ISSN (Print) 0023-4001
ISSN (Online) 1738-0006

Korean J Parasitol Vol. 59, No. 1: 67-76, February 2021
https://doi.org/10.3347/kjp.2021.59.1.67

▣ BRIEF COMMUNICATION

Differentially Expressed Gene Profile of *Acanthamoeba castellanii* Induced by an Endosymbiont *Legionella pneumophila*

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Abstract: *Legionella pneumophila* is an opportunistic pathogen that survives and proliferates within protists such as *Acanthamoeba* spp. in environment. However, intracellular pathogenic endosymbiosis and its implications within *Acanthamoeba* spp. remain poorly understood. In this study, RNA sequencing analysis was used to investigate transcriptional changes in *A. castellanii* in response to *L. pneumophila* infection. Based on RNA sequencing data, we identified 1,211 upregulated genes and 1,131 downregulated genes in *A. castellanii* infected with *L. pneumophila* for 12 hr. After 24 hr, 1,321 upregulated genes and 1,379 downregulated genes were identified. Gene ontology (GO) analysis revealed that *L. pneumophila* endosymbiosis enhanced hydrolase activity, catalytic activity, and DNA binding while reducing oxidoreductase activity in the molecular function (MF) domain. In particular, multiple genes associated with the GO term 'integral component of membrane' were downregulated during endosymbiosis. The endosymbiont also induced differential expression of various methyltransferases and acetyltransferases in *A. castellanii*. Findings herein are may significantly contribute to understanding endosymbiosis of *L. pneumophila* within *A. castellanii*.

Key words: *Acanthamoeba*, *Legionella*, endosymbiosis, differential gene expression

Acanthamoeba spp. is one of the most abundant protozoan in the environment and commonly isolated from soil and water. *Acanthamoeba* spp. trophozoite usually feeds on bacteria, fungi, algae or small organic particles by phagocytosis [1]. However, some bacteria have developed strategies to resist phagocytosis, survive intracellularly and exploit *Acanthamoeba* spp. for multiplication [2]. These bacteria are able to survive in encysted *Acanthamoeba* spp. which protects the endosymbionts from adverse environmental conditions [3]. *Acanthamoeba* spp. not only enables the endosymbionts to persist in the environment but also enhances its pathogenicity [4]. Moreover, since mammalian macrophages and amoebae show similar interactions with endosymbionts, investigating the endosymbiotic relationship between intracellular pathogens and *Acanthamoeba* spp. would contribute to understanding how these organisms behave in the mammalian cells and its evasion of the human immune system.

Acanthamoeba spp. can be a host for a wide range of pathogenic microorganisms such as *Legionella pneumophila*, *Chlamydophila pneumoniae*, *Cryptococcus neoformans*, *Mycobacterium avium*, *Listeria monocytogenes*, and *Pseudomonas aeruginosa*, *etc* [3,5-7]. Among these microorganisms, the interaction between *Acanthamoeba* spp. and *Legionella* spp. is one of the most investigated. After uptake of *Legionella* spp. by *Acanthamoeba* spp., *Legionella* spp. forms a specialized compartment called *Legionella*-containing vacuole (LCV). LCV avoids fusion with lysosomes to deter lysosomal digestion and also inhibits phagosomal maturation, thereby enabling *L. pneumophila* to actively replicate inside the LCV [8].

To date, LCV and a large number of effectors transferred by the intracellular multiplication/defective organelle transport (Icm/Dot) type IV system of *Legionella* spp. have been identified [9-12]. Although the roles of these genes from *Legionella* spp. have been evaluated, little research has been done on genes of *Acanthamoeba* spp. during endosymbiosis with *Legionella* spp. To understand the intracellular survival strategy of

• Received 14 October 2020, revised 15 December 2020, accepted 21 December 2020.
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Legionella spp., inhibition of phagosome lysis in *Acanthamoeba* spp. needs to be studied. In this study, total transcriptional changes of *A. castellanii* in response to survival and replication of *L. pneumophila* during 12 hr and 24 hr were investigated by RNA sequencing analysis.

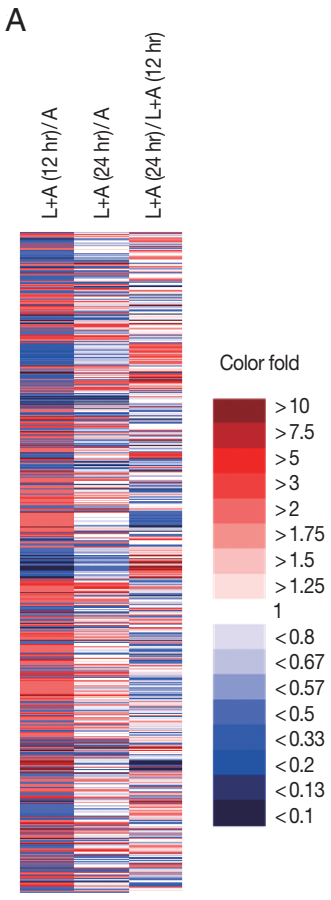
The LCV in the *Legionella*-infected *A. polyphaga* has been reported to remain intact for up to 8 hr post-infection (hr pi), disrupted by 12 hr pi, and eventually lysed to release the intracellular pathogens into the cytoplasm of the amoeba by 18 to 24 hr pi [13]. *L. pneumophila* infection incurred the lysis of more than 80% of *A. polyphaga* at 24 hr pi, and it has also been suggested that the intracellular condition may significantly differ between 12-18 hr pi and 24 hr pi [13]. Contrary to the previous findings, *L. pneumophila*-infected *A. castellanii* in the present study remained intact even at 24 hr pi. Therefore, gene expression patterns at 12 hr pi and 24 hr pi were compared to confirm whether drastic differences were present in *L. pneumophila*-infected *A. castellanii* at these 2 time points.

A. castellanii was infected with *L. pneumophila* [14], and the *Legionella*-infected *Acanthamoeba* (L+A) was incubated for 12 hr and 24 hr at 25˚C incubator. mRNA-Seq reads were mapped using TopHat software [15], and differentially expressed gene were determined based on BEDtools and EdgeR [16-18]. And we used the FPKM (fragments per kilobase of exon per million fragments) as the method of determining the expression level of the gene regions. Gene classification was based on searches done by DAVID (http://david.abcc.ncifcrf.gov/).

RNA samples from different experimental conditions were sequenced to investigate the endosymbiosis-induced gene expression changes in *A. castellanii* (Fig. 1). A total of 7,108 genes whose expressions changed 12 hr pi and 24 hr pi were displayed using a heat map (Fig. 1A). Genes from each group were colorized based on their expression level. Strongly upregulated/downregulated genes, as indicated by intense red/blue colors, were more prevalent in the 12 hr pi group than the 24 hr pi group. Genes whose expression levels changed more than 2 fold were selected for further analysis (Fig. 1B). Among the 7,018 genes, 1,211 and 1,131 genes in the 12 hr pi group were upregulated and downregulated more than 2 fold, respectively. Similarly, 1,321 and 1,379 genes from the 24 hr pi group were upregulated and downregulated more than 2 fold, each respectively. Venn diagram revealed that a fraction of the DEGs found in the 12 hr pi overlapped with the DEGs from 24 hr pi group (Fig. 1C). Our results revealed that 2,342 and 2,700 DEGs in *L. pneumophila*-infected *A. castellanii* at 12 hr pi and 24 hr pi were changed more than 2 fold, respectively. More DEGs were observed at 24 hr pi than at 12 hr pi, which may indicate that more gene involvement is required for survival of *L. pneumophila* in the later stages of infection.

DEGs of *L. pneumophila*-infected *A. castellanii* were assigned an Entrez Gene ID and subsequently classified into 3 domains: biological process (BP), cellular component (CC), and molecular function (MF). Classified DEGs were subdivided further into various gene ontology (GO) terms under each of the domains. In the 12 hr pi group, DEGs were assigned to 10 subcategories in BP, 8 subcategories in CC, and 23 subcategories in MF (Fig. 2). During the 12 hr endosymbiosis, 17 genes in 'regulation of transcription' (domain: BP), 30 genes in the 'nucleus' (domain: CC), and 37 genes in 'DNA binding' (domain: MF) were determined to be the most upregulated genes. Within the CC domain, 200 downregulated genes were involved in the GO term 'integral component of the membrane'. In the 24 hr pi group, DEGs were subdivided into 29, 13, and 41 GO terms under BP, CC, and MF domains, each respectively (Fig. 3). In the BP domain, 26, 39, and 32 genes from the GO terms 'regulation of transcription', 'intracellular signal transduction', and 'cyclic nucleotide biosynthetic process' were drastically downregulated, each respectively. In the CC domain, similar to the 12 hr pi *A. castellanii*, 270 downregulated genes were involved in the GO term 'integral component of membrane'. In the MF domains, downregulated genes associated with the GO terms 'zinc ion binding' and 'protein kinase activity' were 57 and 42, respectively. From each of the domains, 26 genes from 'carbohydrate metabolic process' (domain: BP), 30 genes from the 'nucleus' (domain: CC), and 40 genes from 'DNA binding' (domain: MF) were mainly upregulated.

DEGs from *A. castellanii*, which were upregulated or downregulated more than 10 fold post-infection with *L. pneumophila*, were listed in Tables 1 and 2. In the 12 hr pi *A. castellanii*, 47 out of 1,211 DEGs were upregulated (Table 1) and 90 out of 1,131 DEGs were downregulated more than 10 fold (Table 2). Identities for several most upregulated proteins in this group were 2 hypothetical proteins (1,100 fold and 345 fold), S-adenosylmethionine-dependent methyltransferases (173 fold), and GDPD-mannose-3', 5'-epimerase (87 fold) (Table 1). GO analysis of the assigned Entrez Gene IDs revealed that the DEG which underwent 1,100 fold increase was a hypothetical protein that belonged to the DNA binding (GO: 0003677) category. Similarly, the GDPD-mannose-3',5'-epimerase which was increased 87 fold, was associated with catalyt-



B

	L+A (12 hr)/A	L+A (24 hr)/A	L+A (24 hr)/L+A (12 hr)
No. of up-regulated	1,211	1,321	996
No. of down-regulated	1,131	1,379	980

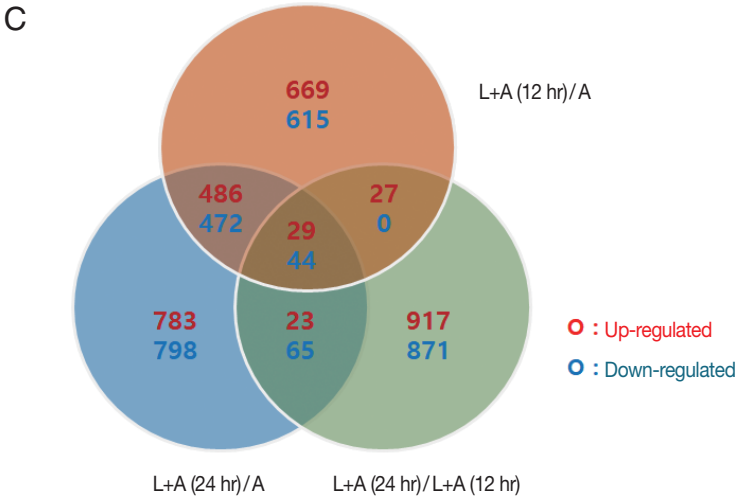


Fig. 1. An overview of significant changes in the gene expression profiles of *A. castellanii*. (A) Gene expressions under different experimental conditions displayed using a heat map. (B) The number of genes with significantly increased or decreased expression (more than 2 fold). (C) Venn diagram showing the number of overlapping genes differentially expressed among 3 experimental conditions. L+A(12 hr)/A; *L. pneumohpila* infected *A. castellanii* for 12 hr/*A. castellanii*, L+A(24 hr)/A; *L. pneumohpila* infected *A. castellanii* for 24 hr/*A. castellanii*, L+A(24 hr)/L+A(12 hr); *L. pneumohpila* infected *A. castellanii* for 24 hr/*L. pneumohpila* infected *A. castellanii* for 12 hr.

ic activity (GO: 0003824). Based on these findings, it can be speculated that these DEGs may be of importance during the initial phase of infection. While xylosyltransferase 1 was downregulated more than 700 fold, sulfiredoxin 1 was downregulated more than 500 fold, and vacuolar sorting-associated protein 13 were downregulated more than 12 fold. (Table 2). GO analysis results revealed that the DEGs downregulated 10 fold or more were predominantly associated with the integral component of membrane (GO: 0016021). Findings are consistent with the changes in DEGs categorized under CC as illustrated in Fig. 3.

Although 132 out of 1,321 DEGs were upregulated and 54 out of 1,379 DEGs were downregulated more than 10 fold in *A. castellanii* 24 hr pi, approximately 60% of these DEGs (78 DEGs and 30 DEGs) were identified as hypothetical proteins. Strong inhibition of DEGs were observed in both 12 hr pi *A. castellanii* (90 DEGs) and 24 hr pi *A. castellanii* (53 DEGs). Among the DEGs demonstrating 10 fold or greater changes, 47 genes were upregulated while 90 genes were downregulated within the initial 12 hr pi (Tables 1, 2). Conversely, by 24 hr pi, 132 upregulated and 54 downregulated DEGs were observed. From these results, we supposed that *L. pneumophila* infection facilitated reduced *A. castellanii* gene expression during the early stage of infection to inhibit phagocytic digestion, while enhancing the expression of specialized *A. castellanii* genes during the late infection stage for LCV lysis and access to host cell machinery for intracellular replication.

Interestingly, *L. pneumophila*-infected *A. castellanii* showed differential expressions of methyltransferase-associated proteins. In addition to the S-adenosylmethionine-dependent

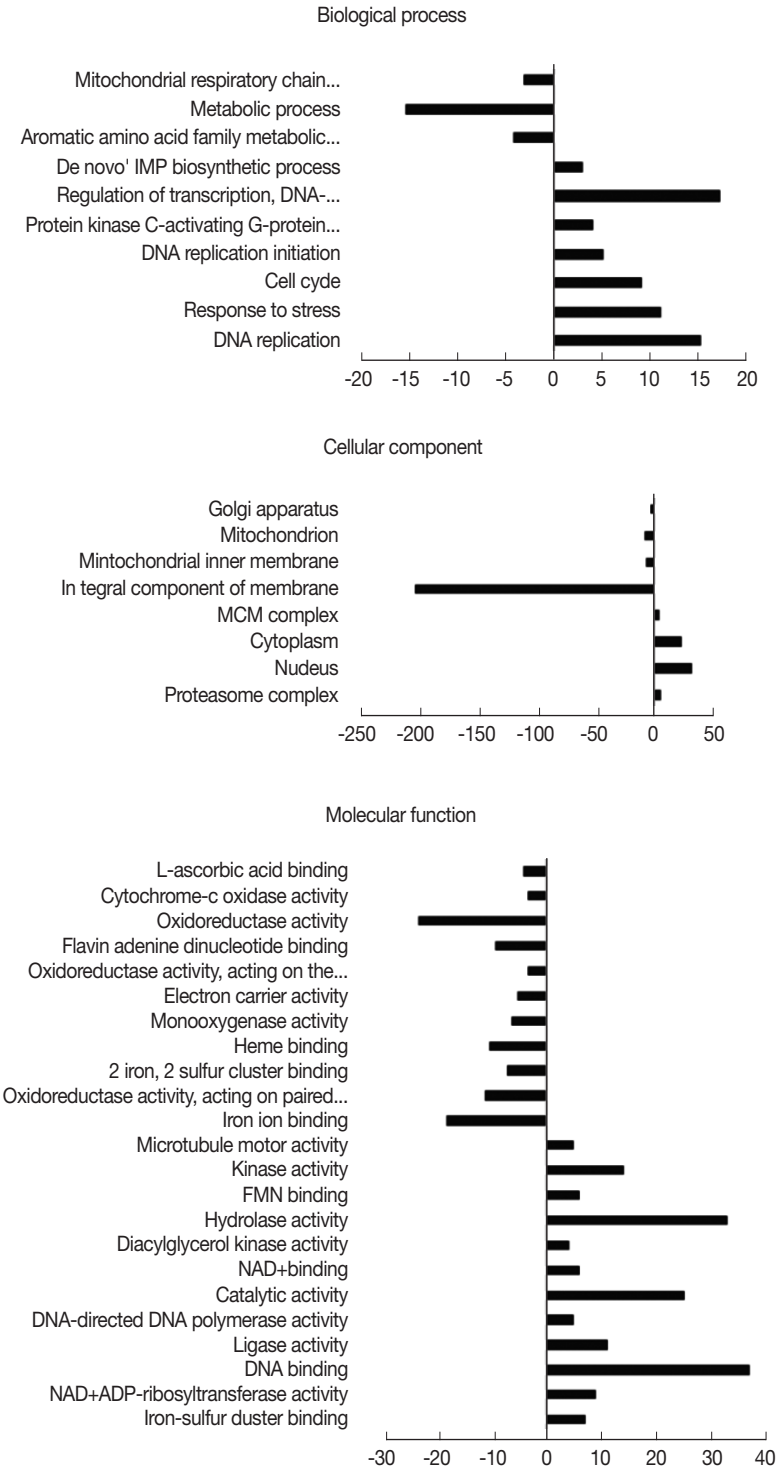


Fig. 2. Distribution of gene ontology (GO) functional classifications. GO analysis of downregulated (left-hand direction) and upregulated (right-hand direction) genes in *A. castellanii* infected with *L. pneumophila* after 12 hr.

methyltransferases and lysine methyltransferase enzyme domain-containing protein (Table 1), 11 DEGs associated with methyltransferase were upregulated, and 19 DEGs were downregulated upon infection with *L. pneumophila* for 12 hr. Furthermore, *L. pneumophila*-infected *A. castellanii* also demonstrated differential expressions of acetyltransferase-associated

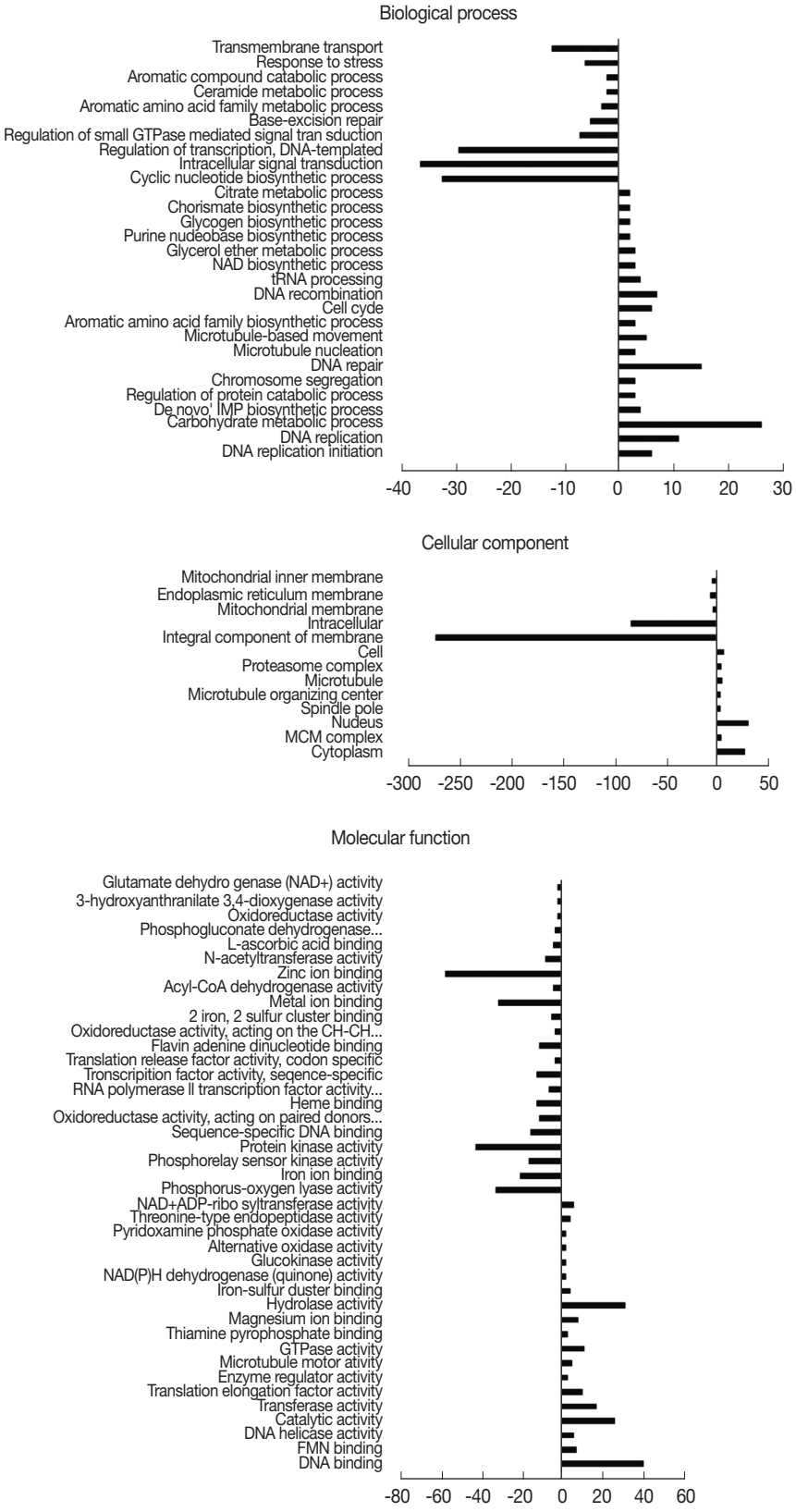


Fig. 3. Distribution of gene ontology (GO) functional classifications. GO analysis of downregulated (left-hand direction) and upregulated (right-hand direction) genes in *A. castellanii* infected with *L. pneumophila* after 24 hr.

Table 1. Genes upregulated more than 10 fold in *A. castellanii* 12 hr pi

Gene symbol	Fold change		Annotation	GO analysis
	L+A(12)/A	L+A(24)/A	Product	Category (Term)
ACA1_328910	1099.734	136.04	hypothetical protein	MF (GO:0003677)
ACA1_183610	345.332	0.920	hypothetical protein	-
ACA1_183700	173.774	0.462	S-adenosylmethionine-dependent methyltransferases	-
ACA1_140050	116.896	15.154	hypothetical protein	CC (GO:0016021)
ACA1_183570	87.745	0.232	GDPD-mannose-3',5'-epimerase	MF (GO:0003824)
ACA1_324870	66.918	38.929	hypothetical protein	-
ACA1_300830	61.232	23.274	permeases of the major facilitator superfamily	CC (GO:0016021)
ACA1_139940	51.100	7.577	hypothetical protein	-
ACA1_183970	50.688	0.239	hypothetical protein	CC (GO:0016021)
ACA1_264780	41.374	2.313	hypothetical protein	-
ACA1_159010	36.741	11.383	NmrAlike family protein	-
ACA1_098380	27.710	0.838	hypothetical protein	-
ACA1_248200	25.965	3.365	phosphotransferase enzyme domain containing protein	-
ACA1_183940	25.733	0.905	GTPase activating Rap/RanGAP domainlike 3, putative	-
ACA1_096640	20.427	7.341	hypothetical protein	-
ACA1_183760	17.942	0.999	lysine methyltransferase enzyme domain containing protein	-
ACA1_376940	17.875	3.938	BNR/Aspbox repeat domain containing protein	-
ACA1_183580	17.602	0.724	S-adenosylmethionine-dependent methyltransferases	-
ACA1_270170	15.076	1.686	von Willebrand factor type A domain containing protein	-
ACA1_158840	15.058	6.332	metal dependent phosphohydrolase	-
ACA1_289630	14.765	11.367	hypothetical protein	-
ACA1_381540	14.601	0.503	hypothetical protein	-
ACA1_224160	14.371	6.761	Sec23/Sec24 beta-sandwich domain containing protein	-
ACA1_175370	14.029	6.388	Erf4 domain containing protein	CC (GO:0016021)
ACA1_140540	13.733	6.286	MORN repeatcontaining protein	-
ACA1_184710	13.687	26.561	Phospholipid methyltransferase domain containing protein	CC (GO:0016021)
ACA1_068540	13.266	5.034	Prokumamolisin, activation domain containing protein	-
ACA1_217750	13.172	4.030	phosphoenolpyruvate carboxykinase (GTP), putative	MF (GO:0016301)
ACA1_116700	13.149	9.620	hypothetical protein	-
ACA1_279740	12.960	5.501	hydrogenase assembly factor, putative	MF (GO:0051536)
ACA1_116690	12.553	5.917	hypothetical protein	CC (GO:0016021)
ACA1_285180	12.470	58.313	DNA breaking-rejoining enzyme domain containing protein	MF (GO:0003677)
ACA1_215790	12.170	8.113	copper/zinc superoxide dismutase	-
ACA1_275740	12.097	4.360	glycerol-3-phosphate dehydrogenase (soluble)	MF (GO:0051287)
ACA1_058320	12.094	4.914	GPR1/FUN34/yaaH family protein	CC (GO:0016021)
ACA1_358270	11.433	7.344	pyridine nucleotidedisulfide oxidoreductase domain containing protein	MF (GO:0016491)
ACA1_067720	11.300	8.046	hypothetical protein	-
ACA1_153710	11.001	0.845	RFX_DNA_binding	BP (GO:0006355)
ACA1_091110	10.950	0.842	hypothetical protein	-
ACA1_256560	10.936	3.671	hypothetical protein	-
ACA1_275730	10.900	2.743	phosphoglycerate mutase family domain containing protein	-
ACA1_165640	10.762	1.971	hypothetical protein	-
ACA1_060580	10.631	4.515	phosphatase	-
ACA1_245710	10.555	7.545	hypothetical protein	-
ACA1_325450	10.443	4.372	CBS domain containing protein	-
ACA1_187310	10.274	6.715	heme NO binding domain containing protein	-
ACA1_238590	10.109	3.454	CBS domain containing protein	-

proteins. Histone acetyltransferase-associated protein was upregulated and 8 other acetyltransferases were downregulated 12 hr pi with *L. pneumophila*. Icm/Dot type IV secretion system and its effectors of *L. pneumophila* modulate host gene expression by altering the chromatin structure or by affecting the activities of transcription factors [19]. Post-translational modifi-

Table 2. Genes downregulated more than 10 fold in *A. castellanii* 12 hr pi

Gene symbol	Fold change		Annotation	GO analysis
	L+A(12)/A	L+A(24)/A	Product	Category (Term)
ACA1_113420	0.001	0.764	hypothetical protein	-
ACA1_112520	0.001	0.493	Cysteine-rich 4 helical bundle widely conserved	-
ACA1_111980	0.003	0.368	EF hand domain containing protein	-
ACA1_112090	0.003	0.454	hypothetical protein	-
ACA1_111740	0.007	0.190	hypothetical protein	-
ACA1_112480	0.007	0.492	Fbox domain containing protein	-
ACA1_376130	0.007	0.386	xylosyltransferase 1, putative	-
ACA1_058410	0.009	0.019	hypothetical protein	-
ACA1_147740	0.010	0.006	CBS domain containing protein	-
ACA1_113310	0.013	0.765	hypothetical protein	-
ACA1_166550	0.017	0.318	hypothetical protein	-
ACA1_374390	0.018	0.924	hypothetical protein	-
ACA1_101570	0.019	1.673	hypothetical protein	-
ACA1_392590	0.020	0.401	hypothetical protein	-
ACA1_060120	0.021	0.548	hypothetical protein	-
ACA1_400130	0.021	0.021	hypothetical protein	-
ACA1_307550	0.022	0.084	Fbox domain containing protein	-
ACA1_230230	0.022	0.022	hypothetical protein	-
ACA1_050390	0.024	2.233	3-oxoacyl-[acyl-carrier protein] reductase	CC (GO: 0016021)
ACA1_112110	0.024	0.463	glycosyl transferase	CC (GO: 0016021)
ACA1_112490	0.024	0.480	hypothetical protein	-
ACA1_063680	0.026	0.072	Reverse transcriptase	-
ACA1_390590	0.027	0.132	Hsp20/alpha crystallin superfamily protein	-
ACA1_063960	0.029	0.078	hypothetical protein	-
ACA1_112130	0.029	0.457	regulator of g protein signaling domain containing protein	-
ACA1_111970	0.029	0.399	sulfiredoxin 1	-
ACA1_158820	0.029	0.646	hypothetical protein	-
ACA1_064370	0.029	0.029	AT Hook plus PHD finger transcription factor family member (athp1), putative	-
ACA1_064780	0.029	0.029	hypothetical protein	-
ACA1_064790	0.029	0.029	hypothetical protein	-
ACA1_340040	0.030	0.275	zinc finger, zz type domain containing protein	-
ACA1_350050	0.030	0.298	hypothetical protein	-
ACA1_112530	0.031	0.499	NLPC_P60 super family	-
ACA1_112180	0.032	0.527	major facilitator subfamily transporter	CC (GO: 0016021)
ACA1_112590	0.034	0.474	WH2 motif domain containing protein	-
ACA1_050380	0.036	1.885	hypothetical protein	-
ACA1_230220	0.037	0.025	hypothetical protein	-
ACA1_199000	0.038	0.229	SnoaL-like domain containing protein	-
ACA1_077210	0.040	0.376	hypothetical protein	-
ACA1_173000	0.043	0.352	Predicted NAD/FAD-dependent oxidoreductase	-
ACA1_326260	0.043	0.073	hypothetical protein	-
ACA1_060740	0.045	0.310	hypothetical protein	-
ACA1_270160	0.046	0.071	fascin subfamily protein	-
ACA1_200180	0.048	0.428	hypothetical protein	-
ACA1_383480	0.051	0.029	hypothetical protein	-
ACA1_155760	0.052	0.145	phosphoribosyltransferase	-
ACA1_207830	0.052	0.426	myotubularins and other putative membrane-associated proteins	-
ACA1_133180	0.052	0.254	Ser/Thr phosphatase family superfamily protein	-
ACA1_077290	0.053	0.638	5'nucleotidase	CC (GO: 0016021)
ACA1_365080	0.053	0.367	hypothetical protein	-

(*Continued to the next page*)

Table 2. Continued

Gene symbol	Fold change		Annotation	GO analysis
	L+A(12)/A	L+A(24)/A	Product	Category (Term)
ACA1_383650	0.055	0.349	Human glyoxalase domain-containing protein 5 and similar proteins	-
ACA1_048480	0.056	0.616	hypothetical protein	-
ACA1_055330	0.059	0.292	N-terminal region of Chorein or VPS13	-
ACA1_346470	0.060	0.450	RUN domain containing protein	-
ACA1_298420	0.062	0.056	hypothetical protein	-
ACA1_197730	0.064	0.823	hypothetical protein	-
ACA1_378930	0.064	0.329	hypothetical protein	-
ACA1_214630	0.067	0.147	hypothetical protein	CC (GO: 0016021)
ACA1_128200	0.068	0.051	CBS domain containing protein	-
ACA1_052800	0.071	0.571	O-methyltransferase family 3 protein	-
ACA1_112560	0.071	0.499	hypothetical protein	-
ACA1_322750	0.072	0.552	Glycosyl hydrolases family 2, TIM barrel domain	-
ACA1_383400	0.072	1.376	hypothetical protein	CC (GO: 0016021)
ACA1_253630	0.073	0.947	hypothetical protein	-
ACA1_391470	0.074	0.821	Hsp20/alpha crystallin superfamily protein	-
ACA1_066110	0.074	0.05	SCP-like extracellular protein domain containing protein	-
ACA1_131790	0.075	0.133	protein from patent family protein	-
ACA1_111930	0.075	0.311	carbonsulfur lyase, putative	BP (GO: 0008152)
ACA1_064380	0.077	0.052	hypothetical protein	-
ACA1_064940	0.077	0.029	Fbox domain containing protein	-
ACA1_323370	0.077	0.011	Hsp20/alpha crystallin superfamily protein	-
ACA1_383750	0.078	0.236	Ubiquitinconjugating enzyme subfamily protein	-
ACA1_006080	0.079	0.210	hypothetical protein	CC (GO: 0016021)
ACA1_180590	0.082	0.546	TRRAP family protein	-
ACA1_112980	0.083	0.755	protein kinase	-
ACA1_077300	0.083	0.247	serine/threonine kinase	CC (GO: 0016021)
ACA1_111880	0.084	0.225	Small acidic protein family	-
ACA1_372720	0.084	0.148	obtusifoliol 14alphademethylase, putative	CC (GO: 0016021)
ACA1_046720	0.086	0.076	Glycosyl hydrolase families	-
ACA1_324050	0.088	0.646	Vacuolar sorting-associated protein 13 [Intracellular trafficking and secretion]	-
ACA1_400540	0.088	0.076	sphingosine hydroxylase	CC (GO: 0016021)
ACA1_290200	0.089	0.329	hypothetical protein	-
ACA1_389110	0.09	1.016	hypothetical protein	-
ACA1_112500	0.094	0.535	TBC domain containing protein	-
ACA1_311650	0.095	0.096	hypothetical protein	-
ACA1_112060	0.096	0.429	O-methyltransferase, putative	-
ACA1_178260	0.097	0.118	cytochrome P450, putative	MF (GO: 0005506)
ACA1_066960	0.098	1.036	hypothetical protein	-
ACA1_046710	0.099	0.126	cytoplasmic protein, putative	-
ACA1_112640	0.100	0.498	MBOAT family protein	CC (GO: 0016021)

cations such as DNA methylation, histone acetylation, and histone methylation have been shown to play a critical role in the epigenetic regulation of eukaryotic gene expression [19,20]. Our results revealed that *L. pneumophila*-infected *A. castellanii* showed differential expressions of 30 kinds of methyltransferase-associated proteins and 9 kinds of acetyltransferase-associated proteins at 12 hr pi. Based on the changes to epigenetic regulatory gene expressions, it can be speculated that *L. pneumophila* can alter the gene expression of *A. castellanii* through epigenetic mechanisms.

A plethora of DEGs induced in *A. castellanii* by the endosymbiont *L. pneumophila* were revealed in this study. However, 38.3% (1,930 of the 5,042) of *A. castellanii* genes were identified to be hypothetical proteins. Proportions of theses hypothetical proteins in the 12 and 24 hr pi groups can be ascribed to the lack of *Acanthamoeba* spp. database. Our investigation of

the DEGs in *A. castellanii* by an endosymbiont provides important information to understanding the survival strategy utilized by notable intracellular pathogen *L. pneumophila* in *A. castellanii*. Future studies investigating the presence of an endosymbiosis-specific gene may help elucidate the underlying mechanism involved in *L. pneumophila* pathogenesis, which would contribute to understanding the inhibition of phagocytosis within *A. castellanii* or even immune evasion mechanism in human macrophages.

ACKNOWLEDGMENT

This work was supported by the National Research Foundation of Korea (NRF) grant funded by Korea government (MIST) (No. 2020R1F1A1068719).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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