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Wild ciliates differ in susceptibility to Legionella pneumophila JR32

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ABSTRACT

We investigated how *Legionella pneumophila* (Lp) JR32 interacts with *Anteglaucoma* CS11A and *Colpoda* E6, two ciliates that we isolated from sewage and sink trap sludge, respectively, using a handmade maze device containing a 96-well crafting plate. Our 18S rDNA-based phylogenetic analysis showed that *Anteglaucoma* CS11A and *Colpoda* E6 formed distinct clades. Scanning electron microscopy showed that *Anteglaucoma* CS11A had a bigger-sized body than *Colpoda* E6 and, unlike *Tetrahymena* IB (the reference strain), neither ciliate produced pellets. Fluorescence microscopic observations revealed that although the intake amounts differed, all three ciliates rapidly ingested LpJR32 regardless of the presence or absence of the *icm/dot* virulence genes, indicating that they all interacted with LpJR32. In co-cultures with *Anteglaucoma* CS11A, the LpJR32 levels were maintained but fell dramatically when the co-culture contained the LpJR32 *icm/dot* deletion mutant instead. *Anteglaucoma* CS11A died within 2 days of co-culture with LpJR32, but survived co-culture with the deletion mutant. In co-cultures with *Colpoda* E6, LpJR32 levels were maintained but temporarily decreased independently of the virulence gene. Concurrently, the *Colpoda* E6 ciliates survived by forming cysts, which may enable them to resist harsh environments, and by diminishing the sensitivity of trophozoites to Lp. In the *Tetrahymena* IB co-cultures with LpJR32 or Δ*icm/dot*, the Lp levels were maintained, albeit with temporal decreases, and the *Tetrahymena* IB levels were also maintained. We conclude that unlike *Tetrahymena* IB with pellet production, *Anteglaucoma* CS11A can be killed by LpJR32 infection, and *Colpoda* E6 can resist LpJR32 infection through cyst formation and the low sensitivity of trophozoites to Lp.

Thus, the two ciliates that we isolated had different susceptibilities to LpJR32 infection.

Keywords

Legionella pneumophila JR32, *Anteglaucoma* CS11A, *Colpoda* E6, infection, susceptibility, co-cultures

INTRODUCTION

Legionella pneumophila (Lp) is ubiquitous in a wide range of natural environments such as soil or pond water, where it interacts with amoebae [1-3]. Amoebae provide Lp with the intracellular niche required for its replication. In the process of adapting to its cellular environment, Lp acquired a set of virulence genes encoding the Icm/Dot system that deliver effector proteins to support its successful phagocytosis and invasion, thereby favoring its growth inside human cells along with its own growth [4-7]. As a human pathogen, Lp can cause life-threatening atypical pneumonia (legionellosis) in immunocompromised patients when aerosols or mine dust contaminated with it are inhaled [8-10]. Lp prefers to colonize plumbing walls or gravel floors in hot springs, and *Legionella* outbreaks frequently occur when people take public baths in circulating water systems or in free-flowing hot springs where aerosols containing Lp are frequently formed [11-13]. Therefore, from a public health perspective, the ability to control Lp requires better understanding of its interactions with amoebae.

Various microorganisms such as predatory ciliates with numerous cilia gather in places where amoebae and Lp interactions occur [14-16], and several studies have shown an association of Lp with ciliates [17, 18]. Meanwhile, the role of these ciliates in maintaining and/or killing Lp is not fully understood, although some ciliates (*Paramecium* and *Tetrahymena*) play a role in the symbiotic interaction of Lp [19-23]. With the exception of *Paramecium* and *Tetrahymena*, research on the interactions of other ciliates with Lp has not progressed because the active movements and the global

diversity with low concentration of ciliates make them difficult to isolate [24, 25].

Ciliates are diverse and over 8,000 species have been reported to date [26-28]. As mentioned above, interactions between Lp and *Paramecium* or Lp and *Tetrahymena* have been investigated, and both ciliates have been found to support the intracellular growth of Lp, albeit differently [19-23]. Some *Paramecium* strains can maintain the survival of Lp symbiotically, depending on the *lefA* gene (*Legionella* endosymbiosis-modulating factor A), a key factor contributing to the life stage change in Lp from endosymbiosis to host lysis, which enables its escape into the outside environment [19]. It has been observed that Lp inhibits phagosome formation by *Paramecium* through a component of the type I secretion system (a TolC-dependent mechanism) [20]. It is also reported that *Tetrahymena* can support the survival of Lp [21, 22]. One way in which *Tetrahymena* does this is by expelling environmental Lp-laden pellets, a finding indicating that it may be a defense mechanism against Lp rather than a symbiotic role [22, 23]. However, studies on how Lp interacts with other ciliates are limited in number.

Here, we assessed the interaction of Lp with two wild ciliates, *Anteglaucoma* CS11A and *Colpoda* E6, which we isolated from sewage and sink trap sludge, respectively, using a handmade maze device fitted with a 96-well crafting plate. We show that unlike *Tetrahymena* IB with pellet production, *Anteglaucoma* CS11A can be effectively killed by Lp infection, whereas *Colpoda* E6 is protected against Lp infection through cyst formation and the low sensitivity of trophozoites to Lp. Thus, these two ciliates are

differentially susceptible to Lp infection.

METHODS

Ciliates

Anteglaucoma CS11A (isolated from sewage) and *Colpoda* E6 (isolated from sink strap sludge) were used in this study (See below for "Isolation methods for ciliates"). These ciliates were maintained in Sonneborn's *Paramecium* medium (SPM, also known as ATCC medium 802) consisting of cerophyl grass powder (Toyotama Healthy Foods Co., Ltd. Japan) in distilled water with live *Enterobacter aerogenes* (our laboratory stock) at 22°C [29]. *Tetrahymena* IB was also maintained in peptone-yeast extract glucose (PYG) broth, which contains peptone (BD, Franklin Lakes, NJ), yeast extract (BD), and glucose (FUJIFILM Wako Pure Chemical Co., Tokyo, Japan) at 15°C, as described previously [30].

Bacteria

The Lp originally derived from the Philadelphia-1 progenitor strain [JR32 (wild-type strain) and its mutant (Δ*icm*/*dot*)] were used in this study [31]. Both strains were genetically modified to carry a GFP-expressing plasmid (pAM239GFP) for easy visualization. The plasmid, which continuously expressed GFP was introduced to LpJR32 and its mutant by electroporation. Also, mCherry-expressing-*Escherichia coli* DH5α (Ec) was constructed by the introduction with a plasmid (pBBR122mCherry)

(Funakoshi, Tokyo, Japan), which continuously expressed mCherry. Lp and Ec were cultured on B-CYE agar (Thermo Fisher Scientific, Waltham, MA) and LB agar plate with or without 10μg/mL of chloramphenicol, respectively, at 37 °C for appropriate period (2 days for Lp, one day for Ec). The mutant with the plasmid (pAM239GFP) was kindly provided by Dr. Nagai (Gifu University, Japan).

Isolation methods for ciliates

Ciliates were isolated according to the procedure with the maze device described in Fig. S1. To make this device inexpensive and easy to use, we used commercially available 96-well culture plates in its operation. We simply broke the walls between the plate wells with a heated spatula (Fig. S2, a photo of the device that we used). Briefly, sink trap sludge samples (n=5) and manhole sewage samples (n=13) were collected from different places in our university, because Lp frequently colonizes in sinks or sewages [32-36]. The sample solutions, which were diluted in Page's amoeba saline (PAS) [37], were cultured with sterilized rice grains at room temperature for 2 weeks. When a large number of ciliates had been reared, a concentrated sample containing >1,000 ciliate cells was placed into the "sample addition hole" of the maze device, which was filled with PAS except for the "sample addition hole", which was partitioned with a cotton plug. After removing the cotton plug-containing partition, the ciliates were cultured for several days at room temperature. Ciliates captured in the "capture hole" were carefully collected, cultured in SPM medium (See above), and isolated by the limiting dilution method. The maze device could be reused approximately three times by performing intermittent sterilization (95°C,

10 min, twice).

Direct sequencing and phylogenetic analysis

Total DNA was extracted from the isolated ciliates using the High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's instructions. Extracted DNA was amplified using Quick Taq HS Dye Mix (TOYOBO, Osaka, Japan) with an universal primer set for each ciliate's 18S rDNA gene (P-SSU-342f ; 5'-CTT TCG ATG GTA GTG TAT TGG ACT AC-3', Medlin B; 5'-TGA TCC TTC TGC AGG TTC ACC TAC-3'), as previously reported [38]. The amplified products were electrophoretically separated and then extracted from the agarose gel using the FastGene Gel/PCR Extraction Kit (NIPPON Genetics, Tokyo, Japan) according to the manufacturer's protocol, and then sequenced by Fasmac (Kanagawa, Japan). The 18S rDNA phylogenetic tree containing the isolated ciliates, which also contains representative ciliates, was constructed by the neighbor-joining method (bootstrap replication value, 500) with MEGA X [39]. Representative sequences were obtained from the National Center for Biotechnology Information (NCBI: (https://www.ncbi.nlm.nih.gov/). All the nucleotide sequence accession numbers are shown in Fig. 1.

Scanning electron microscopy (SEM)

The isolated ciliates were processed according to a previous method [40]. Briefly, they were washed in saline, fixed with 2.5% glutaraldehyde in phosphate-buffered saline for 2 h at room temperature, and then soaked in osmium tetroxide for 1 h at 4°C. The samples

were then dehydrated in ethanol, freeze-dried, and coated with osmium using a plasma osmium coater. The samples were analyzed by SEM (Hitachi S-4800; Hitachi, Tokyo, Japan).

Co-culture experiment with imaging

Our co-culture system was constructed with green fluorescent protein (GFP) or mCherry-expressing Lp (LpJR32 or Δ*icm/dot*) or Ec (10^7 CFU/mL) with or without each ciliate (*Anteglaucoma* CS11A, *Colpoda* E6, *Tetrahymena* IB) (10^3 cell/ml) in 50 mL PAS or CA-1 medium (some experiment), a nutrient-rich medium used for preventing cyst formation [41]. The cultures were maintained at room temperature. Samples were collected immediately (1, 30, 120 min) or daily over 7 days, and used for extraction of DNA, ciliate's counts or imaging with a conventional fluorescence microscope (BIOREVO BZ-9000, Keyence, Osaka, Japan), an Olympus microscope (OKX41) with a fluorescence unit (U-RFLT50, Tokyo, Japan), or a confocal laser fluorescence microscope (TCSSP5 TIRF, Wetzlar, Germany). The amount of amplicon targeting Lp *mip* gene and each ciliate was then quantified (see below).

Pellet production assay

The following experiments were conducted to confirm whether pellet production is a phenomenon peculiar to *Tetrahymena* IB. Briefly, each of the ciliates (*Anteglaucoma* CS11A, *Colpoda* E6, *Tetrahymena* IB) (10^3 cell/ml) was mixed with GFP-expressing Lp (JR32 or Δ*icm/dot*) (10^7CFU/ml) to 10ml of PAS solution in a 15ml-sterilized tube, and

incubated upright without shaking for one day at 22°C. After incubation, the supernatant containing a 50 μl solution was carefully removed, and the remaining solution was fixed with 4% paraformaldehyde. The presence or absence of pellets in the sample was then confirmed under a fluorescence microscope (BIOREVO BZ-9000, Keyence).

Determining Lp amounts using quantitative (q)PCR

The samples obtained from co-culture system were treated with freeze-thawing and then used for DNA extraction [42]. DNA extraction was performed using a Instagene kit (Bio-Rad, Hercules, CA, USA) according to the manufacture protocol. The Lp and ciliate amounts in the co-culture system were quantified by CFX Connect (BioRad) with SYBR Green (KOD SYBR qPCR Mix, TOYOBO) targeting the bacterial *mip* gene (Lg3: 5'-GCT ACA GAC AAG GAT AAG TTG -3', Lg4: 5'- GTT TTG TAT GAC TTT AAT TCA -3') [43]. The aliquot solution of mixed culture of Lp with ciliates was simply used as the sample for qPCR, but not for culture. We adopted the qPCR instead of the CFU to quantify Lp for two main reasons; firstly, it is very difficult to uniformly suspend Lp into ciliates or expelled pellets; and secondly, bacteria, which is given as food during subculture, may also grow together on an agar medium.

Determining ciliate amounts using a cell counting chamber

Ciliate (trophozoite) numbers and cyst numbers were determined using 10μl of culture in a disposable cell counting chamber (Thermo Fisher Scientific, Tokyo, Japan) by the naked eye under a microscope.

Statistical analysis

Data obtained from the co-culture experiments were compared using the Bonferroni correction. *P*-values of less than $p<0.05$ were considered significant. The presence of a correlation for each ciliate's (trophozoite) number and cyst number was determined by Pearson's correlation coefficient test. A correlation coefficient value of >0.3 or <−0.3 with a *P*-value of less than 0.05 was considered significant.

Nucleotide sequence accession numbers

The 18S rDNA sequences from *Anteglaucoma* CS11A and *Colpoda* E6 described in this study have been deposited in the DDBJ GenBank database (https://www.ddbj.nig.ac.jp/index.html) under accession numbers LC573510 and LC573511.

RESULTS

Characterization of the ciliates newly isolated from sewage and sink trap sludge by phylogenetic analysis and SEM observations

First, we successfully isolated two ciliates (*Anteglaucoma* CS11A and *Colpoda* E6). Their taxonomic positions were determined by phylogenetic analysis of their 18S rDNA sequences. As a result, two ciliates, CS11A and E6, were assigned to *Anteglaucoma* (isolated from sewage) (accession number: LC573510) and *Colpoda* (isolated from sink

trap sludge) (accession number: LC573511), respectively. Both fell into distinct clades and their locations in the phylogenic tree differed from those of *Paramecium* and *Tetrahymena*, which are the only ciliates that have been studied for their interactions with Lp [19-23] (Fig. 1). Our SEM observations revealed that unlike *Colpoda* E6 (Fig. 2A and B), *Anteglaucoma* CS11A possesses a large body with numerous thin cilia projecting outwards from it (Fig. 2C and D). Although *Tetrahymena* IB, which has thicker cilia, has been reported to expel pellets [23] (Fig. 2F, arrows), pellet production was not observed in *Colpoda* E6 and *Anteglaucoma* CS11A. To confirm this, we assessed if the pellet production with the packaging of Lp occurred as a phenomenon specific to *Tetrahymena* IB. As a result, in contrast to the other ciliates, the presence of expelled pellets was only observed in the remaining solution of the standing-mixed culture of *Tetrahymena* IB with Lp at 24 h after co-culture (Fig. S3), indicating the pellet production with the packaging of Lp is unique to *Tetrahymena* IB among ciliates used for this study. Thus, based on these morphological differences, the two ciliates we isolated may have different interactions with Lp.

Interactions of the newly isolated ciliates with Lp immediately (1 min, 30 min, 2 h) or after 24 h in co-culture

We next determined whether the wild ciliates (*Anteglaucoma* CS11A and *Colpoda* E6), and the control ciliate (*Tetrahymena* IB), interact with Lp (the LpJR32 wild-type strain or the Δ*icm*/*dot* mutant) using confocal laser microscopy. All three ciliate genera ingested Lp and their features were distinctive at 24 h post-inoculation regardless of whether the

infections involved the wild-type or mutant Lp (Fig. 3). Furthermore, we assessed if these ciliates ingested Lp immediately (1 min, 30 min, 2 h) after co-culture through visualization of Lp in ciliates. As a result, the uptakes of Lp regardless of its mutant in the ciliates (*Anteglaucoma* CS11A and *Colpoda* E6) was observed from 1 min after co-culture, although there was a difference in the amount of uptake as the *Anteglaucoma* CS11A ingested a large amount (Fig. 4). Also, as expected, we confirmed that the uptake of these bacteria into *Tetrahymena* IB normally occurred at 30 min after co-culture (Fig. S4), and as previously reported, the ciliates packaged it in pellets at 24 h after co-culture (Fig. 3C and Fig. S3) [23], thereby confirming that our co-culture conditions were optimal. Notably, *Anteglaucoma* CS11A ingested a large amount of Lp compared with *Colpoda* E6. Together, the findings indicate that some interaction between ciliates and Lp occurred in our co-culture system.

Changes in Lp amounts in the co-culture system

We used qPCR to monitor changes in Lp amounts (the LpJR32 wild-type strain or the JR32Δ*icm*/*dot* mutant) in the ciliates using the co-culture system over 7 days. As a result, the Lp amount in the *Anteglaucoma* CS11A co-culture was constantly maintained over the 7-day period (Fig. 5A), but fell dramatically when the Δ*icm/dot* mutant was substituted for JR32 (Fig. 5B, "+*Anteglaucoma* CS11A"). In contrast with *Anteglaucoma* CS11A, the Lp amount was maintained in the co-culture system with *Colpoda* E6 independently of the virulence genes, but with a temporal decrease occurring one day after starting the culture (Fig. 5, "+*Colpoda* E6"). In addition, the amount of Lp was

maintained in *Tetrahymena* IB irrespective of the virulence genes (Fig. 5, "+*Tetrahymena* IB"). Thus, the findings indicate that the Lp amounts in the co-culture system differed between *Anteglaucoma* CS11A and *Colpoda* E6, although the change of bacterial amount in the culture with the *Colpoda* was similar to those of *Tetrahymena* IB.

Changes in ciliate amounts during co-culturing

Using cell counting, we also monitored changes in ciliate amounts in the co-culture system with Lp (the LpJR32 wild-type strain or the Δ*icm*/*dot* mutant) over 7 days. Although uptake of LpJR32 and the mutant was separately confirmed, no ciliates were observed in the co-culture of *Anteglaucoma* CS11A and LpJR32 (Fig. 6A). While the *Anteglaucoma* CS11A-Δ*icm/dot* co-culture was maintained at levels similar to the bacteria alone control during the culture period (Fig. 6B, "+LpJR32 Δ*icm*/*dot*"), the ciliates were completely killed in the presence of LpJR32 by day 2 (Fig. 6B, "+LpJR32"). Conversely, the morphology of *Colpoda* E6 was maintained even in the presence of LpJR32, a finding similar to that of the "ciliates alone" control (Fig. 7A). Although the ciliate amounts gradually decreased, there was no significant difference in *Colpoda* E6 amounts among the co-cultures, regardless of the presence or absence of the virulence genes (Fig. 7B). Interestingly, when the number of ciliates decreased, the number of cysts tended to increase, although this finding was not statistically significant (Fig. 8). Also, to confirm the sensitivity of Lp to trophozoites, *Colpoda* E6 was co-cultured with LpJR32 or Δ*icm*/*dot* mutant in a nutrient-rich medium, CA-1, which inhibits cyst formation (See the Methods), and the number of ciliates was calculated under the condition suppressive

of cyst formation. However, the trophozoites of ciliates grew well regardless of the presence or absence of Lp during the culture period (Fig. S5), suggesting that along with resistance to Lp by cyst formation, the low sensitivity of trophozoites to Lp also may contribute to their resistance to Lp. In addition, the *Tetrahymena* IB amounts were maintained regardless of the presence or absence of Lp during the culture period (Fig. S6). Thus, we conclude that unlike *Tetrahymena* IB, which can resist Lp by enwrapping it and expelling it in pellets, *Anteglaucoma* CS11A can be completely killed by Lp infection, whereas *Colpoda* E6 resists Lp infection by forming cysts with the low sensitivity of trophozoites to Lp. Therefore, the two ciliates we isolated have distinctive to Lp infection.

DISCUSSION

As mentioned above, Lp is ubiquitous in a wide range of natural environments such as soil or pond water [1-3]. Lp interacts with various protozoa including amoebae and ciliates, many of which are unknown species [26-28]. More importantly, Lp propagating through such interactions can create the ideal conditions for legionellosis in humans [8-10]. However, few studies have been published on the interactions of Lp with ciliates, and those that have been published are limited to *Paramecium* and *Tetrahymena* [16-23]. Therefore, we investigated the interaction of Lp with two wild ciliates, *Anteglaucoma* CS11A and *Colpoda* E6, which we isolated from sewage and sink trap sludge, respectively, using a handmade maze device with a 96-well crafting plate. Our data

clearly shows that unlike *Tetrahymena* IB, *Anteglaucoma* CS11A is killed by Lp infection, whereas *Colpoda* E6 resists Lp infection through cyst formation and the low sensitivity of trophozoites to Lp.

Ciliates are not easy to isolate because they move around actively [24, 25]. Therefore, we created a simple handmade maze device that can discriminate differences in the moving capacities of other microbes such as motile bacteria. Meanwhile, there are some limitations in using the maze device. First, intermittent sterilization (<95°C, 10 mins, twice) of the device deforms it making it unusable after three uses. Second, toxic gas is generated when making the device, so it is necessary to prepare it in a well-ventilated place. Third, because the wall is broken manually, the groove width will subtly differ in each device. Fourth, the separation cannot be done unless the number of ciliates added exceeds $>10^3$ cells, approximately.

We isolated two morphologically distinct ciliates using this device and assigned them as *Anteglaucoma* CS11A and *Colpoda* E6. These ciliates differ from *Paramecium* and *Tetrahymena*, which have been extensively studied for their interactions with Lp. *Anteglaucoma,* which was originally isolated from a farmland pond in China, has been reported as a new ciliate genus in the *Glaucomidae* family, and is recognized by its hectic jerking motion [34]. *Anteglaucoma* CS11A also has an outstanding athletic ability, possibly affording it a predation advantage. In contrast, a large number of studies have been published over a long time period on the ecological and biological properties of

Colpoda, and these support it as a ubiquitous and prosperous genus among known ciliates [45-47]. With its ubiquitous nature, *Colpoda* has been used as an indicator of environmental pollution (e.g., heavy metal pollution) [48, 49]. Because there are currently no studies on the interactions of these ciliates with Lp, this study is the first to discover their interactions.

We found that *Anteglaucoma* CS11A can be killed by LpJR32 depending on whether or not the *icm/dot* virulence genes are present. Although it is well known that *Tetrahymena* ensures bacterial survival by expelling any bacteria (e.g., *Salmonella*, *Mycobacterium*, and Lp) packaged in its fecal pellets as a way for ciliates to discard preys [23, 50, 51], no pellet production but the accumulation of Lp at earlier interaction was seen in the *Anteglaucoma* CS11A-co-cultures, suggesting that the defense mechanism of the ciliates against Lp may be lacking. Also, the exact reason why the Lp infections caused ciliate death in our experiments is not known, but it is possible that some Lp effectors (e.g., those which shut down endoplasmic reticulum–mitochondria interactions), may be involved in this type of cell death [52]. Meanwhile, it was not clear that Lp can replicate inside the ciliates. Thus, *Anteglaucoma* CS11A could be used as a biological indicator to verify the pollution status of Lp in water supplies or hot springs. We also found that *Colpoda* E6 can be maintained in the presence of Lp independently of the *icm/dot* virulence genes through cyst formation. Stimulation of cyst formation is not a sign of Lp infection, because cyst formation is observed even when ciliates are cultured alone. Furthermore, because we observed that despite the presence of Lp, the ciliates mostly consisting of trophozoites

grew well in a nutrient-rich condition that inhibits cyst formation (See Fig. S5), it is possible that along with the transitions to cysts in low-nutrient environments, the low sensitivity of trophozoites to Lp may contribute to their resistance to Lp. In addition, it has been reported that starvation can induce production of the cyst coat protein responsible for cyst formation in *Colpoda steinii* [53].

In conclusion, we successfully isolated two ciliates (*Anteglaucoma* CS11A and *Colpoda* E6) from sewage and sink trap sludge, respectively, using a handmade maze device. Each one differs in its susceptibility to Lp infection. Contrasting with *Tetrahymena* IB with pellet production, *Anteglaucoma* CS11A can be killed by Lp infection, whereas *Colpoda* E6 resists Lp infection through cyst formation and the low sensitivity of trophozoites to Lp (Fig. 9). Our new identification of two ciliates with different susceptibilities to Lp infection provides useful public health information towards the control of human pathogenic Lp.

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Author contribution

HY and TO designed the study. AK and TO carried out the experiments. HY, AK, and TO analyzed the data. SN carried out imaging analysis. MM provided the Lp strains. JT edited the manuscript and made critical suggestions. HY wrote the manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Figure legends

Fig. 1. 18S rDNA-based phylogenetic tree showing the location of *Anteglaucoma* CS11A and *Colpoda* E6 among representative ciliates. The red and blue circles show the locations of *Anteglaucoma* CS11A and *Colpoda* E6, respectively. Small numbers in the phylogenetic tree indicate local bootstrap probability. Numbers after species names are accession numbers in the NCBI database.

Fig. 2. Representative SEM images showing the morphological features of three ciliates. A and B, *Colpoda* E6. C and D, *Anteglaucoma* CS11A. E and F, *Tetrahymena* IB. Arrows, expelled fecal pellets. Images in small dashed boxes (A and C) in the left-hand panels are enlarged in the right-hand panels (B and D). White bars show scales for 5μm (B and D), 10μm (E and F), and 20μm (A and C).

Fig. 3. Representative confocal laser microscopic images showing three the ciliates from this study with distinct features at 24 h after co-culture with GFP-expressing Lp. GFP-Lp, GFP-expressing *L. pneumophila*. A, Images show *Anteglaucoma* CS11A co-cultured with JR32 (up) and JR32Δ*icm/dot* (down). B. Images show *Colpoda* E6 co-cultured with JR32 (up) and JR32Δ*icm/dot* (down). C. Images show *Tetrahymena* IB co-cultured with JR32 (up) and JR32Δ*icm/dot* (down). Each image shows one of the observed 3-5 fields of view. White bars show scales for 10μm.

Fig. 4. Representative fluorescence microscopic images showing the uptake of Lp into each of the ciliates [*Anteglaucoma* CS11A (A) and *Colpoda* E6 (B)] immediately (1, 30, 120 min) after co-culture with mCherry-expressing Lp (the wild-type LpJR32 strain and the Δ*icm/dot* mutant) or mCherry-expressing Ec. mCherry-Lp, mCherry-expressing *L. pneumophila*. mCherry-expressing Ec, mCherry-expressing *E. coli*. Each image shows one of the observed 3-5 fields of view. Black bars show a scale of 10μm.

Fig. 5. Changes in the amount of amplicon targeting Lp *mip* gene in the co-culture system of Lp (JR32 strain and Δ*icm/dot* mutant) with *Anteglaucoma* CS11A and *Colpoda* E6. The co-culture system was maintained for 7 days. “Day 0” means immediately after co-culture. A. Amount of amplicon targeting Lp *mip* gene in the co-culture with LpJR32. B. Amount of amplicon targeting Lp *mip* gene in the co-culture system with the LpJR32Δ*icm/dot* mutant. The data (average ± SD) independently obtained from four experiments were compared using the Bonferroni correction. *, $p<0.05$ vs. “Day0” of each group.

Fig. 6. Changes in the *Anteglaucoma* CS11A cell amounts in the co-culture system of Lp (JR32 and Δ*icm/dot* mutant) with *Anteglaucoma* CS11A. The co-culture system was maintained for 7 days. “Day 0” means 2 h after co-culture. A. Representative images showing the interaction of the ciliate with LpJR32 or Δ*icm/dot* during the culture period. Because the ciliates could not be seen at 2 days after co-culture, no images were obtained after the second day. Each image shows one of the observed 3-5 fields of view. Black bars

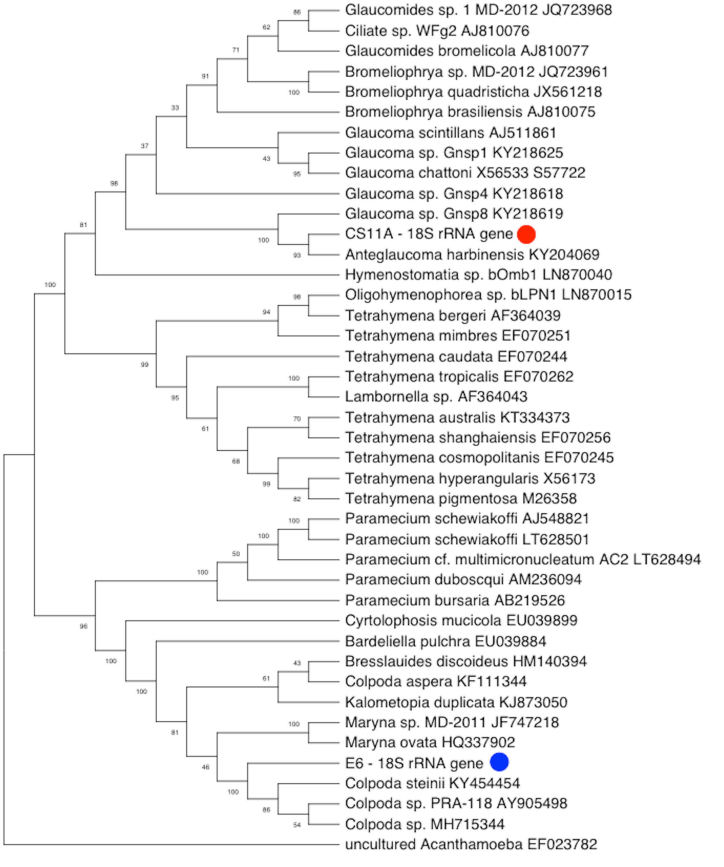
show a scale of 10μm. B. Changes in *Anteglaucoma* CS11A cell amounts in the co-culture system. The data (average ± SD) independently obtained from three experiments were compared using the Bonferroni correction. *, $p<0.05$ vs. "Ciliates alone".

Fig. 7. Changes in *Colpoda* E6 cell amounts in the co-culture system of Lp (JR32 and the Δ*icm/dot* mutant) with *Colpoda* E6. The co-culture system was maintained for 7 days. "Day 0" means 2 h after co-culture. A. Representative images showing the interaction of the ciliate with LpJR32 or mutant LpJR32 during the culture period. Each image shows one of the observed 3-5 fields of view. Black bars show a scale of 10μm. B. Changes in *Colpoda* E6 cell amounts in the co-culture system. The data (average ± SD) were independently obtained from three experiments.

Fig. 8. Changes in the cyst amounts in the co-culture system of *Colpoda* E6 with Lp (JR32 and Δ*icm/dot* mutant). The upper images show the appearances of the cysts from each of the cultures at 7 days post-co-culture. The middle graphs show the changes in cyst numbers over time. "Day 0" means 2 h after co-culture. Black bars show a scale of 10μm. Bottom graphs show the correlations between the ciliate (trophozoites) numbers and cyst numbers in the cultures with or without LpJR32 or the LpJR32Δ*icm/dot* mutant. The data for "Ciliate (trophozoites) numbers" used for correlation in this experiment were the same as that for Fig. 7B. "*r*" shows the correlation values. The data (average ± SD) were independently obtained from three experiments.

Fig. 9. Results summary. Upper illustration shows the fate of *Anteglaucoma* CS11A after interacting with LpJR32. Whether or not this ciliate dies depends on the presence/absence of the *icm/dot* virulence genes. Middle illustration shows the fate of *Colpoda* E6 after interacting with LpJR32. Whether or not this ciliate survives depends on avoiding Lp infection by forming cysts and the low sensitivity of trophozoites to Lp. Bottom illustration shows the fate of *Tetrahymena* IB after interacting with LpJR32. Whether or not this ciliate survives depends on enwrapping Lp in pellets and excreting it outside of its body, as reported previously [22, 23].

Fig. 1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Ciliate sp. WFg2 AJ810076
Glaucomides bromelicola AJ810077
Bromeliophrya sp. MD-2012 JQ723961
Bromeliophrya quadristicha JX561218
Bromeliophrya brasiliensis AJ810075
Glaucoma scintillans AJ511861
Glaucoma sp. Gnsp1 KY218625
Glaucoma chattoni X56533 S57722
Glaucoma sp. Gnsp4 KY218618
Glaucoma sp. Gnsp8 KY218619
CS11A - 18S rRNA gene
Anteglaucoma harbinensis KY204069
Hymenostomatia sp. bOmb1 LN870040
Oligohymenophorea sp. bLPN1 LN870015
Tetrahymena bergeri AF364039
Tetrahymena mimbres EF070251
Tetrahymena caudata EF070244
Tetrahymena tropicalis EF070262
Lambornella sp. AF364043
Tetrahymena australis KT334373
Tetrahymena shanghaiensis EF070256
Tetrahymena cosmopolitanis EF070245
Tetrahymena hyperangularis X56173
Tetrahymena pigmentosa M26358
Paramecium schewiakoffi AJ548821
Paramecium schewiakoffi LT628501
Paramecium cf. multimicronucleatum AC2 LT628494
Paramecium duboscqui AM236094
Paramecium bursaria AB219526
Cyrtolophosis mucicola EU039899
Bardeliella pulchra EU039884
Bresslauides discoideus HM140394
Colpoda aspera KF111344
Kalometopia duplicata KJ873050
Maryna sp. MD-2011 JF747218
Maryna ovata HQ337902
E6 - 18S rRNA gene
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Colpoda sp. PRA-118 AY905498
Colpoda sp. MH715344
uncultured Acanthamoeba EF023782

Fig. 2

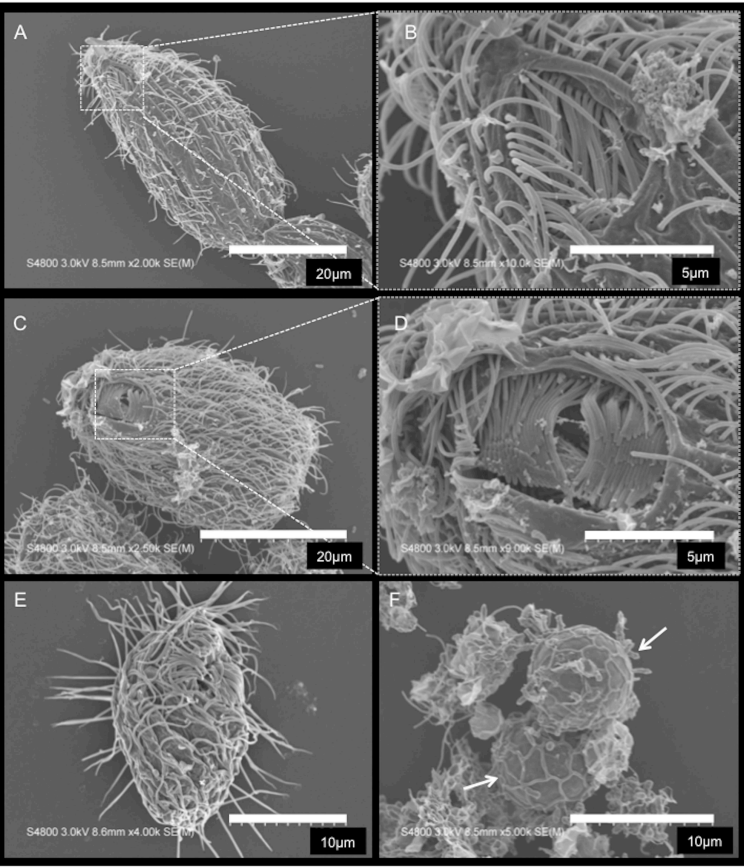

A
S4800 3.0kV 8.5mm x2.00k SE(M)
20μm
B
S4800 3.0kV 8.5mm x10.0k SE(M)
5μm
C
S4800 3.0kV 8.5mm x2.00k SE(M)
20μm
D
S4800 3.0kV 8.5mm x9.00k SE(M)
5μm
E
S4800 3.0kV 8.6mm x4.00k SE(M)
10μm
F
S4800 3.0kV 8.5mm x5.00k SE(M)
10μm

Fig. 3

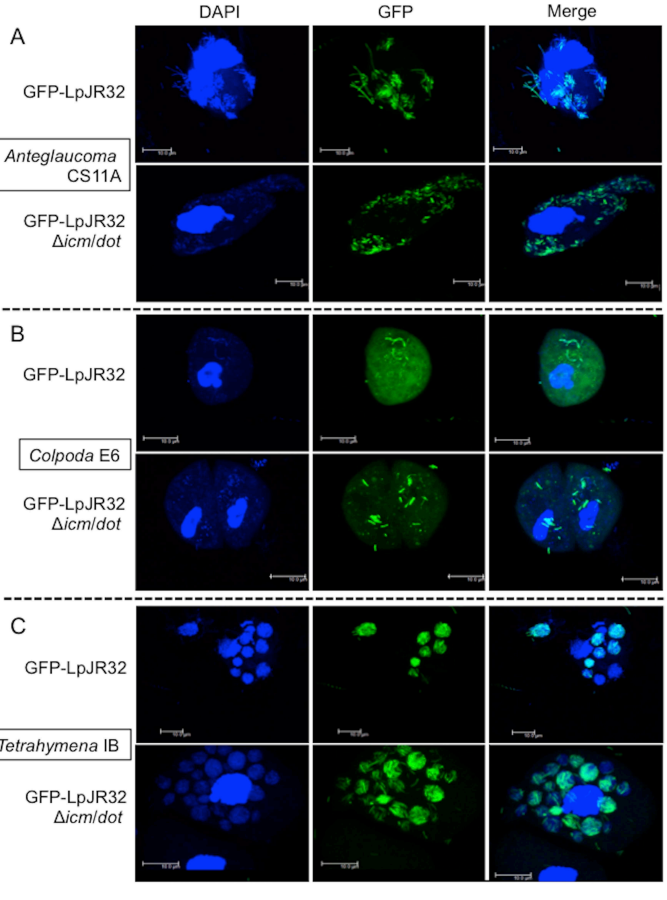

DAPI
GFP
Merge
A
GFP-LpJR32
Anteglaucoma CS11A
GFP-LpJR32 Δicm/dot
B
GFP-LpJR32
Colpoda E6
GFP-LpJR32 Δicm/dot
C
GFP-LpJR32
Tetrahymena IB
GFP-LpJR32 Δicm/dot

Fig. 4

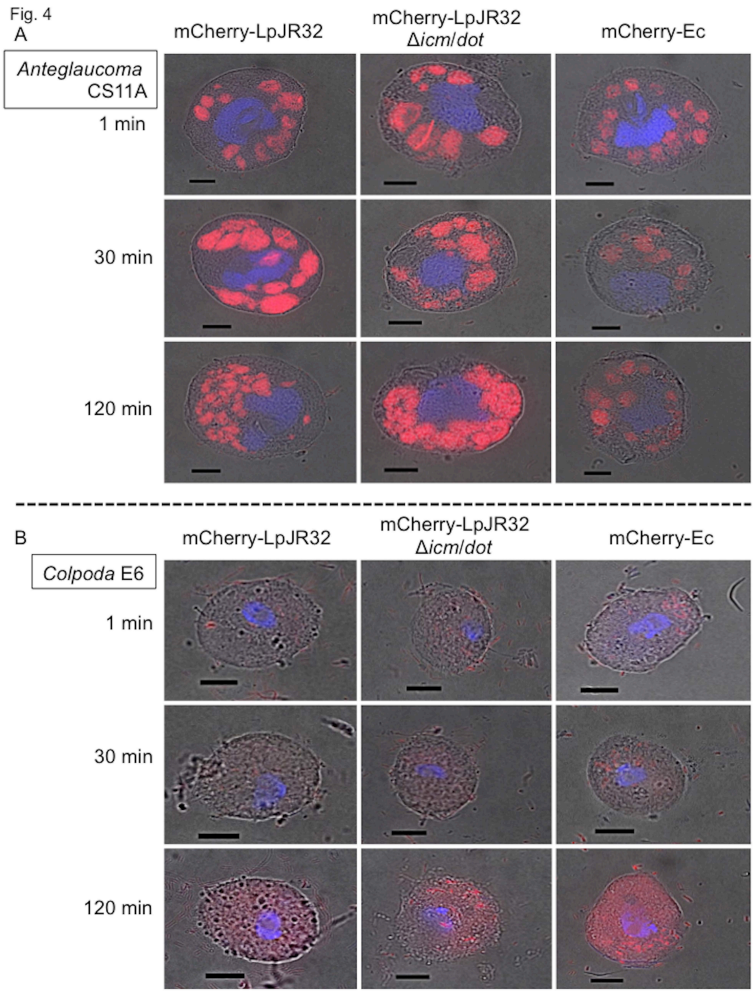

A
mCherry-LpJR32
mCherry-LpJR32 Δicm/dot
mCherry-Ec
Anteglaucoma CS11A
1 min
30 min
120 min
B
mCherry-LpJR32
mCherry-LpJR32 Δicm/dot
mCherry-Ec
Colpoda E6
1 min
30 min
120 min

Fig. 5

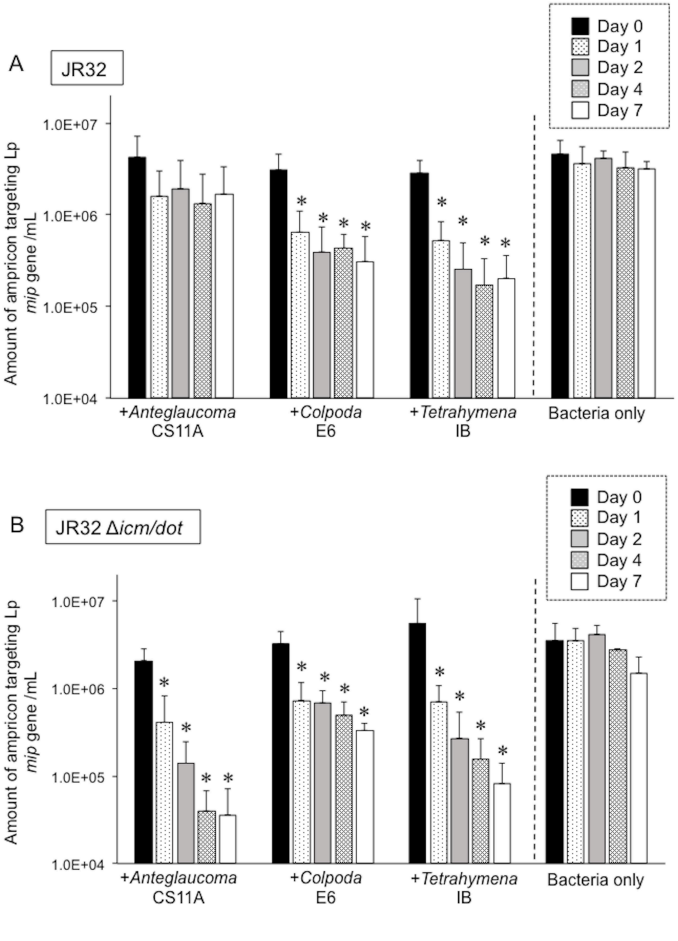
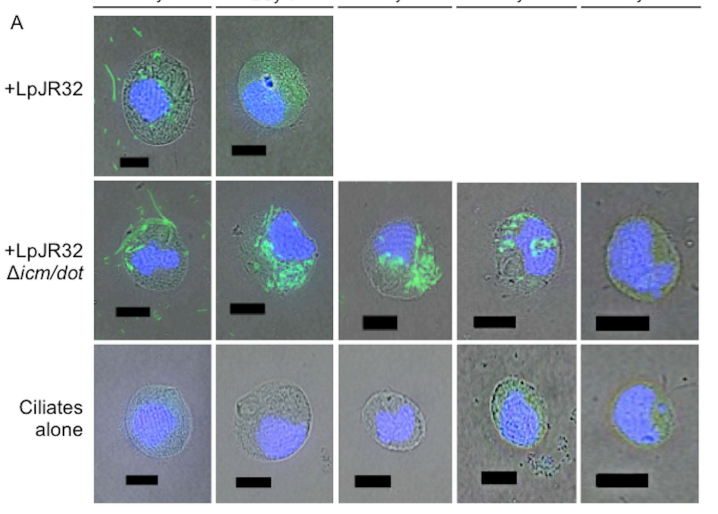

A
JR32
Day 0
Day 1
Day 2
Day 4
Day 7
Amount of ampricon targeting Lp mip gene /mL
1.0E+07
1.0E+06
1.0E+05
1.0E+04
+Anteglaucoma CS11A
+Colpoda E6
+Tetrahymena IB
Bacteria only
B
JR32 Δicm/dot
Day 0
Day 1
Day 2
Day 4
Day 7
Amount of ampricon targeting Lp mip gene /mL
1.0E+07
1.0E+06
1.0E+05
1.0E+04
+Anteglaucoma CS11A
+Colpoda E6
+Tetrahymena IB
Bacteria only

Fig. 6


Day 0
Day 1
Day 2
Day 4
Day 7
A
+LpJR32
+LpJR32
Δicm/dot
Ciliates
alone

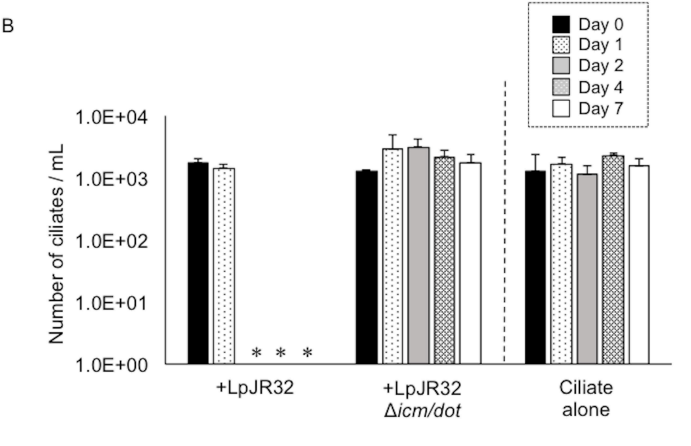
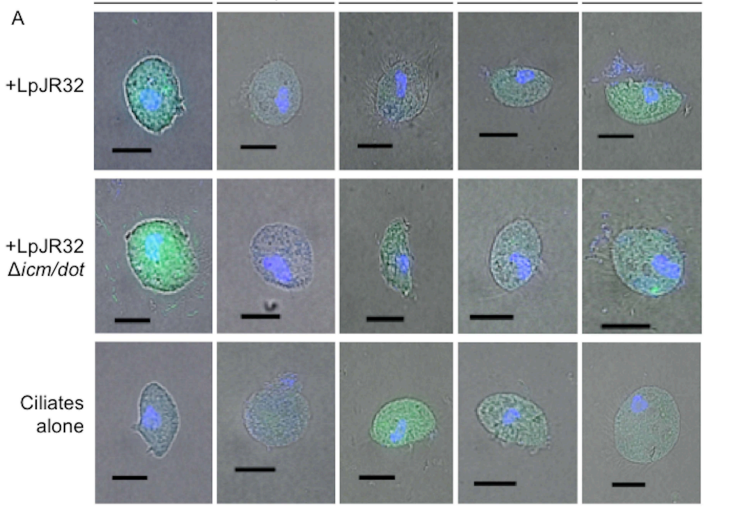

B
Day 0
Day 1
Day 2
Day 4
Day 7
Number of ciliates / mL
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
* * *
+LpJR32
+LpJR32
Δicm/dot
Ciliate
alone

Fig. 7


Day 0
Day 1
Day 2
Day 4
Day 7
A
+LpJR32
+LpJR32
Δicm/dot
Ciliates
alone

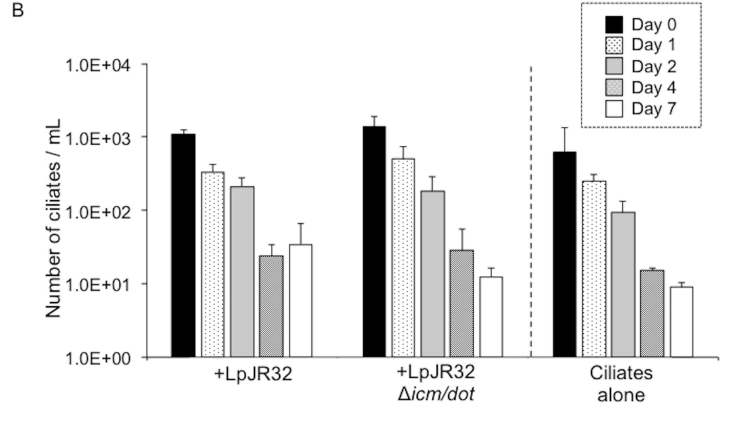

B
Day 0
Day 1
Day 2
Day 4
Day 7
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
Number of ciliates / mL
+LpJR32
+LpJR32
Δicm/dot
Ciliates
alone

Fig. 8

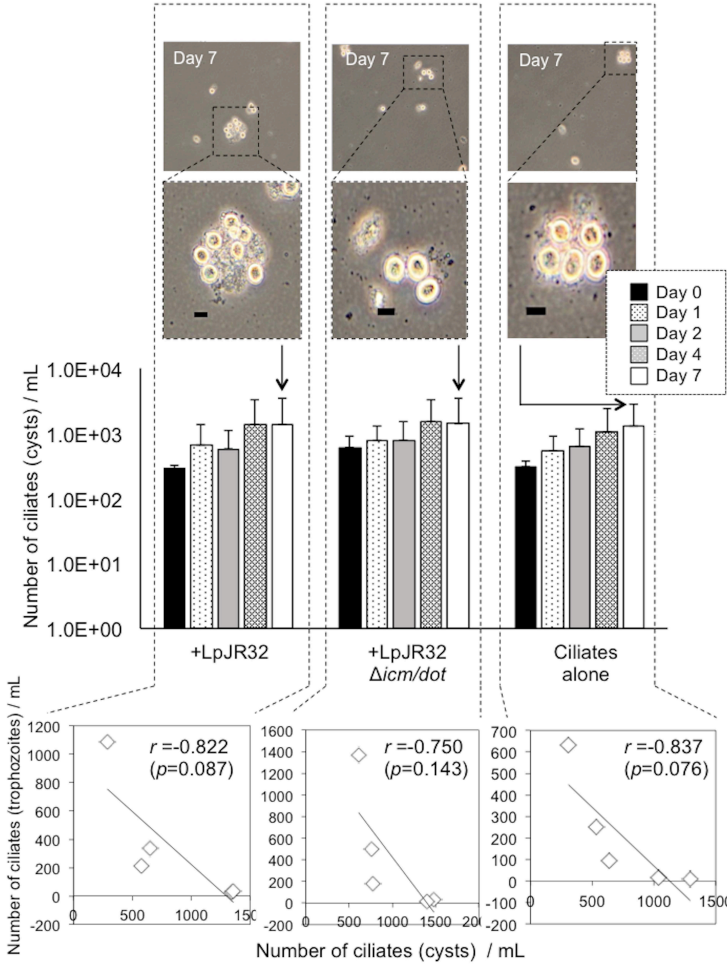
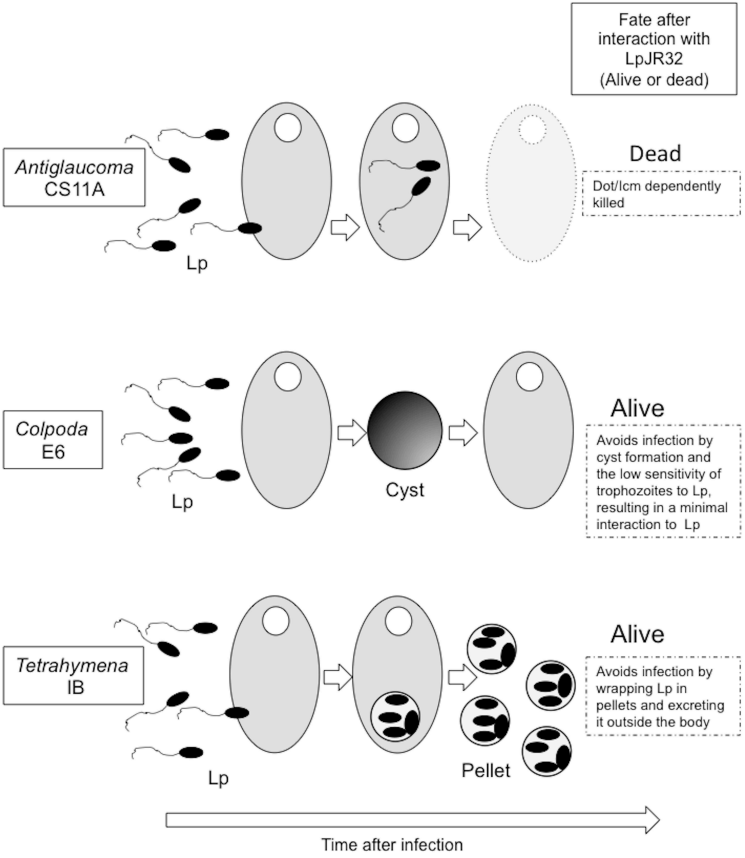
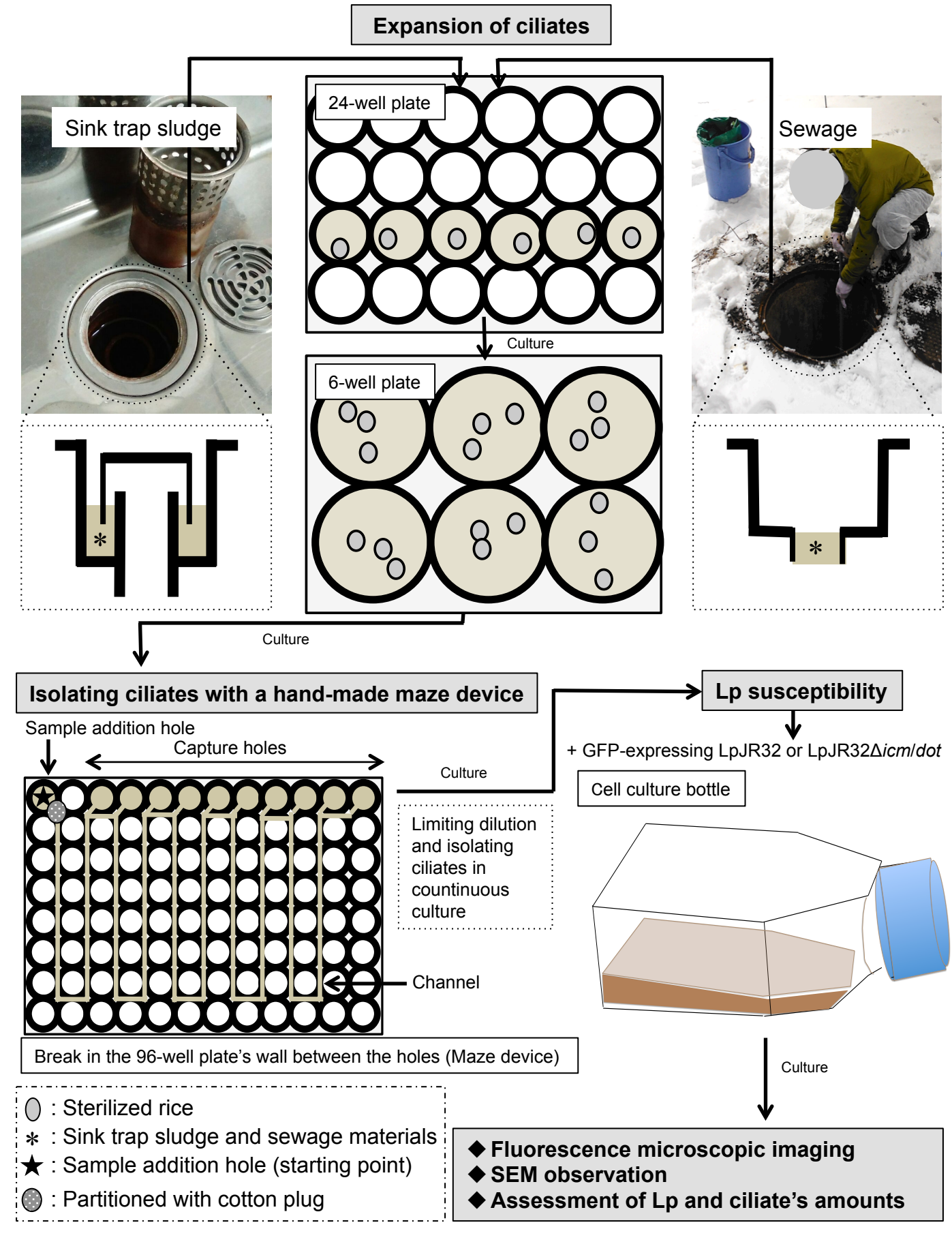

Day 7
Day 7
Day 7
Day 0
Day 1
Day 2
Day 4
Day 7
Number of ciliates (cysts) / mL
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
+LpJR32
+LpJR32
Δicm/dot
Ciliates
alone
Number of ciliates (trophozoites) / mL
r =-0.822
(p=0.087)
r =-0.750
(p=0.143)
r =-0.837
(p=0.076)
Number of ciliates (cysts) / mL

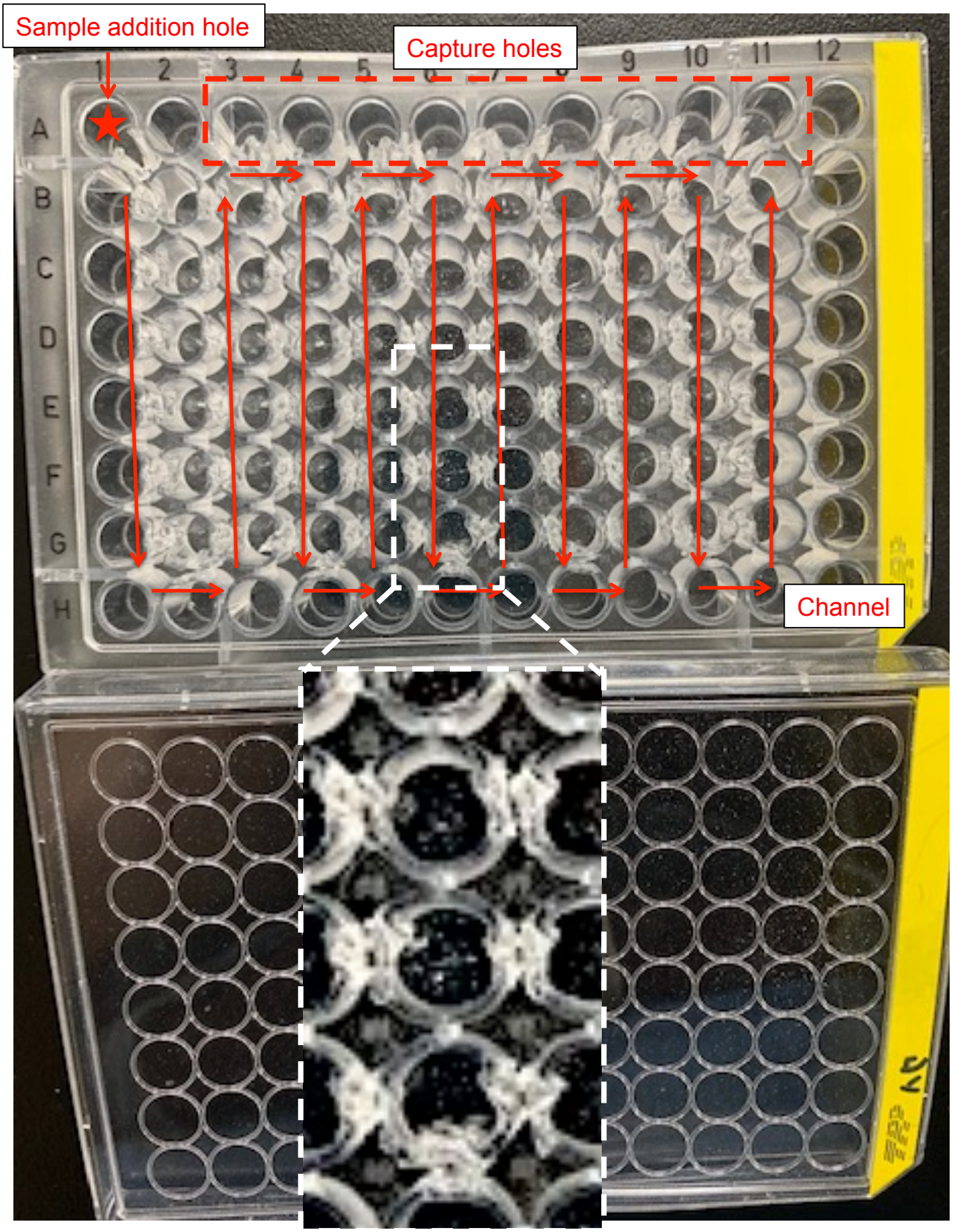
Fig. 9


Fate after interaction with LpJR32 (Alive or dead)
Antiglaucoma CS11A
Lp
Dead
Dot/Icm dependently killed
Colpoda E6
Lp
Cyst
Alive
Avoids infection by cyst formation and the low sensitivity of trophozoites to Lp, resulting in a minimal interaction to Lp
Tetrahymena IB
Lp
Pellet
Alive
Avoids infection by wrapping Lp in pellets and excreting it outside the body
Time after infection

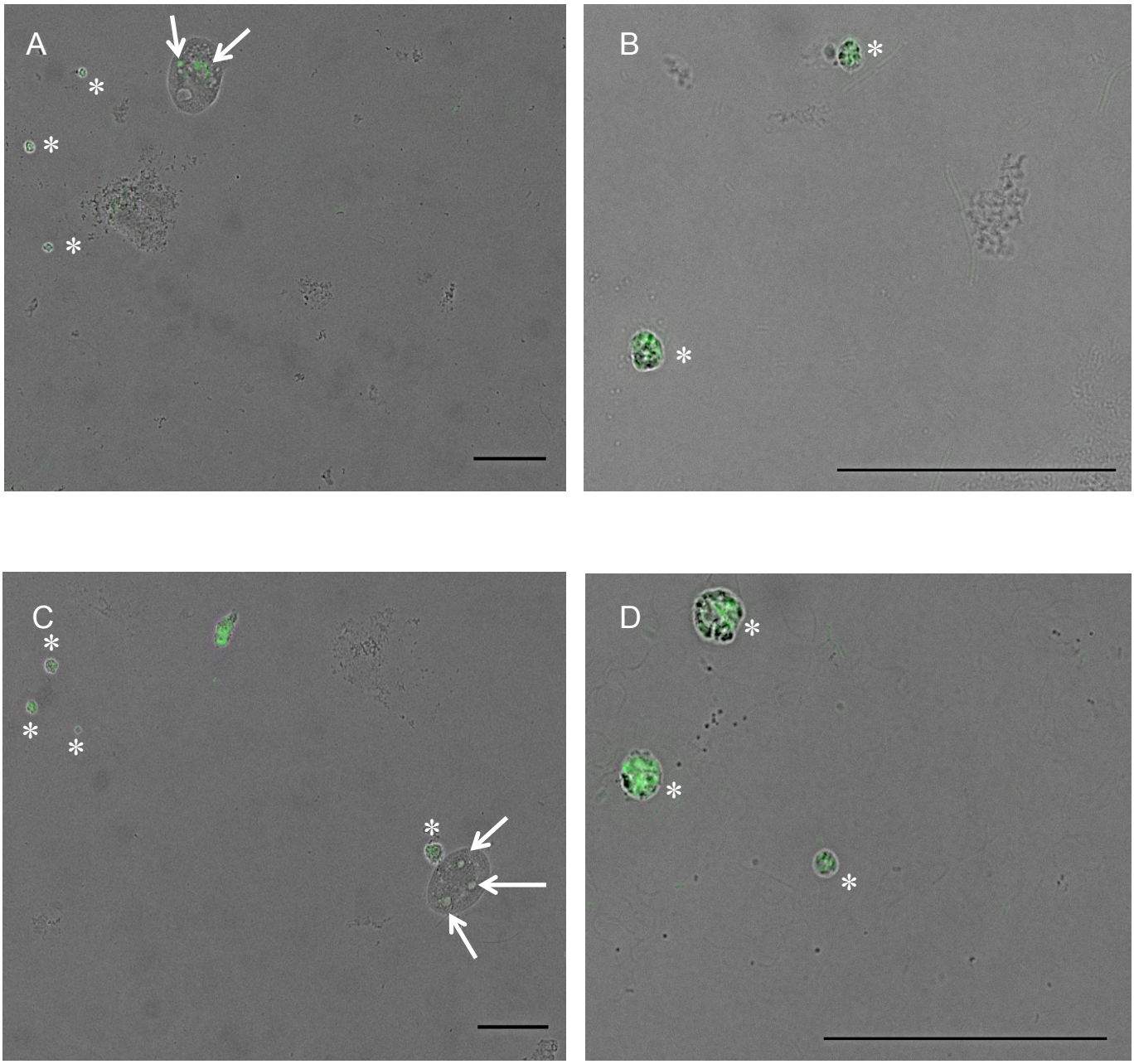
Supporting information Fig. S1



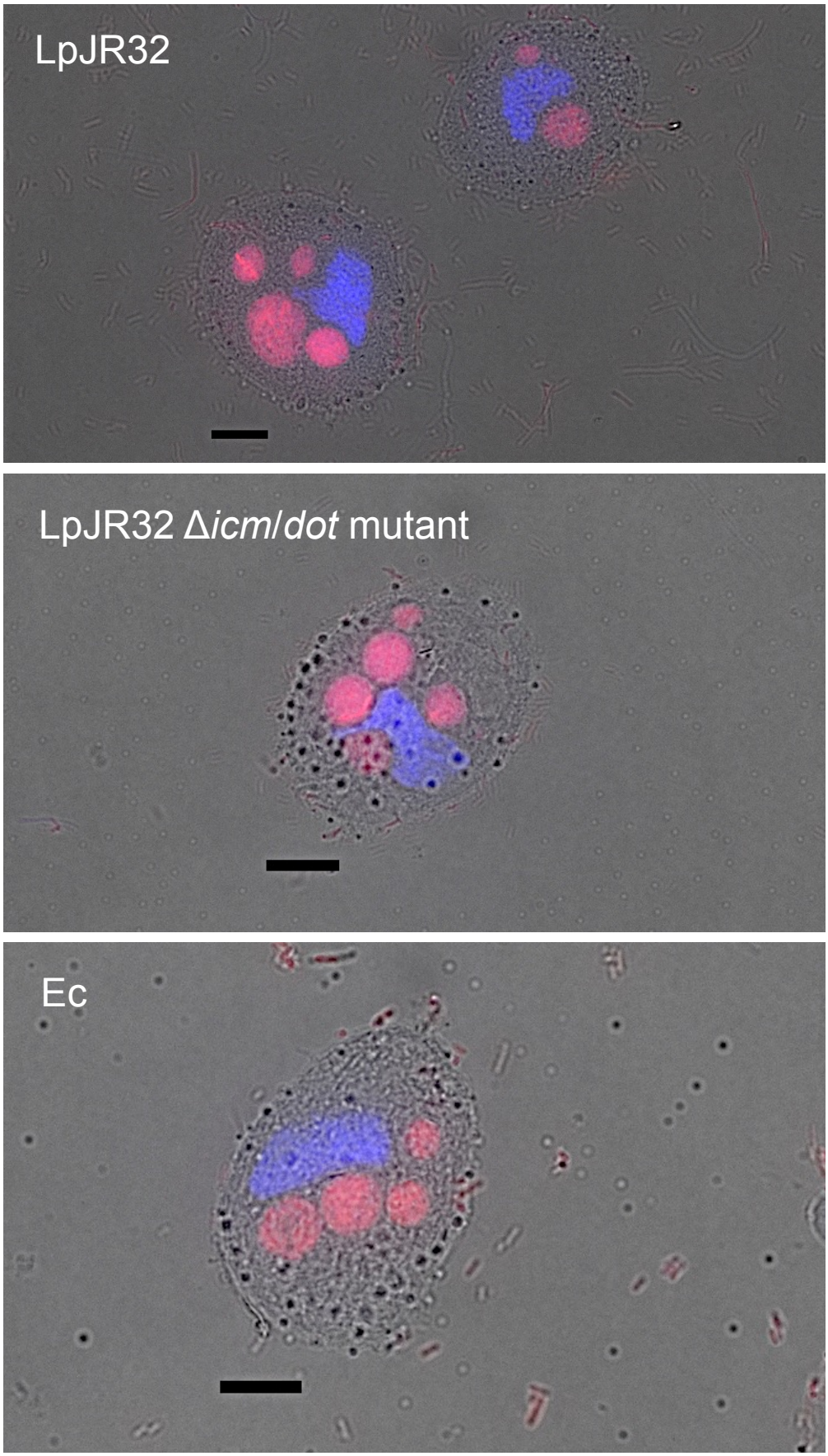
Supporting information Fig. S2



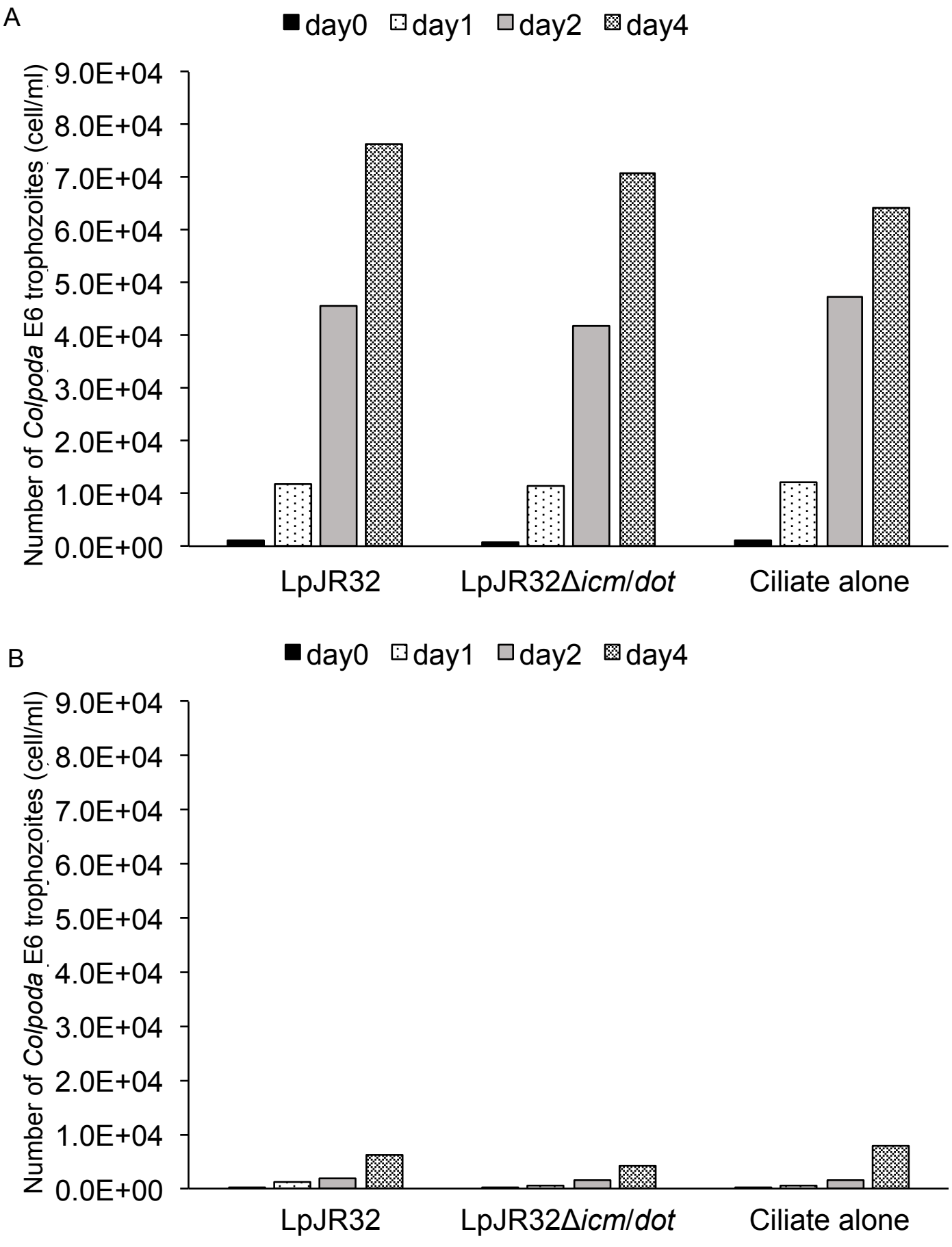
Supplementary Fig. S3



Supplementary Fig. S4



Supplementary Fig. S5



Supporting information Fig. S6