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1 **Wild ciliates differ in susceptibility to Legionella pneumophila JR32**

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1 **ABSTRACT**

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

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

2

3 Keywords

4 *Legionella pneumophila* JR32, *Anteglaucoma* CS11A, *Colpoda* E6, infection,

5 susceptibility, co-cultures

6

7

1 INTRODUCTION

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

15

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

1 diversity with low concentration of ciliates make them difficult to isolate [24, 25].
2
3 Ciliates are diverse and over 8,000 species have been reported to date [26-28]. As
4 mentioned above, interactions between Lp and *Paramecium* or Lp and *Tetrahymena* have
5 been investigated, and both ciliates have been found to support the intracellular growth of
6 Lp, albeit differently [19-23]. Some *Paramecium* strains can maintain the survival of Lp
7 symbiotically, depending on the *lefA* gene (*Legionella* endosymbiosis-modulating factor
8 A), a key factor contributing to the life stage change in Lp from endosymbiosis to host
9 lysis, which enables its escape into the outside environment [19]. It has been observed
10 that Lp inhibits phagosome formation by *Paramecium* through a component of the type I
11 secretion system (a TolC-dependent mechanism) [20]. It is also reported that
12 *Tetrahymena* can support the survival of Lp [21, 22]. One way in which *Tetrahymena*
13 does this is by expelling environmental Lp-laden pellets, a finding indicating that it may
14 be a defense mechanism against Lp rather than a symbiotic role [22, 23]. However,
15 studies on how Lp interacts with other ciliates are limited in number.
16
17 Here, we assessed the interaction of Lp with two wild ciliates, *Anteglaucoma* CS11A and
18 *Colpoda* E6, which we isolated from sewage and sink trap sludge, respectively, using a
19 handmade maze device fitted with a 96-well crafting plate. We show that unlike
20 *Tetrahymena* IB with pellet production, *Anteglaucoma* CS11A can be effectively killed
21 by Lp infection, whereas *Colpoda* E6 is protected against Lp infection through cyst
22 formation and the low sensitivity of trophozoites to Lp. Thus, these two ciliates are

1 differentially susceptible to Lp infection.

2

3

4 **METHODS**

5 **Ciliates**

6 *Anteglaucoma* CS11A (isolated from sewage) and *Colpoda* E6 (isolated from sink strap
7 sludge) were used in this study (See below for “Isolation methods for ciliates”). These
8 ciliates were maintained in Sonneborn's *Paramecium* medium (SPM, also known as
9 ATCC medium 802) consisting of cerophyl grass powder (Toyotama Healthy Foods Co.,
10 Ltd. Japan) in distilled water with live *Enterobacter aerogenes* (our laboratory stock) at
11 22°C [29]. *Tetrahymena* IB was also maintained in peptone-yeast extract glucose (PYG)
12 broth, which contains peptone (BD, Franklin Lakes, NJ), yeast extract (BD), and glucose
13 (FUJIFILM Wako Pure Chemical Co., Tokyo, Japan) at 15°C, as described previously
14 [30].

15

16 **Bacteria**

17 The Lp originally derived from the Philadelphia-1 progenitor strain [JR32 (wild-type
18 strain) and its mutant ($\Delta icm/dot$)] were used in this study [31]. Both strains were
19 genetically modified to carry a GFP-expressing plasmid (pAM239GFP) for easy
20 visualization. The plasmid, which continuously expressed GFP was introduced to
21 LpJR32 and its mutant by electroporation. Also, mCherry-expressing-*Escherichia coli*
22 DH5 α (Ec) was constructed by the introduction with a plasmid (pBBR122mCherry)

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

6

7 **Isolation methods for ciliates**

8 Ciliates were isolated according to the procedure with the maze device described in Fig.
9 S1. To make this device inexpensive and easy to use, we used commercially available
10 96-well culture plates in its operation. We simply broke the walls between the plate wells
11 with a heated spatula (Fig. S2, a photo of the device that we used). Briefly, sink trap
12 sludge samples ($n=5$) and manhole sewage samples ($n=13$) were collected from different
13 places in our university, because Lp frequently colonizes in sinks or sewages [32-36]. The
14 sample solutions, which were diluted in Page's amoeba saline (PAS) [37], were cultured
15 with sterilized rice grains at room temperature for 2 weeks. When a large number of
16 ciliates had been reared, a concentrated sample containing >1,000 ciliate cells was placed
17 into the "sample addition hole" of the maze device, which was filled with PAS except for
18 the "sample addition hole", which was partitioned with a cotton plug. After removing the
19 cotton plug-containing partition, the ciliates were cultured for several days at room
20 temperature. Ciliates captured in the "capture hole" were carefully collected, cultured in
21 SPM medium (See above), and isolated by the limiting dilution method. The maze device
22 could be reused approximately three times by performing intermittent sterilization (95°C,

1 10 min, twice).

2

3 **Direct sequencing and phylogenetic analysis**

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

18

19 **Scanning electron microscopy (SEM)**

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

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

4

5 **Co-culture experiment with imaging**

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

17

18 **Pellet production assay**

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

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

5

6 **Determining Lp amounts using quantitative (q)PCR**

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

18

19 **Determining ciliate amounts using a cell counting chamber**

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

1

2 **Statistical analysis**

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

8

9 **Nucleotide sequence accession numbers**

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

14

15

16 **RESULTS**

17 **Characterization of the ciliates newly isolated from sewage and sink** 18 **trap sludge by phylogenetic analysis and SEM observations**

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

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

17 **Interactions of the newly isolated ciliates with Lp immediately (1 min,** 18 **30 min, 2 h) or after 24 h in co-culture**

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

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

13

14 **Changes in Lp amounts in the co-culture system**

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

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

5

6 **Changes in ciliate amounts during co-culturing**

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

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

11

12

13 **DISCUSSION**

14 As mentioned above, Lp is ubiquitous in a wide range of natural environments such as
15 soil or pond water [1-3]. Lp interacts with various protozoa including amoebae and
16 ciliates, many of which are unknown species [26-28]. More importantly, Lp propagating
17 through such interactions can create the ideal conditions for legionellosis in humans
18 [8-10]. However, few studies have been published on the interactions of Lp with ciliates,
19 and those that have been published are limited to *Paramecium* and *Tetrahymena* [16-23].
20 Therefore, we investigated the interaction of Lp with two wild ciliates, *Anteglaucoma*
21 CS11A and *Colpoda* E6, which we isolated from sewage and sink trap sludge,
22 respectively, using a handmade maze device with a 96-well crafting plate. Our data

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

4

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

14

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

1 *Colpoda*, and these support it as a ubiquitous and prosperous genus among known ciliates
2 [45-47]. With its ubiquitous nature, *Colpoda* has been used as an indicator of
3 environmental pollution (e.g., heavy metal pollution) [48, 49]. Because there are
4 currently no studies on the interactions of these ciliates with Lp, this study is the first to
5 discover their interactions.

6
7 We found that *Anteglaucoma* CS11A can be killed by LpJR32 depending on whether or
8 not the *icm/dot* virulence genes are present. Although it is well known that *Tetrahymena*
9 ensures bacterial survival by expelling any bacteria (e.g., *Salmonella*, *Mycobacterium*,
10 and Lp) packaged in its fecal pellets as a way for ciliates to discard preys [23, 50, 51], no
11 pellet production but the accumulation of Lp at earlier interaction was seen in the
12 *Anteglaucoma* CS11A-co-cultures, suggesting that the defense mechanism of the ciliates
13 against Lp may be lacking. Also, the exact reason why the Lp infections caused ciliate
14 death in our experiments is not known, but it is possible that some Lp effectors (e.g., those
15 which shut down endoplasmic reticulum–mitochondria interactions), may be involved in
16 this type of cell death [52]. Meanwhile, it was not clear that Lp can replicate inside the
17 ciliates. Thus, *Anteglaucoma* CS11A could be used as a biological indicator to verify the
18 pollution status of Lp in water supplies or hot springs. We also found that *Colpoda* E6 can
19 be maintained in the presence of Lp independently of the *icm/dot* virulence genes through
20 cyst formation. Stimulation of cyst formation is not a sign of Lp infection, because cyst
21 formation is observed even when ciliates are cultured alone. Furthermore, because we
22 observed that despite the presence of Lp, the ciliates mostly consisting of trophozoites

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

6
7 In conclusion, we successfully isolated two ciliates (*Anteglaucoma* CS11A and *Colpoda*
8 E6) from sewage and sink trap sludge, respectively, using a handmade maze device. Each
9 one differs in its susceptibility to Lp infection. Contrasting with *Tetrahymena* IB with
10 pellet production, *Anteglaucoma* CS11A can be killed by Lp infection, whereas *Colpoda*
11 E6 resists Lp infection through cyst formation and the low sensitivity of trophozoites to
12 Lp (Fig. 9). Our new identification of two ciliates with different susceptibilities to Lp
13 infection provides useful public health information towards the control of human
14 pathogenic Lp.

15

16

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20

21

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2 GFP-expressing plasmids. We thank Edanz Group
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4

5

6 **Author contribution**

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

10

11 **Conflicts of interest**

12 The authors declare that there are no conflicts of interest.

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1 **Figure legends**

2

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

8

9 **Fig. 2.** Representative SEM images showing the morphological features of three ciliates.
10 A and B, *Colpoda* E6. C and D, *Anteglaucoma* CS11A. E and F, *Tetrahymena* IB. Arrows,
11 expelled fecal pellets. Images in small dashed boxes (A and C) in the left-hand panels are
12 enlarged in the right-hand panels (B and D). White bars show scales for 5 μ m (B and D),
13 10 μ m (E and F), and 20 μ m (A and C).

14

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

22

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

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

16
17 **Fig. 6.** Changes in the *Anteglaucoma* CS11A cell amounts in the co-culture system of Lp
18 (JR32 and $\Delta icm/dot$ mutant) with *Anteglaucoma* CS11A. The co-culture system was
19 maintained for 7 days. “Day 0” means 2 h after co-culture. A. Representative images
20 showing the interaction of the ciliate with LpJR32 or $\Delta icm/dot$ during the culture period.
21 Because the ciliates could not be seen at 2 days after co-culture, no images were obtained
22 after the second day. Each image shows one of the observed 3-5 fields of view. Black bars

1 show a scale of 10 μ m. B. Changes in *Anteglaucoma* CS11A cell amounts in the
2 co-culture system. The data (average \pm SD) independently obtained from three
3 experiments were compared using the Bonferroni correction. *, $p < 0.05$ vs. “Ciliates
4 alone”.

5

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

13

14 **Fig. 8.** Changes in the cyst amounts in the co-culture system of *Colpoda* E6 with Lp
15 (JR32 and $\Delta icm/dot$ mutant). The upper images show the appearances of the cysts from
16 each of the cultures at 7 days post-co-culture. The middle graphs show the changes in cyst
17 numbers over time. “Day 0” means 2 h after co-culture. Black bars show a scale of 10 μ m.
18 Bottom graphs show the correlations between the ciliate (trophozoites) numbers and cyst
19 numbers in the cultures with or without LpJR32 or the LpJR32 $\Delta icm/dot$ mutant. The data
20 for “Ciliate (trophozoites) numbers” used for correlation in this experiment were the
21 same as that for Fig. 7B. “ r ” shows the correlation values. The data (average \pm SD) were
22 independently obtained from three experiments.

1

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

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Fig. 1

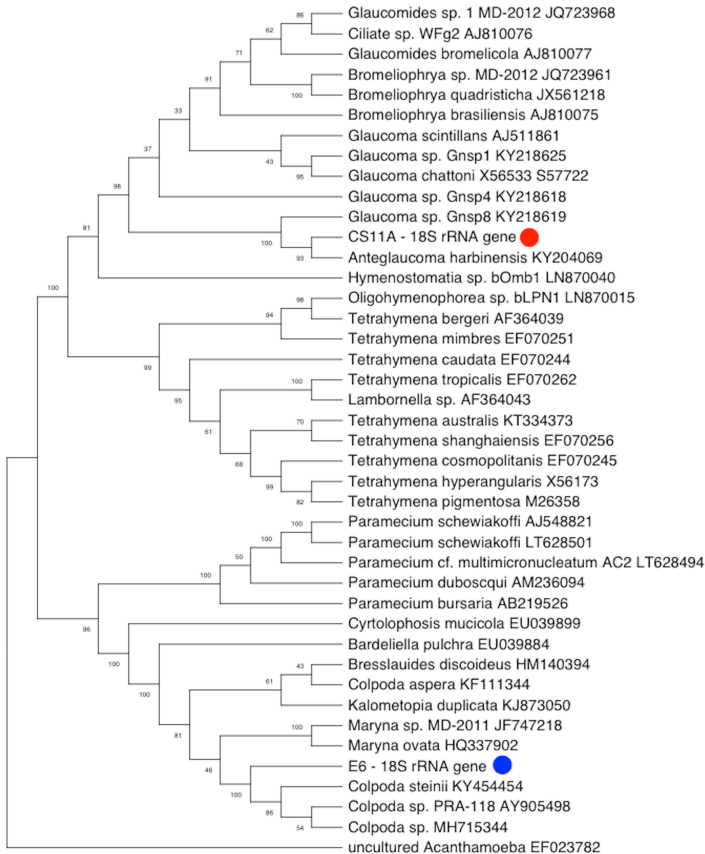


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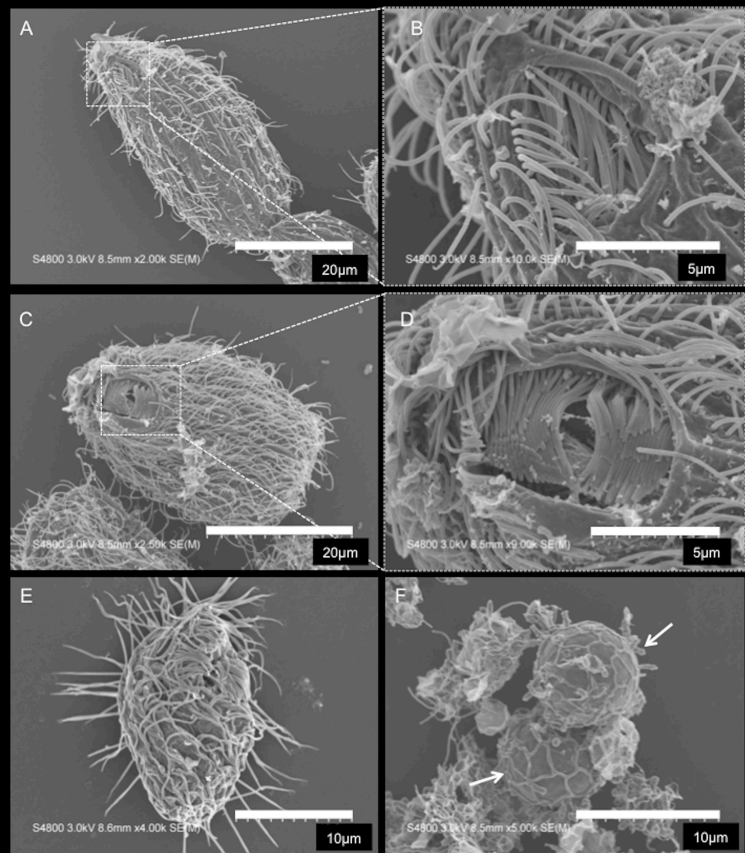


Fig. 3

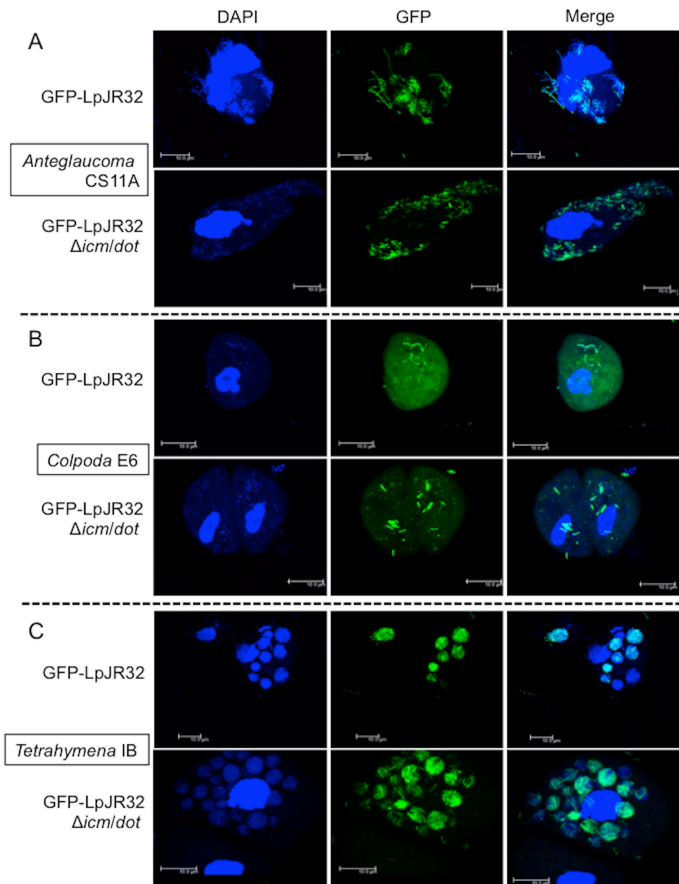


Fig. 4

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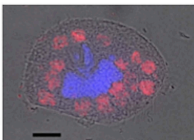
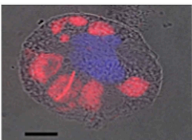
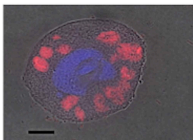
Anteglaucoma
CS11A

1 min

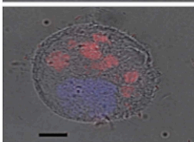
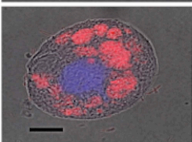
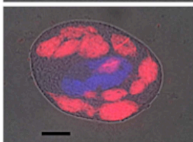
mCherry-LpJR32

mCherry-LpJR32
 $\Delta icm/dot$

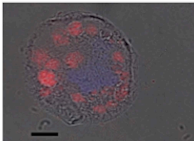
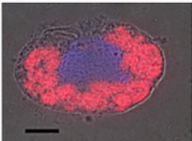
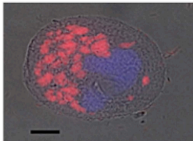
mCherry-Ec



30 min



120 min



B

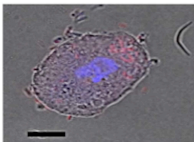
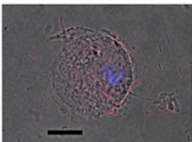
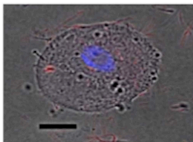
Colpoda E6

1 min

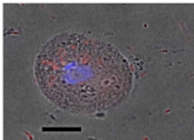
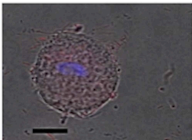
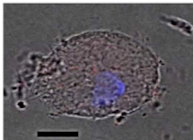
mCherry-LpJR32

mCherry-LpJR32
 $\Delta icm/dot$

mCherry-Ec



30 min



120 min

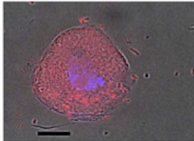
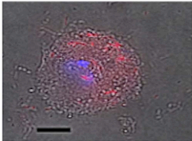
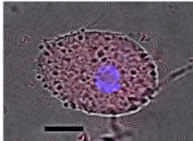


Fig. 5

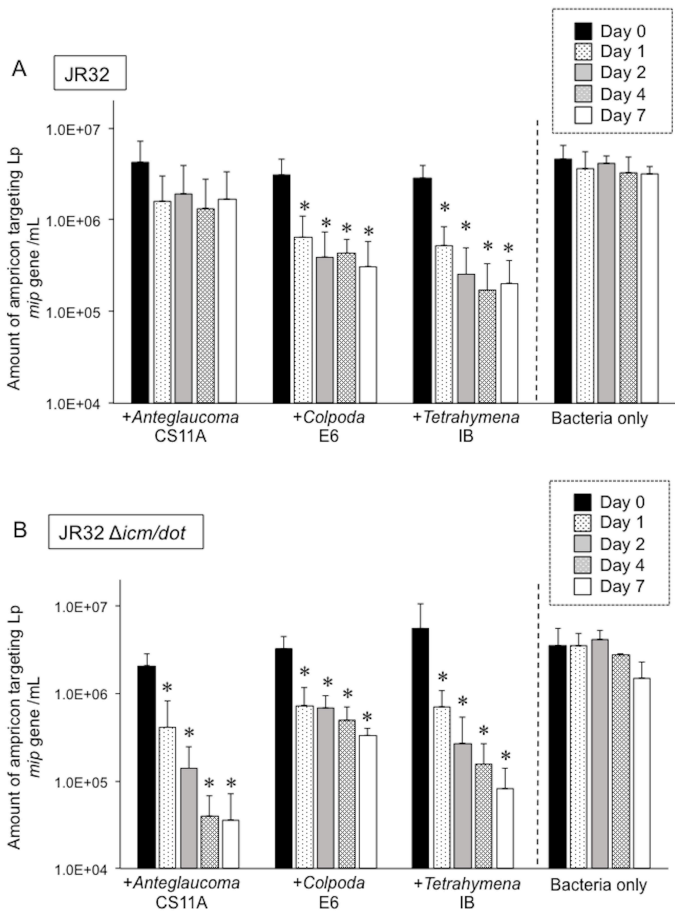


Fig. 6

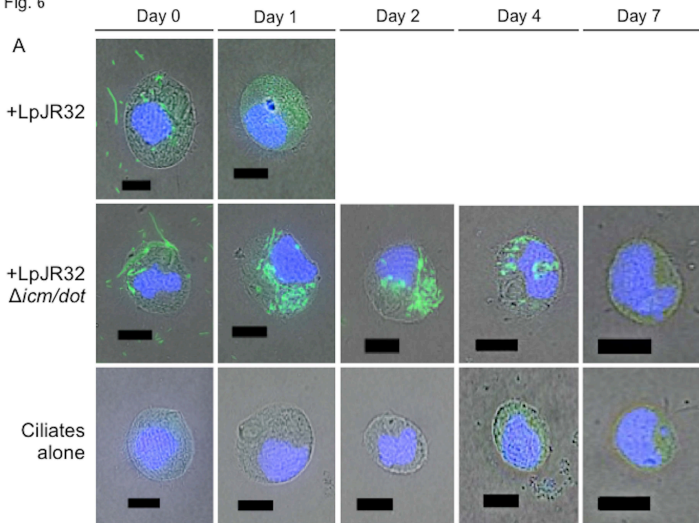
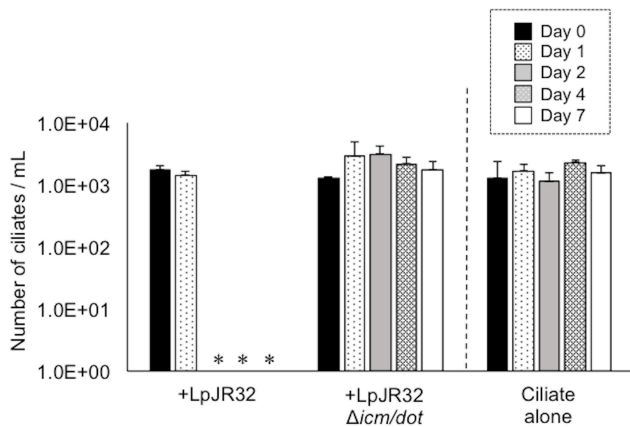
**B**

Fig. 7

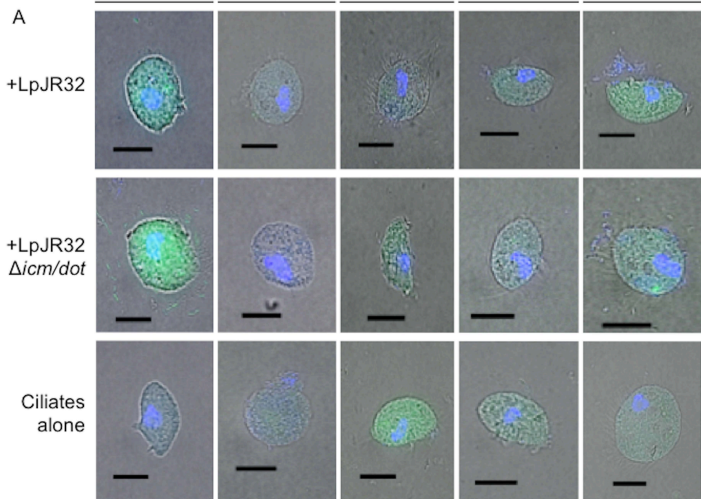
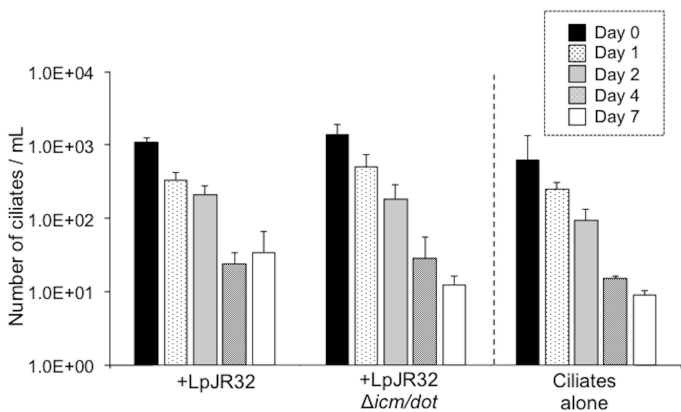
**B**

Fig. 8

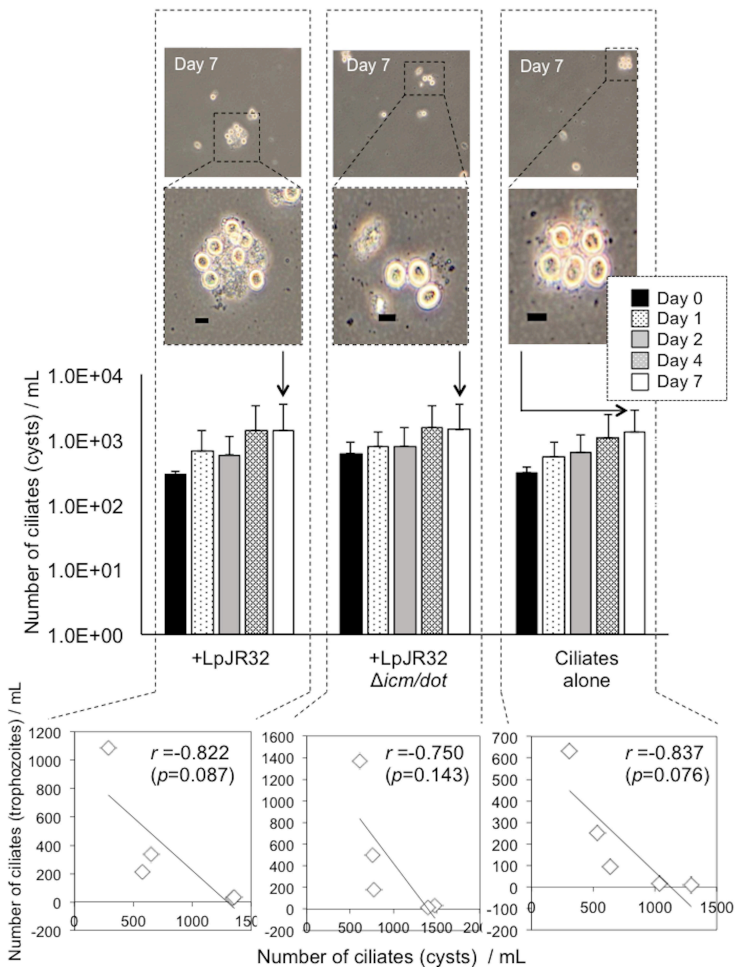
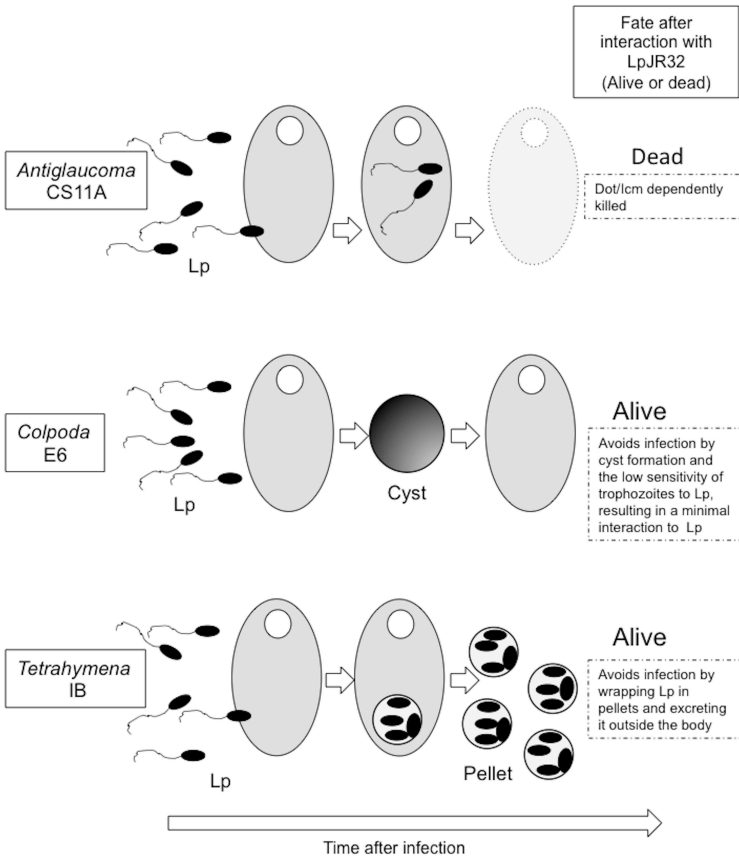
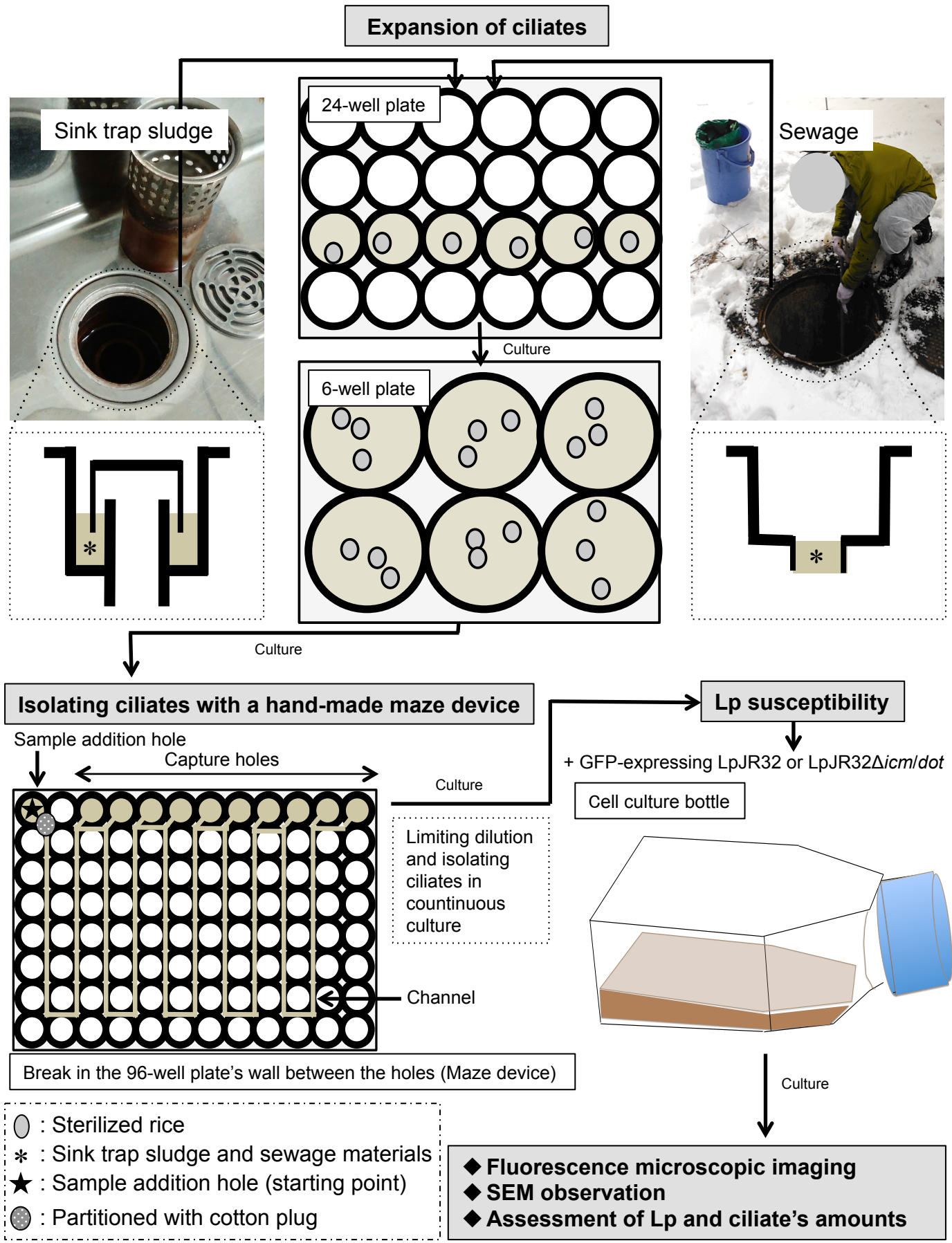
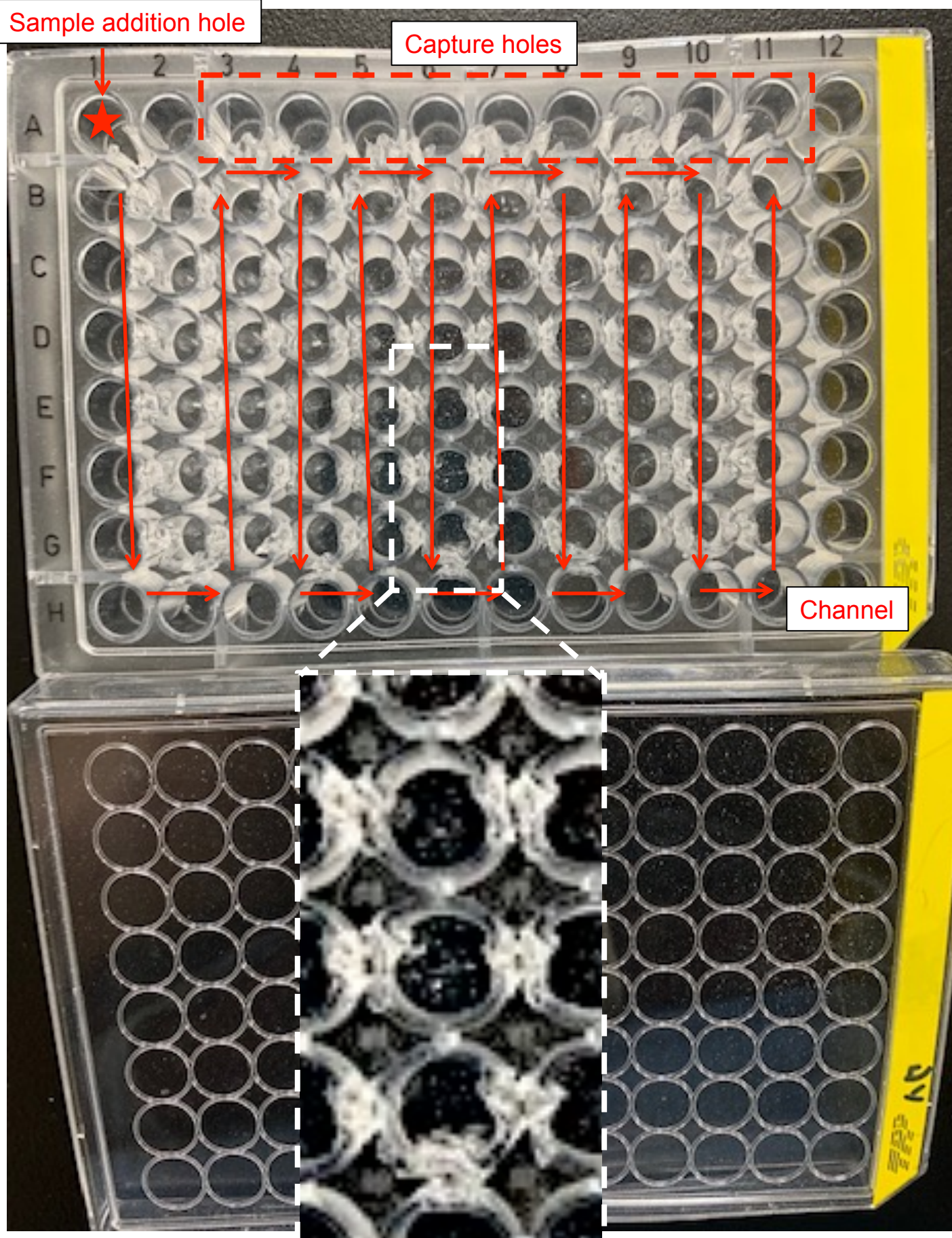


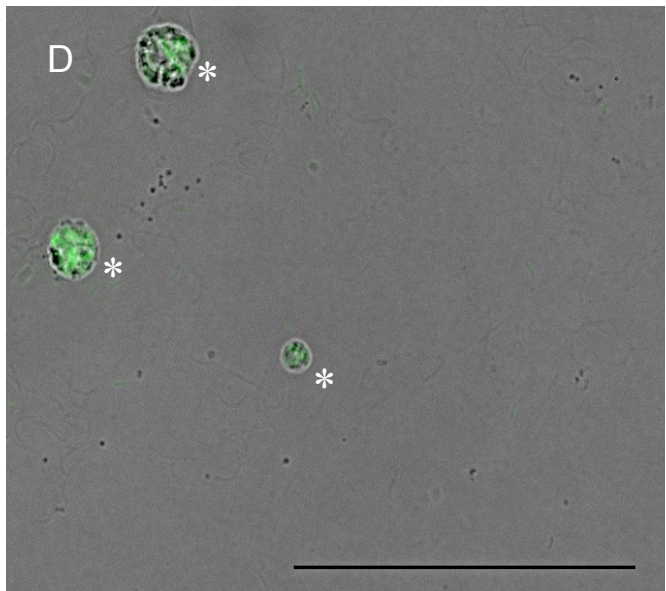
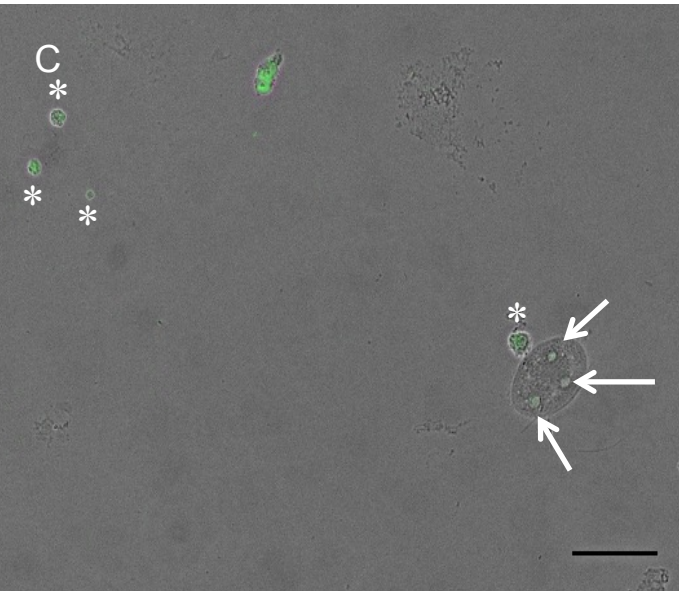
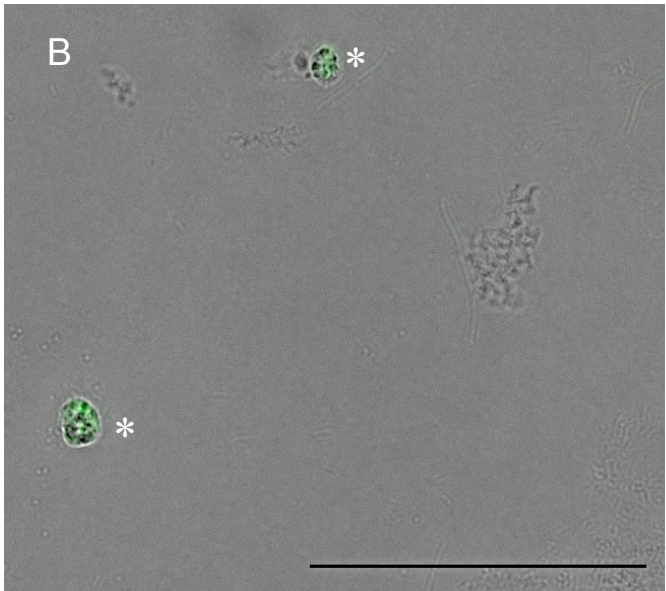
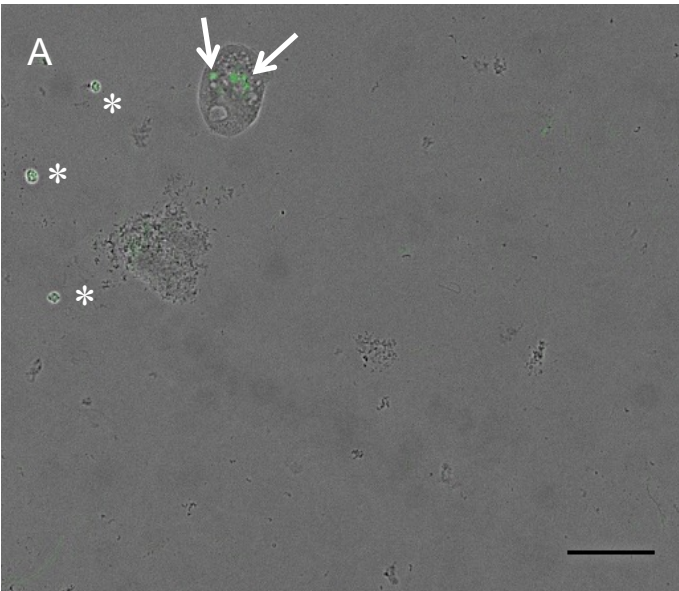
Fig. 9

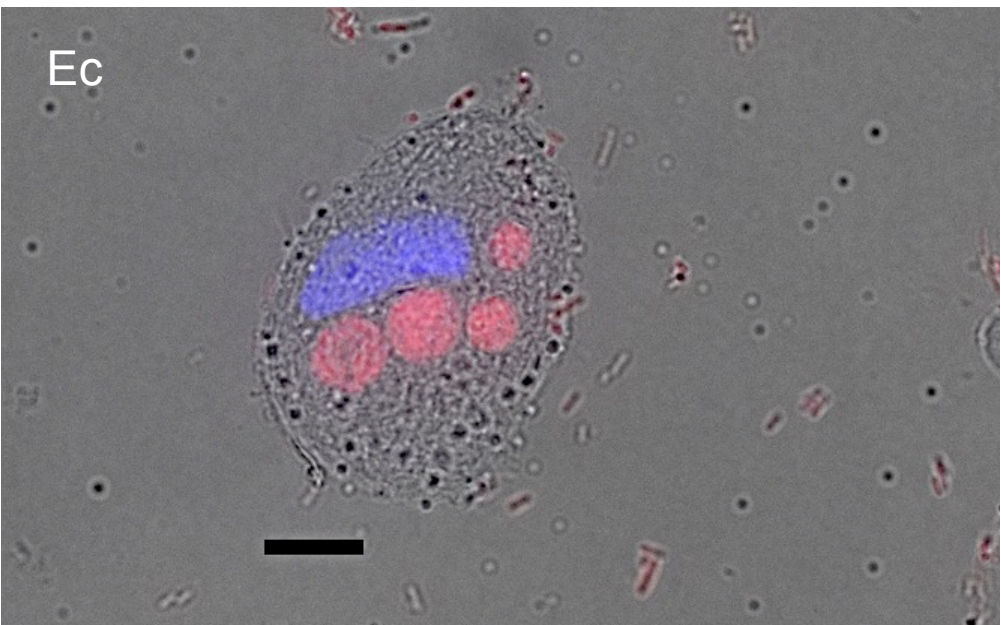
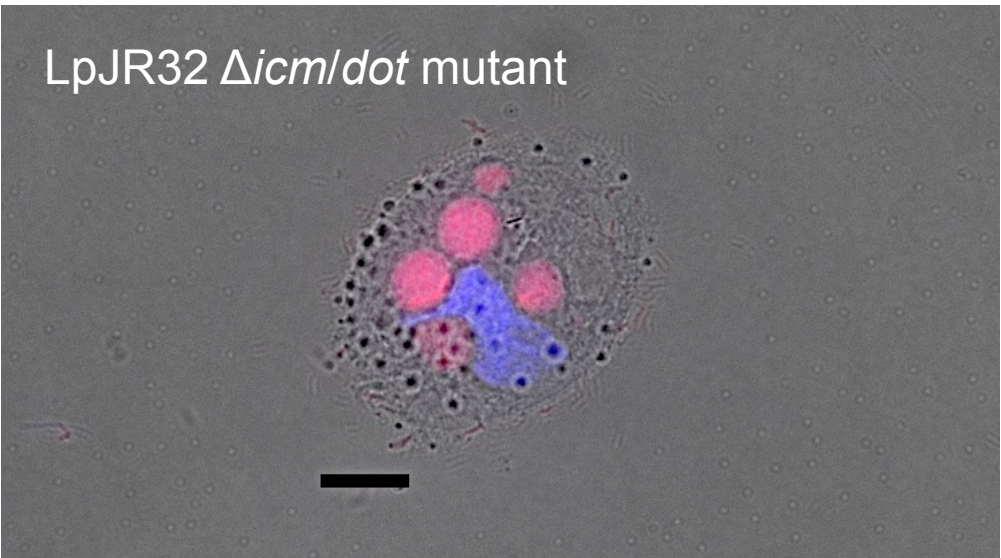
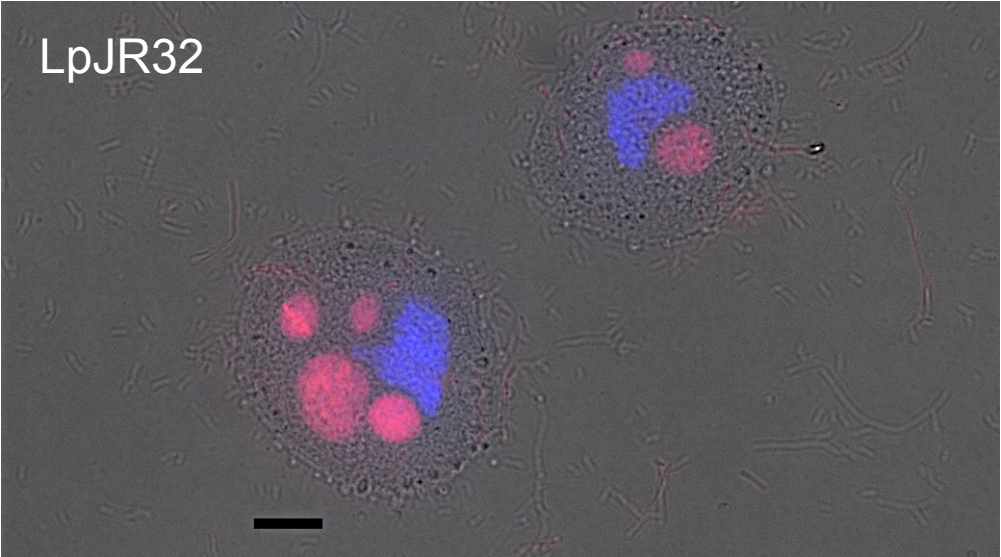






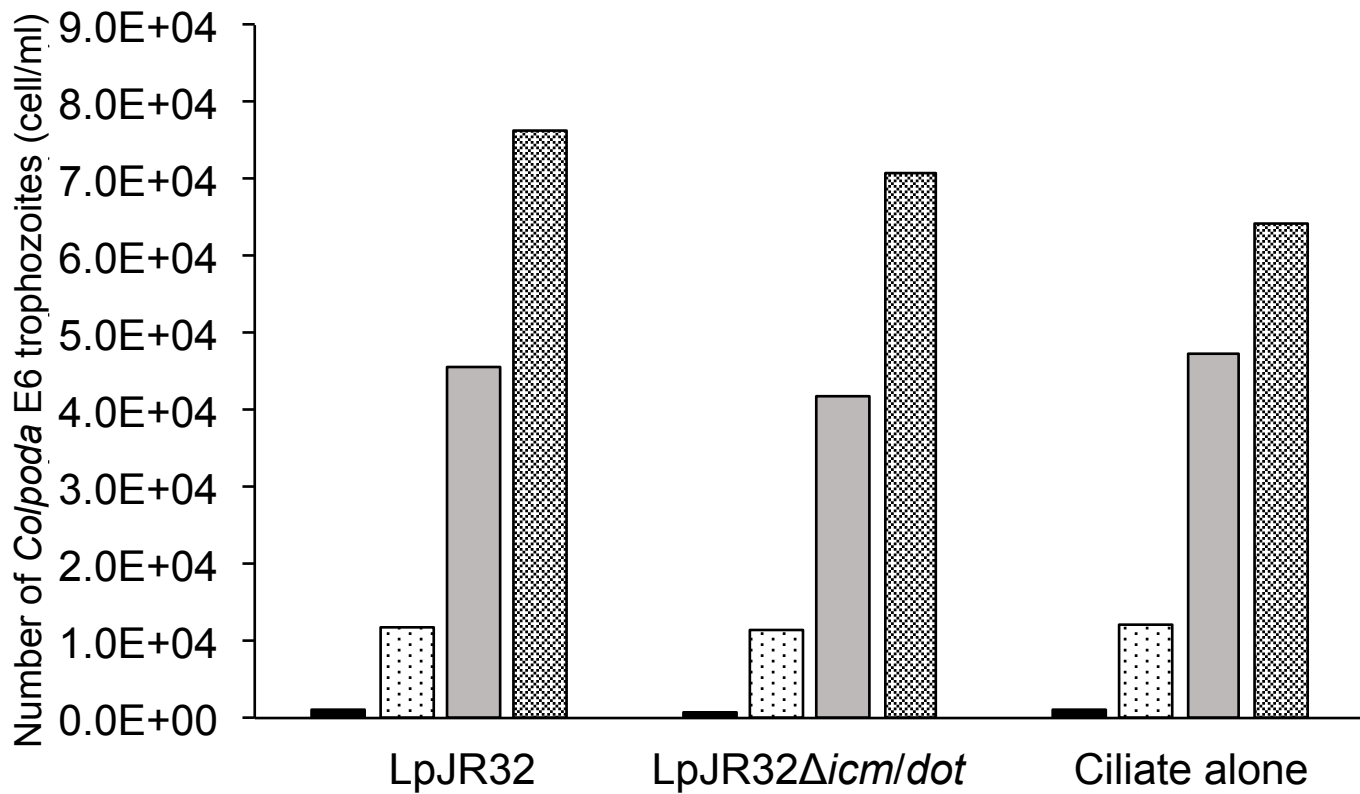
Supplementary Fig. S3





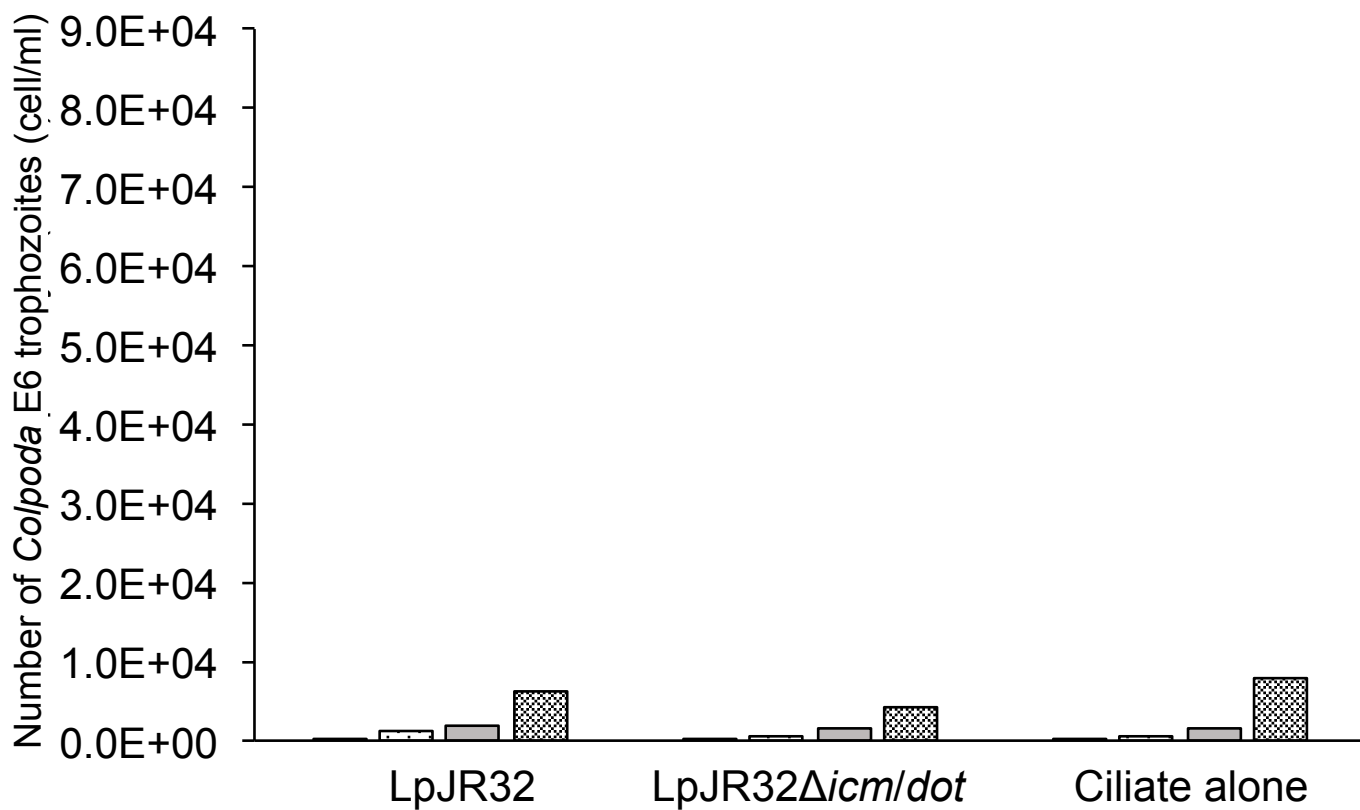
A

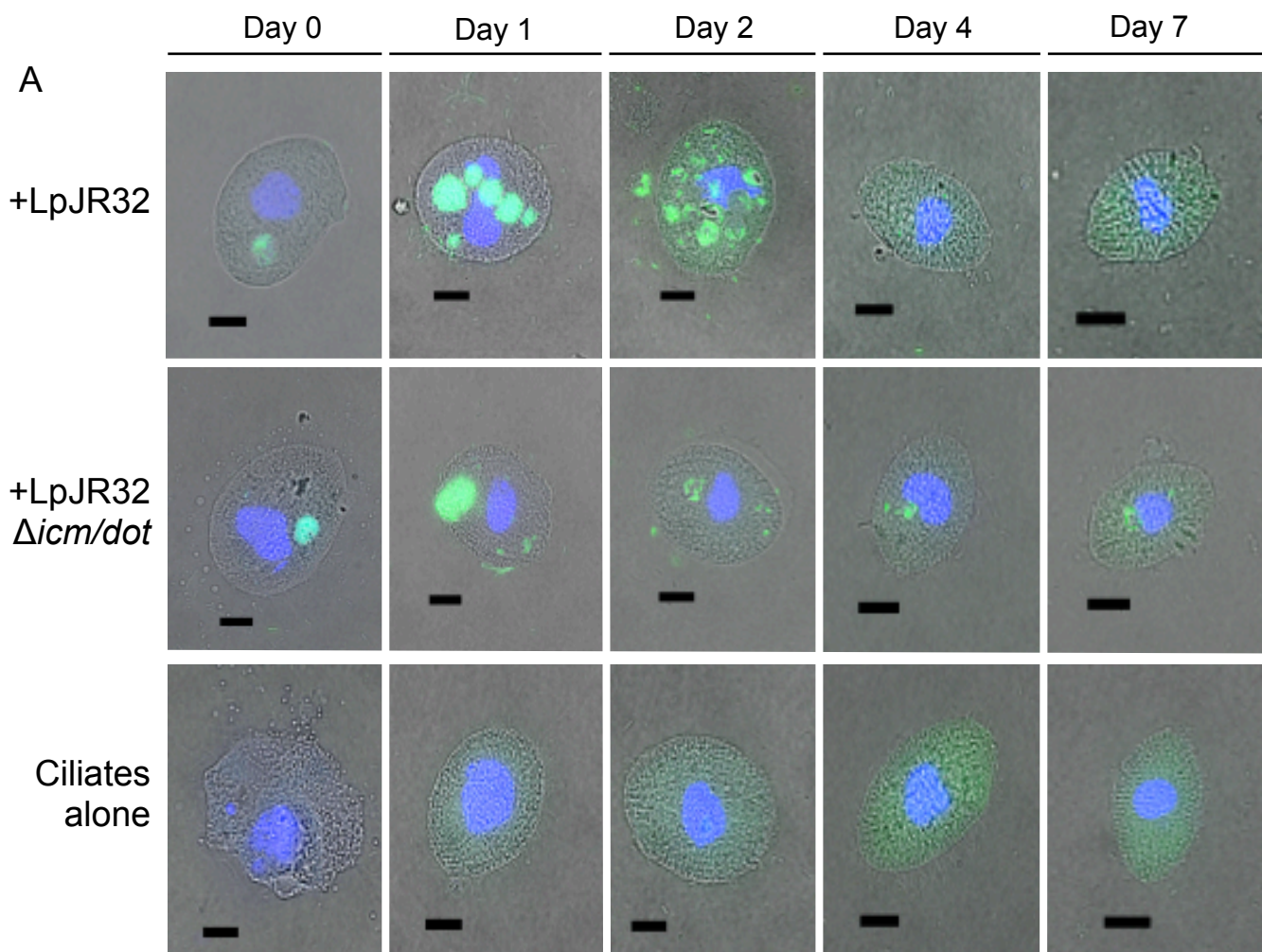
■ day0 □ day1 ▒ day2 ▓ day4



B

■ day0 □ day1 ▒ day2 ▓ day4



**B**