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Original Article

Phagocytosis-associated genes in *Acanthamoeba castellanii* feeding on *Escherichia coli*

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Abstract

Acanthamoeba species are free-living amoebae those are widely distributed in the environment. They feed on various microorganisms, including bacteria, fungi, and algae. Although majority of the microbes phagocytosed by *Acanthamoeba* spp. are digested, some pathogenic bacteria thrive within them. Here, we identified the roles of 3 phagocytosis-associated genes (*ACA1_077100, ACA1_175060*, and *AFD36229.1*) in *A. castellanii*. These 3 genes were upregulated after the ingestion of *Escherichia coli*. However, after the ingestion of *Legionella pneumophila*, the expression of these 3 genes was not altered after the consumption of *L. pneumophila*. Furthermore, *A. castellanii* transfected with small interfering RNS (siRNA) targeting the 3 phagocytosis-associated genes failed to digest phagocytized *E. coli*. Silencing of *ACA1_077100* disabled phagosome formation in the *E. coli*-ingesting *A. castellanii*. Alternatively, silencing of *ACA1_175060* enabled phagosome formation; however, phagolysosome formation was inhibited. Moreover, suppression of *AFD36229.1* expression prevented *E. coli* digestion and consequently led to the rupturing of *A. castellanii*. Our results demonstrated that the *ACA1_077100, ACA1_175060*, and *AFD36229.1* genes of *Acanthamoeba* played crucial roles not only in the formation of phagosome and phagolysosome but also in the digestion of *E. coli*.

Keywords: *Acanthamoeba*, gene expression, gene silencing, phagocytosis, phagolysosome, phagosome, siRNA

Introduction

Acanthamoeba species are free-living protist pathogens that cause *Acanthamoeba* keratitis and granulomatous amoebic encephalitis [1]. *Acanthamoeba* feed on a wide range of microorganisms including bacteria, algae, viruses, and other protists; however, these protozoa can serve as a reservoir host [2]. To date, several endosymbiotic relationships between clinically important pathogens and amoebae have been reported, with the survival of *Legionella pneumophila* on *Acanthamoeba* being a prominent example [3-5]. The intracellular survival of *L. pneumophila, Legionella*-containing vacuole, the defective organelle transport/intracellular multiplication (Dot/Icm) type IV system, and several other effectors of *Legionella* have been identified and their roles have been studied [6-8]. However, the genes associated with phagocytosis and endosymbiosis in *Acanthamoeba* have not been extensively studied.

In our previous study, differentially expressed genes (DEGs) acquired from *A. castellanii*

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Funding acquisition: Kong HH
Investigation: Kim MJ, Moon EK, Jo HJ
Methodology: Kim MJ, Moon EK, Jo HJ, Quan FS
Writing – original draft: Moon EK
Writing – review & editing: Kim MJ, Jo HJ, Quan FS, Kong HH

Conflict of interest
The authors declare no conflict of interest related to this study.

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during the phagocytosis of *E. coli* or endosymbiosis with *L. pneumophila* were compared [9]. Based on the findings of this previous study, it was hypothesized that the genes upregulated in *E. coli*-ingested *Acanthamoeba* were involved in the phagocytosis of *Acanthamoeba* and those upregulated in *L. pneumophila*-ingested *Acanthamoeba* were involved in promoting its survival and endosymbiosis within *Acanthamoeba*. Among the upregulated genes in *L. pneumophila*-ingested *A. castellanii*, *ACA1_114460*, *ACA1_091500*, and *ACA1_362260* were found to be integral to the formation of excretory vesicles containing *L. pneumophila* along with the lysosomal colocalization with the vesicles [10].

While elucidating the mechanism of action for these survival and lysosome colocalization-associated genes would be beneficial, we identified another set of genes with interesting expression patterns which became the focal point of the present study. Specifically, we identified several genes that were upregulated in *E. coli*-ingesting *Acanthamoeba* whose expressions were upregulated following *E. coli* ingestion, but remain unaffected upon *L. pneumophila* ingestion. Given this finding, we anticipated that these genes are essential for phagocytosis and *L. pneumophila* somehow ensures that the expression of *E. coli*-induced genes remains near basal levels to prolong their survival within the host. To confirm this hypothesis, we selected 3 genes that meet these criteria (*ACA1_077100, ACA1_175060*, and *AFD36229.1*) in the present study, and investigated their potential involvement in phagocytosis. Our findings revealed that phagocytosis inhibition could be a mechanism utilized by *L. pneumophila* to promote its growth and survival in host cells.

Materials and Methods

Cell culture and bacterial infection

Acanthamoeba castellanii (ATCC 30868 and ATCC 30011) were obtained from the American Type Culture Collection and cultured axenically in peptone-yeast-glucose (PYG) medium at 25°C. *Escherichia coli* DH5α (Enzynomics, Seoul, Korea) was cultured in tryptone-yeast-NaCl (LB) media at 37°C using a shaking incubator. *Legionella pneumophila* Philadelphia-1 (ATCC 33152) was cultured on a buffered charcoal yeast extract (BCYE) agar plate at 37°C with 5% CO_2. *A. castellanii* was infected by *E. coli* and *L. pneumophila* as previously described [11]. Briefly, *E. coli* and *L. pneumophila* were diluted in PBS until the OD_{600} absorbance reading reached 1 which corresponds to 10^9 CFU/ml [12]. Next, 1×10^7 of *Acanthamoeba* were incubated with 1 ml of *E. coli* and *L. pneumophila* suspension at 37°C with 5% CO_2 for 1 h. After incubation, *Acanthamoeba* was washed with Page's amoeba saline (PAS) and incubated with new PYG media containing 100 μg/ml of gentamicin for 2 h to kill extracellular bacteria. *Acanthamoeba* infected with *E. coli* (A+E) and *L. pneumophila* (A+L) were washed with PAS twice and incubated in fresh PYG media for 12 h, 25°C.

Gene expression analysis by real-time PCR

Target gene expressions were determined by real-time PCR analysis. The total RNA was purified using an RNeasy Mini kit (Qiagen, Hilden, Germany), and the cDNA was synthesized using a RevertAid first-strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Real-time PCR was conducted using a Magnetic Induction Cycler PCR machine (PhileKorea, Seoul, Korea) as previously de-

scribed [13] which included preincubation at 95°C for 1 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 30 sec. All reaction mixtures were made using a Luna Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) with different sense and antisense primers (Supplementary Table S1).

Gene silencing

Small interfering RNAs (siRNAs) targeting *ACA1_077100, ACA1_175060*, and *AFD36229.1* of *Acanthamoeba* were synthesized by Bioneer Inc (Bioneer, Daejeon, Korea), based on their cDNA sequences (Supplementary Table S2). The siRNA (final concentration of 100 nM) was transfected into live *Acanthamoeba* trophozoites at a cell density of 4×10^5 cells using the Effectene transfection reagent (Qiagen, Hilden, Germany) following the manufacturer's protocol. Successful transfection of siRNA was confirmed by observing cells under a fluorescent microscope (Leica, Wetzlar, Germany).

Observation of phagosomes and phagolysosomes

Phagosomes and phagolysosomes of *A. castellanii* containing *E. coli* were observed with Giemsa and LysoTracker staining. *Acanthamoeba* was subjected to siRNA transfection (siRNA-A), followed by *E. coli* (siRNA-A+E) infection as aforementioned. For Giemsa staining assays, cells were fixed with methanol for 5 min and stained with Giemsa solution (Sigma-Aldrich, Burlington, MA, USA) for 10 min. For the LysoTracker stain, cells were stained with 50 μM LysoTracker Red DND-99 (Invitrogen, Carlsbad, CA, USA) for 1 h. Stained cells were washed with PBS, and observed under a fluorescent microscope.

Statistical analysis

Data are presented as mean ± SD from three independent experiments. Student's *t*-tests were performed using GraphPad Prism version 8 (Dotmatics, San Diego, CA, USA). Statistical significance between the means of groups was denoted using an asterisk. *P* values less than 0.05 were considered statistically significant ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$).

Results

Identification of up-regulated genes in *Acanthamoeba* feeding on *E. coli*

Based on our previous study involving DEGs, 3 genes (*ACA1_077100, ACA1_175060*, and *AFD36229.1*) that were upregulated in *A. castellanii* after *E. coli* ingestion but unchanged after *L. pneumophila* ingestion were selected in the present study. To confirm their expression levels, real-time polymerase chain reaction (RT-PCR) was performed using gene-specific primers (Supplementary Table S1). As depicted in Fig. 1A. *ACA1_077100* was upregulated in *Acanthamoeba* that ingested *E. coli* (A+E) but not in *Acanthamoeba* that ingested *L. pneumophila* (A+L). Furthermore, the expression patterns of *ACA1_175060* and *AFD36229.1* were similar to those of *ACA1_077100* in both A+E and A+L (Fig. 1B, C).

siRNA-mediated gene silencing

To investigate the phagocytic roles of *ACA1_077100, ACA1_175060*, and *AFD36229.1*

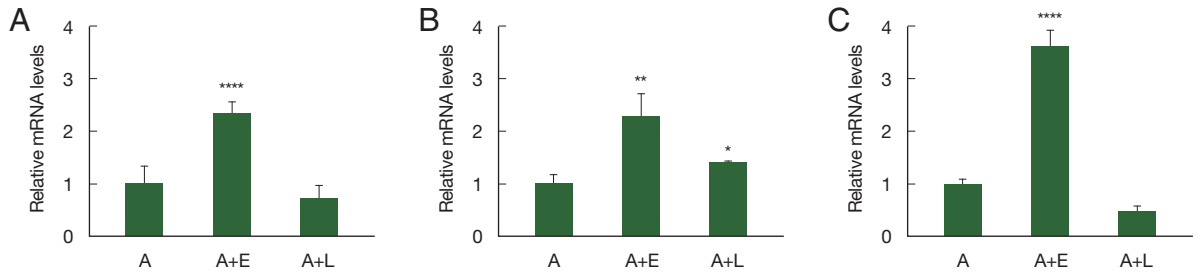

Fig. 1. Real-time polymerase chain reaction (RT-PCR) analysis of 3 differentially expressed genes in *Acanthamoeba*. *A. castellanii* ingested *E. coli* (A+E) and *L. pneumophila* (A+L) for 12 h, and the gene expression ACA1_077100, ACA1_175060, and AFD36229.1 was determined by RT-PCR. (A) *ACA1_077100*, (B) *ACA1_175060*, and (C) *AFD36229.1*. Data are expressed as mean ± SD from 3 individual experiments. Asterisks indicate statistical significance compared to the Acanthamoeba control (A), as determined by Student's *t*-test (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$).

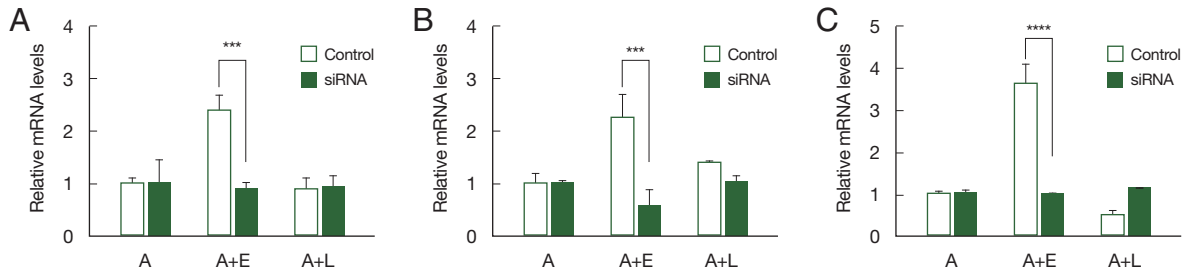

Fig. 2. Real-time polymerase chain reaction (RT-PCR) analysis of the 3 differentially expressed genes in *Acanthamoeba* after transfection of siRNA. *A. castellanii* were transfected with siRNA and coincubated with either *E. coli* (A+E) or *L. pneumophila* (A+L) for 12 h. siRNA-mediated gene silencing was confirmed by RT-PCR. (A) *ACA1_077100*, (B) *ACA1_175060*, and (C) *AFD36229.1*. Data are expressed as mean ± SD from 3 individual experiments. Asterisks indicate that the means are significantly different compared to untransfected *Acanthamoeba* (***$P < 0.001$, ****$P < 0.0001$).

within *A. castellanii*, small interfering RNAs (siRNAs) specific for each gene (Supplementary Table S2) were synthesized. The gene silencing effects of siRNAs were validated using RT-PCR (Fig. 2). The transfection of *ACA1_077100, ACA1_175060*, and *AFD36229.1* sequence-specific siRNAs significantly suppressed the expression of the respective mRNAs in *A. castellanii* during *E. coli* ingestion (Fig. 2A-C).

Effect of gene silencing on the phagosome formation

To visually confirm the effects of *ACA1_077100, ACA1_175060*, and *AFD36229.1* gene silencing in A+E, Giemsa staining was performed (Fig. 3). *E. coli* digestion was observed in the A+E control group because none of the 3 genes was downregulated (Fig. 3B). However, the suppression of *ACA1_077100* in A+E inhibited the formation of phagosome-like structures, with *E. coli* dissemination occurring in the cytosols of *Acanthamoeba* (Fig. 3D). Furthermore, the silencing of *ACA1_175060* and *AFD36229.1* in A+E resulted in the formation of phagosomes containing *E. coli*, however, the phagocytized *E. coli* remained undigested in the phagosomes (Fig. 3E, F). These phenomena were not observed in the *Acanthamoeba* control group (Fig. 3A) and siRNA-transfected *Acanthamoeba* control group (Fig. 3C).

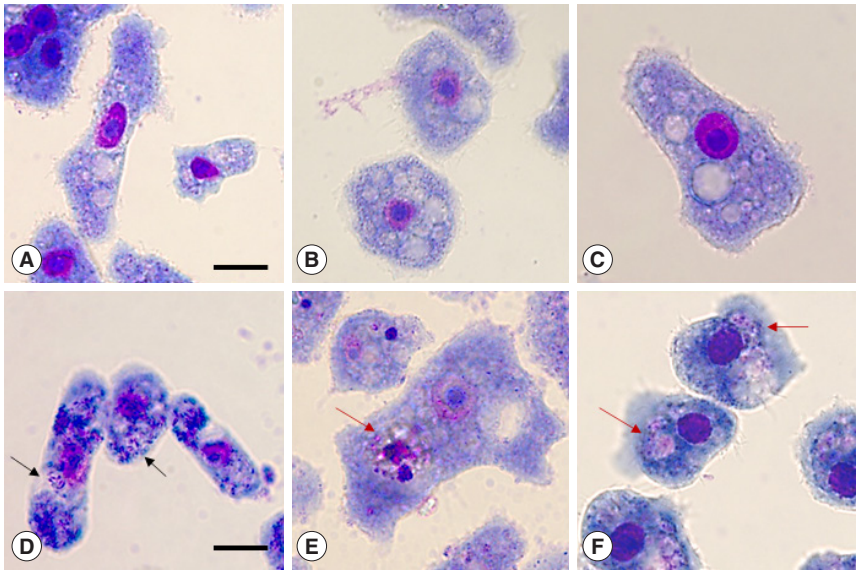

Fig. 3. siRNA-transfected *Acanthamoeba* containing *E. coli*. *Acanthamoeba* that did and did not undergo siRNA transfection were cultured with *E. coli* for 12 h. Those containing *E. coli* were stained with Giemsa staining solution. (A) *A. castellanii* control, (B) *A. castellanii* containing *E. coli*, (C) siRNA-transfected *A. castellanii* control, (D) *ACA1_077100* siRNA-transfected *A. castellanii* containing *E. coli*, (E) *ACA1_175060* siRNA-transfected *A. castellanii* containing *E. coli*, and (F) *AFD36229.1* siRNA-transfected *A. castellanii* containing *E. coli*. Black arrows: *E. coli* in the cytoplasm of *Acanthamoeba*. Red arrows: *E. coli* in the phagosomes of *Acanthamoeba*. Bar = 10 μm.

Effect of gene silencing on the phagolysosome formation and *E. coli* digestion

To verify whether the failure of *E. coli* digestion in the siRNA-transfected *Acanthamoeba* was related to lysosomal acidifications, LysoTracker Red DNA-99 staining and fluorescent microscopy were conducted (Fig. 4). Phagolysosome-like structures were barely detected in uninfected *Acanthamoeba* (Fig. 4A) but were prevalent in A+E (Fig. 4B). Similar to the uninfected control, siRNA transfection in *Acanthamoeba* did not affect LysoTracker staining (Fig. 4C). However, LysoTracker-stained intracellular organelles were not observed in *ACA1_077100*-silenced *Acanthamoeba* (Fig. 4D). Lysosome fusion could not occur because *Acanthamoeba* did not form *E. coli*-containing phagosomes, and *E. coli* was distributed in the cytosols of *Acanthamoeba* (Fig. 4D). These findings suggested that the *ACA1_077100* gene in *A. castellanii* was involved in the formation of the phagosomal membrane during *E. coli* ingestion. Alternatively, silencing of *ACA1_175060* led to the formation of *E. coli*-containing phagosomes (Fig. 4E) but failed to form phagolysosomes (Fig. 4E). The *ACA1_175060* gene in *A. castellanii* was believed to play a role in the formation of the phagolysosome within *Acanthamoeba*. Fig. 4F shows the leakage of proliferating *E. coli* out of *AFD36229.1*-silenced *Acanthamoeba*, possibly indicating the rupturing of *Acanthamoeba*. Bacterial digestion did not occur, despite the fusion of lysosomes with *E. coli*-containing phagosomes, leading to the exponential growth of *E. coli* and subsequent bursting of *Acanthamoeba*. These findings suggest that *AFD36229.1* is crucial for the phagosomal digestion of microbial contents in *Acanthamoeba*.

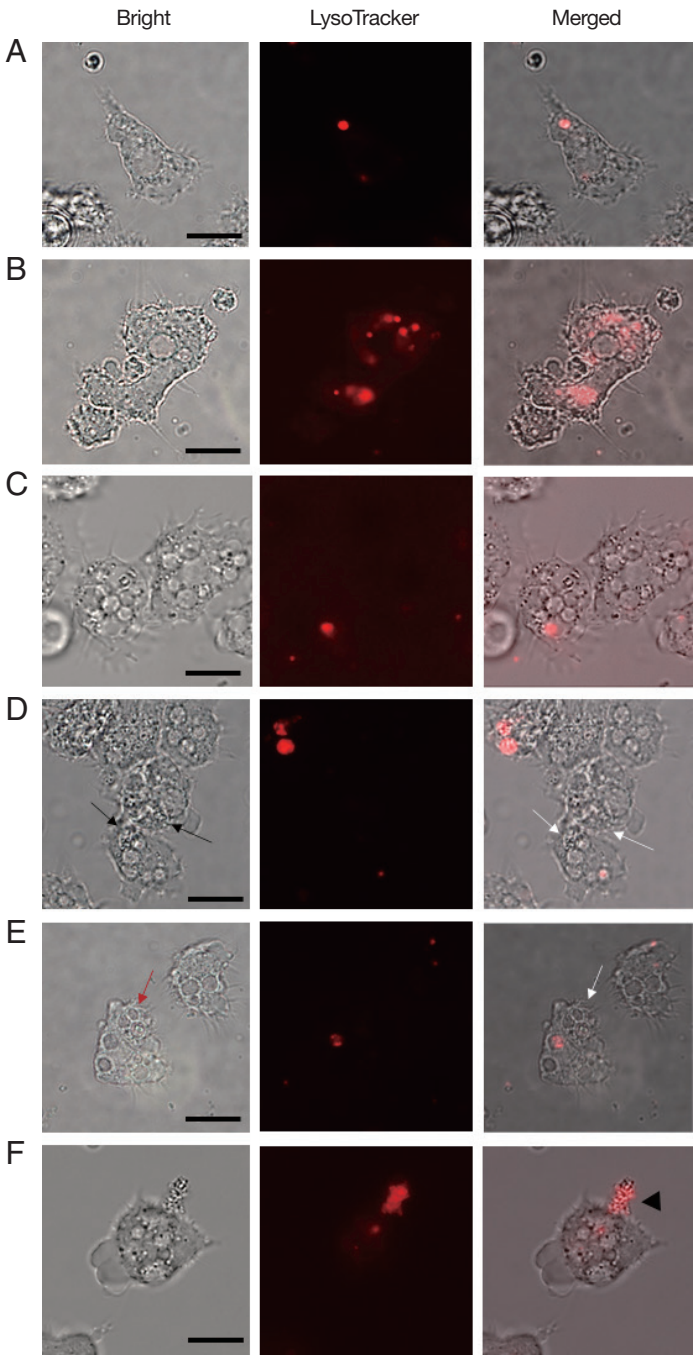

Fig. 4. LysoTracker staining of siRNA-transfected *Acanthamoeba* containing *E. coli*. The *Acanthamoeba* that did and did not undergo siRNA transfection were allowed to ingest *E. coli* for 12 h, and those containing *E. coli* were stained with LysoTracker. (A) *A. castellanii* control, (B) *A. castellanii* containing *E. coli*, (C) siRNA-transfected *A. castellanii* control, (D) *ACA1_077100* siRNA-transfected *A. castellanii* containing *E. coli*, (E) *ACA1_175060* siRNA-transfected *A. castellanii* containing *E. coli*, and (F) *AFD36229.1* siRNA-transfected *A. castellanii* containing *E. coli*. Black arrows, *E. coli* in the cytoplasm of *Acanthamoeba*. Red arrow, *E. coli* in the phagosomes of *Acanthamoeba*; White arrows, *E. coli* in *Acanthamoeba* cytoplasm or phagosomes without lysosomes. Black arrowhead, *E. coli* that burst out of *Acanthamoeba*. Bar = 10 μm.

Discussion

In this study, we investigated the role of 3 genes (*ACA1_077100, ACA1_175060*, and *AFD36229.1*) upregulated in *A. castellanii* feeding on *E. coli*. We observed that the *ACA1_077100* gene in *Acanthamoeba* was involved in the formation of the phagosomal membrane during *E. coli* ingestion. We also observed that the *ACA1_175060* gene played a role in the formation of the phagolysosome within *Acanthamoeba*, and the *AFD36229.1* gene is crucial for the phagosomal digestion of bacteria in *Acanthamoeba*.

The 3 upregulated genes *ACA1_077100, ACA1_175060*, and *AFD36229.1* observed in A+E were identified to be a Rab1/RabD family small GTPase (96% sequence identity to that of *Acanthamoeba* spp.), a vacuolar proton ATPase (99% sequence identity to that of *Acanthamoeba* spp.), and a cyst-specific cysteine proteinase (100% sequence identity to that of *Acanthamoeba* spp.), respectively (Table 1). The Rab family protein is a member of the Ras superfamily of small G proteins; moreover, Rab1/RabD GTPase regulates the transport of proteins from the endoplasmic reticulum to the Golgi bodies, membrane tethering, and vesicle fusion in plants and humans [14,15]. Vacuolar ATPase is a proton pump responsible for controlling the intracellular and extracellular pH of cells [16], and the cyst-specific cysteine proteinase plays an important role in the autophagosomal degradation of mitochondria during *A. castellanii* encystation [17].

Several methods are frequently employed to detect phagosome, as well as phagolysosome formation. Giemsa staining has been traditionally used to observe such phenomena in numerous cell lines [18]. However, due to monochrome staining, accurately differentiating between amoebal mitochondria from *E. coli* was somewhat problematic. To accurately observe phagosomes or phagolysosomes, LysoTracker reagents were used in the present study. LysoTracker reagents are frequently used to stain acidic cellular components, such as phagolysosomes and autophagosomes, and it is suitable for confirming whether the bacterial aggregates observed under the microscope are truly phagolysosomes. In the present study, siRNA-treated *Acanthamoeba* demonstrated impaired phagocytic function. Based on this finding, we reasoned that these genes could be associated with phagocytosis. Additional studies, such as gain of function studies investigating the overexpression of *ACA1_077100, ACA1_175060*, and *AFD36229.1* genes in *A. castellanii* are highly desired. Specifically, exposing *A. castellanii* overexpressing these genes to *L. pneumophila* and subsequently assessing whether phagocytosis of these pathogenic bacteria occurs within the amoeba is worth investigating. Findings from such studies could confirm that *L. pneumophila* downregulates the expression of several genes associated with phagocytosis through an unknown mechanism to prevent their degradation in phagosomes.

In conclusion, our study demonstrated the roles of *ACA1_077100, ACA1_175060*, and *AFD36229.1* genes in *A. castellanii* during phagocytosis of *E. coli*. These upregulated genes in the A+E were heavily involved in phagosome formation, phagolysosomal fusing, and digestion of phagocytized microbial contents. However, these genes were not upregulated in *Acanthamoeba* that consumed *L. pneumophila*. This suggested that the expression of these genes was inhibited by *Legionella* for its survival within *Acanthamoeba*. Confirming *L. pneumophila* digestion within *Acanthamoeba* when these gene are overexpressed would imply that the survival of intracellular pathogen such as *Legionella* and *Mycobacterium* spp.

is inhibited and thereby contribute to preventing the bacetrial infection with *Acanthamoeba*.

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References

1. Siddiqui R, Khan NA. Biology and pathogenesis of *Acanthamoeba*. *Parasit Vectors* 2012;5:6. https://doi.org/10.1186/1756-3305-5-6
2. Diehl MLN, Paes J, Rott MB. Genotype distribution of *Acanthamoeba* in keratitis: a systematic review. *Parasitol Res* 2021;120(9):3051-3063. https://doi.org/10.1007/s00436-021-07261-1
3. Iovieno A, Ledee DR, Miller D, Alfonso EC. Detection of bacterial endosymbionts in clinical acanthamoeba isolates. *Ophthalmology* 2010;117(3):445-452. https://doi.org/10.1016/j.ophtha.2009.08.033
4. Scheikl U, Sommer R, Kirschner A, Rameder A, Schrammel B, et al. Free-living amoebae (FLA) co-occurring with legionellae in industrial waters. *Eur J Protistol* 2014;50(4):422-429. https://doi.org/10.1016/j.ejop.2014.04.002
5. Greub G, Raoult D. Microorganisms resistant to free-living amoebae. *Clin Microbiol Rev* 2004;17(2):413-433. https://doi.org/10.1128/CMR.17.2.413-433.2004
6. Nora T, Lomma M, Gomez-Valero L, Buchrieser C. Molecular mimicry: an important virulence strategy employed by *Legionella pneumophila* to subvert host functions. *Future Microbiol* 2009;4(6):691-701. https://doi.org/10.2217/fmb.09.47
7. Segal G, Feldman M, Zusman T. The Icm/Dot type-IV secretion systems of *Legionella pneumophila* and *Coxiella burnetii*. *FEMS Microbiol Rev* 2005;29(1):65-81. https://doi.org/10.1016/j.femsre.2004.07.001
8. Qiu J, Luo ZQ. Effector translocation by the Legionella Dot/Icm type IV secretion system. *Curr Top Microbiol Immunol* 2013;376:103-115. https://doi.org/10.1007/82_2013_345
9. Moon EK, Kim MJ, Lee HA, Quan FS, Kong HH. Comparative analysis of differentially expressed genes in *Acanthamoeba* after ingestion of *Legionella pneumophila* and *Escherichia coli*. *Exp Parasitol* 2022;232:108188. https://doi.org/10.1016/j.exppara.2021.108188
10. Kim MJ, Moon EK, Jo HJ, Quan FS, Kong HH. Identifying the function of genes involved in excreted vesicle formation in *Acanthamoeba castellanii* containing *Legionella pneumophila*. *Parasit Vectors* 2023;16(1):215. https://doi.org/10.1186/s13071-023-05824-y
11. Mou Q, Leung PHM. Differential expression of virulence genes in *Legionella pneumophila* growing in *Acanthamoeba* and human monocytes. *Virulence* 2018;9(1):185-196. https://doi.org/10.1080/21505594.2017.1373925
12. Harding CR, Schroeder GN, Reynolds S, Kosta A, Collins JW, et al. *Legionella pneumophila* pathogenesis in the *Galleria mellonella* infection model. *Infect Immun* 2012;80(8):2780-2790. https://doi.org/10.1128/IAI.00510-12
13. Moon EK, Kim MJ, Lee HA, Quan FS, Kong HH. Comparative analysis of differentially expressed genes in *Acanthamoeba* after ingestion of *Legionella pneumophila* and *Escherichia coli*. *Exp Parasitol* 2022;232:108188. https://doi.org/10.1016/j.exppara.2021.108188
14. Lycett G. The role of Rab GTPases in cell wall metabolism. *J Exp Bot* 2008;59(15):4061-4074. https://doi.org/10.1093/jxb/ern255
15. Barr FA. Review series: Rab GTPases and membrane identity: causal or inconsequential? *J Cell Biol* 2013;202(2):191-199. https://doi.org/10.1083/jcb.201306010
16. Pamarthy S, Kulshrestha A, Katara GK, Beaman KD. The curious case of vacuolar ATPase: regulation of signaling pathways. *Mol Cancer* 2018;17(1):41. https://doi.org/10.1186/s12943-018-0811-3
17. Moon EK, Hong Y, Chung DI, Kong HH. Cysteine protease involving in autophagosomal degradation of mitochondria during encystation of *Acanthamoeba*. *Mol Biochem Parasitol* 2012;185(2):121-126. https://doi.org/10.1016/j.molbiopara.2012.07.008
18. Corbett Y, Parapini S, Perego F, Messina V, Delbue S, et al. Phagocytosis and activation of bone marrow-derived macrophages by *Plasmodium falciparum* gametocytes. *Malar J* 2021;20(1):81. https://doi.org/10.1186/s12936-021-03589-2