Proteomic Analysis of Growth Phase-Dependent Expression of Legionella pneumophila Proteins Which Involves Regulation of Bacterial Virulence Traits

Tsuyoshi Hayashi1*, Masahiro Nakamichi1,9, Hirotaka Naitou2, Norio Ohashi3,4, Yasuyuki Imai1,4, Masaki Miyake1*

1 Laboratory of Microbiology and Immunology, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka, Japan, 2 Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Shizuoka, Japan, 3 Laboratory of Microbiology, Department of Food and Nutritional Sciences, University of Shizuoka, Shizuoka, Japan, 4 Global COE, University of Shizuoka, Shizuoka, Japan

Abstract

Legionella pneumophila, in particular, is known to become virulent at a post-exponential phase in vitro culture. In this study, we performed a proteomic analysis of differences in expression between the exponential phase and post-exponential phase to identify candidates associated with L. pneumophila virulence using 2-Dimensional Fluorescence Difference Gel Electrophoresis (2D-DIGE) combined with Matrix-Assisted Laser Desorption/Ionization–Mass Spectrometry (MALDI-TOF-MS). Of 68 identified proteins that significantly differed in expression between the two growth phases, 64 were up-regulated at a post-exponential phase. The up-regulated proteins included enzymes related to glycolysis, ketone body biogenesis and poly-3-hydroxybutyrate (PHB) biogenesis, suggesting that L. pneumophila may utilize sugars and lipids as energy sources, when amino acids become scarce. Proteins related to motility (flagella components and twitching motility-associated proteins) were also up-regulated, predicting that they enhance infectivity of the bacteria in host cells under certain conditions. Furthermore, 9 up-regulated proteins of unknown function were found. Two of them were identified as novel bacterial factors associated with hemolysis of sheep red blood cells (SRBCs). Another 2 were found to be translocated into macrophages via the Icm/Dot type IV secretion apparatus as effector candidates in a reporter assay with Bordetella pertussis adenylate cyclase. The study will be helpful for virulent analysis of L. pneumophila from the viewpoint of physiological or metabolic modulation dependent on growth phase.

Introduction

Legionella pneumophila is a causative agent of Legionnaires’ disease that replicates in macrophages in humans [1]. In nature, the bacteria reside and replicate in protozoa [2]. With the inhalation of L. pneumophila-contaminated aerosols, the bacteria can invade the human body and be phagocytosed by phagocytic cells, such as alveolar macrophages. The nascent phagosomes supporting the intracellular survival of L. pneumophila represent unique forms in that they are associated with small vesicles, mitochondria and the rough endoplasmic reticulum [3,4]. L. pneumophila replicates vigorously within this unique compartment, evading lysosomal fusion [5]. After it has replicated enough in phagosomes, the bacteria lyse the macrophage membrane, and infect new host cells. As this bacterial infectious cycle expands, it becomes pathogenic to humans.

The Icm/Dot type IV secretion apparatus is known as a major virulence factor of L. pneumophila [6,7]. The icm/dot system consists of 26 genes located in two separate regions of the genome. Icm/Dot delivers effector proteins into host cells, forming the unique phagosomes in which bacteria can survive and replicate [8]. Recently, many L. pneumophila effector proteins have been identified using several techniques including the Cya (B. pertussis adenylate cyclase toxin) assay system, yeast expression system and β-lactamase reporter system [9–16]. So far, over 140 substrates of Icm/Dot have been identified, but the single mutation of most of these proteins has only minor effects on bacterial growth in host cells, except for LepAB which promotes nonlytic release from protozoa, SidJ which contributes to the trafficking of ER proteins to phagosomes containing bacteria, SdhA which protects macrophages from apoptotic cell death in the early stages of infection, and AnkB which promotes the decoration of the Legionella-containing phagosomes (LCPs) with polyubiquitinated proteins to establish a favorable replicative niche [9,15–20].

Similar to other bacterial pathogens, L. pneumophila has a biphasic life cycle: one is a replicative form in which bacteria
multiply in host cells, and another is a transmissive form in which they escape from infected cells and infect new host cells [21]. In broth culture, \textit{L. pneumophila} at a post-exponential phase when the supply of nutrients becomes limited, not at an exponential phase in which the bacteria vigorously replicate, exhibit transmissive phenotypes (stress resistance, cytotoxicity for macrophages, high motility and evasion of bacteria-containing phagosomes from lysosomal fusion) [22]. Therefore, the \textit{L. pneumophila} biphasic life cycle can be roughly modelled in vitro broth culture, which regards the bacterial form in the exponential phase and post-exponential phase as the replicative form and transmissive form during the infection, respectively [21]. To date, studies have identified virulent phenotypes of this pathogen in post-exponential phase [21,22], the stringent response enzymes RelA and SpoT which monitor bacterial amino acid levels and fatty acid biosynthesis, respectively [23–25], and RpoS, LetA/S, CsrA, and small non-coding RNAs RsmY and RsmZ as regulators of the virulent phenotypes in the post-exponential phase [26–30]. However, a comprehensive analysis of \textit{L. pneumophila} proteins that show growth phase-dependent expression has not been attempted. The proteomic approach is applicable to a comprehensive analysis of bacterial virulence factors, because fluorescence 2-D DIGE can analyze multiple proteins in a single gel and has greater sensitivity, reproducibility and quantitative accuracy than conventional 2-dimensional gel electrophoresis (2-DE).

In this paper, we report the result of a proteomic analysis of the growth phase-dependent expression of \textit{L. pneumophila} in vitro culture. Using 2-D DIGE and MALDI-TOF-MS, we identified 68 protein species which significantly differ in expression between the exponential phase and post-exponential phase. Most of the identified proteins were up-regulated at the post-exponential phase, including metabolic enzymes and proteins related to motility. Moreover, 9 uncharacterized proteins up-regulated at the post-exponential phase were found. The genes encoding 6 of these proteins (\textit{lpg}0634, \textit{lpg}0901, \textit{lpg}1851, \textit{lpg}2275, \textit{lpg}2678, and \textit{lpg}2874) were specific to \textit{L. pneumophila} in several Legionella species, but the single knockout of these 6 genes did not influence bacterial intracellular replication within U937 macrophages and \textit{Acanthamoeba polyphaga}. It was shown that \textit{lpg}0634 and \textit{lpg}0901 partially contributed to hemolysis of SRBCs. And \textit{lpg}1851 and \textit{lpg}2874 were found to be translated into macrophages via the Icm/Dot secretion apparatus, suggesting them to be the effector candidates.

Materials and Methods

Bacterial strains, plasmids, primers and cell culture

The \textit{Legionella} strains used in this study are listed in Table S1. The plasmids and primers are listed in Table S2 and Table S3, respectively. The \textit{Legionella} strains were cultured on charcoal-yeast extract (CYE) agar plates or ACES-buffered yeast extract (AYE) broth with appropriate antibiotics as needed. The human monocytic cell line U937 [31] was maintained in RPMI1640 medium (Sigma, Tokyo, Japan) supplemented with 10% heat-inactivated FBS (Hyclone Laboratories, Inc., U.S.A.). At 48 h prior to infection, the U937 cells were induced to differentiate with 50 ng/ml of phorbol 12-myristate 13-acetate (Sigma). \textit{Axenic} \textit{A. polyphaga} was cultured as adherent cells in PYG medium [32]. All cells were maintained under a humidified atmosphere of 5% CO2 and 95% air at 37°C, as described previously [31].

Sample preparation for 2-D DIGE

\textit{L. pneumophila} Philadelphia 1 JR32 [33] was grown until the exponential phase (OD600 = 1.6) or post-exponential phase (OD600 = 3.8) in AYE broth, and the bacteria (7.5 x 10^6 cells each) were harvested by centrifugation at 7,000 x g for 10 min at 4°C. The bacterial pellets were solubilized with lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 30 mM Tris-HCl (pH 8.5), and centrifuged at 360,000 x g for 1 h at 10°C to eliminate genomic DNA. The supernatants were dialysed in a 7 M urea/ 2 M thiourea solution using a PhosOne mini dialysis kit (GE Healthcare) at 15°C for 16 h. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories).

2-D DIGE

2-D DIGE was performed as described previously [34,35]. Briefly, 30 μg of bacterial protein extract cultured in the exponential phase or post-exponential phase was labeled with 400 pmol of either Cy3 or Cy5 (GE Healthcare). An internal standard was prepared by combining 25 μg of each bacterial protein extract in the exponential phase and the post-exponential phase, followed by labeling with 400 pmol of Cy2 (GE Healthcare). The protein labeling was achieved by incubation on ice in the dark for 30 min. The reaction was quenched by the addition of 10 mM lysine, followed by incubation on ice for a further 10 min. The labeled samples were combined and mixed with an equal volume of lysis buffer containing 2% DTT and adjusted to 450 μL with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.2% DTT, and 0.002% bromophenol blue) and used for 2-DE. The sample solutions were then loaded on non-linear 24 cm IPG strips (pH 3–10) (GE Healthcare) and rehydrated at 20°C for 12 h. First-dimension isoelectric focusing was carried out using the IPGphor IEF system (GE Healthcare) for 9 to 11 h (a total of 45 to 60 kVh). The IPG strips were equilibrated with equilibration buffer (6 M urea, 30% glycerol, 2% SDS, and 50 mM Tris-HCl (pH 8.8)) containing 10 mg/mL of DTT for 15 min, followed by equilibration buffer containing 25 mg/mL of iodoacetamide for a further 15 min. The equilibrated strips were loaded on 10% or 12.5% polyacrylamide gels and the gels were electrophoresed using the Etan DALT 6 unit (GE Healthcare).

Image Analysis and spot picking

The 2-D gel images were scanned using a Typhoon 9410 scanner (GE Healthcare) by selecting adequate excitation and emission wavelengths (Cy2, excitation 488 nm, emission 522 nm; Cy3, excitation 532 nm, emission 580 nm; Cy5, excitation 633 nm, emission 670 nm). The statistical analysis was performed with Decyder software (version 5.02; GE Healthcare). Only those spots with a 1.5-fold change in volume between two populations, with p values less than 0.05 (p<0.05) in Student’s t-test for the variance of these ratios for each protein pair across three independent gels, were defined as significantly different, as described previously [34,35]. For the picking of protein spots of interest, pooled whole protein (500 μg) was separated by 2-DE, and stained with Deep Purple dye (GE Healthcare). The gel was imaged with a Typhoon 9410 scanner using excitation/emission wavelengths of 532/560 nm. The protein spots of interest were picked with an Etan Spotpicker (GE HealthCare).

In-gel digestion and MALDI-TOF-MS analysis

The gel pieces were destained with 50 mM ammonium bicarbonate/50% acetonitrile and dehydrated with acetonitrile. The dried gel pieces were digested with 2.5% trypsin (Promega) in 100 mM ammonium bicarbonate overnight at 37°C. The peptide solutions were desalted and concentrated using μ-C18 Zip Tip (Millipore, Bedford, MA, U.S.A.). The samples were mixed with α-cyano-4-hydroxycinnamic acid (CHCA) matrix and applied
onto a target plate. MALDI-TOF-MS was performed using Ultraflex (Bruker Daltomics). Protein identification was carried out with Mascot software against the sequence databases of NCBI

Southern blot analysis

The Southern blot analysis was performed as described previously [31]. Briefly, chromosomal DNAs of Legionella strains were digested with EcoRI, separated by agarose gel electrophoresis, and transferred to Hybond-N+ (GE Healthcare). Parts of each candidate gene were amplified by PCR with the following primer pairs (Table S3): [lpg0390-F and lpg0390-R for lpg0390, lpg0483-F and lpg0483-R for lpg0483, lpg0584-F and lpg0584-R for lpg0584, lpg0634-F and lpg0634-R for lpg0634, lpg0901-F and lpg0901-R for lpg0901, lpg0992-F and lpg0992-R for lpg0992, lpg1647-F and lpg1647-R for lpg1647, lpg1851-F and lpg1851-R for lpg1851, lpg2275-F and lpg2275-R for lpg2275, lpg2678-F and lpg2678-R for lpg2678, and lpg2874-F and lpg2874-R for lpg2874, and were used as probes. Labelling of DNA probes and detection of signals were performed using the AlkPhos Direct Labelling and Detection System with CDP-Star (GE Healthcare, RPN3600).

Construction of L. pneumophila mutants and their complemented strains

The insertion mutants were constructed in JR32 using allelic exchange, according to a published procedure [36,37]. Briefly, parts of each candidate gene were amplified by PCR with the following primer pairs (Table S3): [lpg0634-F and lpg0634-R for lpg0634, lpg0901-F and lpg0901-R for lpg0901, lpg1647-F and lpg1647-R for lpg1647, lpg1851-F and lpg1851-R for lpg1851, lpg2275-F and lpg2275-R for lpg2275, lpg2678-F and lpg2678-R for lpg2678, and lpg2874-F and lpg2874-R for lpg2874, and ligated to the pGEM®-T Vector System (Promega). The kanamycin resistance cassette (kan-cassette) aphA-3 was cloned into the restriction sites (EcoRI III: lpg0634, EcoRI III: lpg0901, BsrGI I: lpg1647, PshI A: lpg2275, BsaH II: lpg2678, Bgl II: lpg2874) in the uncharacterized protein-coding genes. These plasmids (pMN21, pMN22, pMN23, pMN24, pMN25, and pMN26) were digested with Not I or Xba I, and the resultant fragments were cloned into the Not I or Xba I site of the allelic exchange vector pLAW444 [36]. These plasmids (pMN31, pMN32, pMN33, pMN34, pMN35, and pMN36) were introduced into L. pneumophila JR32 by electroporation with the Gene pulser® II system (BIO-RAD). Then, the bacteria were grown in AYE for the post-exponential phase at a multiplicity of infection (MOI) of 0.5 or 10, respectively. The plate was spun at 250×g for 20 min to synchronize the infection, and the time point at the end of this centrifugation was designated as 0 h. The infected cells were incubated for 1 h at 37°C, and washed three times with culture medium. The cells were incubated for 1 h at 37°C in culture medium containing 50 μg/mL of gentamicin to kill the extracellular bacteria. They were washed again, and then incubated in culture medium for the indicated period. At the appropriate time points, the cells were lysed with distilled water, diluted serially in distilled water, and plated on CYE plates to measure the number of colony forming units (CFUs) per well.

Construction of L. pneumophila producing Cya-fused proteins and producing Cya-fused RalF

Cya-fused proteins were constructed using pcy-aaf [38] that can express the Cya-RalF fusion protein kindly provided by Dr. Hiroki Nagai. Coding region of lpg1851 and lpg2874 was amplified by PCR with the following primer pairs (Table S3): [lpg1851-F and lpg1851-R2 for lpg1851, lpg2874-F2 and lpg2874-R2 for lpg2874]. The PCR products were cloned into the pGEM®-T Vector System to generate pTH12 and pTH14. The Bam HI/Sph I fragment from pTH12 which contain lpg0901, pTH13, and pTH14 was ligated into a Bam HI/Sph I fragment of pcy-aaf to generate pTH23, pTH24, and pTH25, respectively. This procedure was aiming at the production of M45 epitope-tagged Cya-fusion proteins with Lpg0901, Lpg1051 and Lpg2874, respectively. These plasmids were introduced into the JR32 or LELA3118 strain by electroporation. Construction of the fused proteins was assessed by immunoblotting using an affinity-purified rabbit polyclonal antibody raised against the M45 epitope (MDSRDRLPPFETETRIL) [39]. L. pneumophila strains producing Cya-fused RalF were constructed by introducing pcy-aaf to the JR32, LELA3118, MN101 or MN102 strain by electroporation.

Contact-dependent hemolysis assay using SRBCs

As described previously [31], contact-dependent pore formation in membranes was determined by examining the hemolysis of SRBCs in contact with L. pneumophila at a MOI of 10 for 1 h.

Cya reporter assay

The Cya reporter assay was performed as described previously [10]. Briefly, U937 cells (3×10^5 cells per well) were cultured and induced to differentiate in the wells of a 24-well culture plate (Costar 3526, Corning Incorporated, Amsterdam, Netherlands).
The differentiated U937 cells were infected with JR32 (MOI = 50) and LELA3118 (MOI = 100) containing different Cya-fused proteins or Cya-fused RalF. The plate was spun at 250 × g for 10 min to synchronize the infection, then incubated for 1 h at 37°C. After removal of the supernatant, the infected cells were lysed in lysis buffer 1B provided in the cAMP Biotrak EIA System (GE Helthcare, RPN225), and cAMP levels were determined as instructed by the manufacturer.

Results and Discussion

Protein expression profiles of L. pneumophila depends on growth phase

For the proteomic analysis of the growth phase-dependent expression of L. pneumophila whole-cell proteins, 2-D DIGE was performed three times. Representative images of 12.5% polyacrylamide gels are shown in Fig. 1A. To separate proteins with high molecular masses, we used a 10% polyacrylamide gel in other experiments. On this gel, SidE family proteins already known to be substrates of the Icm/Dot system [40] were clearly detected (Fig. 1B). Among 2143 spots detected on the 2-D gel, 105 were found to significantly differ in protein expression between the exponential phase and post-exponential phase. Among these, 82 spots were successfully identified as corresponding to 71 protein species (Table S4), and the identified proteins were functionally classified into the category of metabolic enzyme, motility, substrate of the Icm/Dot type IV secretion system, transcriptional regulator, chaperone, toxin production, or unknown function (Table S5).

Identified proteins

Enzymes. Approximately 60% of the identified proteins were defined as enzymes. Most of the identified enzymes were up-regulated at the post-exponential phase, and categorized as involved in carbohydrate metabolism, amino acid metabolism, and lipid metabolism. Moreover, the identified enzymes were sorted according to KEGG pathway maps of L. pneumophila Philadelphia-1 available at http://www.genome.jp/kegg/[41]. (Fig. 2). The data showed that enzymes related to glycolysis (pyruvate kinase II (YP_094190) and malate dehydrogenase (YP_096964)), the TCA cycle (2-oxoglutarate ferredoxin oxidoreductase beta subunit (YP_094982)), ketone biogenesis (HMG-CoA lyase (YP_095056) and acetoacetate decarboxylase (ADC) (YP_094708)) and poly-3-hydroxybutyrate (PHB) biogenesis (acetoacetyl CoA reductase (YP_095092), acetylactetyl CoA reductase (YP_094601, YP_094602), 3-hydroxyisobutyryl Coenzyme A hydrolase (YP_094904)) were up-regulated at the post-exponential phase. This is the first report that the expression of enzymes related to carbohydrate or lipid metabolism in L. pneumophila species are up-regulated at a post-exponential phase. L. pneumophila is thought to utilize only amino acids as carbon and energy sources [42], but a
study of the whole-genome sequence study reported that this bacterium has genes encoding enzymes involved in carbohydrate metabolism, lipid metabolism, and the TCA cycle [43,44]. Moreover, it is known that \textit{L. pneumophila} utilizes PHB as an energy source for survival in nutrient-poor environments such as tap water [45]. Therefore, \textit{L. pneumophila} may utilize sugars and lipids as energy sources at the post-exponential phase or within host cells in which amino acids are scarce.

![Dendrogram](image)

**Figure 2.** Identified proteins in a schematic overview of metabolic pathways of \textit{L. pneumophila}. Metabolic pathways of \textit{L. pneumophila} related to our results were constructed based on the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database. Some of the identified enzymes are involved in Glycolysis, the TCA cycle, the Synthesis and degradation of ketone bodies, and PHB biogenesis. Eleven identified enzymes are shown in underlined boldface and the reaction steps that they catalyze are shown by black arrows. The numbers in parentheses correspond to the spot numbers in 2D-DIGE gel images of Fig. 1 and the list of identified proteins in Table S4.

doi:10.1371/journal.pone.0011718.g002

| Name   | NCBI accession no. | Average Ratio | Theoretical pl/MW | Cell localization | Description (Homology) |
|--------|---------------------|---------------|-------------------|-------------------|------------------------|
| Lpg0584| YP_094620           | 1.84          | 5.74/25423        | cytoplasm         | PhoU                   |
| Lpg0634| YP_094670           | 2.57          | 5.71/50775        | inner membrane    |                        |
| Lpg0901| YP_094935           | 8.42          | 6.41/25309        | cytoplasm         | NMA0899 (\textit{N. meningitidis}) |
| Lpg0992| YP_095025           | 23.36         | 9.46/31725        | inner membrane    |                        |
| Lpg1647| YP_095674           | 6.62          | 9.06/22349        | periplasmic space | YcE                    |
| Lpg1851| YP_095877           | 3.01, 1.78    | 7.85/25355        | periplasmic space |                        |
| Lpg2275| YP_096287           | 1.85          | 6.19/26349        | periplasmic space | CBU0952 (\textit{C. burnetii}), FTT0975 (\textit{F. tularensis}) |
| Lpg2678| YP_096863           | 7.5           | 5.85/30206        | cytoplasm         | UbiE                   |
| Lpg2874| YP_096888           | 2.22, 1.79    | 5.37/33474        | cytoplasm         |                        |

*Average Ratio: the protein spot intensity at PE to that at E; PE/E.
*Cell localization: cited from Legionella genome project (http://legionella.cu-genome.org/annotation/anno_table.html).
*Description (Homology): the homology predicted by NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

doi:10.1371/journal.pone.0011718.t001
Motility-associated proteins

We identified two flagellar components (FlgA:YP_095785, FlgK:YP_094942) and the type IV pilus (Tip) component PitT (YP_095020). FlgK is a motor switch protein of flagella that regulates bacterial motility, and Salmonella typhimurium FlgK is essential for the assembly, rotation, and switching of the flagellar motor [46]. And bacterial motility and flagella promote the infectivity of bacteria and macrophage cell death [22,47,48]. Two flagellar component proteins were up-regulated at the post-exponential phase, consistent with a previous report that bacterial motility was elevated at the post-exponential phase in vitro [22]. PitT is involved in Tip-mediated motility, and Neisseria gonorrhoeae PitT is essential for Tip-mediated twitching motility [49]. Recently, the presence of type IV Pil-mediated twitching motility in L. pneumophila has been shown [50]. Originally, it has been shown that type IV pilus of L. pneumophila is essential for attachment to mammalian and protozoan cells and associated with natural competence for DNA transfer [51,52]. Therefore, it is suggested that these 3 identified proteins associated with bacterial motility may function as virulence-associated factors [53].

Substrates of Icm/Dot

Figure 1B shows 2D-DIGE gel images of 3 substrates of the Icm/Dot secretion system (SidE, SdeA and SdeC) cultured in the exponential phase and post-exponential phase. These proteins were identified as proteins transferred between bacterial cells, and SdeE and SdeA were identified as proteins known to be translocated into macrophages via the Icm/Dot secretion apparatus [10,40,54]. As noted in Table S4, SdeA (Fig. 1B, spot no. 77) and SdeC (Fig. 1B, spot no. 77, 78, 79), which are paralogous to SidE, were up-regulated at the post-exponential phase, consistent with previous results [10]. Interestingly, in contrast to SdeA and SdeC, it was found that SidE (Fig. 1B, spot no. 80, 81, 82) was down-regulated in post-exponential phase. Many Icm/Dot substrates were up-regulated at the post-exponential phase in vitro [10,54–56], but translocating proteins up-regulated at the exponential phase should exist because of their diverse functions throughout the infection process. Therefore, we predict that SidE participates in different stages of bacterial infection, which differ from the case of translocating proteins such as SdeA and SdeC which are up-regulated at the post-exponential phase.

Uncharacterized proteins

We focused on the uncharacterized proteins which have no homology with any known proteins. In total, 12 proteins were annotated as hypothetical proteins. Eleven of these were up-regulated, and one was down-regulated, at the post-exponential phase (Table S4, spot no. 51). Among the up-regulated proteins, it has been reported that Lpg0390 and Lpg0483 were identified as VipA and LegA12, respectively [11,12]. VipA is a substrate of Icm/Dot that interferes with organelle trafficking in yeast. Therefore, VipA may play a role in manipulating the host secretion pathway in macrophages [11]. LegA12 is an eukaryote-like protein that contains ankyrin repeats [12]. Eukaryotic ankyrin proteins are thought to act as a link between membrane proteins and the cytoskeleton [44]. Ankyrin repeats-containing protein may modify host cell function. Pan et al. identified ankyrin repeats-containing proteins (AnkW, AnkX, AnkY, AnkZ) translocated into host cells, and revealed that AnkX prevented microtubule-dependent maturation of bacteria-containing phagosomes [57]. And Price et al. have recently shown that AnkB mimics the action of host cell F-box proteins promoting the decoration of LCPs with polyubiquitinated proteins to establish a intracellular replicative niche [20]. Table 1 lists 9 uncharacterized proteins up-regulated at the post-exponential phase in this study, except Lpg0390 and Lpg0483.

We performed homology research based on NCBI protein-protein BLAST (Basic Local Alignment Search Tool). Five proteins had homology in other bacterial species. Lpg0390 and Lpg1647 showed high homology to PhoU (identity: 52%) that correlates with phosphate transport regulator [50] and YciE reported to be a base-induced periplasmic protein in Escherichia coli [59], respectively. The other three revealed <35% homology to certain proteins; Lpg2678 had homology to UbiE, a methylase involved in ubiquinone/menaquinone biosynthesis [60], Lpg0901 had homology to the hypothetical protein NMA0899 in Neisseria meningitidis. Lpg2275 was homologous with the hypothetical

![Figure 3. Genomic southern blotting of the genes encoding uncharacterized proteins in L. pneumophila and other Legionella species.](http://example.com/figure3.png)

**Figure 3.** Genomic southern blotting of the genes encoding uncharacterized proteins in L. pneumophila and other Legionella species. Bacterial genomic DNA was digested with EcoRI and hybridized with probes against Lpg0390, Lpg0483, Lpg0564, Lpg0634, Lpg0901, Lpg0992, Lpg1647, Lpg1851, Lpg2275, Lpg2678, and Lpg2874, respectively. Eight genes (Lpg0390, Lpg0483, Lpg0564, Lpg0901, Lpg1647, Lpg1851, Lpg2275, Lpg2678, and Lpg2874) are specific for L. pneumophila among several Legionella species, but 3 genes (Lpg0564, Lpg0992, and Lpg1647) were detectable in other Legionella species except L. pneumophila. doi:10.1371/journal.pone.0011718.g003
proteins CBU0952 and FTT0975 in the intracellular pathogens Coxiella burnetii and Francisella tularensis, respectively, speculating that these proteins might be originally transferred horizontally for parasitism within host cells. Lpg0992 had homology to hypothetical proteins in Congregibacter litoralis, Nitrosococcus oceanii and Aromatoleum aromaticum. In contrast, Lpg0634, Lpg1851, and Lpg2874 had almost no homology with any proteins in other bacterial species. These 9 uncharacterized proteins up-regulated at the post-exponential phase are possible to be novel virulence-associated factors of L. pneumophila, and we therefore focused on them.

Analysis of the genes encoding the uncharacterized proteins in different Legionella species

Virulence factors specific to L. pneumophila are thought to exist because 90% of clinical isolates were L. pneumophila among Legionella species. [61]. Actually, previous studies reported that the effector protein RalF and intracellular growth factor PmiA were specific for L. pneumophila among several Legionella species [31,55,62]. Therefore, we examined whether 9 uncharacterized proteins, Lpg0390 (VipA) and Lpg0483 (LegA12) were specific to L. pneumophila.

A Southern blot analysis was performed to demonstrate the presence or absence of the genes for the uncharacterized proteins in several Legionella strains. The results showed that three genes (lpg0992, lpg0538, and lpg1647) were distributed among several Legionella species. In contrast, 8 genes (lpg0390, lpg0483, lpg0634, lpg0901 lpg1851, lpg2275, lpg2678, and lpg2874) were only detected in L. pneumophila (all strains and serogroups examined) and were not present in other Legionella species (Fig. 3). This result suggested that these genes may be specific for L. pneumophila. As mentioned above, proteins encoded by two (lpg0390 and lpg0483) of 8 genes were identified as VipA and LegA12, respectively [11,12]. Except for these two proteins (Lpg0390 and Lpg0483), we focused on the other 6 L. pneumophila specific genes encoding the uncharacterized proteins.

Six uncharacterized proteins (Lpg0634, Lpg0901, Lpg1851, Lpg2275, Lpg2678, and Lpg2874) are not directly required for bacterial replication within macrophages and protozoa, but Lpg0634 and Lpg0901 are partially associated with the hemolytic activity on SRBCs.

To explore the function of six uncharacterized proteins (Lpg0634, Lpg0901, Lpg1851, Lpg2275, Lpg2678, and Lpg2874) in macrophages and protozoa, we constructed single mutants of each six encoding genes by insertion of the kan-cassette, and tested these strains for intracellular growth within host cells. Six individual mutants (MN101, MN102, MN103, MN104, MN105 and MN106) were compared with a wild type, JR32, and an intracellular growth-deficient dotA mutant, LEFA3118. The data showed that all mutants grew robustly within U937 cells, and A. polyphaga in a time-dependent manner, similar to JR32, suggesting that the single inactivation of each of the genes did not influence bacterial growth within U937 macrophages and protozoa (Fig. 4). This result indicates that not all differentially expressed proteins play the critical role for intracellular replication and survival of bacteria. There may be synergistic effects among their proteins.

It is known that the pore-forming activity of L. pneumophila is not sufficient for or partially independent of their intracellular growth [63]. Therefore, we next examined the pore-forming activity of the mutants by conducting contact-dependent hemolysis assay using SRBCs. It was found that Lpg0634 and Lpg0901 are partially associated with hemolysis of SRBCs (Fig. 5). So far, IcmQ and IcmT are known as pore-forming factors of L. pneumophila [64–67]. The single knockout of each impeded intracellular bacterial replication dependent on the Icm/Dot system, suggesting that the pore-forming activity of IcmQ and IcmT would be probably involved in the function of Icm/Dot as the secretion apparatus. On the other hand, MN101 and MN102, which are single knockout mutants of lpg0634 and lpg0901 respectively, retain all of the intact icm/dot genes, however, the hemolytic activity on SRBCs...
of their mutants decreased. Lpg0634 and Lpg0901 may be novel types of bacterial factors associated with pore-formation in mammalian cells. The function of Lpg0634 and Lpg0901 in host infection is under elucidation.

Lpg1851 and Lpg2874 are translocated into macrophages via Icm/Dot secretion apparatus

It is reported that many known effectors or Icm/Dot substrates localize in the bacterial cytoplasm and they are up-regulated at a post-exponential phase in broth culture [10,54,55], suggesting that translocation of many effectors into the host cells on an early stages of *L. pneumophila* infection would be necessary for causing specific phagocytosis of bacteria and beginning the formation of LCPs. Therefore, we investigated whether the uncharacterized proteins identified in our proteome analysis were translocated into the host cells in an Icm/Dot-dependent manner. We chose three (Lpg0901, Lpg1851 and Lpg2874) of the 9 uncharacterized proteins, because it is predicted on Legionella Genome Project database that these proteins locate in the bacterial cytoplasm and do not have similarity to other known bacterial proteins, as shown in Table 1.

To determine whether Lpg0901, Lpg1851 and Lpg2874 are translocated into host cells, we used a reporter assay system that takes biochemical advantage of the adenylate cyclase domain (Cya) of *Bordetella pertussis*. Cya is activated by calmodulin as a cofactor in the eukaryotic cell cytosol to synthesize cAMP from ATP. Therefore, the translocation of Cya-fused proteins was detected by monitoring the accumulation of cAMP in infected cells [68].

We constructed forms of each of 3 uncharacterized proteins (Lpg0901, Lpg1851 and Lpg2874) fused with Cya, expressed in *L. pneumophila* (JR32 and LELA3118). Then, U937 cells were infected with *L. pneumophila* producing these Cya-fused proteins. After 1 h, levels of cAMP on infection with the wild type JR32 strain producing Cya-Lpg1851 and Cya-Lpg2874 were over 100000 fmol/well (Fig. 6). The level of cAMP resembled that on infection with the JR32 strain producing a Cya-fused form of RalF, an effector protein previously shown to be translocated into host cells, as a positive control [38]. In contrast, when U937 cells were infected with the dotA mutant LELA3118 strain producing

Figure 5. Lpg0634 and Lpg0901 are partially involved in the hemolysis of SRBCs. The hemolytic activity of *L. pneumophila* MN101 (lpg0634<sup>2</sup>), MN102 (lpg0901<sup>2</sup>), TH101 (lpg0634<sup>2</sup> /lpg0634<sup>+<sup>2</sup><sup>+</sup></sup>) and TH102 (lpg0901<sup>2</sup> /lpg0901<sup>+<sup>2</sup>+<sup>2</sup></sup>) was tested by contact-dependent hemolysis of SRBCs. Bacterial contact with SRBCs was performed at a MOI of 10 for 1 h. Wild type JR32 and LELA3118 (dotA<sup>+</sup>) strains were used as positive and negative controls, respectively. PBS (−) was also used as a negative control for the absence of bacteria. The experiment was done in triplicate, and the error bars represent standard deviations. The data shown are representative of at least two independent experiments. Asterisks indicate statistically significant differences (*<sup>p</sup><0.01, compared with JR32 samples by ANOVA followed by Dunnett’s test). Sharp indicates statistically significant differences (#<sup>p</sup><0.01 by Student t test).

doi:10.1371/journal.pone.0011718.g005
and Cya-Lpg2874) independent experiments. NS: not significant.

representative of one (Cya-Lpg0901, Cya-RalF) or three (Cya-Lpg1851 in triplicate, and the data are shown as means ± S.D. ANOVA followed by Dunnett’s test). The experiments were performed (Fig. 6A). Therefore, this result clearly showed that Lpg1851 and Lpg2874 are translocated into U937 cells via the Icm/Dot secretion apparatus. Thus we constructed the MN101 and MN102 strains producing Cya-fused RalF, and we performed the Cya reporter assay as well. As a result, it was shown that MN101 and MN102 can normally translocate the RalF to macrophages and the ability of effector translocation for these mutants is not impaired (Fig. 6B). Therefore, it is likely that the partial defect of hemolytic activity for MN101 and MN102 is independent of effector translocation and the function of the Icm/Dot system.

Concluding remarks

In this study, we comprehensively identified proteins up-regulated in the post-exponential phase in which L. pneumophila shows virulent phenotypes. They included proteins related to sugar utilization, motility, and Icm/Dot-substrates. Regarding high motility of L. pneumophila in the post-exponential phase, previous papers reported an increase of FlaA expression [69–71]. It was confirmed in this study that the expression of several flagella and pili components increased as well. The up-regulation of proteins related to sugar utilization would indicate that the proteins are functional. In addition to circumstances in which the supply of amino acid becomes limited, L. pneumophila might use a pathway of sugar metabolism within intracellular environments in which the source of nutrients is restricted. On the screening of L. pneumophila Icm/Dot substrates based on a comparison of specific amino acid sequences in silico, it had been estimated that there exist ~100 substrates [72]. Recently, a large number of (~140) Icm/Dot substrates have been identified, and some of which were characterized as effectors [8,15]. RalF and DrrA/SidM function as a guanine nucleotide exchange factor of ARF, leading to the interception of host vesicle transport and establishment of bacterial replication-permissive phagosomes [13,55,73]. SidF neutralizes the proapoptotic function of BNIP3 and Bcl-rambo, which is followed by the prevention of macrophage death caused in an early stage of infection [74]. Although few Icm/Dot substrates have been characterized in terms of their biological function, it is supposed that almost all substrates should be injected into host cells to perform certain functions for permitting bacteria replication intracellularly. Further functional analyses of two Icm/Dot substrates, Lpg1851 and Lpg2874, identified in this study are indispensable.

Since the genome sequencing of L. pneumophila has been completed and the post-genome era for the research into this bacteria has begun, several researchers have performed comprehensive analyses with transcriptomics or proteomics [75–78]. In our study, proteomics was shown to be powerful tool for identifying potential virulence factors of L. pneumophila. A comprehensive analysis will help to determine the entire molecular network for L. pneumophila pathogenesis.

Supporting Information

Table S1 Strains used in this study

Table S2 Plasmids used in this study
References

1. Horwitz MA, Silverstein SC (1980) Legionnaires’ disease bacterium (Legionella pneumophila) multiples intracellularly in monocytes. J Clin Invest 66: 441–450.

2. Harb OS, Gao LY, Abu Kwaik, Y (2000) From protozoa to mammalian cells: a new paradigm in the life cycle of intracellular bacterial pathogens. Environ Microbiol 2: 251–265.

3. Horwitz MA (1983) Formation of a novel phagosome by the Legionnaires’ disease bacterium (Legionella pneumophila) in human monocytes. J Exp Med 156: 1191–1311.

4. Swanson MS, Isberg RR (1995) Association of Legionella pneumophila with the macrophage endoplasmic reticulum. Infect Immun 63: 3069–3620.

5. Horwitz MA (1983) The Legionnaires’ disease bacterium (Legionella pneumophila) inhibits phagosome-lysosome fusion in human monocytes. J Exp Med 156: 2108–2126.

6. Berger KH, Isberg RR (1993) Two distinct defects in intracellular growth complemented by a single genetic locus in Legionella pneumophila. Mol Microbiol 7: 7–19.

7. Sadosky AB, Wiater LA, Shuman HA (1993) Identification of Legionella pneumophila genes required for growth within and killing of macrophages. Infect Immun 61: 5361–5373.

8. Ninio S, Roy CR (2007) Effector proteins translocated by Legionella pneumophila: strength in numbers. Trends Microbiol 15: 372–380.

9. Chen J, de Felice KS, Clarke M, Lu H, Anderson OR, et al. (2004) Legionella effectors that promote nonlytic release from protozoa. Science 303: 1358–1361.

10. Burkard JP, Miller JL, Vogel JP (2005) Icm-dependent translocation of SdeA into macrophages by the Legionella pneumophila type IV secretion system. Mol Microbiol 56: 90–103.

11. Sholyt N, Eje JA, Enr SD, Shuman HA (2005) Pathogen effector protein screening in yeast identifies Legionella factors that interfere with membrane trafficking. Proc Natl Acad Sci U S A 102: 4866–4871.

12. de Felice KS, Pampou S, Jovanovic OS, Pericone CD, Ye SF, et al. (2005) Evidence for acquisition of Legionella type IV secretion substrates via interdomain horizontal gene transfer. J Bacteriol 187: 7716–7726.

13. Murata T, Delprato J, Amundson A, Toome DR, Lambright DG, et al. (2006) Legionella pneumophila effector protein DrrA is a Rab1 guanine nucleotide-exchange factor. Nat Cell Biol 8: 971–977.

14. Franco IS, Shuman HA, Charpentier N (2009) The perplexing functions and surprising origins of Legionella pneumophila type IV secretion effectors. Cell Microbiol 11: 1435–1444.

15. Burstein D, Zisuman T, Degtyar E, Viner R, Segal G, et al. (2009) Genome-scale identification of Legionella effector proteins using a machine learning approach. PLoS Pathog 5: e1000508.

16. Ensinger AW, Isberg RR (2009) Legionella pneumophila Dot/Icm translocated substrates: a sum of parts. Curr Opin Microbiol 12: 67–73.

17. Laguna RK, Geacey EA, Li Z, Valtz N, Isberg RR (2006) A Legionella pneumophila-translocated substrate that is required for growth within macrophages and protection from host cell death. Proc Natl Acad Sci U S A 103: 10745–10750.

18. Liu Y, Luo ZQ (2007) The Legionella pneumophila effector SidJ is required for efficient recruitment of endoplasmic reticulum proteins to the bacterial phagosome. Infect Immun 75: 592–603.

19. Al-Khodr S, Price CT, Habyarimana F, Kalia A, Abu Kwaik YA (2008) Dot/Icm-translocated anthrax protein of Legionella pneumophila is required for intracellular proliferation within mammalian macrophages and protozoa. Mol Microbiol 70: 908–923.

20. Price CT, Al-Khodr S, Al-Quadan T, Santei M, Habyarimana F, et al. (2009) Molecular mimicry by an F-box effector of Legionella pneumophila hijacks a conserved polyubiquitination machinery within macrophages and protozoa. PLoS Pathog 5: e1000704.

21. Molodsky AB, Swanson MS (2004) Differentiate to thrive: lessons from the Legionella pneumophila life cycle. Mol Microbiol 53: 29–40.

22. Byrne B, Swanson MS (1998) Expression of Legionella pneumophila virulence traits in response to growth conditions. Infect Immun 66: 3029–3034.

23. Hammer BK, Swanson MS (1999) Co-ordination of Legionella pneumophila virulence with entry into stationary phase by ppGpp. Mol Microbiol 33: 721–731.

24. Dailebyroux ZD, Edwards RI, Swanson MS (2009) Spot governs Legionella pneumophila differentiation in host macrophages. Mol Microbiol 71: 640–658.

25. Edwards RL, Dailebyroux ZD, Swanson MS (2009) Legionella pneumophila couples fatty acid flux to microbial differentiation and virulence. Mol Microbiol 71: 1190–1204.

26. Bachman MA, Swanson MS (2001) RpsS co-operates with other factors to induce Legionella pneumophila virulence in the stationary phase. Mol Microbiol 40: 1201–1214.

27. Hammer BK, Tateda ES, Swanson MS (2002) A two-component regulator induces the transmission phenotype of stationary-phase Legionella pneumophila. Mol Microbiol 44: 107–118.

28. Molodsky AB, Swanson MS (2003) Legionella pneumophila CsrA is a pivotal repressor of transmission traits and activator of replication. Mol Microbiol 50: 445–461.

29. Sabir S, Brüggemann H, Jules M, Lomma M, Albert-Weissenberger C, et al. (2009) Two small mRNAs jointly govern virulence and transcription in Legionella pneumophila. Mol Microbiol 72: 741–762.

30. Kaiss M, Segal G (2009) The LetA/RsmY-Z/Csr regulatory cascade, together with RpsP and RpsA, post-transcriptionally regulates stationary phase activation of Legionella pneumophila Icm/Dot effectors. Mol Microbiol 72: 995–1010.

31. Miyake M, Watanabe T, Koike H, Molermet M, Imai Y, et al. (2005) Characterization of Legionella pneumophila pmkA, a gene essential for infectivity of protozoa and macrophages. Infect Immun 73: 6272–6282.

32. Bouzé JA, Johnson W (1996) Interaction of Legionella pneumophila with Acanthamoeba castellanii upstake by coiling phagocytosis and inhibition of phagosome-lysosome fusion. Infect Immun 64: 669–673.

33. Sadosky AB, Wiater LA, Shuman HA (1993) Identification of Legionella pneumophila genes required for growth within and killing of human macrophages. Infect Immun 61: 5361–5373.

34. Irikura VM, Kihara M, Yamaguchi S, Sockett H, Macnab RM (1993) Mutations in rpoS affect Legionella pneumophila Phagosome-lysosome fusion. Infect Immun 61: 18745–18750.

35. Sadosky AB, Wiater LA, Shuman HA (2004) Differentiate to thrive: lessons from the Legionella pneumophila life cycle. Mol Microbiol 53: 29–40.

36. Wiater LA, Sadosky AB, Shuman HA (1994) Mutagenesis of Legionella pneumophila using Tn5303 diltiaZ identification of a growth-phase-regulated pigment gene. Mol Microbiol 11: 641–653.

37. Yenushalimi G, Zuman T, Segal G (2005) Additive effect on intracellular growth by Legionella pneumophila Icm/Dot proteins containing a lipoxin motif. Infect Immun 73: 7578–7587.

38. Nagai H, Cambronne ED, Kagan JC, Amor JC, Kahan RA, et al. (2005) A C-terminal translation signal required for Dot/Icm-dependent delivery of the Legionella Ralf protein to host cells. Proc Natl Acad Sci U S A 102: 826–831.

39. Kubori T, Matsuhashi Y, Nakamura D, Uralil J, Lara-Tejero M, et al. (1998) Supramolecular structure of the Salmonella typhimurium type III protein secretion system. Science 280: 602–605.

40. Cambronne ED, Roy CR (2007) The Legionella pneumophila IcmSW complex interacts with multiple Dot/Icm effectors to facilitate type IV translocation. PLoS Pathog 3: e188.

41. Kanazawa M, Goto S, Hattori M, Akio Kinosita KF, Ioth M, et al. (2006) From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res 34: 354–357.

42. George JR, Pine L, Reeves MW, Harrell WK (1980) Amino acid requirements for Salmonella typhimurium fliG type III protein secretion. Mol Microbiol 44: 107–118.

43. Chien M, Morozova I, Shi S, Sheng H, Chen J, et al. (2004) Genomics of Salmonella enterica subsp. enterica serovar typhimurium fliC couples transcription to translational regulation of the flagellar motor. J Bacteriol 176: 802–810.

44. Van T, Zamboni DS, Roy CR, Dietrich WF, Vance RE (2006) Flagellin-deficient Legionella mutants evade caspase-1- and Naip5-mediated macrophage immunity. PLoS Pathog 2: e118.

45. Ren J, Zamboni DS, Roy CR, Dietrich WF, Vance RE (2006) Flagellin-deficient Legionella mutants evade caspase-1- and Naip5-mediated macrophage immunity. PLoS Pathog 2: e118.
48. Whitfield NN, Byrne BG, Swanson MS (2010) Mouse macrophages are permissive to motile Legionella species that fail to trigger pyroptosis. Infect Immun 78: 423–432.

49. Mez AJ, So M, Shetz MP (2008) Phosphatase retraction powers bacterial twitching motility. Nature 447: 90–102.

50. Coi1 DA, Anné J (2009) Twitching motility in Legionella pneumophila. FEMS Microbiol Lett 293: 271–277.

51. Stone BJ, Abu Kwaik Y (1998) Expression of multiple pili by Legionella pneumophila identifies and characterizes a type IV pilin gene and its role in adherence to mammalian and protozoan cells. Infect Immun 66: 1768–1775.

52. Stone BJ, Kwaik YA (1999) Natural competence for DNA transformation by Legionella pneumophila and its association with expression of type IV pili. J Bacteriol 181: 1393–1402.

53. Molodtky AB, Shetron-Rama LM, Swanson MS (2005) Components of the Legionella pneumophila flagellar regulon contribute to multiple virulence traits, including lysosome avoidance and macrophage death. Infect Immun 73: 5720–5734.

54. Luo ZQ, Isberg RR (2004) Multiple substrates of the Legionella pneumophila Dot/Icm system identified by interbacterial protein transfer. Proc Natl Acad Sci U S A 101: 8411–8416.

55. Nagai H, Kagan JC, Zhu X, Kahn RA, Roy CR (2002) A bacterial guanine nucleotide exchange factor activates Rac on Legionella phagosomes. Science 295: 679–682.

56. Brüggemann H, Hagman A, Jules M, Simeone O, Dillies MA, et al. (2006) Virulence strategies for infecting phagocytes deduced from the in vivo transcriptional program of Legionella pneumophila. Cell Microbiol 8: 1226–1240.

57. Pan X, Lührmann A, Satoh A, Laskowski-Arce MA, Roy CR (2008) Ankyrin repeat proteins comprise a diverse family of bacterial type IV effectors. Science 320: 1653–1654.

58. Buckles EL, Wang X, Lockatt CV, Johnson DE, Donnenberg MS (2006) PhoU enhances the ability of extraintestinal pathogenic Escherichia coli strain CFT073 to colonize the murine urinary tract. Microbiology 152: 153–60.

59. Stancik LM, Stancik DM, Schmidt B, Barnhart D M, Yoncheva YN, et al. (2009) Comprehensive Analysis of Mutations in the Legionella pneumophila icmT locus that emerged within a highly diverse species. Genome Res 19: 431–441.

60. Young IG, McCann LM, Stroobant P, Gibson F (1971) Characterization and bacteriophage sensitivity of the K-12 accumulating the biquinone precursors 2-octaprenyl-6-methoxy-1,4-benzoquinone and 2-octaprenyl-5-methyl-6-methoxy-1,4-benzoquinone. J Bacteriol 105: 769–778.

61. Banga S, Gao P, Shen X, Fiscus V, Zong WX, et al. (2007) Identification and characterization of a type IV pilin gene and its association with expression of type IV pili. J Bacteriol 189: 1307–1319.

62. Machner MP, Isberg RR (2006) Targeting of host Rab GTPase function by the Icm system identified by interbacterial protein transfer. Proc Natl Acad Sci U S A 104: 5121–5126.

63. Lebeau I, Lammertyn E, De Buck E, Hoëper D, Anne J (2008) Differential 2-D gel-based proteomic analysis of Legionella pneumophila wild type and Tat secretion mutants. Int J Med Microbiol 298: 449–461.