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Abstract: Acanthamoeba isolated from environmental soil harbors the obligate intracellular symbiont Neochlamydia, which has a critical role in host amoebal defense against Legionella pneumophila infection. Here, by using morphological analysis with confocal laser scanning fluorescence microscopy and transmission electron microscopy, proteome analyses with two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and liquid chromatography-mass spectrometry (LC/MS), and transcriptome analysis with DNA microarray, we explored the mechanism by which the Neochlamydia effected this defense. We observed that when rare uptake did occur, the symbiotic amoebae allowed Legionella to grow normally. However, the symbiotic amoebae had severely reduced uptake of Legionella when compared with the aposymbiotic amoebae. Also, in contrast to amoebae carrying the endosymbiont, the actin cytoskeleton was significantly disrupted by Legionella infection in aposymbiotic amoebae. Furthermore, despite Legionella exposure, there was little change in Neochlamydia gene expression. Taken together, we concluded that the endosymbiont, Neochlamydia prevents Legionella entry to the host amoeba, resulting in the host defense against Legionella infection.

Amoebal endosymbiont *Neochlamydia* protects host amoebae against *Legionella pneumophila* infection by preventing *Legionella* entry

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Abstract

Acanthamoeba isolated from environmental soil harbors the obligate intracellular symbiont *Neochlamydia*, which has a critical role in host amoebal defense against *Legionella pneumophila* infection. Here, by using morphological analysis with confocal laser scanning fluorescence microscopy and transmission electron microscopy, proteome analyses with two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and liquid chromatography–mass spectrometry (LC/MS), and transcriptome analysis with DNA microarray, we explored the mechanism by which the *Neochlamydia* effected this defense. We observed that when rare uptake did occur, the symbiotic amoebae allowed *Legionella* to grow normally. However, the symbiotic amoebae had severely reduced uptake of *Legionella* when compared with the aposymbiotic amoebae. Also, in contrast to amoebae carrying the endosymbiont, the actin cytoskeleton was significantly disrupted by *Legionella* infection in aposymbiotic amoebae. Furthermore, despite *Legionella* exposure, there was little change in *Neochlamydia* gene expression. Taken together, we concluded that the endosymbiont, *Neochlamydia* prevents *Legionella* entry to the host amoeba, resulting in the host defense against *Legionella* infection.

Keywords: Symbiosis; *Neochlamydia*; *Legionella*; *Acanthamoeba*; amoebae; defense; entry

1. Introduction

Host-parasite relationships, seen in protozoa and symbiotic bacteria, provide insights into the establishment of parasitism, co-evolution, and pathogenesis [1]. Free-living amoebae, such as *Acanthamoeba*, a representative protozoan, are readily isolated from a wide range of natural environments, such as soil or river water [2-4]. Interestingly, bacterial endosymbionts, in particular environmental chlamydiae, are sometimes found within these amoebae, although further research is needed to examine their role in pathogenesis of disease [5]. To investigate such symbiotic features, we isolated several environmental amoebae harboring endosymbiotic environmental chlamydiae, including *Neochlamydia, Protochlamydia* or *Parachlamydia*, from Sapporo, Hokkaido, Japan [6]. Chlamydiae are divided into pathogenic and environmental species, and both types of chlamydiae implement a unique intracellular developmental cycle consisting of two distinct forms, the elementary body (EB), which is the infectious form to the host cells, and the reticulate body (RB), which is the replicative form within cells, which enters into the surrounding plasma membrane to form the so-called inclusion body [7]. Accumulating studies of pathogenic chlamydiae have demonstrated that this cycle is strictly operated using a type III secretion system for effector molecules, which target host molecules such as actin [8]. Meanwhile, the mechanism by which environmental chlamydiae manipulate host amoebae remains to be fully understood [9].

Among the environmental amoebal isolates collected, an amoeba strain (S13WT amoebae) from environmental soil, harboring the obligate intracellular symbiont, *Neochlamydia* eS13, an environmental chlamydia, is particularly of interest for a number of reasons. First, the endosymbiont has an S13WT amoebal infection rate of

100% , but lacks transferability to other host amoebae [6]. Second, the aposymbiotic amoebae (S13RFP amoebae) grow well when compared with the symbiotic amoebae [10]. Last, the endosymbiont has a critical role for host amoebal defense against *Legionella* infection [11]. Investigation of this particular endosymbiotic relationship may therefore give us insights into not only the biological aspects of endosymbiosis, but also a mechanism for eliminating intracellular pathogens. *Legionella pneumophila*, the causative organism of Legionnaires' disease, encodes two virulence-associated type IV secretion systems (T4SSs), the Dot/Icm type 4B (T4BSS) and the Lvh type 4A (T4ASS) [12]. In particular, T4BSS has a critical role in *Legionella* replication in host cells, both amoebae and macrophages [13, 14]. Furthermore, it has been reported that *Legionella* effector proteins disrupt host cytoskeletal structure by cleaving actin and inhibiting actin polymerization [13, 14]. It is possible that when *Legionella* enters the amoeba, the amoebal symbiont *Neochlamydia* could sense some *Legionella* elements, such as type IV secretion systems or effector molecules, subsequently protecting actin, resulting in defense against infection.

In the present study, we therefore explored the defense mechanism by using morphological analysis with confocal laser scanning fluorescence microscopy and transmission electron microscopy, proteome analyses with two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and liquid chromatography–mass spectrometry (LC/MS), and transcriptome analysis with DNA microarray. Unexpectedly, we determined that the defense occurred secondarily, by prevention of *Legionella* entry to the amoebae, in an endosymbiotic bacteria *Neochlamydia*-dependant manner.

2. Materials and Methods

2.1. Amoebae

Symbiotic S13WT amoebae harboring *Neochlamydia* eS13 and aposymbiotic amoebae established by treatment with rifampicin (RFP; 64μg/ml) (S13 RFP amoebae) were used for this study [6, 10]. *Neochlamydia* eS40CH symbiotic amoebae (S40CH amoebae) isolated from soil [6] were also used for this study. Although the S40CH amoebae were originally infected with two phylogenetically different bacterial endosymbionts, *Neochlamydia* eS40 and an endosymbiont belonging to α-*Proteobacteria* [6], the latter endosymbionts were readily eliminated from the S40CH amoebae after incubation at 4 °C. An amoeba strain (*Acanthamoeba castellanii* C3 ATCC 50739: C3 amoebae), purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA), was used as a reference strain. These amoebae were maintained in peptone-yeast extract-glucose (PYG) medium at 18 °C until use, according to published protocols [6].

2.1. Legionella *strains and others*

L. pneumophila with distinct genetic backbones, but originally derived from the Philadelphia-1 progenitor strain, [JR32 ($T4ASS^+/T4BSS^+/Tra^-$), JR32Δ ($T4ASS^+/T4BSS^-/ Tra^-$), Lp01 ($T4ASS^-/T4BSS^+/Tra^+$), Lp02 ($T4ASS^-/T4BSS^-/Tra^+$)] were used [15, 16]. All the bacteria were genetically modified to carry a GFP-expressing plasmid for ease of visualization. The bacteria were cultured on B-CYE agar (OXOID) at 37 °C for 2 days. *Parachlamydia* Bn_9 (ATCC VR-1476) was purchased from ATCC. The bacteria were propagated and stored at -80°C until used

according to methods described previously [17]. Also, Mimivirus Kasaii, isolated from the mouth of the Arakawa River, which is close to Kasai Rinkai Park in Tokyo [18], was used for this study. The virus was propagated in C3 amoebae, and the supernatant of ameobal lytic culture was used as the solution of virus. GFP-expressing *Escherichia coli* was constructed by transformation of *E. coli* (DH5α) with pBBR122 (Funakoshi) carrying *gfp* gene.

2.3. Legionella *infection of amoebae*

Amoebae were infected with *Legionella* at a wide range of multiplicity of infections (MOI), according to the following conditions. Since two of the *Legionella* strains (Lp01 and Lp02) exhibited low rates of infection of amoebae at low MOI (1–10) in PYG medium, the experiments with these *Legionella* strains were performed at an artificially high MOI (10,000).

TEM: Amoebae (S13WT, S13RFP) (2.0×10^3 cells/well) were incubated in PYG with JR32 (without GFP) (MOI 1) for 2 h at 30 °C, then free bacteria were killed by the addition of gentamycin (50 μg/ml). After washing with PYG medium, the infected amoebae were incubated in the medium for 24 h at 30 °C, and then the amoebae were collected for TEM.

Uptake of *Legionella*: Amoebae (S13WT, S13RFP, C3, S40CH) (2.0×10^4 cells/well) were incubated in PYG with Lp01 or Lp02 (MOI 10,000) for 2–18 h at 30 °C, and then free bacteria were killed by the addition of gentamycin (50 μg/ml). After washing with PYG medium, the infected amoebae were incubated in the medium for up to 3 days. The number of amoebae that ingested *Legionella* was estimated under a conventional fluorescence microscope or confocal laser fluorescence microscope (Bio-Rad).

2D-DIGE: Amoebae (S13WT, S13RFP) (2.0×10^6 cells/well) were incubated in PYG with JR32 (MOI 5) for 2 h at 30 °C, and then free bacteria were killed by the addition of gentamycin (50 μg/ml). After washing with PYG medium, the infected amoebae were incubated in the medium for 24 h at 30 °C, followed by collection for 2D-DIGE.

DNA microarray: Amoebae (S13WT) (1.0×10^6 cells/well) were incubated in PYG with JR32 (MOI 1), JR32Δ (MOI 1), Lp01 (MOI 10,000), or Lp02 (MOI 10,000) for 2 h at 30 °C, and then free bacteria were killed by the addition of gentamycin (50 μg/ml). After washing with PYG medium, the infected amoebae were incubated in the medium for 20 h at 30 °C, followed by collection for DNA microarray (total RNA preparation).

2.4. Uptake of GFP-expressing E. coli *and infection of amoebae with* Parachlamydia *Bn*$_9$ *and Mimivirus Kasaii*

The phagocytic activity of amoebae with GFP-expressing *E. coli* was assessed. In brief, each of the amoebae (S13WT ameobae, S13RFP amoebae, C3 amoebae) (5×10^6 cells/well) were cultured with the bacteria (approximately 5×10^8) at MOI 1,000 in PAS at 30°C. At 48 h after incubation, amoebae were observed under a fluorescence microscope. Also, S13WT amoebae (5×10^5) were incubated with *Parachlamydia* Bn$_9$ at MOI 4 in PYG for 1 h at 30 °C. After washing, the amoebae were cultured at 30 °C for 2 days, and then the amoebae were spotted onto slides and fixed with 70% ethanol. After fixing, the amoebae were stained with anti-*Parachlamydia* antibody for 2h. After washing, the amoebae were stained with FITC-conjugated anti-rabbit antibody (Sigma) for 1 h, and then the amoebae were observed with a fluorescent microscopy (Olympus). Rabbit serum containing anti-*Parachlamydia* Bn$_9$ antibodies against formalin fixed the bacteria was produced by Iwaki (Tokyo, Japan) [19]. In addition, each of the amoebae

(S13WT ameobae, S13RFP amoebae, C3 amoebae) (1×10^6 cells/well) were cultured in PYG with 50μl of virus solution for up to 60 h. Amoebal lysis was observed under a light microscope.

2.5. Transmission Electron Microscope (TEM)

TEM analysis was performed using the following protocol: In brief, amoebal cells were immersed in a fixative containing 3% glutaraldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4, for 24 h at 4 °C. After washing with PBS, the cells were processed by alcohol dehydration and embedding in Epon 813. Ultrathin cell sections were stained with lead citrate and uranium acetate prior to visualization by electron microscopy (Hitachi H7100; Hitachi, Tokyo, Japan) as described previously [6].

2.6. 2D-DIGE and LC/MS

Samples for 2D-DIGE were prepared using the following steps. In brief, amoebal cells with or without *Legionella* (JR32) infection (See above) were disrupted with an ultrasonic disintegrator in Tris-HCl buffer containing urea (8 M), CHAPS (2 %), and Triton-X100 (0.5 %). Supernatants of lysis solutions were collected, and then concentrated by treatment with trichloroacetic acid. Equal amounts of resultant protein samples were labeled covalently with fluorescent cyanine dyes, Cy3 or Cy5. The labeled samples were separately or concurrently loaded on an automated 2D-DIGE system, Auto 2D (SHARP, Tokyo, Japan). The gels were stained with Coomassie Brilliant Blue, and visualized under white light with Image Analyzer. Spots with a difference in gel density between samples were cut out from the gels, and then analyzed by LC/MS (MALDI-TOF). LC/MS was conducted by Genomine (Kyunbuk, Korea).

215

216 *2.7. Colony counts of Legionella*

217 Amoebal cultures were collected and frozen at -80°. The thawed culture solutions
218 were incubated on B-CYE agar (OXOID) at 37 °C for 5 days, then colonies formed on
219 the agar plates were counted.

220

221 *2.8. Transcriptome analysis with DNA microarray*

222 Total RNA was extracted from amoebae harboring *Neochlamydia* eS13 with or
223 without *Legionella* (JR32) infection (at 20 h after the infection) using an RNeasy Mini
224 kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Extracted RNA
225 was treated with DNase (DNA-free; Ambion, Austin, TX). Purified RNAs (100 ng)
226 were amplified with T7 RNA polymerase, and cRNAs were labeled with cyanine 3 CTP
227 using a Low Input Quick Amp WT Labeling Kit (Agilent Technologies, Santa Clara,
228 CA) according to manufacturer's instructions. The amplified-labeled cRNA was
229 hybridized to a 4×44K-1-color eS13 *Neochlamydia* DNA microarray customized with
230 bacterial annotation information (Agilent Technologies) [11], and then analyzed using a
231 microarray scanner (Agilent Technologies). Hokkaido System Science (Sapporo, Japan)
232 carried out the DNA microarray analysis including the customized array and
233 normalization of gene expression. Data were expressed as either fluorescence intensities
234 or a gene expression ratio (relative fold change) of "with *Legionella* infection":
235 "without *Legionella* infection".

236

237 *2.9. Statistical analysis*

238 Comparisons between groups were assessed by Student's *t*-test. Comparisons

among groups (more than three) were corrected for multiple testing using the Bonferroni/Dunn method. Spearman's correlation index *r* was calculated using statistical analysis software (Excel for Mac2011). A *p* value of < 0.05 was considered statistically significant.

3. Results

3.1. Morphological analysis with confocal laser scanning fluorescence microscopy and TEM revealed that after Legionella *entry the bacteria grew similarly in S13WT and S13RFP amoebae*

First, we wished to see if morphological analysis by confocal laser scanning fluorescence microscopy and TEM could provide any insight into the mechanism that could eliminate *Legionella* from S13WT amoebae. As a result, confocal laser scanning fluorescence microscopic observation revealed that although the prevalence of *Legionella* infection was very low in S13WT amoebae compared with S13RFP amoebae (data not shown), the GFP clusters, showing the growth of GFP-expressing *Legionella* Lp01 ($T4ASS^-/T4BSS^+/Tra^+$) inside amoebae, were similar in both S13WT and S13RFP amoebae (Fig. 1A). TEM analysis of amoebae infected with the *Legionella* JR32 strain ($T4ASS^+/T4BSS^+/Tra^-$) also showed that, corresponding to the observation of confocal laser scanning fluorescence microscope, growth and morphology of the bacteria was normal in both infected S13WT and uninfected S13RFP amoebae (Fig. 1B, upper images); the enlarged images also showed that bacterial disruption was minimal (Fig. 1B, lower images). This suggests that the defense mechanism is most likely a defect of the host amoebae, such as phagocytosis, rather than a direct attack of *Neochlamydia* on

Legionella inside the host amoebae.

3.2. S13WT amoebae have a phagocytic defect

To confirm this hypothesis, we next examined phagocytosis by amoebae of GFP-expressing *Legionella* strains with distinct genetic backgrounds (Lp01, T4ASS$^-$/T4BSS$^+$/Tra$^+$; Lp02, T4ASS$^-$/T4BSS$^-$/Tra$^+$), easily visualized in infected amoebae as brightening clusters under a conventional fluorescence microscope. As a result, in contrast to amoebae not carrying the endosymbiont (S13RFP and C3 amoebae), few fluorescent clusters were observed in S13WT amoebae infected with Lp01, 72 h after infection (Fig. 2A). Additionally, clusters of Lp02, which lack T4BSS, were seen in S13RFP and C3 amoebae immediately (6h) after infection, in contrast to S13WT amoebae (Fig. 2B), which did not exhibit fluorescent clusters at any point after infection. We also confirmed that, in contrast to S13RFP and C3 amoebae, the number of *Legionella* CFU in S13WT amoebae was minimal even 3 or 4 days after infection, supporting a defect of phagocytosis in S13WT amoebae (Fig. 2C). A delay in phagocytosis is another possible explanation. To test this possibility, we assessed if the number of GFP clusters observed after *Legionella* ingestion altered depending on incubation time before the treatment with gentamycin. When compared to other amoebae, the number of clusters seen in S13WT amoebae were minimal regardless of the incubation time (Fig. 3), suggesting that the reduced rate of infection was not due to a delay in phagocytosis. Thus, taken together, the results indicated that the S13WT amoebae have a defect in phagocytosis, resulting in minimal ingestion of *Legionella*.

In addition, we also confirmed if the defect on the phagocytosis of S13WT amoebae was unique to *Legionella* infection, through the experiments with

GFP-expressing *E. coli*, *Parachlamydia* Bn_9, and Mimivirus Kasaii. As a result, similar to aposymbiotic S13RFP amoebae, S13WT amoebae normally ingested the *E. coli* (Fig. 4A). Also, Immunofluorescence staining with anti-*Parachlamydia* polyclonal antibodies that the *Parchlamydia* could infect to S13WT amoebae (Fig. 4B). Furthermore, the co-culture of amoebae (S13WT amoebae, S13RFP amoebae, C3 amoebae) with the Mimivirus obviously induced amoebal lysis regardless of amoebal strains until 60 h after incubation, indicating the virus replication (Fig. 4C). Thus, the results indicated that the defect on the phagocytosis of S13WT amoebae was likely specific to *Legionella* infection.

3.3. Proteome analysis with 2D-DIGE and LC/MS revealed that, in contrast to S13RFP amoebae, Legionella didn't disrupt actin of S13WT amoebae harboring Neochlamydia eS13

It has been reported that *Legionella* effector proteins disrupt host cytoskeletal structure by cleaving actin, thus inhibiting actin polymerization [13, 14]. This suggests that in contrast to S13RFP amoebae, *Legionella* exposure should induce little disruption of actin in S13WT amoebae. To confirm this, protein profiles of S13WT and S13RFP amoebae infected with *Legionella* JR32 were comprehensively compared by proteome analysis with 2D-DIGE and LC/MS. The 2D-DIGE gel images showed that the densities of several major spots from S13RFP amoebae were obviously decreased compared with those from S13WT amoebae (Fig. 5A, See gel images). Subsequent LC/MS analysis revealed the spots to be actin (Fig. 5A, See spot matched proteins). Meanwhile, the influence of *Legionella* infection on the protein profiles of S13WT amoebae was minimal (Fig. 5B). Thus, as expected, these results supported the

hypothesis that *Legionella* entry to S13WT amoebae was reduced in the presence of *Neochlamydia* eS13.

3.4. Transcriptome analysis with DNA microarray revealed few changes in the Neochlamydia *eS13 response to* Legionella

If minimal ingestion of *Legionella* by S13WT amoebae occurs, it is possible that the response of *Neochlamydia* eS13 to *Legionella* might be limited. To confirm this possibility, we performed transcriptome analysis of *Neochlamydia* eS13 in host amoebae infected with *Legionella* with distinct genetic backbones [JR32 ($T4ASS^+/T4BSS^+/Tra^-$), JR32Δ ($T4ASS^+/T4BSS^-/ Tra^-$), Lp01 ($T4ASS^-/T4BSS^+/Tra^+$), Lp02 ($T4ASS^-/T4BSS^-/Tra^+$)] [13, 14]. Regardless of *Legionella* strain, no difference between gene expression patterns of *Neochlamydia* eS13 in amoebae infected with each of the *Legionella* strains was observed (Fig. 6), indicating that because of a minimal ingestion of *Legionella* the *Neochlamydia* could not sense *Legionella* T4SSs inside the amoebae Thus, the results supported our hypothesis regarding *Legionella* failure to enter the S13WT amoebae depending on the presence of endosymbiont *Neochlamydia* eS13. All microarray data are deposited in Table S1.

4. Discussion

Legionella, the causative agent of Legionnaires' disease is known to inhabit free-living amoebae such as *Acanthamoeba.* Interestingly, the symbiotic amoeba (S13WT), originally isolated from environmental soil, harboring obligate intracellular symbiont *Neochlamydia* eS13 is resistant to *Legionella* infection, and it has been

previously shown that the endosymbiont plays a critical role in this process [11]. Here, we investigated the defense mechanism in this system, and for the first time showed that the presence of symbiotic *Neochlamydia* eS13 could be responsible for preventing *Legionella* entry, resulting in subsequent resistance to *Legionella pneumoniae* infection.

Accumulated studies have showed that *Legionella* invades host cells, both human cells and protozoa, by exploiting host cellular cytoskeletal proteins such as actin, indicating that entry of *Legionella* to the host cells is critically dependent on phagocytosis via an actin-mediated process [13, 14]. Therefore, we initially speculated that *Neochlamydia* eS13 might block the process of actin modification implemented by *Legionella*. Surprisingly, the results indicated that in contrast to the S13RFP amoebae, that do not carry the endosymbiont, *Legionella* failed to enter the S13WT amoebae because of a defect in phagocytosis, resulting in partial resistance to *Legionella* infection. Meanwhile, since poor phagocytosis by SW13 amoebae was not seen with FITC-latex beads [10] or GFP-expressing *E. coli*, this is likely a *Legionella*-infection specific defect. At present, the mechanism by which the defect in *Legionella* phagocytosis functions remains unknown. Interestingly, it has been reported that a specific event referred to as coiling phagocytosis is required for entry of *Legionella* to host amoebae. Coiling phagocytosis, where *Legionella* is engulfed by processes extending from the host membrane, is dependent on actin processing [20, 21]. Therefore, it is likely that the host amoebal surface structure may be changed depending on the presence of *Neochlamydia* eS13.

While the entry of *Legionella* was severely limited by the phagocytosis defect caused by the presence of *Neochlamydia* eS13, the amoebae were defenseless against accidental entrance of *Legionella*, and, once they entered, allowed them to replicate

(See Fig. 1B, TEM images). In fact, the DNA microarray data suggest that *Neochlamydia* eS13 could not sense virulence-associated T4SSs of *Legionella* inside host amoebae. Meanwhile, the presence of symbionts has been shown to be a hindrance for host amoebae, as growth is more active in the absence of symbionts [10]. In the face of this burden, why the host amoebae allow the symbionts to survive remains unknown. Many predators, including bacteria (such as *Pseudomonas*, *Francisella*, *Simkania*, and *Parachlamydiae*, as well as *Legionella*) [22] and giant viruses (such as *Mimivirus*) [23], which co-inhabit the natural environment of amoebae, prey upon protozoa. However, as expected, our data showed that the symbiotic amoebae harboring *Neochlamydia* eS13 easily allowed the growth of *Parachlamydia* Bn_9 and replication of giant viruses (Mimivirus Kasaii), indicating that symbionts do not provide general protection from predation.

Analysis of the *Neochlamydia* transcriptome in host amoebae infected with *Legionella* with distinct genetic backbones, aimed to determine a *Neochlamydia* response to various components of *Legionella.* However, this analysis revealed that the *Neochlamydia* did not respond specifically to the products of the T4SSs [T4ASS (Lvh) and T4BSS (Dot/Icm)] tested, both of which are critically important for survival of *Legionella* in human macrophages or amoebae [12-14]. Meanwhile, it couldn't deny that *Neochlamydia* responded to general bacterial components of *Legionella* such as cell wall or LPS, rather than T4SSs. Alternatively, it is possible that *Neochlamydia* can sense the presence of bacteria outside host amoebae through bacterial general components such as LPS or peptidoglycan, presumably required for maintaining the homeostasis of host amoebae.

In conclusion, we show here for the first time that the presence of symbiotic

Neochlamydia eS13 could be responsible for restriction of phagocytic activity of the host amoebae, resulting in resistance of the host amoebae to *Legionella* infection. These findings might contribute not only to an understanding of the host-parasite relationship, but also to development of a novel strategy against complicated infectious diseases with intracellular parasites.

Conflict of interest

All authors confirm that there are no conflicts of interest.

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406 **Supplementary data**

407

408 **Table S1.** DNA microarray data after normalization. Data values show the gene
409 expression levels of *Neochlamydia* S13 genes after infection with either JR32, JR32
410 dotA Δ , Lp01, or Lp02.

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413 **References**

414 1. Taylor M, Mediannikov O, Raoult D, Greub G Endosymbiotic bacteria associated
415 with nematodes, ticks and amoebae. FEMS Immunol Med Microbiol 2012; 64:
416 21-31.
417 2. Rivera F, Roy-Ocotla G, Rosas I, Ramirez E, Bonilla P, Lares F Amoebae isolated
418 from the atmosphere of Mexico City and environs. Environ Res 1987; 42:149-154.
419 3. Ettinger MR, Webb SR, Harris SA, McIninch SPC, Garman G, Brown BL
420 Distribution of free-living amoebae in James River, Virginia, USA. Parasitol Res
421 2003; 89: 6-15.
422 4. Górnik K, Kuzna-Grygiel W Presence of virulent strains of amphizoic amoebae in
423 swimming pools of the city of Szczecin. Ann Agric Environ Med 2004; 11: 233-236.
424 5. Horn M, Wagner M Bacterial endosymbionts of free-living amoebae. J Eukaryot
425 Microbiol 2004; 51: 509-514.
426 6. Matsuo J, Kawaguchi K, Nakamura S, Hayashi Y, Yoshida M, Takahashi K, et al.
427 Survival and transfer ability of phylogenetically diverse bacterial endosymbionts in
428 environmental *Acanthamoeba* isolates. Environ Microbiol Rep 2010; 2: 524-533.
429 7. Abdelrahman YM, Belland RJ The chlamydial developmental cycle. FEMS
430 Microbiol Rev 2005; 229:949-959.
431 8. Mueller KE, Plano GV, Fields KA. New frontiers in type III secretion biology: the
432 Chlamydia perspective. Infect Immun 2014; 82: 2-9.
433 9. Horn M Chlamydiae as symbionts in eukaryotes. Annu Rev Microbiol 2008;
434 62:113-131.
435 10. Okude M, Matsuo J, Nakamura S, Kawaguchi K, Hayashi Y, Sakai H, et al.
436 Environmental chlamydiae alter the growth speed and motility of host

437 acanthamoebae. Microbes Environ 2012; 27: 423-429.
438 11. Ishida K, Sekizuka T, Hayashida K, Matsuo J, Takeuchi F, Kuroda M, et al.
439 Amoebal endosymbiont *Neochlamydia* genome sequence illuminates the bacterial
440 role in the defense of the host amoebae against *Legionella pneumophila*. PLoS One
441 2014; 9: e95166.
442 12. Nagai H, Kubori T Type IVB secretion systems of *Legionella* and other
443 gram-negative bacteria. Front Microbiol 2011; 2: 136.
444 13. Guo Z, Stephenson R, Qiu J, Zheng S, Luo ZQ A *Legionella* effector modulates host
445 cytoskeletal structure by inhibiting actin polymerization. Microbes Infect 2014;
446 16:225-236.
447 14. Liu Y, Zhu W, Tan Y, Nakayasu ES, Staiger CJ, Luo ZQ A *Legionella* effector
448 disrupts host cytoskeletal structure by cleaving actin. PLoS Pathog 2017;
449 13:e1006186.
450 15. Samrakandi MM, Cirillo SL, Ridenour DA, Bermudez LE, Cirillo JD Genetic and
451 phenotypic differences between *Legionella pneumophila* strains. J Clin Microbiol
452 2002; 40: 1352-1362.
453 16. Rao C, Benhabib H, Ensminger AW Phylogenetic reconstruction of the *Legionella*
454 *pneumophila* Philadelphia-1 laboratory strains through comparative genomics. PLoS
455 One 2013; 8: e64129.
456 17. Matsuo J, Hayashi Y, Nakamura S, Sato M, Mizutani Y, Asaka M, et al. Novel
457 *Parachlamydia acanthamoebae* quantification method based on coculture with
458 amoebae. Appl Environ Microbiol 2008; 74:6397-404.
459 18. Takemura M, Mikami T, Murono S. Nearly Complete Genome Sequences of Two
460 Mimivirus Strains Isolated from a Japanese Freshwater Pond and River Mouth.

461 Genome Announc 2016; 4. pii: e01378-16.
462 19. Yamane C, Yamazaki T, Nakamura S, Matsuo J, Ishida K, Yamazaki S, et al.
463 Amoebal endosymbiont *Parachlamydia acanthamoebae* Bn_9 can grow in immortal
464 human epithelial HEp-2 cells at low temperature; an in vitro model system to study
465 chlamydial evolution. PLoS One 2015; 10:e0116486.
466 20. Horwitz MA. Phagocytosis of the Legionnaires' disease bacterium (*Legionella*
467 *pneumophila*) occurs by a novel mechanism: engulfment within a pseudopod coil.
468 Cell 1984; 36:27-33.
469 21. Cirillo JD, Falkow S, Tompkins LS. Growth of *Legionella pneumophila* in
470 *Acanthamoeba castellanii* enhances invasion. Infect Immun 1994; 62:3254-3261.
471 22. Greub G, Raoult D Microorganisms resistant to free-living amoebae. Clin Microbiol
472 Rev 2004; 17: 413-433.
473 23. Abergel C, Legendre M, Claverie JM The rapidly expanding universe of giant
474 viruses: Mimivirus, Pandoravirus, Pithovirus and Mollivirus. FEMS Microbiol Rev
475 2015; 39: 779-796.
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Figure legends

Fig. 1. Representative confocal laser fluorescence microscopic and TEM images showing the morphology and ultrastructure of *Legionella*-infected amoebae at 3 days after infection. **A:** the morphologies of *Legionella*-infected amoebae (Lp01: T4ASS$^-$/T4BSS$^+$/Tra$^+$) were observed by confocal laser fluorescence microscopy. S13WT_Lp01, S13WT amoebae infected with Lp01 *Legionella*. S13RFP_Lp01, S13RFP infected with Lp01. Green, GFP-expressing *Legionella*. Blue, DAPI. **B:** the ultrastructure of *Legionella*-infected amoebae (JR32: T4ASS$^+$/T4BSS$^+$/Tra$^-$) was observed under a TEM. Squares indicate regions enlarged below. S13WT_JR32, S13WT amoebae infected with JR32. S13RFP_JR32, S13RFP amoebae infected with JR32. Bars, 2 μm.

Fig. 2. Assessment of phagocytosis by S13WT and S13RFP amoebae of GFP-expressing *Legionella* strains with distinct genetic backgrounds (Lp01, T4ASS$^-$/T4BSS$^+$/Tra$^+$; Lp02, T4ASS$^-$/T4BSS$^-$/Tra$^+$) under a fluorescence microscope. **A.** Fluorescence images showing representative bright clusters of the bacteria observed under a fluorescence microscope (green). Magnification, ×400. **B.** Changes in phagocytic activity over time of these amoebae with the *Legionella* strains (Lp01 and Lp02). Data are the means ± SD from at least three experiments. *, $P < 0.05$ vs. each of the values (S13WT_values) over the time course (6 h, 24 h, or 72 h). **C.** Growth of *Legionella* (Lp01 and Lp02) in each of the amoeba lines. CFU, colony-forming units. Data are the means ± SD from at least three experiments. *, $P < 0.05$ vs. each of the values (S13WT amoebae with Lp01 infection) over the time course (3days or 4 days).

Fig. 3. Assessment of phagocytosis by S13WT and S13RFP amoebae with GFP-expressing *Legionella* (Lp01, T4ASS$^-$/T4BSS$^+$/Tra$^+$; Lp02, T4ASS$^-$/T4BSS$^-$/Tra$^+$) under a fluorescence microscope. Amoebae (S13WT, S13RFP, C3, S40CH) (2.0×10^4 cells/well) were incubated in PYG with Lp01 or Lp02 (MOI 10,000) for 2–18 h at 30 °C before treatment with gentamycin. Changes in phagocytic activity over time of these amoebae with the *Legionella* strains (Lp01 and Lp02) were monitored. Data are the means ± SD from at least three experiments. *, $P < 0.05$ vs. each of the values (S13WT, S13RFP, C3, and S40CH amoebae) at 2 h-incubation.

Fig. 4. Uptake of GFP-expressing *E. coli* and infection of amoebae with *Parachlamydia* Bn_9 and Mimivirus Kasaii. **A:** Images showing uptake of GFP-expressing *E. coli* of amoebae (S13WT amoebae, S13RFP amoebae, C3 amoebae). Each of the amoebae (5×10^5 cells/well) were cultured in PAS with the *E. coli* (approximately 5×10^8) at 30°C. At 48 h after incubation, amoebae were observed under a fluorescence microscope. Magnification, ×600. **B:** Immune staining images showing *Parachlamydia*-infected S13WT amoebae. α*Prachlamydia*, Rabbit serum containing anti-*Parachlamydia* Bn_9 antibodies against formalin fixed the bacteria. **C:** Phase contrast images showing amoebal lysis at 60 h after incubation with the virus. Magnification, ×200.

Fig. 5. Proteome analysis with 2D-DIGE and LC/MS: comparison of protein profiles between S13WT and S13RFP amoebae. **A:** 2D-DIGE-gel image showing the changes of protein profiles between S13WT and S13RFP with *Legionella* infection. Protein lysates were obtained 24 h after infection. Numbers (1–5) show the major spots with a decrease

in protein expression in the S13RFP amoebae. Lower box shows the protein annotation in the NCBI database with accession number, score, coverage and expect value. **B:** 2D-DIGE-gel image showing the changes in protein profiles between S13WT with and without *Legionella* infection. Protein lysates were obtained 24 h after infection.

Fig. 6. Transcriptome analysis by DNA microarray: comparison of gene expression of eS13 *Neochlamydia* between infections with different *Legionella* strains. Total RNA was obtained 12 h after infection. Lower panel shows the difference of genetic backbones among the *Legionella* strains as follows: *Legionella* JR32, $T4ASS^{+}$/ $T4BSS^{+}$/ Tra^{-}; JR32Δ, $T4ASS^{+}$/ $T4BSS^{-}$/ Tra^{-}; Lp01, $T4ASS^{-}$/ $T4BSS^{+}$/ Tra^{+}; Lp02, $T4ASS^{-}$/ $T4BSS^{-}$/ Tra^{+} [12-14].

Figure1

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Fig. 1

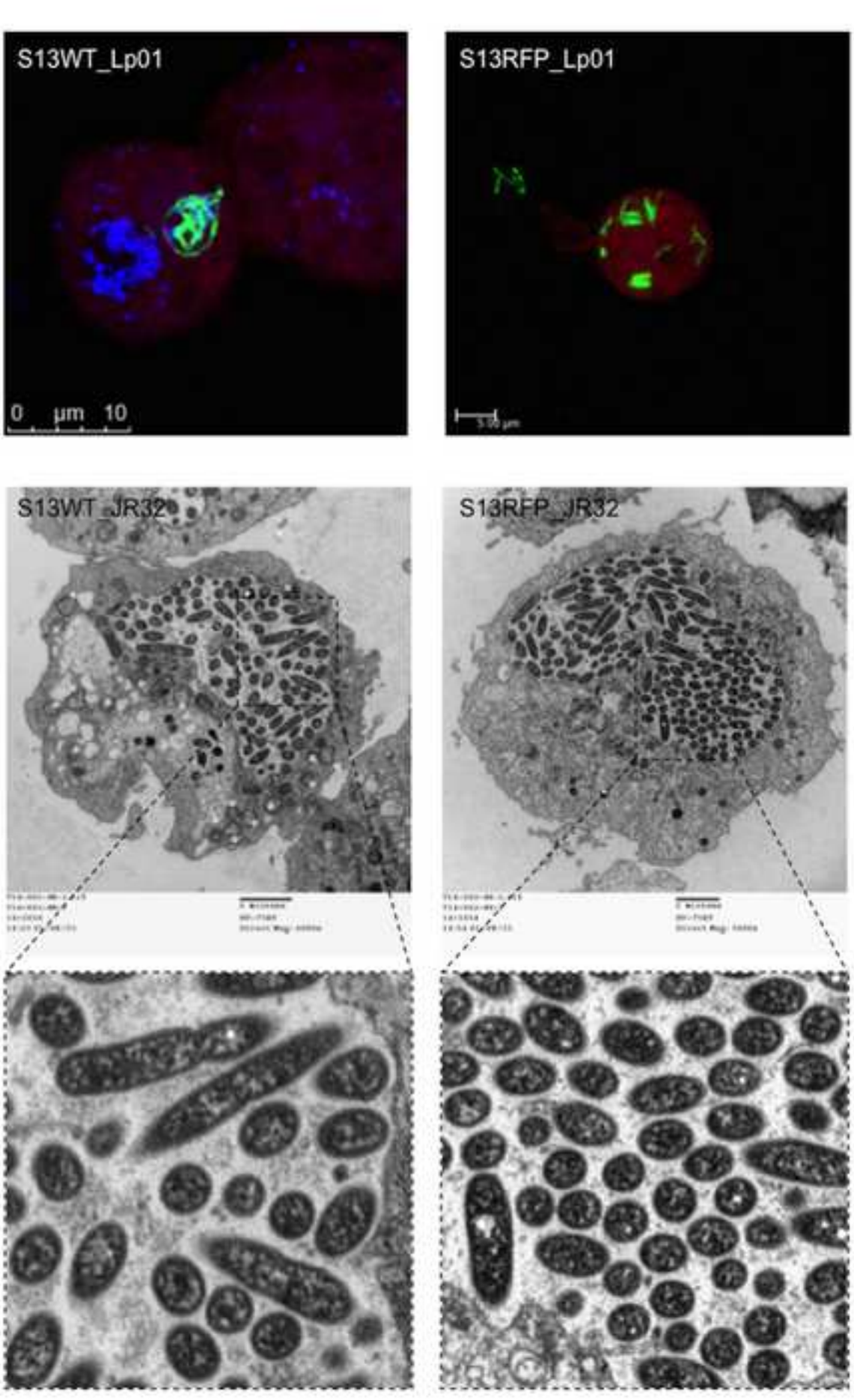

Figure2

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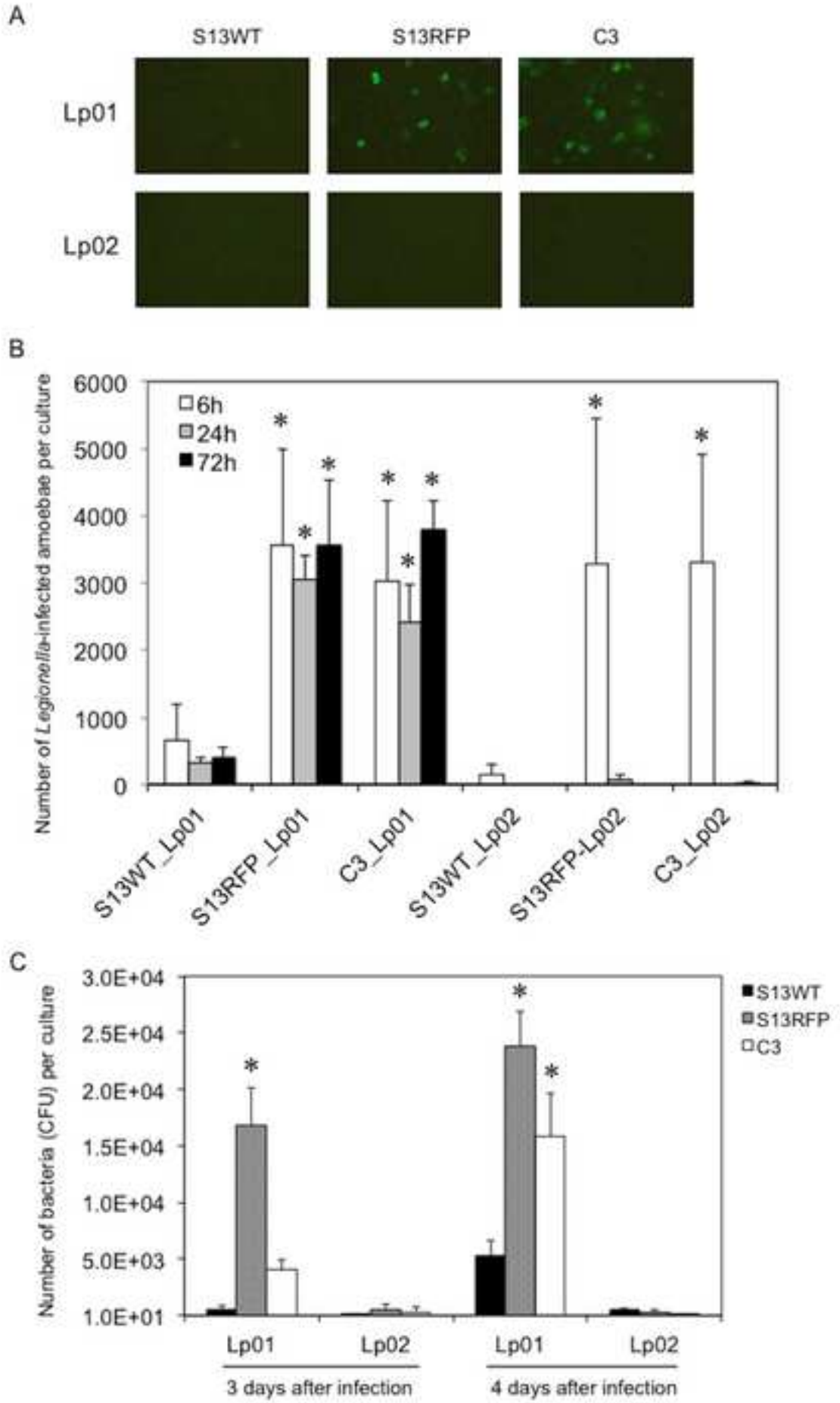
Fig. 2



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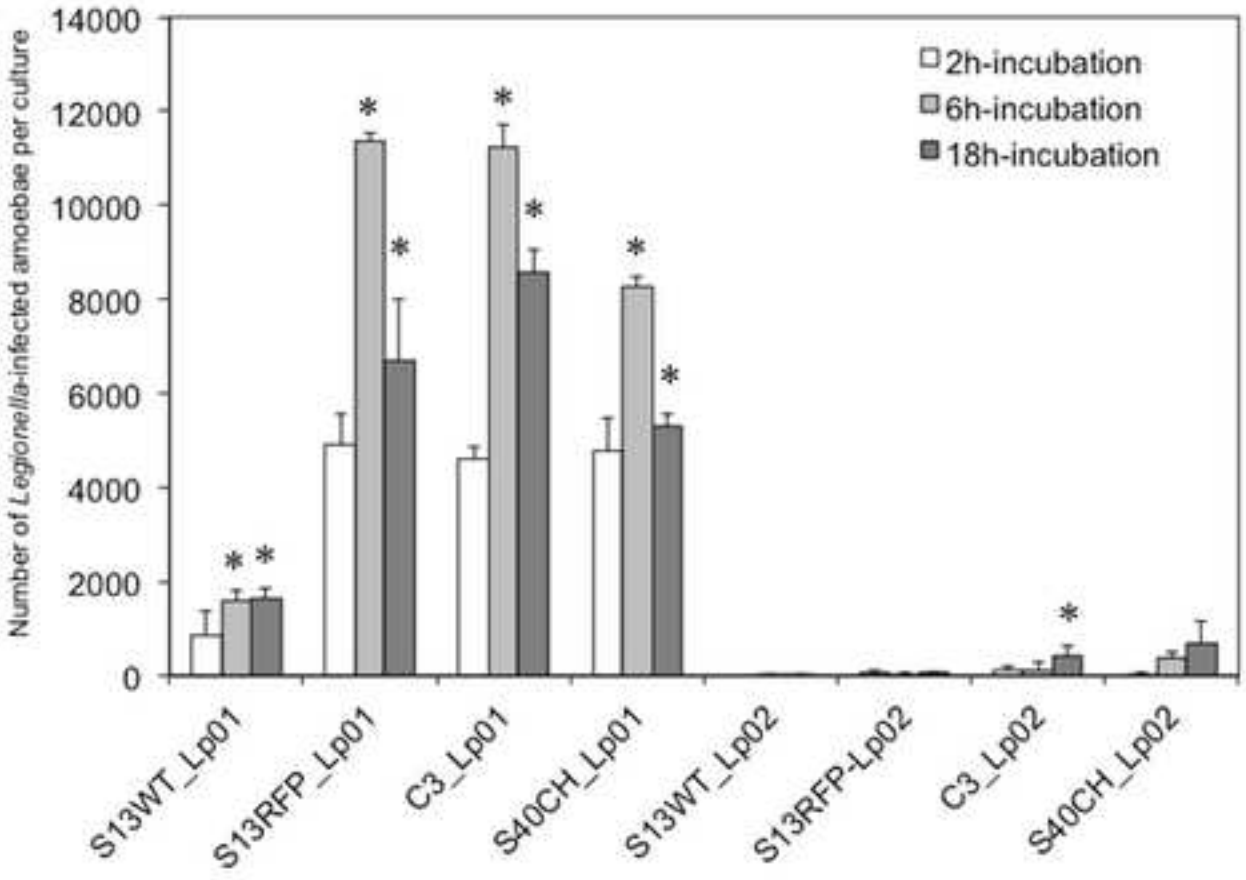

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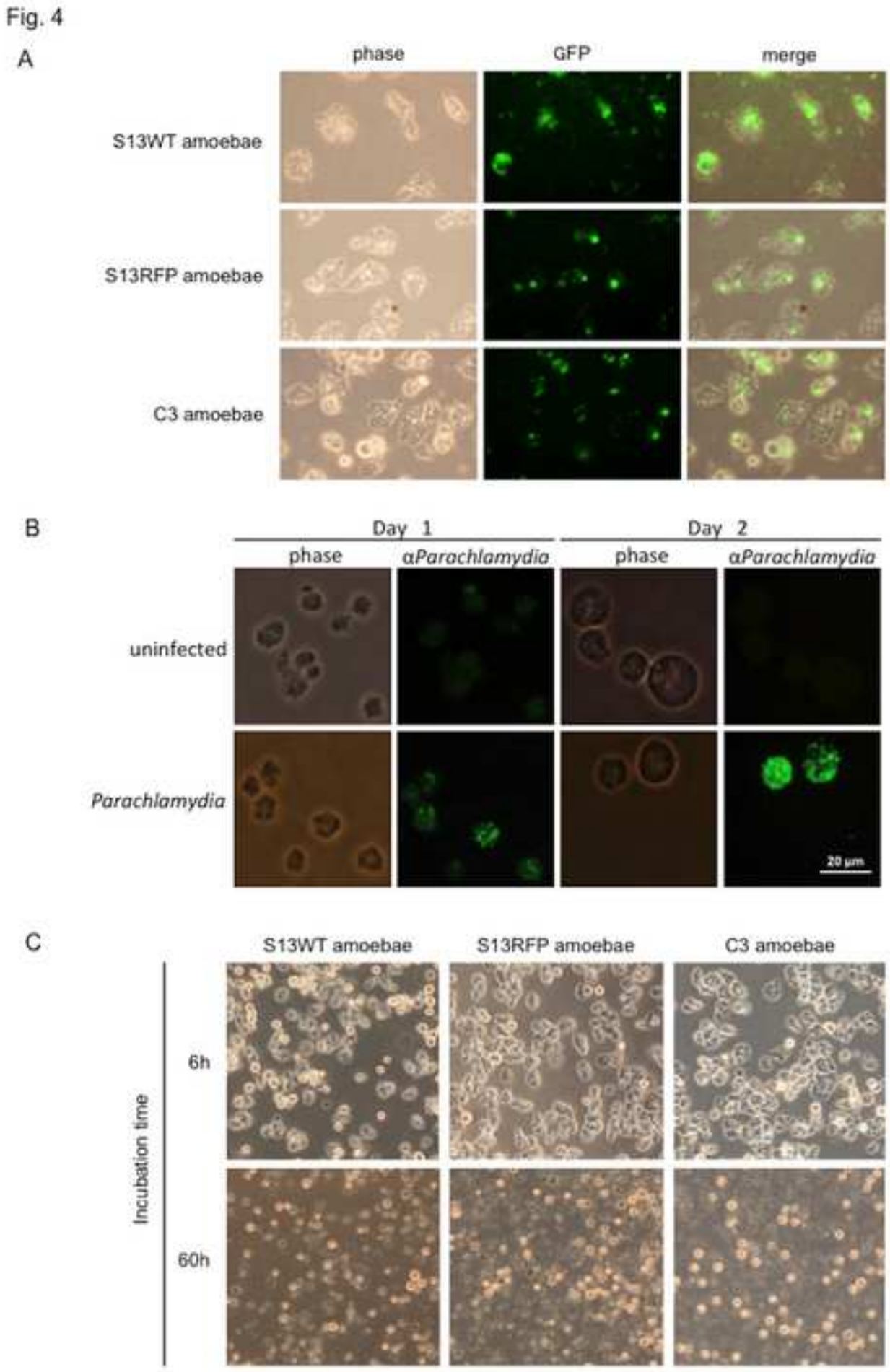

Figure5

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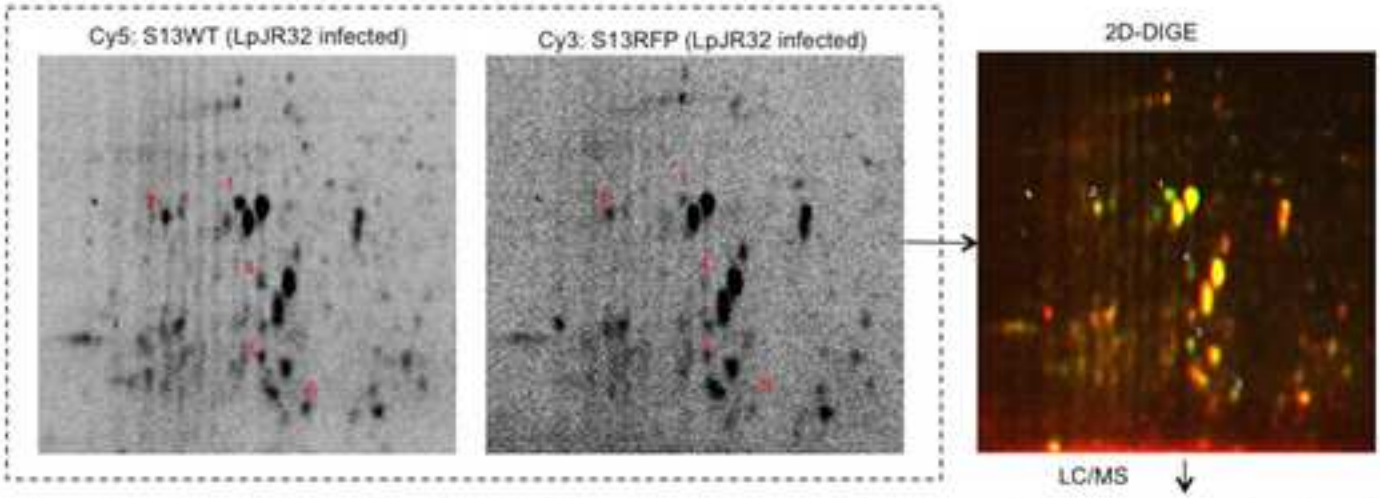
Fig. 5

A



Sopt number	Matched protein	NCBI accession number	Score	Coverage	Expect
1	beta-actin	gi|344944062	148	34%	2.60E-08
2	not determined				
3	actin 1	gi|761949935	130	33%	1.70E-06
4	actin	gi|761949935	115	36%	5.30E-05
5	not determined				

B

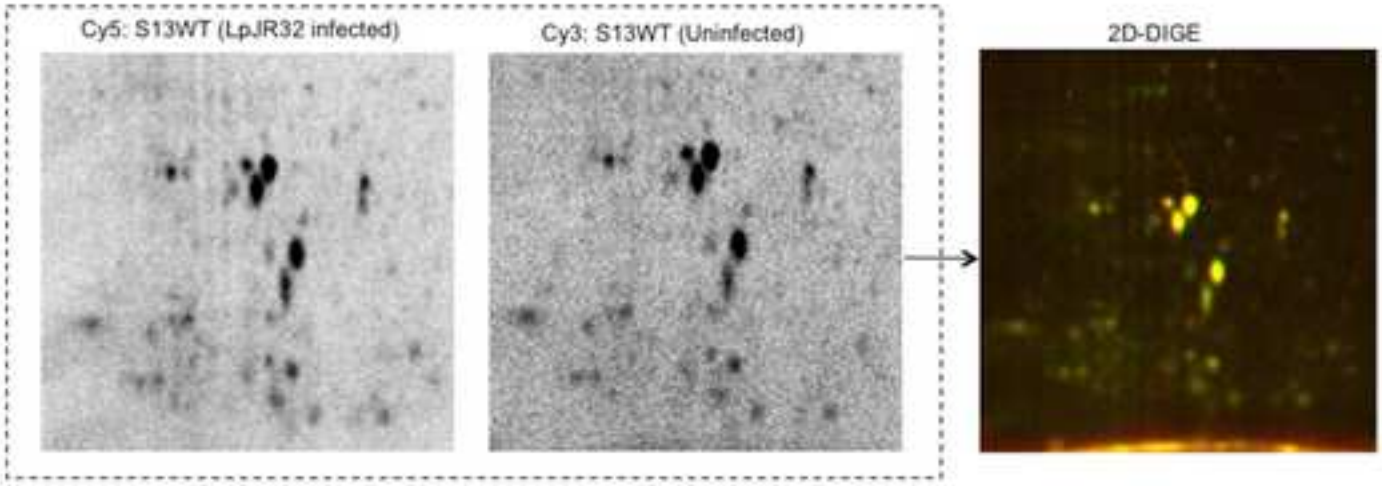


Figure6

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Fig. 6