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Research article

Identification of differentially expressed *Legionella* genes during its intracellular growth in *Acanthamoeba*

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ABSTRACT

Legionella grows intracellularly in free-living amoeba as well as in mammalian macrophages. Until now, the overall gene expression pattern of intracellular *Legionella* in *Acanthamoeba* was not fully explained. Intracellular bacteria are capable of not only altering the gene expression of its host, but it can also regulate the expression of its own genes for survival. In this study, differentially expressed *Legionella* genes within *Acanthamoeba* during the 24 h intracellular growth period were investigated for comparative analysis. RNA sequencing analysis revealed 3,003 genes from the intracellular *Legionella*. Among them, 115 genes were upregulated and 1,676 genes were downregulated more than 2 fold compared to the free *Legionella*. Gene ontology (GO) analysis revealed the suppression of multiple genes within the intracellular *Legionella*, which were categorized under 'ATP binding' and 'DNA binding' in the molecular function domain. Gene expression of alkylhydroperoxidase, an enzyme involved in virulence and anti-oxidative stress response, was strongly enhanced 24 h post-intracellular growth. Amino acid ABC transporter substrate-binding protein that utilizes energy generation was also highly expressed. Genes associated with alkylhydroperoxidase, glucose pathway, and Dot/Icm type IV secretion system were shown to be differentially expressed. These results contribute to a better understanding of the survival strategies of intracellular *Legionella* within *Acanthamoeba*.

1. Introduction

Legionella pneumophila is an intracellular pathogen and the causative agent of a severe form of pneumonia known as the Legionnaires' disease (Fields et al., 2002). *L. pneumophila* utilizes *Acanthamoeba* spp. as a host for survival and replication, but it can also thrive within human macrophage (Iovieno et al., 2010). Some species of *Legionella* cannot be cultured *in vitro* and requires co-cultivation within amoeba (Steinert et al., 1997). *Legionella* avoids the lysosomal degradation by forming a *Legionella*-containing vacuole (LCV), and this intracellular replication within *Acanthamoeba* could increase the virulence of *Legionella* (Gomes et al., 2018).

Several features of *Acanthamoeba* spp. makes it a suitable model organism for studying interactions between intracellular bacteria such as *L. pneumophila* and macrophages. Notably, the infection processes and the intracellular life cycle of *Legionella* within *Acanthamoeba* are similar to those demonstrated within human macrophages (Best and Abu Kwaik, 2018). These features include uptake of *Legionella* by coiling phagocytosis, formation of ribosome-studded phagosome containing *Legionella*, and inhibition of lysosomal fusion with phagosomes (Bozue and Johnson, 1996). Additionally, *L. pneumophila* uses similar genes to multiply in both hosts. The genes of the type IV secretion system required for intracellular growth in human macrophages are also required for intracellular growth in *A. castellanii* (Segal and Shuman, 1999). Pili aid in attachment of flagellated *L. pneumophila* to human macrophage and *A. polyphaga* (Stone and Abu Kwaik, 1998). The ankyrin repeat protein B (AnkB) effector is necessary for intracellular proliferation in *Acanthamoeba* and human macrophage to form the LCV (Richards et al., 2013). Therefore, understanding how intracellular *L. pneumophila* interacts within *Acanthamoeba* could be key to unraveling the intracellular life cycle of *Legionella* and the mechanisms involved in human macrophages.

Multitudes of studies have been conducted to identify factors contributing to *L. pneumophila* pathogenicity in *Acanthamoeba*. CpxRA two-component system was reported to contribute significantly to its

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virulence (Tanner et al., 2016). The nuclease activity of Cas2 protein in *L. pneumophila* was confirmed to be crucial for promoting amoebic infection (Gunderson et al., 2015). The pyroptosis-related gene, *flaA*, and apoptosis-related gene, *vipD* of *Legionella* were upregulated in growing *A. castellanii* (Mou and Leung, 2018). The Dot/Icm effector SdhA of *Legionella*, a virulence factor, was expressed highly in the macrophages upon *Acanthamoeba* infection (Gomes et al., 2018). Higher concentrations of pro-inflammatory cytokines in macrophages infected with *Legionella* were derived from a previous intracellular passage through *Acanthamoeba* (Gomes et al., 2018).

Recently, factors associated with survival and replication of *L. pneumophila* in *Acanthamoeba* are also being investigated since these are essential for its growth in human macrophage. Yet, the genome-wide expression analysis of an intracellular *Legionella* within *Acanthamoeba* remain unreported to date. To this extent, unraveling the regulation of gene expressions and their role in pathogenesis would have a significant impact on public health improvement. In this study, total transcriptional changes to genes involved in survival, replication, and virulence of *L. pneumophila* were investigated during its 24 h intracellular growth phase in *A. castellanii* through RNA sequencing analysis. This analysis demonstrated several genes involved in the survival of intracellular *Legionella* in *Acanthamoeba*.

2. Materials and methods

2.1. Cell cultures

Legionella pneumophila Philadelphia-1 strain (ATCC 33152) was cultured on BCYE (Buffered Charcoal Yeast Extract) agar plate at 37 °C with 5% CO_2. *Acanthamoeba castellanii* Castellani (ATCC 30868) was cultured axenically in PYG (Proteose peptone-Yeast extract-Glucose) medium at 25 °*C incubator*.

2.2. Intracellular growth of Legionella

A. castellanii was infected with *L. pneumophila* as previously described (Mou and Leung, 2018). Monolayers of *Acanthamoeba* in T75 flask were incubated with 1×10^9 of *Legionella* at 37 °C with 5% CO_2 for 1 h. After incubation, *Acanthamoeba* was washed with PAS (Page's Amoeba Saline) and incubated with new PYG media containing 100 μg/ml of gentamicin for 2 h to kill extracellular *Legionella*. *Legionella* infected *Acanthamoeba* was washed with PAS twice and incubated with new PYG media for 24 h at 25 °*C incubator*. To visualize the *Legionella* within *Acanthamoeba,* Giemsa stain was used. For Giemsa stain, *Legionella*-infected *Acanthamoeba* was fixed with methanol for 5 min and stained with Giemsa solution for 10 min.

2.3. RNA isolation

A colony on BCYE agar plate was picked for free-living *Legionella* RNA extraction. For intracellular *Legionella* RNA, *Legionella*-infected *Acanthamoeba* (for 24 h) was incubated with 2 ml of dH_2O for 10 min, and lysed by 20 forced passages through a 26 gauge syringe needle. The lysate was centrifuged at 150 g for 1 min for supernatant acquisition. Total RNA was purified using the RNeasy Mini kit (Qiagen, Hilden, Germany). RNA quality was assessed by Agilent 2100 bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, Netherlands), and RNA quantification was performed using ND-2000 Spectrophotometer (Thermo Inc., DE, USA).

2.4. Library preparation and sequencing

For *Legionella* and *Acanthamoeba* passaged *Legionella* RNAs, rRNA was removed using Ribo-Zero Magnetic kit (Epicentre, Inc., USA) from each 5 μg of total RNA. Construction of the library was performed using SMARTer Stranded RNA-Seq Kit (Clontech lab Inc., CA, USA) according to the manufacturer's instructions. The rRNA-depleted RNAs were used for cDNA synthesis and shearing, following the manufacturer's instruction. Indexing was performed using the Illumina indexes 1–12. The enrichment step was carried out using PCR. Subsequently, libraries were checked using the Agilent 2100 bioanalyzer (DNA High Sensitivity Kit) to evaluate the mean fragment size. Quantification was performed using the library quantification kit using a StepOne™ Real-Time PCR System (Life Technologies, Inc., USA). High-throughput sequencing was performed as paired-end 100 sequencing using HiSeq 2500 (Illumina, Inc., USA).

2.5. Data analysis

Legionella RNA sequence reads were mapped using the Bowtie2 software tool in order to obtain the alignment file. Differentially expressed genes were determined based on counts from unique and multiple alignments using EdgeR within R using Bioconductor. The alignment file also was used for assembling transcripts. Quantile-Quantile normalization method was used for comparison between samples. Gene classification was based on searches done by DAVID (http://david.abcc.ncifcrf.gov/).

3. Results

3.1. Intracellular Legionella

Giemsa staining revealed intracellular *L. pneumophila* in *A. castellanii* at 24 h post infection (Figure 1B). In non-infected *Acanthamoeba*, Giemsa staining showed a nuclear and many mitochondras (Figure 1A). Survival and replication of *Legionella* within *Acanthamoeba* were observed until 24 h post-infection. The plasma membrane of *Acanthamoeba* remains intact for up to 24, however after 24 h post infection, *Acanthamoeba* was lysed and the culture media was contaminated with *Legionella* (data not shown).

3.2. Differentially expressed genes of Legionella during intracellular growth

To better understand the changes in gene expression profiles of intracellular *Legionella* in *Acanthamoeba* (L + A), RNA sequencing analysis was performed and the results were compared with those of free *Legionella* (L). A total of 3,003 genes from *Legionella* were selected, and 1,791 differentially expressed genes (DEGs) were identified. A large number of DEGs (1,676) were downregulated, and only 115 DEGs were upregulated. DEGs of intracellular *Legionella* were classified biological process, cellular component, and molecular function categories based on Gene Ontology (GO) analysis. GO analysis of upregulated genes was shown in Table 1. Among upregulated genes, drastic increase in the number of GO terms were not found. On the contrary, downregulated DEGs were subdivided into various GO terms under each of the GO categories (Table 2). Within the biological process category, 32 genes were involved in 'transcription'. Analysis of the cellular component category revealed that 147 genes were involved in 'cytoplasm'. In the molecular function category, 139 genes were involved in 'ATP binding'', and 79 genes were involved in 'DNA binding'.

3.3. Upregulated genes in intracellular Legionella

Genes upregulated more than 3.5 fold in intracellular *Legionella* during its 24 h growth phase in *Acanthamoeba* were listed in Table 3. Notably, lpg2350-alkylhydroperoxidase, lpg2349-alkylhydroperoxidase, and the lpg0491-amino acid ABC (ATP-binding cassette) transporter substrate binding protein were upregulated 11 fold, 5 fold, and 4 fold, each respectively. The full list of 115 genes upregulated more than 2 fold was described in Supplementary Table 1. Proteins with potential relevance to lpg2350-alkylhydroperoxidase were searched by STRING database (Figure 2A). Ten proteins including AhpD (lpg2349-alkylhydroperoxidase) have been identified to be associated with lpg2350-

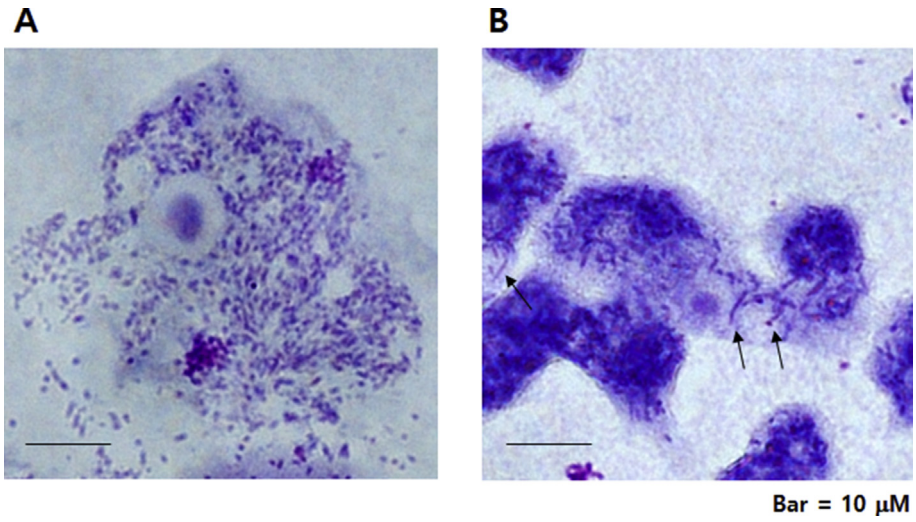


Figure 1. *L. pneumophila* in *A. castellanii* 24 h post-infection. Microscopic images of non-infected *Acanthamoeba* (A) and *Legionella* infected *Acanthamoeba* (B) after Giemsa stain. Arrows indicate *L. pneumophila*.

Table 1. Gene ontology analysis of upregulated genes in *Legionella*.

GO category	GO term	Count
Biological process	GO:0042773~ATP synthesis coupled electron transport	2
	GO:0006412~translation	4
	GO:0015074~DNA integration	2
	GO:0009245~lipid A biosynthetic process	2
	GO:0015986~ATP synthesis coupled proton transport	2
	GO:0006260~DNA replication	2
Cellular component	GO:0045261~proton-transporting ATP synthase complex, catalytic core F (1)	2
	GO:0005840~ribosome	3
Molecular function	GO:0008137~NADH dehydrogenase (ubiquinone) activity	3
	GO:0019843~rRNA binding	4
	GO:0046961~proton-transporting ATPase activity, rotational mechanism	2
	GO:0008080~N-acetyltransferase activity	3
	GO:0016787~hydrolase activity	4
	GO:0003735~structural constituent of ribosome	4
	GO:0016874~ligase activity	2
	GO:0003887~DNA-directed DNA polymerase activity	2
	GO:0046933~proton-transporting ATP synthase activity, rotational mechanism	2
	GO:0003677~DNA binding	6
	GO:0016740~transferase activity	3

alkylhydroperoxidase, and their expression levels were summarized in a table (Figure 2B). A marked increase in lpg2350-alkylhydroperoxidase expression was observed during the 24 h intracellular growth. The lpg2349-alkylhydroperoxidase and lpg1815-hydrogen peroxide inducible genes activator OxyR inductions were also enhanced.

3.4. Glucose pathway and type IV secretion system of Legionella

To understand the intracellular survival and replication processes of *Legionella* in *Acanthamoeba*, the expression levels of genes associated with the glucose pathway and nutritional adaptation were investigated (Figure 3). STRING protein-protein association networks revealed that 12 proteins were associated with glucose metabolism in *Legionella* (Figure 3A). Of these 12, only 2 proteins lpg2486-phosphomannomutase and *edd*-phosphogluconate dehydratase were highly expressed while the remaining 10 were expressed at low levels following 24 h intracellular growth (Figure 3B). Expression levels of Dot/Icm type IV secretion system from *Legionella* which are required for intracellular growth and host cell lysis were summarized in Table 4. DotB and dotC were upregulated while dotD and all icm genes were downregulated during 24 h growth within *Acanthamoeba*.

4. Discussion

Legionella is an intracellular pathogen that controls the expression of various genes to survive and replicate in host cells. After entry into the macrophage, *L. pneumophila* replicated by 4 h within the LCV (Horwitz, 1983). By 18–24 h, *Legionella* is released from the LCV and remains in the cytoplasm of macrophages (Molmeret et al., 2004). At 24 h after infection with *L. pneumophila*, macrophage exhibited apoptotic cell death (Müller et al., 1996). Intracellular replication of *L. pneumophila* showed similar growth kinetics in U937 macrophage and *A. polyphaga* (Molmeret et al., 2004). Therefore, the time point 24 h post-infection with *L. pneumophila* was selected to investigate the factors associated with intracellular growth and killing of the host organism.

In this study, *L. pneumophila* within *A. castellanii* showed 1,676 differentially expressed genes (DEGs) by 24 h post-infection. In comparison to the free *L. pneumophila*, 115 DEGs were upregulated and 1,676 DEGs were downregulated. However, it is noteworthy to mention that

Table 2. Gene ontology analysis of downregulated genes in *Legionella*.

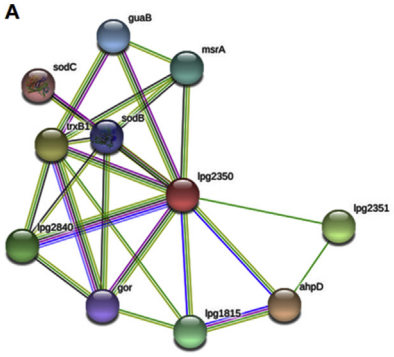
GO category	GO term	Count
Biological process	GO:0006310~DNA recombination	16
	GO:0009116~nucleoside metabolic process	8
	GO:0030091~protein repair	6
	GO:0043087~regulation of GTPase activity	6
	GO:0006629~lipid metabolic process	14
	GO:0006633~fatty acid biosynthetic process	12
	GO:0009405~pathogenesis	10
	GO:0019877~diaminopimelate biosynthetic process	5
	GO:0006782~protoporphyrinogen IX biosynthetic process	5
	GO:0042619~poly-hydroxybutyrate biosynthetic process	6
	GO:0071555~cell wall organization	13
	GO:0006099~tricarboxylic acid cycle	10
	GO:0009073~aromatic amino acid family biosynthetic process	7
	GO:0007049~cell cycle	11
	GO:0008299~isoprenoid biosynthetic process	4
	GO:0006281~DNA repair	15
	GO:0006351~transcription, DNA-templated	32
	GO:0008652~cellular amino acid biosynthetic process	9
	GO:0009435~NAD biosynthetic process	5
	GO:0009231~riboflavin biosynthetic process	5
	GO:0016226~iron-sulfur cluster assembly	5
	GO:0006400~tRNA modification	5
Cellular component	GO:0042597~periplasmic space	9
	GO:0005737~cytoplasm	147
	GO:0005622~intracellular	29
	GO:0009424~bacterial-type flagellum hook	4
Molecular function	GO:0003700~transcription factor activity, sequence-specific DNA binding	24
	GO:0008270~zinc ion binding	32
	GO:0051536~iron-sulfur cluster binding	10
	GO:0008113~peptide-methionine (S)-S-oxide reductase activity	6
	GO:0016829~lyase activity	11
	GO:0003684~damaged DNA binding	7
	GO:0005524~ATP binding	139
	GO:0004222~metalloendopeptidase activity	13
	GO:0000155~phosphorelay sensor kinase activity	12
	GO:0008237~metallopeptidase activity	9
	GO:0050660~flavin adenine dinucleotide binding	20
	GO:0003677~DNA binding	79
	GO:0003676~nucleic acid binding	20
	GO:0033743~peptide-methionine (R)-S-oxide reductase activity	4
	GO:0016987~sigma factor activity	5
	GO:0015197~peptide transporter activity	5
	GO:0016746~transferase activity, transferring acyl groups	10
	GO:0004553~hydrolase activity, hydrolyzing O-glycosyl compounds	6

culture condition, particularly temperature may have influenced the DEGs 24 h post-infection since *Legionella* were cultured at 37 °C whereas *Acanthamoeba* were maintained at 25 °C.

Of the DEGs, *Legionella* gene with the highest upregulation following 24 h intracellular growth in *Acanthamoeba* was alkylhydroperoxidase (Table 3). Alkylhydroperoxidase is an enzyme acting to breakdown toxic peroxide compounds to alcohol and water, and the function is related to the oxidative stress response of several bacterial species (Paterson et al., 2006). In the case of *L. pneumophila*, antioxidant enzymes are of importance since the microbes become exposed to oxidative stress within the intracellular milieu of its host *A. castellanii* (LeBlanc et al., 2006). During the 24 h intracellular growth phase of *Legionella* within *Acanthamoeba*, alkylhydroperoxidases (lpg2350 and lpg2349) were upregulated, which may signify its importance for survival upon exposure to oxidative stress. Upregulation of several genes, including alkylhydroperoxide reductase are documented to be a feature reflecting *L. pneumophila* in its replicative phase (Brüggemann et al., 2006). In line with this notion, alkylhydroperoxide reductase was upregulated more than 5 fold in our study (Figure 2). The hydrogen peroxide-inducible genes activator (*OxyR*) was also upregulated nearly 2 fold in our study. Its overexpression in *L. pneumophila* has been associated with diminished growth as well as negative regulations of *icmR* and *cpxRA* in *A. castellanii* (Tanner et al., 2017). Consistent with this finding, the *icmR* expression in the L + A group deceased to 0.054 (Table 4). Interestingly, superoxide dismutase genes *sodB* and *sodC* were down-regulated in the L + A group whereas the H_2O_2-reducing AhpD and alkylhydroperoxidase were enriched. Previous studies have documented that catalase activity is severely lacking in *L. pneumophila*, which leaves them vulnerable to even low concentrations of H_2O_2 (Hoffman and Pine, 1982; Hoffman et al., 1983). Based on these reports, we speculate that profuse levels of H_2O_2 were present within

Table 3. Genes upregulated more than 3.5 fold in *Legionella*.

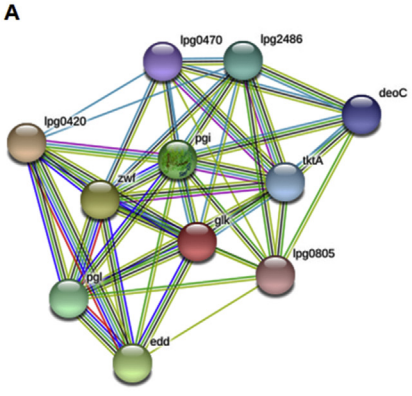
Gene symbol	Fold change	Annotation
	L + A/L	product
lpg2350	11.029	alkylhydroperoxidase
lpg2857	9.470	-
lpg2124	7.240	hypothetical protein
lpg1053	5.912	ATP synthase F0F1 subunit epsilon
lpg2753	5.890	16S ribosomal RNA
lpg0572	5.165	hypothetical protein
lpg2349	5.046	alkylhydroperoxidase
lpg0689	4.995	DNA binding stress protein
lpg1706	4.916	arginine N-succinyltransferase subunit beta
lpg1940	4.882	peptide synthetase
lpg0491	4.237	amino acid ABC transporter substrate-binding protein
lpg0594	4.185	hypothetical protein
nuoM	3.971	NADH dehydrogenase I subunit M
lpg0797	3.828	tRNA-Met
lpg0146	3.807	transposase B, TnpA
mutL	3.715	DNA mismatch repair protein MutL
lpg0166	3.655	hypothetical protein
lpg0433	3.637	hypothetical protein
lpg0189	3.554	hypothetical protein
lpg0705	3.527	transporter component
lpg0039	3.503	hypothetical protein



B

Gene symbol	Fold change L+A/ L	Annotation product
lpg2350	**11.029**	**alkylhydroperoxidase**
msrA	0.132	bifunctional methionine sulfoxide reductase B/A protein
guaB	0.628	inosine-5'-monophosphate dehydrogenase
sodC	0.153	superoxide dismutase
trxB1	0.581	thioredoxin reductase
sodB	0.182	superoxide dismutase
lpg2840	0.133	peroxiredoxin, AhpC/TSA family protein
gor	0.664	glutathione reductase
lpg1815	1.880	hydrogen peroxide-inducible genes activator OxyR
lpg2349	**5.406**	**Alkylhydroperoxide reductase (AhpD)**
lpg2351	0.141	hypothetical protein

Figure 2. The STRING network of alkylhydroperoxidase and their expression levels. The gene networks of lpg2350 alkylhydroperoxidase and lpg2349 alkylhydroperoxidase of *Legionella* generated by the STRING database (A). Colored lines between the gene/protein indicate the various type of interaction evidence. The expression levels of associated genes with alkylhydroperoxidase were summarized in table (B).



B

Gene symbol	Fold change L+A/ L	Annotation product
deoC	0.244	2-deoxyribose-5-phosphate aldolase
lpg2486	1.883	phosphomannomutase
lpg0470	0.471	fructose bisphosphate aldolase
pgi	0.820	glucose-6-phosphate isomerase
tktA	0.751	transketolase
lpg0420	0.289	eda
lpg0420	-	-
zwf	0.863	glucose-6-phosphate 1-dehydrogenase
glk	0.247	glucokinase
lpg0805	0.716	phosphoenolpyruvate synthase
pgl	0.274	6-phosphogluconolactonase
edd	2.976	phosphogluconate dehydratase

Figure 3. Network visualization of glucose pathway in *Legionella* and their expression levels. Network by STRING showed an interaction among the genes associated with glucose metabolism in *Legionella* (A). The expression levels of twelve genes involved in the glucose pathway were present in table (B).

Table 4. Expression levels of Dot/Icm type IV secretion system.

Gene symbol		Fold change	Annotation
		L + A/L	Product
Region I	icmV	0.062	intracellular multiplication protein IcmV
	icmW	0.408	intracellular multiplication protein IcmW
	icmX	0.262	intracellular multiplication protein IcmX
	dotA	0.944	defect in organelle trafficking protein DotA
	dotB	1.279	ATPase
	dotC	2.330	DotC
	dotD	0.180	lipoprotein DotD
Region II	icmT	0.164	IcmT protein
	icmS	0.109	IcmS protein
	icmR	0.054	IcmR
	icmQ	0.617	IcmQ protein
	icmP	0.296	IcmP protein
	icmO	0.070	IcmO protein
	icmM	0.160	IcmM (DotJ)
	icmL	0.249	IcmL protein
	icmK	0.180	IcmK protein
	icmE	0.345	IcmE protein
	icmG	0.075	IcmG protein
	icmC	0.103	hypothetical protein
	icmD	0.113	IcmD protein
	icmJ	0.145	IcmJ protein
	icmB	0.692	IcmB protein
	icmF	0.171	IcmF

A. castellanii, which forced *L. pneumophila* to overexpress H_2O_2-reducing enzymes to cope with this environmental stress.

A key component required for the intracellular growth of *Legionella* is glucose metabolism. Intracellular *L. pneumophila* metabolizes glucose through the Entner-Doudoroff (ED) pathway, and this metabolic process involves glucokinase (*glk*), glucose-6-phosphate dehydrogenase (*zwf*), 6-phosphogluconolactonase (*pgl*), phosphogluconate dehydratease (*edd*), and 2-dehydro-3-deoxy-phosphogluconate aldolase (*edd*) (Harada et al., 2010). DEGs analysis in this study confirmed that *edd* was highly expressed 24 h post-infection (Figure 3). Earlier studies have confirmed the expression of Entner-Doudoroff pathway during the replicative phase of *L. pneumophila in vivo* (Brüggemann et al., 2006) and consistent with this finding, upregulation of *edd* gene was observed in the L + A group. From these findings, it is assumed that *edd* gene of *Legionella* plays an important role in its growth within *Acanthamoeba*.

The intracellular life cycle of *Legionella* is comprised of the replicative phase and the transmissive phase (Byrne and Swanson, 1998). During the replicate phase, Dot/Icm type IV secretion system of *L. pneumophila* can manipulate host cellular functions to support its intracellular growth in the LCV (Coers et al., 1999). Protein translocation by the Dot/Icm complex could occur not only through host phagosomal membranes but also from one bacterial cell to another bacterial cell (Luo and Isberg, 2004). DotB and icmE are important for conjugation-mediated genetic exchange in *L. pneumophila* (Swanson and Hammer, 2000). Recently, a similar study investigating the differential gene expressions of *L. pneumophila* reported strong upregulation of *dotC*, *dotA*, *icmW,* and several other genes involved in stress response during the transmissive phase of *L. pnuemophila* (Weissenmayer et al., 2011). Similar to the previous findings, our results confirmed increased dotB and dotC expressions as well several oxidative stress-related genes from *Legionella* 24 h post-infection in *Acanthamoeba* (Figure 2 and Table 4).

In this study, enrichment of genes associated with pathogenesis was not confirmed. One explanation for the lack of bacterial virulence as observed in the present study stems from the temperature used to culture *L. pneumophila*. Culture condition, especially temperature, is one of the factors affecting the virulence of *L. pneumophila* (Swanson and Hammer, 2000). Evidently, *L. pneumophila* cultured at 24 °C was less virulent than the *L. pneumophila* cultured at 37 °C (Mauchline et al., 1994). Based on this notion, the virulence-associated genes of *L. pneumophila* may have been induced to a lesser extent in our study since they were incubated within *Acanthamoeba* at 25 °C for 24 h.

In summary, we demonstrated the gene expression patterns of *L. pneumophila* 24 h post-infection in *A. castellanii*. Protection against oxidative stress is one of the most important aspects required by *Legionella* to remain viable within *Acanthamoeba*. More studies involving oxidative stress and antioxidant defense system of *Legionella* are necessary to understand the intracellular growth of *Legionella* with *Acanthamoeba*. In addition to this, significantly increased or decreased genes of *Legionella* during intracellular growth need more research. *Acanthamoeba* and human macrophage share similar features that permit the intracellular growth of *Legionella* and therefore, further investigating the genes of *Legionella* used for survival and replication within *Acanthamoeba* could provide important information to understanding its lifestyle and pathogenicity in human macrophage.

Declarations

Author contribution statement

Fu-Shi Quan: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Hyun-Hee Kong: Conceived and designed the experiments; Analyzed and interpreted the data.

Hae-Ahm Lee: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ki-Back Chu: Analyzed and interpreted the data; Wrote the paper.

Eun-Kyung Moon: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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