates. Therefore, I led a project to obtain a more complete list of Dot/Icm substrates involved in the modulation of host functions, which would then pave the road for subsequent activity-based studies. To this end, I constructed more than 600 genes into the pXDC61M vector [43], which is suitable for expression of β-lactamase fusions in *L. pneumophila*, to test for Dot/Icm-dependent protein translocation by the FRET-based beta-lactamase assay. Moreover, because of the availability of a number of effective genetic and genomic tools, yeast is extremely useful in dissecting the molecular functions of pathogenic factors. Therefore, I also constructed all of these pGBKT7 plasmids that express each of these ORFs to test their capability to disrupt cellular processes of *S. cerevisiae*.

Effector Modulation of Host Processes

As more effectors have been identified and more of their functions revealed, it has become clear that elucidating the functions of these substrates will not only allow better understanding of the mechanisms used by *L. pneumophila* to subvert host cellular processes but also could potentially reveal novel host signaling mechanisms that are undetectable or difficult to study under normal physiological conditions [6]. For examples, the small GTPase Rab1, which is a molecular switch involved in regulating vesicle trafficking between ER and Golgi apparatus, is targeted by at least five *L. pneumophila* effectors.

Amongst these effectors, SidM, which harbors both guanine nucleotide exchange factor (GEF) and adenylyl transferase activity, activates Rab1 and locks it in the activated state by transferring an adenylyl group (AMP moiety) from ATP onto the Tyr 77 residue at the switch II region of Rab1, ensuring that it is insensitive to unwanted inactivation by bacterial and host GAP [44-46]. Subsequently, another *L. pneumophila* effector SidD removes the adenylyl group in a manner similar to those of protein phosphatases [45]. Once the adenylyl group is removed, Rab1 then becomes sensitive to inactivation by yet another *L. pneumophila* effector--LepB for its effective removal from LCV membrane [47]. Amazingly, another effector pair, which also functions in a Yin & Yang manner, provides the bacterium additional layer of subversion of Rab1 function by transferring/removing a phosphocholine group to Rab1 [48, 49]. These studies provided us a fascinating example of how studies of bacterial effector functions could open the door to the previous unappreciated posttranslational modifications.

Further analysis of the biochemical functions of *Legionella* effectors revealed that this bacterium also subverts a variety of other host cell processes beyond membrane trafficking [6]. For instance, when ectopically expressed, Dot/Icm substrates LegK1 and LnaB modulate the activation of NF-κB pathway [31, 50]; Also, *L. pneumophila* codes for multiple factors to further support sustained activation of NF-κB pathways by inhibiting host protein synthesis, thus preventing efficient biosynthesis of IκB, the inhibitor of NF-κB [51]. Further, this bacterium injects a V-ATPase specific inhibitor SidK into the host, which, when loaded into macrophages, inhibits vacuole acidification [38]. Last but not the least, *L. pneumophila* codes for a factor named SdhA, which maintains the membrane integrity of the LCV, allowing the bacterium to evade multiple host innate immunity surveillance pathways to establish a replicative niche inside host cells [52].

One other significant aspect of the cell biology during Legionella infection is phosphatidylinositol (PI) metabolism. It is well established that PIs play essential roles in a broad spectrum of cellular processes, such as remolding cytoskeleton, defining organelle identity and relaying cellular signaling events [53, 54]. Distribution of particular species of PIs in subdomains of cellular membranes licenses different biological events by recruiting effector proteins through binding of their cytoplasmic head groups to the cytosolic proteins or the cytosolic domains of membrane proteins [54]. As for bacterial infection process, PIs have been shown to be of particular importance, as PI species such PI3P is important for fusing the nascent phagosomes to lysosome, where non-pathogenic bacteria are normally degraded. Therefore, it is not surprising that many pathogens code for virulence factors to subvert PI metabolism for their benefit. For instance, *Shigella flexneri* encodes a PI-4-phosphatase IpgD to reduce the level of phosphatidylinositol 4,5-bisphosphate (PIP(2)) at the plasma membrane, thereby inhibiting host T cell migration [55]. On the other hand, *Salmonella typhimurium* delivers the PI phosphatase SopB into the host to hydrolyze PI(3,4,5)P_3, which contributes to the localized membrane ruffling to facilitate bacterial entry into nonphagocytic cells [56, 57]. Manipulation of host PI metabolism is also key to the establishing the replicative vacuole of *Legionella*—the *Legionella* containing vacuole (LCV) [58, 59]. One of the PI species, PI(4)P, has been suggested to be enriched on LCV [60]. Several Legionella effectors which requires PI4P to anchor onto the membrane of LCV, such as SidM/DrrA, SidC, and SdcA have been identified, and SidF, the effector protein(s) that contributes to PI(4)P enrichment on LCV also been recently described [60]. Taken together, studies of the biochemical functions of *Legionella* effectors had added an amazing page to our understanding of host-microbe-interactions, and will continue to shed light on the intriguing war between these two forces.

Despite the copious advances made on the biochemical functions of *L. pneumophila* effectors, one problem lingers in the field of *Legionella* biology: the

functional redundancy among the Dot/Icm substrates. *L. pneumophila* translocates more than 300 Dot/Icm substrates into the host cells, but deleting one or more of these substrates rarely results in detectable intracellular growth defect [6, 61, 62]. Further, ablating the effector proteins that have similar biochemical consequences or share the same host target does not result in defects in intracellular colonization either [62, 63]. More strickingly, SdhA, SidJ and MavN are the only three effectors whose deletion causes severe deficiency in *L. pneumophila* intracellular replication [64, 65]. The striking functional redundancy in *L. pneumophila* is further demonstrated by a recent study which revealed that seven distinct genomic clusters containing 27.1% of the *Legionella* genome are dispensable for bacterial growth *in vitro*. More remarkably, *L. pneumophila* mutant lacking six of such clusters, which contain more than 30% of the known Dot/Icm substrates, showed no significant growth defects in murine macrophages. However, *L. pneumophila* mutants lacking five such clusters showed differential defects in colonizing distinct amoebae species. This evidence, together with the low GC content and marked variability of these cluster, prompted the authors to propose that these clusters were acquired by acquired by the bacterium horizontally to maintain optimal growth in a vast variety of amoebae hosts [66]. Although this report shed light on the possible origin of the functional redundancy of *L. pneumophila*, the reason why this bacterium acquires and maintains such a large repertoire of Dot/Icm substrates remains to be one of the most intriguing challenges in the field.

The functional redundancy has and will continue to puzzle the field of *L. pneumophila*. This problem is, however, not unsolvable, especially considering the emergence of novel strategies such iMAD (Insertional mutagenesis and depletion) [67]. The iMAD approach, on the other hand, is based on the hypothesis that *Legionella* effector proteins target multiple host pathways, therefore inactivating one host pathway or effector proteins targeting the same pathway may not suffice to cause defects in intracellular growth. However, in

infection, inactivating one host pathway by siRNA and blunting *Legionella* effector proteins targeting another pathway via transposon mutagenesis may allow for identification of mutants with appreciable growth defect *in vivo* [66]. Using iMAD, the Isberg group identified 55 Dot/Icm effectors important for manipulating distinct host pathways [66]. This approach not only allows for surmounting the functional redundancy of *Legionella* effectors, but also provides information on the common host targets shared by distinct Dot/Icm substrates, as well as the functional prediction of uncharacterized effector proteins that are in the same functional group with the effectors with known functions [66]. iMAD, together with other emerging approaches, demonstrated that although the functional redundancy among Legionella effectors is a limiting factor for *Legionella* study, it could be a window of opportunities to further our understanding in the biology of this fascinating bacterium.

Host Cell Death Pathways and *L. pneumophila* Infection

The host cell death pathways are integrated components of the innate immune system that defends against infection and they have become among the most important foci of the field, because they are one of the main determinants of the outcome of host and pathogen interaction. Upon entering the host cell, the pathogen will need to take advantage of the available nutrition from the host for its multiplication [68]. As the nutrition inside of the cell is depleted, the pathogen will need to leave the host and gain access to a new one to start a new cycle of infection [69]. Premature host cell death could be detrimental to the pathogen as it could remove the infected niche, kill the pathogen or exposes the pathogen to addition layer of immune surveillance [69]. Therefore, it is not surprising that host cell death is one of the effector mechanisms of the immune response. On the other hand, host cell death could also be beneficial to the pathogen as it could lead to the deletion of immune cells that are “designed” to clear the infection [70].

Different types of host cells death could result in distinct influence on the outcome of infection [71]. For example, apoptosis is licensed by the activation of the executioner caspases (cysteine-aspartic proteases)-caspase-3, 6, or 7, which cleave various cellular substrates, such as PARP (poly-(ADP-ribose) polymerase) and ICAD (inhibitor of caspase activated DNase) [71]. These events are then followed by characteristic chromatin condensation and fragmentation, cell shrinkage, and the formation of apoptotic bodies [71]. At the end of this process, the lipid phosphatidylserine, which is normally on the cytoplasmic surface of cells, redistributes to the extracellular surface of the apoptotic bodies, marking these structures available for phagocytosis by cells such as macrophages and neutrophils [71]. Because the cellular content is not released into the extracellular milieu, apoptosis is considered as a non-inflammatory and more “silent” form of cell death [72].

On the contrary, pyrotopsis is a rapid, lytic cell death that releases inflammatory contents into the extracellular spaces [73]. This form of programmed cell death is initiated by the engagement of one or more of the pattern recognition receptors (PRRs) to the pathogen associated molecular pattern (PAMP) [74]. The engagement of the PRRs with their cognate ligands recruits caspase-1/11 to form the high molecular weight protein complexes termed inflammasomes, which are platforms for activating caspase-1 by inducing its dimerization and autoproteolysis [75]. The activated caspase-1/11 not only promotes the pore formation on the plasma membrane of the dying cell, but also induces the maturation and active secretion of inflammatory cytokines such as IL-18 and IL-1β into the extracellular milieu to attract additional layers of immune defense. Therefore, pyroptosis is generally considered as an inflammatory form of cell death [74].

In addition to the two distinctive pathways, necroptosis also plays important roles during infection. Once considered as a passive, unregulated form

of cell death, necroptosis has now been established to be tightly controlled by defined molecules [76]. The major governors of necroptosis are the kinase RIP1 (receptor interacting protein) and RIP3 [76]. Stimuli such as the engagement of TNF-α to TNFR, for examples, triggers the formation of the RIP1/RIP3/MLKL (mixed lineage kinase domain like) necroptosome, which promotes the rapid, lytic cell death followed the loss of the plasma membrane integrity [76]. The loss of plasma membrane integrity is accompanied by the dynamic release of CDAMP (cell death associated molecular pattern), which strongly attracts immune cells [77].

In addition to these conventional pathways, accumulating evidence suggests the existence of yet another form of cell death--lysosomal cell death, which plays a pivotal role in numerous pathological conditions, such as stroke, acute pancreatitis, and parasitic and viral infections [78]. Similar to the roles of mitochondria in apoptosis and necroptosis, lysosomes, is the central signaling organelle for the lysosomal cell death pathway [79]. Upon perceiving stresses, the lysosome membranes destabilize, leading to the release of acidic content as well as various hydrolases including the members of the cathepsin protein family into the cytosol [80]. Some cathepsins are stable at physiological pH, thereby can process various cellular substrates upon translocation and initiate lysosomal cell death [81, 82]. Apparently, not all the cathepsins play equal roles in such cell death, but rather, certain cathepsins are selectively released into the cytosol, or some of them play a more dominant role than others [82]. For example, cathepsin B plays a dominant role in tumor necropsies induced by Granulysin, while cathepsin D is more important in triggering apoptosis when T lymphocytes enter the early commitment phase [83, 84].

The release of cathepsins into cytosol could also activate downstream pathways shared by conventional apoptosis, pyroptosis, and necroptosis. For example, cathepsin B and cathepsin D are capable of cleaving BID into its

mature form (tBID), which inserts into the outer membrane of mitochondria to trigger mitochondria outer membrane permeabilization (MOMP) in a Bax dependent manner [85, 86]. The permeabilized mitochondria then release cytochrome c into the cytosol, where it initiates the assembly of the multi-protein complex termed apoptosome to activate the initiator caspase, caspase-9. Activated caspase-9 then activates the executioner caspase, caspase-3 [86]. Depending on the specific signaling context, cathepsins redistribution into the cytosol could also lead to either pyroptosis via the activation of the NLRP3 inflammasome by ROS burst, or the caspase-independent necrotic cell death when caspases are inhibited [78]. Therefore, lysosome membrane permeabilization could commit the cell to the “point of no return”, as inhibiting a single cell death regulator cannot effectively block such cell death. The importance of such a form of cell death during microbial infections has been documented, such as those by *Mycobacterium tuberculosis* and HIV. The outcome of this type of cell death, however, could vary, as it could play important roles in limiting viral infection or allowing the bacteria to egress from the dying host [87, 88].

Subversion of Host Cell Death Pathways by *L. pneumophila*

Since host cell death is of such importance to microbial infection, it is thereby not surprising that these pathways are the “hotspots” for pathogen manipulations [72]. For instance, *Coxiella burnetii*, a pathogen that is closely related to *L. pneumophila*, translocates multiple virulence factors through its Dot/Icm apparatus to inhibit host apoptosis. One such factor is AnkG, which antagonizes the host apoptosis pathway possibly by binding to the host mitochondria protein p32 [89]. *Anaplasma phagocytophilum*, another intracellular vacuolar pathogen, injects a Dot/Icm substrate, Ats-1, into the host cell, which translocates to host mitochondria and interferes host apoptosis induction [90]. Further, *Mycobacterium tuberculosis* codes for a Type I NADH-dehydrogenase that suppresses host apoptosis induction [91].

Pyroptosis pathway is also targeted by various pathogens. *Yersinia pestis* for example, codes for a T3SS effector YopM, which structurally mimics the substrates of caspase-1, inhibits caspase-1 activity through direct binding [92]. This bacterium also injects another T3SS effector protein, YopK, into host cells to mask the T3SS translocon from the recognition by the inflammasome [93]. Moreover, ExoU, a phospholipase A_2 translocated by the T3SS of *P. aeruginosa*, also dampens the inflammasome activity, although the mechanisms of action is still unknown [94]. Further, pneumolysin, the pore-forming toxin coded by *S. pneumonia* has also been implicated in blunting the caspaspe-1 activation [95]. This is surprising, however, because the pore-formation activity of other bacterial toxins triggers the activation of NLRP3 inflammasome [95].

To avoid premature termination of bacterial replication, apoptosis inhibition is important for the establishment of the replicative vacuole of *L. pneumophila*. Consistent with this notion, permissive cells infected by *L. pneumophila* exhibit remarkable resistance to exogenous cell death stimuli, and such resistance requires a functional Dot/Icm system [96, 97]. This anti-apoptotic phenotype appears to be the result of the collective functions of several effector proteins, such as SdhA, LegK1, Lnab, and SidF [50, 98, 99]. Intriguingly, despite their capability to resist cell death stimuli, the cells harboring actively replicating *L. pneumophila* also contain high levels of active caspase-3 [100, 101], indicating the presence of a balance between the pro-death and pro-survival signaling. Clearly, in permissive mammalian cells, the activation of caspase-3 does not lead to restriction of *L. pneumophila* replication. Interestingly, the effect of such activation becomes apparent in the interactions between *L. pneumophila* and dendritic cells (DC). Infection of DCs by *L. pneumophila* causes extensive apoptotic cell death in a process that is dependent upon caspase-3 or Bax/Bak, two critical regulatory proteins in the mitochondrial cell death pathway [102]. Despite a plethora of literature documenting the anti-apoptotic pathways induced by *Legionella*, relatively little is known about the bacterial factors involved in

activating caspase-3, or the mechanisms behind it [103, 104]. Therefore, investigation is warranted to identify these factors and their molecular mechanism of caspase-3 activation. To achieve this goal, I performed a screening to identify Dot/Icm substrates capable of activating this enzyme upon transient expression in mammalian cells. These efforts led to the identification of five proteins with such activity. Among these five proteins, I chose to focus on VipD, because VipD bears significant homogy to the putative conserved patatin-like phospholipase A_2, and that our laboratory has the necessary reagents and expertise to study such proteins.

Patatins are a family of proteins that catalyze the hydrolysis of phospholipids at the *sn-2* position, liberating a fatty acid and a 2-lysophospholipid molecule. Patatin-like-proteins (PLPs) are found in both eukaryotes and bacteria and share the conserved GxSxG and DGx (x: any amino acid) catalytic dyad motifs that are essential for their phospholipase activity [105]. PLPs play critical roles in diverse aspects of lipid metabolism and signal transduction, such as membrane trafficking, inflammatory response and apoptosis. Many pathogens code for PLPs to facilitate their infection and dissemination [106]. For example, ExoU, a phospholipase A2 from *Pseudomonas aeruginosa* plays an important role in diverse aspects of its interactions with hosts such as the inhibition of caspase-1 mediated inflammatory responses [94]. *L. pneumophila* is predicted to code for at least 11 PLPs, but the activity of most of these proteins has not yet been characterized [107]. The Dot/Icm substrate VipD is one such predicted PLP that was first identified in a screening for *L. pneumophila* Dot/Icm effectors by their ability to interfere with membrane trafficking in the budding yeast [108]. This protein harbors a phospholipase A2 motif, which has been shown to be essential for the activity of ExoU from *P. aeruginosa* [30].

Immune Recognition and *L. pneumophila* Infection

A fundamental aspect of immune system lies in its capability to differentiate “self” from “non-self”. The innate immune system branch achieves such recognition by employing an arsenal of germ-line coded PRRs (Pattern-Recognition-Receptor) distributed on different cellular locales to perceive the presence of the MAMPs (Microbe-Associated-Molecular-Pattern) from microbes, in order to initiate and calibrate appropriate immune responses [109].

The PRRs can be categorized based on the subcellular location where the receptor initiates signaling. Toll-like-receptors (TLRs) and C-type lectins are among the most well characterized membrane associated receptors that mainly function to recognize extracellular ligands (some TLRs are located in endosomes that recognize intracellular ligands) [110]. These receptors recognize a wide ranges of ligands. For example, the mouse genome codes for 12 TLRs, and the ligands to these receptors includes bacterial lipopolysaccharides (LPS), peptidoglycans, triacyl lipopeptides, and flagellin, as well as the zymosan of fungi [111]. On the other hand, C-type lectins are a family of carbohydrate-binding proteins, and the most prominent member of this family is the Dectin-1, which recognizes exogenous β-glucans [112]. Upon engaging their agonists, these receptors activates downstream transcription factors, such as NF-κB and interferon-regulatory-factors (IRFs), to promote the production of inflammatory cytokines and type-I interferon, respectively [111]. TLR4, for example, turns on the expression of inflammatory cytokines after encountering its cognate substrate--LPS by activating the NF-κB and Ap-1 pathway in a TIRAP (Toll-Interleukin 1 Receptor (TIR) Domain Containing Adaptor Protein) and Myd88 (Myeloid Differentiation Primary Response 88) dependent manner [113]. A second pathway is triggered as the LPS-bound TLR-4 is internalized into the endosomal network and regulates Type I Interferon expression vis two adaptor TRIF (TIR-domain-containing Adapter-inducing Interferon-β) and TRAM(TRIF-Related Adaptor Molecule) dependent manner [114].

The cytosol of mammalian cell is considered as the “sacred temple” that is highly sensitive to foreign biomolecules. It is therefore not surprising that mammal code for a huge number of cytoplasmic PRRs, including the NOD-like receptors (NLRs), pyrin and HIN domain containing family (PYHIN), RIG-I-like receptors (RLRs) and a large number of cytosolic nucleic acid sensors, to guard the cytosol from invading microbes [111]. The downstream signaling cascades of cytoplasmic PRRs are more diverse than those of TLRs. NOD1 and NOD2, members of the NLR family, for instance, activate NF-κB and IFN-β production upon the engagement of their cognate ligands [115]. Other NLRs, however, drive the assembly of a large protein complex termed the inflammasome, which causes pyroptotic cell death, and the secretion of mature inflammatory cytokines, such as IL-1β and IL-1α and IL-18 [116]. The diverse ligand recognition capacity of NLRs makes them one of the most important guardians of the cytoplasm. For example, NLRP1b responds to the protease activity of the lethal toxin of *B. anthracis* and infection by *Toxoplasma gondii* [117], whereas the AIM2 inflammasome responds to cytosolic DNA molecules, which contributes to the recognition of vacuolar bacteria that aberrantly enter the cytosol, such as the *sdhA* mutant of L*. pneumophila* [118, 119]. With the help of the NAIP (baculoviral IAP repeat-containing protein 1, neuronal apoptosis inhibitory protein) proteins for expanded specificity, the NLRC4 inflammasome responds to many bacterial MAMPs, such as the rod and needle proteins of bacterial Type III secretion system as well as flagellin [120]. The inflammasome that has the widest range of MAMP sensing is the NLRP3 inflammsome, which can be activated by bacterial, fungal, viral pathogens, as well as pore formation toxin, protein amyloid, extracellular ATP, and inorganic substances such as silica and alum [116].

Other cytoplasmic PRRs such as RLRs, including retinoic acid-inducible gene I (RIG-I), LGP2 and melanoma differentiation-associated protein 5 (MDA5), detect nucleic acid and their derivatives, which are one of most important categories of MAMPs [111]. RIG-I, for example, recognizes cytoplasmic dsRNA

to promote the prion conversion of MAVS (Mitochondrial antiviral-signaling protein), which signals to upregulate the production of IFN-β [121]. Of particular interest is the long-anticipated revelation of the major cytoplasmic dsDNA sensor, cGAS (Cyclic GMP-AMP synthase). This sensor is special in that it is an enzyme itself, which catalyzes the formation of cyclic GMP-AMP upon recognizing dsDNA. The cyclic GMP-AMP is then sensed by STING (STimulator of INterferon Genes), which signals the production of type I IFN [122, 123]. In the case of *L. pneumophila*, induction of type I interferon by macrophages only occurs when the cells are challenged with bacterial strains harboring a functional Dot/Icm machinery. Considering that the infection by this bacterium is associated with potential leak of DNA into the cytosol via the Dot/Icm transporter, and that the type I IFN response is STING-dependent, it has been proposed that the ligand(s) responsible for the activation is bacterial DNA [119, 124, 125].

MAMPs are not the only microbe-associated features that the immune systems are sensing [126]. The observation that different plants respond distinctively to the same pathogen has prompted the plant pathologists to postulate the “gene-for-gene” hypothesis, which suggests that resistant plants codes for the “resistant gene (R gene)” that is required for the recognition of the virulence factor “avirulence gene (Avr gene)” [126]. Since the presence of the Avr gene is sufficient to trigger immune responses from the resistant plants, it was proposed that the R gene product directly recognizes the gene product of the Avr, which is similar to the PRR-PAMP paradigm in metazoans [126]. However, as the interactions between Avr and R genes have rarely been demonstrated, and that the Avr genes usually code for bacterial virulence factors with distinct biochemical activities, Dangl and other researchers hypothesized that the R genes are not recognizing the Avr genes per se, but their biochemical consequences in the plant cells [127]. This hypothesis was further supported by the observation that Avr genes lacking their enzymatic activities are not immunogenic [128]. Thus, the immunity triggered by perceiving the biochemical

consequences of bacterial effectors is defined as effector-triggered immunity (ETI) [128]. Evidence supporting this theory is plentiful in plants: R gene RPS2 senses the proteolytic cleavage of RIN4 by the *Pseudomonas syringae* effector AvrRpt2, and another R gene RMP1 recognizes the kinase activity of *P. syringae* T3SS substrates AvrB and AvrRPM1 [127]. Despite being well established in plants, whether the metazoan immune system functions in a similar way was not clear until recently. It was reported that *Escherichia coli* CNF (cytotoxic necrotizing factor-1) modifying host proteins RAC2 actives NF-κB pathway, and that pore formation activities by various bacterial toxins trigger inflammasome activation in a NLRP3 dependent manner [129, 130]. A recent report describing that the GAP (GTPase Activating Protein) activity of the *Yersinia pestis* T3SS effector YopE triggers killing of *Yersinia* in macrophages lent further support to the presence of ETI in mammals [131]. One of the first R gene equivalences in mammals was also recently identified: Pyrin, the defining member of the Pyrin-domain-containing protein (PYD) family, detects various bacterial modifications, such as ampylation, ADP-ribosylation, deamindation, glucosylation, on small GTPase RhoA at its switch I region and activates caspase-1 [132]. Taken together, ETI constitutes a newly emerging and import branch of innate immune surveillance in both plants and metazoans.

ETI also contributes to immune recognition of *L. pneumophila*. It has been shown that infection of macrophages by wild type *L. pneumophila* but not a mutant deficient in Dot/Icm induces both the MAP kinase pathway (transcriptionally) and the NF-κB pathway [65, 133, 134]. Interestingly, protein synthesis inhibition conferred by five Dot/Icm substrates is part of the signals sensed by both the NF-κB and the MAP kinase pathways [135, 136]. Prolonged activation of the NF-κB pathway is achieved by the delayed re-synthesis of IκB, the labile inhibitory protein of NF-κB [136]. The MAP kinase response, on the other hand, is “frustrated”, because the genes involved in this pathway are transcribed but not translated. The “frustrated” host responses triggers host

signaling pathways that allow selective translation of at least two inflammatory cytokines, IL-1α and IL-1β, in a MyD88 dependent manner, providing further evidence of ETI in metazoan [99].

Studying how *L. pneumophila* manipulates host pathways during infection is undoubtedly important to the understanding of *Legionella* and host interactions; also essential is the host immune responses elicited during infection, which seems to have equally great impact on the outcome of infection. Toll-like receptors play salient roles in innate immune defenses against *L. pneumophila*. TLR4 for examples, contributes significantly to the immune responses to *L. pneumophila*, as mouse macrophages lacking a functional TLR4 allele showed increased permissiveness to this bacterium [137]. Further, analysis of a regional outbreak of Legionnaires' diseases showed that TLR4 polymorphisms is associated with the resistance to the disease [138]. Another TLR, TLR5, which recognizes bacterial flagellin and promotes TNF-α, IL-6, and other chemokines [139]. Consistent with the important role of TLR5 in innate recognition of *L. pneumophila*, individuals that bear non-functional TLR5 allele are more susceptible to the infection by this bacterium [140].

Studies using mice lacking MyD88$^{-/-}$, the common signaling adaptor for TLR pathways, lent further support to the importance of TLR receptors in host defenses against *L. pneumophila*. MyD88 deficient mice are impaired in production of TNF-α, IL-6 and IFN-γ, cytokines that are important in eliminating infected cells and controlling systematic bacteria dissemination. As a result, mice lacking MyD88 fail to clear *L. pneumophila* lung infection and eventually succumb to acute pulmonary infection, with bacteria spreading to lymph nodes and the spleen [141].

NOD receptors also contribute to *L. pneumophila* recognition, at least at later stages of infection [142]. Although both of the receptors could recognize

heat-killed *Legionella*, NOD1 seems to play a dominant role in sensing *Legionella*, as mice lacking NOD1 are impaired in neutrophils infiltration and the ability to clear *L. pneumophila* in the lungs, while the mice lacking NOD2 respond normally to this bacterium [142]. Despite the apparent importance of NOD1 in *Legionella* recognition, the identity of the ligand and the molecular mechanisms of NOD1 sensing remain elusive.

Despite the fact that *L. pneumophila* dedicates almost 10% of the its total genomic capacity to code for virulence factors, most of the inbred mice strains are refractory to its infection, making the search for an appropriate animal/cellular model to study its pathogenesis frustrating [143]. This phenomenon, however, also hints that *L. pneumophila* could be a useful tool to dissect immune pathways that are difficult to study because they are not induced by other more adapted pathogens. For example, BMDMs (Bone Marrow Derived Macrophage) derived from C57BL/6 mouse challenged by *L. pneumophila* results in bacterial clearance accompanied by extensive, lytic host death termed pyroptosis, suggesting the involvement of active host immune responses [144]. However, macrophages from one particular strain, the A/J mouse, allow intracellular replication of *L. pneumophila* [143]. Taking advantage of the sharply different immune responses of the two mouse strains to *L. pneumophila*, Dietrich *et al*. mapped the genetic elements responsible for the permissiveness of A/J mice to the Lgn1 locus, which was later pinpointed to the gene *Naip5* [145, 146]. The very same phenotype also was utilized to identify the bacterial factor that engages Naip5—flagellin, the conserved component of the bacterial flagellum [147, 148]. Further investigation of this pathways revealed that, upon engaging flagellin, Naip5 recruits and activates NLRC4, which recruits caspase-1 into inflammasome [149]. Formation of the NLRC4 inflammasome leads to the oligomerization and proteolytic activation of caspase-1, which promotes the processing and secretion of inflammatory cytokines (IL-18 and IL-1β) accompanied by rapid, lytic pyrototic cell death [150]. These signaling events eventually cause the removal of the

replicative niche and further recruitment of other immune cells [151]. These exciting successes in revealing previously underappreciated immune pathways by studying *L. pneumophila* infection clearly demonstrate the great potential of investigating host responses using less adapted pathogens. A schematic presentation of the immune surveillance engaged by *L. pneumophila* is illustrated in **Fig. 1-2**.

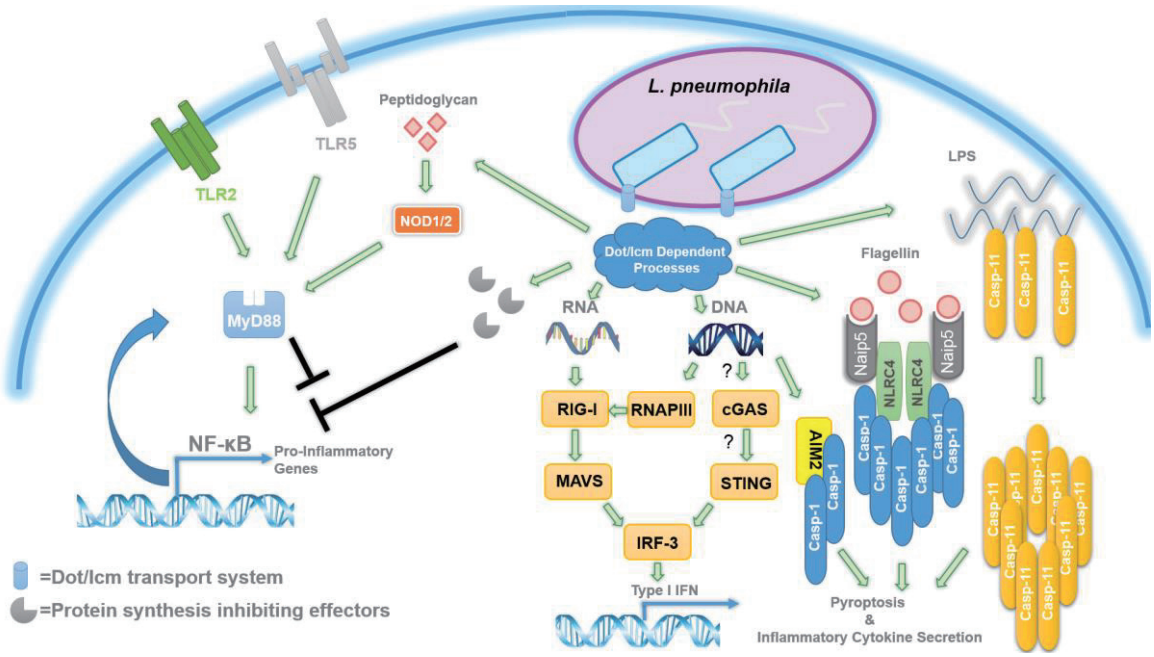


Fig 1-2. Innate immune recognition of *L. pneumophila* A schematic presentation of the immune surveillance engaged by *L. pneumophila*. Note that the inhibition of protein synthesis conferred by the *L. pneumophila* is sensed by the host via a MyD88 dependent mechanism. Host perception of these activities promotes selective translation of pro-inflammatory genes such as IL-1α. RIG-I, retinoic acid-inducible gene 1; IRF-3, Interferon regulatory factor 3; STING, stimulator of interferon genes; MAVS, Mitochondrial antiviral-signaling protein; RNAPIII, RNA polymerase III; cGAS, cyclic GMP-AMP synthase; NOD1/2, Nucleotide-binding oligomerization domain-containing protein 1/2; TLR2, Toll like receptor 2; TLR5, Toll like receptor 5; MyD88, Myeloid differentiation primary response gene 88; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Naip5, NLR family, apoptosis inhibitory protein 5; NLRC4, NLR family CARD domain-containing protein 4; AIM2, Absent in melanoma 2; Casp-1, capase-1; Casp-11, caspase-11; IFN. Interferon; LPS, lipopolysaccharide.

Legionella and Underappreciated Host Immune Signaling

Despite these exciting progresses, most of our understanding of *L. pneumophila* pathogenesis was based on the use of laboratory strains such as Lp02 and JR32 [152]. These laboratory strains were constructed from the original clinical isolate Philadelphia-1, necessitated by the need of effective genetic manipulations [152]. Further, because environmental bacteria are considered the primary source of outbreaks of Legionnaires' disease, many studies have directed to analyze *L. pneumophila* isolated from natural or man-made water systems [153, 154]. Although some of these studies have examined intracellular bacterial growth of these environmental isolates in cultured human macrophages, few of them have determined the response of different hosts such as primary murine macrophages to challenge of these bacteria [154-156]. Last but not the least, because the primary evolutionary pressure for virulence of *L. pneumophila* comes from amoebae hosts, *L. pneumophila* does not seem to have evolved significant immune-evasive mechanisms. As a result, challenge of immune cells such as primary macrophages with this bacterium could lead to strong immune responses often not seen with more adapted pathogens. We recently found that LPE509, a strain recently isolated from a man-made facility was unable to replicate in A/J mouse macrophages, which is permissive to most, if not all laboratory strains [157]. As host cell death plays indispensable roles in limiting bacterial replication in the case of a resistant host challenged by *L. pneumophila* expressing flagellin, we set out to test whether host cell death plays a role in the growth restriction of LPE509. Our results revealed that infection by this strain in BMDM induces rapid and extensive cell death in a manner independent of the known mechanisms of cell death associated with by *L. pneumophila* [157]. These results suggested the strain LPE509 is capable of triggering a unique cell death pathway, probably by activating an immune surveillance system. In order to identify the factor encoded by LPE509 responsible for this cell death phenotype, I screened for LPE509 mutants that are capable of growing in A/J macrophages and revealed that a mutation on the bacterial ribosome protein S12, *rpsL*, allows

LPE509 to replicate in similar efficiency as laboratory strains. I demonstrate that RpsL, after accessing the host cytosol presumbly by the Dot/Icm secretion system of *L. pneumophila*, engages host immune systems and triggers the lysosomal and apoptotic cell death.

CHAPTER II: COMPREHENSIVE IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF SUBSTRATES OF THE DOT/ICM TYPE IV SECRETION SYSTEM OF LEGIONELLA PNEUMOPHILA

Abstract

The vacuolar pathogen *Legionella pneumophila* utilities a Type IV Secretion System (Dot/Icm) to translocate a large cohort of substrates into the host cells to coordinate various cellular pathways, such as intracellular trafficking, protein synthesis and host cell death pathways. A large number of Dot/Icm (Defect in Organelle Trafficking/ IntraCellular Multiplication) substrates have been identified by various strategies, based on one or more characteristics associated with the tested proteins. Given the high level of diversity exhibited by the identified proteins, it is possible that some substrates have been missed in these screenings. To better understand the interaction between host and *L. pneumophila*, we took a systematic approach to test Dot/Icm dependent translocation of *L. pneumophila* hypothetical *orfs* larger than 300 base pairs. The transfer of the fusions into mammalian cells was determined using the β-lactamase reporter substrate CCF4-AM. 164 Dot/Icm substrates were identified in this screen and expanded the list of known Dot/Icm substrates by 70 effectors. Our effort has identified a large number of Dot/Icm substrates and facilitated future studies on the biology of *L. pneumophila.*

Introduction

As a “master manipulator”, *L. pneumophila* subverts various host processes in order to thrive intracellularly. Such subversion is made possible by

Dot/Icm of the bacterium, which injects a large number of virulence factors into the host [61]. Despite much exciting process made in understanding the biology of *L. pneumophila*, our knowledge of this bacterium is far from complete. Part of the reason is due to the lack of a comprehensive list of effector proteins translocated by the Dot/Icm of *L. pneumophila* [6]. Much effort have been dedicated in developing methods for measuring the translocation of Dot/Icm substrates into the host cells: such as immune-staining for the presence of effector protein(s) on LCV, cellular fractionation, CYA-based Elisa assay, and intra-bacterial protein transfer [158]. A recently developed beta-lactamase-based assay has been shown to be useful in detecting protein transfer by specialized bacterial secretion system [34]. This assay takes advantage of the fluorescence resonance energy transfer (FRET) between the coumarin and fluorescein fluorophores of the substrate CCF4-AM, which is disrupted when the beta-lactamase fusion protein is delivered by the bacteria into the host cytosol. These features make beta-lactamase assay amenable to high throughput screening while retaining high sensitivity.

Most screens for Dot/Icm substrates have chosen candidate genes with: features that are present mostly in eukaryotic organisms [35]; physical interactions with components of the Dot/Icm apparatus [29, 36]; or capabilities to disrupt cellular processes in *S. cerevisiae* [37-39]; or expression regulation profiles similar to known effectors [40, 41]. Self-learning computing strategies to search for protein harboring one or more features mentioned above have also been shown to be useful in such screens [42]. Although these methods and translocation assays have led to the identification of 204 protein substrates translocated by Dot/Icm apparatus by 2013, it is likely that these screens have missed some substrates with undefined features, as these screens are of a biased nature [6]. To this end, we initiated a genome wide screen, which surveyed hypothetical genes larger than 300 bp for novel effectors of *L. pneumophila*, using beta-lactamase assay as the readout.

Functional redundancy amongst Dot/Icm substrates makes *L. pneumophila* recalcitrant to many studies aiming at elucidating the roles of these proteins in infection [159]. For example, *L. pneumophila* codes for at least six virulence factors to interfere Rab1 function, and deleting some of these factors may not result in detectable phenotype [160]. Therefore, a comprehensive list of *L. pneumophila* effectors would certainly facilitate the functional studies of these protein, especially in the context of infection.

Results

Construction of a library expressing *Legionella*-protein-β-lactamase fusions

In order to obtain a more complete list of protein substrates transferred by the *L. pneumophila* Dot/Icm system, I initiated a comprehensive screen to test Dot/Icm-dependent translocation of hypothetical proteins in strain Philadelphia-1. β-lactamase was chosen as the reporter because this system is applicable for large-scale screens while holding comparable, if not higher sensitivity than other systems such as the adenylate cyclase (Cya) assay [34]. In this method, each candidate gene is amplified and fused to TEM1 (β-lactamase), and the bacterial strain expressing the fusion protein is used to infect differentiated human U937 cells. Infected U937 are then loaded with CCF4-AM which, when excited at 409 nm, emits green fluorescence (520 nm) due to fluorescence resonance energy transfer (FRET) between the coumarin and fluorescein fluorophores. Delivery of the β-lactamase fusion protein into host cells leads to cleavage of the β-lactam ring of CCF4-AM, releasing the two fluorophores and changing the fluorescence emission from green to blue (447 nm) when excited at the same wavelength. Translocation detected by this reporter can be easily quantitated by the percentage of infected cells emitting blue fluorescence signals [34].

To construct the fusion library, I first retrieved all the open reading frames larger than 300 base pairs annotated as hypothetical genes from the *L. pneumophila* Philadelphia 1 genome (**Table 1**). We chose 300 bp as the cut off parameters because most of the bacterial effector proteins discovered so far have specific enzymatic activity, and the probability that a gene smaller than 100 amino acids codes for an enzyme is relatively low. After eliminating genes that have been reported as substrates of the Dot/Icm system at the time the project was initiated, a total of 833 candidate genes were obtained (**Table 1**). To make the process more amenable for large scale cloning, I first modified pXDC61 plasmid in such a way that BamHI and SalI site could be used as "universal sites" to make in frame fusion of the genes of interest to TEM1 in this vector. I then examined whether there are BamHI/SalI sites on these genes. These two sites were used to design primers for the genes that cannot be digested by BamHI/SalI. For those which have one or more BamHI/SalI sites, BglII and XhoI were used as compatible site. For very limited number of genes that have 2 or more of these sites, alternative sites such as HindIII and NotI were used for cloning. To clone the genes, I designed primer pairs to amplify each open reading frame by PCR, used BamHI/SalI or BglII/XhoI to digest the inserts, and ligated them individually into pXDC61M to generate translational fusions with the upstream β-lactamase gene (**Fig. 2-1, A**). A total of 798 plasmids expressing the β-lactamase fusion were constructed. To examine the quality of the library, we randomly chose 30 *L. pneumophila* strains harboring the plasmid to detect the expression of the fusion proteins with an antibody specific for TEM1. Although the level of expression varies, a protein corresponding to the expected sizes of the chimeras was detected in most of the samples (**Fig. 2-1, B**), indicating that we have successfully constructed a library expressing β-lactamase fusions in *L. pneumophila*.

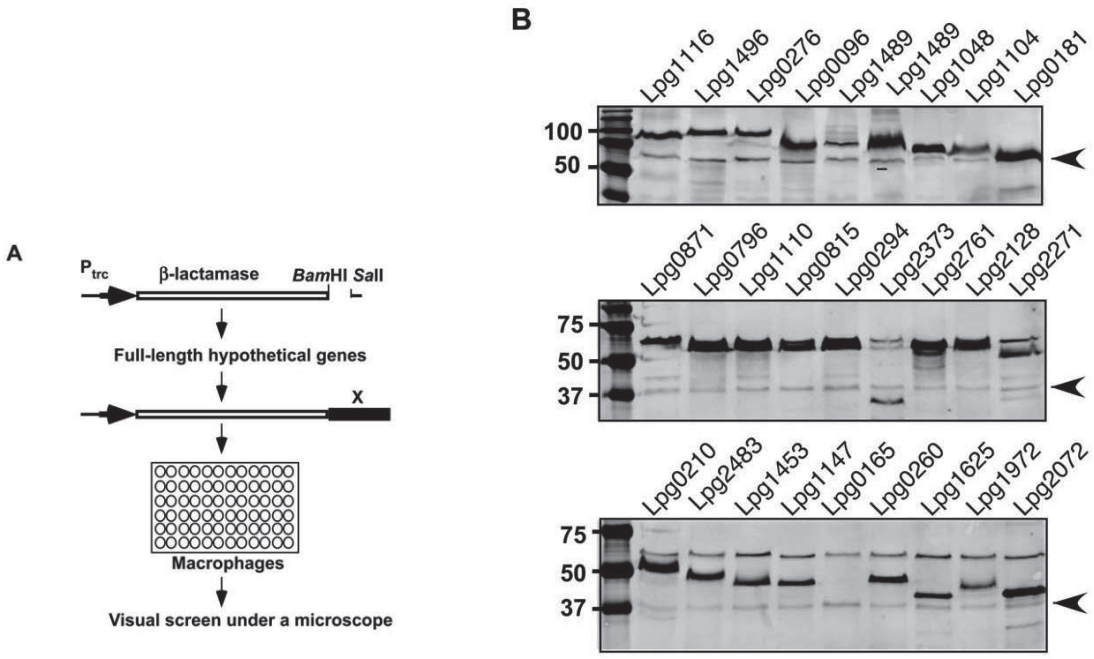


Figure 2-1. Construction of a library expressing fusions of β-lactamase and *L. pneumophila* hypothetical proteins. A. A schematic structure of the fusion proteins and the screening strategy. In most cases, the gene was fused with the b-lactamase by inserting into the vector as a BamHI/SalI fragment. After infection, samples were loaded with the CCF4-AM dye and were inspected under a fluorescence microscope. **B.** Evaluation of the library for expression of the fusion proteins. Plasmids directing expression of β-lactamase fusions were introduced into a wild type L. pneumophila strain. Total cell lysates of bacteria grown in the presence of IPTG were used to examine the steady state of the fusion proteins by immunoblot via a anti-beta-lactamase antibody. In each image, the detection of a protein nonspecifically recognized by the antibody (arrow) was used as a loading control.

Identification of proteins transferred by the Dot/Icm system

After verifying the expression of the fusion proteins in *L. pneumophila*, we infected U937 macrophages with *L. pneumophila* strains expressing β-lactamase fusions grown to post exponential phase. Experiment was done in 96-well format to facilitate high-throughput screening. A group of 24 bacterial strains expressing β-lactamase were used to infected host cells for each screen. One day before the experiment, differentiated human U937 cells were plated into each well of the 96-well plate at the density of $1x10^5$ cells per well. *L. pneumophila* strains expressing individual β-lactamase fusions were cultured in AYE in the presence of IPTG to induce expression of fusion protein. On the day of the experiment, *L. pneumophila* strains grown to post exponential phase were used to infect differentiated U937 cells at the MOI of 20. One hour after infection, cells were loaded with CCF4-AM dye and incubated for an additional 2 hours at room temperature. Translocation of the β-lactamase chimera was assessed by the presence of cells emitting blue fluorescence signals. Each infection experiment is triplicated. In each experiment, we used the TEM-RalF and TEM1-FabI chimera as the positive and negative controls, respectively [34]. Samples were visually inspected under a fluorescence microscope and strains that gave more than 5% of blue cells were retained for further study. Under this experimental condition, more than 95% of the cells infected by wild type *L. pneumophila* strain expressing the TEM-RalF fusion emits blue fluorescence, whereas similar infections with the strain expressing TEM- FabI resulted in no blue cells, which are consistent with the results from an earlier study [34]. Infections were repeated at least twice for strains that gave positive translocation results. Constructs harboring genes exhibiting detectable transfer were introduced into the dot/icm-deficient strain Lp03 and the resultant strains were similarly tested for delivery the β-lactamase fusions into host cells. None of these fusions caused detectable translocation in this dot/ icm-deficient strain.

In this study, 798 of the 832 analyzed orfs were successfully fused to the carboxyl end of β-lactamase (**Table 1)**. In the 798 proteins tested in this study, a total of 164 proteins that consistently promoted translocation of TEM translational fusions in a Dot/Icm-dependent manner were obtained (**Tables 2** and **3**). Among these, 94 proteins had been reported as Dot/Icm substrates by the time of this study (**Table 3**), further validating the reliability of our screen strategy. Thus, our efforts have added 70 proteins to the inventory of the substrate pool of the *L. pneumophila* Dot/Icm transporter (**Table 2**). The transfer efficiencies of these proteins vary greatly, ranging from 5% to 95% (**Fig. 2-2**). Among these, 5 proteins exhibited transfer efficiencies comparable to that of RalF, causing more that 90% of infected cells to emit blue fluorescence signals (**Fig. 2-2**). These 5 proteins do not share any detectable common features. Instead, the primary sequence of the C-terminal portion of Lpg2844 is quite different from all known substrates (see below). Twenty-five proteins converted 50%–80% of the green cells into blue cells, while 13 proteins exhibited translocation efficiencies between 20% to 45%, and 27 proteins showed low transfer efficiencies with less than 20% blue cells in the samples (**Fig. 2-2** and **Table 2**).

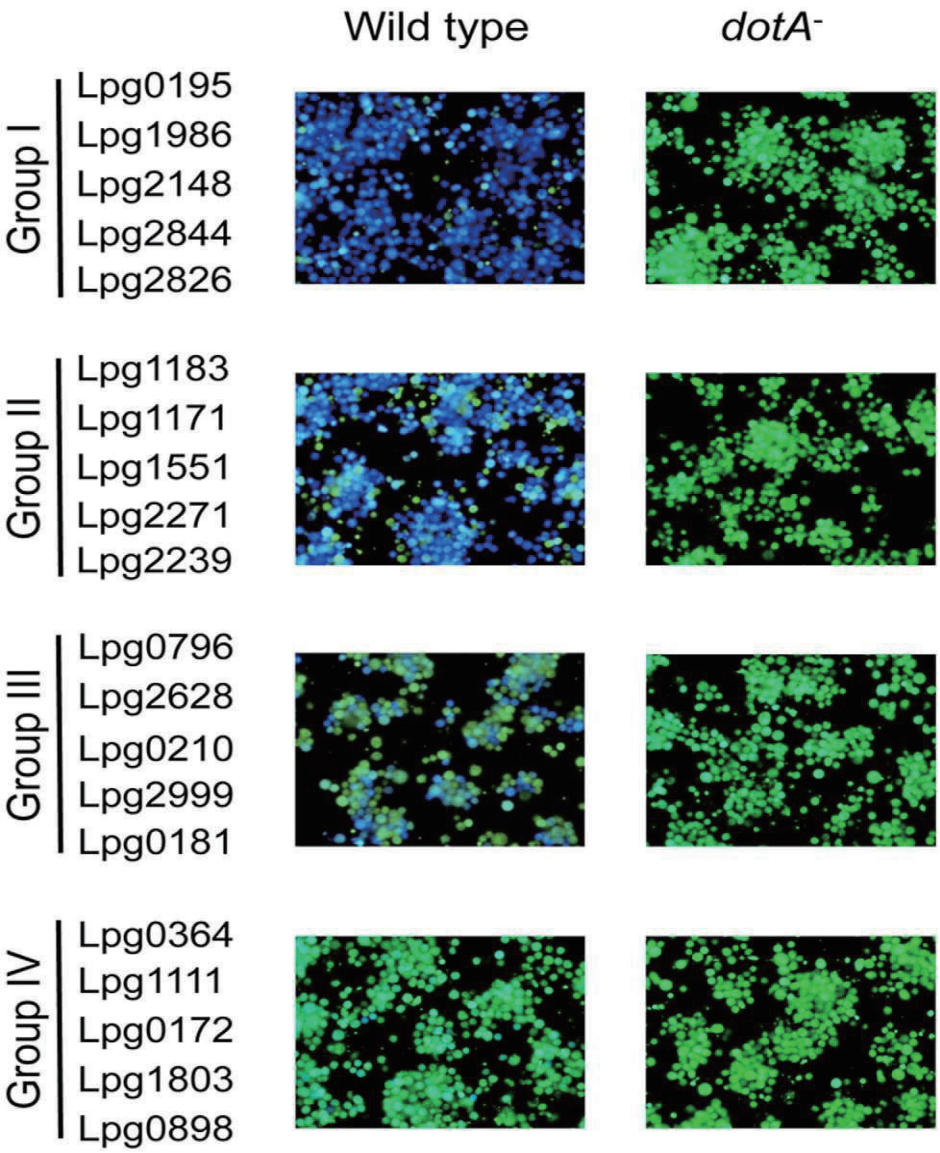


Figure 2-2. Dot/Icm-dependent translocation of substrates. Effectors identified in this study were divided into four groups according to their transfer efficiencies: 5 genes from each group were shown as representatives. U937 cells seeded in 96-well plates were infected with wild type or dot/ icm-deficient *L. pneumophila* strains expressing a gene fusion and images were acquired 2 hrs after CCF4-AM loading with a DP72 color camera (Olympus). Group I, genes with translocation efficiency greater than 90%; Group II, genes with translocation efficiency between 50% and 80%; Group III, genes with translocation efficiency between 20% and 45% and Group IV, genes with translocation efficiency less than 20%.

Although all candidates were annotated as hypothetical proteins in the genome database of the Philadelphia 1 strain, careful bioinformatic analysis revealed that a small fraction of them harbor motifs of known or putative functions (**Table 2**). Further, most of these proteins have a homolog in the genome of the Paris, Lens or the Corby strain; the number of proteins without a detectable homolog are 7, 12 and 13 for these three strains, respectively (**Table 2**). Two proteins, Lpg1083 and Lpg1684 are specific for the Philadelphia-1 strain (**Table 2**). In some cases, two or more genes were clustered in a specific region in the chromosome (**Table 2**), a common phenomenon in gene organization of *L. pneumophila* type IV substrates [29] that often accounts for the remarkable plasticity of genomes of this organism [161, 162].

Many factors could influence the translocation efficiency of an effector protein as demonstrated in the β-lactamase assay, and one of the important parameter is the stability of the fusion protein. To examine whether the difference in transfer efficiency among proteins was due to the stability of the fusion proteins, we examined the levels of the fusion proteins in several strains from each group. In general, there was no clear correlation between the steady state levels of the hybrids and translocation efficiencies. For example, the steady state levels of the TEM-Lpg0021 and TEM-Lpg0181 were among the highest in these strains, but their transfer efficiencies were not the highest (**Fig. 2-3**). On the other hand, the poorly expressed TEM-Lpg2555 and TEM-Lpg2874 were translocated at high efficiency (**Fig. 2-3**). To further determine to what extent the lack of detectable translocation was a result of a failure to express the protein fusions, we examined the β-lactamase fusions in 27 transfer deficient strains. Although the protein levels vary, all of these strains produced readily detectable fusion proteins (**Fig. 2-3**). Therefore, the level of fusion protein expressed in *L. pneumophila* is not the sole factor determining the translocation competency of a particular substrate.

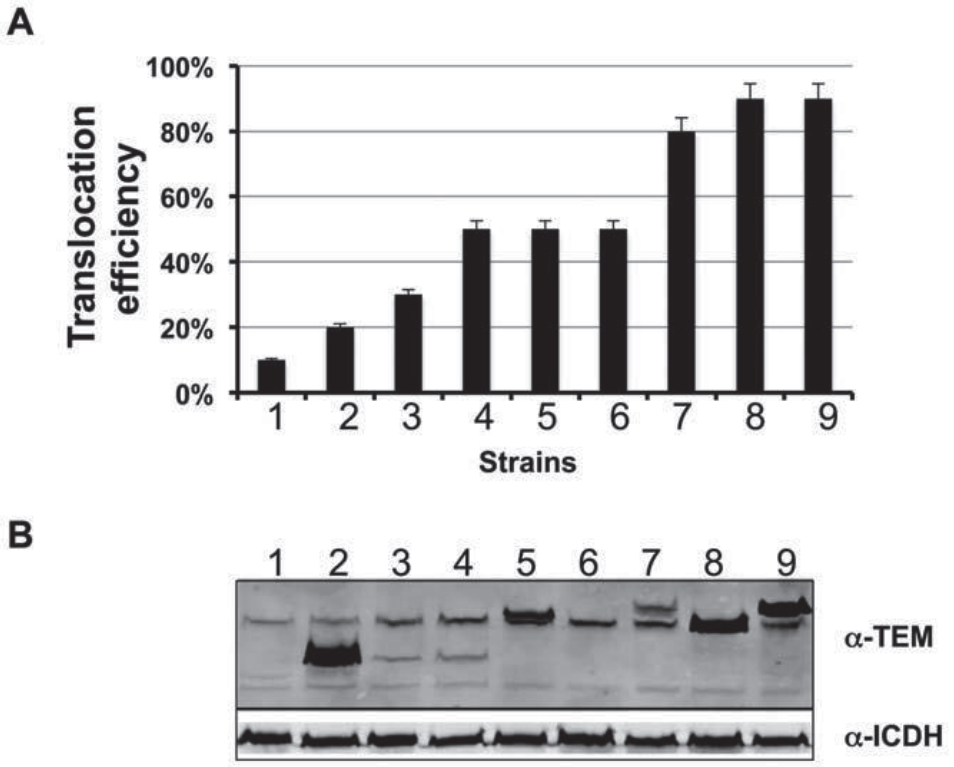


Figure 2-3. Translocation efficiency does not correlate with levels of b-lactamase fusion expressed in *L. pneumophila*. A. The translocation efficiency of 9 substrates in the β-lactamase assay. After CCF4-AM loading, macrophages infected with bacterial strains expression fusions between β-lactamase and individual genes were inspected under a fluorescence microscope, translocation efficiencies were obtained by enumerating cells emitting blue and green fluorescence signals, respectively. Experiments were performed in triplicates and at least 300 cells were counted each sample. Similar results were obtained in at least two independent experiments. **B.** The levels of the fusion proteins in *L. pneumophila* strains used for infections shown in **A.** Bacterial cells equivalent to one OD600 unit were lysed in 200 ml of SDS loading buffer, 15 ml of boiled supernatant were resolved by SDS-PAGE. After transferring to nitrocellulose membranes, the fusion protein was detected with a β-lactamase specific antibody by immunoblot. The isocitrate dehydrogenase (ICDH) was probed as a loading control. Samples: 1. Lpg1776; 2, Lpg0021; 3, Lpg2425; 4, Lpg1147; 5, Lpg0181; 6, Lpg2555; 7, Lpg2874; 8, Lpg0405; 9, Lpg0195.

Recognition of diverse translocation signals by the Dot/ Icm transporter

In our efforts to identify common features in the last 100 amino acids of the substrates that may be important for Dot/Icm-dependent protein translocation, we found that a few proteins that have amino acid composition greatly different from the known features. One such example is Lpg2844, a 361 aa protein in which more than 1/3 of the residues are serine. Interestingly, the last 100-aa region of this protein contains few of the features known to be important for Dot/Icm-dependent translocation. A hydrophobic residue (methionine) at the -3rd position [163] is the only recognizable characteristic associated with translocation found in this region of this protein (**Fig. 2-4, A**). Full-length Lpg2844 promoted the translocation of β-lactamase with 85% efficiency (**Table 2** and **Fig. 2-4, B&D**). Importantly, a chimera containing β-lactamase fused to the last 100 amino acids of Lpg2844 promoted translocation at efficiencies only marginally lower than those of full-length proteins, indicating that like other Dot/Icm substrates, signals important for translocation localized to the C-terminal portion of this protein (**Fig. 2-4, B–D**). As expected, a fusion that contained Lpg2844 lacking the last 100 amino acids failed to promote translocation at a detectable level (**Fig. 2-4, B&D**). These data indicate that the Dot/Icm transporter is capable of recognizing diverse features in the C-terminal portions of its substrates and that in some cases such differences do not affect translocation efficiencies of the proteins.

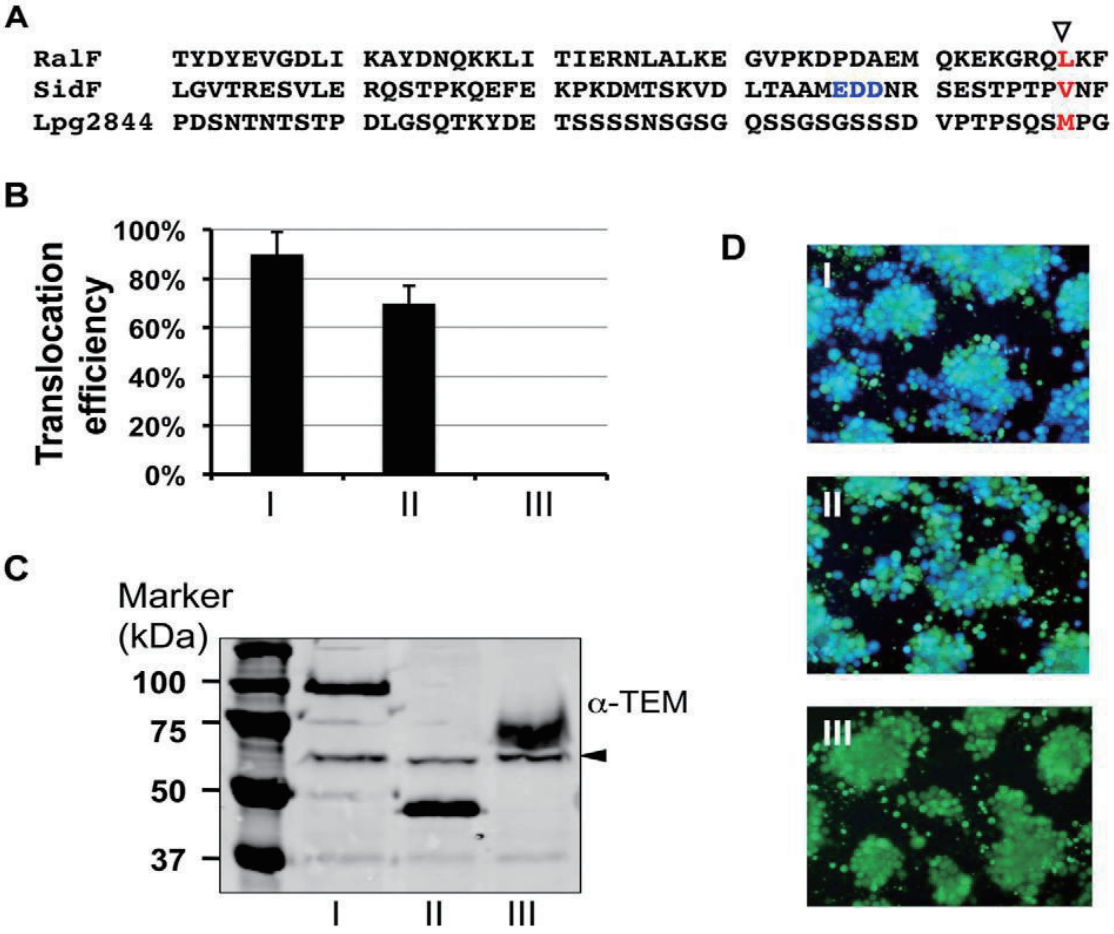


Figure 2-4. Diverse features presented in the C-terminal end of Dot/Icm substrates. A. Alignment of the last 50 amino acids of three well established effectors and the new substrate Lpg2844 to highlight the features important for translocation found in Dot/Icm substrates, including: i) The hydrophobic residue at the -3rd position (red) [163] and ii) the E-block [164] (the three blue residues in SidF). Note the different amino acids composition in Lpg2844. B–D. A region containing the last 100 amino acids of Lpg2844 is important and sufficient for promoting translocation. Bacterial strains expressing fusions of b-lactamase to full-length Lpg2844 (I), its last 100 aa (II) or a fragment lacking the last 100 aa (III) were used to infect macrophages and infected cells were loaded with the CCF4-AM dye. Translocation efficiency shown in **B.** was obtained as described in **Fig. 1-3**, data shown are the average of three independent experiments done in triplicates; stable expression of the fusions by *L. pneumophila*, equal amount of protein samples resolved by SDS-PAGE was probed for the fusions with a β-lactamase specific antibody. The ca. 60-kDa non-specific band detected by the antibody was used as a loading control (arrow in panel **C**). Representative images of infected cells loaded with CCF4-AM (**D**). Similar results were obtained in at least two independent experiments

Discussion

Almost four decades after its initial discovery, *L. pneumophila* continues to be the “hot spot” of microbe-host interaction studies. This is not due to that this bacterium is any more lethal than many other pathogens such as *Yersinia pestis*, but lies in the fact that it is both an excellent “cell biologist”, as this bacterium encodes multiple virulence factors to efficiently manipulate host intracellular cellular processes, and a “terrible immunologist” because it triggers many underappreciated immune responses that are not stimulated or even silenced by more adaptive pathogens [99, 165].

Arguably the most important virulence determinant of *L. pneumophila* is the Dot/Icm secretion system. *L. pneumophila* utilizes this system to deliver a large cohort of substrates in order to create a replication niche inside of many different hosts [158]. Functional studies of the protein delivered into the host by specialized translocation system has proved to be extremely useful in dissecting the biology of both the pathogen and host [6]. Studying the molecular functions of the Dot/Icm substrates had led to the revelation of novel posttranslational modifications such as the ampylation and the phosphocholination, as well as intriguing strategies such the “meta-effector” utilized by the bacterium to coordinate host cellular processes [49, 62, 158]. However, our understanding of this field is far from complete because many Dot/Icm substrates could be “missed” in the previous screening due to the potentially biased nature of these screening. To gain more insights into the functions of these substrates and the revolutionary pressure that drives the diversity of them, we initiated the genome-wide screening for a more complete list of the substrates of the Dot/Icm system.

The genome-wide screening for new effector was effective, as it did not rely on potentially biased parameters to choose candidates and it utilized a high-throughput yet sensitive beta-lactamase assay as the readout. This screen led to the identification of additional 70 effectors to the *L. pneumophila* Dot/Icm

substrate inventory [158]. The overlap between the proteins identified in this study and other studies validates the effectiveness of our methods. The identification of these new effectors also raises exciting possibilities to uncover novel effector functions, as the predicted activities of these effectors cover acyltransferases, peptidases, Rho-GEF (Guanine nucleotide exchange factors), hydrolases, and methyltransferases [158]. Despite the comprehensiveness of the screening, it is not without limitations. First, we only selected the candidate genes bigger than 300bp, since most of the effectors harbor enzymatic activities, and the probability of smaller genes coding for enzymes is lower. However, at least one *L. pneumophila* effector, Lpg0045, is smaller than this threshold [166]. Second of all, we excluded the genes that have been annotated by proteins with known functions. One exception will be the flagellin, which is thought to be translocated into the host by the Dot/Icm system [147]. Third, although we showed that the protein level does not necessarily correlate with the level of translocation, it is possible that some of our constructs were not expressed well enough to allow detectable translocation. In agreement with this concern, eight proteins that were shown capable of promoting translocation of SidCΔ100 did not show positive signals in our assay, even though the beta-lactamase fusion of seven of these proteins were stably expressed in *L. pneumophila*. Further, since the translocation signal has not been unequivocally determined, it is possible that some translocation signals are located at other parts of the protein other than the C-terminus. If this were the case, our N-terminus beta-lactamase fusion might not work. Clearly, there are different limitations that could be applied to different systems that measure substrate translocation, our the beta-lactamase reporter system is of no exception. Nevertheless, the fact that we have re-identified 94 Dot/Icm substrates that have been reported prior to our study validated the reliability of our reporter system [158, 167]. The newly identified effectors in this report not only facilitated the research of *L. pneumophila*, but also showed the great potential of this method in high-throughput-screening of translocation

system substrates in other microbes, such as *Coxiella burnetii*, *Salmonella Typhymurium* and *Helicobacter pylori* [167].

The number of the Dot/Icm substrates coded by *Legionella pneumophila* far exceeds that of any bacterium studied so far. Such a large repertoire may explain the lack of intracellular growth defects when one or more effectors are deleted from the genome of the bacterium. The obvious question that follows is, what is the evolutionary pressure that drives the bacterium to acquire and maintain so many effectors in its genome? A recent study aimed at this question revealed that seven distinct genomic clusters containing 27.1% of the *Legionella* genome are dispensable for in bacterial growth in vitro and in murine macrophages. However, *L. pneumophila* mutants lacking five such clusters showed differential defects in colonizing distinct amoebae species. This evidence, together with the low GC content and marked variability of these clusters, prompted the authors to propose that these clusters were acquired by the bacterium horizontally to maintain optimal growth in a wide variety of amoebae hosts [66]. Although this bacterium could encode such a large number of Dot/Icm substrates to conquer a wide variety of amoebae hosts in the natural environment, other bacteria, such as *Pseudomonas aeruginosa*, whose virulence also depends on a functional secretion system (Type III Secretion System), harbors significantly fewer effectors, yet colonize hosts that range from plants to mammals [168]. Therefore, an alternative explanation could be that the Dot/Icm apparatus is more promiscuous than other secretion systems and “accidentally” translocates bacterial proteins into the host [148]. This is in agreement with the observation that this bacterium delivers bacterial flagellin into the host with no obvious benefits [148]. Even if the large number of *L. pneumophila* Dot/Icm effectors are the result of the promiscuousness of the translocation system, it does not reduce the significance of our study in any way. On the contrary, this could lead to the identification of more conserved bacterial molecules that are under surveillance by the host cells, thus the identification of previously

understudied immune pathways that are not elicited by more adaptive pathogens. Regardless the driving force for acquiring such a large repertoire of effectors, the study aimed at elucidating the biochemical and cell biological functions of these substrates will definitely contribute to the better understanding of the biology of both the pathogen and the host.

CHAPTER III: STUDY OF THE OF DOT/ICM SUBSTRATES CAPABLE OF ACTIVATING CASPASE-3

Abstract

Permissive macrophages challenged by *L. pneumophila* are resistant to exogenous cell death stimuli despite harboring high levels of active caspase-3. Although several reports have described the *L. pneumophila* effectors that contribute to pro-survival signaling cascade, the Dot/Icm substrates that fuel caspase-3 activation have not been documented, nor have the mechanisms underlying such activation. Taking advantage of the expended list of *L. pneumophila* Dot/Icm effectors, we initiated a screening to survey all known effectors for their ability to activate caspase-3. Our screening led to the discovery that at least five Dot/Icm substrates, Lpg0716, Lpg0898, Lpg1831, Lpg1625 and Lpg2716, could activate caspase-3 when expressed in mammalian cells. We further demonstrated that one of these effectors, VipD (lpg2831), is a phospholipase A2 that hydrolyzes phosphatidylethanolamine (PE) and phosphocholine (PC) on the mitochondrial membrane in a manner that appears to require host cofactor(s). The lipase activity is essential to its ability to activate caspase-3 and leads to the production of free fatty acids and 2-lysophospholipids, which destabilize the mitochondrial membrane. Mitochondria membrane destabilization may contribute to cytochrome c release and subsequent activation of caspase-3. Moreover, whereas caspase-3 activation does not seem to play significant roles in limiting *L. pneumophila* replication in permissive macrophages, it is essential to restricting bacterial growth in dendritic cells. We further demonstrated that genetic ablation of all five caspase-3 activating effectors dampens apoptosis activation in dendritic cells. Our result

demonstrated that the activation of host apoptosis pathway is a collective effect of multiple effectors with diverse biochemical activities.

Introduction

Host cell death pathways are essential determinants of the outcome of infection [169]. It is not surprising that numerous pathogens utilize various strategies to manipulate these processes to their benefit. For instance, *Coxiella burnetii*, a pathogen that is closely related to *L. pneumophila*, translocates multiple virulence factors through the Dot/Icm to inhibit host apoptosis [170]. *Yersinia pestis*, on the other hand, codes for a T3SS effector YopM, which structurally mimics the substrates of caspase-1, to inhibit caspase-1 activity through direct binding [92]. Similar to many other vacuolar pathogens, *L. pneumophila* actively thwarts host cell death to avoid premature termination of bacterial replication. It is therefore almost “cliché” that permissive macrophages infected by *L. pneumophila* showed strong resistance to exogenous cell death stimuli [100]. However, it is intriguing that these macrophages also harbor active caspase-3, the executioner of cellular apoptosis [100]. The importance of this phenomeon was demonstrated in dendritic cells, which die of apoptosis soon after *L. pneumophila* infection [104]. Suppressing cellular apoptosis allows *L. pneumophila* to replicate in dendritic cells, which otherwise are not permissive to this bacterium [104]. Although the *L. pneumophila* effectors contributing to the suppression of host cell death have been well documented, almost nothing is known about the Dot/Icm substrates that activate caspase-3. Therefore, we attempted to take advantage of the newly available list of *L. pneumophila* effectors and initiated a screen to identify bacterial factors that contribute to caspase-3 activation.

Results

Identification of Dot/Icm substrates capable of activating caspase-3

Many host cell signaling cascades including the host cell death pathways, are modulated by numerous effector proteins of *L. pneumophila*. Despite the fact that permissive cells that harbor actively replicating *L. pneumophila* are resistant to exogenous cell death stimuli, these cells also contain active caspase-3, the executioner caspase [100]. The caspase-3 activation in *L. pneumophila* infection could be the result of the innate immune responses to the biochemical activities of the Dot/Icm substrates, or the consequences of one or more Dot/Icm substrates directly activating this enzyme. The recent availability of a more complete list of Dot/Icm substrates made the latter possibility more practical to test [171]. To identify the Dot/Icm substrates capable of activating caspase-3, I utilized two independent approaches: The first is a commercially available Caspase-Glo 3/7 Assay System (Promega), a luminescence assay that measures the activity of caspase-3. In the presence of active caspase-3, the proluminescent luciferase substrate containing the DEVD caspase-3 cleavage site will be released for luciferase to produce light. To identify caspase-3 activating effectors using this system, I transfected 293T cells with the GFP-candidate-gene fusion constructs in a 96-well format (to facilitate the high throughput screening) using Lipofectamine 2000, 24 hours later, cells were lysed to measure the caspase-3 activity. One protein, Lpg0898, capable of activating caspase-3 was identified using the Caspase-Glo 3/7 Assay System, providing evidence that *L. pneumophila* does code for factors that activate caspase-3. However, deleting Lpg0898 did not ablate caspase-3 activation *L. pneumophila* during infection, suggesting that *L. pneumophila* codes for more effectors capable of activating caspase-3. To achieve a more comprehensive screening, I used an immunoblot-based method to probe for the presence of activated caspase-3 (p-17 and p-19) in 293T cells ectopically expressing the candidate effectors. The tested candidate genes included those that showed

marginally higher reading than control in the Caspase-Glo 3/7 Assay, and also ones that have been shown to be toxic to mammalian cells, also found in earlier studies. 100μg total lysate of each sample was resolved by SDS-PAGE gels. And the immunoblot-based method led to the identification of five Dot/Icm substrates, Lpg0716 [158], Lpg0898(Ceg18) [41], Lpg1625 (Lem12) [42], Lpg2176 (LegS2) [35], and Lpg2831 (VipD) [30, 108] that are able to induce caspase-3 activation in 293T cells under transfection condition (**Fig. 3-1**). Although some of these proteins are toxic to mammalian cells, expression of GFP fusions of these proteins was readily detectable (**Fig. 3-1,** middle panel). Sequence analyses revealed that Lpg2716 (LegS2) is a sphingosine-1-phosphate lyase, which has been shown to target to the mitochondria during bacterial infection [172]. Further, the product of sphingosine-1-phosphate lyase, sphingosine-1-PO_4, has been shown to cooperate Bak and Bax to induce the onset of apoptosis [173]. Lpg2831 (VipD) possesses a domain found in the catalytic motif of members of the patatin-like phospholipase A_2 [30, 108]. On the other hand, Lpg0716, Lpg0898 (Ceg18), Lpg1625 (Lem12) and Lpg1803 harbor no motif suggestive of any potential biochemical activity.

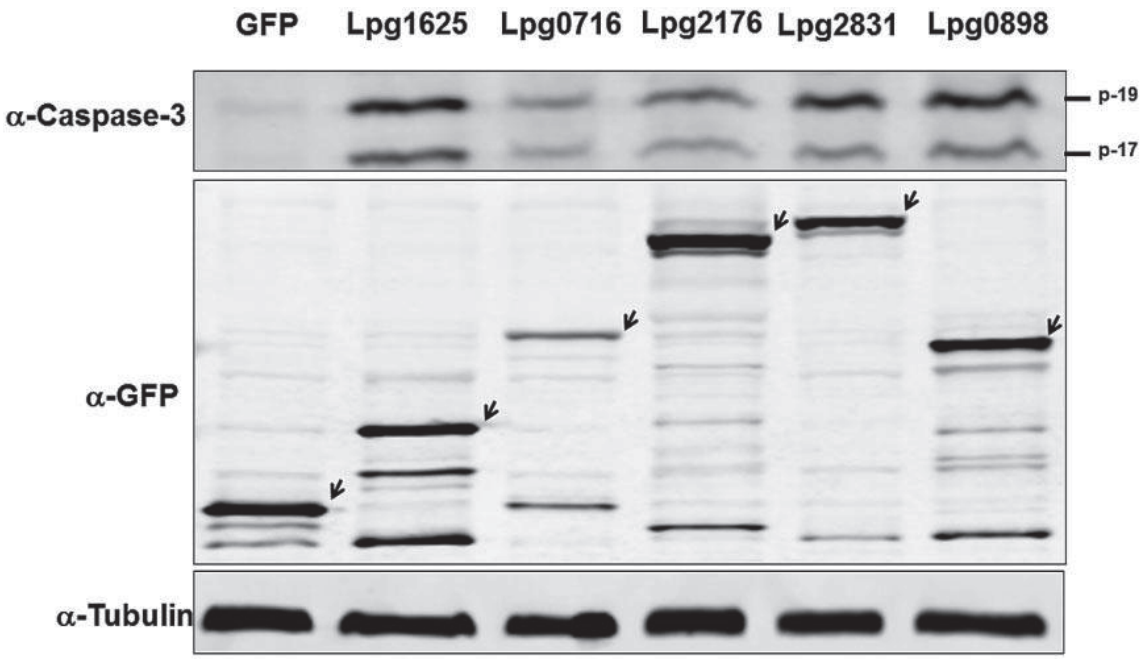


Fig. 3-1 Activation of caspase-3 by *L. pneumophila* Dot/Icm substrates. Lysates of HEK293T cells transfected to express GFP or its fusions of indicated proteins for 24 hr were probed for active caspase-3 with a specific antibody (upper panel). The p-17 and p-19 bands are the processed mature form of caspase-3. The expression of the GFP fusions in these samples was probed with a GFP specific antibody (middle panel). Arrows indicate the band corresponding to the GFP fusion proteins. Tubulin was probed as a loading control (lower panel).

Some of the *Legionella* caspase-3 activators localize to the mitochondrion

The mitochondrion is the central regulator for the intrinsic apoptotic pathway. The sphingosine-1-phosphate lyase Lpg2176 (LegS2) had been shown to localize to this organelle [172]. We thus examined whether other identified caspase-3 activators also target to mitochondria. Cells transfected to transiently express GFP fusions of each of these proteins were stained with an antibody specific for Cyto c oxidase (COX4I1). We found that like GFP-Lpg2176, in cells expressing GFP-Lpg1625, the GFP signal exclusively localized to the mitochondria and the patterns of the signal were identical to those of COX4I1 (**Fig. 3-2**). Most, but not all of GFP-Lpg0898 localized to the mitochondria (**Fig. 3-2**). However, the expression level of GFP-0716 is low and the GFP signals appeared to localize to the cytosol. GFP-2831(VipD) mostly occupied the plasma membrane of transfected cells, and did not significantly colocalize with the COX4I1 staining signals (**Fig. 3-3**).

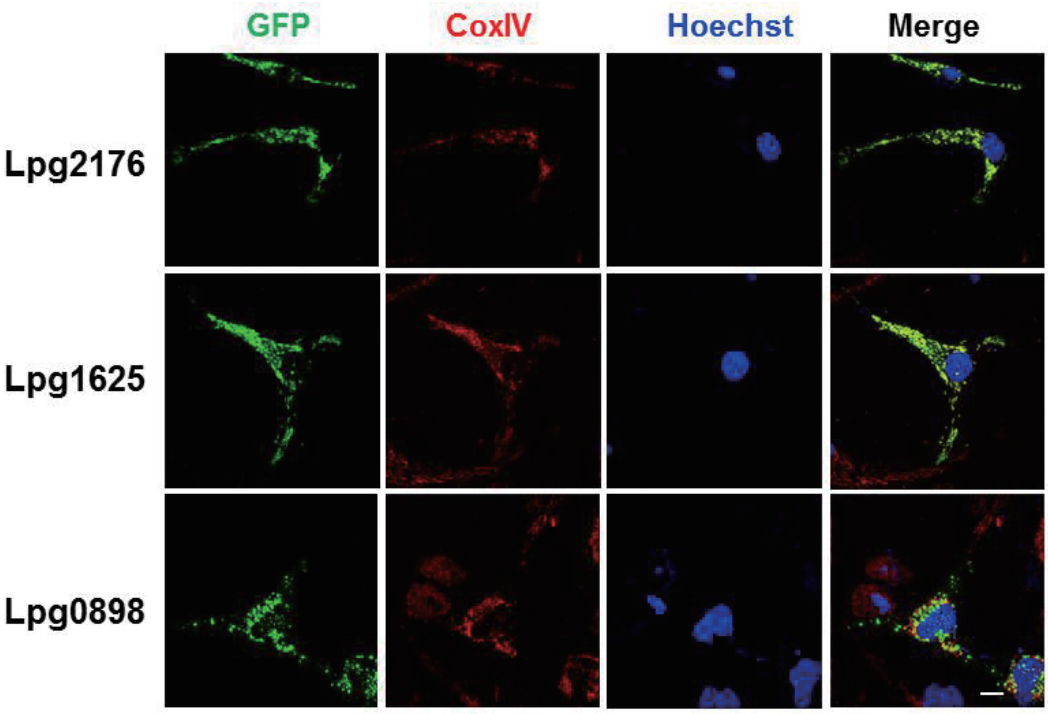


Fig. 3-2 Some of the caspase-3 activators localized to the mitochondrion. Hela cells transfected to express GFP fusion of the indicated proteins were stained with antibody specific for the mitochondrial protein COX4I1. Samples were analyzed using an Olympus IX-81 fluorescence microscope for images acquisition. Images were pseudocolored with the IPLab software package. Bar: 20 μm.

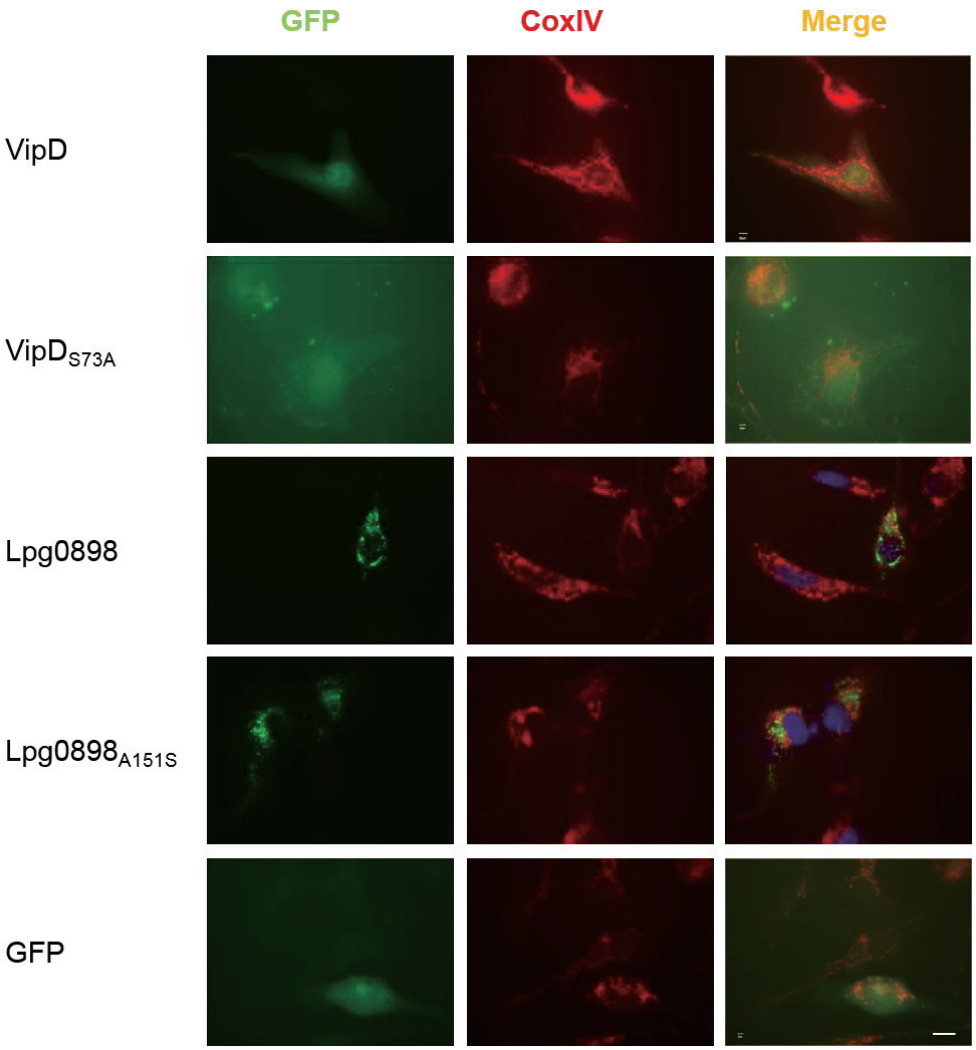


Fig. 3-3 GFP-VipD localizes in the cytoplasm of mammalian cell. Hela cells transfected to express GFP, GFP-0898, GFP-0898$_{A151S}$, GFP-VipD or GFP-VipD$_{S73A}$ for 18 hr were fixed and stained for the mitochondrial protein COX4I1. Samples were analyzed using an Olympus IX-81 fluorescence microscope for images acquisition. Images were pseudocolored with the IPLab software package. Bar: 20 μm.

We also found that protein Lpg0898 and Lpg2831 (VipD) caused significant rounding of mammalian cells (**Fig. 3-2**). To rule out the possibility that the cytotoxicity of these proteins interferes with the proper cellular localization, we isolated a non-toxic mutant of Lpg0898, $Lpg0898_{A151S;}$ and this mutant still partially localized to the mitochondria (**Fig. 3-1**). For Lpg2831, we determined whether the putative phospholipase A2 is responsible for the cell rounding phenotype by mutating serine 73 in the predicted catalytic motif to alanine. Although this mutation did not reveal the targeting of Lpg2831(VipD) to a specific organelle, it abolished its ability to cause the cell rounding phenotype in mammalian cells (**Fig. 3-4**). These results suggest that mitochondrion is the site of action for some of the caspase-3 activators. Our observations also suggest that the putative phospholipase A2 activity of VipD is important for its role in caspase-3 activation. Because of the potential biochemical activity and the availability of many useful reagents for VipD [30], I chose to focus on this protein for further study.

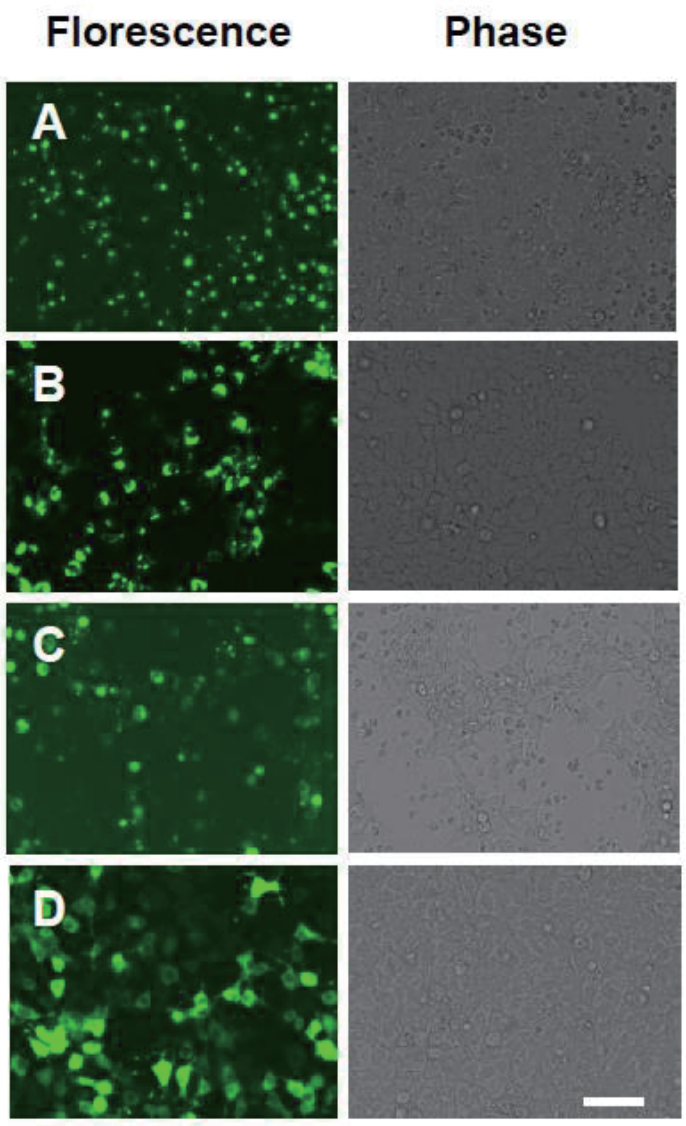


Fig. 3-4 Cell rounding phenotype caused by Lpg0898 and VipD and their nontoxic mutants. 293T cells transfected to express GFP-Lpg0898 (A), GFP-Lpg0898 $_{A151S}$ (B), GFP-VipD (C) or GFP-VipD$_{S73A}$ (D) for 24 hr. Images were acquired with an Olympus IX-81 fluorescence microscope equipped with a CCD camera. Note the cell rounding phenotype caused by wild type Lpg0898 and VipD. Bar: 50 μm.

VipD induces the release of Cyto c from mitochondria

The fact that VipD$_{S73A}$ is no longer toxic to mammalian cells prompted me to hypothesize that the putative phospholipase A2 activity may be important for VipD's capability to activate caspase-3. Lysates of 293T cells transfected to express VipD or its mutant were probed for active caspase-3. Whereas expression of GFP-VipD consistently led to production of the p-17 and p-19 fragments of caspase-3, cells expressing GFP or GFP-VipD$_{S73A}$ did not produce detectable active caspase-3 (**Fig. 3-5, A**). Thus, the putative PLA2 activity of VipD is essential for its role in the activation of caspase-3.

In the intrinsic apoptotic pathway, the release of the cytochrome c from mitochondria is a prerequisite for caspase-3 activation; I then asked whether VipD is able to cause Cyto c release *in vivo*. Cells transfected with appropriate constructs were permeabilized with mitochondrial buffer (20 mM HEPES, pH 7.4, 250 mM sucrose, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 100 µg/µl digitonin) on ice for 5 mins, which selectively disrupts plasma membrane while leaving the mitochondrial membrane intact [174]. The resulting cytosolic fraction was separated from the mitochondria-rich membrane fraction by low speed centrifuge and tested for the presence of Cyto c. Expression of wild type VipD, but not the S73A mutant or GFP alone led to Cyto c release (**Fig. 3-5, B**).

Although VipD is capable of causing cytochrome c release under transfection conditions, it was not clear whether that was a direct or indirect consequence of its biochemical activity. To test these two possibilities, I further examined the activity of VipD using a cell-free system. To purify mitochondria from 293T cells, cells were suspended in hypotonic solution followed by mechanical homogenization to remove the plasma membrane, and the heavy membrane fraction and nucleus were removed by low speed centrifugation. Mitochondria were then pelleted and washed per manufacturer's instruction

(Sigma) and further purified by sucrose-gradient centrifuge. Recombinant VipD was added to purified mitochondria and the release of Cyto c was monitored as mentioned above after 2 hr of incubation. As little as 0.65 μg His_6-VipD caused significant Cyto c release (total volume of 100μl), and 2.5 μg protein was sufficient to disperse more than 90% of Cyto c from 100 μg of mitochondria (**Fig. 3-5, C**). Interestingly, addition of VipD also led to the release of SOD2, a mitochondria matrix resident protein, into the supernatant, suggesting the integrity of the mitochondrial inner membrane was also compromised in this process (**Fig. 3-5, C**). Consistent with the *in vivo* results, as much as 40 μg $VipD_{S73A}$ failed to cause significant Cyto c release (**Fig. 3-5, C**, last lane).

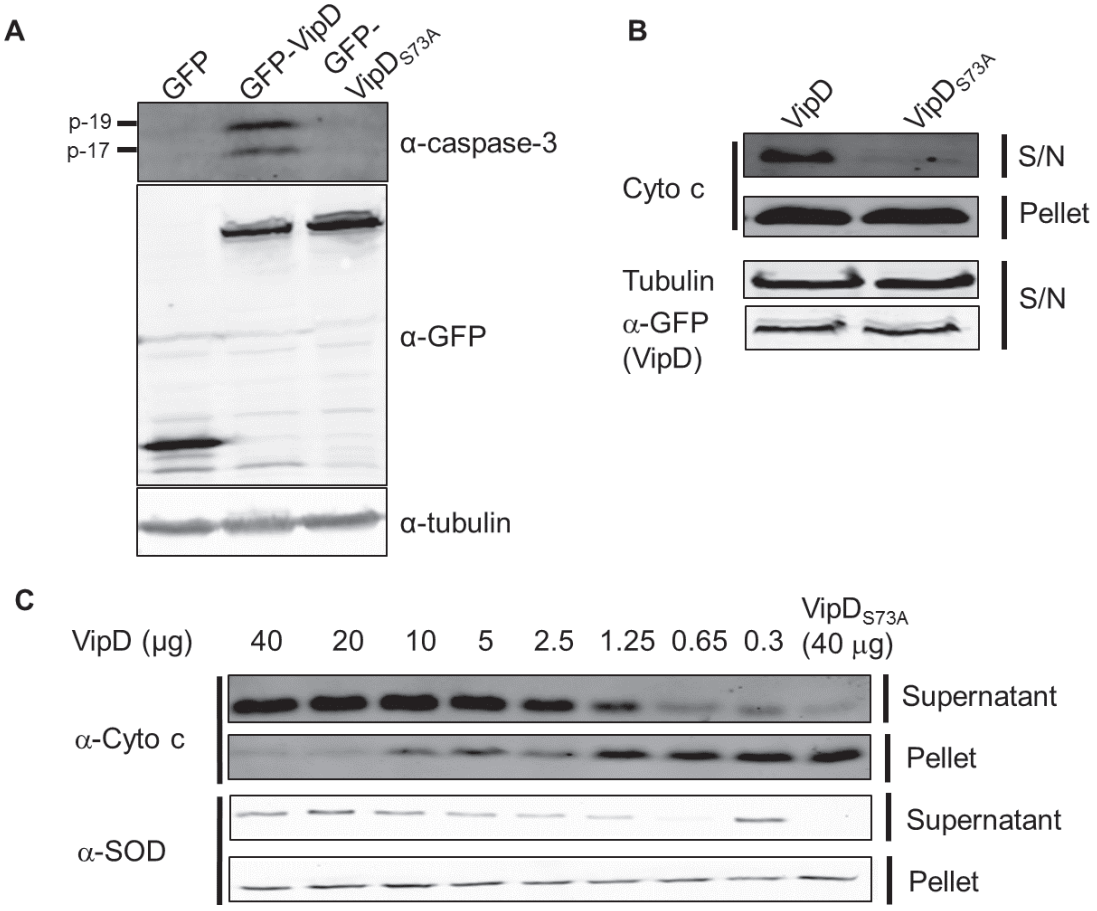


Fig. 3-5 VipD-induced caspase-3 activation and Cyto c release require the phospholipase motif. **A**. The phospholipase motif is essential for the activation of caspase-3. 100μg lysates of HEK293 cells transfected to express GFP (1st lane), GFP-VipD (2nd lane) or GFP-VipD$_{S73A}$ (3rd lane) for 24 hr were probed for active caspase-3. Note the high level expression of GFP-VipD$_{S73A}$ (3rd lane, middle panel). Tubulin was probed as loading controls (lower panel). **B**. VipD-induced Cyto c release *in vivo*. 50μg soluble fraction and mitochondrial fraction of HEK293 cells transfected to express GFP-VipD or GFP-VipD$_{S73A}$ for 24 hr were probed for Cyto c. The expression of the GFP fusions was also analyzed. Tubulin present in the soluble fraction was detected as a loading control. **C.** VipD-induced Cyto c release from purified mitochondria. Indicated amounts of His$_6$-VipD or His$_6$-VipD$_{S73A}$ were incubated with 50μg mitochondria at 30°C for 2 hr. The presence of Cyto c in the soluble fractions was detected by immunoblotting. Note that little Cyto c release was induced by 40μg His$_6$-VipD$_{S73A}$ (last lane).

The disruption of the mitochondrial inner membrane potential ($\Delta\psi_m$) often precedes the release of Cyto c [175]. I thus examined whether VipD disrupted the integrity of the mitochondrial inner membrane. Hela cells transfected to produce VipD, $VipD_{S73A,}$ or GFP were stained with the mitochondrial transmembrane potential sensitive dye, tetramethylrhodamine methyl ester perchlorate (TMRM), and the fluorescence intensity of mitochondrion (*Fm*) and cytosol (*Fc*) reflected by the TMRM staining was quantified. TMRM images were thresheld to isolate the mitochondrial florescence (*Fm*), and the florescence intensity of areas of one square micrometer was measured using the software ImageJ (NIH). Cytoplasmic florescence (*Fc*) is determined in hand-drawn regions in close proximity to the mitochondria. The value of $\Delta\psi_m$ was obtained with the formula $\Delta\psi_m$=[(Fm-(2/3)Fc)/(1/3) Fc]. Expression of VipD but not its S73A mutant compromised the integrity of the mitochondrial inner membrane (**Fig. 3-6**). Taken together, these results indicate that induction of caspase-3 activation by VipD is mediated by its putative PLA2 activity, which triggers the mitochondrial inner membrane permeabilization (MOMP) and the subsequent release of Cyto c.

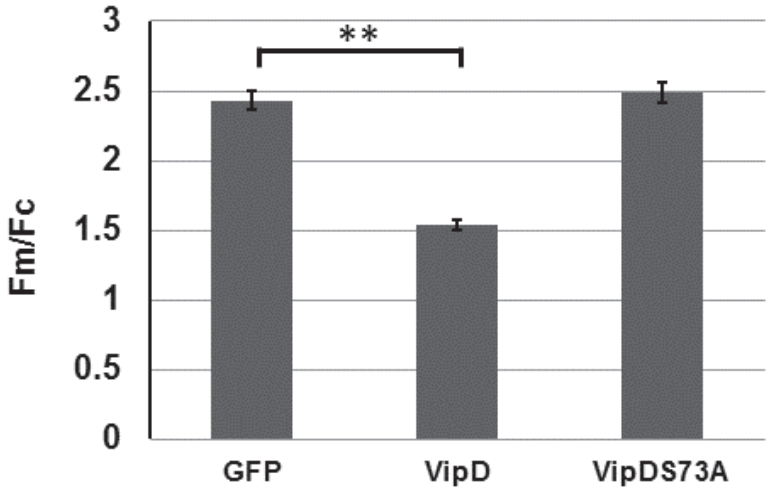


Fig. 3-6 VipD compromises the mitochondrial inner membrane integrity. Culture medium of HEK293T cells transfected with indicated constructs for 18 hr was replaced with medium containing 20 nM TMRM for 20 min. Dye loaded samples were then mounted on a Teflon holder for image acquisition using a Nikon Eclipse Ti microscope (60x oil Plan Apo objective). TMRM images were thresheld to isolate the mitochondrial florescence (*Fm*), and the florescence intensity of areas of one square micrometer was measured using the software ImageJ (NIH). Cytoplasmic florescence (*Fc*) is determined in hand-drawn regions in close proximity to the mitochondria. $\Delta\psi_m$ is then determined as the ratio of *Fm/Fc* using the following formula: [(Fm-(2/3)Fc)/(1/3) Fc]. At least 300 mitochondria in each sample were analyzed and the statistical analysis was performed using SPSS 20.0 (IBM). **, $p<0.001$.

VipD-mediated mediated Cyto c release is independent of Bax and Bak

Since Bax and Bak, the two pro-death, pore-forming proteins important for MOMP [175] appear essential for cell death induction during *L. pneumophila* infection [102], I sought to determine whether these two proteins are necessary for the activity of VipD in caspase-3 activation. To this end, I transiently expressed VipD in both WT mouse embryonic fibroblasts (MEFs) and deficient in Bax and Bak. Similar levels of activated caspase-3 could be detected in both MEFs **(Fig. 3-7** upper left panel,). Of note is that the Mirus transfection reagent nonspecifically caused caspase-3 activation in this cell line (**Fig. 3-7, A**, upper left panel, 1st lane). Although caspase-3 activation occurred in samples that received no DNA or received the catalytically inactive VipD$_{S73A}$ mutant, the amount of active caspase-3 in the sample expressing wild type VipD is consistently higher (**Fig. 3-7, A**&**B**). Importantly, in the Bax/Bak$^{-/-}$MEFs, similar activation of caspase-3 was detected, but only in samples expressing wild type VipD (**Fig. 3-7, A**, upper right panel). Similarly, purified His$_6$-VipD but not the VipD$_{S73A,}$ was able to induce Cyto c release from mitochondria isolated from both wild type and *bak*/*bax*$^{-/-}$ MEFs (**Fig. 3-7, C**). Thus, VipD appears to directly act on the mitochondrial membranes to compromise its integrity, leading to Cyto c release and the subsequent caspase-3 activation.

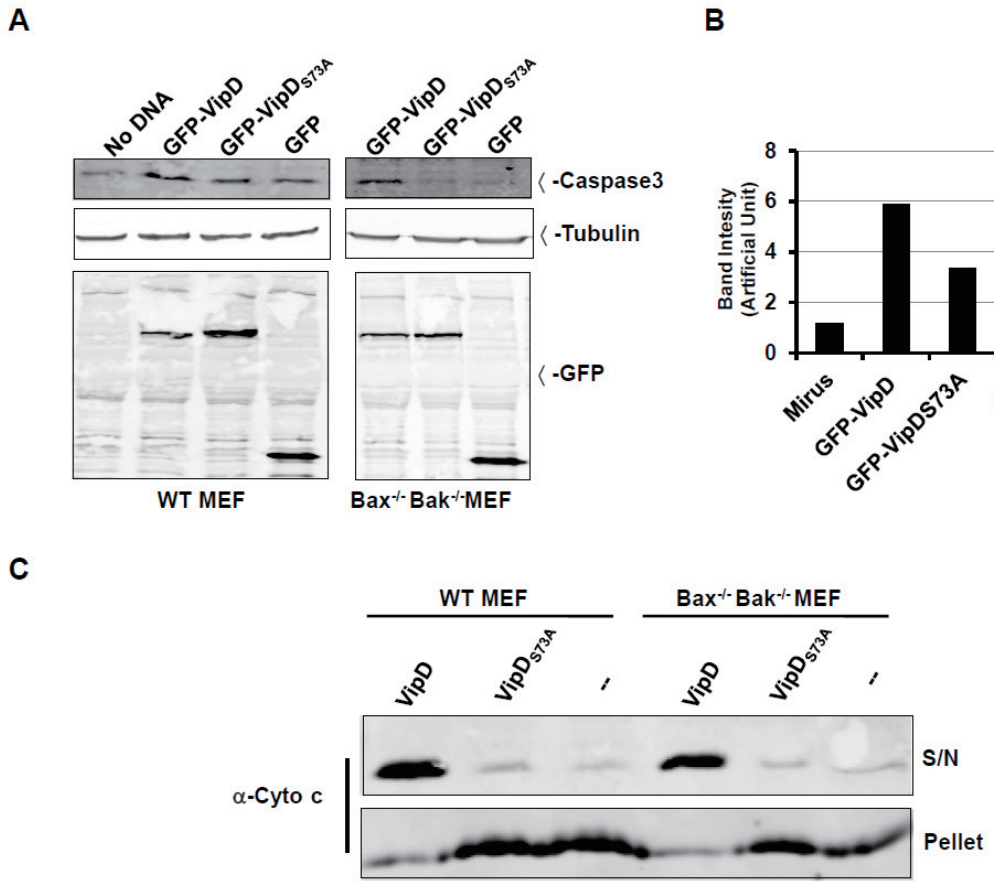


Fig. 3-7 VipD-induced Cyto c release is independent of Bax and Bak. A. Induction of caspase-3 in MEFs by transient expression. Wild type and *bax/bak*$^{-/-}$ MEFs were transfected with plasmid DNA that allows the expression of GFP-VipD, GFP-VipD$_{S73A}$ and indicated controls for 24 h, and the lysates of these samples were analyzed for activated caspase-3 by immunoblot (upper panel). The levels of the GFP fusions were probed by an antibody specific for GFP (lower panel) and tubulin was detected as a loading control (middle panel). Note that in wild type MEFs, caspase-3 can be activated by the Mirus transfection reagent (Thermo Scientific, Waltham, MA), which is the only one that allows efficient transfection of these cells (lane 1). **B**. Quantitation of the caspase-3 in panel A. Quantitation was obtained by measuring the band intensity using the Odyssey imaging system. Similar results were obtained in two independent experiments. **C**. Induction of Cyto c release by VipD from mitochondria isolated from MEFs. 40μg His$_6$-VipD or His$_6$-VipD$_{S73A}$ was added to 50μg mitochondria purified from wild type mouse embryonic fibroblast (MEF) or from MEF of *bak*/*bax*$^{-/-}$ mouse. After incubation at 30°C for 3 hr, the release of Cyto c was evaluated by probing its presence in the soluble fraction by immunoblot.

Inhibition of VipD-mediated Cyto c release by two PLA_2 inhibitors

The observation that the predicted PLA2 active site of VipD was important for the induction of Cyto c release strongly suggests that VipD is a phospholipase A2. We thus examined this activity by determining the effects of two specific phospholipase A2 inhibitors, Methyl arachidonyl fluorophosphonate (MAFP) [176] and (R)-Bromoenol lactone ((R)-BEL) [177], on VipD-mediated Cyto c release. As little as 0.2 μM MAFP almost completely abolished Cyto c release caused by VipD (**Fig. 3-8**); (R)-BEL also blocked VipD activity, but with a relatively lower potency; 10 μM of this compound was required to exert detectable inhibitory effects (**Fig. 3-8**). Because both MAFP and (R)-BEL are highly selective, active site directed, irreversible inhibitors of PLA_2 [176, 177], these results further suggest that VipD is a phospholipase A2.

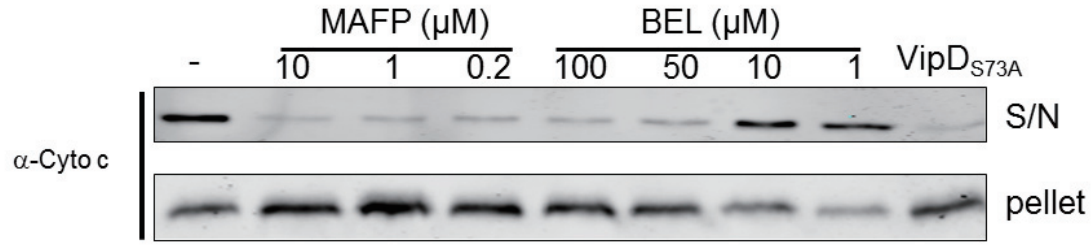


Fig. 3-8 Inhibition of VipD-induced Cyto c release by two inhibitors for phospholipase A2. The inhibitors MAFP or (R)-BEL was added to suspensions of 50μg purified mitochondria at indicated concentrations simultaneously with 42 μg of His_6-VipD. After incubation for 3 hr at 30°C, samples were subjected to centrifugation to obtain the soluble fraction and the insoluble membrane pellet and each fraction was analyzed for the presence of Cyto c. Samples receiving His_6-VipD (first lane) or His_6-$VipD_{S73A}$ (last lane) but no inhibitor were included as controls.

VipD does not indiscriminately disrupt biological membranes

Phospholipase A2s have a relatively broad substrate spectrum and are capable of disrupting the integrity of membranes from different sources [178, 179]. To determine whether VipD-mediated Cyto c release is due to indiscriminate membrane disruption, we first examined whether VipD compromises the integrity of the plasma membrane. Hela cells were transfected with GFP-VipD for 24 hours and the integrity of the plasma membrane (PM) was assessed with low concentration (4 μM) of ethidium bromide (EtBr), a dye impermeable to intact PM. Treatment with 0.2% TritonX-100 effectively facilitated the uptake of EB. However, although exhibiting the cell rounding phenotype, cells expressing GFP-VipD were not detectably permeable to this dye (**Fig. 3-9, A**). We also tested the effects of exogenous VipD on PM integrity. Inclusion of His_6-VipD in cell culture medium at 10 μM, which is 2-fold higher than the concentration necessary for effective Cyto c release, did not detectably disrupt the PM (**Fig. 3-9, B**). Taken together, these results indicate that VipD specifically targets mitochondrial membranes but not the plasma membrane.

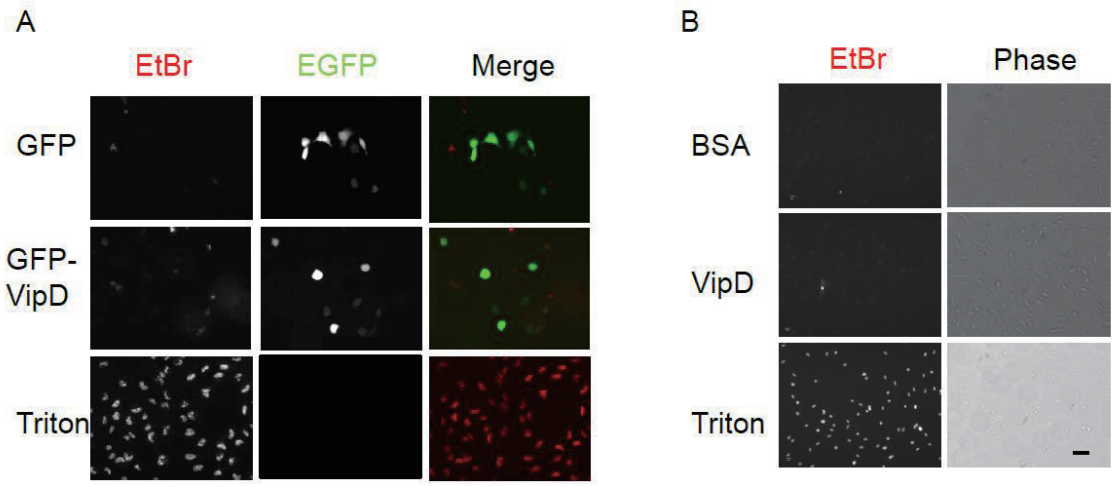
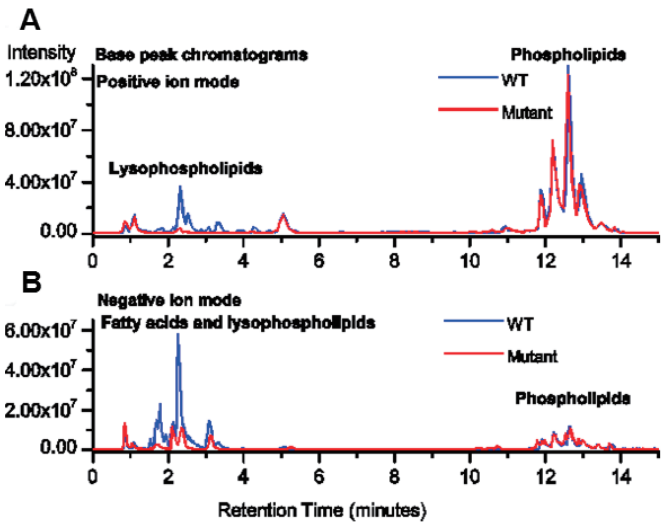


Fig. 3-9 VipD does not cause damage in plasma membrane. A. Hela cells transfected to express GFP or GFP-VipD for 24 hr were washed and stained with 4 μM EtBr. Retaining of the dye by the cells was analyzed by microscopic analysis using an Olympus IX-81 fluorescence microscope equipped with a CCD camera. Untransfected cells treated by Triton-100 were included as a control (lower panel). Note that the nuclei of cells treated by Triton-100 were stained by the dye. **B.** His_6-VipD or BSA was added to culture medium at a final concentration of 8 μg/ml. After 3 hr of incubation, the integrity of the plasma membrane was assessed by the retaining of EtBr using an Olympus IX-81 fluorescence microscope equipped with a CCD camera. Bar: 60 μm.

VipD hydrolyzes two phospholipids on mitochondrial membranes

The specific disruption of mitochondrial membranes by VipD suggests that its substrates are associated with this organelle. To identify these substrates, we extracted total lipids from the mitochondria treated with His_6-VipD or His_6-$VipD_{S73A}$ by the Folch method [180] and subjected these samples to mass spectrometry analysis. Briefly, chloroform, methanol, and water with a volume ration of 8:4:3 were mixed in a separation funnel, allowed to stand to obtain a biphasic system. The upper phase was used as the pure-solvent-upper-phase. The mitochondria samples were placed into a glass homogenizer with 5 drops of ice-cold chloroform-methanol extraction solvent (2:1) and manually homogenized with a pestle. The volume of extraction solvent was slowly increased to 0.5-mL in order to achieve complete homogenization. Homogenates were filtered through a lipid-free paper and mixed with 0.2 volumes of H_2O, and the mix is allowed to separate into 2 phases. After removing the upper phase, the lower phase was rinsed 3 times with the pure-solvent-upper-phase. The lower organic phase containing the total lipids was collected, dried under a stream of nitrogen, and subjected to mass spectrometry analysis. 2-lysophosphatidylcholine and 2-lysophosphatidylethanolamine, the products of phospholipase A_2, increased 18 and 16-fold, respectively in samples receiving VipD but not in those receiving $VipD_{S73A}$, indicating that phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are substrates of VipD (**Fig. 3-10**).



C

m/z	RATIO WT/ST3A	species
317.3035	2.666666667	GL (O-16:0-0:0-0:0)
464.3113	16.81081081	LYSO PE (18:2) ETHER
466.3268	6.696165192	LYSO PE (18:1) ETHER
468.3064	5.325	LYSO PC (14:0)
468.3064	18.18181818	LYSO PE (17:0)
480.3061	13.15384615	LYSO PE (18:1)
482.322	7.574074074	LYSO PE (18:0)
494.3217	8.317757009	LYSO PC (16:1)
496.3374	8.774509804	LYSO PC (16:0)
522.3528	8.387096774	LYSO PC (18:1)
524.3687	4.761904762	LYSO PC (18:0)
482.3581	2.13	LYSO PC (16:0) ETHER

Fig. 3-10 Identification of the natural substrates of VipD. Total lipids from mitochondria treated with His_6-VipD or His_6-$VipD_{S73A}$ were extracted using the Folch method. The composition of the lipids was profiled by mass spectrometry on an LTQ Orbitrap instrument. Shown are representative base peak chromatograms of mitochondrial lipid extracts obtained in positive (A) and negative (B) ion modes using reversed-phase chromatography. Lysophospholipid species with significant increase in His_6-VipD treated samples detected by LC-MS/MS (C).

Despite extensive efforts, we were unable to detect the phospholipase activity of His_6-VipD in biochemical assays using synthetic substrates, which was reported in a recent study on the structure of this protein [181] (**Fig. 3-11, A**). To test the possibility that VipD needs co-factor(s) from the host, we treated isolated mitochondria with proteinase K prior to examining VipD-mediated Cyto c release. Exposure of mitochondria to 1 µg/ml of this proteinase abolished VipD-mediated Cyto c release (**Fig. 3-11, B**). The loss of this activity is not caused by the potential residual proteinase activity as VipD recovered from this reaction was able to compromise membranes in fresh mitochondrial samples (**Fig. 3-11, B**). These results validate the potential need for host factors for the lipase activity of VipD.

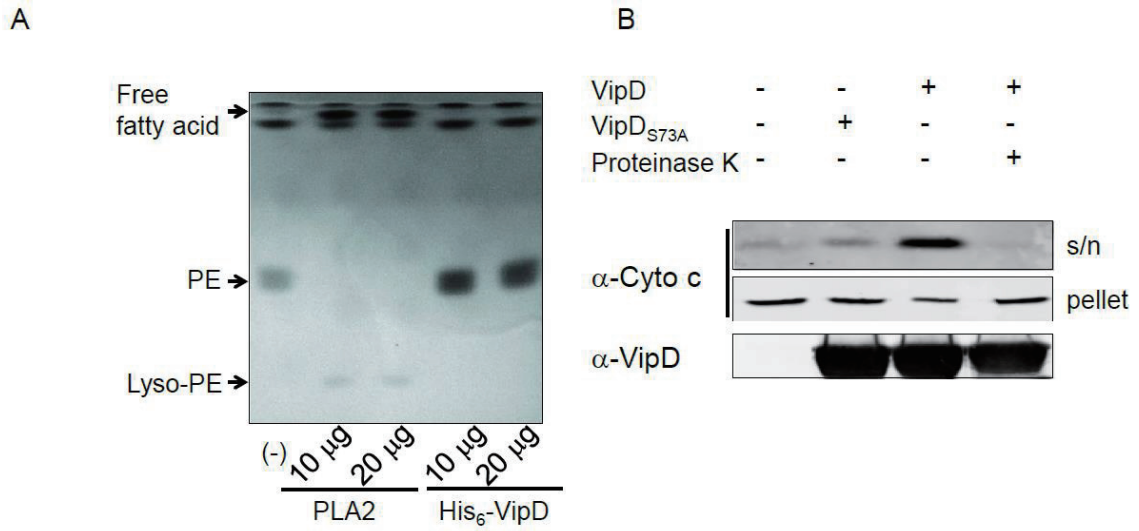


Fig. 3-11 A. VipD did not detectably hydrolyze phospholipids in reactions with defined components. His_6-VipD or commercial phospholipase A_2 was added to liposome established with phosphoethanolamine and the reactions were allowed to proceed for 3 hr at 30°C. Total lipids extracted from the reactions were separated on thin-layer chromatograph (TLC) Silica gel 60 F_{254} (EMD) and lipids were detected using iodine vapor. **B.** Mitochondria were treated with proteinase K or buffer before incubated with VipD or its mutant. The release of cytochrome c from mitochondria was detected via immunoblot.

Lysophospholipids and fatty acids can mimic the effect of VipD in inducing cytochrome c release

It is well-documented that phospholipids adopt a cone shape conformation when it is inserted in to the lipid bilayer [182]. The 2-lysophospholipid and fatty acid molecules produced by phospholipase A2 from phospholipids adopt an inverted cone shape and cone shape, respectively, making them unsuitable in forming lipid bilayer [183]. Membrane damage and the subsequent Cyto c release caused by phospholipase A2 can be the result of either direct lipid degradation [184] or the formation of curvature or lesions on the mitochondrial membranes by the lipids and fatty acids [185]. To determine whether the products of VipD contribute to such damage, we prepared a mixture of lysophospho-ethanolamine and linolenic acid at a 1:1 ratio and examined the effects of the mixed compounds on purified mitochondria. This mixture is capable of recapitulating the effects of VipD on mitochondria, and at least 9 μM of the mixed compounds were required to induce Cyto c release to the level induced by 40 μg of His_6-VipD (**Fig. 3-12**). Thus, VipD-mediated production of lysophospholipids and fatty acids contributes to the disruption of mitochondrial membranes and the subsequent Cyto c release.

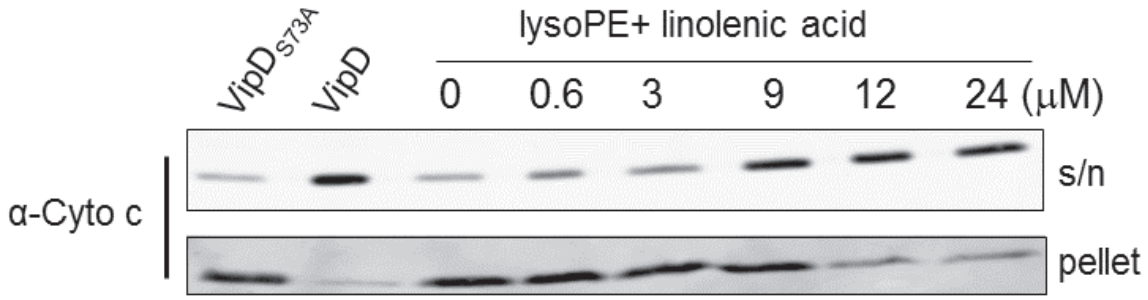


Fig. 3-12 A mixture of lyso-PE and linolenic acid induces Cyto c release from mitochondria. 5μl of linonelic acid and 7.5 mg of lysophosphoethanolamine were dispersed in 5-ml of L buffer. The mixture was brought to dryness under gentle nitrogen stream and was reconstituted with 5-ml L buffer; the indicated amounts of lipid mixture was then added to 50μg purified mitochondria, and incubated at 30°C for 2 hr. The samples were then separated by centrifugation, soluble or pellet fraction was resolved on SDS-PAGE and probed for Cyto c.

The caspase-3 activating effectors are required for maximal induction of cell death in dendritic cells

To determine whether these effectors proteins contribute to caspase-3 activation during *L. pneumophila* infection, we constructed a series of mutants lacking one or more of these genes. To assess their roles in caspase-3 activation during *L. pneumophila* infection, non-polar deletions of each of these genes and the combination of all of them were constructed. The deletion strain, along with WT *L. pneumophila* and a Dot/Icm mutant cultured to post-exponential phase were used to infect U937 at an MOI of 1 [102]. Infected cells were lysed six hours after infection to assay for caspase-3 activity. Similarly prepared bacterial cells were used to infect mouse bone marrow-derived macrophages to evaluate intracellular replication. Deletion of single caspase-3 activator or all five of these genes (Lpg0898, Lpg1625, Lpg0716, Lpg2716, and Lpg2831) did not cause a detectable defect in caspase-3 activation (**Fig. 3-13, A**). This was not due to the fact that U937 being a more permissive host, as the primary mouse macrophages challenged by these strains also showed no defect in caspase-3 activation (**Fig. 3-13, B**). Consistent with this observation, the Δ5 mutant did not display any defect or enhancement in intracellular bacterial growth (**Fig. 3-13, B**).

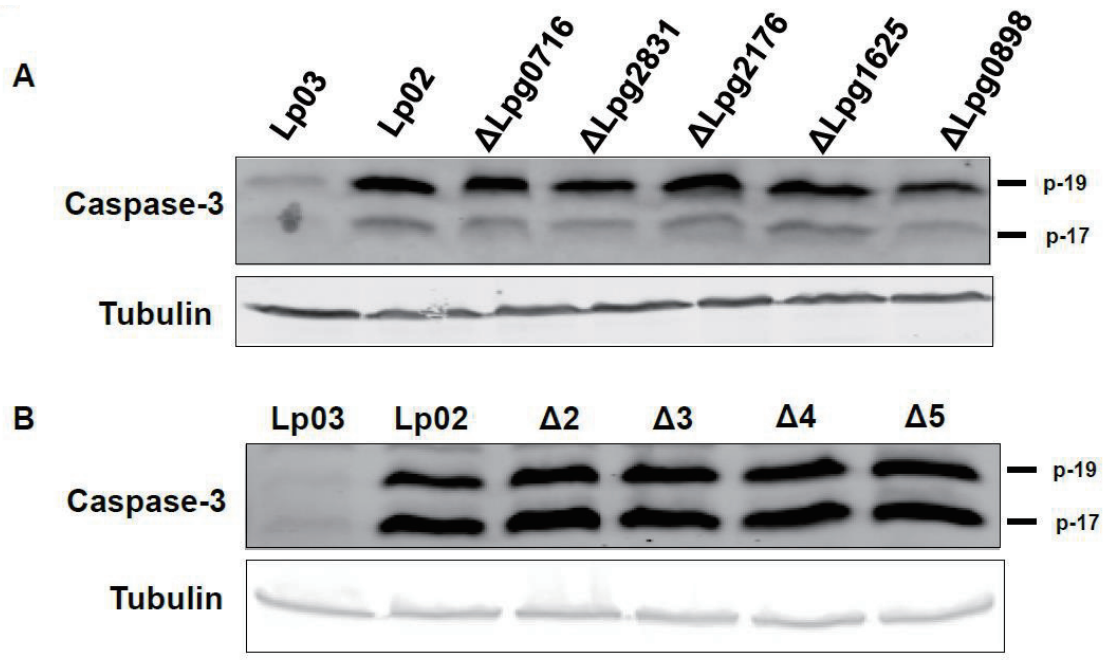


Fig. 3-13 Deletion of caspase-3 activators has little impact on the activation of this caspase by *L. pneumophila*. A & B. Differentiated U937 cells were infected by indicated *L. pneumophila* strains for 6 hr, lysates of infected samples resolved by SDS-PAGE were probed for active caspase-3. **B.** *L. pneumophila* deletion strain lacking one or more of the caspase-3 activating effectors still potently induced caspase-3 activation. A/J mouse macrophages were infected by indicated *L. pneumophila* strains for 6 hr, lysates of infected samples resolved by SDS-PAGE were probed for active caspase-3. Similar results were obtained in multiple independent experiments. Strains: Δ2 (Lp02Δ0898, Δ1625); Δ3 (Lp02Δ0898, Δ1625, Δ0716); Δ4 (Lp02Δ0898, Δ1625, Δ0716, Δ2831); Δ5 (Lp02Δ0898, Δ1625, Δ0716, Δ2831, Δ2176). The wild type strain Lp02 and Lp03, the mutant deficient for the Dot/Icm transporter was used as controls.

Because dendritic cells (DCs) are more sensitive to cell death induced by *L. pneumophila* [102], and that cell death seems to be important in restricting bacterial intracellular replication, we then sought to determine whether any of these effectors contribute to the cell death induced in DCs. Although the Δ5 mutant did not productively replicate in DCs, comparing to the wild type strain, it was not efficiently killed by these cells (**Fig. 3-14, A**). The mutant survived in rates very similar to a mutant deficient in the Dot/Icm transporter. Evaluation of the apoptotic status of infected DCs revealed that less than 5% of the cells harboring the Δ5 mutant stained positively by the TUNEL reagent, whereas more than 25% of the cells infected by the wild type strains are apoptotic (**Fig. 3-14, B-C**). Thus, deletion of the five genes only partially abolished the ability of *L. pneumophila* to induce cell death in DCs. Together with the fact that this mutant still grew robustly in macrophages, cells that are less sensitive to cell death induction by the bacteria, these data suggest the existence of yet unidentified caspase-3 activating effectors. Alternatively, activation of caspase-3 by *L. pneumophila* could be a result of the collective effects of many effectors, which when expressed individually did not cause detectable activation.

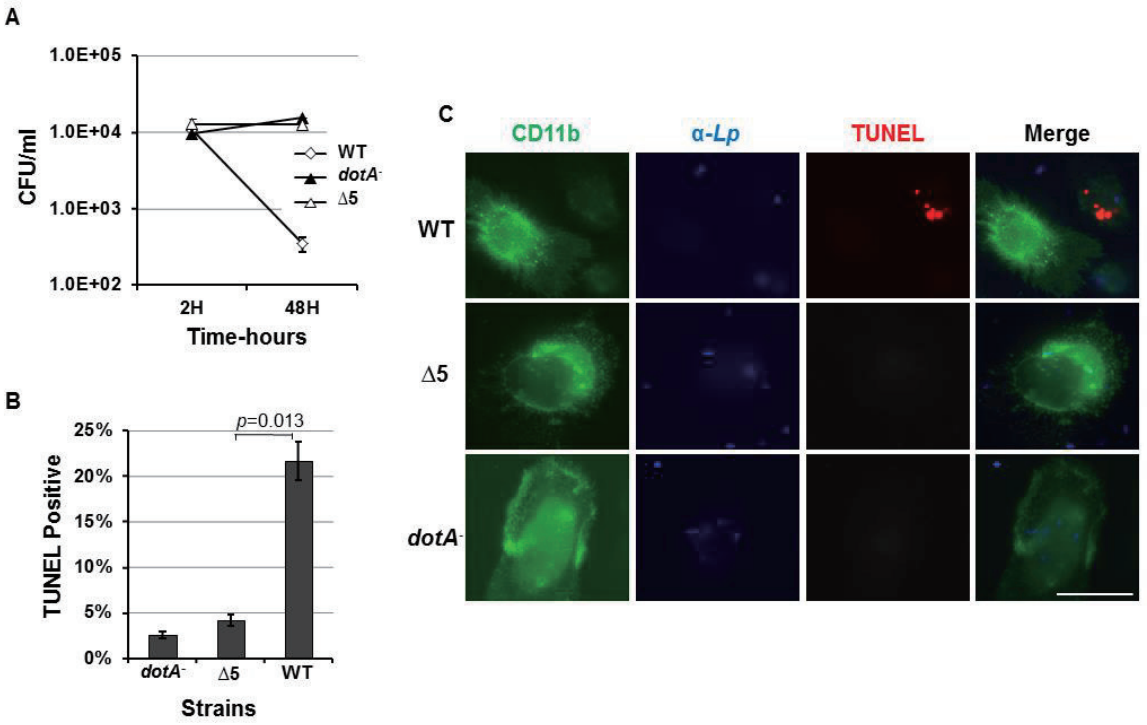


Fig. 3-14 The five caspase-3 activating proteins are important for maximal cell death induction in dendritic cells. A. Dendritic cells prepared from bone marrows of A/J mice used infected with indicated bacterial strains and the total bacterial counts were determined at indicated time points. Note the drastic drop in the number of survived wild type bacteria over the 48-hr infection duration. **B-C**. DCs similarly infected as panel A were subjected to immunostaining to label the cells, the bacteria and the apoptotic status of the infected cells with specific antibodies. Infected cells stained positively by the TUNEL reagent were enumerated under an Olympus IX-81 fluorescence microscope. At least 200 infected cells were scored from each sample done in triplicate. Representative images of the cells were acquired from these samples. Similar results were obtained in three experiments done with independently isolated DCs.

Discussion

Host cell death plays an indispensable roles in shaping the outcome of infection, in which *L. pneumophila* achieves an intriguing balance between pro-death pathways and pro-survival signaling cascade. Permissive macrophages infected by *L. pneumophila* are resistant to cell death stimuli, yet harbor high levels of activated caspase-3, the executioner caspase in apoptotic process [186]. Although caspase-3 activation does not seem to play a significant role in restricting *L. pneumophila* in macrophages, it does seem to be important for limiting replication of this bacterium in dendritic cells [104]. Since this phenomenon requires a functional Dot/Icm secretion system, it could be possible that a subset of Dot/Icm substrates, or the host responses against these substrates, trigger the activation of the caspase-3. To test this hypothesis, I initiated a screening to identify Dot/Icm substrates capable of activating caspase-3. Using the combination of western blotting and a luminescence-based activity assay, five Dot/Icm substrates were identified to be capable of activating caspase-3 when ectopically expressed. Of these five effectors, I chose to focus on VipD for detailed analysis because of the presence of a conserved phospholipase A2 domain [30, 187] and the availability of the useful reagents in the lab. We showed that when ectopically expressed, VipD causes mitochondria outer membrane permeabilization and cytochrome c release into the cytosol. Further, recombinant VipD is capable of liberating cytochrome c from purified mitochondria in a Bax/Bak independent manner, suggesting mitochondria membrane permeabilization is a direct consequence of the activity of VipD. Because mutating the putative catalytic residue Ser73 or application of phospholipase inhibitor abolished the capability of VipD to activate caspase-3 and liberate cytochrome c, we reason that VipD is a putative phospholipase A2. The capability of VipD to hydrolyze phosphatidylcholine (PC) and phosphatidylethanolamine (PE) on the mitochondria membrane lends further support to this hypothesis. However, despite repeated attempts, we could not detect appreciable phospholipase activity when VipD was incubated with purified

phospholipids. It seems that VipD is active only in the presence of a proteinaceous molecule on mitochondria, indicating that a eukaryotic co-factor is necessary for the phospholipase activity of VipD.

Interestingly, another report investigating the biochemistry function of VipD showed that VipD is a phospholipase A1 instead of phospholipase A2 [187]. Although the products of these two closely resemble enzymes are both lysophospholipids, the mechanisms of action of these two enzymes are different, as phospholipase A1 targets the SN-1 acyl bond on the phospholipids while phospholipase A2 cleaves the SN-2 bond. The two different products of phospholipase A1 and phospholipase A2, however, cannot distinguished by mass spectrometry analysis such the one we utilized to identify the product of VipD. Gasper *et al.* reached the conclusion that VipD is a phospholipase A1 using phospholipase A1-specific phospholipase inhibitors and a fluorescence-substrate-based phospholipase activity assay. In this assay, a fluorescent moiety capable of FRET (fluorescence resonance energy transfer) is covalently attached to the phospholipid substrates on the SN-1 position. The phospholipase activity is measured by the alteration of FRET emission prior and after the enzymatic cleavage of the substrates. Due to the specific position of the fluorescent moiety, the alteration of FRET is only detected in the presence of an active phospholipase A1, but not phospholipase A2. Therefore, this assay could be more specific than the mass spectrometry analysis [187]. On the other hand, Gasper *et al.* also demonstrated that the eukaryotic protein Rab5 is necessary for the phospholipase activity of VipD, which is consistent with our observation. They further showed that VipD is activated on endosome, where Rab5 is located, to hydrolyze PI(3)P to protect *L. pneumophila* from endosomal fusion [187]. These observations are inconsistent with our observation that VipD is involved in the activation of caspase-3 and contribute to the cell death response elicited in dendritic cells. Several lines of evidence suggest that VipD has functions beyond interfering with endosomal trafficking. First, VipD adopts an evenly diffusing

cellular distributing pattern other than co-localizing with endosomal compartments when expressed in eukaryotic cells. The discrepancy may be the results of 1) the utilization of different cell lines, plasmids that drives heterologous expression of VipD; 2) the transfection reagents that deliver VipD-constructs into the cells; 3) the sampling time post transfection. The diffused distribution pattern may contribute to the protein localization in mitochondria where it stimulates caspase-3 activation. Second, although it was shown that Rab5 could activate VipD, the possibility that VipD is activated by other mammalian proteins present on mitochondria cannot be excluded (**Fig. 1-17 B**). Third, despite these inconsistencies, Gasper *et al*. did observe that VipD expression in mammalian cells caused cell rounding and decreased cell viability, which is line with our observation that VipD causes cell death. The cell death caused by VipD cannot be simply explained by the PI(3)P hydrolyzing activity, especially if VipD exclusively localizes on the endosomal membrane. Taken together, it is possible that VipD contributes to the interference with the endosomal trafficking and to the induction of cell death. Such a phenomenon is not without precedent, as ExoU, the phospholipase A_2 coded by *P. aruoginosa*, bears functions from causing cell death to dampening inflammasome activation; a *Salmonella* effector protein, SopB, contributes to both bacterial invasion and the maturation of the *Salmonella*-containing-vacuole [56, 94].

In this study, we identified five Dot/Icm substrates that are capable of activating caspase-3. However, the mechanisms underlying the activation by four other effectors remains elusive. One possibility is that caspase-3 activation is an “unintended” consequence of the biochemical activities of these five Dot/Icm substrates. Alternatively, *L. pneumophila* could encode effectors to directly activate caspase-3. Considering that the major evolutionary pressure of *L. pneumophila* comes from protozoan amoebae, which do not seem to contain caspase-3, this possibility is unlikely to be the case. Another possibility is that caspase-3 activation is a result of effector-triggered-responses (ETR), which

refers to the phenomenon in which the biochemical activity of the virulence factors, but not the virulence factors per se, is recognized by the host immune system. Such paradigm has been well established in plant-pathogen interactions [188], as well as in metazoa [136]. For example, at least five *L. pneumophila* effectors are capable of inhibiting host protein synthesis, and the consequence of such inhibition includes prolonged activation of the NF-κB pathways, up-regulation of anti-apoptotic genes, and activation of the MAPK pathway [136]. Inhibition of protein translation has been shown to trigger the MyD88 dependent "frustrated responses", which allow the host to overcome translation inhibition and selectively translate certain transcripts such as the inflammatory cytokines IL-1α [99]. More examples of ETR in metazoans comes from the Pyrin inflammasome, which detects various bacterial modifications, such as ampylation, ADP-ribosylation, deamindation, glucosylation, on small GTPase RhoA at its switch I region and activates caspase-1 [132]. These observations prompted us to propose that caspase-3 activation is a result of host sensing of the biochemical activity of at least five effectors from *L. pneumophila*. In line with this hypothesis, activation of caspase-3 is essential in host cell death and bacterial replication restriction in dendritic cells [104]. Although similar phenomena were not observed in macrophages, the different responses may result from the differing level of sensitivity to active caspase-3 in the two cell types, or the differences in the abundance of the host molecules that detect the effector activity. Despite its frequent association with cell death, activation of caspase-3 in infection does not always translate into host cell death [189]. *S. Typhimurium* activates this enzyme by a Type III secretion system effector SipA without triggering host apoptotic cell death. The activated caspase-3 in turn processes SipA into two functionally distinct domains [190]. It will therefore be interesting to determine whether caspase-3 activation also contributes to the pathogenesis of *L. pneumophila*, because the caspase-3 recognition motif "DEVD" could indeed be found on some proteins encoded by this bacterium. Nonetheless, we demonstrate that five *L. pneumophila* effectors contribute to caspase-3 activation, cell death and bacterial

replication restriction during infection. Further investigation is warranted to study the mechanisms of action of these five effectors to activate caspase-3, and whether more effectors contribute to such activities during infection. It will be interesting to further determine whether these effectors directly trigger caspase-3 activation, or caspase-3 stimulation is a result of the effector-triggered-responses to the biochemical consequences of these effectors. If the latter possibility is true, it will certainly be compelling to determine whether the ETR is responding to a common activity that is shared among these effectors, as in the case of host response to the effectors involved in inhibiting protein translation.

CHAPTER IV: PROBING HOST IMMUNE RESPONSES USING *LEGIONELLA* ENVIRONMENTAL ISOLATES

Abstract

Although environmental *Legionella* are responsible for most of the Legionnaires' disease outbreaks so far, most of our knowledge of *Legionella* pathogenesis was acquired using laboratory strains. To investigate how mouse macrophages respond to *Legionella* environmental isolates, we analyzed the intracellular growth of these isolates in A/J mouse macrophages, which is permissive to most, if not all, laboratory strains. None of the *Legionella* environmental isolates were able to replicate in A/J mouse macrophages. Further investigations into one of the isolates, LPE509, showed that such growth restriction is not due to the lack of important pathogenic determinants, as this strain is competent for replication in two protozoan hosts and the human macrophage U937 cell line. Moreover, the inability of intracellular growth of LPE509 is accompanied by host cell death of unique nature, as it is independent of most signaling components known to control *Legionella* infection. In an attempt to screen for the unique properties of LPE509 responsible for host cell death and growth restriction, we found that a spontaneous streptomycin resistant mutant had gained the ability to overcome the growth restriction in macrophages. Analysis of the mutant strain revealed that a lysine to arginine substitution on position 88 of the 30S ribosome small subunit S12 (RpsL) severely attenuates the host cell death induction and enables the bacterium to replicate in macrophages. We also demonstrate that RpsL is a Microbe-Associated-Molecular-Pattern (MAMP), which is sensed intracellularly and triggers the permeabilization of lysosomal membranes. Upon the loss of lysosome

membrane integrity, various hydrolases are released into the cytosol, where they process diverse substrates and cause cell death. Amongst these hydrolases, cathepsin B plays an important role in RpsL sensing, as genetically ablating this gene dampens host cell death induced by recombinant $RpsL_{WT}$ and *Legionella* expressing wild type RpsL. Our results suggeste that RpsL is a microbial associated molecular pattern (MAMP) that triggers a unique cell death pathway in mouse macrophages to limit the spread of invading bacterial pathogens.

Introduction

Host cell death is an important determinant of outcome of an infection. At the early stage of infection, host responses such as secretion of inflammatory cytokines, recruitment of immune cells, and production antimicrobial molecules are initiated as attempts to clear the infection [191]. If the infection is not resolved, host cell death could be an effective defense mechanism, as the demise of host cells could remove the niche of infection and expose the pathogens to additional layers of immune surveillance [191]. Exposing the pathogens is important to the host defense, as the release of PAMPs that accompanys this process could help the training of incoming immune cells, such as macrophages and dendritic cells, for a more robust immune response [192, 193]. Further, phagocytosis of dead infected cells helps to boost the activation of adaptive immune system through antigen presentation [194]. On the other hand, host cell death could also lead to elimination of key immune cells, resulting in the impairment of immune responses, leading to successful evasion of the host defenses by the pathogen and systematic spread of the infection [195].

The primary evolutionary pressure for *L. pneumophila* is believed to come from its environmental amoebae, its natural hosts [159]. As an opportunistic pathogen, *L. pneumophila* does not seem to have evolved to evade the immune surveillance of mammalian hosts [159]. It is therefore not surprising that immune cells challenged by *L. pneumophila* mount robust immune responses that are

otherwise silent or not detectable during infection by better adapted pathogens [147]. For example, taking advantage of the strong cell death phenotype associated with *L. pneumophila* infected BMDM derived from C57BL/6 but not A/J mouse, investigators revealed that the bacterial flagellin is recognized by the the NLR receptor--Naip5 [143]. Further investigation of this pathways demonsrated that, flagellin, engaged by Naip5, recruits and activates NLRC4, which leads to caspase-1 activation and subsequent pyrototic cell death [149]. These signaling events eventually result in removal of the replicative niche and further recruitment of other immune cells [151].

Almost all of the disease outbreaks in history have been caused by environmental or clinical isolates of pathogens [159]. However, despite the importance of the environmental isolates, most of our understanding on the pathogenesis of *L. pneumophila* has been based on studies of laboratory strains such as Lp02 and JR32 [152], which were acquired through the domestication of the original clinical isolate Philadelphia-1 [152]. The important roles of the environmental isolates in Legionnaires' disease have prompted researchers to analyze the intracellular bacterial growth of these isolates in cultured human macrophages, but few of them have determined the response of different hosts such as primary murine macrophages to challenge of these bacteria [154-156].

The lack of knowledge of how *L. pneumophila* environmental isolates interact with mammalian hosts prompted us to investigate the immune responses to these bacteria. We recently found that LPE509, a strain from a man-made facility was unable to replicate in A/J mouse macrophages, which is permissive to most, if not all laboratory strains [157]. Because host cell death is important to restrict bacterial replication in the case of resistant host challenged by flagellin expressing *L. pneumophila*, we set out to test whether host cell death plays a role in the growth restriction of LPE509. We found that infection by this strain in BMDM induces rapid and extensive cell death, in a manner independent of the

known mechanisms of cell death induced by *L. pneumophila* [157]. These results suggest that LPE509 is capable of triggering a unique cell death pathway, probably by activating an immune surveillance system. To identify the bacterial factor responsible for this cell death phenotype, we screened for LPE509 mutants that of growing in A/J macrophages and revealed that a mutation on the bacterial ribosome protein S12, *rpsL*, enables LPE509 to overcome the intracellular growth restriction. Further analysis demonstrates that RpsL, after reaching the host cytosol presumbly by the Dot/Icm secretion system of *L. pneumophila*, engages host immune pathways and triggers lysosomal and apoptotic cell death. Our results established RpsL as a ligand capable of triggering a unique immune recognition by inducing lysosomal cell death.

Results

Several environmental *L. pneumophila* strains are unable to replicate in A/J macrophages

Although it has been almost four decades since *L. pneumophila* was found to be a human pathogen, most of the research on this particular pathogen was done using the “domesticated” strains for more amenable genetic manipulations. Few studies had analyzed how primary macrophages responsd to clinical or environmental isolate of *L. pneumophila*, which are responsible for most of the Legionella outbreaks in history [154-156]. To fill in this gap in our knowledge, Dr. Lili Tao isolated several serogroup 1 *L. pneumophila* strains from hospital water distribution systems in Shanghai, China and tested the interactions between these strains and mouse macrophages [196]. As shown in **Fig. 4-1**, A/J macrophages allowed for efficient intracellular replication by strain JR32, a derivative of the original Philadelphia-1 strain to replicate (at a multiplicity of infection (MOI) of 0.05) [197]. As expected, the *dotA* mutant of JR32, which is deficient in the ability to translocate substrates of the Dot/Icm type IV system, cannot replicate in A/J macrophages. To our surprise, under similar conditions,

none of the 4 environmental isolates yielded productive bacterial replication throughout the entire experimental duration, despite a similar level of bacterial uptake (**Fig. 4-1**). Moreover, whereas the Dot/Icm mutant of JR32 was not effectively cleared by A/J macrophages, all four environmental were readily cleared by 72 hours post infection (**Fig. 4-1**). These results indicate under the infection conditions used, none of these four environmental *L. pneumophila* strains can replicate in mouse macrophages that are permissive for standard laboratory strains.

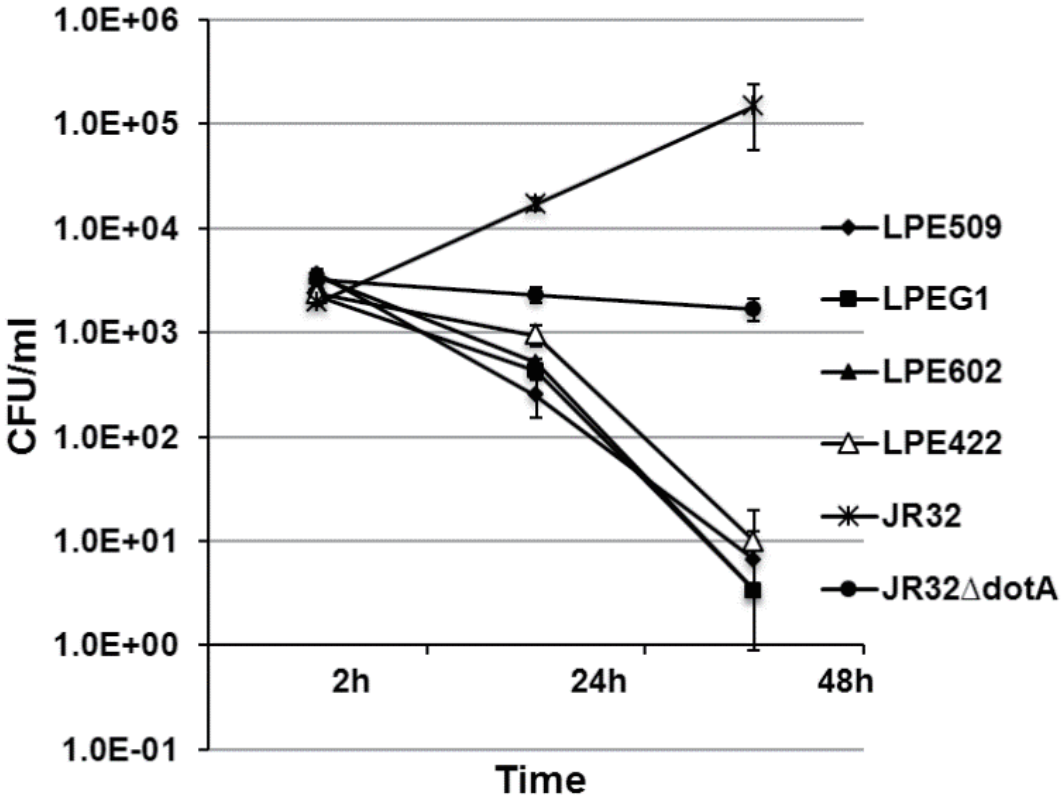


Fig. 4-1 Intracellular replication of several *L. pneumophila* environmental isolates in A/J mouse macrophages. Bone marrow-derived macrophages were challenged with indicated bacterial strains grown to post-exponential phase at an MOI of 0.05. Infections were synchronized two hour after adding the bacteria. At indicated time points, total bacterial counts (colony-forming-unit) were determined by plating appropriately diluted saponin solubilized infected cells onto bacteriological medium. Infections were performed in triplicate and data shown were from one representative of three independent experiments with similar results.

Vacuoles containing LPE509 are targeted properly in A/J macrophage

To determine the mechanism underlying the growth defect of LPE509 in A/J macrophage, we analyzed the trafficking of its phagosome in this host. Cells infected with LPE509, its *dotA* mutant and appropriate control strains for 1 hr were stained for the lysosomal marker LAMP-1. Similar to wild type JR32, positive staining signals for LAMP-1 were absent in about 82% of the vacuoles formed by strain LPE509 (**Fig. 4-2A**). Deletion of *dotA* led to acquisition of this marker by the bacterial vacuoles (**Fig. 4-2A**). We also examined the recruitment of Rab1, the small GTPase important for *L. pneumophila* infection [198] to vacuoles of LPE509. About 60% of phagosomes containing LPE509 stained positively for Rab1, which was abolished by the deletion of the *dotA* (**Fig. 4-2B**). Thus, vacuoles containing strain LPE509 are not delivered into the lysosomal network and the defect in intracellular growth is not caused by its inability to manipulate the cellular processes such as recruitment of Rab1.

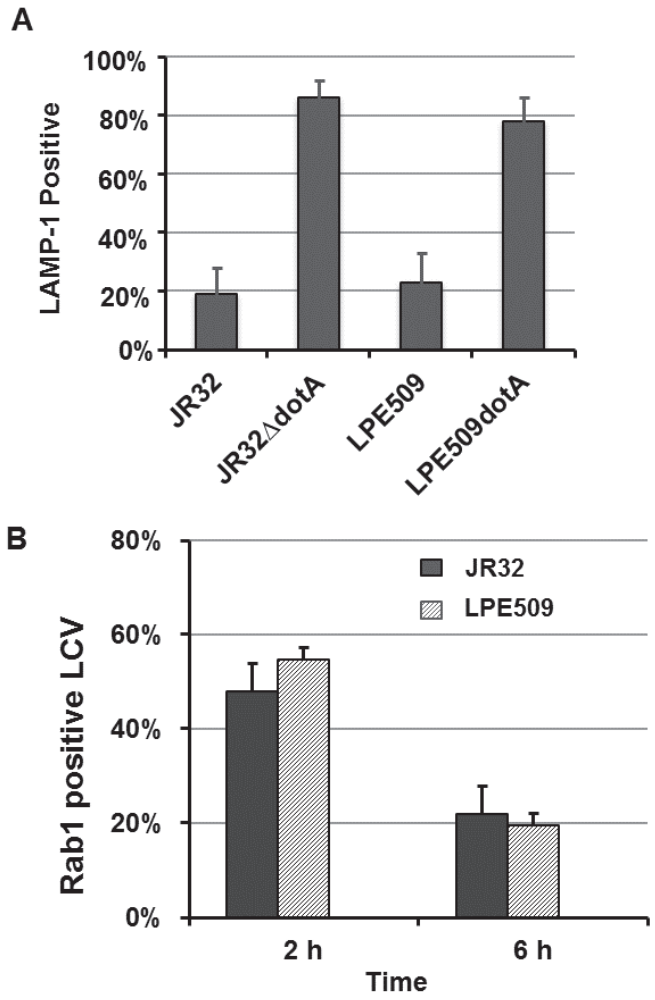


Fig. 4-2 Intracellular trafficking of strain LPE509 in A/J macrophages. Bone marrow-derived macrophages seeded on glass coverslips were infected with properly grown bacterial strains at an MOI of 2. One hour after infection, samples were washed with medium and were fixed at the indicated time points. After labeling extracellular and intracellular bacteria differently by immunostaining, the host proteins LAMP-1 (A) and Rab1 (B) were stained with specific antibodies. Data were collected using an IX-81 Olympus microscope by inspecting bacterial vacuoles for the association of the protein staining signals. All infections were performed in triplicate and at least 100 bacterial phagosomes were scored for each sample. Data shown were the averages of two independent experiments.

A fraction of internalized LPE509 bacteria establish larger vacuoles in A/J macrophages

In permissive host, after a brief lag phase of 4-6 hours, *L. pneumophila* begins to replicate and the size of the vacuole expanses. By 14 hours post infection, the replicating vacuole can contain more than 10 bacteria [6]. The observation that phagosomes of LPE509 were properly targeted in macrophages indicates that some of them may develop into large vacuoles containing multiple bacteria when infection proceeds to later phase. We thus determined the distribution of vacuoles formed by this strain 14 hr after uptake. In infections using strain JR32, more than 63% of the vacuoles were large phagosomes containing ≥10 bacteria. The fraction of small (1-3 bacteria/vacuole) and medium size (4-9 bacteria/vacuole) phagosomes was about 19% and 17%, respectively (**Fig. 4-3**). In contrast, the majority (~58%) of the LPE509 vacuoles at this time point contained 1-3 bacteria; about 22% of them were intermediate vacuoles with 4-9 bacteria and only about 18% were large vacuoles containing more than 10 bacteria (**Fig. 4-3**). Thus, strain LPE509 was able to form replicative vacuoles in A/J macrophages but further development of most of these vacuoles did not occur.

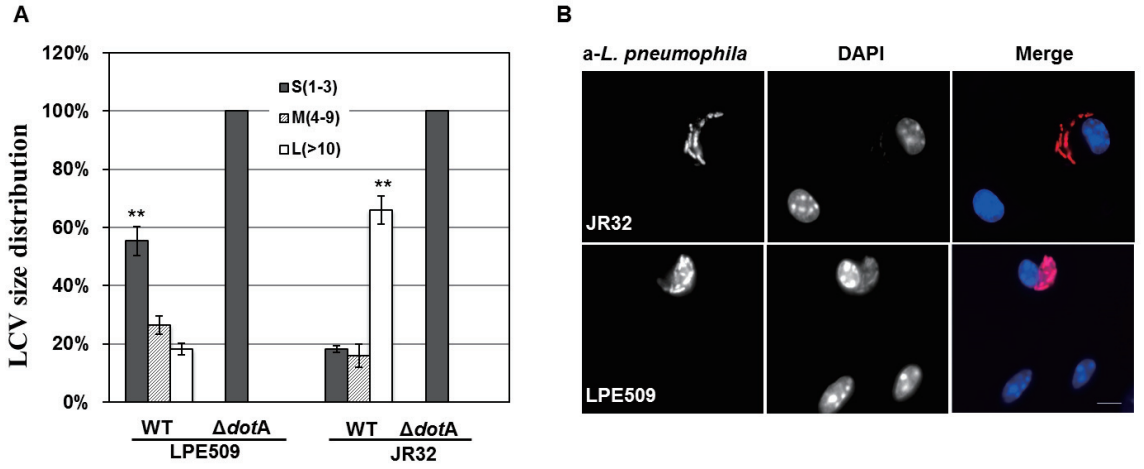


Fig. 4-3 The formation of replicative vacuoles by strain LPE509 in macrophages. A/J mouse macrophages on glass coverslips were infected with appropriately grown bacterial strains at an MOI of 1. Infections were synchronized one hour after bacterial challenge and were allowed to proceed for an additional 13 hours. Samples were then fixed and subjected to immunostaining to label intracellular and extracellular *L. pneumophila* with different fluorescent colors. When necessary, samples were further staining with DAPI to highlight the nuclei of host cells. The bacterial phagosomes were scored under a fluorescence microscope according to the number of bacteria they harbored. Phagosomes containing more than 10 bacteria were categorized as large; those with 4-9 bacteria were called medium and those with 1-3 bacteria were classified as small vacuoles. **A**. The distributions of vacuole sizes of strain JR32 and LPE509 and their derivatives defective in the Dot/Icm transporter. **, $p<0.001$ for values of big or small vacuoles between strain JR32 and LPE509. **B**. Representative images of large vacuoles by both bacterial strains. Note the condensed nucleus of the cell infected by strain LPE509. Bar, 10 □m.

LPE509 flagellin is not responsible for its intracellular replication restriction in A/J macrophages

The subtle balance of host cell death controls the outcome of the infection. The phenotypes associated with the interactions between strain LPE509 and A/J macrophages are reminiscent of those seen in infections of C57BL/6 macrophages with strain Philadelphia 1, in which sensing of flagellin by NAIP5 leads to pyroptosis and termination of intracellular bacterial replication [120, 199]. To examine whether the defects were caused by the recognition of LPE509 flagellin by NAIP5 of A/J mice, we constructed LPE509Δ*flaA*, a mutant that no longer expressed flagellin (**Fig. 4-4A**). Challenge of A/J macrophages with this mutant at a low MOI (0.05) did not lead to productive intracellular bacterial growth (**Fig. 4-4B**). These results indicate that the lack of intracellular growth of LPE509 in A/J macrophages was not due to variations in flagellin that can be recognized by NAIP5 of A/J to cause pyroptosis.

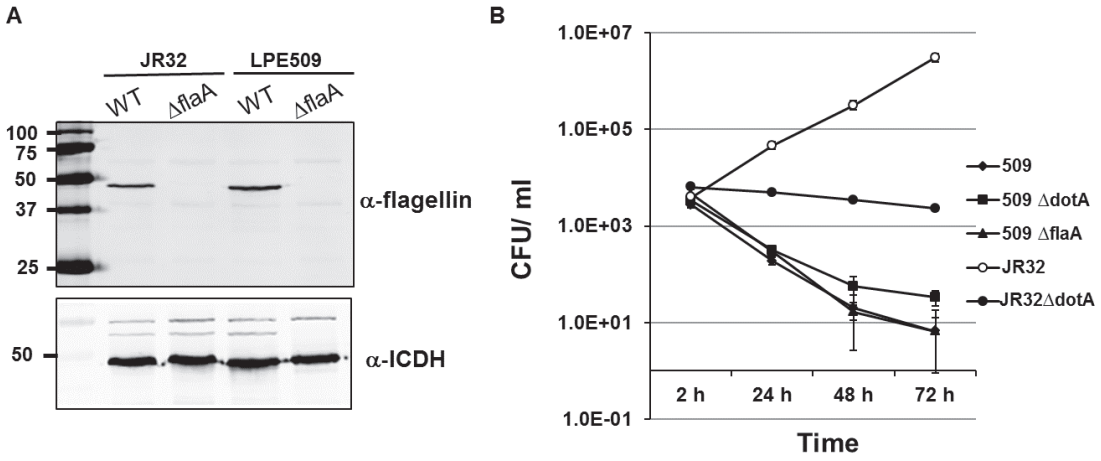


Fig. 4-4 Intracellular growth of a mutant of strain LPE509 lacking flagellin in A/J macrophages. **A**. Expression of flagellin by the testing bacterial strains. Cells of indicated strains grown to post-exponential phase were lysed with Laemmli sample buffer and total protein was resolved by SDS-PAGE. Flagellin was detected by a specific antibody and the isocitrate dehydrogenase (ICDH) was probed as a loading control (lower panel). **B**. Testing bacterial strains grown in AYE broth to post-exponential phase were used to infect bone marrow-derived macrophages from A/J mice at an MOI of 0.05. Infections synchronized 2 hrs after adding bacteria were lysed with saponin at indicated time points and total bacterial counts were determined by plating appropriate diluted lysates onto bacteriological medium. Infections were performed in triplicate and similar results were obtained in three independent experiments.

LPE509 causes extensive membrane damage in A/J macrophages

To determine the phenotypic changes associated with infection of A/J macrophages by LPE509, we examined the morphology of these cells infected with a moderate MOI (2). Under this condition, strain JR32 did not cause discernible change in cell morphology (**Fig. 4-5A,** top panel). However, challenge with LPE509 led to extensive cell rounding and no such phenotype was detected in infections with the strain LPE509Δ*dotA* (**Fig. 4-5A,** middle and lower panels, respectively). We further examined the integrity of plasma membrane of macrophages infected with these strains. Infected macrophages were stained simultaneously by propidium iodide (PI), which is membrane impermeable and generally excluded from viable cells and by Hochest 33342, which allows the staining of nuclei of both live and dead cells. In samples receiving LPE509 at an MOI of 1, about 45% of macrophages became permeable to PI. In infections using strain JR32, only approximately 6% of the cells were stained positively by PI (**Fig. 4-5, B-C**). The *dotA* mutants of neither strain caused detectable membrane damage under this infection condition (**Fig. 4-5 B-C**).

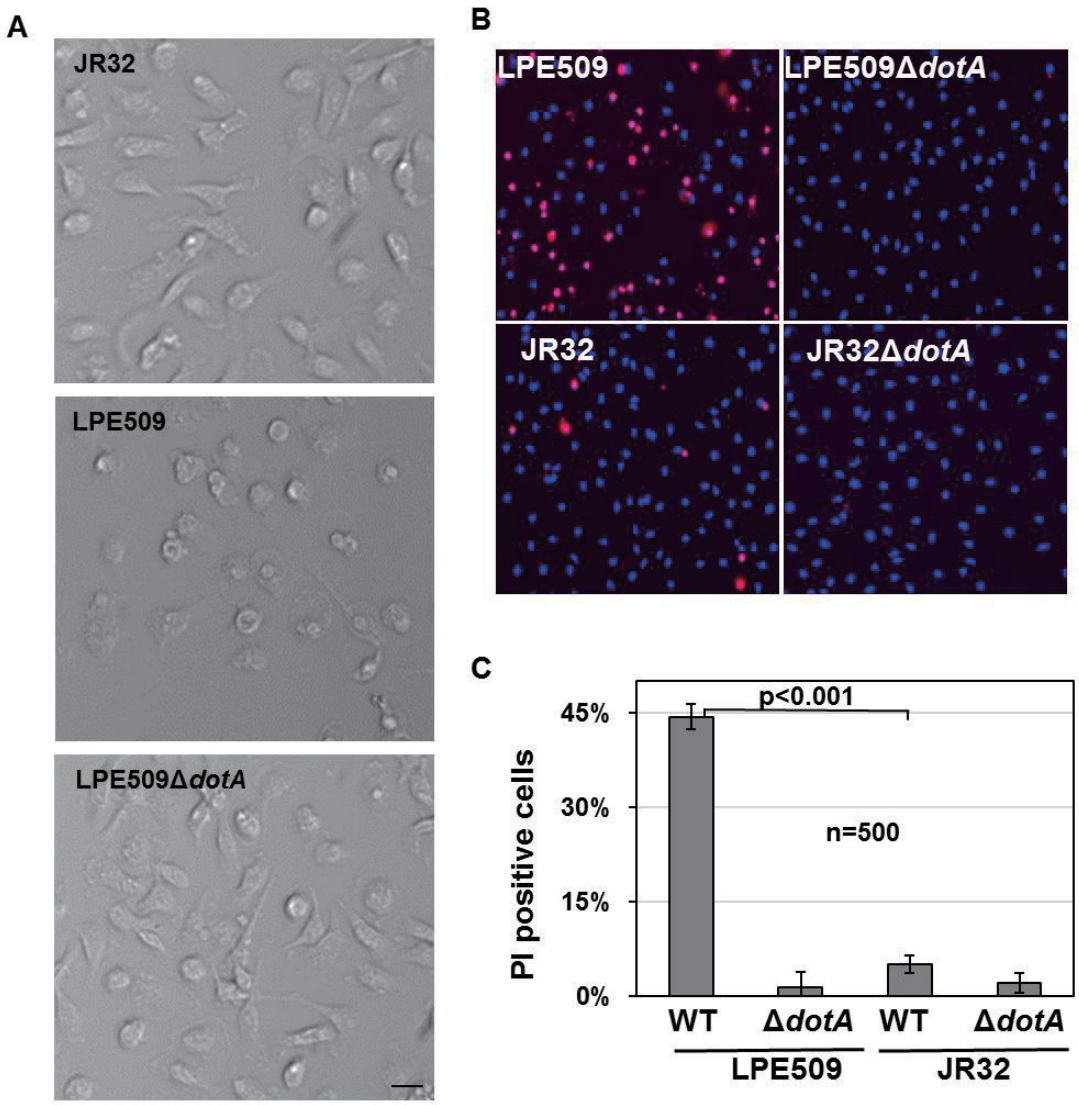


Fig. 4-5 Infection by strain LPE509 damages macrophage morphology and membrane integrity. Bone marrow derived macrophages from A/J mice were challenged with indicated bacterial strains grown to post-exponential phase at an MOI of 1 for 2 hrs. **A-B**. Cell morphology and membrane integrity of infected samples was examined under a microscope. Similarly infected samples were simultaneously stained with propidium iodide (PI) and Hoechst 33342 and were analyzed by imaging with a fluorescence microscope. Bar, 10μm. **C**. Quantitation of membrane damaged cells. Membrane damage was determined by positive staining by PI under a microscope, at least 500 cells were scored in each sample for infections done in triplicate. Data shown are from one representative experiment from three independent experiments with similar results.

At 14 hr post-infection, a portion of vacuoles containing more than 10 bacteria were established but the total bacterial counts at 24 hrs actually decreased, suggesting that some of the bacteria are not viable (**Figs. 4-1, 4-3**). We thus used TUNEL staining to examine whether these cells were undergoing cell death, which is an effective way for macrophages to eliminate internalized and replicating bacteria. At 14 hr post-infection, about 40% of cells infected by strain LPE509 stained positively for TUNEL and deletion of *flaA* did not significantly reduce the rate of TUNEL positive cells (**Fig. 4-6, A-B**). For cells harboring JR32, less than 20% were TUNEL positive (**Fig. 4-6, A-B**). Disruption of the Dot/Icm transporter abolished the ability of both strains to induce cell death (**Fig. 4-6, A-B**).

We further examined the cytotoxicity of LPE509 to A/J macrophages by measuring membrane integrity of infected cells using LDH release as an indicator. At an MOI of 1, LDH release induced by strain LPE509 was significantly higher than that caused by strain JR32 (**Fig. 4-6C**). Deletion of *flaA* abolished the ability of strain JR32 to induce cell death. However, although deleting *flaA* in strain LPE509 detectably reduced the cytotoxicity, this mutant still significantly caused LDH release when infection was performed with an MOI of 1 (**Fig. 4-6C**). Again, the type IV secretion system in both strains is essential for the cytotoxicity (**Fig. 4-6C**). Taken together, these results indicate that strain LPE509 is more cytotoxic than strain JR32 to A/J macrophages. Importantly, the cytotoxicity of strain LPE509 on these macrophages requires the Dot/Icm transporter but not flagellin.

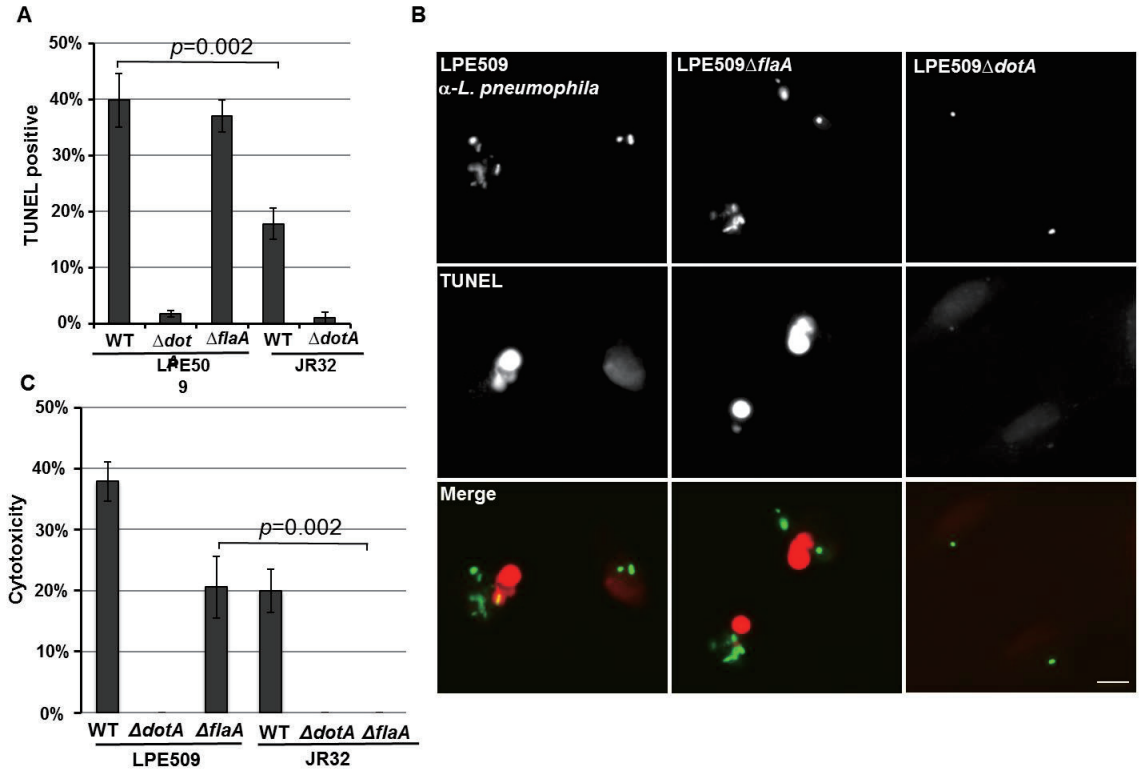


Fig. 4-6 Infection by LPE509 caused extensive cell death on A/J macrophages. A-B. Bone marrow-derived macrophages from A/J mice were infected by indicated *L. pneumophila* strains at an MOI of 1 for 14 hrs and infected cells were immunostained to identify extracellular and intracellular bacteria followed by TUNEL staining to obtain representative images of the nuclei of infected cells. The label of host nuclei by the TUNEL reagent was inspected and scored under a fluorescence microscope. Bar, 10 □m. **C**. Cells infected with the similar bacterial strains at an MOI of 1 were assayed for the release of LDH. Each experiment was performed in triplicate and similar results were obtained in four independent experiments.

The cytotoxicity induced by LPE509 is independent of caspase 1 and caspase 11 and can be inhibited by glycine

Two pathways are known to be important for the activation of pyroptosis in response to infection of murine macrophages by *L. pneumophila* with a functional Dot/Icm system. The first is the NLRC4/NAIP5 inflammasome pathway, which senses flagellin by the leucine-rich repeat containing protein (NLR) NLR family, apoptosis inhibitory protein 5 (NAIP5) [120, 199] and induces the activation of caspas-1 and the subsequent pyroptosis. The second is the caspase 11-dependent pathway, which senses the cytosolic LPS that is probably released during vacuolar bacteria entering host cytosol [200, 201], and activates caspase-1 by proteolytic cleavage, which is followed by the rapid and lytic pyroptotic cell detah. To determine whether either of these two pathways is involved in the cell death induced by strain LPE509, we examined the intracellular growth and cytotoxicity of this strain in macrophages from mice deficient in both caspase 1 and caspase 11 (Casp1/11$^{-/-}$). Consistent with earlier results, macrophages from C57BL/6 or Casp1/11$^{-/-}$ supported robust intracellular growth the *flaA* mutant of JR32 [147, 148], but no productive intracellular growth of LPE509Δ*flaA* was detected (**Fig. 4-7, A-B**). Further, mutants without a functional Dot/Icm transporter were unable to cause cytotoxicity in C57BL/6 macrophages (**Fig. 4-7, C**). Importantly, whereas the *flaA* mutant of JR32 almost completely failed to cause cytotoxicity on C57BL/6 macrophages, the flagellin-deficient mutant of LPE509 is still able to induce LDH release from these cells at levels similar to its wild type parent strain (**Fig. 4-7, C**).

When macrophages from Casp1/11$^{-/-}$ mice were tested, we found that strain LPE509Δ*flaA* still strongly induced cytotoxicity (**Fig. 4-7, D**). In contrast, the *flaA*-deficient strain of JR32 failed to induce membrane damage and LDH release (**Fig. 4-7, D**). Taken together, these results indicate that the rapid cell death induced by strain LPE509 occurs in a way that requires the Dot/Icm system but not caspase 1 nor caspase 11. Notably, we consistently observed that strain

JR32 still caused considerable LDH release from Casp1/11$^{-/-}$ macrophages (**Fig. 4-7, D**). Although JR32 and Lp02 are derivatives of strain Philadelphia 1, the latter strain was not able to induce cytotoxicity on macrophages from Casp1/11$^{-/-}$ mice [200, 201]. Such differences may result from the genetic variations between these strains. For example, the type IVB secretion system Lvh found in JR32 [202] is absent in the genome of Lp02 [159, 203].

Pathogen-induced pyroptosis can be prevented by exogenous glycine, which blocks the formation of non-specific plasma membrane leaks for small ions such as sodium [204, 205]. We thus determined the nature of cell death induced by infections with our bacterial strains in macrophages from caspase1/11$^{-/-}$ mice. Macrophages infected by strains LPE509, JR32 and LPE509Δ*flaA* all exhibited extensive cytotoxicity (**Fig. 4-7, D**). On the other hand, similar to results in previously experiments, JR32Δ*flaA* failed to induce LDH release in these cells (Fig. 8D). Importantly, glycine treatment significantly blocked cytotoxicity induced by these bacterial strains, including LPE509Δ*flaA* (**Fig. 4-7, D**). Interestingly, glycine cannot rescue the defect in intracellular growth of LPE509 in A/J macrophages (**Fig. 4-8**). This result is consistent with the fact that exogenous glycine functions to prevent non-specific plasma membrane damage, which is the consequence of the cell death initiation [204, 205]. Taken together, these results suggest that cytotoxicity induced by strain LPE509Δ*flaA* is caused by a form of cell death that may differ from classical pyroptosis involving in caspase-1.

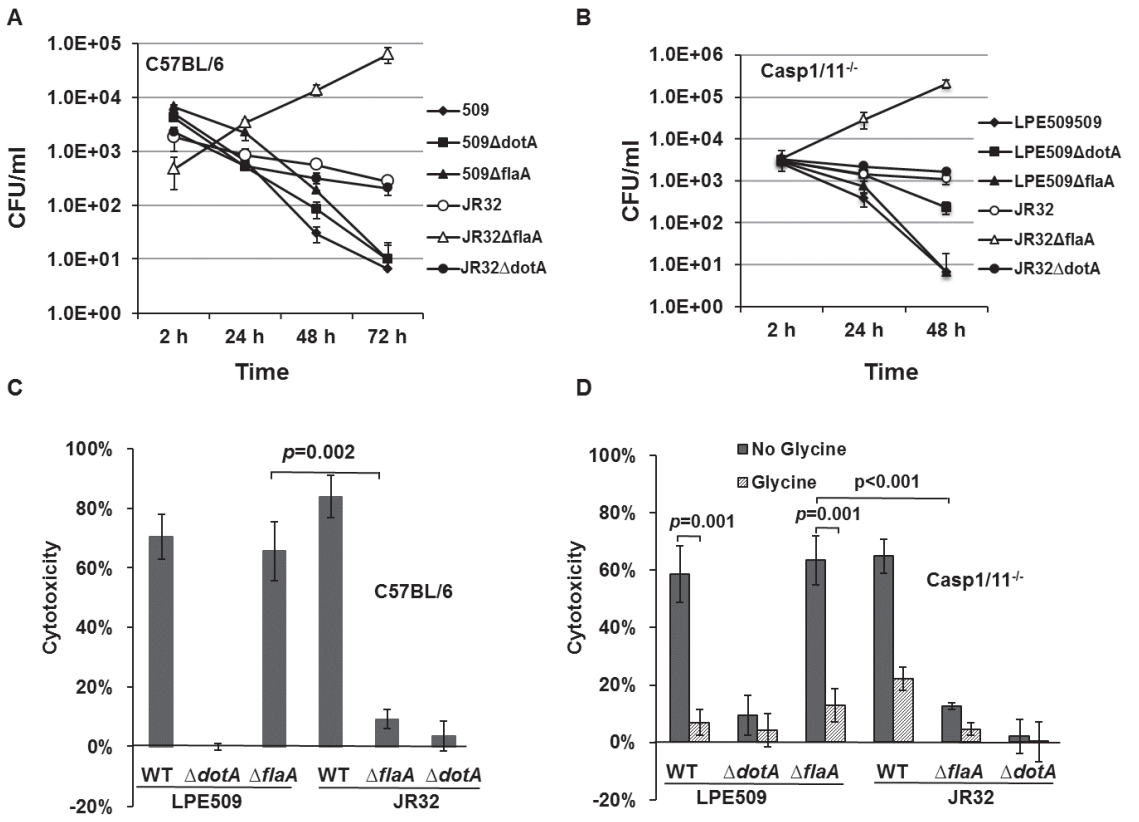


Fig. 4-7 Intracellular growth and cytotoxicity of strain LPE509 in macrophages from mouse deficient in caspase 1 and caspase 11. A-B. Bone marrow-derived macrophages from C57BL/6 (A) mice and capase1/11-deficient mice (Casp1/11$^{-/-}$) were infected with indicated bacterial strains at an MOI of 0.05 and intracellular replication of the bacteria was determined by obtaining total colony-forming-unit (CFU) at a 24-hr interval. **C-D**. The cytotoxicity of the relevant bacterial strains to these cells was examined by measuring the release of lactate dehydrogenase (LDH) upon be challenged by these bacterial at an MOI of 1 for 4 hrs. All assays were performed in triplicate and similar results were obtained in four independent experiments.

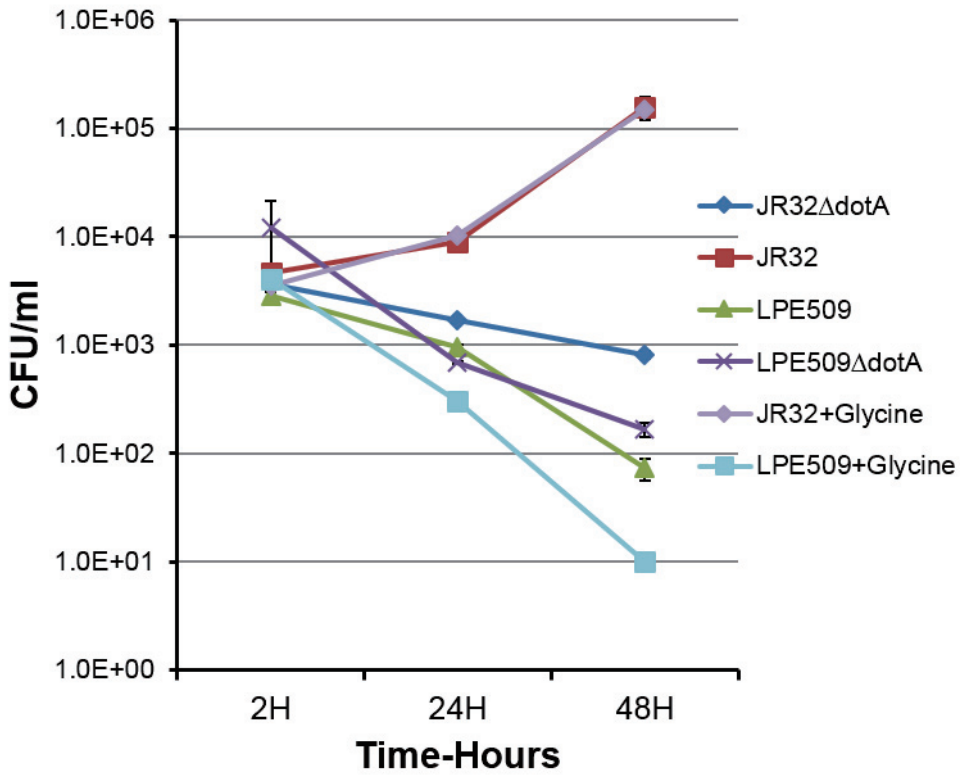


Fig. 4-8 Glycine cannot rescue the intracellular growth defect of LPE509 in A/J macrophages. A/J macrophages were challenged with indicated bacterial strains grown to postexponential phase at an MOI of 0.05. Glycine was added at a final concentration of 20 mM to a subset of samples throughout the entire experimental duration. Total bacterial counts were determined at indicated time points. Infection was performed in triplicate and similar results were obtained in three independent experiments.

A K88R mutation in *rpsL* allows *L. pneumophila* strain LPE509 to replicate in mouse primary macrophages

Our previous studies found that the environmental *L. pneumophila* strain LPE509 cannot replicate in primary mouse macrophages; instead, it induces extensive macrophage death by a mechanism that is independent of the several factors involved in cell death during infection [157]. We hypothesized that this strain codes for unique factor(s) capable of triggering a signaling cascade that leads to cell death and that mutants lacking such factor(s) may enable the bacterium to replicate in macrophages, presumably by evading a yet unidentified immune surveillance mechanism. To identify such factor(s), we attempted to generate an insertion mutant library of LPE509 with a transposon introduced by bi-parental mating. To acquire an antibiotic resistance marker for eliminating the *E. coli* cells harboring the transposon, we first isolated a spontaneous streptomycin resistant ($Strep^R$) mutant of LPE509, which was used for subsequent production of mutant pools by transposition. These mutants were then used to infect BMDMs, and bacterial yields at 72 hr post-infection were determined. Unexpectedly, we found that robust growth occurred in all mutant pools derived from one particular $Strep^R$ clone of strain LPE509 (**Fig. 4-9**). Further analyses indicated that the parental strain used for mutant production has gained the ability to grow in BMDMs (**Fig. 4-10A**). These results suggest that that the growth phenotype may result from the $Strep^R$ property of the bacteria. Because multiple mutations in the ribosomal protein RpsL (Lpg0324) and 16S rRNA can lead to $Strep^R$ [206], we next examined a number of such mutants for intracellular growth in BMDMs to determine whether all mutations that confer $Strep^R$ result in the gain of intracellular replication. From 8 such mutants tested, we found that 4 mutants have overcome the restriction whereas the other 4 failed to replicate in macrophages similar to the wild type (**Fig. 4-9 & 4-10A**). Sequencing analysis of the *rpsL* gene in these mutants revealed that in the 4 mutants capable of replicating in BMDMs, an A to G mutation occurred at nucleotide 263 which caused a Lys to Arg substitution in the 88^{th} residue (K88R)

of the protein. In the 4 Strep^R mutants unable to grow in BMDMs, an A to T mutation at nucleotide 129 that led to a Lys to Asn substitution (K43N) in residue 43 was detected (**Fig. 4-10B**). To rule out the possibility that additional mutations contribute to this phenotype, we introduced the *rpsL*$_{K88R}$ allele into strain LPE509 by gene replacement to produce LPE509*rpsL*$_{K88R}$; in BMDMs, this strain grew at rates comparable to the laboratory strain Lp02, which increased almost 1000-fold after 72 hrs of incubation (**Fig. 4-10C).** Consistent with earlier observations [157], wild type bacteria were steadily cleared by macrophages within the same experimental duration (**Fig. 4-10C**). These results suggest that the K88R mutation in RpsL (*rpsL*$_{K88R}$) is specific for overcoming the growth restriction of strain LPE509 in BMDMs. To further rule out the possibility that Strep^R *per se* is responsible for the intracellular growth phenotype, we introduced a plasmid expressing a streptomycin 3`-adenyltransferase that confers resistance to this antibiotic into strains LPE509 [207]. This strain has a minimal inhibitory concentration (MIC) of 30 µg/ml (**Table 4-1**), and cannot productively replicate in BMDMs (**Fig. 4-11)**. Taken together, these results indicate that a K88R mutation in the ribosome protein RpsL is sufficient to allow strain LPE509 to replicate in BMDMs and that resistance to Strep does not necessarily confer *L. pneumophila* the ability to overcome the restriction imposed by the macrophages.

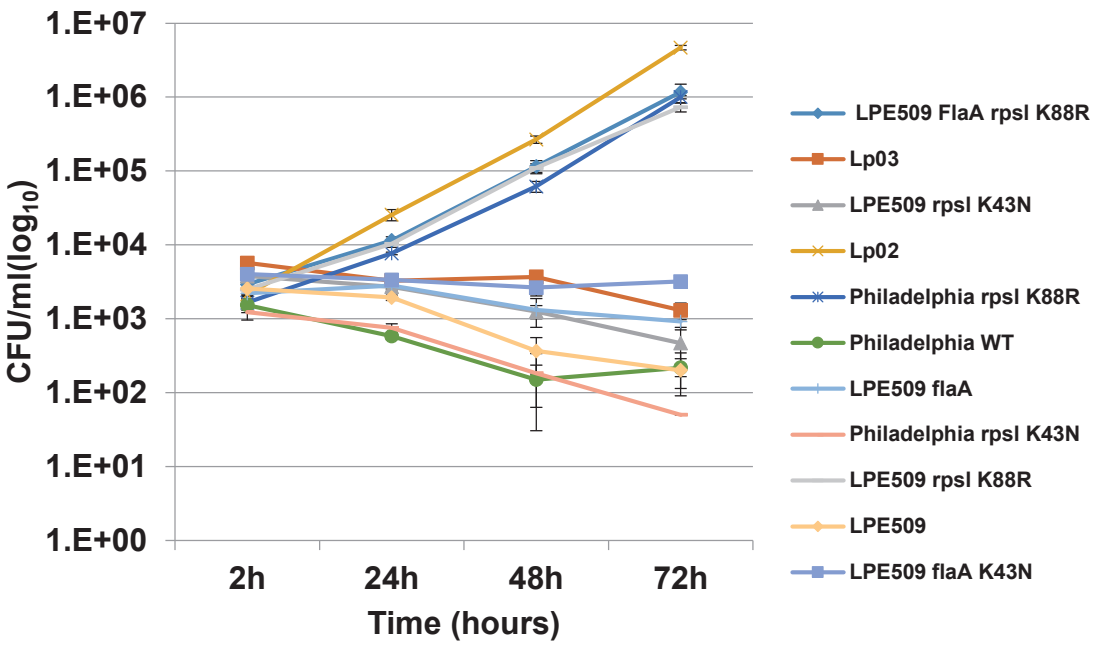


Fig. 4-9 Some streptomycin resistant mutants of *L. pneumophila* could replicate in A/J mouse macrophages. Bone marrow-derived macrophages from A/J mouse were challenged with indicated bacterial strains grown to post-exponential phase at an MOI of 0.05. After synchronization 2 hrs after infection, total bacterial counts (colony-forming-unit) were determined by plating appropriately diluted saponin solubilized infected cells onto bacteriological medium. Infections were performed in triplicate and data shown were from one representative of multiple experiments with similar results

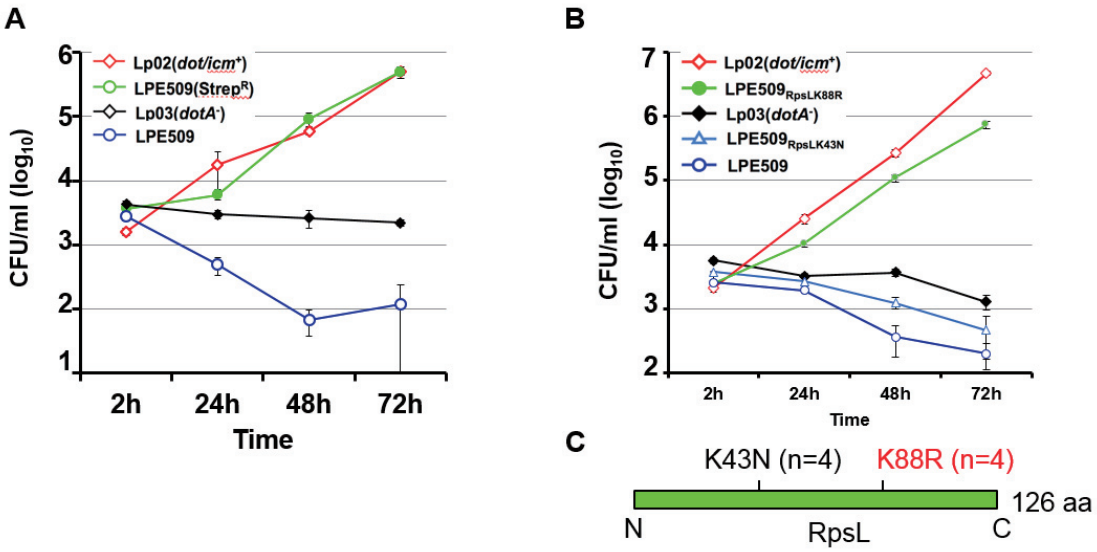


Fig. 4-10 A streptomycin resistant mutant of *L. pneumophila* could replicate in A/J mouse macrophages. A & B. Bone marrow-derived macrophages from A/J mouse were challenged with indicated bacterial strains grown to post-exponential phase at an MOI of 0.05. After synchronization 2 hrs after infection, total bacterial counts (colony-forming-unit) were determined by plating appropriately diluted saponin solubilized infected cells onto bacteriological medium. Infections were performed in triplicate and data shown were from one representative of multiple experiments with similar results. **C.** Diagram representation of the *rpsL* gene of the spontaneous strep^R *L. pneumophila* mutants tested for intracellular growth in A/J BMDMs.

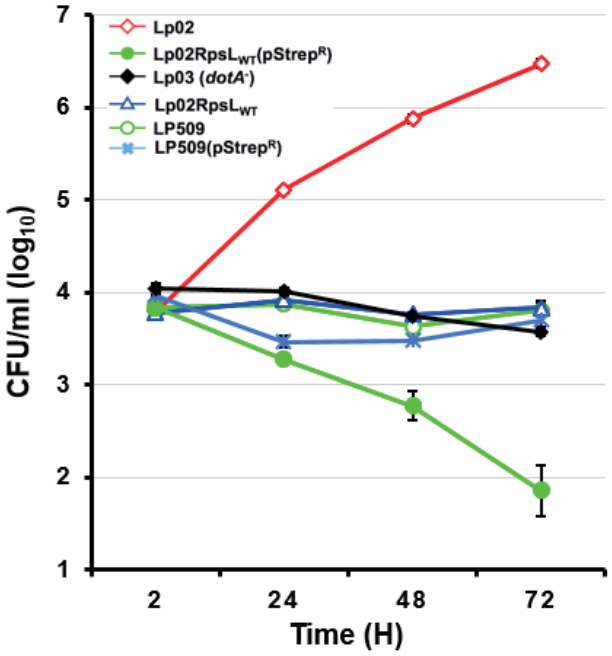


Fig. 4-11 The inability of *rpsL*$_{WT}$ *L. pneumophila* strains to replicate in A/J BMDMs is not due to the presence of the residual streptomycin in the cell culture medium. The intracellular growth of Lp02, LPE509 and their counterpart carrying plasmid pRB970, which constitutively expresses a streptomycin 3'-adenylyltransferase, was tested in A/J mouse macrophage. The streptomycin resistance conferred by this plasmid (MIC=30μg/ml) did not allow Lp02 or LPE509 to replicate in A/J mouse macrophages.

Strains	Streptomycin MIC_{50} (mg/L)
LPE509 *rpsL*$_{WT}$	0.16
LPE509 *rpsL*$_{K88R}$	>100
LPE509 *rpsL*$_{K43N}$	>100
LPE509 *rpsL*$_{WT}$+pRG970	30
Lp02 *rpsL*$_{WT}$	0.16
Lp02 *rpsL*$_{K88R}$	>100
Lp02 *rpsL*$_{WT}$+pRG970	30

Table. 4-1 MIC of *L. pneumophila* strains A suspension of each isolate was prepared in AYE/AYET medium. A final inoculum of $1x10^5$ CFU/ml of indicated strain was added to each well of a 96 well microtiter plate containing serially diluted streptomycin in AYE/AYET. Plates were incubated at 37°C in a humidified incubator for 3 days. MIC was determined as the lowest concentration of streptomycin with no detectable bacterial growth in AYE/AYET broth.

RpsL is responsible for intracellular growth restriction of *L. pneumophila* in primary mouse macrophages

RpsL is a component of the 30S subunit of the ribosome involved in protein translation accuracy and is highly conserved among bacteria [208]. The fact that the $RpsL_{K88R}$ mutation is required for *L. pneumophila* to grow in BMDMs suggests that the commonly used laboratory strains (all resistant to streptomycin) such as Lp02, JR32 and AA100 could harbor the same mutation [152]. Thus, we sequenced the *rpsL* gene of these strains and found that a K88R mutation indeed exists in each of them. This mutation was identified in strains Lp02 and JR32 in a recent study [152]. The requirement of the K88R mutation for growth also suggests that BMDMs should be restrictive to the original streptomycin sensitive isolates. To test this hypothesis, we examined the growth of the original isolate of strain Philadelphia 1 (Phil 1). As expected, unlike its derivative Lp02, this strain was unable to replicate in BMDMs (**Fig. 4-12, A**). Similar results were obtained from the wild type strain Paris and strain Thunderbay [209], a more recent clinical strain (**Fig. 4-13**). Similarly, a K88R mutation allowed both strains to grow in BMDMs (**Fig. 4-13**). Importantly, robust growth occurred when the *rpsL* gene of strain Phil 1 was replaced by homologous recombination with $rpsL_{K88R}$ but not with $rpsL_{K43N}$ (**Fig. 4-11, A**). To further confirm that RpsL is the sole factor that accounts for the phenotype, we replaced $rpsL_{K88R}$ in strain Lp02 with a wild type allele to produce Lp02$rpsL_{WT}$; this strain retained the ability to replicate in the cultured human macrophage cell line U937 (**Fig. 4-14**), but has lost the ability to grow in BMDMs (**Fig. 4-12, B**). Again, introduction of the streptomycin adenyltransferase gene into this strain conferred antibiotics resistance but not intracellular replication (**Table 4-1; Fig. 4-11**), further supporting the notion that wild type RpsL restricts bacterial replication in these cells. Taken together, these results indicate that wild type RpsL is the genetic determinant that governs the outcome of macrophage infection by *L. pneumophila*: the WT protein restricts intracellular growth of *L. pneumophila* in BMDMs, and that such restriction occurs both clinical and environmental isolates.

Because the genomic information of strain Lp02 has been independently verified [210] and this strain is more genetically amenable, we chose to use this strain and its isogenic strain Lp02*rpsL*$_{WT}$ in all subsequent experiments.

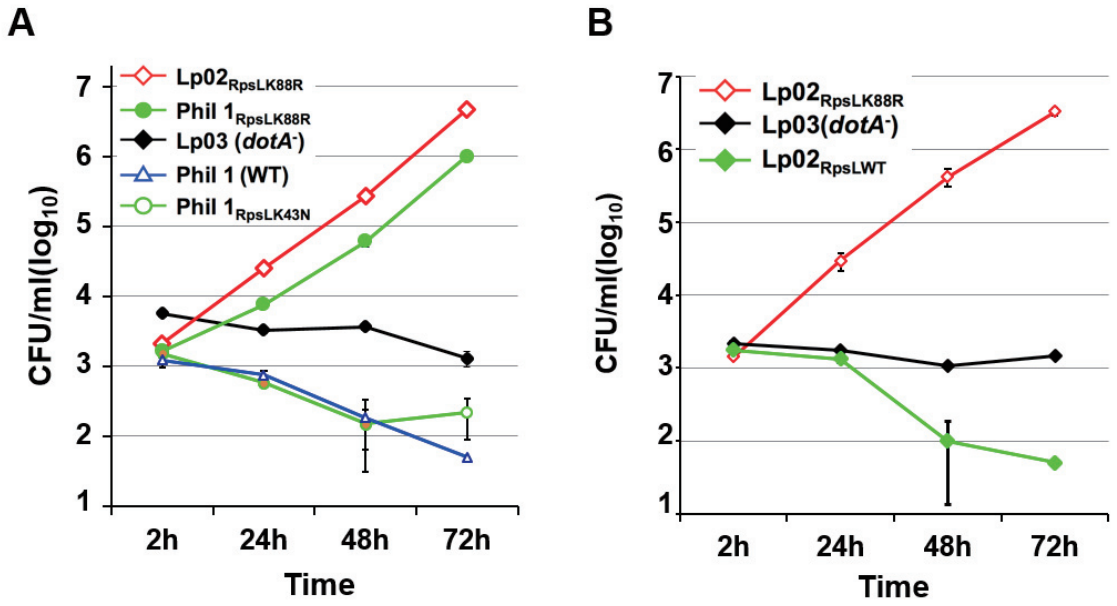


Fig. 4-12 RpsL determines intracellular replication of *L. pneumophila.* A & B. Bone marrow-derived macrophages from A/J mouse were challenged with indicated bacterial strains grown to post-exponential phase at an MOI of 0.05. After synchronization 2 hrs after infection, total bacterial counts (colony-forming-unit) were determined by plating appropriately diluted saponin solubilized infected cells onto bacteriological medium. Infections were performed in triplicate and data shown were from one representative of multiple experiments with similar results.

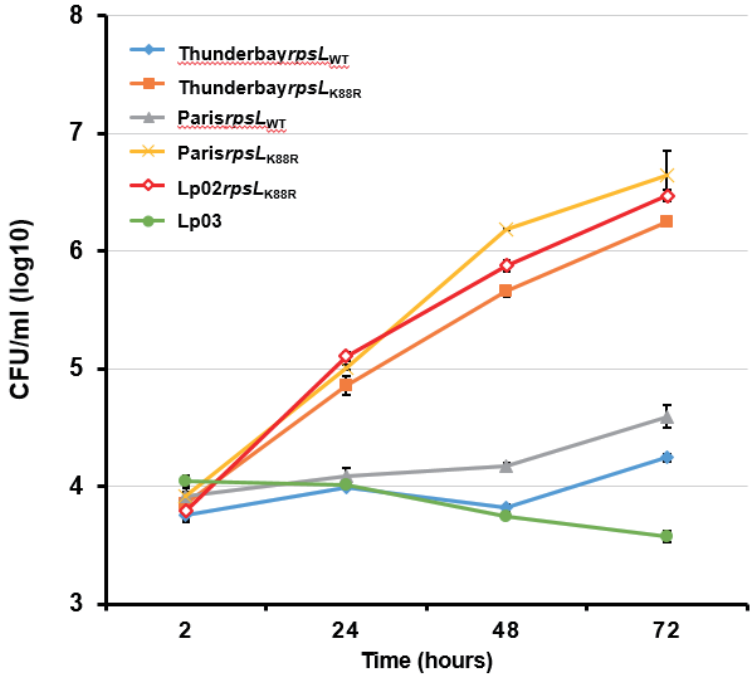


Fig. 4-13 K88R mutation on *rpsL* allows *L. pneumophila* clinical isolates to replicate in A/J mouse macrophages. K88R mutation was introduced into wild type *L. pneumophila Paris* and *L. pneumophila Thunderbay* clinical isolates via homologues recombination. Bone marrow-derived macrophages from A/J mouse were challenged with indicated bacterial strains at an MOI of 0.05. After synchronization, at indicated time points, total bacterial counts (colony-forming-unit) were determined by plating appropriately diluted saponin solubilized infected cells onto bacteriological medium. Infections were performed in triplicate and data shown were from one representative of multiple experiments with similar results.

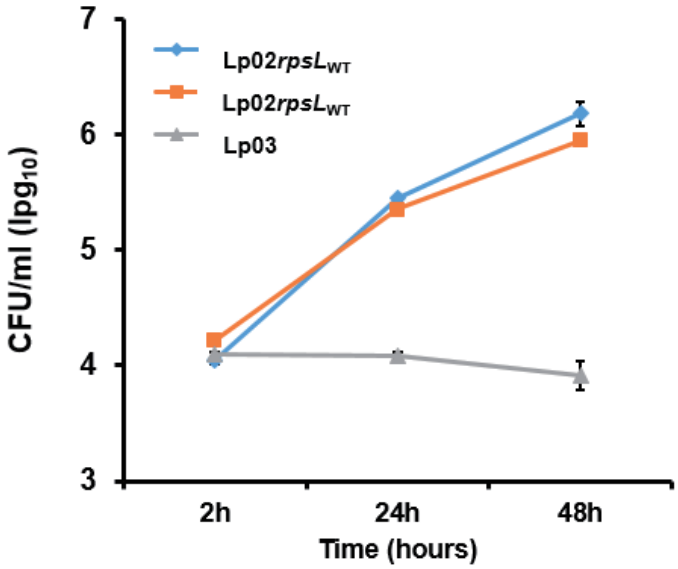


Fig. 4-14 Lp02*rpsL*$_{WT}$ could replicate efficiently in human U937 cells. 4x10^5 human U937 cells were challenged with indicated bacterial strains at an MOI of 0.05. After synchronization, at indicated time points, total bacterial counts (colony-forming-unit) were determined by plating appropriately diluted saponin solubilized infected cells onto bacteriological medium. Infections were performed in triplicate and data shown were from one representative of multiple experiments with similar results. This result indicates that the inability of Lp02*rpsL*$_{WT}$ to replicate in primary mouse macrophages is not due to a compromised Type IV secretion system.

Infection by *L. pneumophila* expressing wild type RpsL leads to macrophage cell death

Our previous study with strain LPE509 indicated that host cell death accompanied the restriction of intracellular growth [157], we thus determine whether a similar event occurred in infections with strain Lp02*rpsL*$_{WT.}$ Comparing to infections with strain Lp02*rpsL*$_{K88R}$, significantly higher levels of lactate dehydrogenase (LDH) was detected in culture supernatant of BMDMs challenged with strain Lp02*rpsL*$_{WT}$ (**Fig. 4-15, A**). Single cell analysis by TUNEL staining revealed that at 12 hrs post-infection, close to 50% of the macrophages infected with Lp02*rpsL*$_{WT}$ stained positive for apoptosis, whereas only about 10% Lp02*rpsL*$_{K88R}$ infected cells appeared apoptotic (**Fig. 4-15, B**). It is established that after a 4-6 hours of lag phase after bacterial uptake, *L. pneumophila* stars to replicate and expands the size of the LCV. 12 hours post infection, the LCV could accommodate more than 10 bacteria [157]. Unlike bacteria of strain Lp02*rpsL*$_{K88R}$ that can be distinctly stained by the anti-Legionella antibody, the staining signals of bacteria of strain Lp02*rpsL*$_{WT}$ appeared blurry and diffusing, which may result from the debris from dying bacterial cells (**Fig. 4-15, C**). Kinetics experiments revealed that such differences became apparent within 8 hrs post-infection (**Fig. 4-16**).

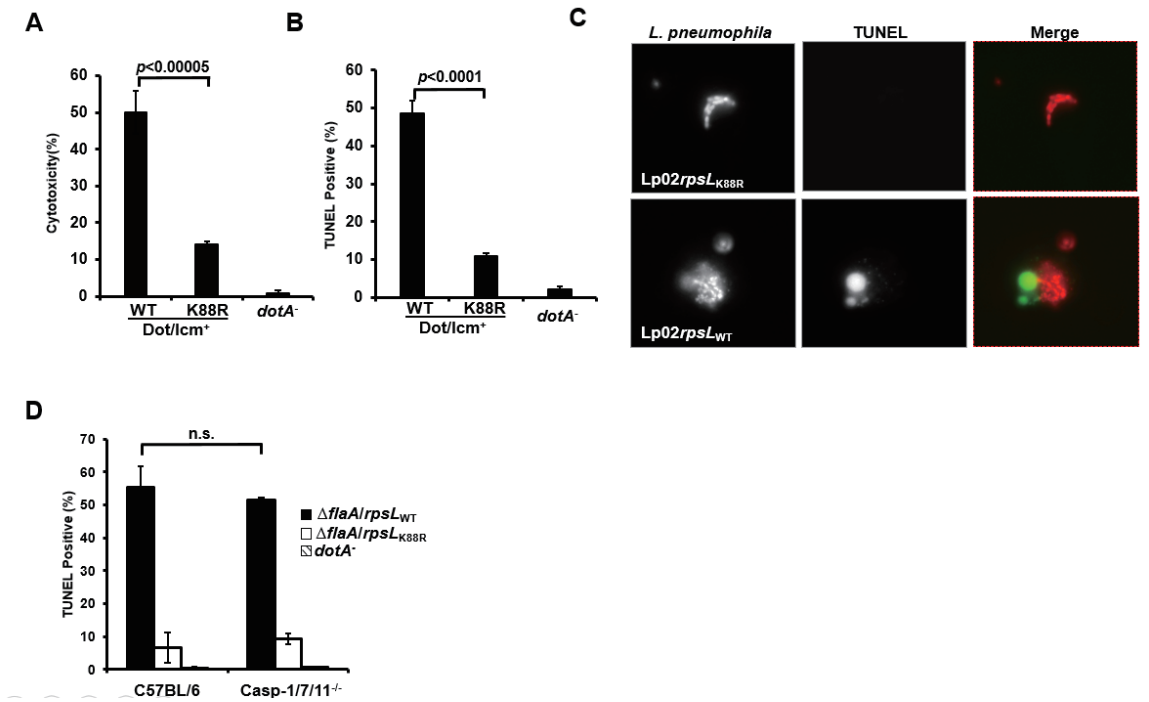


Fig. 4-15 Infection by *L. pneumophila* bearing rpsL$_{WT}$ results in macrophage cell death A. A/J mouse macrophages on glass coverslips were infected with appropriately grown bacterial strains at an MOI of 1. Infection was synchronized and LDH release was determined 6 hours post infection. **B.** Infection was done similarly to **(A).** 12 hours post infection, macrophages were fixed and stained with TUNEL. **c.** Representative images of **(B). D.** C57BL/6 and caspase-1/7/11$^{-/-}$ BMDMs were challenged with indicated strains. 12 hours post infection, macrophages were fixed and stained with TUNEL.

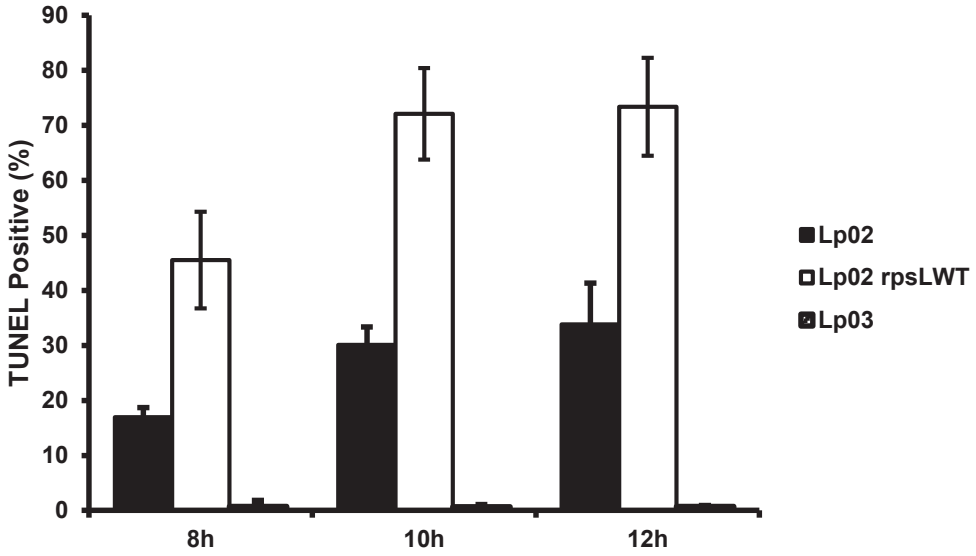


Fig. 4-16 Infection by *L. pneumophila* bearing rpsL$_{WT}$ results in macrophage cell death A/J mouse macrophages on glass coverslips were infected with appropriately grown bacterial strains at an MOI of 1. Infection was synchronized and LDH release was determined at the indicated time point.

Inflammatory caspases such as caspase 1, 7 and 11 have been demonstrated to play important role in macrophage cell death associated with *L. pneumophila* infection [211], we thus examined whether any of these caspases are involved in the cell death induced by *L. pneumophila* expressing wild type RpsL by constructing a mouse line lacking caspase 1, 7 & 11 (Caspase-1/7/11$^{-/-}$, generous gift by Dr. Russell Vance at University of California, Berkeley). Challenge of macrophages from these mice with Lp02*rpsL*$_{WT}$ *ΔflaA* still led to cell death as measured by LDH release and by TUNEL staining. In contrast, infection by the Lp02*rpsL*$_{K88R}$ *ΔflaA* strain caused significantly less death in these macrophages (**Fig. 4-15, D**). Consistent with this observation, BMDMs lacking caspase-1/7/11 did not support the growth of strain Lp02*rpsL*$_{WT}$ Δ*flaA* (**Fig. 4-17**). Thus, macrophage cell death is linked to the intracellular growth restriction of *L. pneumophila* expressing wild type RpsL. Moreover, the cell death occurred in a mechanism independent of caspases 1, 7 and 11.

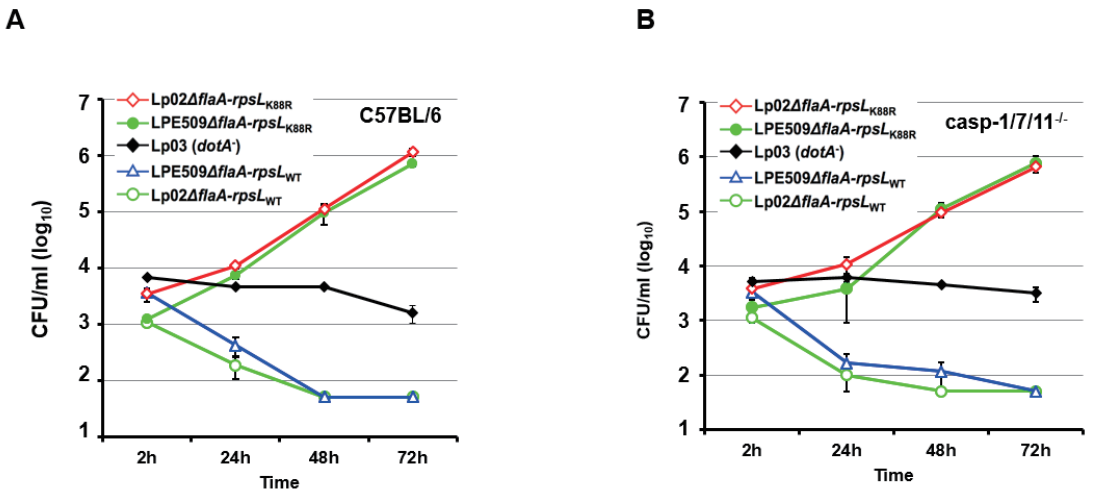


Fig. 4-17 Caspase-1, 7 and 11 are dispensable for growth restriction of *L. pneumophila* bearing *rpsL*$_{WT}$. Bone marrow-derived macrophages from C57L/B6 or caspase-1/7/11$^{-/-}$ mice were challenged with indicated bacterial strains grown to post-exponential phase at an MOI of 0.05. After synchronization, at indicated time points, total bacterial counts (colony-forming-unit) were determined by plating appropriately diluted saponin solubilized infected cells onto bacteriological medium. Infections were performed in triplicate and data shown were from one representative of multiple experiments with similar results.

RpsL directly induces cell death in macrophage

There are several possibilities that could account for the cell death phenotypes that are associated with the genotypes of RpsL. First of all, as we know, the major role of RpsL is to control translation accuracy [212]. It is therefore possible that the K88R mutation, which is known to confer mildly higher translational accuracy, may cause fewer mistranslated proteins, which might be the cause of host cell death and bacterial replication restriction. However, this may not be the case, because *L. pneumophila* expressing $RpsL_{K43N,}$ a mutation known to impose even higher translation accuracy to the ribosome [213], cannot grow in BMDMs. Second, as the Type IV Translocation System (Dot/Icm) is known to be promiscuous in substrate recognition, RpsL could get access to the host cytosol and recognized by the host immune surveillance pathways [214]. The fact that non-effector proteins such as bacterial flagellin are translocated into the host cytosol by this very Dot/Icm and recognized as MAMPs, together with the high level conservation of RpsL among phylogenetically diverse bacteria, prompted us explore this possibility. To this end, we purified recombinant Sumo-$RpsL_{WT}$ and Sumo-$RpsL_{K88R}$ with a procedure involved in the removal of endotoxin (LPS) (Methods) and delivered the proteins into macrophages by transfection [215]. When directly added to the BMDMs culture, neither protein caused detectable cell death (**Fig. 4-18, A-B**). However, when a transfection reagent was used, Sumo-$RpsL_{WT}$ triggered significantly higher amount of LDH release than similarly treated samples receiving Sumo-$RpsL_{K88R}$ (**Fig. 4-18, A**). Similar results were obtained when cell death was evaluated by TUNEL staining, in which close to 30% of cells receiving wild type RpsL were apoptotic and less than 10% cells were TUNEL positive in samples transfected with Sumo-$RpsL_{K88R}$ (**Fig. 4-18, B-C**). Consistent with the genetic results, Sumo-$RpsL_{K43N}$ was able to induce macrophage death at rates comparable to the wild type protein (**Fig. 4-18, D**). Importantly, wild type RpsL but not the K88R mutant of RpsL from *Escherichia coli* induced cell death in macrophages (**Fig. 4-18, E)**, which is consistent with the high-level conservation (91% identity) of this protein from

these two bacteria. Further, the cell death induced is specific for RpsL, as Sumo-RpsM, another ribosomal protein, was unable to induce macrophage death (**Fig. 4-18, E**). Finally, because dsRNA delivered into macrophages is known to trigger cathepsin-dependent apoptotic cell death [Rintahaka, 2011 #2779], we determined the potential effects of contaminated dsRNA by treating the proteins with RNAase III. This treatment did not alter the cell death inducing ability of $RpsL_{WT}$, indicating that dsRNA did not contribute to the cell death (**Fig. 4-18, F**). On the other hand, treatment of protease K led to abolishment of the cell death-inducing ability of the protein samples, further supporting the proteinaceous nature of the cell death stimuli (**Fig. 4-18, F**). Consistent with our previous observation that macrophage cell death induced by *Legionella* strains expressing $RpsL_{WT}$ is independent of caspase 1, 7 and 11, purified $RpsL_{WT}$ could efficiently induce cell death in Caspase-1/7/11$^{-/-}$ macrophages. This observation also supports the fact that LPS did not contribute to RpsL cytotoxicity (**Fig. 4-18, G**).

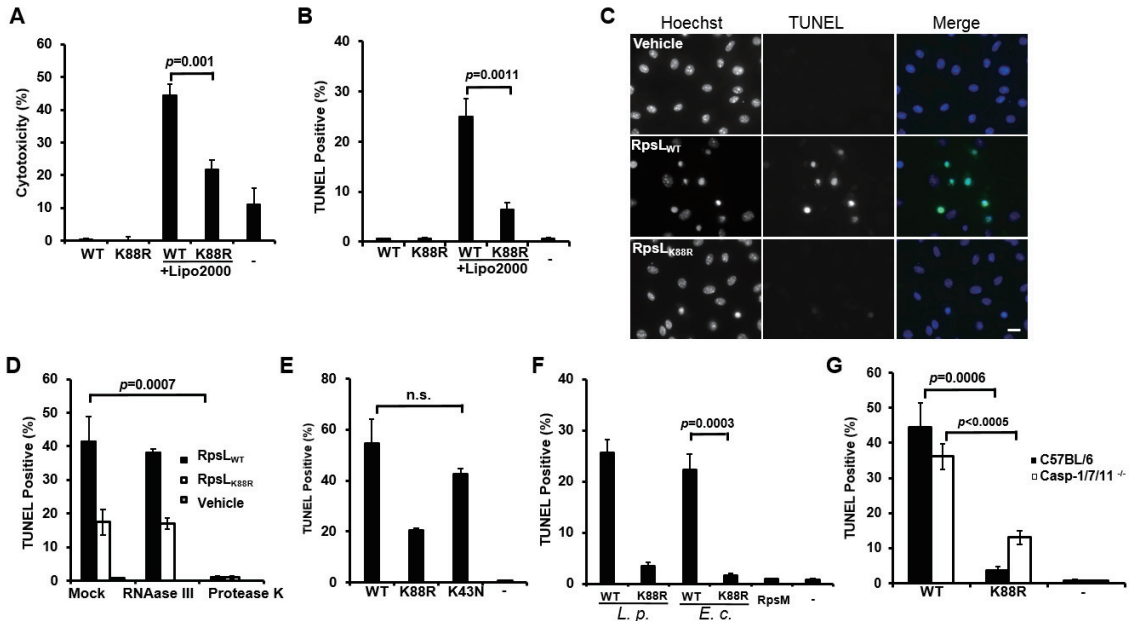


Fig. 4-18 RpsL directly induces cell death in macrophage A. 12.5 µg purified RpsL protein was then either directly added to culture supernatant or delivered intracellularly into A/J BMDM using Lipofectamine 2000. LDH release was measured 6 hours post transfection. **B.** Purified RpsL proteins were added to culture supernatant or delivered intracellularly into A/J BMDM similarly to **(A)**. 6 hours post transfection, macrophages were fixed and stained with TUNEL. **c.** Representative images of **(B). D&E.** Indicated proteins were delivered intracellularlly into A/J BMDM. 6 hours post transfection, macrophages were fixed and stained with TUNEL. **F.** Mock, RNAase III or Protease K treated RpsL proteins were transfected into A/J BMDM. Cell death was measured by TUNEL staining 6 hours post transfection. **G.** C57BL/6 and caspase-1/7/11$^{-/-}$ BMDMs were transfected with indicated RpsL proteins and cell death was measured by TUNEL staining 6 hours post transfection.

The fact that *L. pneumophila* strains harboring wild type *rpsL* were able to replicate in alternative hosts such as U937 and Hela cells (**Fig. 4-14, Fig. 4-19, A** and [157]) suggests that RpsL cannot induce death in these cells. In agreement with this notion, Hela cells transfected with Sumo-RpsL did not detectably stain positive for apoptosis (**Fig. 4-19, B**). The lack of cell death is not due to deficiency in protein delivery because a similar procedure successfully transfected purified beta-galactosidase into these cells (**Fig. 4-19, C**). Taken together, these results establish that wild type RpsL is able to directly induce cell death in macrophages, whilst the K88R mutant triggers similar responses in a less efficient manner.

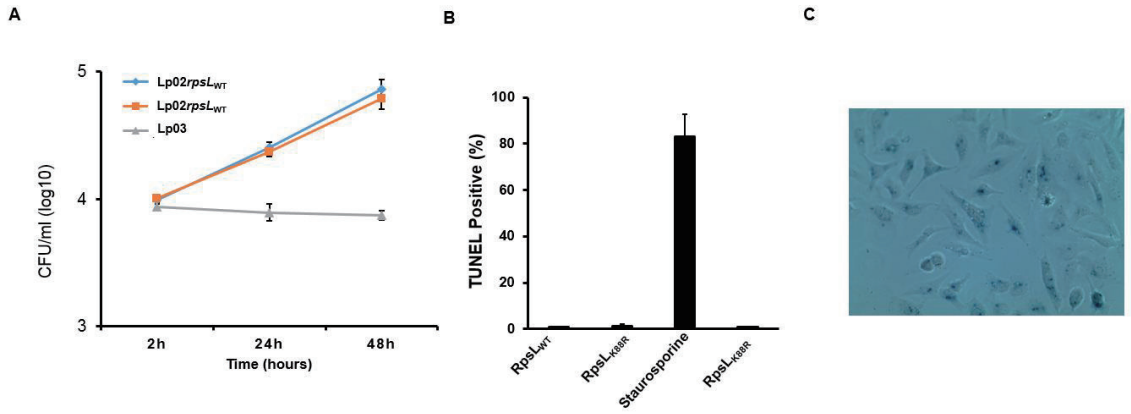


Fig. 4-19 Hela cells are not responsive to RpsL A. The intracellular growth of indicated strains in Hela cells were determined similarly as aforementioned. **B.** Hela cells were either treated with 1μg/ml STS or transfected with indicated proteins using Lipofectamine as aforementioned. Hela cells were then fixed at 6 hours post transfection and stained with TUNEL. The percentage of TUNEL-positive cells in each sample were numerated. **C.** $2x10^5$ Hela cells were transfected with 2μg beta-galactosidase. 6 hours later, the presence of intracellular beta-galactosidase was visualized using a beta-galactosidase staining kit per manufacturer's instruction.

RpsL causes lysosome membrane permeabilization in macrophages

That macrophages from mice lacking the three inflammatory caspases are still sensitive to RpsL suggests that the cell death is not caused by canonical pyroptosis. We thus explored the involvement of alternative cell death pathways [216]. Lysosome is a acidic organelle which contains a variety of hydrolases, and has long been implicated in functions other than the garbage disposal inside of the cell [81]. Stress stimuli could trigger selective or complete permeabilization of lysosome membrane, leading to the release of hydrolases such cathepsins (e.g. capthepsin B, D, or L) from the lysosomal lumen into the cytosol, which could induce apoptotic or pyrototic cell death through caspase activation, or necroptosis when caspase activation is inhibited [217, 218]. If RpsL activates the lysosomal cell death pathway, inhibiting lysosomal acidification should reduce its cytotoxicity. Thus, we treated macrophages with the lysosome acidification inhibitor bafilomycin A1 (BFA1) (which targets the vacuolar ATPase responsible for regulating organelle pH [218]) prior to RpsL transfection. Cell death was almost completely abrogated in samples pre-incubated with BFA1 (**Fig. 4-20, A**). To eliminate the potential non-specific effects of this compound, we co-transfected RpsL with SidK, a *L. pneumophila* effector known to inhibit v-ATPase. Similarly, cell death was blocked in samples receiving both proteins (**Fig. 4-20, B**).

We next examined the integrity of lysosomal membranes in macrophages receiving RpsL by using acridine orange (AO), a lysotrophic dye that when concentrated in lysosome emits orange florescence upon excitation by blue light, but green florescence when redistributed into the cytosol and nucleus upon the loss of lysosome membrane integrity [219]. In samples treated with the transfection reagent, essentially all macrophages emitted orange fluorescence (**Fig. 4-20, C-D**); in samples receiving RpsL, close to 40% of the cells gave green fluorescence signals, indicating the loss of lysosomal membrane integrity (**Fig. 4-20, C-D**). $RpsL_{K88R}$ detectably induced the redistribution of AO, but at significantly

lower rates (**Fig. 4-20, C-D**). Similar results were obtained in infected BMDMs in which Lp02*rpsL*$_{WT}$ caused significantly higher lysosome damage strain Lp02*rpsL*$_{K88R}$ (**Fig. 4-20, E-F**).

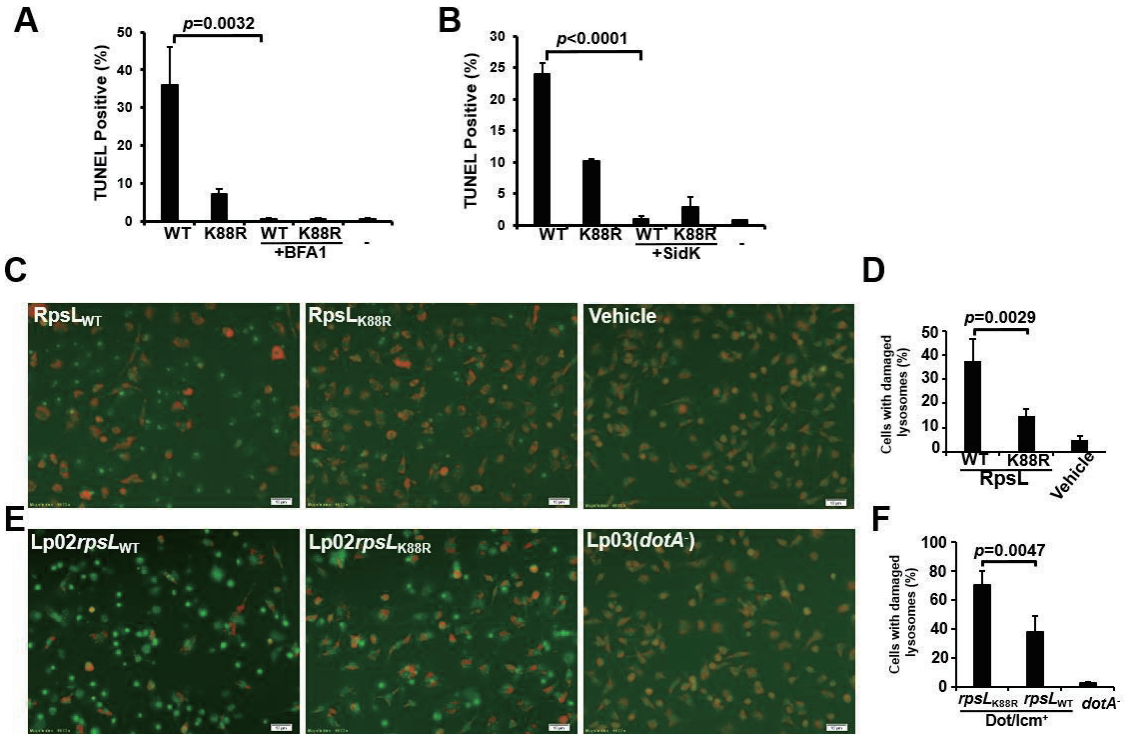


Fig. 4-20 Lysosome cell death pathway is involved in RpsL recognition. A. A/J BMDM were treated with lysosome acidification inhibitor bafilomycin A1 (20nM) for 1 hour before protein transfection. Cell death was measured by TUNEL staining 6 hours post transfection. **B.** Purified RpsL proteins were delivered with buffer or equal amount of SidK proteins into A/J BMDM. Cell death was measured by TUNEL staining 6 hours post transfection. **C&D.** Indicated proteins were transfected into A/J BMDM. 8 hours later, cells were stained with 5 μg/ml AO in 37°C for 15 min before imaging by a fluorescent microscope. Data shown are the percentage of cells with damaged lysosome (loss of red florescence) from one representative from multiple experiments with similar results. **E&F.** A/J BMDMs were challenged with indicated strains at an MOI of 1. Extracellular bacteria were removed with extensive washes 1 hour post infection, and cells were then strained with AO 8 hours later. Cells with ruptured lysosomes were quantitated.

Release of cathepsins into the cytoplasm could lead to the activation of the mitochondria cell death pathway by cleaving the BH3-only protein BID, which subsequently inserts into the mitochondria and induces cytochrome c release and caspase activation [78]. Cathepsin B has also been shown to activate caspase-2, which in turn induces cytochrome c release [220].If lysosome permeabilization occurs upon the detection of RpsL, apoptosis likely will be activated, which could eliminate infected macrophages. Pretreating macrophages with a specific inhibitor of cathepsin B, but not cathepsin D, significantly dampens the responses against RpsL, suggesting lysosome permeabilization acts upstream of apoptosis and cathepsin B plays a major role in this process (**Fig. 4-21, A**). Consistent with this notion, marked increase of cytosolic cathepsin B activity was detected in macrophages transfected $RpsL_{WT}$ but not its K88R mutant (**Fig. 4-21, B**). Further, genetically ablating cathepsin B blunts apoptotic signaling upon RpsL sensing, as we observed a marked decrease of apoptotic cells in cathepsin $B^{-/-}$ BMDMs receiving $RpsL_{WT}$ (**Fig. 4-21, C**). This is not due to a general defect in the apoptotic pathway, as the pathways activated upon staurosporine treatment are intact in both C57BL/6 and cathepsin $B^{-/-}$ BMDMs; nor to the non-specific effects resulting from cathepsin deletion, because BMDMs lacking another cathepsin, cathepsin H, respond normally to RpsL (**Fig. 4-21, C**). Moreover, A/J BMDMs challenged with Lp02*rpsL*$_{WT}$ showed significantly increased level of cleaved BID and activated caspase-3 than those challenged with Lp02*rpsL*$_{K88R}$. Lp02*rpsL*$_{WT}$ infected macrophages also harbor significantly more cleaved product of one of the best characterized caspase-3 substrates, cPARP (cleaved Poly (ADP-ribose) polymerase), further strengthening the notion that cathepsin B activates the BID-Caspase-3 pathway once it is released from lysosome upon RpsL sensing (**Fig. 4-21, D**). Correspondingly, we also observed marked increase of activated caspase-3 and cPARP in macrophage transfected with $RpsL_{WT}$ but not $RpsL_{K88R}$, further strengthening that RpsL is a MAMP that governs that infection outcome of *L. pneumophila*.

In permissive host cells, *L. pneumophila* resides in an intracellular compartment that escapes the endocytic pathway and recruits organelles such as mitochondria and ER-derived small vesicles [221]. After a lag phase of 4-6 hours, bacteria start to replicate and form spacious vacuoles that contain more than 10 bacteria [222]. If infected cells undergo apoptosis, large vacuoles cannot form, and bacterial replication will be limited. We thus examined the formation of bacterial vacuoles by relevant *L. pneumophila* strains. A/J BMDM challenged with Lp02*rpsL*$_{WT}$ has significantly fewer large vacuoles than those challenged by Lp02*rpsL*$_{K88R}$ (**Fig. 4-21, F**). Further, inhibition of cathepsin B by CA-074ME partially reverses such defects, further supporting the notion that lysosome membrane permeabilization is involved in RpsL induced cell death (**Fig. 4-21, F**).

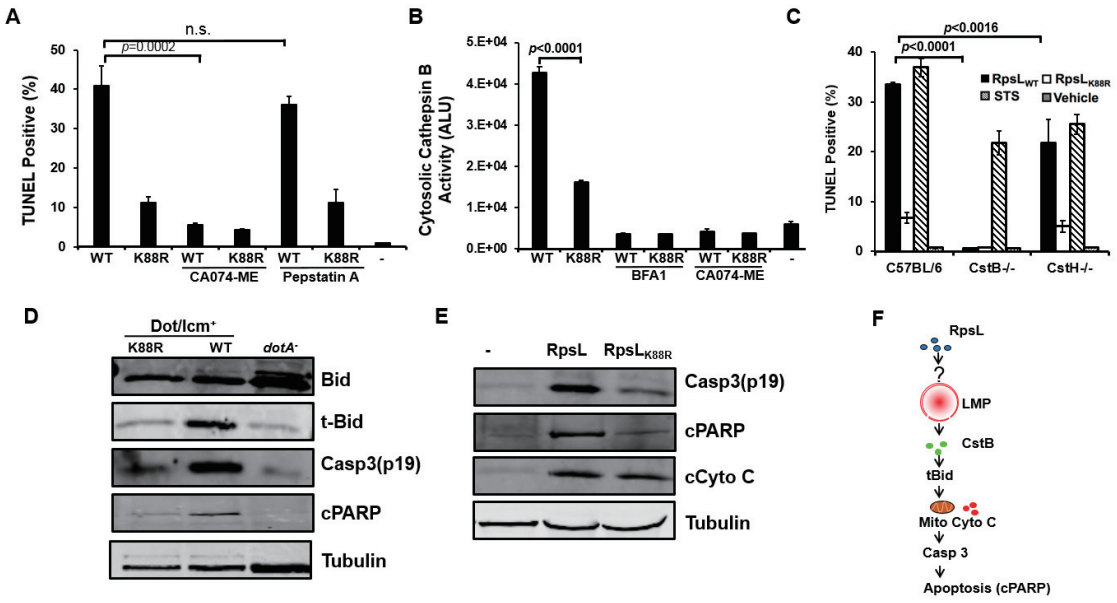


Fig. 4-21 RpsL causes lysosome membrane permeabilization in macrophages A. A/J BMDM were treated with cathepsin B inhibitor CA074-ME (25μM) and cathepsin D inhibitor pepstatin A (25μM) 1 hour before protein transfection. Cell death was measured by TUNEL staining 6 hours post transfection. **B.** A/J BMDMs pretreated with indicated inhibitors were transfected with the indicated proteins. 6 hours post transfected, BMDMs were partially permeabilized with 100μg/ml saponin and the cytosolic cathepsin B activity was determined with z-FR-AMC. **C.** C57BL/6, cathepsin $B^{-/-}$ and cathepsin $H^{-/-}$ BMDMs were transfected with indicated RpsL proteins or treated with 1μg/ml staurosporine (STS). 6 hours later, cell death was measured by TUNEL staining. **D.** A/J BMDMs were challenged with indicated strains at an MOI of 1 for 8 hours. BMDMs were lysed and 100μg total proteins were resolved in SDS-PAGE. Activated caspase-3 (p19), tBID, and cleaved-PARP (cPARP) were detected with specific antibody. Tubulin was probed as a loading control. **E.** A/J BMDMs were transfected with indicated proteins or treated with 1μg/ml staurosporine. 100μg total proteins were resolved in SDS-PAGE. Activated caspase-3 (p19) and cleaved-PARP (cPARP) were detected with specific antibody. Tubulin was probed as a loading control. **F.** Diagram showing the possible downstreaming signaling pathway activated upon macrophage sensing of RpsL.

Sample	Protein Name	DB Peptide	Variable Modifications
WT	30S ribosomal protein S1	VKDLPGVR	Thiomethylation@3
KR	30S ribosomal protein S1	VKDLPGVR	N/A

Fig. 4-22 Mass spectrometry analysis of the presence of thiomethylation on RpsL. Purified recomboinant $RpsL_{WT/K88R}$ proteins were subjected to mass spectrometry analysis to identify the post-translational modification-thiomethylation on RpsL. These results indicated that $RpsL_{WT}$ could be readily thiomethylated on D89 residue while the $RpsL_{K88R}$ lacks such modification.

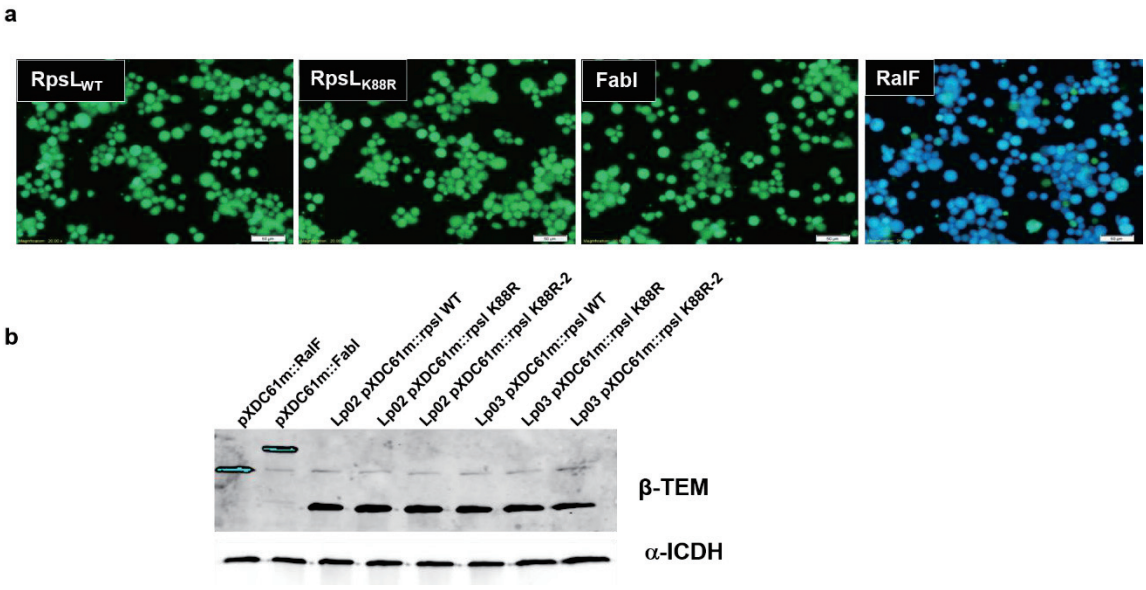


Fig. 4-23 RpsL is not detectably translocated into host cells by *L. pneumophila*. A. RpsL and RpsL$_{K88R}$ were fused to b-lactamase on a plasmid, respectively and the resulting constructs were transformed into *L. pneumophila* strains. The bacterial strains were used to determine Dot/Icm-dependent protein translocation with the CCF4 substrate with an established protocol. Strains expressing RalF or FabI were used as positive and negative controls, respectively. Note the robust translocation by the RalF construct indicated by the appearance of blue cells after bacterial infection. **B.** Expression of the relevant fusions in *L. pneumophila*. The bacterial cells used for infection in (**A**) were probed for the expression of the fusion with a b-lactamase specific antibody. The isocitrate dehydrogenase (ICDH) was probed as a loading control.

Discussion

Our study demonstrates that environmental isolates of *L. pneumophila* cannot replicate in A/J mouse macrophages, which is permissive to most, if not all laboratory strains. The inability of environmental strains to replicate in A/J macrophages was accompanied by rapid and extensive host cell death [223]. LPE509-elicited cell death is of unique nature, as it is independent of *L. pneumophila* factors that are known to be involved in host cell death induction, such as flagellin, and factors shown to protect bacterium from host cell death surveillance components through maintaining the integrity of the LCV, such as SdhA [201]. The uniqueness of LPE509-elicited cell death also applies to the host pathways engaged by this bacterium. Two major cell death pathways have been demonstrated to be important in restricting intracellular *L. pneumophila*. The first one is governed by the non-permissive allele of Naip5, which recognizes flagellin presumably translocated into the host cytosol by the Dot/Icm of *L. pneumophila*, where it induces the assembly Naip5-NLRC4-Caspase-1 inflammasome [120, 224]. The formation of inflammasome activates caspase-1, leading to a rapid and lytic form of cell death termed pyroptosis, as well as the maturation of inflammatory cytokines IL-1α and IL-1β [120]. The other parallel pathway involves the collaboration of a cluster of guanylate-binding proteins (small interferon-inducible GTPases) and yet another caspase, caspase-11 [225]. During infection of vacuolar Gram-negative bacteria, these guanylate-binding proteins are recruited to the damaged bacterial phagosomes and are required for the lysis of bacterial vacuoles. The lysis of the bacterial vacuoles then leads to the release of LPS, which activates caspase-11 [226]. Caspase-11, being the only component known so far that are both the receptor and executioner molecule in a same immune surveillance pathway, binds to cytosolic LPS and oligomerizes into the non-canonical inflammasome, which allows the activation of caspase-11 [226, 227]. Activated caspase-11 could induce pyroptosis alone or process caspase-1 for its activation, to accelerate the cell death process and to induce the secretion of mature inflammatory cytokines [228]. As these two

inflammatory pathways have been shown to be important for controlling *L. pneumophila* infection, we first tested whether these components are important to the cell death induced by LPE509. We found that mouse macrophages lacking caspase-1/11 still respond to and restrict the intracellular growth of LPE509, suggesting the involvement of other cell death pathways. Further, genetic ablation of caspase-3, the executioner caspase of the classic apoptotic pathway, or the application of necrostatin-1, a potent inhibitor of the necroptosis pathway [229], was unable to dampen the responses or rescue the intracellular growth of LPE509. Finally, the cell death triggered by LPE509 displays both apoptotic and pyroptotic features, as it is companied by loss of plasma membrane integrity, positive TUNEL staining and the formation of membrane blebs (**Fig. 4-3**). These results suggested 1) LPE509 codes for a unique factor that stimulates host immune responses, and 2) these responses are governed by alternative cell death pathways. Thus, we postulate that murine macrophages code for receptor(s) that can detect one or more factors specifically coded for by LPE509 and that the engagement of the receptor with the ligand leads to the activation of enzymes such as caspases capable of causing membrane permeabilization, DNA fragmentation and the subsequent cell death.

In our attempt to generate a transposon-mediated mutant library of LPE509 for the identification of the unique factor(s) of this bacterium, we obtained a spontaneous streptomycin resistant mutant that was capable of overcoming the growth restriction imposed by the A/J macrophages. Further analysis showed that a K88R mutation on the conserved bacterial ribosomal component RpsL in this particular strain. This allele also dictates the ability of different environmental and clinical strains of *L. pneumophila* to replicate in A/J mouse macrophages. Several lines of evidence below favor the hypothesis that RpsL is a MAMP directly responsible for the macrophage death induced by wild type *L. pneumophila* infection:

First, all examined *L. pneumophila* strains expressing wild type RpsL failed to replicate in A/J macrophages, while a K88R mutation allowed all these strains to overcome such restriction. The drastic difference between these strains is not simply due to the increased bacterial resistance to streptomycin, as *rpsL*$_{K43N}$, a mutation that confers similar streptomycin resistance (**Table 4-1**), failed to support the replication of *L. pneumophila* in mouse macrophages. Moreover, the strain Lp02*rpsL*$_{WT}$ harboring pRG970, a plasmid that confers the bacterium streptomycin resistance, also fails to replicate in A/J mouse macrophages. These lines of evidence strongly argue against the involvement of streptomycin in clearing the bacteria. Second, the severity of host cell death by *L. pneumophila* infection depends on the genotype of *rpsL* in these strains. Third, intracellular delivery of purified WT RpsL protein recapitulates the host responses that are elicited during the infection by *L. pneumophila* carrying WT RpsL, such as lysosome membrane permeabilization, DNA fragmentation, and loss of plasma membrane integrity. Interestingly, the region surrounding the K88 residue (KVDL), is highly conserved even among phylogenetically distant bacteria, such *E. coli, S. Typhimurium,* and *B. subtilis*, which is consistent with our results that RpsL of *E. coli* also stimulates similar host responses as *L. pneumophila* RpsL does. Many *rpsL* homologues in eukaryotes harbor a Gly (mitoribosome) or Gln (cytoribosome) residue at position 87 instead of Lys, although the K43N mutation of *rpsL* could also confer resistance against streptomycin [230]. If RpsL$_{WT}$ is immune-stimulating, these mutations could be an effective mechanism to avoid autoimmunity. Together, we reason that RpsL, a conserved bacterial ribosomal protein, is a MAMP that is recognized by mouse macrophage by a putative cytosolic receptor.

RpsL is not the first ribosome component shown to trigger immune response. In plants, EF-Tu, an accessory ribosomal protein is recognized by the receptor kinases EFR, a member of the PRRs, to induce antibacterial immunity [231]. In mice, sensing the 23S rRNA by TRL13 leads to the induction of

cytokines such as interleukin-1β [232]. Interestingly, methylation of an adenosine in the antibiotic-binding site of 23S rRNA camouflages it from TLR13, and bacteria carrying this modification are resistant to antibiotics such as erythromycin [233]. Similar to this observation, lysine88 of RpsL is directly involved in binding to streptomycin, and mutation of this residue to arginine confers resistance to the antibiotic [206]; clearly this mutation also made the protein less recognizable by macrophages. Consistent with this notion, nonpathogenic *E. coli* strains harboring *rpsL*$_{K88R}$ survived significantly better than their wild type counterparts in macrophages [234], likely due to less immune activation by RpsL$_{K88R}$ from mutant bacteria. The K88R mutation in RpsL may represent an evolved mechanism that enables the bacteria to gain resistance to both antibiotics and the innate immunity. Alternatively, antibiotics and host innate immune sensors appear to have converged to recognize the same constrained epitope of the pathogen.

Although our results suggest that RpsL is likely to be recognized by host immune system, how this molecule is sensed is yet to be determined. There are two possible mechanisms of immune recognition of RpsL. First, the distinct architecture of RpsL could be the trigger of immune responses, as it shares limited homology with its eukaryotic counterpart. A similar scenario could be found in recognition of profilin by TLR11/12, which is also distantly related to its mammalian counterpart [111]. The K88R mutation escapes recognition probably due to the altered structure of the mutation, making it a less suitable ligand for the putative receptor. Second, a unique posttranslational modification is responsible for the detection. One possible candidate is methylthiolation, which is present on the Gln89 residue on RpsL. The only known biomolecule harboring this modification in eukaryotic cells is tRNA [235]. Although Gln89 mutation could not be tolerated, mutation of the adjacent amino acid Leu to Phe or Tyr causes the loss of methylthiolation on Gln89, possibly due to steric hindrance. Therefore, it is also possible that K88R mutation, which is also immediately adjacent to

Gln89, prevents proper methylthiolation on Gln89. If this were the case, the bacterium would gain the ability to escape immune recognition and gain resistance to streptomycin without paying the “hefty price tag” of a lethal mutation. In agreement with this hypothesis, mass spectrometry analysis showed that methylthiolation was only detected on $RpsL_{WT}$, but not its K88R counterpart (**Fig. 4-22**). Further, although the significance of methylthiolation on Gln89 is yet to be elucidated, it would be interesting to determine whether the loss of the enzyme responsible for this modification, RimO, would impact the ability of RpsL to induce host responses.

Our hypothesis that RpsL is a universal MAMP seems to be difficult to reconcile with the fact that *L. pneumophila* is the only bacterium known to display such distinct a cell death phenotype. This difference most likely results from the promiscuous nature in substrate recognition of the Dot/Icm system of *L. pneumophila*. This transporter recognizes hundreds of substrates with diverse primary sequences in the region essential for translocation [158, 236]. It is therefore possible that *L. pneumophila* Dot/Icm system may “accidentally” deliver RpsL into the host cytosol to elicit immune responses, although the relative contribution of bacterial lysis cannot be ruled out. Such a phenomenon is not without precedent, as the Dot/Icm system has been implicated in the delivery of bacterial flagellin into the host cell, where it is sensed by the Naip5-NLRC4 inflammasome and thus dictates the susceptibility of mouse line to *L. pneumophila* infection [147]. Flagellin from other bacteria is similarly potent in the activation of the NLRC4 inflammasome but does not exhibit such distinct phenotypes in infections [120]. It is notable that Dot/Icm-dependent flagellin translocation has not yet been experimentally demonstrated with standard reporters. Similarly, we cannot detect Dot/Icm-dependent delivery of RpsL into host cells (**Fig. 4-24**). Thus, although these proteins can be robustly sensed by the immune recognition systems, the amount delivered by the transporter is beyond the sensitivity of current methods for measuring protein translocation.

The lysosome is emerging as a signal integration center for a wide variety of stress stimuli, such as oxidative and osmatic stress, various lipids, engagement of certain cell death receptors, ischemic injury, and lysosomal detergent [78]. Upon the perception of various stresses, the membrane of lysosome permeabilizes (LMP) and releases various hydrolases from the lumen of lysosome into the cytosol. As some of these hydrolases, such as cathepsins B and D, which are catalytically active even at neutral pH, could process various molecules in cytosol, to activate the intrinsic apoptosis pathways in apoptosis-competent cells, or caspase-independent cell death when caspases are inhibited [78]. Although most of the lysosomal contents are leaked into the cytosol in case of extensive LPM, in the scenario of limited LMP, certain cathepsins are selectively released into the cytosol. This notion is supported by the observation that genetic ablation or specific inhibitor(s) of one or more cathepsins can confer significant protection against cell death [78]. For example, cathepsins B is selectively released and plays a dominant role in chemotherapy-triggered NLPR3 inflammasome activation and IL-1β secretion, as well as in TNF-α induced cellular apoptosis in fibrosarcoma cells [237, 238]. In scenarios such as the caspase-8 mediated inflammation resolution in neutrophils, cytosolic cathepsin D plays the dominant role [239]. This is in line with our observation that the host perception of RpsL triggers the onset of LMP, which selectively releases cathepsin B into the cytosol to activate the intrinsic apoptosis pathways. Our observations are akin to those of bacterial flagellin, which also induces LMP upon entry into the host cytosol [216]. Additional examples of LMP as an immune response to MAMP come from the HIV protein Nef, which causes LMP when present in cytosol, and may contribute to the massive $CD4^+$ T cell upon HIV infection [87]. It seems that lysosome is becoming one signal integrated center for host response to MAMPs. The future challenges will be the identification of the receptors for these MAMPs and how the relevant signaling pathways govern the selective release of certain cathepsins.

The important role of cathepsin B in host responses to RpsL is seemingly irreconcilable with the inability of *ctsB*$^{-/-}$ macrophages to support intracellular growth of *L. pneumophila* expressing the wild type RpsL. The apparent complex roles of cathepsin B in lysosomal cell death may provide explanations to this conundrum. For instance, cathepsin B could be the signaling component upstream of LMP [240], the amplifier of signaling cascade [241], a signal relaying molecule [237], or the terminal executioner in the cell death pathway [242]. If cathepsin B functions in one of the branches in the signaling cascade that governs the responses against RpsL, it is possible that genetic ablation of this enzyme could not support the growth of *L. pneumophila* carrying RpsL$_{WT}$ allele. This scenario is akin to the Naip5-NLRC4-caspase-1 axis of inflammasome which responds to flagellin, as delecting caspase-1 leads to only limited replication of *L. pneumophila* that expresses flagellin, while ablating Naip5 or NLRC4 greatly alleviates the growth restriction [147, 243]. It is also possible that the arrest of bacterial replication occurred prior to macrophage death. One focus in future study will be the mechanism underlying these two phenotypes.

RpsL induced cell death only happens in macrophages. Such specificity suggests that RpsL do not directly damage lysosomal membrane but instead that the components that govern the responses to RpsL may not be present or present at a lower abundance in other cell types. Such scenario is not without precedent: for instance, Pyrin, the NLR sensor for pathogen-mediated modifications on small GTPase RhoA, could be detected only in primary macrophages, but not in immortalized macrophages, dendritic cells, and dendritic cell line [132]. It is likely that RpsL engages this putative receptor to activate or to initiate the production of molecules capable of causing LMP. Alternatively, immortalized cell lines may have a stronger pro-survival signaling profile than primary macrophages, which is critical for their survival in culture. These signaling cascades could potentially antagonize the pathways activated by RpsL, making these cells phenotypically irresponsive to RpsL stimulation.

That RpsL-induced macrophage death occurs independent of several inflammatory caspases is consistent with the observation that we did not detect significant induction of inflammatory cytokines in macrophages upon RpsL delivery. The lysosomal cell death process is involved in the activation of the NLRP3 inflammasome induced by cholesterol crystals [244], as well as the induction of the inflammatory, caspase-1/11 independent cell death in response to cytosolic flagellin [216]. Whether RpsL-induced cell death is accompanied by any inflammation awaits further investigation. Clearly, identification of the putative receptor for RpsL will greatly facilitate the research to elucidate the signaling pathway that leads to LMP.

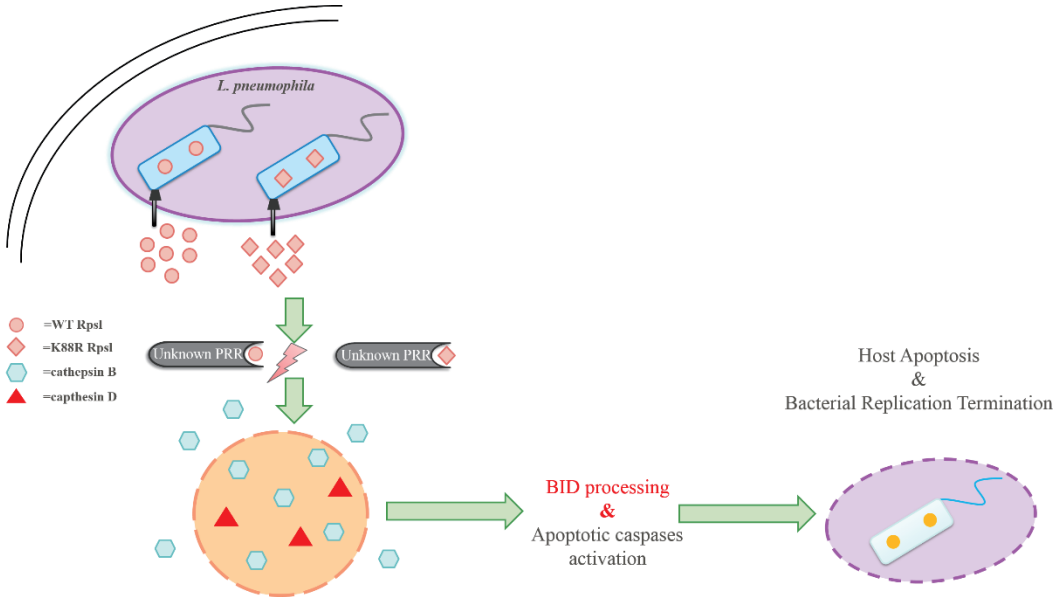


Fig. 4-23 A schematic presentation of the model of host perception of RpsL In this model, bacterial ribosomal protein RpsL is leaked into the cytosol presumbly due to the promiscuous nature of the Dot/Icm secretion system. After accesses to host cytosol, RpsL engages host immune systems and triggers permeabilization of the lysosomal membrane, allowing the release of various hydrolases including cathepsin B into the cytosol. Cathepsin B then processes BID into tBID, allowing it to insert into the outer membrane of mitochondria, inducing the cytochrome c and intiating the classic apoptotic cascade. These events eventually leads to host cell death and termination of bacterial replication.

We favor a model in which RpsL reaches the host cytosol via the Dot/Icm of *L. pneumophila*. Upon accessing the cytosol, RpsL is recognized by a putative receptor, which initiates the signaling cascade that leads to the permeabilization of the lysosomal membrane. Compromised lysosomal membranes allows for the release of lysosomal hydrolases into the cytosol. One of these hydrolases, cathepsin B, activates the cellular apoptosis pathway by processing the BH3-only protein BID. Processed BID (tBID) then inserts into the mitochondria membrane and liberates the cytochrome c into the cytosol, which subsequently activates caspase-3, resulting in host cell death (**Fig. 4-23**).

CHAPTER V: MATERIALS AND METHODS

Bacterial strains, cell culture and media

The *L. pneumophila* strains used are derivatives of the strain Lp02 (thyA Δ(hsdR-lvh) rpsL), and were grown on charcoal-yeast extract (CYE) solid medium or ACES-yeast extract (AYE) [10]; Lp03 contains the dotA mutation [10]. The growth media were supplemented with thymidine at 100 mg/ml when appropriate. For infection experiments, the *L.pneumophila* strains used in all assays were grown to post-exponential phase (OD600<3.4–3.7) unless stated otherwise. Antibiotics were used at the following concentrations with E. coli strains: ampicillin 100 mg/ml and chloramphenicol, 30 mg/ml. For *L. pneumophila* strains, chloramphenicol was used at 5 mg/ml. U937 cells were cultured in RPMI medium supplemented with 10% FBS prior to being induced by phorbol myristate acetate (PMA) (0.1 mg/ml). For assays in 96-well microtiter plates, differentiated U937 cells were plated in 96-well having optically clear bottoms in a density of $1x10^5$/well.

Non-polar deletions of each or the combination of lpg0716, lpg0898, lpg1625, lpg2716, and lpg2831 were constructed as described elsewhere [29]. In each case, primers were designed such that the open reading frame was replaced by a polypeptide consisting of 32 amino acids: the first and last 15 residues of the protein separated by two residues encoded by the restriction enzyme used for plasmid construction. The sequences of the primers used were listed in **Appendix Table 1**.

293T cells and mouse embryonic fibroblasts (MEFs) were cultured in DMEM medium supplemented with 10% FBS; U937 cells were maintained in RPMI-1640 medium supplemented with 10% FBS prior to being induced by

phorbol myristate acetate (PMA) (0.1 μg/ml) [5]. Bone marrow-derived macrophages were prepared according to an established protocol [245]. All cell lines were maintained at 37 °C within a humidified atmosphere and with 5% CO2.

Dendritic cells derived from A/J mouse bone marrow were cultured as previously described [246]. Maturing, nonadherent DCs were plated onto poly-lysine treated coverslips seeded in 24-well plates) at a concentration of $1x10^5$ cells per well. 12 hr later, DCs were challenged with properly grown *L. pneumophila* strains at an MOI of 25. The plates were centrifuged at 150xg for 10 minutes. After two hours, extracellular bacterial growth was stopped by addition of 50 μg/ml gentamicin for 1 hour. DCs were then washed 3 times with PBS to remove residual antibiotic and cultured in fresh medium for the indicated length of time. At indicated time point, DCs were lysed in 0.02% saponin, and dilutions of lysate were plated on CYE agar to determine bacterial count.

For single cell analysis, DCs were infected by indicated strains of *L. pneumophila* for 8 hours. Infected samples were incubated with FITC-conjugated anti-CD11b antibody (Miltenyi Biotec, Auburn, CA) for 10 minutes in 4 °C to identify DCs. Infected samples were then fixed with 4% paraformaldehyde for 20 minutes at room temperature, washed 3 times with RPMI-1640 and permeabilized in RPMI-1640 containing 0.5% saponin for 15 min. *L. pneumophila* were stained as described elsewhere [29]. Apoptotic DCs were identified using In Situ Cell Death Detection Kit (Roche) per manufacturer's instruction.

Allele exchange of the rpsL gene was performed using the standard method [62].

Briefly, the open reading frame of rpsL (lpg0324) together with 300bp flanking sequence was amplified from genomic DNA of wild type or rpsLK88R mutant strain of LPE509. The DNA digested with appropriate restriction enzymes was ligated to the pir protein-dependent plasmid pSR47s [Rankin, 2002 #16], to produce pZLrpsL and pZLrpsLK88R, respectively. The primers used were: 5'-

CTGGAGCTCAAAAGAAAACGTGATGGTAG-3' and 5'CTGGTCGACGTACTTCCACACTTGGGCGA-3'. Plasmids were introduced into appropriate L. pneumophila strains by electroporation, and transformants were streaked onto CYE plate supplemented with 5% sucrose and the desired strains were screened by the gain or loss of resistance to streptomycin followed by DNA sequencing of the locus.

A/J and C56BL/6 mice were purchased from the Jackson laboratory (Bar Harbor, Maine). Caspase 1/11-/- and Caspase 1/7/11-/- [247] and the corresponding C56BL/6 control mice were generously supplied by Dr. R. E. Vance (UC Berkeley). The cathepsin B and cathepsin H knock mice (cstB-/-, cstH-/-) were generous supplied by Dr. Joyce Jones (NYU School of Medicine). Bone marrow-derived macrophages were prepared from 6-10-week old female mice with L-supernatant as described previously [245]. Macrophages were seeded in 24-well plate the day before infection. Cell density of $4x10^5$/well was used for intracellular bacterial growth and LDH release assay, whereas $2x10^5$/well was used for immunofluorescence staining and other single cell-based assays. L. pneumophila used for infection was grown in AYE broth to post exponential phase based on both optical value (OD600=3.4-4.0) and bacterial motility.

Antibody and Western blotting

The antibody against b-lactamase was purchased from Abcam (Cambridge, MA). The antibody specific for the isocitrate dehydrogenase (ICDH) of *L. pneumophila* was described elsewhere [20]. To detect the expression of the fusion proteins in *L. pneumophila*, strains harboring the plasmids were grown in CYE medium supplemented with thymidine (200 mg/ml), chloramphenicol 5 mg/ml and IPTG (0.5 mM) to post exponential phase (OD600 = 3.4–3.7). Cells equal to 1 OD unit were withdrawn and dissolved in 200 ml of SDS loading buffer. After boiling for 5 min, cleared supernatant was resolved by SDS-PAGE. To detect proteins of interest in mammalian cells, cells were lysed with mammalian

cell lysis buffer or RIPA buffer in the presence of protease inhibitor cocktail (CalBiochem). The lysate were then cleared at top speed for 10 mins at 4°C. 100μg total protein was then mixed with SDS loading buffer and boiled for 10 minutes. SDS-PAGE separated proteins were transferred to nitrocellulose membranes and were incubated with appropriately diluted primary antibodies in PBS before incubating with IRDye-conjugated secondary antibodies. The signals were detected by a Li-COR's Odyssey system (LI-COR Biosciences, Lincoln, NE). The antibody for detecting cleaved caspase-3 was from Cell Signaling (#9664) and was used at 1:1000. The SOD2 antibody from Abcam was used at 1:2000. The Cyto c antibody from Santa Cruz Biotechnology (#sc-13560) was used at 1:3000. The VipD antibody [30] was a kind gift from Dr. Ralph Isberg (Tufts Medical School). The antibody against GFP described earlier [38] was used at 1:20,000. The His-tag antibody from Sigma (#H1029) was used at 1:40,000; the SdhA [52] was used as 1:5,000 and the flagellin antibody [148] was used at 1:5,000. Anti-cleaved PARP was purchased from Abcam (ab2317). Anti-mouse-BID was purchased from R&D System (MAB860).

For immunostaining, mouse macrophages were infected with *L. pneumophila* at an MOI of 2. Samples were collected 1 hour after infection for LAMP-1 staining, 2 hours and 6 hours after infection for Rab1 staining. After fixation, samples were first stained for bacteria using anti-*L.pneumophila* antibodies [38] at a dilution of 1:10,000. Extracellular and intracellular bacteria were distinguished using secondary antibodies conjugated to distinct fluorescent dyes. Cyto c oxidase (COX4I1) was detected with an antibody from Cell Signaling at a 1:500 ratio for immunostaining. LAMP-1 was labeled with antibody clone IDB4 (Development Studies Hybridoma Bank, Iowa City, IA) at a dilution of 1:10. Rab1 was labeled with antibody (Santa Cruz Biotechnology, sc-599) at a 1:200 dilution. Processed coverslips were mounted on glass slides with an anti-fade reagent (Vector laboratories, CA). For infection center, mouse macrophages were infected at an MOI of 1; extracellular bacteria were removed by washing the cells with PBS for three times. Samples were collected at 14 hours after infection.

After fixation, extracellular and intracellular *Legionella* were stained with an anti-Legionella antibody followed with secondary antibody conjugated with fluorescein isothiocyanate (FITC) and Texas red, respectively. Samples were examined by visual inspection with an Olympus IX-81 fluorescent microscope. Infection centers were classified into 3 categories as small (1-3 bacteria), medium (4-9 bacteria) and large (≥10 bacteria). Average distribution of the infection centers was determined from triplicate infections of each bacterial strain.

Construction of β-lactamase fusion library

To accommodate the fusion of genes to the b-lactamase gene as BamHI/SalI DNA fragments in the first open reading frame, we inserted a DNA fragment obtained from annealing oligos

5`-CGGATCCCTGCAGGCGGCCGCGTCGACT-3` and

5`-CATGGCCTAGGGACGTCCGCCGGCGCAGCTGAGATC-3` into KpnI and XbaI digested pDXC61 [34] to give pDXC61M. To make the fusion library, open reading frames larger than 300 base pairs that code for hypothetical proteins were retrieved from the genome of L. pneumophila strain Philadelphia 1. 19-base primers were designed to amplify the entire gene by PCR with the Pfu UltraII high fidelity DNA polymerase (Agilent, Santa Clara, CA). In each case, DNA sequences recognized by the restriction enzymes BamHI and SalI were added to the 5` and 3` primers, respectively (Table S1). For genes whose sequences contain the BamHI or SalI recognition site, sequences for BglII or XhoI were added. For a number of genes that harbor one or more of these sites, other restriction enzymes were used (Table S1). After digestion with the appropriate restriction enzymes the PCR products were inserted into similarly digested pDXC61M. Plasmids containing correct inserts were introduced into *L. pneumophila* strains by electroporation. To make β-lactamase fusions with specific regions of genes, the target regions were amplified by PCR with the appropriate primers (Table S6) and were inserted into pDXC61M as described above.

Screen for fusions that transfer the β-lactamase into mammalian cells

To test Dot/Icm-dependent transfer of the fusion proteins into host cells, *L. pneumophila* strains expressed the fusions grown to post exponential phase in the presence of 0.5 mM IPTG were used to infect monolayers of U937 cells seeded in 96-well plates at an MOI of 20. One hour after infection, the CCF4-AM substrates (Invitrogen, Carlsbad, CA) were mixed with medium in the wells. After further incubation for 2 hours at 25°C, infected cells were visually inspected under a Nikon IX-80 fluorescence microscope equipped with a b-lactamase FL-Cube (U-N41031, Chroma Technology Corp, Bellows Falls, VT). Images of infected cells were obtained by a DP-72 color fluorescence camera (Olympus). The percentage of infected cells was determined by counting the number of cells emitting blue fluorescence in specified areas of the wells. Experiments were performed in triplicate and in each sample and at least 300 cells were counted.

Mutagenesis

The serine residue (S73) critical for the putative phospholipase of VipD is mutated to alanine using primers vipDS73AF and vipDS73AR (Table S2) with the Pfu UltraII (Agilent Technologies) DNA polymerase. Mutants of Lpg0898 no longer toxic to yeast were isolated by mutagenizing pGBKT7m-0898 with hydroxylamine using an established protocol [248]. Treated DNA was introduced into yeast strain W303, colonies appeared on selective medium were screened for the presence of full-length Lpg0898 by immunoblot with a Gal4 specific antibody (sc-577, Santa Cruz Biotechnology). All mutations were determined by double strand DNA sequencing.

Library construction and screen for caspase-3 activators

A library for expression of GFP fusions of experimentally confirmed Dot/Icm substrates in mammalian cells was constructed by inserting individual genes [158] into vector pEGFP-C1 (Clontech). These genes were listed in Table S4 of reference [158]. 293T cells were transfected with individual plasmid

harboring the GFP fusion using Lipofactamine-2000 (Invitrogen) per manufacturer's instructions. Twenty-four hours after transfection, cells were lysed and the cellular caspase-3 activity was measured using the Caspase-Glo 3/7 Assay Systems (Promega, Madison, WI) according to protocols supplied by the manufacturer. Alternatively, transfected cells were lysed with the RIPA buffer (1% NP-40, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1 mM EDTA) supplemented with a protease inhibitor cocktail (Millipore, PA) 24 hr after transfection. The presence of activated caspase-3 (p17 and p19 fragments) was detected by immunoblot.

Measurement of mitochondrial transmembrane potential

Mitochondrial transmembrane potential (Δψm) is measured by determining the mitochondrial-to-cytoplasmic fluorescence ratio (Fm/Fc) using tetramethylrhodamine methyl ester perchlorate (TMRM) with established methods [249]. Briefly, Hela-60 cells were exposed to 20 nM of TMRM in DMEM+10% FBS medium for 20 minutes in a CO_2 incubator of 37 °C to allow dye equilibration. The medium is then replaced with 5 nM of TMRM in DMEM+10% FBS medium for imaging. After loading with the mitochondrial dye, coverslips were mounted on a Teflon holder and the images were acquired using a Nikon Eclipse Ti microscope (60x oil Plan Apo objective). TMRM images were thresheld to isolate the mitochondrial florescence (*Fm*), and the florescence intensity of areas of one square micrometer was measured using the software ImageJ (NIH). Cytoplasmic florescence (*Fc*) is determined in hand-drawn regions in close proximity to the mitochondria. $\Delta\psi_m$ is then determined as the ratio of *Fm/Fc* using the following formula: [(Fm-(2/3) Fc)/(1/3) Fc]. At least 300 mitochondria in each sample were analyzed and the statistical analysis was performed using SPSS 20.0 (IBM).

In vivo cytochrome c release assay

293T cells transfected to express individual GFP fusion of testing proteins were harvested and washed twice with PBS. $1X10^7$ Cells were then permeabilized for 5 min on ice with a mitochondrial buffer (20 mM HEPES, pH 7.4, 250 mM sucrose, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 100 µg/µl digitonin) at a concentration of about 3.5 x 10^7 cells/ml [250]. The cytosolic fraction was separated from mitochondrial fraction by centrifugation at 600x*g* for 10 min at 4 °C. 50 µg of soluble cytosolic proteins (supernatant) and mitochondrial proteins (pellet) were then resolved on 15% SDS-PAGE gel. The presence of cytochrome c (Cyto c) in these samples was detected by immunoblot.

In vitro cytochrome c release assay

We isolated mitochondria from HEK293T cells using a Mitochondria Isolation Kit (Sigma) per manufacturer recommendations. 100 µg purified mitochondria were then incubated with VipD or its mutant proteins in a mitochondrial storage buffer (Sigma) at 30 °C for 2 hr. The samples were then cleared at 13,000x*g* for 10 min at 4 °C. The resulting supernatant and pellet were then resolved on 15% SDS-PAGE gels and Cyto c was detected by immunoblot. The matrix protein SOD2 was probed to monitor the integrity of mitochondrial inner membrane.

Lipid extraction

Lipids from 200 µg purified mitochondria that had been incubated with purified His_6-VipD or His_6-$VipD_{S73A}$ were extracted using the Folch method [180]. Briefly, chloroform, methanol, and water with a volume ration of 8:4:3 were mixed in a separation funnel, allowed to stand to obtain a biphasic system. The upper phase was used as the pure-solvent-upper-phase. The mitochondria samples were placed into a glass homogenizer with 5 drops of ice-cold chloroform-methanol extraction solvent (2:1) and manually homogenized with a pestle. The volume of extraction solvent was slowly increased to 0.5-mL in order to achieve

complete homogenization. Homogenates were filtered through a lipid-free paper and mixed with 0.2 volumes of H_2O, and the mix is allowed to separate into 2 phases. After removing the upper phase, the lower phase was rinsed 3 times with the pure-solvent-upper-phase. The lower organic phase was collected, dried under a stream of nitrogen, and stored at -80°C.

Liquid chromatography mass spectrometry

Lipid extracts from samples treated by VipD or its S73A mutant were profiled using the LTQ Orbitrap instrument to determine their lipid contents. The analysis was performed using a Dionex 3000 Ultimate LC system (Dionex, Sunnyvale, CA) interfaced to an externally calibrated LTQ Orbitrap hybrid mass spectrometer with an electrospray ion source (Thermo Scientific, San Jose, CA). A 1-µL aliquot of lipid extract dissolved in 50 µL of methanol was injected onto a Kinetex™ C18 column (100 mm × 2.1 mm, 2.6 µm particle size) equipped with an inline filter from Phenomenex (Phenomenex, Torrance, CA). Mobile phase A was 10 mM ammonium acetate in water:methanol (10%:90%, v:v), and mobile phase B was 10 mM ammonium acetate in isopropanol:methanol (50%:50%, v:v). The column was maintained at 25 ºC and the gradient conditions were a linear ramp of 30% B to 100% B in 15 minutes using a flow rate of 250 µL/min.

The mass spectrometer was operated in an automated data dependent mode alternating between an FT-MS scan in the Orbitrap and a collision-induced dissociation (CID) scan in the linear ion trap. The scan in the Orbitrap was from *m/z* 300 to *m/z* 2000 at 15000 resolving power. The precursor ions were isolated in the linear trap using the data-dependent acquisition mode with a 2 *m/z* isolation width to select automatically and sequentially the three most intense ions from each survey scan. The isolated ions were collisionally activated in the linear trap at 35 % normalized collision energy. The cycle was continuously repeated throughout the entire separation with the dynamic exclusion set to 45 seconds for a repeat count of 2. Performing the initial mass scanning in the

Orbitrap offered high mass accuracy of the selected precursor ions. The analysis was performed twice, once in positive ion mode and again in negative ion mode.

Lipid preparation

Five µl of lysophosphoethanolamine (Avanti Lipids, Alabaster, AL) and 7.5 mg linolenic acid (Sigma, St. Louis, MI) were dissolved in 2-ml of chloroform: methanol 3:1 solvent, which are then dried to a thin film under a gentle nitrogen stream for at least 2 hours to remove residual organic solvent. The resulting thin film of lipids were then dispersed in 5-ml of L buffer (50 mM Tris·Cl 7.5, 150 mM NaCl) at 37 °C using a tip-sonicator with 20% vibration until optical clarity is achieved.

Phospholipase activity assay and thin-layered chromatography

Phosphatidylcholine (PC) and phosphatidylserine (PS) were purchased from NOF America Corp and phosphatidylethanolamine (PE) were obtained from Sigma. Lipids were dissolved in 100% chloroform to a final concentration of 100 mg/ml. To prepare PC or PE only liposomes, 40 µg of each lipid was dried in a Speed-Vac and rehydrated with enzyme buffer (20 mM Tris·Cl pH 7.4, 150 mM NaCl, and 4 mM $CaCl_2$) at RT for 30 min followed by two 30s incubation in bath sonicator (Branson 220). For PC:PS or PE:PS, liposomes were made in a ratio of 3:1 containing a total lipids of 40 µg.

For testing the phospholipase activity of His_6-VipD, each reaction was prepared by mixing PC, PC:PS, PE, or PE:PS liposomes with 20 µg of enzyme and incubated for 3 hr at 30°C. The reaction was quenched with the addition of 4 volumes of chloroform/methanol (2:1 by volume). Total lipids were extracted from the lower organic phase and subjected to Speed-Vac drying. The dried pellet was resuspended in 10 µl of methanol/isopropanol/acetic acid (5/5/2) and spotted onto a TLC Silica gel 60 F_{254} (EMD) that was pretreated by soaking in methanol/water (3:2) containing 1% potassium oxalate and then 1 h drying in a 65°C oven. The TLC plates were developed in a solvent system consisting of 1-

propanol/2 M acetic acid (65%:35%) and the final products were visualized using iodine vapor (Alfa Aesar). Phospholipase A_2 from bovine pancreas (Sigma) was used as a positive control in the experiment.

Elimination of potential co-factors associated with mitochondria by proteinase K

We treated mitochondria (100 μg/sample) purified from HEK293T cells with proteinase K at a concentration of 1μg/ml or buffer at 25°C for 30 minutes. Proteinase K was then removed by washing three times with the storage buffer (Sigma) containing 2 mM PMSF. Mitochondria were then incubated with VipD or its mutant proteins in mitochondrial storage buffer (Sigma) at 30 °C for 2 hr. The release of cytochrome c from mitochondria was detected as described above.

TUNEL staining

$2x10^5$ mouse macrophages were infected with an MOI of 1, and incubated for 14 hours after infection. Samples were fixed and stained with intracellular and extracellular bacteria as described above, and then stained with TUNEL using the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN). Fifty microliter TUNEL reaction mixture was added to each coverslip and incubated at room temperature for 30 min. After three times washes with PBS, coverslips were mounted with anti-fade reagent.

Propidium iodide and Hoechst 33342 staining

$2x10^5$ mouse macrophages were infected with an MOI of 1, and incubated for 2 hr after infection. After centrifugation at 200*g* for 5 min, the culture medium was removed, and 0.5 ml of the dye solution containing 500 nM propidium iodide and 1:10,000 Hoechst was then added into each well. The plate was incubated in the dark for 30 min before image acquisition using an Olympus IX-81 fluorescence microscope.

LDH release assay and glycine protection

LDH release during infection was determined using the CytoTox 96 Assay (Promega, WI). Macrophages were infected at an MOI of 1 on 24-well plates, and incubated in 37°C for 2 or 4 hr. After centrifugation at 200*g* for 5 min, 50-µl supernatant of each well was transferred to a new 96-well enzymatic assay plate. Fifty microliter reconstituted Substrate Mix was then added to each well of the plate, and incubated in dark at room temperature for 30 min. The enzymatic reaction was stopped by adding 50-µl stop solution to each well. The absorbance 490 was measured using a Biotek microplate reader. Total LDH release was determined by complete lysis of the cells using a lysis solution provided by the Kit, and spontaneous LDH release was determined by using the supernatant of cells without infection. For glycine protection, glycine was added to tissue culture medium at a final concentration of 20 mM 1hr prior to infection and was kept in infection samples throughout the entire experimental duration. The percentage of LDH release was calculated with the follow formula: LDH release (%)=(Experimental LDH release-Spontaneous LDH release)/(Total LDH release-Spontaneous LDH release)*100.

Intracellular protein delivery

Recombinant proteins were purified as previously described [Yang, 2013 #2612]. Briefly, recombinant proteins bound to Ni-NTA columns were subjected to extensive organic solvent wash (50x bed volume with wash buffer containing 60% isopropanol) to remove the majority of endotoxin contaminants. If necessary, BMDMs were treated with lysosome acidification inhibitor bafilomycin A1 (20 nM), cathepsin B inhibitor CA-074ME (25 µM), cathepsin D inhibitor pepstatin A (25 µM) or DMSO (vehicle) for 1 hour before protein transfection. The purified proteins were then introduced into macrophages using Lipofectamine 2000 as follows: For transfection of $2x10^5$ macrophages, 12.5-µg recombinant protein suspended in 50-µl RPMI-1640 and 2-µl Lipofectamine 2000 suspended in 50-µl RPMI-1640 were equilibrated at RT for 5 min, then mixed at RT for 30 min. The

mixture was then directly applied to the macrophage cultures.

Acridine orange staining

BMDMs plated on glass coverslips at a density of $2x10^5$/well were either transfected with appropriate proteins or infected with different *L. pneumophila* strains at an MOI of 1. For infection, the samples were washed 3 times with warm PBS to synchronize the infection 2 hrs after adding the bacteria. The transfected/infected samples were incubated for 8 hrs. After centrifugation at 200*g* for 5 min, the culture medium was removed, and 0.5 ml of the dye solution containing acridine orange (5 μg/ml) was added into each well. The plate was incubated at 37°C for 15 min before imaging using a fluorescent microscope (Olympus IX-81).

Cytosolic cathepsin B activity

$4x10^5$ A/J BMDMs were plated in 24-well plates. RpsL proteins were delivered into the cells as described above. 6 hours post protein transfection, cytosolic cathepsin B activity was determined as previously described [Newman, 2009 #2798]. Briefly, macrophages were wash with phenol red and HEPES free DMEM (Invitrogen, OR). 100μg/ml saponin was added to each well and samples are incubated for 10 mins on ice. Cell lystae was cleared at 2,000 rpm for 10 min at 4°C in flat-bottom 96-well plates. 10μl of the cell lysate was then added to 90 μl of the cathepsin B substrate buffer (100 μM zRR-AMC, 50 mM sodium acetate [pH 6.0], 4 mM EDTA, 10 mM dithiothreitol, 1 mM Pefablock). The generation of free AMC was determined by recording fluorescence (excitation, 355 nm; emission, 460 nm) at 30° in a TECAN multiplate reader. Cathepsin B activity was determined by measuring the increase in AMC fluorescence.

Caspase-3 activity assay

$4x10^5$ A/J BMDMs were plated in 24-well plates. RpsL proteins were delivered into the cells as described above. 6 hours post protein transfection,

macrophages were lysed according to manufacturer's instruction (Caspase-3 Fluorometric Assay Kit, R&D). Cleared lysate was then incubated with DEVD-AFC in 37° for 2 hours. And the generation of free AFC from DEVD-AFC cleavage was determined by recording fluorescence (excitation, 355 nm; emission, 460 nm) at 30° in a TECAN multiplate reader. Caspase-3 activity was determined by measuring the increase in AFC fluorescence.

REFERENCES

REFERENCES

1. McDade, J.E., et al., *Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory disease.* N Engl J Med, 1977. **297**(22): p. 1197-203.
2. Stone, B.J., A. Brier, and Y.A. Kwaik, *The Legionella pneumophila prp locus; required during infection of macrophages and amoebae.* Microb Pathog, 1999. **27**(6): p. 369-76.
3. Horwitz, M.A. and S.C. Silverstein, *Legionnaires' disease bacterium (Legionella pneumophila) multiples intracellularly in human monocytes.* J Clin Invest, 1980. **66**(3): p. 441-50.
4. Horwitz, M.A., *The Legionnaires' disease bacterium (Legionella pneumophila) inhibits phagosome-lysosome fusion in human monocytes.* J Exp Med, 1983. **158**(6): p. 2108-26.
5. Tilney, L.G., et al., *How the parasitic bacterium Legionella pneumophila modifies its phagosome and transforms it into rough ER: implications for conversion of plasma membrane to the ER membrane.* J Cell Sci, 2001. **114**(Pt 24): p. 4637-50.
6. Isberg, R.R., T.J. O'Connor, and M. Heidtman, *The Legionella pneumophila replication vacuole: making a cosy niche inside host cells.* Nat Rev Microbiol, 2009. **7**(1): p. 13-24.
7. Marra, A., et al., *Identification of a Legionella pneumophila locus required for intracellular multiplication in human macrophages.* Proc Natl Acad Sci U S A, 1992. **89**(20): p. 9607-11.
8. Mintz, C.S., J.X. Chen, and H.A. Shuman, *Isolation and characterization of auxotrophic mutants of Legionella pneumophila that fail to multiply in human monocytes.* Infect Immun, 1988. **56**(6): p. 1449-55.
9. Horwitz, M.A., *Characterization of avirulent mutant Legionella pneumophila that survive but do not multiply within human monocytes.* J Exp Med, 1987. **166**(5): p. 1310-28.
10. Berger, K.H. and R.R. Isberg, *Two distinct defects in intracellular growth complemented by a single genetic locus in Legionella pneumophila.* Mol Microbiol, 1993. **7**(1): p. 7-19.
11. Vogel, J.P., C. Roy, and R.R. Isberg, *Use of salt to isolate Legionella pneumophila mutants unable to replicate in macrophages.* Ann N Y Acad Sci, 1996. **797**: p. 271-2.
12. Vogel, J.P., et al., *Conjugative transfer by the virulence system of Legionella pneumophila.* Science, 1998. **279**(5352): p. 873-6

13. Segal, G. and H.A. Shuman, *Possible origin of the Legionella pneumophila virulence genes and their relation to Coxiella burnetii.* Mol Microbiol, 1999. **33**(3): p. 669-70.
14. Sampei, G. and K. Mizobuchi, *The organization of the purL gene encoding 5'-phosphoribosylformylglycinamide amidotransferase of Escherichia coli.* J Biol Chem, 1989. **264**(35): p. 21230-8.15. Christie, P.J. and J.P. Vogel, *Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells.* Trends Microbiol, 2000. **8**(8): p. 354-60.
16. Sexton, J.A., et al., *Legionella pneumophila DotU and IcmF are required for stability of the Dot/Icm complex.* Infect Immun, 2004. **72**(10): p. 5983-92.
17. Vincent, C.D., et al., *Identification of the core transmembrane complex of the Legionella Dot/Icm type IV secretion system.* Mol Microbiol, 2006. **62**(5): p. 1278-91.
18. Low, H.H., et al., *Structure of a type IV secretion system.* Nature, 2014. **508**(7497): p. 550-3.
19. Kubori, T., et al., *Native structure of a type IV secretion system core complex essential for Legionella pathogenesis.* Proc Natl Acad Sci U S A, 2014. **111**(32): p. 11804-9.
20. Ninio, S., et al., *The Legionella IcmS-IcmW protein complex is important for Dot/Icm-mediated protein translocation.* Mol Microbiol, 2005. **55**(3): p. 912-26.
21. Sutherland, M.C., et al., *The Legionella IcmSW complex directly interacts with DotL to mediate translocation of adaptor-dependent substrates.* PLoS Pathog, 2012. **8**(9): p. e1002910.
22. Bitar, D.M., M. Molmeret, and Y.A. Kwaik, *Structure-function analysis of the C-terminus of IcmT of Legionella pneumophila in pore formation-mediated egress from macrophages.* FEMS Microbiol Lett, 2005. **242**(1): p. 177-84.
23. Krysan, P.J., J.C. Young, and M.R. Sussman, *T-DNA as an insertional mutagen in Arabidopsis.* Plant Cell, 1999. **11**(12): p. 2283-90.
24. Shrivastava, R. and J.F. Miller, *Virulence factor secretion and translocation by Bordetella species.* Curr Opin Microbiol, 2009. **12**(1): p. 88-93.
25. Vogel, J.P. and R.R. Isberg, *Cell biology of Legionella pneumophila.* Curr Opin Microbiol, 1999. **2**(1): p. 30-4.
26. Sexton, J.A. and J.P. Vogel, *Type IVB secretion by intracellular pathogens.* Traffic, 2002. **3**(3): p. 178-85.
27. Stebbins, C.E. and J.E. Galan, *Structural mimicry in bacterial virulence.* Nature, 2001. **412**(6848): p. 701-5.
28. Nagai, H., et al., *A bacterial guanine nucleotide exchange factor activates ARF on Legionella phagosomes.* Science, 2002. **295**(5555): p. 679-82.
29. Luo, Z.Q. and R.R. Isberg, *Multiple substrates of the Legionella pneumophila Dot/Icm system identified by interbacterial protein transfer.* Proc Natl Acad Sci U S A, 2004. **101**(3): p. 841-6.

30. VanRheenen, S.M., et al., *Members of a Legionella pneumophila family of proteins with ExoU (phospholipase A) active sites are translocated to target cells.* Infect Immun, 2006. **74**(6): p. 3597-606.
31. Huang, L., et al., *The E Block motif is associated with Legionella pneumophila translocated substrates.* Cellular microbiology, 2011. **13**(2): p. 227-45.
32. Chen, J., et al., *Legionella effectors that promote nonlytic release from protozoa.* Science, 2004. **303**(5662): p. 1358-61.
33. Bardill, J.P., J.L. Miller, and J.P. Vogel, *IcmS-dependent translocation of SdeA into macrophages by the Legionella pneumophila type IV secretion system.* Mol Microbiol, 2005. **56**(1): p. 90-103.
34. Charpentier, X., et al., *Chemical genetics reveals bacterial and host cell functions critical for type IV effector translocation by Legionella pneumophila.* PLoS Pathog, 2009. **5**(7): p. e1000501.
35. de Felipe, K.S., et al., *Evidence for acquisition of Legionella type IV secretion substrates via interdomain horizontal gene transfer.* J Bacteriol, 2005. **187**(22): p. 7716-26.
36. Zusman, T., E. Degtyar, and G. Segal, *Identification of a hypervariable region containing new Legionella pneumophila Icm/Dot translocated substrates by using the conserved icmQ regulatory signature.* Infect Immun, 2008. **76**(10): p. 4581-91.
37. Campodonico, E.M., L. Chesnel, and C.R. Roy, *A yeast genetic system for the identification and characterization of substrate proteins transferred into host cells by the Legionella pneumophila Dot/Icm system.* Mol Microbiol, 2005. **56**(4): p. 918-33.
38. Xu, L., et al., *Inhibition of host vacuolar H+-ATPase activity by a Legionella pneumophila effector.* PLoS Pathog, 2010. **6**(3): p. e1000822.
39. Shen, X., et al., *Targeting eEF1A by a Legionella pneumophila effector leads to inhibition of protein synthesis and induction of host stress response.* Cell Microbiol, 2009. **11**(6): p. 911-26.
40. Rasis, M. and G. Segal, *The LetA-RsmYZ-CsrA regulatory cascade, together with RpoS and PmrA, post-transcriptionally regulates stationary phase activation of Legionella pneumophila Icm/Dot effectors.* Mol Microbiol, 2009. **72**(4): p. 995-1010.
41. Altman, E. and G. Segal, *The response regulator CpxR directly regulates expression of several Legionella pneumophila icm/dot components as well as new translocated substrates.* J Bacteriol, 2008. **190**(6): p. 1985-96.
42. Burstein, D., et al., *Genome-scale identification of Legionella pneumophila effectors using a machine learning approach.* PLoS Pathog, 2009. **5**(7): p. e1000508.
43. de Felipe, K.S., et al., *Legionella eukaryotic-like type IV substrates interfere with organelle trafficking.* PLoS Pathog, 2008. **4**(8): p. e1000117.
44. Muller, M.P., et al., *The Legionella effector protein DrrA AMPylates the membrane traffic regulator Rab1b.* Science, 2010. **329**(5994): p. 946-9.

45. Chen, Y., et al., *Structural basis for Rab1 de-AMPylation by the Legionella pneumophila effector SidD.* PLoS Pathog, 2013. **9**(5): p. e1003382.
46. Rigden, D.J., *Identification and modelling of a PPM protein phosphatase fold in the Legionella pneumophila deAMPylase SidD.* FEBS Lett, 2011. **585**(17): p. 2749-54.
47. Ingmundson, A., et al., *Legionella pneumophila proteins that regulate Rab1 membrane cycling.* Nature, 2007. **450**(7168): p. 365-9.
48. Campanacci, V., et al., *Structure of the Legionella effector AnkX reveals the mechanism of phosphocholine transfer by the FIC domain.* EMBO J, 2013. **32**(10): p. 1469-77.
49. Tan, Y., R.J. Arnold, and Z.Q. Luo, *Legionella pneumophila regulates the small GTPase Rab1 activity by reversible phosphorylcholination.* Proc Natl Acad Sci U S A, 2011. **108**(52): p. 21212-7.
50. Ge, J., et al., *A Legionella type IV effector activates the NF-kappaB pathway by phosphorylating the IkappaB family of inhibitors.* Proc Natl Acad Sci U S A, 2009. **106**(33): p. 13725-30.
51. Fontana, M.F., et al., *Secreted Bacterial Effectors That Inhibit Host Protein Synthesis Are Critical for Induction of the Innate Immune Response to Virulent Legionella pneumophila.* PLoS pathogens, 2011. **7**(2): p. e1001289.
52. Creasey, E.A. and R.R. Isberg, *The protein SdhA maintains the integrity of the Legionella-containing vacuole.* Proc Natl Acad Sci U S A, 2012. **109**(9): p. 3481-6.
53. Odorizzi, G., M. Babst, and S.D. Emr, *Phosphoinositide signaling and the regulation of membrane trafficking in yeast.* Trends Biochem Sci, 2000. **25**(5): p. 229-35.
54. Di Paolo, G. and P. De Camilli, *Phosphoinositides in cell regulation and membrane dynamics.* Nature, 2006. **443**(7112): p. 651-7.
55. Konradt, C., et al., *The Shigella flexneri type three secretion system effector IpgD inhibits T cell migration by manipulating host phosphoinositide metabolism.* Cell Host Microbe, 2011. **9**(4): p. 263-72.
56. Bakowski, M.A., V. Braun, and J.H. Brumell, *Salmonella-containing vacuoles: directing traffic and nesting to grow.* Traffic, 2008. **9**(12): p. 2022-31.
57. Patel, J.C., et al., *Diversification of a Salmonella virulence protein function by ubiquitin-dependent differential localization.* Cell, 2009. **137**(2): p. 283-94.
58. Hubber, A. and C.R. Roy, *Modulation of host cell function by Legionella pneumophila type IV effectors.* Annu Rev Cell Dev Biol, 2010. **26**: p. 261-83.
59. Ham, H., A. Sreelatha, and K. Orth, *Manipulation of host membranes by bacterial effectors.* Nat Rev Microbiol, 2011. **9**(9): p. 635-46.
60. Weber, S.S., et al., *Legionella pneumophila exploits PI(4)P to anchor secreted effector proteins to the replicative vacuole.* PLoS Pathog, 2006. **2**(5): p. e46.

61. Isaac, D.T. and R. Isberg, *Master manipulators: an update on Legionella pneumophila Icm/Dot translocated substrates and their host targets.* Future Microbiol, 2014. **9**(3): p. 343-59.
62. Tan, Y. and Z.Q. Luo, *Legionella pneumophila SidD is a deAMPylase that modifies Rab1.* Nature, 2011. **475**(7357): p. 506-9.
63. Wenhan Zhu, L.H., FoSheng Hsu, Yuxin Mao and Zhao-Qing Luo, *Induction of host cytochrome c release and caspase-3 activation by a bacterial phospholipase* 2013.
64. Laguna, R.K., et al., *A Legionella pneumophila-translocated substrate that is required for growth within macrophages and protection from host cell death.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(49): p. 18745-50.
65. Losick, V.P. and R.R. Isberg, *NF-kappaB translocation prevents host cell death after low-dose challenge by Legionella pneumophila.* J Exp Med, 2006. **203**(9): p. 2177-89.
66. O'Connor, T.J., et al., *Aggravating genetic interactions allow a solution to redundancy in a bacterial pathogen.* Science, 2012. **338**(6113): p. 1440-4.
67. O'Connor, T.J. and R.R. Isberg, *iMAD, a genetic screening strategy for dissecting complex interactions between a pathogen and its host.* Nat Protoc, 2014. **9**(8): p. 1916-30.
68. Labbe, K. and M. Saleh, *Cell death in the host response to infection.* Cell Death Differ, 2008. **15**(9): p. 1339-49.
69. Aachoui, Y., et al., *Inflammasome-mediated pyroptotic and apoptotic cell death, and defense against infection.* Curr Opin Microbiol, 2013. **16**(3): p. 319-26.
70. Doitsh, G., et al., *Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection.* Nature, 2014. **505**(7484): p. 509-14.
71. Fink, S.L. and B.T. Cookson, *Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells.* Infect Immun, 2005. **73**(4): p. 1907-16.
72. Lamkanfi, M. and V.M. Dixit, *Manipulation of host cell death pathways during microbial infections.* Cell host & microbe, 2010. **8**(1): p. 44-54.
73. Bergsbaken, T., S.L. Fink, and B.T. Cookson, *Pyroptosis: host cell death and inflammation.* Nat Rev Microbiol, 2009. **7**(2): p. 99-109.
74. Cunha, L.D. and D.S. Zamboni, *Subversion of inflammasome activation and pyroptosis by pathogenic bacteria.* Front Cell Infect Microbiol, 2013. **3**: p. 76.
75. Lage, S.L., G.P. Amarante-Mendes, and K.R. Bortoluci, *Evaluation of pyroptosis in macrophages using cytosolic delivery of purified flagellin.* Methods, 2013. **61**(2): p. 110-6.
76. Linkermann, A. and D.R. Green, *Necroptosis.* N Engl J Med, 2014. **370**(5): p. 455-65.

77. Linkermann, A., et al., *Two independent pathways of regulated necrosis mediate ischemia-reperfusion injury.* Proc Natl Acad Sci U S A, 2013. **110**(29): p. 12024-9.
78. Aits, S. and M. Jaattela, *Lysosomal cell death at a glance.* J Cell Sci, 2013. **126**(Pt 9): p. 1905-12.
79. Jaattela, M., *Multiple cell death pathways as regulators of tumour initiation and progression.* Oncogene, 2004. **23**(16): p. 2746-56.
80. Turk, B. and V. Turk, *Lysosomes as "suicide bags" in cell death: myth or reality?* J Biol Chem, 2009. **284**(33): p. 21783-7.
81. Repnik, U., et al., *Lysosomes and lysosomal cathepsins in cell death.* Biochim Biophys Acta, 2012. **1824**(1): p. 22-33.
82. Guicciardi, M.E., M. Leist, and G.J. Gores, *Lysosomes in cell death.* Oncogene, 2004. **23**(16): p. 2881-90.
83. Zhang, H., et al., *Granulysin induces cathepsin B release from lysosomes of target tumor cells to attack mitochondria through processing of bid leading to Necroptosis.* J Immunol, 2009. **182**(11): p. 6993-7000.
84. Bidere, N., et al., *Cathepsin D triggers Bax activation, resulting in selective apoptosis-inducing factor (AIF) relocation in T lymphocytes entering the early commitment phase to apoptosis.* J Biol Chem, 2003. **278**(33): p. 31401-11.
85. Blomgran, R., L. Zheng, and O. Stendahl, *Cathepsin-cleaved Bid promotes apoptosis in human neutrophils via oxidative stress-induced lysosomal membrane permeabilization.* J Leukoc Biol, 2007. **81**(5): p. 1213-23.
86. Cirman, T., et al., *Selective disruption of lysosomes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins.* J Biol Chem, 2004. **279**(5): p. 3578-87.
87. Laforge, M., et al., *DRAM triggers lysosomal membrane permeabilization and cell death in CD4(+) T cells infected with HIV.* PLoS Pathog, 2013. **9**(5): p. e1003328.
88. Lee, J., et al., *Macrophage apoptosis in response to high intracellular burden of Mycobacterium tuberculosis is mediated by a novel caspase-independent pathway.* J Immunol, 2006. **176**(7): p. 4267-74.
89. Luhrmann, A., et al., *Inhibition of pathogen-induced apoptosis by a Coxiella burnetii type IV effector protein.* Proc Natl Acad Sci U S A, 2010. **107**(44): p. 18997-9001.
90. Niu, H., et al., *Anaplasma phagocytophilum Ats-1 is imported into host cell mitochondria and interferes with apoptosis induction.* PLoS Pathog, 2010. **6**(2): p. e1000774.
91. Velmurugan, K., et al., *Mycobacterium tuberculosis nuoG is a virulence gene that inhibits apoptosis of infected host cells.* PLoS Pathog, 2007. **3**(7): p. e110.
92. LaRock, C.N. and B.T. Cookson, *The Yersinia virulence effector YopM binds caspase-1 to arrest inflammasome assembly and processing.* Cell Host Microbe, 2012. **12**(6): p. 799-805.

93. Dewoody, R., et al., *YopK regulates the Yersinia pestis type III secretion system from within host cells.* Mol Microbiol, 2011. **79**(6): p. 1445-61.
94. Sutterwala, F.S., et al., *Immune recognition of Pseudomonas aeruginosa mediated by the IPAF/NLRC4 inflammasome.* J Exp Med, 2007. **204**(13): p. 3235-45.
95. Littmann, M., et al., *Streptococcus pneumoniae evades human dendritic cell surveillance by pneumolysin expression.* EMBO Mol Med, 2009. **1**(4): p. 211-22.
96. Abu-Zant, A., et al., *Anti-apoptotic signalling by the Dot/Icm secretion system of L. pneumophila.* Cellular microbiology, 2007. **9**(1): p. 246-64.
97. Banga, S., et al., *Legionella pneumophila inhibits macrophage apoptosis by targeting pro-death members of the Bcl2 protein family.* Proc Natl Acad Sci U S A, 2007. **104**(12): p. 5121-6.
98. Losick, V.P., et al., *LnaB: a Legionella pneumophila activator of NF-kappaB.* Cellular microbiology, 2010. **12**(8): p. 1083-97.
99. Asrat, S., A.S. Dugan, and R.R. Isberg, *The Frustrated Host Response to Legionella pneumophila Is Bypassed by MyD88-Dependent Translation of Pro-inflammatory Cytokines.* PLoS Pathog, 2014. **10**(7): p. e1004229.
100. Gao, L.Y. and Y. Abu Kwaik, *Activation of caspase 3 during Legionella pneumophila-induced apoptosis.* Infect Immun, 1999. **67**(9): p. 4886-94.
101. Abu-Zant, A., et al., *Incomplete activation of macrophage apoptosis during intracellular replication of Legionella pneumophila.* Infect Immun, 2005. **73**(9): p. 5339-49.
102. Nogueira, C.V., et al., *Rapid pathogen-induced apoptosis: a mechanism used by dendritic cells to limit intracellular replication of Legionella pneumophila.* PLoS Pathog, 2009. **5**(6): p. e1000478.
103. Gao, L.Y. and Y. Abu Kwaik, *Activation of caspase 3 during Legionella pneumophila-induced apoptosis.* Infection and immunity, 1999. **67**(9): p. 4886-94.
104. Nogueira, C.V., et al., *Rapid pathogen-induced apoptosis: a mechanism used by dendritic cells to limit intracellular replication of Legionella pneumophila.* PLoS pathogens, 2009. **5**(6): p. e1000478.
105. Banerji, S. and A. Flieger, *Patatin-like proteins: a new family of lipolytic enzymes present in bacteria?* Microbiology, 2004. **150**(Pt 3): p. 522-5.
106. Sitkiewicz, I., K.E. Stockbauer, and J.M. Musser, *Secreted bacterial phospholipase A2 enzymes: better living through phospholipolysis.* Trends Microbiol, 2007. **15**(2): p. 63-9.
107. Banerji, S., P. Aurass, and A. Flieger, *The manifold phospholipases A of Legionella pneumophila - identification, export, regulation, and their link to bacterial virulence.* Int J Med Microbiol, 2008. **298**(3-4): p. 169-81.
108. Shohdy, N., et al., *Pathogen effector protein screening in yeast identifies Legionella factors that interfere with membrane trafficking.* Proc Natl Acad Sci U S A, 2005. **102**(13): p. 4866-71.
109. Abt, M.C., et al., *Commensal bacteria calibrate the activation threshold of innate antiviral immunity.* Immunity, 2012. **37**(1): p. 158-70.

110. Lee, M.S. and Y.J. Kim, *Pattern-recognition receptor signaling initiated from extracellular, membrane, and cytoplasmic space.* Mol Cells, 2007. **23**(1): p. 1-10.
111. Broz, P. and D.M. Monack, *Newly described pattern recognition receptors team up against intracellular pathogens.* Nat Rev Immunol, 2013. **13**(8): p. 551-65.
112. Brown, G.D., *Dectin-1: a signalling non-TLR pattern-recognition receptor.* Nat Rev Immunol, 2006. **6**(1): p. 33-43.
113. Kagan, J.C. and R. Medzhitov, *Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling.* Cell, 2006. **125**(5): p. 943-55.
114. Kagan, J.C., et al., *TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta.* Nat Immunol, 2008. **9**(4): p. 361-8.
115. Strober, W., et al., *Signalling pathways and molecular interactions of NOD1 and NOD2.* Nat Rev Immunol, 2006. **6**(1): p. 9-20.
116. Lamkanfi, M. and V.M. Dixit, *Mechanisms and functions of inflammasomes.* Cell, 2014. **157**(5): p. 1013-22.
117. Cirelli, K.M., et al., *Inflammasome sensor NLRP1 controls rat macrophage susceptibility to Toxoplasma gondii.* PLoS Pathog, 2014. **10**(3): p. e1003927.
118. Rathinam, V.A., et al., *The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses.* Nat Immunol, 2010. **11**(5): p. 395-402.
119. Ge, J., et al., *Preventing bacterial DNA release and absent in melanoma 2 inflammasome activation by a Legionella effector functioning in membrane trafficking.* Proc Natl Acad Sci U S A, 2012. **109**(16): p. 6193-8.
120. Zhao, Y., et al., *The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus.* Nature, 2011. **477**(7366): p. 596-600.
121. Cai, X., et al., *Prion-like polymerization underlies signal transduction in antiviral immune defense and inflammasome activation.* Cell, 2014. **156**(6): p. 1207-22.
122. Sun, L., et al., *Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway.* Science, 2013. **339**(6121): p. 786-91.
123. Wu, J., et al., *Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA.* Science, 2013. **339**(6121): p. 826-30.
124. Monroe, K.M., S.M. McWhirter, and R.E. Vance, *Identification of host cytosolic sensors and bacterial factors regulating the type I interferon response to Legionella pneumophila.* PLoS Pathog, 2009. **5**(11): p. e1000665.
125. Lippmann, J., et al., *Dissection of a type I interferon pathway in controlling bacterial intracellular infection in mice.* Cell Microbiol, 2011. **13**(11): p. 1668-82.

126. Stuart, L.M., N. Paquette, and L. Boyer, *Effector-triggered versus pattern-triggered immunity: how animals sense pathogens.* Nat Rev Immunol, 2013. **13**(3): p. 199-206.
127. Chisholm, S.T., et al., *Host-microbe interactions: shaping the evolution of the plant immune response.* Cell, 2006. **124**(4): p. 803-14.
128. Jones, J.D. and J.L. Dangl, *The plant immune system.* Nature, 2006. **444**(7117): p. 323-9.
129. Boyer, L., et al., *Pathogen-derived effectors trigger protective immunity via activation of the Rac2 enzyme and the IMD or Rip kinase signaling pathway.* Immunity, 2011. **35**(4): p. 536-49.
130. Gurcel, L., et al., *Caspase-1 activation of lipid metabolic pathways in response to bacterial pore-forming toxins promotes cell survival.* Cell, 2006. **126**(6): p. 1135-45.
131. Wang, X., et al., *The GAP Activity of Type III Effector YopE Triggers Killing of Yersinia in Macrophages.* PLoS Pathog, 2014. **10**(8): p. e1004346.
132. Xu, H., et al., *Innate immune sensing of bacterial modifications of Rho GTPases by the Pyrin inflammasome.* Nature, 2014.
133. Abu-Zant, A., et al., *Anti-apoptotic signalling by the Dot/Icm secretion system of L. pneumophila.* Cell Microbiol, 2007. **9**(1): p. 246-64.
134. Shin, S., et al., *Type IV secretion-dependent activation of host MAP kinases induces an increased proinflammatory cytokine response to Legionella pneumophila.* PLoS Pathog, 2008. **4**(11): p. e1000220.
135. Fontana, M.F., et al., *Secreted bacterial effectors that inhibit host protein synthesis are critical for induction of the innate immune response to virulent Legionella pneumophila.* PLoS Pathog, 2011. **7**(2): p. e1001289.
136. Fontana, M.F., S. Shin, and R.E. Vance, *Activation of host mitogen-activated protein kinases by secreted Legionella pneumophila effectors that inhibit host protein translation.* Infect Immun, 2012. **80**(10): p. 3570-5.
137. Yoshida, S., et al., *Association of Lps gene with natural resistance of mouse macrophages against Legionella pneumophila.* FEMS Microbiol Immunol, 1991. **4**(1): p. 51-6.
138. Hawn, T.R., et al., *Toll-like receptor 4 polymorphisms are associated with resistance to Legionnaires' disease.* Proc Natl Acad Sci U S A, 2005. **102**(7): p. 2487-9.
139. Hawn, T.R., et al., *Altered inflammatory responses in TLR5-deficient mice infected with Legionella pneumophila.* J Immunol, 2007. **179**(10): p. 6981-7.
140. Hawn, T.R., et al., *A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to legionnaires' disease.* J Exp Med, 2003. **198**(10): p. 1563-72.
141. Archer, K.A. and C.R. Roy, *MyD88-dependent responses involving toll-like receptor 2 are important for protection and clearance of Legionella pneumophila in a mouse model of Legionnaires' disease.* Infect Immun, 2006. **74**(6): p. 3325-33.

142. Berrington, W.R., et al., *NOD1 and NOD2 regulation of pulmonary innate immunity to Legionella pneumophila.* Eur J Immunol, 2010. **40**(12): p. 3519-27.
143. Yamamoto, Y., et al., *Growth of Legionella pneumophila in thioglycolate-elicited peritoneal macrophages from A/J mice.* Infect Immun, 1988. **56**(2): p. 370-5.
144. Yamamoto, Y., et al., *Interaction of Legionella pneumophila with peritoneal macrophages from various mouse strains.* Adv Exp Med Biol, 1988. **239**: p. 89-98.
145. Luo, Z.Q., *Legionella secreted effectors and innate immune responses.* Cell Microbiol, 2012. **14**(1): p. 19-27.
146. Diez, E., et al., *Birc1e is the gene within the Lgn1 locus associated with resistance to Legionella pneumophila.* Nat Genet, 2003. **33**(1): p. 55-60.
147. Ren, T., et al., *Flagellin-deficient Legionella mutants evade caspase-1- and Naip5-mediated macrophage immunity.* PLoS Pathog, 2006. **2**(3): p. e18.
148. Molofsky, A.B., et al., *Cytosolic recognition of flagellin by mouse macrophages restricts Legionella pneumophila infection.* J Exp Med, 2006. **203**(4): p. 1093-104.
149. Lightfield, K.L., et al., *Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin.* Nature immunology, 2008. **9**(10): p. 1171-8.
150. Lightfield, K.L., et al., *Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin.* Nat Immunol, 2008. **9**(10): p. 1171-8.
151. von Moltke, J., et al., *Recognition of bacteria by inflammasomes.* Annu Rev Immunol, 2013. **31**: p. 73-106.
152. Rao, C., H. Benhabib, and A.W. Ensminger, *Phylogenetic reconstruction of the Legionella pneumophila Philadelphia-1 laboratory strains through comparative genomics.* PLoS One, 2013. **8**(5): p. e64129.
153. Molmeret, M., et al., *Different growth rates in amoeba of genotypically related environmental and clinical Legionella pneumophila strains isolated from a thermal spa.* Epidemiol Infect, 2001. **126**(2): p. 231-9.
154. Qin, T., et al., *High prevalence, genetic diversity and intracellular growth ability of legionella in hot spring environments.* PLoS One, 2013. **8**(3): p. e59018.
155. Izu, K., et al., *Grouping of 20 reference strains of Legionella species by the growth ability within mouse and guinea pig macrophages.* FEMS Immunol Med Microbiol, 1999. **26**(1): p. 61-8.
156. Messi, P., et al., *Protozoa and human macrophages infection by Legionella pneumophila environmental strains belonging to different serogroups.* Arch Microbiol, 2013. **195**(2): p. 89-96.
157. Tao, L., et al., *Induction of rapid cell death by an environmental isolate of Legionella pneumophila in mouse macrophages.* Infect Immun, 2013. **81**(9): p. 3077-88.

158. Zhu, W., et al., *Comprehensive identification of protein substrates of the Dot/Icm type IV transporter of Legionella pneumophila.* PLoS One, 2011. **6**(3): p. e17638.
159. Ensminger, A.W., et al., *Experimental evolution of Legionella pneumophila in mouse macrophages leads to strains with altered determinants of environmental survival.* PLoS Pathog, 2012. **8**(5): p. e1002731.
160. Neunuebel, M.R. and M.P. Machner, *The taming of a Rab GTPase by Legionella pneumophila.* Small GTPases, 2012. **3**(1): p. 28-33.
161. Cazalet, C., et al., *Evidence in the Legionella pneumophila genome for exploitation of host cell functions and high genome plasticity.* Nat Genet, 2004. **36**(11): p. 1165-73.
162. Ninio, S., J. Celli, and C.R. Roy, *A Legionella pneumophila effector protein encoded in a region of genomic plasticity binds to Dot/Icm-modified vacuoles.* PLoS Pathog, 2009. **5**(1): p. e1000278.
163. Nagai, H., et al., *A C-terminal translocation signal required for Dot/Icm-dependent delivery of the Legionella RalF protein to host cells.* Proc Natl Acad Sci U S A, 2005. **102**(3): p. 826-31.
164. Huang, L., et al., *The E Block motif is associated with Legionella pneumophila translocated substrates.* Cell Microbiol, 2011. **13**(2): p. 227-45.
165. Fortier, A., E. Diez, and P. Gros, *Naip5/Birc1e and susceptibility to Legionella pneumophila.* Trends Microbiol, 2005. **13**(7): p. 328-35.
166. Schroeder, G.N., et al., *Legionella pneumophila strain 130b possesses a unique combination of type IV secretion systems and novel Dot/Icm secretion system effector proteins.* J Bacteriol, 2010. **192**(22): p. 6001-16.
167. Chen, C., et al., *Large-scale identification and translocation of type IV secretion substrates by Coxiella burnetii.* Proc Natl Acad Sci U S A, 2010. **107**(50): p. 21755-60.
168. He, J., et al., *The broad host range pathogen Pseudomonas aeruginosa strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes.* Proc Natl Acad Sci U S A, 2004. **101**(8): p. 2530-5.
169. Ashida, H., et al., *Cell death and infection: a double-edged sword for host and pathogen survival.* J Cell Biol, 2011. **195**(6): p. 931-42.
170. Niu, H., et al., *Anaplasma phagocytophilum Ats-1 is imported into host cell mitochondria and interferes with apoptosis induction.* PLoS pathogens, 2010. **6**(2): p. e1000774.
171. Zheng, D.Q., et al., *Comparative functional genomics to reveal the molecular basis of phenotypic diversities and guide the genetic breeding of industrial yeast strains.* Appl Microbiol Biotechnol, 2013. **97**(5): p. 2067-76.
172. Degtyar, E., et al., *A Legionella effector acquired from protozoa is involved in sphingolipids metabolism and is targeted to the host cell mitochondria.* Cell Microbiol, 2009. **11**(8): p. 1219-35.

173. Chipuk, J.E., et al., *Sphingolipid metabolism cooperates with BAK and BAX to promote the mitochondrial pathway of apoptosis.* Cell, 2012. **148**(5): p. 988-1000.
174. Figueira, T.R., et al., *Safranine as a fluorescent probe for the evaluation of mitochondrial membrane potential in isolated organelles and permeabilized cells.* Methods Mol Biol, 2012. **810**: p. 103-17.
175. Jiang, X. and X. Wang, *Cytochrome C-mediated apoptosis.* Annu Rev Biochem, 2004. **73**: p. 87-106.
176. Deutsch, D.G., et al., *Methyl arachidonyl fluorophosphonate: a potent irreversible inhibitor of anandamide amidase.* Biochem Pharmacol, 1997. **53**(3): p. 255-60.
177. Balsinde, J., et al., *Regulation and inhibition of phospholipase A2.* Annu Rev Pharmacol Toxicol, 1999. **39**: p. 175-89.
178. Rehfeldt, W., K. Resch, and M. Goppelt-Struebe, *Cytosolic phospholipase A2 from human monocytic cells: characterization of substrate specificity and Ca(2+)-dependent membrane association.* Biochem J, 1993. **293 (Pt 1)**: p. 255-61.
179. Putz, T., et al., *Bee venom secretory phospholipase A2 and phosphatidylinositol-homologues cooperatively disrupt membrane integrity, abrogate signal transduction and inhibit proliferation of renal cancer cells.* Cancer Immunol Immunother, 2007. **56**(5): p. 627-40.
180. Folch, J., M. Lees, and G.H. Sloane Stanley, *A simple method for the isolation and purification of total lipides from animal tissues.* J Biol Chem, 1957. **226**(1): p. 497-509.
181. Ku, B., et al., *VipD of Legionella pneumophila Targets Activated Rab5 and Rab22 to Interfere with Endosomal Trafficking in Macrophages.* PLoS Pathog, 2012. **8**(12): p. e1003082.
182. Barr, F.A. and J. Shorter, *Membrane traffic: do cones mark sites of fission?* Curr Biol, 2000. **10**(4): p. R141-4.
183. Caccin, P., O. Rossetto, and C. Montecucco, *Neurotoxicity of inverted-cone shaped lipids.* Neurotoxicology, 2009. **30**(2): p. 174-81.
184. Grandbois, M., H. Clausen-Schaumann, and H. Gaub, *Atomic force microscope imaging of phospholipid bilayer degradation by phospholipase A2.* Biophys J, 1998. **74**(5): p. 2398-404.
185. Rigoni, M., et al., *Equivalent effects of snake PLA2 neurotoxins and lysophospholipid-fatty acid mixtures.* Science, 2005. **310**(5754): p. 1678-80.
186. Molmeret, M., et al., *Activation of caspase-3 by the Dot/Icm virulence system is essential for arrested biogenesis of the Legionella-containing phagosome.* Cell Microbiol, 2004. **6**(1): p. 33-48.
187. Gaspar, A.H. and M.P. Machner, *VipD is a Rab5-activated phospholipase A1 that protects Legionella pneumophila from endosomal fusion.* Proc Natl Acad Sci U S A, 2014. **111**(12): p. 4560-5.
188. Thomma, B.P., T. Nurnberger, and M.H. Joosten, *Of PAMPs and effectors: the blurred PTI-ETI dichotomy.* Plant Cell, 2011. **23**(1): p. 4-15.

189. Zakeri, Z. and R.A. Lockshin, *Cell death: history and future.* Adv Exp Med Biol, 2008. **615**: p. 1-11.
190. Srikanth, C.V., et al., *Salmonella pathogenesis and processing of secreted effectors by caspase-3.* Science, 2010. **330**(6002): p. 390-3.
191. Lamkanfi, M. and V.M. Dixit, *Manipulation of host cell death pathways during microbial infections.* Cell Host Microbe, 2010. **8**(1): p. 44-54.
192. Cheng, S.C., et al., *mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity.* Science, 2014. **345**(6204): p. 1250684.
193. Saeed, S., et al., *Epigenetic programming of monocyte-to-macrophage differentiation and trained innate immunity.* Science, 2014. **345**(6204): p. 1251086.
194. Albert, M.L., *Death-defying immunity: do apoptotic cells influence antigen processing and presentation?* Nat Rev Immunol, 2004. **4**(3): p. 223-31.
195. McMichael, A.J., et al., *The immune response during acute HIV-1 infection: clues for vaccine development.* Nat Rev Immunol, 2010. **10**(1): p. 11-23.
196. Tao, L.L., et al., *Genetic diversity of Legionella pneumophila serogroup 1 in hospital water distribution systems in Shanghai.* Zhonghua Jie He He Hu Xi Za Zhi, 2011. **34**(2): p. 100-3.
197. Sadosky, A.B., L.A. Wiater, and H.A. Shuman, *Identification of Legionella pneumophila genes required for growth within and killing of human macrophages.* Infect Immun, 1993. **61**(12): p. 5361-73.
198. Hardiman, C.A., et al., *The role of Rab GTPases in the transport of vacuoles containing Legionella pneumophila and Coxiella burnetii.* Biochem Soc Trans, 2013. **40**(6): p. 1353-9.
199. Kofoed, E.M. and R.E. Vance, *Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity.* Nature, 2011. **477**(7366): p. 592-5.
200. Case, C.L., et al., *Caspase-11 stimulates rapid flagellin-independent pyroptosis in response to Legionella pneumophila.* Proc Natl Acad Sci U S A, 2013. **110**(5): p. 1851-6.
201. Aachoui, Y., et al., *Caspase-11 protects against bacteria that escape the vacuole.* Science, 2013. **339**(6122): p. 975-8.
202. Segal, G., J.J. Russo, and H.A. Shuman, *Relationships between a new type IV secretion system and the icm/dot virulence system of Legionella pneumophila.* Mol Microbiol, 1999. **34**(4): p. 799-809.
203. O'Connor, T.J., et al., *Minimization of the Legionella pneumophila genome reveals chromosomal regions involved in host range expansion.* Proc Natl Acad Sci U S A, 2011. **108**(36): p. 14733-40.
204. Brennan, M.A. and B.T. Cookson, *Salmonella induces macrophage death by caspase-1-dependent necrosis.* Mol Microbiol, 2000. **38**(1): p. 31-40.
205. Frank, A., U. Rauen, and H. de Groot, *Protection by glycine against hypoxic injury of rat hepatocytes: inhibition of ion fluxes through nonspecific leaks.* J Hepatol, 2000. **32**(1): p. 58-66.

206. Springer, B., et al., *Mechanisms of streptomycin resistance: selection of mutations in the 16S rRNA gene conferring resistance.* Antimicrob Agents Chemother, 2001. **45**(10): p. 2877-84.
207. Van den Eede, G., et al., *Broad host range and promoter selection vectors for bacteria that interact with plants.* Mol Plant Microbe Interact, 1992. **5**(3): p. 228-34.
208. Pelchovich, G., et al., *The contribution of common rpsL mutations in Escherichia coli to sensitivity to ribosome targeting antibiotics.* Int J Med Microbiol, 2013. **303**(8): p. 558-62.
209. Khan, M.A., et al., *Comparative Genomics Reveal That Host-Innate Immune Responses Influence the Clinical Prevalence of Serogroups.* PLoS One, 2013. **8**(6): p. e67298.
210. Chien, M., et al., *The genomic sequence of the accidental pathogen Legionella pneumophila.* Science, 2004. **305**(5692): p. 1966-8.
211. Akhter, A., et al., *Caspase-7 activation by the Nlrc4/Ipaf inflammasome restricts Legionella pneumophila infection.* PLoS Pathog, 2009. **5**(4): p. e1000361.
212. Sreevatsan, S., et al., *Characterization of rpsL and rrs mutations in streptomycin-resistant Mycobacterium tuberculosis isolates from diverse geographic localities.* Antimicrob Agents Chemother, 1996. **40**(4): p. 1024-6.
213. Kurland, C.G., *Translational accuracy and the fitness of bacteria.* Annu Rev Genet, 1992. **26**: p. 29-50.
214. Franchi, L., et al., *Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages.* Nat Immunol, 2006. **7**(6): p. 576-82.
215. Franken, K.L., et al., *Purification of his-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent.* Protein Expr Purif, 2000. **18**(1): p. 95-9.
216. Lage, S.L., et al., *Cytosolic flagellin-induced lysosomal pathway regulates inflammasome-dependent and -independent macrophage responses.* Proc Natl Acad Sci U S A, 2013. **110**(35): p. E3321-30.
217. Repnik, U., M. Hafner Cesen, and B. Turk, *Lysosomal membrane permeabilization in cell death: Concepts and challenges.* Mitochondrion, 2014.
218. Yoshimori, T., et al., *Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells.* J Biol Chem, 1991. **266**(26): p. 17707-12.
219. Lipton, P., *Lysosomal membrane permeabilization as a key player in brain ischemic cell death: a "lysosomocentric" hypothesis for ischemic brain damage.* Transl Stroke Res, 2013. **4**(6): p. 672-84.
220. Yeung, B.H., D.C. Huang, and F.A. Sinicrope, *PS-341 (bortezomib) induces lysosomal cathepsin B release and a caspase-2-dependent mitochondrial permeabilization and apoptosis in human pancreatic cancer cells.* J Biol Chem, 2006. **281**(17): p. 11923-32.

221. Derre, I. and R.R. Isberg, *Legionella pneumophila replication vacuole formation involves rapid recruitment of proteins of the early secretory system.* Infect Immun, 2004. **72**(5): p. 3048-53.
222. Sturgill-Koszycki, S. and M.S. Swanson, *Legionella pneumophila replication vacuoles mature into acidic, endocytic organelles.* J Exp Med, 2000. **192**(9): p. 1261-72.
223. Ma, J., et al., *Genome Sequence of an Environmental Isolate of the Bacterial Pathogen Legionella pneumophila.* Genome Announc, 2013. **1**(3).
224. Fortier, A., E. Diez, and P. Gros, *Naip5/Birc1e and susceptibility to Legionella pneumophila.* Trends in microbiology, 2005. **13**(7): p. 328-35.
225. Meunier, E., et al., *Caspase-11 activation requires lysis of pathogen-containing vacuoles by IFN-induced GTPases.* Nature, 2014. **509**(7500): p. 366-70.
226. Kayagaki, N., et al., *Non-canonical inflammasome activation targets caspase-11.* Nature, 2011. **479**(7371): p. 117-21.
227. Shi, J., et al., *Inflammatory caspases are innate immune receptors for intracellular LPS.* Nature, 2014.
228. Wang, S., et al., *Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE.* Cell, 1998. **92**(4): p. 501-9.
229. Degterev, A., et al., *Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury.* Nat Chem Biol, 2005. **1**(2): p. 112-9.
230. Bottger, E.C., et al., *Structural basis for selectivity and toxicity of ribosomal antibiotics.* EMBO Rep, 2001. **2**(4): p. 318-23.
231. Zipfel, C., et al., *Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation.* Cell, 2006. **125**(4): p. 749-60.
232. Li, X.D. and Z.J. Chen, *Sequence specific detection of bacterial 23S ribosomal RNA by TLR13.* Elife (Cambridge), 2012. **1**: p. e00102.
233. Oldenburg, M., et al., *TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification.* Science, 2012. **337**(6098): p. 1111-5.
234. Miskinyte, M. and I. Gordo, *Increased survival of antibiotic-resistant Escherichia coli inside macrophages.* Antimicrob Agents Chemother, 2013. **57**(1): p. 189-95.
235. Song, H., et al., *Regioselectivity of the oxidative C-S bond formation in ergothioneine and ovothiol biosyntheses.* Org Lett, 2013. **15**(18): p. 4854-7.
236. Lifshitz, Z., et al., *Computational modeling and experimental validation of the Legionella and Coxiella virulence-related type-IVB secretion signal.* Proc Natl Acad Sci U S A, 2013. **110**(8): p. E707-15.
237. Bruchard, M., et al., *Chemotherapy-triggered cathepsin B release in myeloid-derived suppressor cells activates the Nlrp3 inflammasome and promotes tumor growth.* Nat Med, 2013. **19**(1): p. 57-64.

238. Foghsgaard, L., et al., *Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor.* J Cell Biol, 2001. **153**(5): p. 999-1010.
239. Conus, S., et al., *Caspase-8 is activated by cathepsin D initiating neutrophil apoptosis during the resolution of inflammation.* J Exp Med, 2008. **205**(3): p. 685-98.
240. Werneburg, N.W., et al., *Tumor necrosis factor-alpha-associated lysosomal permeabilization is cathepsin B dependent.* Am J Physiol Gastrointest Liver Physiol, 2002. **283**(4): p. G947-56.
241. Oberle, C., et al., *Lysosomal membrane permeabilization and cathepsin release is a Bax/Bak-dependent, amplifying event of apoptosis in fibroblasts and monocytes.* Cell Death Differ, 2010. **17**(7): p. 1167-78.
242. Vancompernolle, K., et al., *Atractyloside-induced release of cathepsin B, a protease with caspase-processing activity.* FEBS Lett, 1998. **438**(3): p. 150-8.
243. Roy, C.R. and D.S. Zamboni, *Cytosolic detection of flagellin: a deadly twist.* Nat Immunol, 2006. **7**(6): p. 549-51.
244. Duewell, P., et al., *NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals.* Nature, 2010. **464**(7293): p. 1357-61.
245. Swanson, M.S. and R.R. Isberg, *Association of Legionella pneumophila with the macrophage endoplasmic reticulum.* Infect Immun, 1995. **63**(9): p. 3609-20.
246. Inaba, K., et al., *Isolation of dendritic cells.* Curr Protoc Immunol, 2009. **Chapter 3**: p. Unit 3 7.
247. Broz, P., et al., *Differential requirement for Caspase-1 autoproteolysis in pathogen-induced cell death and cytokine processing.* Cell Host Microbe, 2010. **8**(6): p. 471-83.
248. Luo, Z.Q., et al., *Mutational analysis of TraR. Correlating function with molecular structure of a quorum-sensing transcriptional activator.* J Biol Chem, 2003. **278**(15): p. 13173-82.
249. Verburg, J. and P.J. Hollenbeck, *Mitochondrial membrane potential in axons increases with local nerve growth factor or semaphorin signaling.* J Neurosci, 2008. **28**(33): p. 8306-15.
250. Du, H., et al., *Cyclophilin D deficiency attenuates mitochondrial and neuronal perturbation and ameliorates learning and memory in Alzheimer's disease.* Nat Med, 2008. **14**(10): p. 1097-105.
251. Huang, L., et al., *The E Block motif is associated with Legionella pneumophila translocated substrates.* Cell Microbiol, 2010. **13**(2): p. 227-45.
252. Kubori, T., A. Hyakutake, and H. Nagai, *Legionella translocates an E3 ubiquitin ligase that has multiple U-boxes with distinct functions.* Mol Microbiol, 2008. **67**(6): p. 1307-19.
253. Heidtman, M., et al., *Large-scale identification of Legionella pneumophila Dot/Icm substrates that modulate host cell vesicle trafficking pathways.* Cell Microbiol, 2008.

254. Zusman, T., et al., *The response regulator PmrA is a major regulator of the icm/dot type IV secretion system in Legionella pneumophila and Coxiella burnetii.* Mol Microbiol, 2007. **63**(5): p. 1508-23.
255. Pan, X., et al., *Ankyrin repeat proteins comprise a diverse family of bacterial type IV effectors.* Science, 2008. **320**(5883): p. 1651-4.
256. Conover, G.M., et al., *The Legionella pneumophila LidA protein: a translocated substrate of the Dot/Icm system associated with maintenance of bacterial integrity.* Mol Microbiol, 2003. **48**(2): p. 305-21.
257. Liu, Y. and Z.Q. Luo, *The Legionella pneumophila effector SidJ is required for efficient recruitment of endoplasmic reticulum proteins to the bacterial phagosome.* Infect Immun, 2007. **75**(2): p. 592-603.
258. Machner, M.P. and R.R. Isberg, *Targeting of host Rab GTPase function by the intravacuolar pathogen Legionella pneumophila.* Dev Cell, 2006. **11**(1): p. 47-56.
259. Ivanov, S.S., et al., *Lipidation by the host prenyltransferase machinery facilitates membrane localization of Legionella pneumophila effector proteins.* J Biol Chem, 2010. **285**: p. 34686-98.

APPENDIX

APPENDIX

Table 1. Candidate genes and the corresponding primers used in the screening for Dot/Icm substrates of *L. pneumophila*

Gene	5' primer	3' primer
lpg0333	ctgggatccATGCCTACTTTCAGATCA	ctggtcgacTCATAAATTAGGTGAGTT
lpg1858	ctgggatccATGATTAACTGTAAAGGG	ctggtcgacTTAAGCTTGTTGTACCGC
lpg1891	ctgggatccATGTTGAATGAAAGGGAT	ctggtcgacTTATCTGGTTGCCTTCAA
lpg1980	ctgggatccATGATAAAAAAAATTACT	ctggtcgacTTATTCTATGCCTTCATT
lpg2058	ctgggatccATGGGGTTGCTTATGAAT	ctggtcgacTTACTTTAGTCCTAGTGC
lpg2312	ctgggatccATGAGAATAAAAATAATT	ctggtcgacTTAATGAGTTTGCATTTG
lpg0364	ctgggatccATGGACGAAAGGCGAAAA	ctggtcgacTTATGTTTTATTTTTTCG
lpg2407	ctgggatccATGTATAATGTCGTCAGT	ctggtcgacTTATTGTTTTAAGTTTGC
lpg0115	ctgggatccATGTTTATTGTGCAATTA	ctggtcgacTCAACATAATTTGCCTTC
lpg2185	ctgggatccATGCTCCTGAAAGTGCCT	ctggtcgacCTAACCGCTTATCACCGA
lpg1451	ctgggatccATGGATGATCCGGATGCG	ctggtcgacTTAGATTTTAGTATATTT
lpg2296	ctgggatccATGAAAGGCCTTTTTTTT	ctggtcgacCTACAGTTTTCTTGCCAT
lpg0075	ctgggatccATGACCAATATTAAACTC	ctggtcgacTTATGGATTGCTATCCGT
lpg0153	ctgggatccATGAAACCCACTCTATTT	ctggtcgacCTATGGGCGAGCCTCAAG
lpg1309	ctgggatccATGGCAAACCAAACACGT	ctggtcgacTCAAGTGCGCTTAAATTT

lpg1327	ctgggatccATGGTGATTTATGAAGTA	ctggtcgacTTACTTTGATATAATTTC
lpg1476	ctgggatccATGCCAATTTATGAATAT	ctggtcgacTCACTCGCTATCACCTTT
lpg1885	ctgggatccATGAGCACACCCAAATGG	ctggtcgacCTAATCACAGTTTTGGCT
lpg1932	ctgggatccATGCACTTGAAATCTTGG	ctggtcgacCTAAGATATTCGAGCAGG
lpg1997	ctgggatccATGAGCAAGCATCAGGGT	ctggtcgacTCAATGAGCATCCAGATC
lpg2264	ctgggatccATGGGCAAAGACAATCTT	ctggtcgacCTAAAAATGCTTGCGTAA
lpg2948	ctgggatccATGAGTAACCATTTACAA	ctggtcgacCTATGAACAGACTTTGAT
lpg0197	ctgggatccATGACAATGATGATTACA	ctggtcgacTCAGATAATCACTGCCGT
lpg0810	ctgggatccATGAAAGCAAGACTCATT	ctggtcgacTTAATTAATAACGAAAAT
lpg1278	ctgggatccATGATCATCCGGAATTAT	ctggtcgacTTATGGGTTACGATTTAC
lpg1681	ctgggatccATGAAACTTAAATACAGA	ctggtcgacCTAGTGGGCAATCGGTGG
lpg1686	ctgggatccATGAAAATAGTAAGTGCT	ctggtcgacTTAAAAACAACGTTTGGC
lpg2633	ctgggatccATGTTTGTGACTTTTAGT	ctggtcgacTCAACCTGGTGATTTTGA
lpg1289	ctgggatccATGAAAAGAATGATCGGT	ctggtcgacTTAGCTTGGAGAATCTGC
lpg1802	ctgggatccATGTCGGATTGTCTATAT	ctggtcgacTTACAGACCCTTTGCCAA
lpg1826	ctgggatccATGTACATGCTAAGACAT	ctggtcgacTTACCGAATCAAAATATC
lpg2503	ctgggatccATGAAACCGCTATCCAGA	ctggtcgacTTATTCGCTCGCCAGGAG
lpg0367	ctgggatccATGGTTCCAATACAAACA	ctggtcgacTTATATGTGAATATTTTG
lpg0613	ctgggatccATGGTCAGGATATTTTTA	ctggtcgacTTATCTGCAAAATTGCTC
lpg0990	ctgggatccATGATAATGCTTTGCGGT	ctggtcgacTTAATACAGTTCCGATGC
lpg2931	ctgggatccATGGAGCCTTTTATGAAT	ctggtcgacCTAGTGAGACGGGATAGT
lpg0997	ctgggatccATGAAACGAATATTATGC	ctggtcgacTTAGGGCTTCCTCACAAA

lpg1520	ctgggatccATGACAAAAAAATCGCTA	ctggtcgacTTAATCTTTTATCTTTCC
lpg1902	ctgggatccATGGCGCGCTTTTCCAGA	ctggtcgacTTAACCTAAAACGCTGAT
lpg2921	ctgggatccATGAATGAGCAATTAAAC	ctggtcgacCTACGATTCTTTTGTATA
lpg2935	ctgggatccATGAGTGATCATATTAAA	ctggtcgacTTACAGATTACTTTCAAC
lpg0538	ctgggatccATGATCATGCTACGAGCT	ctggtcgacTTAACATTGAACACCCAA
lpg2202	ctgggatccATGGGAGCTTTGGCATTA	ctggtcgacTTATACATCAACAACACA
lpg0509	ctgggatccATGGCTCATTATCTTGGT	ctggtcgacCTAGTGCAAATAGGAATG
lpg0817	ctgggatccATGAGCAAGCAAAATTTA	ctggtcgacTTACTCCGGTTCCATACT
lpg0884	ctgggatccATGCAGCTCAGAACCATG	ctggtcgacTTACCAGCAAAAGTGAAT
lpg1262	ctgggatccATGGATGAGTATTTTAAA	ctggtcgacTTATGAAACTTTCTTTTT
lpg1377	ctgggatccATGCTTGAAAAAAATCCT	ctggtcgacTCAGGAGTTACCAGGGTG
lpg2755	ctgggatccATGGATATTAATCAAAAT	ctggtcgacTTACTCTCCTTCTTTATC
lpg0306	ctgggatccATGGCCCTTCCATTCAGA	ctggtcgacCTATAGTTTTGCACTGGA
lpg0406	ctgggatccATGTCAGACAAATTTTCA	ctggtcgacTTACTTGCTAAATTCCTC
lpg0573	ctgggatccATGGCCCTTCCATTCAGA	ctggtcgacCTATAGTTTTGCACTGGA
lpg1075	ctgggatccATGGAAGAGTATTATGAC	ctggtcgacTCAATACACAGCACGCTT
lpg2518	ctgggatccATGTCAATGACTCTCTCG	ctggtcgacTTAGAAAGAATAATGGTA
lpg2750	ctgggatccATGGCCCTTCCATTCAGA	ctggtcgacCTATAGTTTTGCACTGGA
lpg2765	ctgggatccATGAATTGTTTGTTTTGC	ctggtcgacCTAACCTGGCGGCCAAGT
lpg2724	ctgggatccATGAATAATACGATCGAT	ctggtcgacTCATTCAGGCAATTTATC
lpg0305	ctgggatccATGGCTAGCAGCTTTTCC	ctggtcgacTCAGAAGTGCGAATGCTG
lpg0572	ctgggatccATGGCTAGCAGCTTTTCC	ctggtcgacTCAGAAGTGCGAATGCTG

lpg2379	ctgggatccATGAAACGCTGCCCCATT	ctggtcgacTTAGAGTACACTAAACCA
lpg2751	ctgggatccATGGCTAGCAGCTTTTCC	ctggtcgacTCAGAAGTGCGAATGCTG
lpg0220	ctgggatccATGAATATAATCAAAGTA	ctggtcgacCTAAGAGAAATAATCGAC
lpg1004	ctgggatccATGTTCTTAAGATTTAAT	ctggtcgacTCAGCATGATTTTTTCTC
lpg1887	ctgggatccATGAAAAAAGTGATATTG	ctggtcgacCTATTGGTTAGCCTTTTG
lpg0035	ctgggatccATGTCTAAATTTTTTACT	ctggtcgacTTAAGTAGAACAAACTGC
lpg0039	ctgggatccATGTTAGTGTTTGGTCTT	ctggtcgacTCATTTTTTATTTTTTTC
lpg0563	ctgggatccATGACAAACAATAAAGAT	ctggtcgacTTATTTCTTTACTTCAGC
lpg2994	ctgggatccATGACCCAAGAAAAAGGA	ctggtcgacCTAGCATCCTGCATCAAA
lpg2978	ctgggatccATTTCTACAAAAAATCAG	ctggtcgacTCAATTCCGGTAATATTC
lpg0994	ctgggatccATGAGTCAGCCTTCTTCG	ctggtcgacTCAAACATGAGGCTCTCC
lpg0995	ctgggatccATGAAAACAACGCTTCGC	ctggtcgacCTATATTTTGAACTTGGT
lpg1240	ctgggatccATGATACTAGCAAACAAA	ctggtcgacTCAAGCGTGTTCATGGAT
lpg1458	ctgggatccATGAATATCAATCTAGAA	ctggtcgacTTAGAAGATAAACCCTGC
lpg1710	ctgggatccATGAAAAGGAATGCATTG	ctggtcgacTTATTTTCGCATGTAATT
lpg2364	ctgggatccATGGTCAGATTCCATTTA	ctggtcgacTTATTTGGGCTTTAGTTG
lpg2365	ctgggatccATGAAAAAATCCTTAACA	ctggtcgacTTATAATAATTCCTCTAA
lpg2520	ctgggatccATGCAAACGACTAAAGCT	ctggtcgacTTACTCATCATCCATATG
lpg1078	ctgggatccATGGATTTTTGGATAGAA	ctggtcgacCTAAACCGCTTCTTGATG
lpg1279	ctgggatccATGAAAAAATTATTCAGC	ctggtcgacTTAGTCTGAGGGGTTGGC
lpg2072	ctgggatccATGCATCAGCCAGACATC	ctggtcgacCTATAGTGATTCCTGTTG
lpg2169	ctgggatccATGTTAAGTGACCCTAAT	ctggtcgacTTACGTTCGTTTTAGACA

lpg0624	ctgggatccATGATGAAAAAAATTAAT	ctggtcgacTCATGCTTTATCATTGAG
lpg0896	ctgggatccATGCAACGCATCTTTAAG	ctggtcgacTTATAAACCATTATCAAC
lpg1385	ctgggatccATGTCAAGGAAATTACTT	ctggtcgacCTATTTGGGAATTAGATG
lpg1586	ctgggatccATGATTACAGCAATTCAT	ctggtcgacTTAAAATACCAATTGATA
lpg1972	ctgggatccATGAAAAGACTAATTATC	ctggtcgacTCAAATTTTGTTTATTAA
lpg0433	ctgggatccATGTCTCATCCTCATTAT	ctggtcgacTCAAGCATGACAACCAAG
lpg1132	ctgggatccATGTTTCGTTATTTTAAA	ctggtcgacTCAGCCACTCAAAGCGTC
lpg1112	ctgggatccATGATATTAAGACTAATC	ctggtcgacTTAATCTTTTAGTAACTC
lpg1281	ctgggatccATGAATAAGTACTTAGTG	ctggtcgacTTAACTCTCAGGGGATTC
lpg1892	ctgggatccATGGGATTTTCTGCTTTT	ctggtcgacTTATTGATTGCAGGTTAT
lpg2328	ctgggatccATGCCTGCTTTCAAAAAA	ctggtcgacTTAAAGACCAAATGCTTT
lpg2460	ctgggatccATGTATATACAAAAATTA	ctggtcgacCTAGGTTAGCTGATTTGA
lpg0374	ctgggatccATGAAAAAATGGACAGTA	ctggtcgacTTATTCAATGTTGATAGA
lpg0589	ctgggatccATGAGAAGCATACGACTC	ctggtcgacTTATTCAGGTAGATCGAT
lpg0798	ctgggatccATGAAAAGATATTATTCT	ctggtcgacTTAAGCCGAAGGATCAGC
lpg0539	ctgggatccATGAACAAACTAAATATA	ctggtcgacTCAGTTTTTAAATAATTT
lpg1046	ctgggatccATGGAGGAATTTCCAATG	ctggtcgacTTATAAGGTCTTTTTCTT
lpg1261	ctgggatccATGATAGATATTAAAATG	ctggtcgacTCATGCGAAATGTTTGTC
lpg1505	ctgggatccATGAACACTGCATTCCGT	ctggtcgacTTATGAACAGTATGGTAC
lpg0623	ctgggatccATGTTAACTACATTTCTA	ctggtcgacTTATTTCAAATAAACATA
lpg1625	ctgggatccATGCAAAATGAGAAAGAA	ctggtcgacTTAGCTGTTTTTTGATAC
lpg1809	ctgggatccATGAATTTAATGCTTAAA	ctggtcgacCTAGTTTTTAGTAATTTC

lpg2395	ctgggatccATGAATGGGACTCACAAG	ctggtcgacTCAGGCATACATCGTTTG
lpg2867	ctgggatccATGACTGAAATAAACAAA	ctggtcgacCTACACTAAATTCCTAAT
lpg0046	ctgggatccATGAACTGTTTTCCTTTC	ctggtcgacTTAACTCATTGAGAAAGA
lpg0260	ctgggatccATGTTTGGTTTTTTTAGT	ctggtcgacTTACATCGTATGCTGAAC
lpg0559	ctgggatccATGTTTAATAAATTTTTA	ctggtcgacTTAGTGGTGGCATATAAA
lpg0562	ctgggatccATGACTCGACTTATAAAA	ctggtcgacTTAGACTTTAAACATTTT
lpg1431	ctgggatccATGAAGACTAAAATCATA	ctggtcgacTTAATATACATCGCTTCC
lpg2283	ctgggatccATGGATGAAAAAAAAGAT	ctggtcgacTTAAATCGATAAGGCGCT
lpg1485	ctgggatccATGCAAGAATCAATTCAC	ctggtcgacTCAATGGGAAAACAGGTA
lpg1779	ctgggatccATGACACATATCTTTCTG	ctggtcgacCTAATTCTGATGTTTTAA
lpg2187	ctgggatccATGAAATCTTTAAGGATG	ctggtcgacTTACTGAGCCTGGTTAAC
lpg0614	ctgggatccATGCACAAGTTAATTGAG	ctggtcgacTCAATGATAAACTGCCCT
lpg0722	ctgggatccATGAAGTTAAAATCATTG	ctggtcgacTTATCTGGTTGATAACTC
lpg2211	ctgggatccATGGTACAACATGATATA	ctggtcgacCTAAATCAATTGACTGGC
lpg1082	ctgggatccATGACAAAATTGGGGTTA	ctggtcgacCTATGTGATTGTACGGGT
lpg2479	ctgggatccATGAGGAGTCCAGTTTTG	ctggtcgacTTAACGACAGGCCCAAAA
lpg2539	ctgggatccATGAAACTTATAATTAAT	ctggtcgacTTACATATCAGGATTCAT
lpg1002	ctgggatccATGTTGAGAACAACCATT	ctggtcgacTCATTGTGCTCTCCTTGC
lpg2005	ctgggatccATGCTTTTAACCTTGGCT	ctggtcgacTTAAGAGACTACGAGCAA
lpg2579	ctgggatccATGGATATTCGATGTAAA	ctggtcgacCTATAATAGGCTTTTTAT
lpg1832	ctgggatccATGAAAAAGATGTTTTTA	ctggtcgacTTACCCGAATACTTTAAA
lpg2598	ctgggatccATGTTATTGAATTTGTTT	ctggtcgacTTAGTGAATTGTTAATTC

lpg2605	ctgggatccATGAGTTTTTCTCTTTCT	ctggtcgacTTAATCATCCATTTCCGC
lpg0598	ctgggatccATGAAATTAATATGGATA	ctggtcgacTCATAAACACAGGGATCT
lpg1171	ctgggatccATGAGATTTCAAGATTCT	ctggtcgacTTAGAAAGTAATTTTCAT
lpg2920	ctgggatccATGACCAATATCGATGTT	ctggtcgacTCATTCATGCTCTTTCAG
lpg1494	ctgggatccATGATTAAAATTCACTAC	ctggtcgacTTAGCTTTTTGCTTGGTT
lpg2602	ctgggatccATGAAATCAATCAGTCAA	ctggtcgacTTATTCACTTTGAGCTAA
lpg0873	ctgggatccATGTATCTTAGCCAGTTA	ctggtcgacTTATGCAGTACAAGTTGC
lpg0428	ctgggatccATGTTTATCATTGTAATT	ctggtcgacTCATGTTAATTGATGCAA
lpg1390	ctgggatccATGATGCATTTACAAGAG	ctggtcgacTTAAGAAGTTTGTTTTTT
lpg0999	ctgggatccATGATTAGGATTTTGCTC	ctggtcgacTTACTGCCATACAGCCCC
lpg1383	ctgggatccATGAAGGTAGAAATCTAT	ctggtcgacCTATGATAATTCCTCAAT
lpg2534	ctgggatccATGCAATTGACTCAATTT	ctggtcgacCTAACTGATCCTCAATAA
lpg0266	ctgggatccATGAAAAGGATTTTTTTA	ctggtcgacCTAACCGACACTAAAATT
lpg0526	ctgggatccATGATTATAATATTCCAA	ctggtcgacTTAAACTTTTTTAAAACC
lpg0723	ctgggatccATGAAATACCTCATTCCC	ctggtcgacTTAACTATGGCCATGAAT
lpg2413	ctgggatccATGAAAAAATCCAGTCCT	ctggtcgacTTAACTTTTTAGACCAAC
lpg1055	ctgggatccATGTTAAAAAGGAATGCA	ctggtcgacTTATTGGTTTTTCATCAT
lpg1582	ctgggatccATGAGTTGTTTTGAAAAG	ctggtcgacTCATTGTATGTATACTAA
lpg1620	ctgggatccATGTGGTTATTGGAGAGA	ctggtcgacTTACATCACCTCTGGCTG
lpg2601	ctgggatccATGGCTGAAAATACTACA	ctggtcgacTTAGTGTGGTTCATTCAT
lpg0407	ctgggatccATGCGTCTTTTGTTTATC	ctggtcgacCTATTGCGGCGATAACAG
lpg1091	ctgggatccATGTTTTTTGATGACACT	ctggtcgacTCACTTACTACATTTATA

lpg2359	ctgggatccATGACTATAAAAGAACAA	ctggtcgacTCAGCTTAATTTCGCCTT
lpg2774	ctgggatccATGATAAATGATGACTTG	ctggtcgacCTAGACTGTCAAATTTGC
lpg0089	ctgggatccATGACGATCTGGATTGAT	ctggtcgacTTATTTGCTTTTTGCAAG
lpg0883	ctgggatccATGTACTTACGTTTACTC	ctggtcgacTTAAAGCTTGGGATCAGG
lpg1742	ctgggatccATGTCATTTTTGTATGCG	ctggtcgacTCAGGCAATCGCTCTTTG
lpg0740	ctgggatccATGAAAAAATTAATTGTT	ctggtcgacTTAATTCACAACACGCCA
lpg1578	ctgggatccATGAGGAACCCGAAAAAG	ctggtcgacTTATATACTATTTTTGTT
lpg2453	ctgggatccATGTCGTTTAATAATTTC	ctggtcgacCTAGAGAACAGATGGTCT
lpg0182	ctgggatccATGCTTAATAGTAGCTCC	ctggtcgacCTAACGAGGCGAGGGCGT
lpg1030	ctgggatccATGAAGCACATTACTTTG	ctggtcgacTTAATTAGGGACATCTGA
lpg0165	ctgggatccATGCCAGATATTACACAT	ctggtcgacTTAATGATTTGTTTTGGC
lpg1274	ctgggatccATGAAACAAATTAAAGGA	ctggtcgacTTAAATTTTTTGAGGGTC
lpg1527	ctgggatccATGCAGCATAATATATAC	ctggtcgacCTAAGCTGTTAGGTCAAA
lpg1061	ctgggatccATGAGCAAGAATAAGAAT	ctggtcgacTTAATGGATTGAATCAGT
lpg1099	ctgggatccATGGAGCATAGTGGGTAT	ctggtcgacTTATGATGTGAATTTAAC
lpg1270	ctgggatccATGTCACCTGAAAAAATT	ctggtcgacTTATTCATTAAAGAGCCC
lpg1716	ctgggatccATGAGAGGACATATGATT	ctggtcgacTTATGTTGGGTTTGGACT
lpg0161	ctgggatccATGTTGCCAGTTATTGAT	ctggtcgacTTATTGACCGATAATCTT
lpg0717	ctgggatccATGAAAAAAATACTGTAT	ctggtcgacTTATTGAATTCCAAACCA
lpg2125	ctgggatccATGAACAAAGCCGATTTA	ctggtcgacTTACATATTCAGAGTAGG
lpg0091	ctgggatccATGAGAATTTTGCTCTCT	ctggtcgacTTATTTCTCCGGCAATAC
lpg1740	ctgggatccATGAAAACAATTAGGCTG	ctggtcgacTCACATCTCTATACCAAG

lpg2164	ctgggatccATGGTAAGCTGGGATCTA	ctggtcgacTCACTTATTCCTGCGCTT
lpg1376	ctgggatccATGCTAAAAATTACCATT	ctggtcgacCTATTTATGATAAGGATG
lpg2071	ctgggatccATGAATGCGTCAGGCGCA	ctggtcgacCTACCTGATAGCCAAAAT
lpg0381	ctgggatccATGAGTCCATTAACCCAA	ctggtcgacTTATTTTATAACTTTTAA
lpg1117	ctgggatccATGGCTATTTACAAATTT	ctggtcgacTTAATTAGAGAGTTTGGT
lpg1468	ctgggatccATGAGGAATTATATTTTT	ctggtcgacTTACCAGTTAGTCCCACT
lpg2320	ctgggatccATGTTTCTCGAACCAGGT	ctggtcgacTTACAAATTAACATTCAC
lpg2375	ctgggatccATGCGTAGCAGAACAGAA	ctggtcgacTTAGAGAATGCTGCATTG
lpg2860	ctgggatccATGAAGAACCAGAAAAAA	ctggtcgacTTACTCCTGATAGTCACT
lpg0021	ctgggatccATGGATGAACAAATCAGT	ctggtcgacTCATTTTATTAAAGACCT
lpg0159	ctgggatccATGAAAGAAAAGACCGCG	ctggtcgacTTATATTGGTGACAATAA
lpg1605	ctgggatccATGTATTTTTCATTAAAT	ctggtcgacCTAATAACCTCGCCAAAA
lpg1896	ctgggatccATGACTCTTTGCCCCTGT	ctggtcgacTCATTTATCATGACAGTT
lpg0051	ctgggatccATGTGGCTTGACTACCTC	ctggtcgacTTATTTGTAGGTAATAAA
lpg0383	ctgggatccATGATGCGAAATAGGGAT	ctggtcgacTTATAATGCATTGAGCAA
lpg1776	ctgggatccATGCAGTCTAAAATACAC	ctggtcgacTTATCTATGTCCATGAAC
lpg2445	ctgggatccATGAATCGCTTCATTCGT	ctggtcgacTTAGTGTTTTTCATCGAG
lpg2717	ctgggatccATGGAAAAAGTTAACTTG	ctggtcgacCTACGTTCTTTGCGAGTG
lpg1585	ctgggatccATGTCATTAATTAATTAT	ctggtcgacTCACCATTTTAGGTAAAT
lpg2110	ctgggatccATGGTTAACAATTTTATA	ctggtcgacTTATAAGTCTTTAGGGTC
lpg2542	ctgggatccATGCTGAGTAGATATGTA	ctggtcgacCTACCACGTGTTGCACTG
lpg2434	ctgggatccATGACGATCATGATGAAC	ctggtcgacTTAATACGGCATTCCATT

lpg1041	ctgggatccATGTTTATGTTTCACAGT	ctggtcgacCTATTGATTGGGTGACTG
lpg2591	ctgggatccATGGCCGCGCAATTAGAT	ctggtcgacCTAAACAGTGGGTCTGTT
lpg2818	ctgggatccATGAAACAACTAATATTA	ctggtcgacCTACTGCATAGTCAAATC
lpg0235	ctgggatccATGAAGAAGGCATTTAGA	ctggtcgacTCAGTTGGCATCAGGCAA
lpg1147	ctgggatccATGTTATTTTTTAGTTCA	ctggtcgacTTATATCTTTATTGTTAA
lpg1144	ctgggatccATGGATTTAATTTTTAGT	ctggtcgacTTACATTGTAAAACGGCT
lpg0837	ctgggatccATGACCCAGCTAAAGGTT	ctggtcgacTCATGATTTCTTTTCAGG
lpg0895	ctgggatccATGAAATTTGATATTCAA	ctggtcgacTTATTCCTCTTTTAAAGG
lpg2246	ctgggatccATGAAGAAATGGAATAGC	ctggtcgacTTAAGGACTATTGTATAG
lpg2165	ctgggatccATGTCGGTAATAAATGGT	ctggtcgacTTAAAATTGTTTGCCAAA
lpg0152	ctgggatccATGTCTAATGTATCTATT	ctggtcgacTTATAATTTCTTTTTATA
lpg1453	ctgggatccATGCCTAGATGGTTTAGA	ctggtcgacTTAACCATTGACCAACAC
lpg1090	ctgggatccATGCCGAATGGATTGTGG	ctggtcgacTTAAAAAATAACGAGTCG
lpg2109	ctgggatccATGAAAGAAAAAAAAAGG	ctggtcgacTTATTCTGTAAAATCCTT
lpg0741	ctgggatccATGCTTTCGATTAATGAG	ctggtcgacTCAGGAGTGAATATAATT
lpg0764	ctgggatccATGGATACTGTAATTCGT	ctggtcgacTTAGAGGGTATTCTCCTT
lpg1895	ctgggatccATGATGAAAAGGAAGTTC	ctggtcgacTTATTGAAAAGGATTGTC
lpg0085	ctgggatccATGAGATCAAAATTTGTT	ctggtcgacTTATTTTTCATTGTTCAC
lpg0434	ctgggatccATGGCCCAGGAACTTGTA	ctggtcgacTTATTGAAACCATTTGAT
lpg1031	ctgggatccATGGTAATACCTCATTTG	ctggtcgacTTAATTTGGTTTTAAATC
lpg1883	ctgggatccATGAATCGAATCAAACTT	ctggtcgacTCAATCAGGGTGTTGGAG
lpg1015	ctgggatccATGAAAAGTCCTCCACGT	ctggtcgacTTAGAAAGGAAAAGGAGG

lpg2209	ctgggatccATGATGGCCATATTTAAA	ctggtcgacTTATTGAGATGCATTCTT
lpg2692	ctgggatccATGCCAACAACCTTTGAA	ctggtcgacTTATGGACTAACATGGGA
lpg1876	ctgggatccATGATGGGTATTAGCATC	ctggtcgacCTATTCATTCAAATTTCC
lpg2663	ctgggatccATGATTCAAACCAATTGG	ctggtcgacCTAGGTATCAACATGGGA
lpg0202	ctgggatccATGATTATTAATCCTGTG	ctgggatccATGATTATTAATCCTGTG
lpg0787	ctgggatccATGATATCTGAATCAGTC	ctggtcgacTCAAACTTCAACACCAAA
lpg1429	ctgggatccATGTCTATTAATTATGAA	ctggtcgacTCATAATAAATTTTCCAG
lpg2200	ctgggatccATGAGTGCAAAACAAAAA	ctggtcgacTTAAAGTTGCATACCCAT
lpg2916	ctgggatccATGACATGGTTAGGAAAC	ctggtcgacTTACTCAGCACTTTTGTT
lpg0424	ctgggatccATGAAAAAAATAAAATCT	ctggtcgacTTAAATGGTTAACAAGGC
lpg2403	ctgggatccATGTTTGGTGAATTATTT	ctggtcgacCTATTGCATTAGTCCAAA
lpg0023	ctgggatccATGAAATCGTTTATTGAG	ctggtcgacTTAAGATTTGGATTTTGT
lpg2893	ctgggatccATGAAAATAGCAAGTAAT	ctggtcgacCTAAACCCTATTCACCTC
lpg0301	ctgggatccATGTCCAAACTTGTATAC	ctggtcgacTCAATAATGATAATGTCT
lpg1992	ctgggatccATGAAAGGATACTGCCTT	ctggtcgacTCAATCAATCTTAGGGCT
lpg2266	ctgggatccATGCTCTATCATGATCCA	ctggtcgacCTAGGGTATAGAAATGGA
lpg2420	ctgggatccATGAAAAAAGACATCACT	ctggtcgacTTAAAACCTTAATCTCAT
lpg1814	ctgggatccATGATTAGATTATTAATA	ctggtcgacTCATGAAGCTATTTTATG
lpg2885	ctgggatccATGGAGTTTTTTATGTGG	ctggtcgacTTAGATTCTAAGTTCTGT
lpg1066	ctgggatccATGATATTGAATGATATC	ctggtcgacTTAAACTGCATGACTGTG
lpg1587	ctgggatccATGGCTAGAACTCTATCA	ctggtcgacCTATATTTTCCACTTGAT
lpg1995	ctgggatccATGAAAAAAACCGATTCC	ctggtcgacTCAACCAGTTAATATTTC

lpg2443	ctgggatccATGATAAGGAAGTCAGAA	ctggtcgacTTATTTACGGTCATAAAT
lpg2483	ctgggatccATGAGAATTTTGGAAAAA	ctggtcgacTCAGCTTTTGCCAGTCAG
lpg1365	ctgggatccATGAATAAACTGCCACTA	ctggtcgacCTATTTATTAGAATTGAT
lpg2281	ctgggatccATGCTGCAAAGAGTTTTA	ctggtcgacTTAAAACCATGCTCTTCT
lpg2519	ctgggatccATGCTGATTTTGATTCAA	ctggtcgacTTAGTTTGAACTCAATGT
lpg2571	ctgggatccATGGACCCAGTATTAGAA	ctggtcgacTTAATGACGTTCCCACCT
lpg0082	ctgggatccATGAGTCAAATTAAAACG	ctggtcgacCTAAAGTGCGGGGGAAGC
lpg1213	ctgggatccATGGTAGCGGATTTAATG	ctggtcgacTCATCCATTTTTACTTAT
lpg1926	ctgggatccATGACTATGCCAGTACAT	ctggtcgacTTAGTCAGCACGGCTTTT
lpg2386	ctgggatccATGACCATCTACTTTTTG	ctggtcgacCTATGCAAACTGAAGCTG
lpg2491	ctgggatccATGAACGCTATACGTGCT	ctggtcgacTTACCGGGTTAAATCAAA
lpg2733	ctgggatccATGGCTAATTCACAAGAA	ctggtcgacCTAGAGTTTGGCCGTTTT
lpg0171	ctgggatccATGAAAGCAAAATACGAC	ctggtcgacCTACAATGGCTCACATTG
lpg0254	ctgggatccATGAGTGAATATTTGGTT	ctggtcgacCTACATGAGCACACAAAC
lpg0838	ctgggatccATGAATATTACCAAACAA	ctggtcgacTTAACCTTTAGCTGGGTC
lpg2550	ctgggatccATGAACTTGATGCTTGGA	ctggtcgacTCATTTAGCAGAGCAATC
lpg2992	ctgggatccATGAACATGAATCTAAAA	ctggtcgacCTAACGTATATATTGGAA
lpg0487	ctgggatccATGGAATATCTAACTCCC	ctggtcgacTCAACACGCCCTATAATT
lpg1645	ctgggatccATGAATGTCTATATTAAA	ctggtcgacTTATTTTTTCCGTGTAGC
lpg1954	ctgggatccATGCTTCCCAGTTCAAAA	ctggtcgacTTATGCTTCGGTAGGAAA
lpg2016	ctgggatccATGGCTGGTTTAACTGCT	ctggtcgacTTATAATAACTTCCAGGG
lpg0076	ctgggatccATGTCTTTAGATAATGAT	ctggtcgacTCAATGGGTTGTTCCAGA

lpg0453	ctgggatccATGGCTGATCCGACTTGT	ctggtcgacTTAAGTACCATATAAAGT
lpg1001	ctgggatccATGAAGAAATTTCTGGAT	ctggtcgacTTATTCATTGCCCACCTC
lpg2560	ctgggatccATGAAAACATATACAAAA	ctggtcgacTCATATCGTCATCGCCAA
lpg0087	ctgggatccATGCAAGAAAGTTTTGAT	ctggtcgacCTAGTCAGAACAGTACAA
lpg0405	ctgggatccATGCTGTATAATTTAACA	ctggtcgacTCAGGATATTGCTCGATT
lpg1124	ctgggatccATGCGTATGAAAATGTTT	ctggtcgacTTATGGATTGCAATAAGA
lpg1446	ctgggatccATGCATGAGATGATTGAC	ctggtcgacTCACTGCTCATTTTGCGT
lpg0852	ctgggatccATGAGCAATTTTAGAAAT	ctggtcgacTTATCTGTAACAAATAAT
lpg0967	ctgggatccATGCTACCTTTTAAATAT	ctggtcgacTTAGAACCTGGGGCCGTG
lpg1526	ctgggatccATGTTTATCTTTAAACGA	ctggtcgacTTAAGATGCTCCTTTTCT
lpg0268	ctgggatccATGAATCAAGATATGGAA	ctggtcgacCTATAAAAAAATAAGTTG
lpg1410	ctgggatccATGAAGATTCGATTTACT	ctggtcgacTTACCACCAGGAACGTGA
lpg0375	ctgggatccATGTATTCATCATTATTT	ctggtcgacCTAGGTGTTGAATGCGAT
lpg1647	ctgggatccATGAATATGAAACGGATT	ctggtcgacTTATCCATATTTCTTATC
lpg2993	ctgggatccATGATTCAGTTAGAAGAA	ctggtcgacTTATCCTAAAACTTGGCC
lpg2044	ctgggatccATGAGAGTTCTCAGTAGT	ctggtcgacCTAATCACGTTTAATATG
lpg2561	ctgggatccATGGTGGAAAATCTCTGC	ctggtcgacTTAAGATATAACAGGAAC
lpg0521	ctgggatccATGACTCCTCATATTATT	ctggtcgacCTAAGATAAGTATCCAGG
lpg2086	ctgggatccATGAAAAAGCTTCTGTTG	ctggtcgacTCATACAGTCACCTCATT
lpg2380	ctgggatccATGCAAAGACCATTGGCC	ctggtcgacTCAAAAATTTAATCGCTT
lpg2268	ctgggatccATGAAATTATGTACTAAA	ctggtcgacTCATCGGTGAACTTTATG
lpg1933	ctgggatccATGATTTATAAGATAACA	ctggtcgacTCAGAGAGGATTGCAATC

lpg2371	ctgggatccATGCTTCTAAAATACTTA	ctggtcgacTTAATCCTCCCAGTGCCC
lpg2444	ctgggatccATGTCTTCTGTTAAGCTT	ctggtcgacTCATAGACTATTGGTATG
lpg0287	ctgggatccATGCGAACTTTAGCGAAA	ctggtcgacTTACTCTTTAGCGCGAGA
lpg0556	ctgggatccATGGATATTAAAAAACTT	ctggtcgacTTAACGATCATCCAGGCT
lpg1754	ctgggatccATGGAGGAATATAAAATA	ctggtcgacTCATCGCCAAAAAGAAAT
lpg0210	ctgggatccATGAAAAATATATATCCA	ctggtcgacTTAGGGTTGAGGGCAGTT
lpg2904	ctgggatccATGATTATGTTAAAAGAA	ctggtcgacTTAATTGCCTTCATCATC
lpg2938	ctgggatccATGACTGCATTAATTTAT	ctggtcgacTTACTCAACCAAATGGGT
lpg1689	ctgggatccATGTATCACTATTTATTC	ctggtcgacTCATGTTTTGATTAATGA
lpg2344	ctgggatccATGACTCGATTCATAATG	ctggtcgacTTATGGTTTGTTGCCAAA
lpg2158	ctgggatccATGGTTATGTCGCTCTCT	ctggtcgacTCAATTTATTTTACAGTG
lpg2455	ctgggatccATGACGAAAATTGTTTAT	ctggtcgacTTAATGATGGGGCTGTAT
lpg0998	ctgggatccATGGCAGTAAAGCAATAT	ctggtcgacCTATAAGTATTTCTTAAA
lpg2199	ctgggatccATGTACCCAAATTTCTTG	ctggtcgacTTAAAAATCTAAAGTATT
lpg0732	ctgggatccATGAGAATTACATTACTT	ctggtcgacTTACTTATAATTTCCTTG
lpg1065	ctgggatccATGAAGAAAAGAGAAAAA	ctggtcgacTCAAGGAGTGTGTTCATG
lpg1111	ctgggatccATGCCAACTTATTTTGAT	ctggtcgacTTAATGTGAGTGTCCATG
lpg2205	ctgggatccATGCAAGATTCCTTTTTC	ctggtcgacTTAAGAGACTATAAGAGA
lpg2461	ctgggatccATGGATAATAGGAGAAAA	ctggtcgacTTAAGAAGGGCTATCAAA
lpg0880	ctgggatccATGAAAATGAAATTACAA	ctggtcgacTTATAAGTTGACAGTAAA
lpg2170	ctgggatccATGTATTTTTTAAAAAGG	ctggtcgacTTAACGAGGATGTTTCCA
lpg1267	ctgggatccATGTGGCTTCAAAGGAAG	ctggtcgacTTACGTCACTCCATCTTG

lpg1752	ctgggatccATGCGGTTCTTGCGGGTG	ctggtcgacCTAAAACACGCTATTAAG
lpg0796	ctgggatccATGTTTAAAAAATTGACT	ctggtcgacTTATTTTCCGAGTGTATT
lpg1975	ctgggatccATGGGTTATAATGAAACC	ctggtcgacTTATTTGATGCAATTCAA
lpg1991	ctgggatccATGACTTATTCTTTTTCC	ctggtcgacTCAACCCCCTTTTTCATA
lpg2257	ctgggatccATGAATTATTTCATTGGG	ctggtcgacTTACGGTGTGGATATTTT
lpg2271	ctgggatccATGCCTTCTTACAACAAT	ctggtcgacTTAGACGGTTTGTAAATT
lpg2497	ctgggatccATGATGCTCGATGTAATT	ctggtcgacTTATAAAGAGGAAACTCC
lpg0901	ctgggatccATGCCTCAAAGACAATCT	ctggtcgacTTAATAGTGCAAATGTAT
lpg1633	ctgggatccATGAAAGCAGTCGCAAAT	ctggtcgacTCAATTTCCAGTTGATGA
lpg1606	ctgggatccATGATTGATACAATACAG	ctggtcgacTCATTTCAGCTTTTCCAA
lpg2408	ctgggatccATGCTCACCAAAAATCTG	ctggtcgacTTAAGCAATAAGCATGGG
lpg2643	ctgggatccATGCCTTTTTGTTTTTCC	ctggtcgacTTAAATCATTAAAACAAT
lpg1851	ctgggatccATGTCATTTGAATTGGTC	ctggtcgacTTAAAGGCGATGATTTGC
lpg2128	ctgggatccATGCCCCAGACTTTGGAT	ctggtcgacTTATGTAGAATGCCCTGA
lpg2740	ctgggatccATGACAAATAGAGAACGT	ctggtcgacTCATAAGTCTCCTTCGTT
lpg2761	ctgggatccATGCAACATATGGAGAGT	ctggtcgacTTATTTAAGTTTGTTATT
lpg2162	ctgggatccATGACGGATGTTCGACAA	ctggtcgacTCAGAAGATCCTTGGTTT
lpg2543	ctgggatccATGACTTTGATATTGAAA	ctggtcgacCTAAGCACTTACTGCGGC
lpg1230	ctgggatccATGTGCGGTCGTTTTGCT	ctggtcgacTTATTCAGATAATGGCTC
lpg2373	ctgggatccATGCCTATGTCTGATTTT	ctggtcgacTTACGTTTCTCCTGCTTC
lpg0401	ctgggatccATGGAGTCCCTTATGCCT	ctggtcgacTTAATGGAACCCATGCCC
lpg0292	ctgggatccATGCCAAAACAAATCAAA	ctggtcgacTCATATTTTTAAAGTCTT

lpg1604	ctgggatccATGTATTCCTATATAGAT	ctggtcgacTTAAATATTGGTAATTTT
lpg1697	ctgggatccATGAATACATTATTTACT	ctggtcgacTTATGCTACAAGATAACC
lpg1083	ctgggatccATGGCGCTAATTGATCAA	ctggtcgacTTATATCCCTATGTGTGA
lpg2097	ctgggatccATGAATTCAATGGATATG	ctggtcgacTTAAGCCAGTATATCCTC
lpg1823	ctgggatccATGAGAAAACTGGTATTA	ctggtcgacTTAAACAACACATTGTTG
lpg2669	ctgggatccATGACGCAGCGCAATTTG	ctggtcgacTTAACACAGCCTTCCTCC
lpg0294	ctgggatccATGCCAAAATACACACTG	ctggtcgacCTATCTGATGGATTGAGT
lpg2494	ctgggatccATGACTATGAAATTTCCT	ctggtcgacTCATACCAGTCCAATCAA
lpg0252	ctgggatccATGGTGATAATAGTCAGC	ctggtcgacTTATACCATTAATTTATT
lpg0596	ctgggatccATGAGAGAAGGCTTGCGA	ctggtcgacCTATCTTTTTTTCATTAT
lpg1110	ctgggatccATGGTCAGATTTTTCAAA	ctggtcgacTTAATAAGATAGCTCGCA
lpg2507	ctgggatccATGCCTGAAGAGACGAGC	ctggtcgacTCAATTAAATAAGGCATT
lpg0815	ctgggatccATGAAATGGGTATTGGTT	ctggtcgacCTAGGCATGAAGCAACAT
lpg2030	ctgggatccATGATGGCAAAAGATTAT	ctggtcgacTTATCCGCCAACGCTGCG
lpg0042	ctgggatccATGAATAGATATTCCATA	ctggtcgacTTAAGTGTCTTTTGTGAT
lpg2275	ctgggatccATGGAGCATATTATGAGA	ctggtcgacTTAATCTCCCTGTTTGCG
lpg2682	ctgggatccATGGCAAAAAAAAGATTA	ctggtcgacTCAGTGGATTTCATGATT
lpg1243	ctgggatccATGGAAGATATTCAAAGA	ctggtcgacCTACATATAATAGTTTAC
lpg1679	ctgggatccATGAATAGTCAATCAGAT	ctggtcgacTTAACTTATTACCCACTC
lpg2762	ctgggatccATGAAGATTTTATATGTA	ctggtcgacTTAATTACCCTTTCTCAA
lpg2954	ctgggatccATGCTTGATTTCATATCG	ctggtcgacTTAGTTTTCTTTCTTATG
lpg0380	ctgggatccATGCCTATGATTGACCTA	ctggtcgacTCACTCTTTAGGTTCAAT

lpg0769	ctgggatccATGTCAACTGTCGCGCAA	ctggtcgacTTATATGTGAATTTCTAA
lpg1563	ctgggatccATGCTAGAAATGATTCAT	ctggtcgacTTAATTCAATCTTAAACG
lpg2221	ctgggatccATGACGGAGCGCAAGAAA	ctggtcgacCTATTCAGGAACAGGTCT
lpg0036	ctgggatccATGAATTATCAATACTCT	ctggtcgacTTATTCTGTTAATAATAG
lpg0168	ctgggatccATGAAAAACAATAGACAT	ctggtcgacTTACTTAGTCGTGTTTTT
lpg0246	ctgggatccATGTATAAAAATATACTG	ctggtcgacTTAAATGGACATACGAAT
lpg0830	ctgggatccATGAACTATTTTGGCAAA	ctggtcgacTTAAGCACCCTTTTTATT
lpg1332	ctgggatccATGAAAAATAGATTTGCC	ctggtcgacTTACTCATTAATCCAGAT
lpg0409	ctgggatccATGCCTAGTTTAACCTGT	ctggtcgacTTATATGGTTTTTTCATT
lpg2557	ctgggatccATGTGGTATTATTTTCAA	ctggtcgacTTAGGAACGCATCGCTAA
lpg0056	ctgggatccATGGATATTATAATGATT	ctggtcgacCTACGAGTTTTTCGGTTC
lpg0898	ctgggatccATGCACTTAACAAGAGAG	ctggtcgacTTAATGAATAGCCAATGA
lpg2585	ctgggatccATGAAAGAAATCAGCGCT	ctggtcgacTTATTCGACTGGAAAATT
lpg0054	ctgggatccATGGAATTGATTTTAATA	ctggtcgacTTAAGCATGAGCTTGTTC
lpg1595	ctgggatccATGGAGCAAAAATCAAAA	ctggtcgacTTATTGCTTAAAATCAGC
lpg1990	ctgggatccATGCTAAATAAAATAATT	ctggtcgacTTAGGGAATCGTATTATT
lpg2572	ctgggatccATGTTTATGAGCTATTAT	ctggtcgacTTACTGAGCAAATAAAAC
lpg2936	ctgggatccATGGCTGTGAGAACAATA	ctggtcgacTTATAGATCACCTCCGGC
lpg0731	ctgggatccATGAAGAAGTTAATTCAA	ctggtcgacTCAGGTTGACTTGTCAAA
lpg2884	ctgggatccATGACATTAAAACAATTT	ctggtcgacTTACTTTGAGCCAGTGAG
lpg0969	ctgggatccATGGTAAGTTTGGAGCAT	ctggtcgacTTATATATCAAGCTTTAT
lpg1113	ctgggatccATGGGTATCACTAATGAC	ctggtcgacTTATTTCAGGAAATCATG

lpg1709	ctgggatccATGAGTATCCGAGTCATG	ctggtcgacTTAATATTCAGCAACATA
lpg0058	ctgggatccATGAATCATACTTCAATT	ctggtcgacTTACATAAGCAACATGAA
lpg0377	ctgggatccATGAACAATTTACCCCCT	ctggtcgacTTACCTCTCTGATTTTTG
lpg1286	ctgggatccATGGCTGGTCATAGTAAA	ctggtcgacTTAATTCATTGATTCTAA
lpg1931	ctgggatccATGTCCAGGGCTAAATTT	ctggtcgacTTAGCATGATGCAATTAC
lpg1466	ctgggatccATGCATGACCATATGATA	ctggtcgacCTATATTTGGCAAAATGC
lpg1551	ctgggatccATGAGGAGTTACCCAATG	ctggtcgacTCAAGGTCTGCTTGGTCT
lpg1947	ctgggatccATGTTTTTTAAACGTGAA	ctggtcgacTTAAGAAAGTCTATGAGT
lpg2592	ctgggatccATGATAATTGATTGGCCT	ctggtcgacTTAAAATAATTTTTCATA
lpg2628	ctgggatccATGGCATTCATTAGACAT	ctggtcgacTTATCTGAATCCCAAAGC
lpg0973	ctgggatccATGAAATTAGTTTTTAAC	ctggtcgacTTAGATTGAATCTTCTTT
lpg2577	ctgggatccATGTTAGTAACTCCAAGG	ctggtcgacTTATATCCAATCAGGTAA
lpg0034	ctgggatccATGAAACTACACTCTATC	ctggtcgacTCAGTTCTCATGGATAAA
lpg1043	ctgggatccATGAATGCGCAAGGACGT	ctggtcgacTCATTGAAACCACCTTAA
lpg0893	ctgggatccATGGAGTCTGTTATGAAA	ctggtcgacTTATACTGCTTGATTTTC
lpg1644	ctgggatccATGTATTTAAGTTTGCCT	ctggtcgacTCATGAAAAACGCCAATT
lpg1968	ctgggatccATGGGGAAATTTTATATG	ctggtcgacTCATATGTTGGTTATCAC
lpg0080	ctgggatccATGAACAGATTAAAATTT	ctggtcgacTTAAACTCTAGGGGATAA
lpg1121	ctgggatccATGTTTAAAGAAATTATC	ctggtcgacTTAAATTATATTCACATT
lpg1158	ctgggatccATGATTATATATTTATCA	ctggtcgacTTAGCTGCCGGTTGACGA
lpg1343	ctgggatccATGAAACTGATAATGGAT	ctggtcgacCTAACTAACTCCAGTATT
lpg1409	ctgggatccATGACAATACTTGTTTTT	ctggtcgacTCAGAGCATTTTTTTGAG

lpg1366	ctgggatccATGTTAACTTTGTTATCG	ctggtcgacTTAATGGATTCGAATAAA
lpg1622	ctgggatccATGAAGGACACATTCATG	ctggtcgacTCATCTGGAATAGACTTT
lpg1960	ctgggatccATGTTTTTCTCTAAAGAT	ctggtcgacTTATTTGGGTTTTGGCAT
lpg2342	ctgggatccATGAATCAAATTAAATGG	ctggtcgacCTATGAATTGATCTGTGA
lpg0282	ctgggatccATGTCAAGACGTGAGTGG	ctggtcgacTTATTTTTCGCCGGTTAC
lpg1772	ctgggatccATGTCTTCGCAAACCAAT	ctggtcgacCTAAATTTTCTTAACTAT
lpg0666	ctgggatccATGCGTAATACTCTGCTT	ctggtcgacTCATATAGCCTCCAGTGA
lpg0101	ctgggatccATGCTGAAAAAAATATTG	ctggtcgacTTAAGAATATAAATAGCT
lpg1149	ctgggatccATGACTGTGCAAAAATAC	ctggtcgacTTATCCATGAGAAATATT
lpg2067	ctgggatccATGACAAAAATTACAACA	ctggtcgacTTATTCTTGTCTACCACG
lpg2301	ctgggatccATGAAAAAGATGGCGAAA	ctggtcgacTTAGGGCCCCCGGGGGGT
lpg0201	ctgggatccATGAATAAGATGATAATT	ctggtcgacTTATTTTCTGGGCGTCGA
lpg2969	ctgggatccATGACGGCAGGGGGTACA	ctggtcgacTCAAAACACAAAGCTTTG
lpg1209	ctgggatccATGAAAAGCTTACCAATC	ctggtcgacTCATGGCAAATATATTCT
lpg1741	ctgggatccATGAAAAAATTGATTTAT	ctggtcgacCTATATAACACTTATACA
lpg2253	ctgggatccATGAAATCGCTATTGACT	ctggtcgacCTAATTGCGACGAATACG
lpg0649	ctgggatccATGAATACAAGGGAGACT	ctggtcgacTTATAATTCTAACTCAAC
lpg1445	ctgggatccATGAATATCGATTTGCAG	ctggtcgacTCATGCAGCCTGCAAATG
lpg2678	ctgggatccATGCCCAGTACTTACATT	ctggtcgacTTAATTAAGTTTTGCTAT
lpg1233	ctgggatccATGAAAAAAATTATCACA	ctggtcgacTCACTTTAGCTCAGAATA
lpg2604	ctgggatccATGATCATGCTGAAACAA	ctggtcgacTTAGGAACACTGGTTTAA
lpg0107	ctgggatccATGGAATGCTTAAGTTTC	ctggtcgacCTAAAAATGGAAATTCAT

lpg1820	ctgggatccATGATCCTGGTGGTGAGG	ctggtcgacCTATTCCATAGTTAGTTT
lpg1996	ctgggatccATGTTACGTGGTTTAAAA	ctggtcgacTCATAAATTTCGTACCGA
lpg2425	ctgggatccATGGATATGAGAGAAAAA	ctggtcgacTCACATAGGCGCAACAAG
lpg2870	ctgggatccATGATATCCGGTGAGTTA	ctggtcgacTCACAAAAACATATGAAT
lpg0981	ctgggatccATGATGAGAAAATACAGC	ctggtcgacTTACTCATTGAGATACCT
lpg1460	ctgggatccATGAATGTCATTATAAAC	ctggtcgacTTAATCAGCATGCGTCAG
lpg1484	ctgggatccATGCCTTCAATGGAAATG	ctggtcgacTTAGATTCTTAACACTTG
lpg2845	ctgggatccATGACAATGAAAAATGAA	ctggtcgacTCATTTGCTTTTATTAAT
lpg1528	ctgggatccATGAAACGTTATGTTTTT	ctggtcgacTTATATTTTCCTTTGTAA
lpg1436	ctgggatccATGGAATGCCATTTTGTG	ctggtcgacTTAATACTGTGGTTTACA
lpg0065	ctgggatccATGATGTCTTACATGCTT	ctggtcgacCTATTTTTTACGTAATAC
lpg1000	ctgggatccATGAATAAATTGTCTACC	ctggtcgacTTAAAATCTCCCTAAATC
lpg1268	ctgggatccATGAATACGGAGAAACTA	ctggtcgacCTACTTCGCATTTAAGGT
lpg0676	ctgggatccATGCATAAAATCATAAAT	ctggtcgacCTAGGCATAACAGGCAGA
lpg2411	ctgggatccATGTGGATTTTTAAAGAT	ctggtcgacTTAAGTATACGATGGTCC
lpg1465	ctgggatccATGGACAGAAAAATGATT	ctggtcgacTTAATTAAATTGTATAGT
lpg2339	ctgggatccATGTTTTTTATGTATTTT	ctggtcgacTTATAAAATTCTCTCAAT
lpg2356	ctgggatccATGGCGCTTTTTATATTT	ctggtcgacTTATTCCTTTTCTTTGAC
lpg2541	ctgggatccATGCTAATGGAATTCGAA	ctggtcgacTTACATTATTGTACAACG
lpg1482	ctgggatccATGGGTAAGGTAAACGCA	ctggtcgacTTATTTTGAATTAGATCC
lpg2325	ctgggatccATGAGTTATCAAAAACCA	ctggtcgacCTATATTTTGAGTGGCAA
lpg0675	ctgggatccATGAAGCCAGGAAGTCTG	ctggtcgacTCATTCAGGACAGTTGTT

lpg2417	ctgggatccATGATAAAAGATGTCTTT	ctggtcgacTTATATCGAGTAATTAAT
lpg2551	ctgggatccATGACAATAACTACTATA	ctggtcgacCTATCTAATGCTATGTCC
lpg0518	ctgggatccATGACAGACCTTAGAACA	ctggtcgacTTAGTTGAATTGATAAGC
lpg0698	ctgggatccATGTCTTCCAATCCTTCT	ctggtcgacTCAAATTTTTTCATCCTC
lpg2048	ctgggatccATGCTCTTTTATATCCAG	ctggtcgacTTAATGATTAAAAAAATG
lpg0219	ctgggatccATGGAGGTGACCCCTATG	ctggtcgacTTAAGGATTACAATAAGG
lpg2107	ctgggatccATGACTTTGAACCCGCTA	ctggtcgacTCATGCATTTGCTACCCC
lpg2555	ctgggatccATGGAGTTAACCATGGCC	ctggtcgacTTAAATCATTCTATTGAG
lpg2979	ctgggatccATGAATTTCAATATTATT	ctggtcgacTTATTCTTTTTCAAGAGT
lpg2946	ctgggatccATGTTTTACTATCGCGCA	ctggtcgacCTAGATCTCATTGTAAAA
lpg0043	ctgggatccATGGTTAGCTATTTATTT	ctggtcgacCTAAATTGAATTGGATTC
lpg2684	ctgggatccGTGTTTTCGCCTGAAAAA	ctggtcgacTTATAAGTTGAGTAATTT
lpg2757	ctgggatccATGTCTAAATCCATATTT	ctggtcgacTCATGATTGATTATCTGC
lpg0393	ctgggatccATGCTTTCATCAACAAAG	ctggtcgacTTAAACTTTGGGATTCTT
lpg0189	ctgggatccATGAAACCAGGTTATTTA	ctggtcgacTTACAAACAATGTTTATT
lpg0982	ctgggatccATGCAGGAAAAATGCAGA	ctggtcgacTTAACTCGAAAGATCTAT
lpg2011	ctgggatccATGATTCATAGTATGACG	ctggtcgacTTACTCAAAATTCTGAAT
lpg0667	ctgggatccATGTATTCAAATCATTCT	ctggtcgacTTACGCATAATGATTCAT
lpg1685	ctgggatccATGCAATGTGCTATCGAG	ctggtcgacTTAGTAAAAACCTGGGCG
lpg1617	ctgggatccATGGATAAGAACCTAAAA	ctggtcgacTTAGTCATTAATTTCGCA
lpg0044	ctgggatccATGGATTCTCTAGATTTA	ctggtcgacTTAATAAAATTCAACTAT
lpg1951	ctgggatccATGGGGAGCTTCATGACA	ctggtcgacTCATATTTTTTTAGTCAG

lpg2053	ctgggatccATGAAATGGCAAATTCCC	ctggtcgacTTATATGCTTTTTTCAAA
lpg0166	ctgggatccATGGCAGGAACAAGTTTA	ctggtcgacTTACTTCGTATTCATTAG
lpg1016	ctgggatccATGGATTGTCTTGGAGAC	ctggtcgacCTATCTGTTTACTGTGAC
lpg1549	ctgggatccATGGACGAGATCAAAAAA	ctggtcgacTTAGCGCTCTCCAATCGT
lpg1668	ctgggatccATGTCAAGGACGACAAAA	ctggtcgacTCATGATTTATAAGACAA
lpg2874	ctgggatccATGAATATATTTCTAAAG	ctggtcgacTTACAATGTATGAGTATT
lpg1317	ctgggatccATGAAACAAAAAATTGAT	ctggtcgacTTATTTTTTCAAATCAAA
lpg1594	ctgggatccATGCAAGTTATACCTTTC	ctggtcgacCTATAAATCCAAATAACG
lpg1670	ctgggatccATGGGTAGCTCCTATAAT	ctggtcgacTTAAATAGACAAAGTAGG
lpg0889	ctgggatccATGCAAAAAAATAAACAA	ctggtcgacTTATTCCTGTAAAGAGGC
lpg2254	ctgggatccATGCCTGGTCCCCATTCT	ctggtcgacTCATACGGCTACCTTTTC
lpg0432	ctgggatccATGGAATCAATTATGCAT	ctggtcgacTTAATAACGACCTTCAAA
lpg2962	ctgggatccATGGATGAGCAAAAAATG	ctggtcgacTTATTTTTTTATCAATGT
lpg0902	ctgggatccATGACTATGGAACCAGTG	ctggtcgacTTATGCAAAAATTAGTCT
lpg1136	ctgggatccATGAAGAAAATCATTTTA	ctggtcgacTTATTTAAAACGAGAATA
lpg2481	ctgggatccATGACTTCAGTCATGCCT	ctggtcgacCTAGGCTGTCTCAGGTTG
lpg2858	ctgggatccATGACTGACGGAGTTGTA	ctggtcgacTTAGCCACGAGACCTCCG
lpg0030	ctgggatccATGCTCTGTAATGGGAGT	ctggtcgacTCATTTTAAAAAATTAAC
lpg2404	ctgggatccATGGCAACGACATTAATT	ctggtcgacTCATTGAGAGATCACTCC
lpg0017	ctgggatccATGGAATTACTTATGCGC	ctggtcgacTTAACCGGACGTAGCATG
lpg1042	ctgggatccATGGTTAACCTGTCACAT	ctggtcgacTCAATGATGCTTATAAAA
lpg1728	ctgggatccATGTATTTGATGAGAGAA	ctggtcgacTCATTTGAGCACCATTTT

lpg2351	ctgggatccATGCAAAATATTTGGCTT	ctggtcgacTTATAAACCAGGTTTCAA
lpg0064	ctgggatccATGAGCAGTTTGAAAAAT	ctggtcgacTCACGCAGCTTTTTTCAT
lpg2854	ctgggatccATGATGTGGCGGGTTTTA	ctggtcgacTCATTGATACCACCCACG
lpg2462	ctgggatccATGTCAAAACAAGAAAAT	ctggtcgacCTATTTTTCAGCTATTAC
lpg2498	ctgggatccATGACAATAACTCATACA	ctggtcgacTTAATTTAAAAAGCTTAA
lpg1803	ctgggatccATGAGTCAATATACAAGG	ctggtcgacTCATAATTTATTGACCTT
lpg0431	ctgggatccATGCTTATGTCTATGTCG	ctggtcgacTCAATGCTGAAGTATCAT
lpg1498	ctgggatccATGAAAGGCCTATTCATG	ctggtcgacTCACAAACGCTTCTCTGC
lpg2370	ctgggatccATGAAACACTGCCCTATT	ctggtcgacTTAATCAAAAAAATTTAA
lpg0016	ctgggatccATGGCGCCAGTGCTTATG	ctggtcgacCTAACCATCGGCGCATAA
lpg0191	ctgggatccATGATATCCCAAAAAGAA	ctggtcgacTTATTTTAACTGAGGCTT
lpg1656	ctgggatccATGTATCATTTGATACGG	ctggtcgacTTATTTATATTCACTGAC
lpg1152	ctgggatccATGGCGCTTATCTATCAG	ctggtcgacTTATTGCTGGATTGTTTT
lpg2847	ctgggatccATGCTAAAGCGGTGCTAC	ctggtcgacCTAGGGAAACTGCTTATT
lpg2950	ctgggatccATGGAATATGGCAACCTT	ctggtcgacTTAAATGTAATAAAATTT
lpg1101	ctgggatccATGACTACGTCGGTGTCT	ctggtcgacCTACCTCATTGCAAGCTT
lpg1137	ctgggatccATGATTCAAAGAGGATTT	ctggtcgacTTATTTCTTGGTAGGGGA
lpg0620	ctgggatccATGAAATCAAGAGAAAAA	ctggtcgacTTATAAAAAGGGATTACT
lpg0988	ctgggatccATGACTAATACTGTCTTT	ctggtcgacCTATCGTTTTTGAGGTTG
lpg1238	ctgggatccATGTCCTCACCACCTGAT	ctggtcgacTTATCGGTAAAAATTTAT
lpg0160	ctgggatccATGAACTTAAAAAAAGAA	ctggtcgacTTATAAATTATTTAGCCT
lpg2827	ctgggatccATGGATATGGATTTTTGC	ctggtcgacTTAATAAAAAATCTCAGC

lpg0200	ctgggatccATGTTACCGTTTATTTTC	ctggtcgacCTAGTGATAATTTTCTGA
lpg1738	ctgggatccATGAAAATAAAGTTTTTG	ctggtcgacCTAATTAAATCGGTACTC
lpg1104	ctgggatccATGAATCAAAAGCAAATA	ctggtcgacTCAAGCTAAATGAAAATG
lpg2085	ctgggatccATGAAAGCCTTTTTTATT	ctggtcgacTTAAAAGGCGCAACAATT
lpg0804	ctgggatccATGTTTAAACGCATAGGA	ctggtcgacTTATCCTATCATGGGTTG
lpg1189	ctgggatccATGGAAAGAAGGACAAAT	ctggtcgacTCACCAAAAGGCATTAAT
lpg0195	ctgggatccATGGAGAATGATATGCCT	ctggtcgacTTACATTTTTTTTGATAG
lpg1817	ctgggatccATGATTCCTTTTGGGAAG	ctggtcgacCTATCCCAAATTAGTAAC
lpg0926	ctgggatccATGACTAGATTAGAAGAG	ctggtcgacTTAATCCCCATGGCCATT
lpg2171	ctgggatccATGAGCACTGATAATTTA	ctggtcgacTCAAAATAGTCCTTGTGT
lpg0716	ctgggatccATGTTTGATGATACTAAT	ctggtcgacTCAGCAGAGAGATAATTC
lpg1822	ctgggatccATGGTATGCAGGGAGGAA	ctggtcgacCTAGAATCTTAAACCACA
lpg1316	ctgggatccATGCACGAATGCGTGATA	ctggtcgacCTAATAACTTTTTCGCAG
lpg0033	ctgggatccATGGGATGGACTGATCGA	ctggtcgacTTACTGTAATGAATGGCT
lpg1084	ctgggatccATGAATATGCCTGAACTA	ctggtcgacCTAATTTTTCTGTTTGAT
lpg1489	ctgggatccATGCGTAGAAGTAAAGCA	ctggtcgacTTAATGATGCAAGGAGGT
lpg0767	ctgggatccATGAACCCATTAAATGGT	ctggtcgacTTATCCTTGTAAAATTGT
lpg1481	ctgggatccATGAAAACTATTTTTGCA	ctggtcgacTTATAGCTTTTGTCTATT
lpg2435	ctgggatccATGATAACTAACAACATA	ctggtcgacTTATTCAGCTAACTCAAG
lpg2952	ctgggatccATGCCAAGAGCTAGGTTC	ctggtcgacTTAACCAACAGACATGGA
lpg1944	ctgggatccATGCTATACTTGCTGATA	ctggtcgacTTAAATTAACTGATTCAA
lpg0439	ctgggatccATGGCCGATCATTCCCCC	ctggtcgacTTATGCGAAGGGATTTAT

lpg2210	ctgggatccATGACGAATGATCATTAC	ctggtcgacTTATTTTGCCTGATTAAT
lpg0267	ctgggatccATGTCAATATCATTTAAG	ctggtcgacCTAAAACCACTTCTTTTT
lpg0134	ctgggatccATGCGAATAGCGACATTA	ctggtcgacCTAAGGTTGTTTTAAAAT
lpg0564	ctgggatccATGAAACATACTTACAAT	ctggtcgacCTATCGAATATCAAAAAG
lpg2942	ctgggatccATGAATAAAAAAAGCATT	ctggtcgacCTAAACAATATAACTTGC
lpg0739	ctgggatccATGTCAAGACCTACACGT	ctggtcgacTTATTTGTAACGTACTCT
lpg0670	ctgggatccATGCATCTTTGCCTTATA	ctggtcgacTTATAAATATTCATCCAG
lpg0059	ctgggatccATGCAGTTCTGCCAGATG	ctggtcgacCTACCTTTGGAGTAGAAC
lpg0227	ctgggatccATGTCATATTCAATAGAT	ctggtcgacTCAGTATAAGATTCGTCT
lpg1661	ctgggatccATGAATACTGTTCCTTTA	ctggtcgacTTATATTCGAATAAATAC
lpg0514	ctgggatccATGAAACATTTTGATTTT	ctggtcgacTTAATTTTGAGCTAACTT
lpg2219	ctgggatccATGGAGAAAGACAGGATG	ctggtcgacCTACGAAAGAGTTGAGGT
lpg0527	ctgggatccATGGAAGTCTTTATGATC	ctggtcgacTCAGTTACTCATGATTTT
lpg0279	ctgggatccATGATGAAAATTGAATCA	ctggtcgacTTATGGACTCTCTAACAG
lpg1423	ctgggatccATGATTAATTTATGGCCA	ctggtcgacTTAATCCCCTTCCAAACC
lpg1190	ctgggatccATGAAAGCGAAAGTTATT	ctggtcgacTTATACCAATGCCAATAA
lpg0096	ctgggatccATGCCTAAAGTTATTATT	ctggtcgacCTATGAATTATGGAGTGG
lpg1994	ctgggatccATGAAATCGAAATTAATT	ctggtcgacCTACTTCAACTGTGCCAA
lpg0178	ctgggatccATGAACAGGCCCGTTGTC	ctggtcgacCTAAAATGTTTGGATAAC
lpg0113	ctgggatccATGGTTGTAGAATTGGAT	ctggtcgacTTATTTATTAATAATGTC
lpg2731	ctgggatccATGAGCAATACAGAATAC	ctggtcgacTTAATTACCCTTAAACAA
lpg0742	ctgggatccATGAGTGTCTGGCTATTT	ctggtcgacTCATGTCATTTTAGAAGC

lpg0378	ctgggatccATGTCTTCTTACAACGAA	ctggtcgacTCAGGGGGTAAATTGTTC
lpg0955	ctgggatccATGGCATTATTTTCTTCT	ctggtcgacTTAGATGGATTCAGACTC
lpg1263	ctgggatccATGAAAAAAAGTATAAAA	ctggtcgacCTAATCAGAATTAATTTT
lpg0886	ctgggatccATGTGTGCAGCACACCAC	ctggtcgacTTAACCTAACTCGGCATT
lpg2811	ctgggatccATGGAGCTAACAATTATG	ctggtcgacTCATACCGTTTTCCTTTT
lpg1621	ctgggatccATGCACACAAAAAAGGAT	ctggtcgacTTAGTTTATTTTAAGTTT
lpg0081	ctgggatccATGCGGTCTAAATTTTTT	ctggtcgacCTAGAGTTTAATGCTCGA
lpg0077	ctgggatccATGAAAAGGGAAATTGAT	ctggtcgacTTATTCGCCACGCATCAA
lpg2525	ctgggatccATGAAAGAAAAATACAAA	ctggtcgacTTATAAAATAGAACAAAA
lpg2927	ctgggatccATGGAGGTTGTCTTGTTT	ctggtcgacTCAGGCACCGCGTTTTGC
lpg0625	ctgggatccATGTGCTATCATTTAATC	ctggtcgacCTAAGCCTTATTAGCCGA
lpg2080	ctgggatccATGAAAAAAATTCTTCTG	ctggtcgacTCACCTCATTTGATTAGC
lpg0809	ctgggatccATGTTCAAACCCCAAGGA	ctggtcgacTTATTTTTTCTGTGAAAA
lpg0176	ctgggatccATGAGCGCACAAGTTGCA	ctggtcgacTCATAACTGCGCCAACTC
lpg1810	ctgggatccATGAATTGGGATGTTATT	ctggtcgacTTATTTAGTCGGTGCTGG
lpg2644	ctgggatccATGATACATCGAAATAAA	ctggtcgacTTAAAAGGCTCTTACAGC
lpg0276	ctgggatccATGATAAATAAAAGTCGT	ctggtcgacTTACACGGTAATCATAAC
lpg2958	ctgggatccATGCATCGGGATTGCGTC	ctggtcgacCTAATAAACCGACTGATC
lpg2812	ctgggatccATGAGAAAAAAGCCTCTA	ctggtcgacTCAATTAGTTTCCGCTGT
lpg2225	ctgggatccATGAATTGGAAAAGGTTT	ctggtcgacTTATCTCTCAGCAATAAC
lpg0900	ctgggatccATGGCTTTTAAACTCTGG	ctggtcgacTCAATGGATTAGCTCTTT
lpg1469	ctgggatccATGAGTAAGGTTATTCAC	ctggtcgacTCATATTTTTTCTCCTTT

lpg1718	ctgggatccATGATTATTTTATATGAT	ctggtcgacTCAAAAAAACTTGCTTTC
lpg1702	ctgggatccATGTCACTTGCTACTTAT	ctggtcgacTTAATGCCGGACGGCTGG
lpg1615	ctgggatccATGACTAATCGTTTGCCC	ctggtcgacTTATTCTCCTTTTTCATT
lpg0686	ctgggatccATGTTTATTAGTAACAGT	ctggtcgacTTAGTTCTCATCAATGCT
lpg1855	ctgggatccATGTTGCAGAAGTTAAAT	ctggtcgacCTATTTCCTTTCTATTTT
lpg1183	ctgggatccATGTGCATTGAGAAAATA	ctggtcgacTTATGATTTCATGACATA
lpg2815	ctgggatccATGCTATTGATAATCATT	ctggtcgacTCATTTGCCAGAAGAGAG
lpg0690	ctgggatccATGGCAGCACCATTGGCT	ctggtcgacCTACCATTTCAGCAAGGG
lpg0221	ctgggatccATGAGTAGCCACATCACA	ctggtcgacTCACTTAATTACAGACCA
lpg1739	ctgggatccATGATTAATAAAGAGGAC	ctggtcgacTTACTTGCTTCTATGAAC
lpg1116	ctgggatccATGATGCGATATTTATTA	ctggtcgacTTACTCACAAACACCATT
lpg0724	ctgagatctATGCGCGACTCACTTAAG	ctggtcgacTTACCTTGTTTTTATAAT
lpg0984	ctgggatccATGATTAAACATTATATC	ctgctcgagTCATAGTCGACTTCCAAT
lpg2149	ctgagatctATGATGAGTCGCGAAAAA	ctggtcgacTTAAGAGTAGTCGGGTGC
lpg2436	ctgagatctATGACTCGAAATTCAGTG	ctggtcgacCTACTCTATAGTCATTTC
lpg1011	ctgagatctATGAATAATATTAAATAT	ctggtcgacTTATAATGCGTTTAATAC
lpg2569	ctgagatctATGTCCAAATCTAACAAT	ctggtcgacTTAATCCATCAAGTTCTT
lpg2902	ctgagatctATGTTTTACGGCGATACA	ctggtcgacCTACAAATTTTGCAAGGT
lpg0882	ctgagatctATGACACTGCAACGTTCC	ctggtcgacTTAGTTAAAGCACAAACA
lpg1207	ctgggatccATGCCAGTTAAAAAGCTT	ctgctcgagCTATAGCGTCACCAATGT
lpg0119	ctgagatctATGGGAAAGAATATGGTG	ctggtcgacCTAAACAAGGAGACGCCA
lpg0582	ctgagatctATGACAGGTCTTTATTGT	ctggtcgacCTAAATTTCAACGCCTAC

lpg2017	ctgggatccATGGAAAAAGCTATGAAA	ctgctcgagTCAATTATTTCCGATGGA
lpg1663	ctgagatctATGATTATGTTTGAGTCT	ctggtcgacTTAAACTGAGGCCGAGTT
lpg1499	ctgagatctATGACAAACGTGAGTTTC	ctggtcgacTTATTGATTTAAATTTTC
lpg0410	ctgggatccATGAAAAAACCATATAAA	ctgctcgagTTATCCGCTCTTAATTTC
lpg2150	ctgggatccATGTCATGCTATATTTTG	ctgctcgagTTAAAGTCTCTGGTTCAT
lpg0993	ctgggatccATGTTTGAATATTGGAAA	ctgctcgagTTAATGAGTTTCATGGGA
lpg0285	ctgagatctATGGTTATCATTGGAATA	ctggtcgacTCAAATTCGATGAAACTC
lpg0264	ctgagatctATGTTTTTACAAGTTATT	ctggtcgacTTATGACGCAAGGACTAA
lpg0172	ctgggatccATGCTTTACAATAAATGG	ctgctcgagTTAGGATAGTGTTGGAAT
lpg2953	ctgggatccATGGAGCATTATCAGGAA	ctgctcgagTTATCCTGGTTTCAGACC
lpg0585	ctgagatctATGATTAAGCGATGGCTA	ctggtcgacTTATTTGGTGGTCCCAGA
lpg2106	ctgggatccATGCATGAATTACTTAAA	ctgctcgagTTATTGAGCATTATTGCC
lpg2182	ctgagatctATGATACTGGAGAATGAA	ctggtcgacTTATTTGATAGCCTCTAA
lpg0516	ctgagatctATGAGGAATCACTACATG	ctggtcgacTCATATTGTAATCCCCTG
lpg2419	ctgggatccATGAATAAAAAGTTGAGT	ctgctcgagTTACACTGTATACTTAAA
lpg2999	ctgagatctATGAAAACTAAATCATGG	ctggtcgacTTAGGTTTCAGGGTACAT
lpg2409	ctgagatctATGACAAACAGTAACCAT	ctggtcgacTCATTTTCGATATTTGTT
lpg0992	ctgggatccATGGCTACACCCGTCCTG	ctgctcgagTCATCGGCTGTACCTCGC
lpg2803	ctgagatctATGGATAAATTATACAGC	ctggtcgacTTAATTCCTCAAATCGGG
lpg1102	ctgagatctATGGTAATCACAATGTCC	ctggtcgacTTATATAGGTTCTTTTTT
lpg0871	ctgagatctATGAGATTCAATCATTTT	ctggtcgacTCAAGCTTGCGGTTGTAA
lpg2505	ctgagatctATGATAAAAGGAAAACTT	ctggtcgacTTATAAAATAATTGGTCG

lpg2327	ctgagatctATGGCAAAATTCACCGTA	ctggtcgacTTATTTGGGTGATGAAGC
lpg0181	ctgagatctATGTATAGTAAATATCCA	ctggtcgacCTAATCATAAACTGGCTT
lpg1928	ctgggatccATGTTTCATGACTTGTTG	ctgctcgagTTAAGTTTTTTTTGAAAA
lpg2143	ctgagatctATGATTTTGAATATTACG	ctggtcgacTTACGGTCTAAAAATCTC
lpg0247	ctgagatctATGCGTGTATTTTATGGC	ctggtcgacTTACCGTTGATGACAAAC
lpg1982	ctgggatccATGGTCGACATACGCTCT	ctgctcgagCTAAATAAATTCGATTTT
lpg0256	ctgagatctATGGTTCCCGTGGGCATT	ctggtcgacTTACATCTCATTCAACCT
lpg2917	ctgagatctATGAATCAGAAATCCTTT	ctggtcgacCTAGGCAGGATATTTAAA
lpg0151	ctgagatctATGAAAAAGATAATCCTA	ctggtcgacTTAGCCTGAAAAACTAAT
lpg2241	ctgagatctATGAAACAAAAGGAATTA	ctggtcgacTTATTCATGAAGGGCACG
lpg0187	ctgagatctATGAATGGATTTCATTTT	ctggtcgacTTAAGTTACATTGCTTGA
lpg0770	ctgagatctATGTCAAATACTGTTTTA	ctggtcgacTTATTTAATCAGGCAATC
lpg0205	ctgagatctATGACGACACTGTGTTTT	ctggtcgacTCAGAGATTGGCAGAATC
lpg0265	ctgagatctATGACCAATACAACAGAT	ctggtcgacTTAATTGTTATTTTTGGA
lpg0968	ctgggatccATGTCTTTTATCAAGGTA	ctgctcgagTTAAAGGCTTAGGCTTTC
lpg0597	ctgggatccATGATGACAAAAACAAAT	ctgctcgagTTATACTGTTTCCATGGT
lpg1859	ctgagatctATGTCTAATGAAAATGAA	ctggtcgacTTAATGTGCGTGTAAATC
lpg2079	ctgggatccATGAGTGAAATGGCTGTT	ctgctcgagTCATTTTACGTCCTTGTT
lpg2244	ctgagatctATGCATAAAGTCATATCT	ctggtcgacTTAGGTTGGTTTCACTAT
lpg0644	ctgagatctAGCACGACGGTGACCACC	ctggtcgacTCAAGGCCGGAATCCACC
lpg2077	ctgagatctATCGCCGAAGGTTTTGAA	ctggtcgacCTAACGATCCATTTCTAT
lpg2087	ctgggatccATGGATGCAGCAGTCACA	ctggtcgacTTAAACATAGCCATACAC

lpg2504	ctgggatccATGACTAAAATATACTTA	ctggtcgacTCAAAATACCAGTATCGA
lpg1479	ctgggatccATGCGCCAACTATTACGA	ctggtcgacTCATTCTTCGGTGGTTTG
lpg0519	ctgaagcttATGATAATATACTCTTCG	ctggcatgcTTAGCCTAAAAGGGAACT
lpg0957	ctgtctagaATGAAAATGGAACTTCTG	ctgctgcagTCATTTTACAACTCCTTC
lpg1909	ctgtctagaATGAATCACATGGCAATC	ctgctgcagTTAATATGGGCACTTGGC
lpg2767	ctgtctagaATGAAGCTATTCTCTTTT	ctgctgcagTTACTGCTGATAGCTAAC
lpg2806	ctgtctagaATGGGAATTACAAAAGAC	ctgctgcagTTAAATTTTAGGGACATC
lpg2078	ctgtctagaATGATGGCGGTAAATCCT	ctgctgcagTTATAAAAACTCAACAAG
lpg0198	ctgtctagaATGAACATTGTAAGAATA	ctggtcgacTTAAGCCGTGGCAGCCTG
lpg0135	ctgtctagaACAACGCTTAAAGCACCC	ctggtcgacTTACAATTTGGTAAATTC
lpg3000	ctgggatccATGGTCTACTCAAAACAAAG	cgtgtcgacCTAATTGGGAGTGGCTAAGG
lpg2976	ctgagatctATGAGTATTACAGCCCAGGA	cgtgtcgacTTAGGGCAAAATTGTAATTA
lpg2975	ctgcccggggATGGGTTTGCCCAAAAAAGC	cgtgtcgacTTAGACCACCAAGCTCGTAA
lpg2959	ctgggatccATGAAGTTTAAAAAAATTAT	cgtgtcgacTTAGAAATAAACACCAATTT
lpg2939	ctgagatctATGAAAATGAATATTTTTCA	cgtgtcgacTCATTTTTTCAAACTATCCA
lpg2913	ctgggatccATGAAAATTTCTACTCATGA	cgtgtcgacTTAAACCCCAAATGTCTGCC
lpg2912	ctgggatccATGTCGTATTCAAAAATCGA	cgtgtcgacTTAGATGGAAGCTCTTTCAC
lpg2907	ctgggatccATGAAAAAAAGTATAAAAAA	cgtgtcgacCTAATCAGAATTAATTTTAT
lpg2888	ctgggatccATGGCAACCAGTAGTTTAAT	cgtgtcgacTTATATGCTGGGATTGTTTT
lpg2879	ctgggatccCTGTTGCAGGAGAGCATTAT	cgtgtcgacTTATTTAGTCATGCTCAGTT
lpg2877	ctgggatccATGAATCAATCGTTATCACC	cgtgtcgacTTAATTCCTCTCTGTTTGCA
lpg2864	ctgggatccGTGATTATGAATTTAAAACA	cgtgtcgacTTATAAATTCACTCTCTTAT

lpg2862	ctgggatccATGAGCGAACAATATTGGCG	cgtgtcgacTTATTTTGATAATCGTGCTT
lpg2856	ctgggatccATGGATCAAGCAAAGGGGGA	cgtgtcgacTCAGCTATCATGACTTAAGC
lpg2853	ctgggatccGTGGTATCAATGAAGAAATT	cgtgtcgacCTAAAATGGATTAATAGGAG
lpg2844	ctgggatccATGGCAGAAACTCCAAAACC	cgtgtcgacCTATCCAGGCATAGACTGAC
lpg2832	ctgggatccATGACTAATAAAACTCAAGA	cgtgtcgacTCATTCCTGGCTCAAGTGTG
lpg2828	ctgggatccATGAAAATTAGTGAATTAAA	cgtgtcgacCTAATTTCTTAGTAAAGGAT
lpg2826	ctgggatccATGCTTATGATCATGGCAAG	cgtgtcgacTTATAAATTGTTTCTGTACA
lpg2804	ctgagatctATGTATAAAATATGTACTTA	cgtgtcgacCTAACATAAGCTATGGATTG
lpg2759	ctgggatccATGCTTGAGGAGAAAGGGAT	cgtgtcgacCTAATTTGGTTCTTCAATGA
lpg2758	ctgggatccATGAAAAAAAAGCATTTGAT	cgtgtcgacTTATTGAATAGGATAAGCCA
lpg2745	ctgggatccATGCTTGTTGGTTCAATTTG	cgtgtcgacTCAGGTCGGAGCGCCTATAT
lpg2744	ctgggatccATGTCAAATCTAATAGAACT	cgtgtcgacTCAACGTAACGCAGCACTTG
lpg2719	ctgggatccATGAGATTATTATCTTTTAA	cgtgtcgacTTAAGAAGGTTGTGTCATCC
lpg2718	ctgggatccATGCCAAAACGTCTCATTAA	cgtgtcgacTCATTTGCCCAGAGACGCCT
lpg2638	ctgagatctATGAAATATGCTGTTACATT	cgtgtcgacCTACGTTGCCAAAGGTTGTG
lpg2637	ctgggatccATGTTTTTAACCATAAATGA	cgtgtcgacCTAGTGTAGCCCGTTGGAAT
lpg2627	ctgggatccATGCAAGAAATTGATGTCCT	cgtgtcgacCTACACTACTTGTCCGGCAG
lpg2622	ctgggatccATGAGTAAGTTAATTAATTT	cgtgtcgacTTAAGCTGCTTTTTCTTTCA
lpg2603	ctgggatccTTGTATTCTGATTTTTTTTT	cgtgtcgacCTAAATGCTTACATTAGGGC
lpg2582	ctgggatccATGTCGGTCGAGATGAGCAA	cgtgtcgacTCATATTTTTATATCCAATA
lpg2568	ctgggatccATGAATATCACGCATATTAT	cgtgtcgacTCAAGAATGTTTATCCCAAA
lpg2552	ctgggatccATGAAAGAAATAGTTAGTTT	cgtgtcgacTTATGGCGAGGGGGATGTCA

lpg2546	ctgggatccTTGGAGACGAAAATGAGCAT	cgtgtcgacTTATAAGTTTAGAGTTCTTG
lpg2538	ctgggatccTTGATTCCAGTTCCGGTGAT	cgtgtcgacTTAACGAAGAATTAATCTAT
lpg2529	ctgggatccGTGGTGAGGGTTTTGAATTT	cgtgtcgacTTACATTGCAGCACGCTTAG
lpg2527	ctgggatccTTGTTATATTTTGTACTATG	cgtgtcgacCTATTGTAAACGAGCGCTTT
lpg2526	ctgggatccTTGAGATTGTTTCAGTACAA	cgtgtcgacTTACTGAGGACCCGATTTTT
lpg2523	ctgggatccATGAAAACCAAACTAAACAA	cgtgtcgacTTATAGTTTAACCCACATAT
lpg2508	ctgggatccATGTTTAGTTATCTGGATAA	cgtgtcgacTCATAAAGGCGACTGCGATG
lpg2464	ctgggatccATGAGCATAATGGGGAGAAT	cgtgtcgacTTATTTTATCTTAATGGTTT
lpg2456	ctgagatctATGTTGACTCCTCCGCCTGA	cgtgtcgacTTAAATGGTTTTCCCTGGTT
lpg2433	ctgggatccTTGGACATCCATATGACGCC	cgtgtcgacCTAGGGGAGTGATGTAAGGC
lpg2424	ctgggatccATGAAAGAAATTATCGAGTT	cgtctcgagTTAACCTAAAAATAAAGATT
lpg2423	ctgggatccATGAATAGAATTAAAACAAC	cgtgtcgacTCAACTAGCATTTTGGCATT
lpg2422	ctgagatctATGAGCACATCAGAGAAAGA	cgtctgcagTTAGTTCAATTTAACCTGAC
lpg2410	ctgggatccATGAAAACAAAGCAAGAAGT	cgtgtcgacTTAATTCGGTTTATTATTGG
lpg2406	ctgggatccATGAAAAAGAAAGACGAGCA	cgtgtcgacTCATTGTCCCAGGGAAGTAA
lpg2400	ctgggatccATGAATTACAAGCTAACATT	cgtgtcgacTCAGAGAGGTTTGCAGGTTT
lpg2399	ctgcccggggATGAAAAAATGGATTATCAG	cgtgtcgacCTAATTCAATAACGCTGGAT
lpg2385	ctgcccggggATGATAGAAATGGTTCAATT	cgtgtcgacTTAGTTCAACTGCATTCCTA
lpg2382	ctgggatccATGGAAAATAAAACAATGGC	cgtgtcgacTTATACCTTATTCAAAGAAG
lpg2372	ctgggatccTTGAAGGTTAAATATAGCCT	cgtgtcgacTTACTGCCCATGGTCAATGA
lpg2313	ctgagatctATGTCGCCATCAGAATCAGG	cgtctcgagTTAAACCAACAAAAATCGAA
lpg2248	ctgggatccATGGCGAAGACAATTAAGGC	cgtgtcgacCTAAGGAACATATCTGTTGG

lpg2242	ctgagatctATGCCAAGCGTGCAGCAGGA	cgtgtcgacTTAAAATATCGTGCCATCAC
lpg2239	ctgagatctATGAAAATGAACGAATATAT	cgtgtcgacCTAGAGGCCCGCTGCTGAAA
lpg2223	ctgagatctATGGTGGATGAGAAAAATTA	cgtgtcgacTTATTGAGGTAGCAGGCTAT
lpg2220	ctgagatctATGAAAAAAATTGTTTACGT	cgtgtcgacCTACTGCTGACTTTCAGGGA
lpg2207	ctgagatctATGATTATTTATCTGCTATG	cgtgtcgacTTATAGCCCTAGCCACCGAA
lpg2206	ctgagatctATGAACCGCATGGCACTTTT	cgtgtcgacCTAGCTGTGTGTAATATGCA
lpg2184	ctgagatctATGGCTAGTTGTGAATGGAT	cgtgtcgacCTAACTCAATGTATGAAAGG
lpg2166	ctgagatctATGATTGATATGATTTCAAT	cgtgtcgacTTACATCAAATTTTGAAGGG
lpg2161	ctgagatctATGTTTGCCGAAATACCCAA	cgtgtcgacTCAAACCTGGTTTTGTAACA
lpg2160	ctgcccggggCTGTTCTCAGCTAATCAATC	cgtgtcgacTTACATAGGACTTGCAACAC
lpg2148	ctgggatccTTGATTGTGCGTGGCATTAA	cgtgtcgacTTAGCTTGGCCCCTTTTTAT
lpg2147	ctgggatccATGACAACTTCCAAGCTTGA	cgtgtcgacTCACTTATCACGAAGAACTA
lpg2076	ctgggatccATGGCCAAACTAAATCCAAT	cgtgtcgacCTATTTACCTTTAGTAACTA
lpg2073	ctgggatccATGAGAATATTGAATAAGAG	cgtgtcgacTTACCTGCTATTTTTATCAA
lpg2060	ctgggatccATGGAATTTTGTAGGAATTT	cgtgtcgacTTAGAAGCTGTTTATTGAGA
lpg2050	ctgggatccATGAAATCCAAACTTGAATA	cgtgtcgacTTAAAATGGGATATACAGCT
lpg1986	ctgggatccATGCCAAAATTTTCACTTCA	cgtgtcgacTTATTGGCCTCTCATAGGTA
lpg1979	ctgggatccATGAAAACCTTAACGACCCG	cgtgtcgacTCATCGCTGGATTACAGGAT
lpg1969	ctgggatccATGGAGTCTGTATTTTCAGG	cgtgtcgacTTAGTAGCCGTAATTTACTC
lpg1966	ctgggatccATGATAAAGGACATTCTCAA	cgtctcgagTTAGTTTCTGCAAAACCCAA
lpg1965	ctgagatctTTGCTGAAATTTTACTCAGA	cgtgtcgacTTATTTATTCTCTGGAGTGA
lpg1964	ctgggatccATGAAAATTTACAGTATAGT	cgtgtcgacTTAGGTAAAAGTATAGGATA

lpg1963	ctgggatccATGCAAGAAAAAATTATCAA	cgtgtcgacCTAGATATTCGTAATTCCTT
lpg1961	ctgagatctTTGTTATGCGAGAGTTTCAT	cgtgtcgacTCAGTATGGTTTTTCCCCAT
lpg1959	ctgggatccATGTTAGTTTCCAATACAAT	cgtgtcgacTTAAATGGATACCCTATGAT
lpg1958	ctgggatccCTGTTTCAAATAGGAGAGCA	cgtgtcgacTTAGCATTTCAATAGCGATG
lpg1953	ctgggatccTTGATTCATTATGTATCCTT	cgtgtcgacTTATAGCTTAATATCAAAAG
lpg1949	ctgggatccATGGCTATGGAAGACGAATC	cgtgtcgacCTAATTTATTTTATGAGACT
lpg1934	ctgggatccATGGTGAGGATGAAAATGAT	cgtgtcgacCTAGAGCTTTATTGAAGTAA
lpg1930	ctgggatccTTGTACCCATTCATCCTTAA	cgtctcgagCTAGCGTTTAAAGCCTGAAA
lpg1925	ctgggatccATGGACGTTGGCCAAAGAT	cgtctgcagTTAAACTTTTACTGTCATTC
lpg1924	ctgggatccATGCCTTTAAATTTACCGCC	cgtgtcgacCTATTGTAGATTAGGTTTTG
lpg1918	ctgggatccATGAAAATATTTAAGTTTAG	cgtgtcgacTTAATTAAAATAGGGCTTCA
lpg1907	ctgggatccATGGTTAGGGAGTGTACAAG	cgtgtcgacCTAGCAAAGCCTTTGATGCT
lpg1901	ctgagatctATGGTTCACTTTCAAAAAAT	cgtgtcgacTCATTGATCATGAGAAATTT
lpg1890	ctgggatccATGATCTTTGAGTTAAATAT	cgtgtcgacCTATTTGGGGATTGCCGTT
lpg1888	ctgggatccATGCTCCAATTTTTTGAGAA	cgtgtcgacTCAAGAACAAACAAAACAGG
lpg1881	ctgggatccTTGGGCCAAATGGCAATGTT	cgtgtcgacTCAACGTGAAAAAGGAGAAC
lpg1798	ctgggatccATGCCACCACCATATAGTAC	cgtgtcgacTTAAGTCAGCTTTGCACTAT
lpg1797	ctgggatccATGTTTAAATTTATTTATTG	cgtgtcgacCTACCTCAGGGAATTATGAT
lpg1751	ctgggatccATGACTTTTACTCCGCCCGA	cgtgtcgacTCAAACAAATAAAGACGTAG
lpg1717	ctgagatctATGCCAGTTCGTTATTCAGG	cgtgtcgacTTAAATGGCCAATGCAGTAG
lpg1705	ctgggatccATGGTTAAATTATATAAGGA	cgtgtcgacTCATTTTATTAGATCCTCTA
lpg1692	ctgggatccATGGCACGCAGCAAAGAAGA	cgtgtcgacCTAACTCGGACCACTCGTAC

lpg1687	ctgggatccGTGTCCCATAATTATTTTCG	cgtgtcgacTTATTTATTGTTCAAACAAC
lpg1684	ctgagatctATGAAATCTGTTGATGAAAT	cgtgtcgacTCACGGTTTTGTAACCATAT
lpg1683	ctgggatccATGAAAGGCAAGTTAACAGG	cgtgtcgacCTATTTTACCTTAATGCCAC
lpg1667	ctgggatccCTGGAATACAAGGAGGTCAC	cgtgtcgacTTAGGTTCCCAAATCGAAAC
lpg1666	ctgggatccATGCTAAAATTTGTCATTTA	cgtgtcgacCTAATAAAATGAACATTGTT
lpg1665	ctgcccggggATGAACAAAAACCCATTCTC	cgtctcgagTTAATCTTCTTCTCTAGACA
lpg1660	ctgcccggggATGTTAACTTTATCACTTGC	cgtgtcgacCTAATGGAGATGGACACTAT
lpg1654	ctgagatctATGATGGATGAAGTAAAGAG	cgtgtcgacTTATGGGCGCAAATCGACAT
lpg1639	ctgagatctTTGGCAGGTATATACTTAAA	cgtgtcgacCTAATCTCCAATAATAGAGG
lpg1603	ctgagatctATGATTGTTATCTTCGTCCA	cgtgtcgacTCAATCTATCTTTTTCCCAG
lpg1602	ctgagatctATGCGCGTTGAACTATTTAA	cgtgtcgacTCAGCCGAGAATATTCTTGA
lpg1598	ctgcccggggATGTGGTATGACACTCTATT	cgtgtcgacTTAAAGCTTTCTTACGCATA
lpg1588	ctgggatccATGTCTCATTCCAAAAGAGA	cgtgtcgacTTATTTTGGTAATGAACCGA
lpg1496	ctgggatccATGGTTACGAAAATAATTTG	cgtgtcgacTCAAATACCATATTGATTTG
lpg1491	ctgggatccGTGATAGCCATATTAAATTT	cgtgtcgacTTATGGTTTAAAATTTTTTA
lpg1488	ctgggatccATGAAAGAGCAACAAAAGGC	cgtgtcgacCTAACAGTTTTTTTGACTCA
lpg1449	ctgggatccATGAAAGAAAAAATGATATT	cgtgtcgacTTAAGGAGTGAAGACAGATT
lpg1426	ctgggatccATGACCCCACAGCACGTCAT	cgtgtcgacTCATACGCCAAGGCCAAACC
lpg1403	ctgggatccATGAGTTTATATATCGGTTT	cgtgtcgacTCAATATGATTTTATTATAG
lpg1387	ctgggatccATGATTGAAGTAAATATTTG	cgtgtcgacTTAAGCACCTCGGGTAGGAG
lpg1368	ctgcccggggATGAAAGCAAGAAGGGATCA	cgtgtcgacCTACCCTACTGAAGGCAACC
lpg1354	ctgggatccATGCCAAAATTAATTTTATT	cgtgtcgacTCAATTTTTTAATAGCCTTA

lpg1329	ctgggatccTTGCTCATCACATTTCATAA	cgtgtcgacTTATCCATGAAATAAATAGG
lpg1326	ctgggatccATGCTGTATATAAGAAATTA	cgtgtcgacTTAAAAATTCAGTATGGTTT
lpg1318	ctgcccggggATGAACAAAATACTGCTTTT	cgtgtcgacCTACTCCATATAATCTACCG
lpg1290	ctgggatccATGCCTCAAATCCTAAATGC	cgtgtcgacTTAAATAGGAAATCCTCTTG
lpg1273	ctgggatccATGAAAATTTTTAAAGGATT	cgtgtcgacTTAACTCATTATAGATGCTG
lpg1269	ctgggatccATGATTATGTTTTTAGTGTC	cgtgtcgacTTAAGAAATTATTGTTGATT
lpg1265	ctgggatccTTGTTGGGTGTTTATGGAAT	cgtgtcgacTTATCTACTGAGACTCACAC
lpg1234	ctgggatccATGAAAATATACTCATTTGA	cgtgtcgacTTAGCTCTTTGAGAATTCAC
lpg1228	ctgggatccATGAACGAATTATCACTTCA	cgtgtcgacTTAATTTTGGCTTTCATTAT
lpg1227	ctgggatccATGAAAACGGTAAATAGTCA	cgtgtcgacTTACGCCCTATATAGGGATG
lpg1169	ctgggatccTTGTTCCGGAGTGTTGTTGT	cgtgtcgacTTATTTTATTATTTCTGTTG
lpg1166	ctggagctcAATTGCTTTCTCAAGGAGTGAG	cgtgtcgacTTAAAAGTTCAGTAATTCCT
lpg1154	ctgggatccATGATGAGAATGTTTAGTGT	cgtgtcgacTTAAAGGCCAGGCACAAATT
lpg1151	ctgggatccCTGACTAACATTGTCTATAT	cgtgtcgacTTATTGTTTCATTCCTGGTT
lpg1148	ctgggatccCTGTATATGGATTTAGGGAG	cgtgtcgacCTATGTTTTAGGACCGCTTG
lpg1145	ctgggatccTTGGCTGATTCCAGATCATC	cgtgtcgacTTATAGACCAAAATCCGGAT
lpg1129	ctgggatccATGCCTACTGGAATCGTATT	cgtgtcgacCTAGCCTCCTTTGGTTGGAT
lpg1120	ctgggatccATGACAACACCACTATTCCC	cgtgtcgacTTAGTTTTTAGTCTTAGTTT
lpg1109	ctgggatccATGAAATATTTTTTAAAACT	cgtgtcgacTTAAAGACTAAAGGACGCTT
lpg1106	ctgagatctATGCGAAATAATAAAATGGG	cgtgtcgacTTATAGTTTGGCCGAGGTTA
lpg1103	ctgggatccATGGTTATGAAAACGATGCG	cgtgtcgacTTATTTATTCATTGCTTCTT
lpg1074	ctgggatccATGTCCAAACGTGTTACTAC	cgtgtcgacTTACTCTGATAAATCAAATT

lpg1073	ctgggatccCTGTCTAAAAAACAAAGGGT	cgtgtcgacCTAGCCATCTTTGATTGCCT
lpg1032	ctgagatctATGGACAAGCAAGACATCGA	cgtgtcgacTTAATCGGGATCCTTATAGA
lpg0974	ctgggatccATGAATTTAGTGCATGAAAT	cgtgtcgacTCATTTGTCTTCTATAAGTT
lpg0963	ctgggatccATGCGCTATATACTGCCAGC	cgtctcgagTCAAATAGTTGCTAACAATG
lpg0956	ctgggatccATGAAAACTGCCCATCTTAT	cgtgtcgacTTAATCCACTAACTGACTAA
lpg0944	ctgggatccATGGCTATTGCCCCCCAACA	cgtgtcgacTTATGTGAGGTTAGGTCTGG
lpg0941	ctgggatccATGCCTGATTTTGAAACTAC	cgtgtcgacTTAGATAGTCAGTGTATGTT
lpg0921	ctgggatccATGACTATAGAAAGGGAATT	cgtgtcgacTTAAGTTCCATTATAAACCA
lpg0788	ctgagatctCTGTTATTTATGTCTGATAT	cgtgtcgacTCATGCTTTGTTCTCATTAT
lpg0774	ctgggatccATGAACAAATATGGTGGTGC	cgtgtcgacTTAATTAAAGTTTTTCAAGG
lpg0771	ctgggatccTTGATAACAAATATGAAATC	cgtgtcgacTTAAACGTCTGAGTTTTTGA
lpg0766	ctgggatccGTGAAAATTCTTGTTACTGC	cgtctcgagTTAAGGTTCTCCTGTTAATA
lpg0733	ctgggatccTTGGACAAGGAACAAGGAGC	cgtctcgagTTAAATAGATCTTATAGAGA
lpg0696	ctgggatccATGAAGTTACGCTATATTAT	cgtgtcgacCTATTTCAATTCATTTTTAT
lpg0695	ctgggatccTTGGTAAAAATTATGCCAAA	cgtgtcgacTTACCATTTTAATTTCAAGG
lpg0684	ctgggatccATGGCTAGTTGTGAATGGAT	cgtgtcgacCTAACTCAATGTATGAAAGG
lpg0647	ctgggatccATGGCTAGTTGTGAATGGAT	cgtgtcgacCTAACTCAATGTATGAAAGG
lpg0645	ctgggatccATAAGTGGATTAGGTGGAAA	cgtgtcgacTTACTGTTCTACTATCAAGT
lpg0642	ctgagatctATGAAAACTCAGCGAATTAT	cgtgtcgacCTATTTCAAGTTGAATTGTG
lpg0634	ctgggatccTTGGAGACTAACATGACGTT	cgtgtcgacTTATACGGTCGGTTTGCTGG
lpg0550	ctgggatccATGAGCATTAATAAAATAGA	cgtgtcgacTTAAGTGTATTTCAATCGCT
lpg0519	ctgggatccGTGATAATATACTCTTCGTC	cgtcccgggTTAGCCTAAAAGGGAACTAA

lpg0483	ctgggatccATGGATTTTGTAAGTGAAAT	cgtgtcgacTTATTTTAGGACAACTCGTG
lpg0437	ctgggatccATGCAAAACTTAGATGAGAT	cgtgtcgacTTAGCACCCATAAACAGTTC
lpg0365	ctgggatccGTGGTTGATTTTGATAAACC	cgtgtcgacTCAAGGTGAAATCATTGTTG
lpg0284	ctgagatctATGTTCACAAAATCCTGTGC	cgtgtcgacTTAAAATTTAAATAAATTCT
lpg0269	ctgggatccTTGGTGAATCAAAACGAATC	cgtgtcgacCTAGTTCCTTTTAATCTTAA
lpg0259	ctgggatccATGAAAAGGGAATTTTTTCA	cgtgtcgacCTATATCAACTCAATATCAA
lpg0258	ctgagatctATGAAAATTCCATTTGAAGG	cgtgtcgacTTAAAACCGTAAGTTAAGCA
lpg0236	ctgagatctATGCGTTATACTAATATTGA	cgtgtcgacTTAAAACGTAAAATTCCATG
lpg0209	ctgagatctATGTTAAGTAAAGAAAAAAT	cgtgtcgacTTACAACTTTAACTGGCTAG
lpg0203	ctgggatccATGCCATTAGATAAGTTAAT	cgtctcgagTTAATAATCATTACACTCTC
lpg0196	ctgggatccTTGTTGCTTGGAGATTTTAT	cgtgtcgacTTAACTTATGGTTAAACCTA
lpg0150	ctgggatccGTGAGTTTAGCTTTGCCTGA	cgtgtcgacTTATTTTGTAACAAACTGGA
lpg0149	ctgggatccATGGCATACACGAAATGGAC	cgtgtcgacTCAATTGCCAGTTTTAATAA
lpg0148	ctgggatccATGAGCCAAGATAGTTTTCA	cgtgtcgacTCATTTCCCATTGGGGGATT
lpg0140	ctgggatccATGACACCGATTATTAGTCA	cgtgtcgacTTAAAAGGTGAACGCTGGAA
lpg0130	ctgggatccATGGGAAAAGGTATAATTTT	cgtgtcgacTTACACATAAACCAAGCCAC
lpg0112	ctgggatccTTGTTTATTTATAATTCATG	cgtgtcgacTTAAAGACTAGGATTTAAAA
lpg0110	ctgggatccATGATTCAGTTAGGCGATTT	cgtgtcgacTCAGCCAAGTTGATGTTCGC
lpg0090	ctgggatccATGAAGCAGTTAATAAGTAT	cgtgtcgacCTACATTATACTTTCAAAGT
lpg0086	ctgggatccCTGATAGCCCCATACCTATA	cgtgtcgacCTAAGCTAAACCCAGAATTG
lpg0084	ctgggatccATGAAAAATAACAAACGTCA	cgtgtcgacTCATTTCATCGCACTCGCAA
lpg0062	ctgcccggggTTGATTAGAATTGGGACATA	cgtgtcgacTTAAATACTATTTGAAAAAA

lpg0041	ctgggatccATGCTGCTTTGTTCACTTGC	cgtctcgagTTATAAAATACTGGGGATAG
lpg0012	ctgggatccATGAACACAACGGAACATAC	cgtgtcgacTCAGACTCCCAAATTCAACC
lpg0008	ctgggatccATGACGTTTACTTGCGATGA	cgtgtcgacTTACTTGCCAAATAACCCAC

Table 2. Characteristics of Dot/Icm substrates identified in this study

	Lpg number	Size (base pair)	Translocation Efficiency (%)	Putative motif/domain
1	Lpg0021	480	5%	N/A
2	Lpg0046	399	5%	N/A
3	Lpg0130	2496	80%	N/A
4	Lpg0172	726	10%	N/A
5	Lpg0181	912	50%	N/A
6	Lpg0260	399	5-10%	N/A
7	Lpg0364	306	5%	N/A
8	Lpg0375	600	5-10%	N/A
9	Lpg0405	591	5-10%	N/A
10	Lpg0716	1014	50%	N/A
11	Lpg0796	651	50%	N/A
12	Lpg0967	594	50-60%	N/A
13	Lpg1083	684	30%	N/A
14	Lpg1106	1275	50%	N/A
15	Lpg1124	591	5-10%	N/A
16	Lpg1137	969	10-20%	Translocase of the mitochondrial inner

				membrane (TIM)
17	Lpg1147	504	50%	N-acetyltransferase super family
18	Lpg1171	420	80%	N/A
19	Lpg1449	2613	50%	DUS-FNM phosphate-binding super family[1]
20	Lpg1453	519	5-10%	N/A
21	Lpg1484	810	20-50%	N/A
22	Lpg1578	450	5-10%	N/A
23	Lpg1639	1317	20%	N/A
24	Lpg1654	1119	40%	Tetratricopeptide repeat
25	Lpg1661	1119	5-10%	Acyltransferase family
26	Lpg1666	1404	50%	N/A
27	Lpg1667	1392	80%	IgA Peptidase M64[2]
28	Lpg1670	891	5-10%	N/A
29	Lpg1684	1398	80%	D123 super family[3]
30	Lpg1685	870	40%	N/A
31	Lpg1692	1311	80%	N/A
32	Lpg1716	459	20%	N/A

33	Lpg1776	648	5-10%	N/A
34	Lpg1803	936	20%	Rho_GEF[4]
35	Lpg1888	1332	5%	PLDc Super family[5]
36	Lpg1907	1806	90%	Lipase Family
37	Lpg1924	2793	80%	N/A
38	Lpg1959	1995	70%	N/A
39	Lpg1986	2853	90%	TraI_TIGR Domain[6]
40	Lpg2050	1059	10-20%	PKC_like family, CRIK subfamily[7]
41	Lpg2148	1281	90%	N/A
42	Lpg2149	360	20-30%	N/A
43	Lpg2223	1224	80%	N/A
44	Lpg2239	3858	80%	N/A
45	Lpg2271	651	80%	N/A
46	Lpg2359	444	5-10%	N/A
47	Lpg2370	939	20%	HipA Super family[8]
48	Lpg2372	1269	5%	N/A
49	Lpg2382	1455	15%	N/A
50	Lpg2434	492	5%	Cupin super family
51	Lpg2443	558	5-10%	N/A

52	Lpg2461	639	5-10%	N/A
53	Lpg2505	888	20-30%	N/A
54	Lpg2508	2424	10%	N/A
55	Lpg2538	1416	75%	N/A
56	Lpg2539	408	20%	N/A
57	Lpg2546	1410	10-20%	N/A
58	Lpg2555	855	30%	HAD-like superfamily[9]
59	Lpg2628	753	50%	N/A
60	Lpg2637	1212	75%	N/A
61	Lpg2692	531	5-10%	N/A
62	Lpg2745	1968	60%	N/A
63	Lpg2826	1734	90%	Peptidase C26
64	Lpg2832	1641	40%	N/A
65	Lpg2844	1086	90%	N/A
66	Lpg2885	555	20%	N/A
67	Lpg2888	1914	10-20%	N/A
68	Lpg2912	1488	80%	N/A
69	Lpg2936	735	5-10%	RNA methyltransferase super family
70	Lpg3000	1839	50%	N/A

Notes:

[1] DUS-FNM phosphate-binding super family: Dihydrouridine synthase-like (DUS-like) FMN-binding domain

[2] IgA Peptidase M64: Highly selective metallo-endopeptidases that cleaves IgA

[3] D123 superfamily: This family contains a number of eukaryotic D123 proteins

[4] Rho GEF: Rho GTPase Guanine exchange factor

[5] PLDc family: Phospholipase D family

[6] TraI TIGR Domain: Conjugative transfer relaxase protein TraI

[7] PKC like super family: Protein Kinase C superfamily

[8] HipA family: High-frequency-persistence mutants (Hip) A family

[9] HAD like superfamily: Haloacid dehalogenase-like hydrolases superfamily

[10] ZnMc superfamily: Zinc-dependent metalloprotease, astacin like subfamily or peptidase family M12A

Table 3. Experimentally confirmed protein substrates of the Dot/Icm transporter

	Lpg number	Alias	Size	Reference
1	Lpg0008	ravA	1260	[251]
2	Lpg0012	CegC1	1575	[41]
3	Lpg0021	N/A	480	This study
4	Lpg0030	ravB	915	[251]
5	Lpg0038	LegA10	1521	[35]
6	lpg0041	N/A	2187	[41]
7	Lpg0045	N/A	210	[252]
8	Lpg0046	N/A	399	This study
9	Lpg0059	Ceg2	1107	[42]
10	Lpg0080	Ceg3	765	[42]
11	Lpg0081	N/A	1326	[252]
12	Lpg0090	Lem1	4125	[42]
13	Lpg0096	Ceg4	1194	[42]
14	Lpg0103	VipF	858	[108]
15	Lpg0126	CegC2	3306	[41]
16	Lpg0130	N/A	2496	This study
17	Lpg0160	ravD	978	[251]
18	Lpg0170	ravC	807	[251]
19	Lpg0171	LegU1	564	[35]
20	Lpg0172	N/A	726	This study

21	Lpg0181	N/A	912	This study
22	Lpg0191	Ceg5	954	[42]
23	Lpg0195	ravE	1005	[251]
24	Lpg0196	ravF	1275	[251]
25	Lpg0210	ravG	624	[251]
26	Lpg0227	Ceg7	1104	[41]
27	Lpg0234	SidE	4542	[29]
28	Lpg0240	Ceg8	777	[42]
29	Lpg0246	Ceg9	723	[253]
30	Lpg0260	N/A	399	This study
31	Lpg0276	LegG2	1497	[35]
32	Lpg0284	Ceg10	1122	[254]
33	Lpg0285	Lem2	684	[42]
34	Lpg0294	N/A	693	[42]
35	Lpg0364	N/A	306	This study
36	Lpg0365	N/A	2688	[252]
37	Lpg0375	N/A	600	This study
38	Lpg0376	SdhA	4287	[29]
39	Lpg0390	VipA	1056	[108]
40	Lpg0401	Ceg11	675	[251]
41	Lpg0402	LegA9	1701	[35]
42	Lpg0403	LegA7	672	[255]
43	Lpg0405	N/A	591	This study

44	Lpg0422	LegY	1347	[35]
45	Lpg0436	LegA11	807	[35]
46	Lpg0437	Ceg14	2001	[42]
47	Lpg0439	Ceg15	1050	[251]
48	Lpg0483	LegA12	1485	[35]
49	Lpg0515	LegD2	930	[35]
50	Lpg0518	N/A	846	[252]
51	Lpg0519	Ceg17	2214	[42]
52	Lpg0621	SidA	1422	[29]
53	Lpg0634	N/A	1347	[252]
54	Lpg0642	WipB	1572	[20]
55	Lpg0695	LegA8	2847	[35]
56	Lpg0696	Lem3	1713	[42]
57	Lpg0716	N/A	1014	This study
58	Lpg0733	ravH	1449	[251]
59	Lpg0796	N/A	651	This study
60	Lpg0898	Ceg18	729	[41]
61	Lpg0926	ravI	1011	[251]
62	Lpg0940	LidA	2190	[256]
63	Lpg0944	ravJ	1176	[251]
64	Lpg0945	LegL1	888	[35]
65	Lpg0963	N/A	1242	[252]
66	Lpg0967	N/A	594	This study

67	Lpg0968	SidK	1722	[38]
68	Lpg0969	ravK	741	[251]
69	Lpg1083	N/A	684	This study
70	Lpg1101	Lem4	969	[42]
71	Lpg1106	N/A	1275	This study
72	Lpg1108	ravL	882	[251]
73	Lpg1109	ravM	2061	[251]
74	Lpg1110	Lem5	696	[42]
75	Lpg1111	ravN	639	[251]
76	Lpg1120	Lem6	1767	[42]
77	Lpg1121	Ceg19	771	[42]
78	Lpg1124	N/A	591	This study
79	Lpg1129	ravO	1560	[251]
80	Lpg1137	N/A	969	This study
81	Lpg1144	CegC3	504	[41]
82	Lpg1145	Lem7	2310	[42]
83	Lpg1147	N/A	504	This study
84	Lpg1148	N/A	1512	[252]
85	Lpg1152	ravP	857	[251]
86	Lpg1154	ravQ	1083	[251]
87	Lpg1158	N/A	768	[252]
88	Lpg1166	ravR	2013	[251]
89	Lpg1171	N/A	420	This study

90	Lpg1183	ravS	1893	[251]
91	Lpg1227	VpdB	1794	[30]
92	Lpg1273	N/A	1065	[252]
93	Lpg1290	Lem8	1587	[42]
94	lpg1312	legC1	3405	[35]
95	Lpg1316	ravT	1023	[251]
96	Lpg1317	ravW	888	[251]
97	Lpg1328	LegT	2463	[35]
98	Lpg1355	SidG	2922	[29]
99	Lpg1426	VpdC	884	[30]
100	Lpg1449	N/A	2613	This study
101	Lpg1453	N/A	519	This study
102	lpg1483	LegK1	1587	[35]
103	Lpg1484	N/A	810	This study
104	Lpg1488	LegC5	2595	[35]
105	Lpg1489	ravX	1032	[251]
106	Lpg1491	Lem9	1242	[42]
107	Lpg1496	Lem10	1797	[42]
108	Lpg1551	ravY	747	[251]
109	Lpg1578	N/A	450	This study
110	Lpg1588	LegC6	2019	[35]
111	Lpg1598	Lem11	1062	[42]
112	Lpg1602	LegL2	1284	[35]

113	Lpg1621	Ceg23	1317	[254]
114	Lpg1625	Lem12	393	[42]
115	Lpg1639	N/A	1317	This study
116	Lpg1642	SidB	1251	[29]
117	Lpg1654	N/A	1119	This study
118	Lpg1660	LegL3	1479	[35]
119	Lpg1661	N/A	1119	This study
120	Lpg1666	N/A	1404	This study
121	Lpg1667	N/A	1392	This study
122	Lpg1670	N/A	891	This study
123	Lpg1683	ravZ	1509	[251]
124	Lpg1684	N/A	1398	This study
125	Lpg1685	N/A	870	This study
126	Lpg1687	mavA	1203	[251]
127	Lpg1689	N/A	624	[252]
128	Lpg1692	N/A	1311	This study
129	Lpg1701	LegC3	1680	[35]
130	Lpg1702	PpeB	1638	[162]
131	Lpg1716	N/A	459	This study
132	Lpg1717	N/A	1689	[252]
133	Lpg1718	LegAS4	1635	[35]
134	Lpg1751	N/A	1311	[252]
135	Lpg1752	N/A	648	[251]

136	Lpg1776	N/A	648	This study
137	Lpg1797	rvfA	1281	[251]
138	Lpg1798	marB	1197	[251]
139	Lpg1803	N/A	936	This study
140	Lpg1851	Lem14	663	[42]
141	Lpg1884	YlfB	1215	[37]
142	Lpg1888	N/A	1332	This study
143	Lpg1890	LegLC8	1722	[35]
144	Lpg1907	N/A	1806	This study
145	Lpg1924	N/A	2793	This study
146	Lpg1933	Lem15	615	[42]
147	Lpg1947	Lem16	750	[42]
148	Lpg1948	LegLC4	1005	[35]
149	Lpg1949	Lem17	1341	[42]
150	Lpg1950	RalF	1122	[28]
151	Lpg1953	LegC4	2283	[35]
152	Lpg1958	LegL5	1629	[35]
153	Lpg1959	N/A	1995	This study
154	Lpg1960	LirA	771	[36]
155	Lpg1962	LirB	564	[36]
156	Lpg1963	LirC	2100	[36]
157	Lpg1964	LirD	1311	[36]
158	Lpg1965	LirE	2967	[36]

159	Lpg1966	LirF	1566	[36]
160	Lpg1969	PieE	1908	[162]
161	Lpg1972	PieF	375	[162]
162	Lpg1976	LegG1	858	[35]
163	Lpg1978	SetA	1932	[253]
164	Lpg1986	N/A	2853	This study
165	Lpg2050	N/A	1059	This study
166	lpg2131	LegA6	540	[35]
167	Lpg2137	LegK2	1614	[35]
168	Lpg2144	LegAU13	516	[35]
169	Lpg2147	mavC	1449	[251]
170	Lpg2148	N/A	1281	This study
171	Lpg2149	N/A	360	This study
172	Lpg2153	SdeC	4599	[29]
173	Lpg2155	SidJ	2619	[257]
174	lpg2156	SdeB	5178	[36]
175	Lpg2157	SdeA	4518	[29]
176	Lpg2166	Lem19	1260	[42]
177	Lpg2176	LegS2	1824	[35]
178	Lpg2199	mavD	633	[251]
179	Lpg2200	CegC4	534	[41]
180	Lpg2215	LegA2	1593	[35]
181	Lpg2216	Lem20	1770	[42]

182	Lpg2223	N/A	1224	This study
183	Lpg2224	PpgA	1779	[162]
184	Lpg2239	N/A	3858	This study
185	Lpg2248	Lem21	2235	[42]
186	Lpg2271	N/A	651	This study
187	Lpg2298	YlfA	1275	[37]
188	Lpg2300	LegA3	1401	[35]
189	Lpg2311	Ceg28	3504	[251]
190	Lpg2322	LegA5	1923	[35]
191	Lpg2327	N/A	891	[252]
192	Lpg2328	Lem22	384	[42]
193	Lpg2344	mavE	627	[251]
194	Lpg2351	mavF	921	[251]
195	Lpg2359	N/A	444	This study
196	Lpg2370	N/A	939	This study
197	Lpg2372	N/A	1269	This study
198	Lpg2382	N/A	1455	This study
199	Lpg2391	sdbC	1305	[251]
200	Lpg2392	LegL6	885	[35]
201	Lpg2400	LegL7	1065	[35]
202	Lpg2406	Lem23	1098	[42]
203	Lpg2407	N/A	306	[252]
204	Lpg2409	Ceg29	816	Zusman, 2007 #477]

205	Lpg2410	VpdA	1998	[30]
206	Lpg2411	Lem24	828	[42]
207	Lpg2416	LegA1	1107	[35]
208	Lpg2420	N/A	552	[251]
209	Lpg2422	Lem25	2622	[42]
210	Lpg2424	mavG	1347	[251]
211	Lpg2425	mavH	807	[251]
212	Lpg2433	Ceg30	1761	[42]
213	Lpg2434	N/A	492	This study
214	Lpg2443	N/A	558	This study
215	Lpg2444	mavI	615	[251]
216	Lpg2452	LegA14	2766	[35]
217	Lpg2456	LegA15	1413	[35]
218	Lpg2461	N/A	639	This study
219	Lpg2464	SidM	1944	[258]
220	Lpg2465	SidD	1521	[29]
221	Lpg2490	LepB	3882	[32]
222	Lpg2498	mavJ	933	[251]
223	Lpg2504	SidI	2899	[39]
224	Lpg2505	N/A	888	This study
225	Lpg2508	SdjA	2421	[257]
226	Lpg2509	SdeD	1195	[29]
227	Lpg2510	SdcA	2727	[29]

228	Lpg2511	SidC	2751	[29]
229	Lpg2523	Lem26	2340	[42]
230	Lpg2525	mavK	1355	[251]
231	Lpg2526	mavL	1368	[251]
232	Lpg2527	N/A	1677	[252]
233	Lpg2529	Lem27	1719	[42]
234	Lpg2538	N/A	1416	This study
235	Lpg2539	N/A	408	This study
236	Lpg2541	N/A	834	[259]
237	Lpg2546	N/A	1410	This study
238	Lpg2552	N/A	1668	[251]
239	Lpg2555	N/A	855	This study
240	Lpg2556	LegK3	1386	[35]
241	Lpg2577	mavM	759	[251]
242	Lpg2584	SidF	2736	[29]
243	lpg2588	LegS1	1255	[35]
244	Lpg2591	Ceg33	495	[41]
245	Lpg2603	Lem28	1305	[42]
246	Lpg2628	N/A	753	This study
247	Lpg2637	N/A	1212	This study
248	Lpg2638	mavV	1383	[251]
249	Lpg2692	N/A	531	This study
250	Lpg2694	LegD1	858	[35]

251	Lpg2718	WipA	1560	[20]
252	Lpg2720	LegN	1029	[35]
253	Lpg2744	N/A	1170	[252]
254	Lpg2745	N/A	1968	This study
255	Lpg2793	LepA	3453	[32]
256	Lpg2804	Lem29	1407	[42]
257	Lpg2815	mavN	2052	[251]
258	Lpg2826	Ceg34	1734	[42]
259	Lpg2828	N/A	1257	This study
260	Lpg2829	SidH	6675	[29]
261	Lpg2830	LegU2	738	[35]
262	Lpg2831	VipD	1863	[108]
263	Lpg2832	N/A	1641	This study
264	Lpg2844	N/A	1086	This study
265	Lpg2862	LegC8	1911	[35]
266	Lpg2874	N/A	885	[251]
267	Lpg2879	mavO	1752	[251]
268	Lpg2884	mavP	738	[251]
269	Lpg2885	N/A	555	This study
270	Lpg2888	N/A	1914	This study
271	Lpg2912	N/A	1488	This study
272	Lpg2936	N/A	735	This study
273	Lpg2975	mavQ	2616	[251]

274	Lpg2999	LegP	798	[35]
275	Lpg3000	N/A	1839	This study

Table 4. Strains used in this thesis

Bacterial Strains	Plasmid	Bacterial Strains	Plasmid
XL1Blue	pZL507::lpg1667	XL1Blue	p4xFlag::lpg0898 A151S
XL1Blue	pQE30::lpg1667	XL1Blue	pGEX6P1::lpg0160
XL1Blue	pGBKT7::GFP-lpg0898	XL1Blue	pGEX6P1::lpg0514
XL1Blue	pGBKT7::lpg1667	XL1Blue	pGEX6P1::lpg1183
XL1Blue	pGBKT7:: lpg0898 ΔC40	XL1Blue	pGEX6P1::lpg0096
XL1Blue	pGBKT7:: lpg0898 ΔC60	XL1Blue	pSB157m::lpg0898 A151S
XL1Blue	pGBKT7:: lpg0898 ΔC80	XL1Blue	pSB157m::lpg0898 A156S
XL1Blue	pGBKT7:: lpg0898 ΔC20	XL1Blue	P424 GPD::lpg0898
XL1Blue	pQE30:: lpg0898 ΔC60	XL1Blue	pQE30::lpg0096
XL1Blue	pQE30:: lpg0898 ΔC40	XL1Blue	pQE30::lpg1183
XL1Blue	pQE30:: lpg0898 ΔC20	XL1Blue	pQE30::lpg0160
XL1Blue	pQE30:: lpg0898 ΔC80	XL1Blue	pQE30::lpg1661
XL1Blue	pEGFPC1::lpg0898 A151S	XL1Blue	pQE30::lpg0514 5E4 means lpg0514
XL1Blue	pGBKT7::lpg0898 C156S	XL1Blue	pZL507::lpg1661
XL1Blue	pEGFPC1::lpg0898	XL1Blue	pQE30:: lpg0898

	C156S		ΔN20
XL1Blue	pGEX6P1::lpg0898 ΔC20	XL1Blue	pQE30:: lpg0898 ΔN40
BL21	pQE30:: lpg0898 ΔC20	XL1Blue	pQE30:: lpg0898 ΔN60
XL1Blue	p4xFlag::lpg0898 C156S	XL1Blue	pQE30:: lpg0898 ΔN80
XL1Blue	pGEX6P1:: lpg0898 ΔN40	XL1Blue	pGEX6P1:: lpg0898 ΔN20
XL1Blue	pGBKT7:: lpg0898 ΔN20	XL1Blue	pEGFPC1::lpg0716
XL1Blue	pLV-CMV-lpg0898-GFP	XL1Blue	pEGFPC1::lpg0969
XL1Blue	pLV-CMV-lpg0898-GFP A151S	XL1Blue	4xFlag::lpg0969
XL1Blue	pGBKT7:: lpg0898 ΔN40	XL1Blue	4xFlag::lpg0969 H95A
XL1Blue	pGBKT7:: lpg0898 ΔN60	XL1Blue	pET28::Sumo:lpg2831 VipD
XL1Blue	pGBKT7:: lpg0898 ΔN80	XL1Blue	pET28::Sumo:lpg2831 VipD S73A
XL1Blue	pEGFPC1::lpg0422	XL1Blue	pET28::Sumo:lpg2831 VipD D288A
XL1Blue	pQE30-GST-Annexin	XL1Blue	pGEX6P1::sidK Δ1-7aa
XL1Blue	pGEX6P1::lpg1661	XL1Blue	pXDC61M:lpg0324 rpsL WT

XL1Blue	pEGFPC1::lpg1483	XL1Blue	pXDC61M:rpsL K88R
XL1Blue	pEGFPC1::lpg2482	DH5α	pSR47S::+-300bp of rpsL WT for knock in purpose
XL1Blue	pXDC61M::SidC	DH5α	pSR47S::+-300bp of rpsL K88R
XL1Blue	pEGFPC1::lpg2144	DH5α	pSR47S::+-300bp of rpsL K43N
XL1Blue	pEGFPC1::lpg2139	DH5α	pSR47S::+-300bp of rpsL K43R
XL1Blue	pEGFPC1::lpg1328	XL1Blue	pEGFPC1::rpsL WT
XL1Blue	pEGFPC1::lpg2155	XL1Blue	pEGFPC1::rpsL K88R
XL1Blue	pEGFPC1::lpg2298	XL1Blue	pMSCV2.2-GFP-Kozak sequence-RpsL WT
XL1Blue	pEGFPC1::lpg2515	XL1Blue	pMSCV2.2-GFP-Kozak sequence-RpsL K88R
XL1Blue	pEGFPC1::lpg1701	XL1Blue	pMSCV2.2-rpsL K88R
XL1Blue	pEGFPC1::lpg2588	XL1Blue	pQE30::lpg1298
XL1Blue	pEGFPC1::lpg2157	XL1Blue	pQE30::lpg2283
XL1Blue	pEGFPC1::lpg2452	XL1Blue	pQE30::lpg1171
XL1Blue	pEGFPC1::lpg2391	XL1Blue	pQE30::lpg0191
XL1Blue	pEGFPC1::lpg1850	XL1Blue	pGEX6P1::lpg0898

XL1Blue	pQE30-GST-lpg0096	XL1Blue	pGEX6P1::lpg1803
XL1Blue	pQE30-GST-lpg1661	XL1Blue	pGEX6P1::lpg1171
XL1Blue	pQE30-GST-lpg0518	XL1Blue	pGEX6P1::lpg0190
XL1Blue	pEGFPC1::lpg0275	XL1Blue	pGEX6P1::lpg2283
XL1Blue	pGEX6P1-6xHis-lpg0518	XL1Blue	pGEX6P1::lpg1798
XL1Blue	pGEX6P1-6xHis-lpg0160	XL1Blue	pGEX6P1::RhoA
XL1Blue	pQE30-GST-lpg1183	XL1Blue	pGEX6P1::lpg0081
XL1Blue	pEGFPC1::Annexin II (human)	XL1Blue	pGEX6P1::lpg0195
XL1Blue	pQE30:::Annexin II (human)	XL1Blue	pGEX6P1::lpg1551
XL1Blue	pGADGH::Annexin II (human)	XL1Blue	pGEX6P1::lpg1183
XL1Blue	P4xFLAG::Annexin II (human)	XL1Blue	pEGFPC1::lpg2079
XL1Blue	pQE30-GST- Annexin II (human)	XL1Blue	pEGFPC1::lpg2577
XL1Blue	pSR47s::Δlpg0130	XL1Blue	pEGFPC1::lpg1084
XL1Blue	pJB908FF::lpg0898	XL1Blue	pEGFPC1::lpg1582
XL1Blue	pQE30::lpg0898	XL1Blue	pEGFPC1::lpg0898
XL1Blue	pEGFPC1::lpg0191	XL1Blue	pEGFPC1::lpg1803
XL1Blue	pEGFPC1::lpg1171	XL1Blue	pGBKT7::lpg1667

XL1Blue	pEGFPC1::lpg2283	XL1Blue	pGEX6P1::lpg1667
XL1Blue	pEGFPC1::lpg1633	XL1Blue	pEGFPC1:lpg1798 1-269
XL1Blue	pEGFPC1::lpg1489	XL1Blue	pEGFPC1:lpg1667
XL1Blue	pZL507::lpg1667	XL1Blue	pQE30:: lpg1798 1-269
XL1Blue	pZL507::GFP-lpg1667	XL1Blue	pGEX6P1:: lpg1798 1-269
XL1Blue	pEGFPC1::lpg1667	XL1Blue	Mouse short Bcl-2 ORF
XL1Blue	pSB157::Flag lpg0898	XL1Blue	Mouse long Bcl-2 ORF
XL1Blue	pQE30::lpg1667	XL1Blue	MGC Human BCLAF1 Sequence-Verified cDNA
XL1Blue	pJB2581::lpg0767	XL1Blue	MGC Human BCL2L1 Sequence-Verified cDNA
XL1Blue	pJB2581::lpg1489	XL1Blue	Incyte Partially Sequenced Human SRI cDNA
XL1Blue	pJB2581::lpg2815	XL1Blue	pEGFPC1-Sorcin
XL1Blue	pJB2581::lpg0439	XL1Blue	4xFlag::Bcl-XL
XL1Blue	pJB2581::lpg0926	XL1Blue	pGAPDH::Sorcin
XL1Blue	4XFlag::lpg1798	XL1Blue	pEGFPC1::lpg0898 ΔC20
XL1Blue	4XFlag::lpg1667	XL1Blue	pEGFPC1::lpg0898 ΔC40

XL1Blue	pEGFPC1::cdc42	XL1Blue	pEGFPC1::lpg0898 ΔN60
XL1Blue	pEGFPC1::lpg1798	XL1Blue	pEGFPC1::lpg0130
XL1Blue	pGBKT7::GFP-lpg0898	XL1Blue	pJB908FF::lpg0130
XL1Blue	pEGFPC1::lpg0898 ΔC60	DH5α	pSR47s::lpg0898 +-1000bp +Gentamycin
XL1Blue	pEGFPC1::lpg0898 1-80	XL1Blue	pEGFPC1::lpg2831 VipD
XL1Blue	pEGFPC1::lpg0898 1-60	XL1Blue	pEGFPC1::lpg2831 VipD 1-380
XL1Blue	pEGFPC1::lpg0898 1-40	XL1Blue	pEGFPC1::lpg2831 VipD 381-613
XL1Blue	4XFlag::Bcl-XL	XL1Blue	pGEX6P1::lpg2831 VipD 1-380
XL1Blue	pEGFPC1::lpg0898 ΔC60	XL1Blue	pGEX6P1::lpg2831 VipD 381-613
XL1Blue	Shuttle plasmid for Adeonvirus lpg0898 A151S	XL1Blue	pGEX6P1::lpg2831 VipD
XL1Blue	Shuttle plasmid for Adeonvirus lpg0898 WT	XL1Blue	pGEX6P1::lpg2831 VipD S73A
XL1Blue	pEGFPC1::lpg0898 ΔN20	XL1Blue	pGEX6P1::lpg2831 VipD D288A
XL1Blue	4xFlag::Bcl-2 Long Human	XL1Blue	pEGFPC1::lpg2831 VipD S73A
XL1Blue	4xFlag::Bcl-2 Short Human	XL1Blue	pEGFPC1::lpg2831 VipD D288A

XL1Blue	pERFPC1::sorcin 2	XL1Blue	pET28::Sumo:lpg1625
XL1Blue	4XFlag::sorcin	DH5α	pSR47s::Δlpg1803
XL1Blue	pYESNTA::lpg0898 A151S	DH5α	pSR47s::Δlpg2176
XL1Blue	pGADGH:Bcl-2 Long Human	DH5α	pSR47s::Δlpg1625
XL1Blue	pJB908::5xFlag::lpg0898	DH5α	pSR47s::Δlpg0716
XL1Blue	pLKO-puro	DH5α	pSR47s::Δlpg2831
XL1Blue	pET28::Sumo:lpg0898	XL1Blue	pEGFPC1::lpg0898 A151S
XL1Blue	pEGFPC1::lpg2176	XL1Blue	pEGFPC1::lpg1803
XL1Blue	pEGFPC1::lpg1625	W303	pSB157::lpg1803
XL1Blue	pZL507::rpsL WT	W303	pYESNTA::lpg2283
XL1Blue	pZL507::rpsL K88R	W303	pYESNTA::lpg1171
XL1Blue	pET28::Sumo::rpsL WT	Y381	p157m::lpg0898
XL1Blue	pET28::Sumo::rpsL K88R	Y381	p157m::lpg0898
XL1Blue	pET28::Sumo::rpsL K43N	Y381	p157m::SidL
XL1Blue	pET28::Sumo::rpsM	W303	pYESNTA::lpg1803
XL1Blue	pHR`-CMV-R8.20vpr	W303	pYESNTA::lpg0191
XL1Blue	pCMV-VSVG	W303	pYESNTA::lpg2283

XL1Blue	pLV-CMV	W303	pYESNTA::lpg1171
XL1Blue	pLV-CMV-GFP	W303	pSB157::lpg0898
XL1Blue	pLV-CMV-GFP-RpsL WT	W303	pSB157::lpg1171
XL1Blue	pLV-CMV-GFP-RpsL K88R	W303	pSB157:: lpg1803
XL1Blue	pLV-CMV -RpsL WT	W303	pSB157:: lpg2283
XL1Blue	pLV-CMV -RpsL K88R	W303	pSB157:: lpg0191
XL1Blue	pMSCV2.2	W303	pSB157:: Flag lpg0898
XL1Blue	pVSV-G from Russel Lab	W303	pYESNTA::lpg0191
XL1Blue	pMSCV2.2-rpsL WT	W303	pYESNTA::lpg1803
XL1Blue	pET28::Sumo::rpsL D89A	Lp02 Δlpg0716 lpg0898 lpg1803	pJB908
XL1Blue	pET28::Sumo::rpsL K88P	Lp02 ΔsidF lpg0130	pJB908
W303	pSB157::lpg0191	Lp02 Δlpg1803 lpg0898 lpg2831 lpg1625	pJB908
BY4741	N/A	Lp02 Δlpg1803 lpg0898 lpg2176 lpg1625	pJB908

BY4741 ΔYca1	N/A	Lp02 Δlpg1803 lpg0898 lpg2176 lpg1625 lpg2831	pJB908
W303	pSB157:: lpg2283	Lp02 ΔVipD family	pJB908
Lp03	pZL507::lpg1667	LPE509	N/A
Lp03	pZL507::lpg1798	LPE509 ΔflaA	N/A
Lp02 Δlpg0130	N/A	Lp02 ΔsdhA	pJB908
Lp02 Δlpg0898 complemented	pJB908::lpg0898	LPE509 Spontaneous rpsl K88R mutant	N/A
Lp02 Δlpg0130	pJB908	LPE509 ΔflaA Spontaneous K88R mutant	N/A
Lp02	pJB908::5xFlag::lpg0898	Lp Philadelphia-1 Spontaneous K88R mutant	N/A
Lp02 Δlpg0898	pJB908	LPE509 Spontaneous rpsl K43N mutant	N/A

Lp02 Δlpg1803	pJB908	LPE509 ΔflaA Spontaneous K43N mutant	N/A
Lp02 Δlpg2176 lpg0898	pJB908	Lp02 ΔflaA RpsL K43R knock in mutant	pJB908
Lp02 Δlpg1803 lpg0898	pJB908	Lp02 Δlpg0716	pJB908
Lp02 Δlpg1625 lpg0898	pJB908	Lp Philadelphia -1 Spontaneous K43N mutant	N/A
Lp02 Δlpg0716 lpg0898	pJB908	LPE509 rpsl K43N knock in mutant	N/A
Lp02 pXDC61M::RpsL WT	pXDC61M::RpsL WT	Lp02 RpsL K43N knock in mutant	pJB908
Lp02 pXDC61M::RpsL K88R	pXDC61M::RpsL K88R	Lp02 RpsL K43R knock in mutant	pJB908
LPE509 ΔflaA K43N knock in mutant	N/A	Lp02 ΔflaA RpsL WT knock in mutant	pJB908
Lp Philadelphia-1	N/A	Lp02 ΔflaA RpsL K43N	pJB908

K43N knock in mutant		knock in mutant	
LPE509 rpsl K88R knock in mutant	N/A	Lp02 pZL507::rpsL WT	pZL507::rpsL WT
LPE509 ΔflaA K88R knock in mutant	N/A	Lp02 pZL507::rpsL K88R	pZL507::rpsL K88R
Lp Philadelphia-1 K88R knock in mutant	N/A	Lp02 RpsL WT knock in mutant	pJB908

VITA

VITA

Wenhan Zhu was born in Guangzhou, Guangdong, China. He attended the Sun-Yat-Sen (Zhong Shan) University in 2004 to study Biotechnology in the Department of Biochemistry and obtained the Bachelor Degree of Science in 2008. He joined Dr. Zhao-Qing Luo's Lab in 2009 and started his project on the comprehensive identification of the Dot/Icm substrates of *L. pneumophila*. With stong interests in host cell death as an integrated component of host immune defense, he screened for and identified five Dot/Icm substrates that could acivtate caspase-3. He then later collaborate with Dr. Lili Tao on the project to dissect how mouse macrophages respond to environmental isoaltes of *L. pneumophila*. He then identified bacterial ribosomal protein RpsL as a potential MAMP (Microbe Associated Molecular Pattern).