Ciliates Expel Environmental *Legionella*-laden Pellets for Stockpiling Food

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ABSTRACT

When the ciliate *Tetrahymena* is cultured with *Legionella pneumophila*, they expel bacteria packaged in free spherical pellets. Why the ciliates expel these pellets remains unclear. Hence, we determined optimal conditions for pellet expulsion, and assessed whether they contribute to maintenance of growth and survival of ciliates. When incubated environmental *L. pneumophila*, the ciliates maximally expelled the pellets at 2 days after infection. Heat-killed bacteria failed to produce pellets from ciliates, and there was no obvious difference in pellet production among the ciliates or bacterial strains. Morphological studies with assessment of lipid accumulation showed that pellets contained tightly packed bacteria with rapid lipid accumulation and were composed of the layers of membranes; bacterial culturability in the pellets rapidly decreased in contrast to that in ciliate-free culture, although the bacteria maintained membrane integrity in the pellets. Furthermore, ciliates newly cultured with pellets were maintained and grew vigorously compared with those without pellets. In contrast, a human *L. pneumophila* isolate killed ciliates 7 day post-infection in a Dot/Icm dependent manner and pellets harboring this strain did not support ciliate growth. Also, pellets harboring the human isolate were resuscitated by co-culture with amoebae, depending on Dot/Icm expression. Thus, while ciliates expel pellet-packaged environmental *L. pneumophila* for stockpiling food, the pellets packaged the human isolate are harmful on ciliate's survival, possibly connecting clinical significance.

INTRODUCTION

Legionella pneumophila causes the respiratory infection Legionnaires' disease in susceptible humans. It is a Gram-negative bacterium that has evolved as an intracellular pathogen of amoebae such as *Acanthamoenba* that are found in a wide range of natural environments, such as soil and freshwater, providing an intracellular environment that is required for bacterial replication (6, 12, 15, 28, 36, 40). *L. pneumophila* is also widely distributed in natural environments (15, 36), and therefore can be responsible for a common, life-threatening atypical pneumonia in immunocompromised patients, through inhalation of contaminated aerosols or mine dust (31, 32). Therefore, interaction between *L. pneumophila* and protozoa such as amoebae, to control dissemination of the bacteria or prevent infection, has been investigated (1, 3, 21). *L. pneumophila* intracellular multiplication also has been shown to be dependent on the *dot*/*icm* genes, which encode a type IV translocation apparatus that delivers effector proteins required for phagocytosis, invasion, and initiation of bacterial growth inside amoebae or macrophages (11, 19, 22, 35).

As well as amoebae, ciliated protozoa *Tetrahymena*, which are bacterial feeders, inhabit a wide range of natural environments, including soil and freshwater (13, 25, 34, 37). *Tetrahymena*, depending on the incubation temperature, can support the growth of *Legionella* (1, 2, 16). In fact, it has also been shown that while *Tetrahymena* supports multiplication of *L. pneumophila* at temperatures around 35°C, lower temperatures of 20–25°C do not support the intracellular growth of bacteria (1, 16, 20). When the bacteria are ingested, the ciliates expel *L. pneumophila* packaged in free spherical pellets,

wrapped in membrane (2, 14, 20), suggesting a possible role of pellets on *L. pneumophila* survival in harsh environments. *Legionella* pellets are clusters of up to 100–200 *L. pneumophila* cells kept together by outer membrane fragments derived from a few digested *Legionellae* reflecting massive ingestion by *Tetrahymena*, and perhaps a ciliate-derived material from the lumen of food vacuoles (2). As well as *L. pneumophila*, it has been reported that many bacteria, such as *Escherichia coli* or *Salmonella*, engulfed into amorphous vesicles of ciliates, are not digested, and it is believed these bacteria continue to survive in the pellets after expulsion to the outer environment (4, 18, 27). However, whether the pellets contribute to maintain growth and survival of the ciliates themselves still remains unknown.

In the present study, we therefore determined optimal conditions for expulsion of the pellets expelled from ciliates co-cultured with *L. pneumophila* [five environmental strains, a human isolate (JR32) and its Dot/Icm translocation-defective mutant] and then assessed whether the pellets contributed to maintenance of the ciliates.

MATERIALS AND METHODS

Bacteria and culture conditions.

L. pneumophila [environmental strains: Lp768, Lp920, Lp923, Lp924 and Lp926; human isolate Philadelphia I: JR32 (Wildtype) (39) and the null mutant (Dot/Icm translocation-defective mutant) of JR32 in *dot*/*icm* genes encoding a type IV secretion system required for intracellular growth (Mutant) (26)] was cultured on BCYE agar

(Sigma, St Louis, MO) at 37°C in an atmosphere of 5% CO_2 for 2 days. The environmental strains were isolated from showerhead (Lp768 strain) or several cooling towers (the other strains: Lp920, Lp923, Lp924, Lp926). A laboratory strain of *E. coli* (isolated from a patient with urethritis) was also used for this study, and the bacteria were cultured in Luria Broth (LB) containing 1% NaCl (Wako), 1% peptone (Difco) and 0.5% yeast extract (Difco) at 37°C. These bacteria were collected, washed and suspended in Page's modified Neff's amoeba saline (PAS) (29), and then used for the following experiments.

Protozoa and culture conditions.

Protozoa [ciliates: *Tetrahymena thermophila* inbred strain B (TIB) (Gifted from Dr Sugai of Ibaraki University, Japan), *Tetrahymena thermophila* SB021 (TSB) (Gifted from Dr. Yomo of Osaka University, Japan), *Tetrahymena pyriformis* (TP) (Gifted from Dr. Sonobe of Hyogo University, Japan), *Tetrahymena* sp. (TS) (Gifted from Dr. Tukii of Hosei University, Japan); free-living amoebae: *Acanthamoeba castellani* (a reference strain C3, purchased from ATCC)] were used for this study. Protozoa were maintained in peptone–yeast extract glucose broth (PYG) containing 0.75% peptone (Difco), 0.75% yeast extract (Difco), and 1.5% glucose (Wako) (PYG) at 30°C, as described previously (23). The protozoa were collected, washed and suspended in PAS, and then used for the following experiments.

Induction of pellets.

The *L. pneumophila* concentration was adjusted by using the optical density method with a spectrophotometer (24). The concentration of protozoa was also determined using the

modified trypan blue dye exclusion method (trypan blue assay) [trypan blue solution (Sigma) containing 0.6% ethanol stains viable protozoa] (24). The number of pellets was determined with haemocytometers. The bacteria (1ml, 10^{5-9} CFU/ml) were equally mixed with ciliates (1ml, 10^5 cells/ml) at an MOI of 1–10,000 in 24-well plate, and then cultured for up to 7 day at 4, 15 or 30°C under a normal atmosphere. As a control, the bacteria (1ml, 10^{5-9} CFU/ml) were also incubated without ciliates (1ml, PAS alone). At several time points, samples were collected, and bacterial CFUs (See below **"Assessment of bacterial culturability and viability"**), protozoa and pellet numbers were monitored.

Morphological analysis of pellets.

Either *E. coli* (clinical isolate) or *L. pneumophila* (Lp926) (1ml, 10^9 CFU/ml) were equally mixed with ciliates (TP) (1ml, 10^5 cells/ml) of an MOI of 10,000 in 24-well plate, and cultured for 2 days at 30°C under a normal atmosphere. After centrifugation (100×g for 5 min), morphology of pellets in the sediment was assessed by Gimenez staining (Nikken Biomedical Laboratory, Kyoto, Japan) and transmission electron microscopy (TEM). Gimenez staining was performed according to the manufacturer's protocol. TEM was performed as previously described (23). In brief, the pellets within cultures were immersed in a fixative containing 3% glutaraldehyde in 0.1 M PBS, pH 7.4, for 24 h at 4°C. After a brief wash with PBS, the fixed sediments were processed for alcohol dehydration and embedded in Epon 812. Ultrathin sections of the sediment were stained with lead citrate and uranium acetate before viewing by TEM (Hitachi H7100; Hitachi, Tokyo, Japan).

Assessment of lipid accumulation.

To clarify the relocation of membrane lipid to the pellets, the bacteria (Lp926) (1ml, 10^9 CFU/ml) were equally mixted with ciliates (TP) (1ml, 10^5 cells/ml) in the presence of FM4-64FX dye (final concentration 5μg/ml) (Invitrogen, Carlsbad, CA), which is a specific fluorescence dye (excitation, 510 nm; emission, 625 nm) for membrane lipid, according to the manufacturer's protocol (41), and cultured for 24h at a normal atmosphere. Either ciliates or pellets in the cultures were observed under a fluorescence microscope.

Enrichment of pellets.

The whole culture solution obtained from the culture of bacteria (Lp926; MOI of 10,000) with ciliates (TP) (or without ciliates, used as a control) at 2 days after incubation was used for this enrichment protocol. The enrichment of pellets expelled from ciliates was performed by centrifugation (100 × *g* for 5 min) at 4°C. The supernatant contained mostly bacteria and ciliates, and the sediment contained the pellet, at a recovery rate of approximately 85%. The collected pellets were suspended in PAS, the concentration was adjusted by counting on haemocytometers (See above "**Induction of pellets**"), and then used for the experiment below (pellet solution). As a control, the whole culture solution of the bacteria without ciliates was also treated in a similar way, and used for the experiment below (bacterial solution).

Assessment of bacterial culturability and viability.

The number of *L. pneumophila* in culture with or without ciliates was assessed by CFU assay with BCYE agar. Before plating on the agar, the culture of ciliates with bacteria was subjected to bead beating, as described previously (24). The bacterial membrane integrity

as a possible indicator for bacterial viability was also confirmed with fluorescence microscopy by using a LIVE/DEAD reduced biohazard viability/cytotoxicity kit (Molecular Probes, Eugene, OR), according to the manufacturer's instructions. The numbers of staining expelled pellets were counted under a fluorescence microscope. It was estimated by observing three to five randomly selected fields containing more than 200 pellets under a fluorescence microscope.

Resuscitation and growth of *L. pneumophila* by co-culture with amoebae.

Resuscitation of *L. pneumophila* by co-culture with amoebae was performed according to the method previously described (39). In brief, the above-mentioned samples (100μl: "pellet solution" or "bacterial solution") were added in axenic cultures of amoebae adjusted at 10^5 cells in 1 ml of PYG or PAS prepared in 24-well plates, and then incubated for up to 72 h. After incubation, the amoebae were collected, subjected to bead beating, and cultured on BCYE agar for 5 days. It was also assessed if the *L. pneumophila* (Lp920, Lp923, Lp924, Lp926, Lp768, JR32Wildtype, JR32Dot/Icm mutant) could simply grow in ameobae by monitoring with the CFU assay.

Monitoring ciliate growth in the presence of pellets.

To clarify whether the pellets could contribute to maintenance of growth and survival of ciliates, the number of ciliates newly cultured with enriched pellets was compared with that without pellets (See above **"Induction of pellets"**). The ciliates were adjusted to a concentration of 1–1,000 cells mixed with different amounts of pellet solution, 1 μl (containing approximately 1,000 pellets) to 100 μl (containing approximately 100,000 pellets), and cultured in PAS (1ml) of 24-well plate for 3 days. As a control, the bacterial

solution (1-100μl) equivalent amount of the pellet solution obtained from the culture of bacteria alone without ciliates (See above "**Enrichment of pellets**") was also mixed with the ciliates, and cultured.

Statistical analysis.

The influence of the pellets on survival of ciliates was analyzed by Fisher's exact test (two-way ANOVA; Statview, Abacus Concepts Inc., Piscatway, NJ, USA). Comparison of bacterial or ciliates numbers was also assessed by an unpaired *t* test (Statview, Abacus Concepts Inc.). A p value <0.05 was considered significant.

RESULTS

Morphological traits of expelled pellets.

We attempted to confirm using the cultures of ciliates (TP) with environmental *L. pneumophila* (Lp926) whether the ciliates could expel pellets containing *L. pneumophila*. When incubated with *L. pneumophila*, the ciliates constantly expelled free spherical pellets laden with bacteria, with a surrounding membrane, at 24 h after incubation (Fig. 1A and B). However, when mixed with *E. coli*, typical pellets were not observed, although atypical excretory substances bundled with *E. coli* were sometimes seen (Fig. 1C). Thus, these results indicated that our experimental conditions adequately controlled the production of pellets specifically laden with *L. pneumophila*.

Expelling pellets of ciliates with lipid accumulation. We confirmed through a

preliminary experiment that the production of pellets could be observed at approximately 6 h (data not shown). Interestingly, TEM observation also demonstrated environmental *L. pneumophila*-laden pellets consisting of multiple plasma membranes. The data therefore suggest rapid accumulation of lipid from ciliate plasma membrane to nascent pellets that originated in ciliates. To assess this possibility, we examined if a fluorescent dye, FM1-43FX, which is specific for plasma membrane lipid, could accumulate in pellets laden with *L. pneumophila* (Lp926). As expected, the fluorescent dye was accumulated rapidly and surrounded the plasma membrane of the ciliates at 30 min or earlier after addition (Fig. 2A–C, orange color). At 8 h after incubation, the dye rapidly moved selectively to nascent pellets laden with bacteria (Fig. 2D–G); the arrows show representative vesicles, not laden with bacteria without fluorescence dye, suggesting that rapid accumulation of membrane lipid is specific to vesicles harboring *L. pneumophila*. At 24 h, the pellets that were formed in ciliates, with a fluorescence signal, were expelled, containing the bacteria (Fig. 2H).

Optimal conditions for production of pellets.

To determine optimal conditions for the production of pellets, we monitored the number of pellets and ciliates under different culture conditions and temperature or MOI (*L. pneumophila*: Lp926). As shown in Fig. 3, the production of the pellets expelled by the ciliates clearly changed depending on culture temperature or MOI, and at 30°C with an MOI of 10,000, the production of pellets in culture reached a maximum of 10^5–10^6 per culture. Thus, production of pellets dramatically changed depending on MOI and culture

temperature. With the use of different strains (*L. pneumophila*: Lp768, Lp920, Lp923, Lp924 strains), no difference in pellet production was observed (Fig. S1).

Role of pellet expulsion in ciliate survival or replication in a co-culture system.

To assess if co-cultured with the environmental *L. pneumophila* (Lp926) the pellet production altered ciliate growth or survival, the number of ciliates in culture was monitored for up to 7 days. As shown in Fig. 4, there was no significant change, and the number of ciliates was maintained during the culture period, regardless of different MOI or culture temperature. Moreover, when we used different environmental strains (*L. pneumophila*: Lp768, Lp920, Lp923, Lp924), no difference in ciliate numbers was found (Fig. S2). The results suggest that expulsion of pellets laden with environmental *L. pneumophila* may be required for normal maintenance of growth and survival of ciliates.

Survival of bacteria in membrane-wrapped pellets expelled by ciliates.

We assessed if the environmental *L. pneumophila* packaged in the pellets expelled into the culture supernatant were still viable, as based on two different markers, culturability and bacterial membrane integrity. As shown in Fig. 5, although the culturability of *L. pneumophila* in the absence of ciliates was maintained during culture, the culturability of all the bacterial strains in the presence of ciliates significantly decreased during the course of the experiment. To confirm bacterial death in the pellets, we observed bacterial membrane integrity by using LIVE/DEAD staining, under a fluorescence microscope. Contrary to our expectation, fluorescence signals showed membrane integrity of the

bacteria in the pellets (Fig. 6A and B; green color), although *L. pneumophila* in the heat-treated pellets completely lost membrane integrity (Fig. 6B; red color). It was also confirmed that bacterial membrane integrity was maintained during the 7-day culture period, although no association with amount of the pellets (Fig. 6B and C). Since it is well known that *Acanthamoenba* found in a wide range of natural environments such as soil and freshwater provides an intracellular environment for *L. pneumophila* that is required for bacterial replication (6, 12, 15, 28, 36, 40), we also assessed whether either the bacteria in the pellets "pellet solution" or the bacteria alone "bacterial solution" could grow when co-cultured with amoebae. As a result, the human isolate JR32 packed in the pellets only were resuscitated by amoeba co-culture depending on Dot/Icm expression (Fig. A and B), indicating that the human isolate was still alive into the egested pellets. Meanwhile, the environmental strains failed to proliferate in the test amoeba when simply co-culture with amoebae (Fig. 7C).

Effect of pellets in supporting growth and survival of ciliates.

To assess the possible beneficial effect of the pellets expelled from ciliates co-cultured with environmental *L. pneumophila* for maintaining ciliate growth and survival, growth of ciliates newly cultured with enriched pellets was compared with that of ciliates without pellets. As expected, the growth of ciliates was significantly enhanced when the pellets (pellet solution) were added to the cultures, compared to the growth with addition of the simple bacterial culture (bacterial solution), although it was limited only as an addition of the pellets to low numbers of ciliates (Fig. 8). The results suggest that ciliates can expel

the pellets to ward off hostile bacteria, such as environmental *L. pneumophila*, and might be utilized for stockpiling food.

Characterization of pellets expelled from ciliates co-cultured with either human isolate JR32 or its null mutant in *dot*/*icm* genes.

We finally assessed if pellets expelled from ciliates co-cultured with the human isolate *L. pneumophila* JR32 or its null mutant in *dot*/*icm* genes, which encode a type IV secretion system required for intracellular growth, could support survival for the ciliates. As expected, the pellet productions of human isolate JR32 and its null mutant occurred similarly with maximum pellet production occurring 1 day post-infection (Fig. 9A). However, while the pellets laden with JR32 maintained bacterial membrane integrity, membrane integrity was not maintained in the pellet of the null mutant (Fig. 9B). Meanwhile, in contrast to both the null mutant and Lp926, human isolate JR32 finally killed ciliates at 7 day after incubation (Fig. 9C). Furthermore, while the pellet of the null mutant worked for stockpiling food, the pellet of JR32 completely killed ciliates in co-culture (Fig. 10). Thus, it appears that the pellets packaged the human isolate are harmful on ciliate's survival in a Dot/Icm dependent manner, although further study with other human isolates is needed.

DISCUSSION

Although *L. pneumophila* is commonly seen in soil and freshwater environments

worldwide (15, 36), it is well known that the bacteria require an unusual combination of nutrients that are rarely found in natural environments such as soil or freshwater (6, 30). Therefore, to prevent competition with other bacteria that are rapidly and freely growing in the natural environments, it is believed that *L. pneumophila* has evolved in free-living protozoa such as amoebae, thus providing sequestering niches to the bacteria (6, 13, 15, 17, 28, 36, 40). In fact, through evolution, bacteria have acquired a functional Dot/Icm type IV secretion apparatus that deliver effector proteins to the host cells for bacterial survival (11, 19, 22, 35). Meanwhile, several studies have shown that, when infected with *L. pneumoniae* or *Salmonella*, protozoa such as amoebae or ciliates expel the bacteria packaged in spherical pellets surrounded by plasma membrane (4, 14, 18, 28). Morphological studies have indicated that these pellets are laden with possibly viable bacteria; therefore, it is thought that they have an important role as a shelter for bacterial survival in harsh environments (2, 14, 20). However, why the ciliates have to expel the pellets after infection with bacteria remains unclear. We therefore assessed whether the pellets contribute to growth maintenance and survival of the ciliates, which are representative protozoa that are distributed in natural environments along with *L. pneumophila*.

We first tried to explore the unique features of pellets expelled from environmental *Legionella*-infected ciliates. Our results were similar to those in other studies although lacking assessment of bacterial viability in pellets (2), and morphological observations revealed free spherical pellets laden with *Legionella*, wrapped in a surrounding membrane. However, they were not seen in atypical excretory substances bundled *E. coli*,

indicating that this packing and expulsion might be required for inactivation of *Legionella* in ciliates. The ciliates rapidly began to expel the *Legionella*-containing pellets at 6 h after infection, followed by maximum production at 2 days after incubation, although the production of pellets changed depending on culture temperature and MOI. Meanwhile, it is intriguing that there were no significant differences in the level of pellet production between the strains of ciliates or environmental bacteria, which suggests that, rather than providing for survival of environmental *Legionella* in ciliates, expulsion of pellets provides an advantage for feeding or survival of ciliates in a natural environment that lacks sufficient nutrients. To assess this hypothesis, we examined whether expulsion of pellets affected ciliate survival. As expected, we confirmed that there was no significant decrease in the number of ciliates, regardless of different MOI, culture temperature, or the environmental *Legionella* strains used. Thus, these results also support our hypothesis that the production of pellets possibly provides some growth and survival advantage to ciliates. In addition, the fluorescence dye, FM4-64FX, which is specific for plasma membrane lipid, showed that the lipid present on the cell surface of ciliates was rapidly and constantly relocated to the environmental bacteria-laden pellets, but not to vesicles without bacteria. This suggests that the mechanism for handling the bacteria properly may also be required for rapid accumulation of lipid in the pellets.

As expected, addition of pellets packed with the environmental *L. pneumophila* to ciliate culture supported ciliate growth and survival, suggesting that ciliates briefly expel the pellets for warding off the *L. pneumophila* and might utilize the packaged pellets for stockpiling food. It is clear that natural environments such as soil or pond water without a

constant nutrient source represent harsh conditions, which hardly support protozoa or bacteria. Therefore, such interaction of protozoa with bacteria-laden pellets may be crucial for maintaining ecosystems, and ciliates might need to use environmental *Legionella* as a food source, which can have a strong cytotoxic effect against ciliates. Enhancement of ciliate growth only occurred when pellets were added to low numbers of ciliates. Although the exact reason remains unknown, it seems that culture of high numbers of ciliates with bacteria (not pellets) resulted in formation of new pellets that were able to support ciliate survival.

Contrary to our expectation, while the pellet of the mutant *Legionella* in *dot*/*icm* definitely worked for stockpiling food as well as the environmental *Legionella* used for this study, the pellet of a human isolate JR32 wildtype completely killed ciliates in co-culture, suggesting inherent difference of these pellets. Unfortunately, at present time, it is not clear how the JR32 wildtype pellets differ from the other environmental stain pellets used for this study. However, since accumulated studies have demonstrated that different strains of *L. pneumophila* have a different cohort of type IV effectors and that many effectors exhibit host specific tropism (7, 8, 9, 10, 33, 38), it is likely strain-dependent differences in the effector molecules harbored by JR32 and the environmental strains via the co-evolution of different *L. pneumophila* strains with their various protozoan host. In addition, although the environmental strains failed to proliferate in the test amoeba when simply co-culture with amoebae, it cannot rule out the possibility that the environmental strain pellets may be resuscitated by other host amoebae that are actually permissive for their replication. Thus, whether the

environmental bacteria used for this study are still alive in the egested pellets remains to clarify by using other suitable amoeba strain.

In conclusion, we demonstrated that while ciliates expel pellet-packaged environmental *L. pneumophila* for stockpiling food, the pellets packaged the human isolate (JR32) are harmful on ciliate's survival and are able to be resuscitated by amoeba co-culture which may have clinical significance. Although further study with other *L. pneumophila* or amoebal strains is needed, our results provide not only a new insight into a complicated host–parasite interaction formed between microorganisms that are present in natural environments, but also a hint for developing infection control of *L. pneumophila* as a human pathogen.

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Figure legends

Fig. 1. Morphology of expelled pellets. Bacteria (*E. coli* or environmental *L. pneumophila*: Lp926) were mixed with ciliates (TP) at an MOI of 10,000, and cultured for up to 2 day at 30°C in a normal atmosphere. (A) Gimenez staining of expelled pellets from ciliates at 2 days after the incubation. Arrows, pellets expelled from ciliates cultured with *L. pneumophila*. Magnification, ×400. (B) Representative TEM image showing *L. pneumophila* morphology in a pellet expelled from ciliates at 2 days after the incubation. Bar, 500 nm. (C) Representative TEM image showing *E. coli* morphology in an atypical pellet expelled from ciliates. Bar, 500 nm.

Fig. 2. Selective accumulation of plasma membrane lipid relocated from surface of ciliates to pellets laden with bacteria. The cultures of ciliates (TP) with environmental *L. pneumophila* (Lp926) (at MOI 10,000) were incubated with FM 4-64FX dye for 24 h at 30°C in a normal atmosphere. (A–C) Representative images showing lipid located on surface of ciliates immediately after incubation with bacteria. Phase-contrast image (A). Green color, fluorescent image (B). Merge (C). Magnification, ×200. (D–G) Representative images showing lipid relocated on pellets laden with bacteria at 8 h after incubation. Phase-contrast image (D). Orange color, fluorescent image (E). Merge (F). (G) Enlarged view of the square in panel (F). Arrows, pellets without bacteria lacking fluorescence. Magnification, ×200. (H) Representative images showing lipid accumulation in pellets expelled from ciliates at 24 h after incubation. Magnification,

×1,000.

Fig. 3. Changes in the number of pellets expelled from ciliates with environmental *L. pneumophila* under various conditions. *L. pneumophila* (Lp926) was mixed with ciliates (TP, TS, TIB, TSB) at an MOI of 1–10,000, and cultured for up to 7 days at 4, 15 or 30°C in a normal atmosphere. Each plot shows an average obtained from at least two experiments. Average value of plots at the same time point in parentheses was compared with value at immediately after incubation. *, $p<0.05$ versus each value immediately after incubation.

Fig. 4. Changes in the number of ciliates when cultured with environmental *L. pneumophila* under various conditions. See the legend of Fig. 3. Each plot shows an average obtained from at least two experiments.

Fig. 5. Changes in the culturability of environmental *L. pneumophila* in cultures without (A) or with ciliates [supernatant of "pellet solution" (B), "pellet solution" (C)]. *L. pneumophila* (Lp768, Lp920, Lp923, Lp924, Lp926) was mixed without or with ciliates (TP) at an MOI of 10,000, and cultured for up to 7 days at 30°C in a normal atmosphere. The enrichment of pellets was simply performed by centrifugation. See Materials and Methods "Enrichment of pellets". Each plot shows an average of CFU obtained from at least two experiments. Average value of plots at the same time point in parentheses was compared with value at immediately after incubation. *, $p<0.05$ versus each value

immediately after incubation.

Fig. 6. Representative images and change in the number of pellets showing viability of environmental *L. pneumophila* in pellets. *L. pneumophila* (Lp926) was mixed without or with ciliates (TP) at an MOI of 10,000, and cultured for up to 7 days at 30°C in a normal atmosphere. Bacterial membrane integrity as a possible indicator for bacterial viability was confirmed with fluorescence microscopy by using a Live/Dead kit. (A) Representative LIVE/DEAD images at 24 h after incubation. Green color, *L. pneumophila* with stable bacterial membrane integrity. Magnification, ×100. (B) Change in the number of pellets showing viability of environmental *L. pneumophila* in pellets. Upper images, representative pellet images with laden *L. pneumophila* with stable bacterial membrane integrity (Green color), and heat-treated pellet laden with bacteria (80°C, 20 min) (Red color). Magnification, ×1,000. The change indicates the percentage in the number of pellets showing viability of environmental *L. pneumophila* in pellets with or without the heat treatment. The data shown represent the means ± standard deviation (SD), obtained from at least three independent experiments performed in triplicate. The numbers of staining expelled pellets were estimated under a fluorescence microscope (See Material and Methods "Assessment of bacterial culturability and viability"). (C) Changes in the total number of pellets expelled from ciliates with environmental *L. pneumophila*. The data shown represent the means ± SD, obtained from at least three independent experiments performed in triplicate. *, $p<0.05$; significantly different from each data at immediately (0 day) after incubation.

Fig. 7. Resuscitation of *L. pneumophila* (JR32, the mutant, Lp926) by co-culture with amoebae. Either "pellet solution" (A) or "bacterial solution (B) was added in axenic cultures of amoebae adjusted at 10^5 cells in 1 ml of PYG or PAS prepared in 24-well plates, and then incubated for up to 72 h. See Materials and Methods "Resuscitation of *L. pneumophila* by co-culture with amoebae". Culturability of the bacteria as simply incubated with amoebae was assessed by the CFU assay (C). The data shown represent the means ± SD, obtained from at least three independent experiments performed in triplicate. *, $p<0.05$; significantly different from each data at immediately (0 day) after incubation.

Fig. 8. Effect of pellets expelled from ciliates incubated with environmental *L. pneumophila* (Lp926) on supporting the growth and survival of ciliates. The number of ciliates newly cultured with enriched pellets was compared with that without pellets. Each bar shows an average obtained from at least two experiments. The influence of the pellets on survival of ciliates was analyzed by Fisher's exact test (two-way ANOVA). *, $p<0.05$, significant difference in growth of ciliates with or without enriched pellets.

Fig. 9. Characterization of pellets expelled from ciliates exposed with either human isolate JR32 or its null mutant in *dotA*/*icm* genes. *L. pneumophila* (JR32, the mutant, Lp926) was mixed with ciliates (TP) at an MOI of 10,000, and cultured for up to 7 days at 30°C in a normal atmosphere. (A) Changes in the number of pellets expelled from ciliates

with human isolate *L. pneumophila*. The data shown represent the means ± SD, obtained from at least three independent experiments performed in triplicate. *, $p<0.05$; significantly different from each data at immediately (0 day) after incubation. (B) Gimenez and LIVE/DEAD representative images of expelled pellets from ciliates incubated with either JR32 (Wildtype) or the mutant (Mutant) at 2 days after the incubation. (C) Changes in the number of ciliates when cultured with *L. pneumophila*. The data shown represent the means ± SD, obtained from at least three independent experiments performed in triplicate. *, $p<0.05$; significantly different from each data at immediately (0 day) after incubation.

Fig. 10. Effect of pellets expelled from ciliates incubated with human isolate *L. pneumophila* [JR32 (Wildtype) and the mutant (Mutant)] on supporting the growth and survival of ciliates. The number of ciliates newly cultured with enriched pellets was compared with that without pellets. Each bar shows an average obtained from at least two experiments. The influence of the pellets on survival of ciliates was analyzed by Fisher's exact test (two-way ANOVA). *, $p<0.05$, significant difference in growth of ciliates with or without enriched pellets.

Legend to supporting figures

Fig. S1. Changes in the number of pellets expelled from ciliates with environmental *L. pneumophila* (Lp768, Lp920, Lp923, Lp924). Each of the strain was mixed with ciliates (TP) at an MOI of 10,000, and cultured for up to 7 days at 30°C in a normal atmosphere. Each plot shows an average obtained from at least two experiments. Average value of plots at the same time point in parentheses was compared with value at immediately after incubation. *, $p<0.05$ versus each value immediately after incubation.

Fig. S2. Changes in the number of ciliates when cultured with environmental *L. pneumophila* (Lp768, Lp920, Lp923, Lp924). Each of the strain was mixed with ciliates (TP) at an MOI of 10,000, and cultured for up to 7 days at 30°C in a normal atmosphere. Each plot shows an average obtained from at least two experiments. Average value of plots at the same time point in parentheses was compared with value at immediately after incubation.

FIG. 1

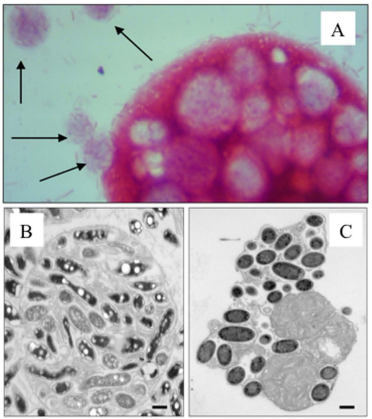


FIG. 2

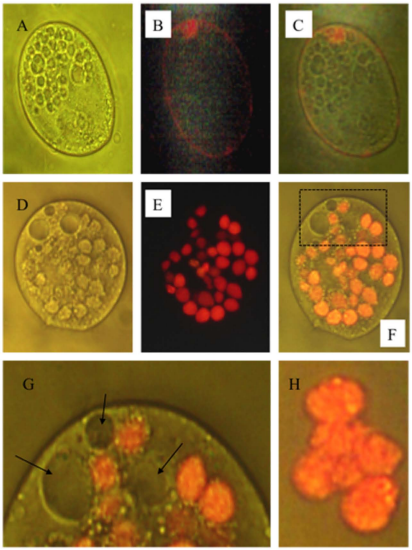
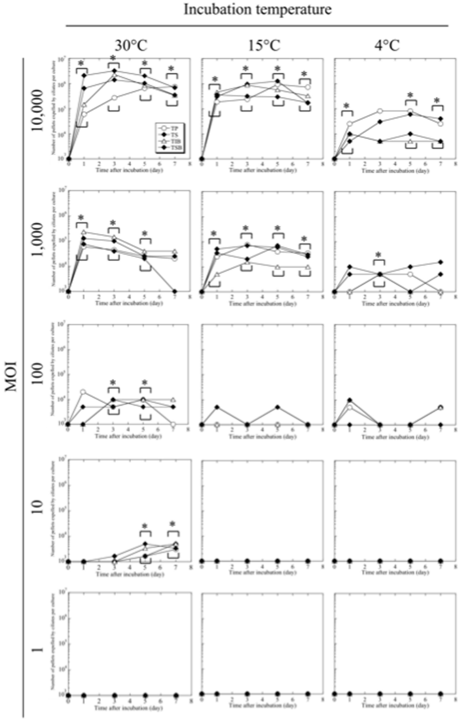


FIG. 3



Incubation temperature
30°C
15°C
4°C
MOI
10,000
1,000
100
10
1
TP
TS
TBB
TSB
Time after incubation (day)
Number of pellets expelled by ciliates per culture

FIG. 4

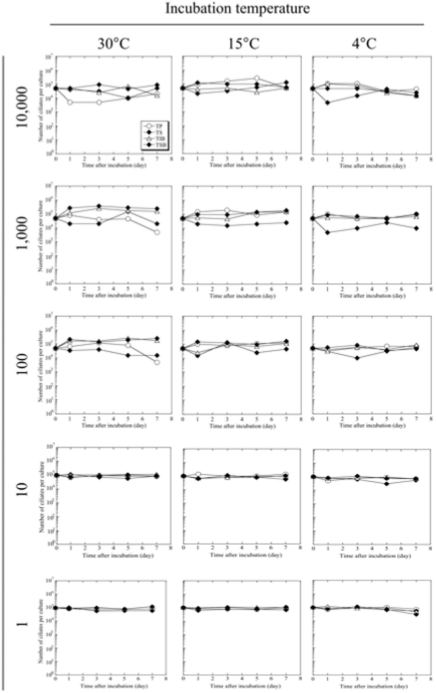

Incubation temperature
30°C
15°C
4°C
MOI
10,000
1,000
100
10
1
Number of cilates per culture
Time after incubation (day)
TP
TS
TSB
TSD

FIG. 5

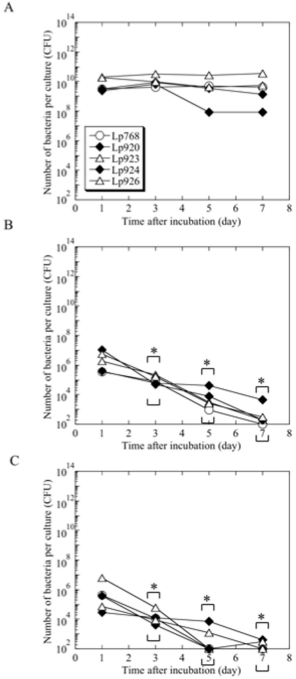

A
B
C
Number of bacteria per culture (CFU)
Time after incubation (day)
Lp768
Lp920
Lp923
Lp924
Lp926
*

FIG. 6

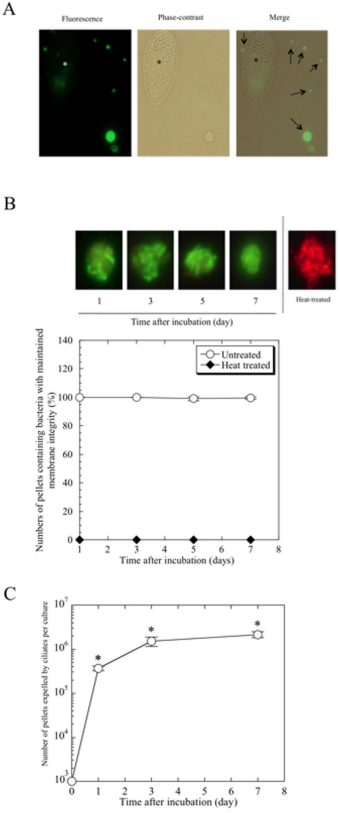
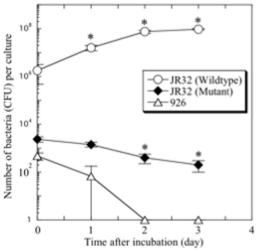
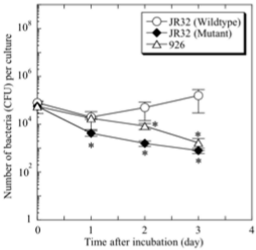

A
Fluorescence
Phase-contrast
Merge
B
1
3
5
7
Heat-treated
Time after incubation (day)
Numbers of pellets containing bacteria with maintained membrane integrity (%)
Untreated
Heat treated
Time after incubation (days)
C
Number of pellets expelled by ciliates per culture
Time after incubation (day)

FIG.7

A


Number of bacteria (CFU) per culture
Time after incubation (day)
JR32 (Wildtype)
JR32 (Mutant)
926

B


Number of bacteria (CFU) per culture
Time after incubation (day)
JR32 (Wildtype)
JR32 (Mutant)
926

C

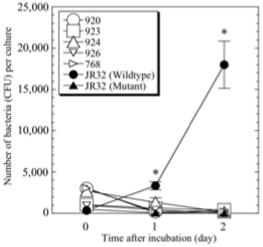

Number of bacteria (CFU) per culture
Time after incubation (day)
920
923
924
926
768
JR32 (Wildtype)
JR32 (Mutant)

FIG. 8

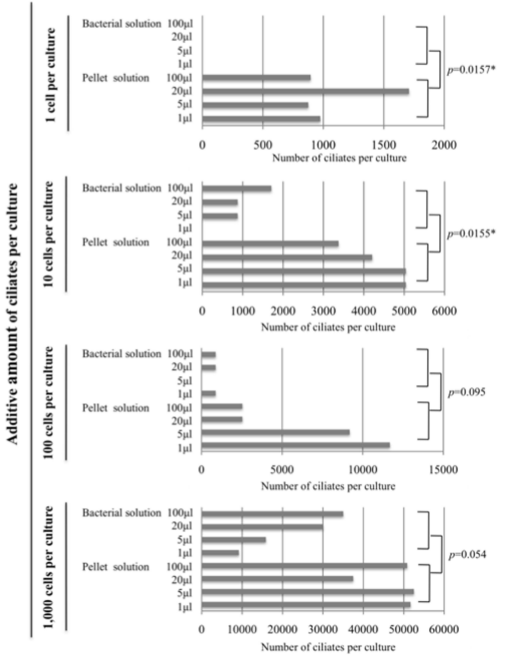
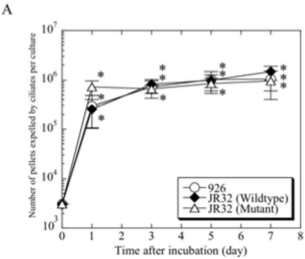
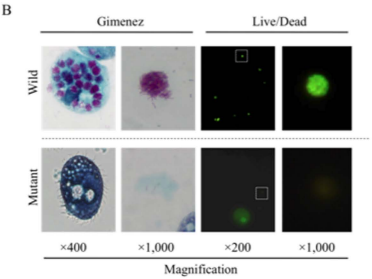

Additive amount of ciliates per culture
1 cell per culture
Bacterial solution 100µl
20µl
5µl
1µl
Pellet solution 100µl
20µl
5µl
1µl
p=0.0157*
0
500
1000
1500
2000
Number of ciliates per culture
10 cells per culture
Bacterial solution 100µl
20µl
5µl
1µl
Pellet solution 100µl
20µl
5µl
1µl
p=0.0155*
0
1000
2000
3000
4000
5000
6000
Number of ciliates per culture
100 cells per culture
Bacterial solution 100µl
20µl
5µl
1µl
Pellet solution 100µl
20µl
5µl
1µl
p=0.095
0
5000
10000
15000
Number of ciliates per culture
1,000 cells per culture
Bacterial solution 100µl
20µl
5µl
1µl
Pellet solution 100µl
20µl
5µl
1µl
p=0.054
0
10000
20000
30000
40000
50000
60000
Number of ciliates per culture

FIG. 9


A
Number of pellets expelled by ciliates per culture
Time after incubation (day)
926
JR32 (Wildtype)
JR32 (Mutant)


B
Gimenez
Live/Dead
Wild
Mutant
×400
×1,000
×200
×1,000
Magnification

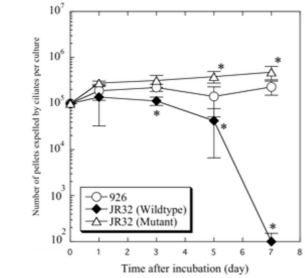

C
Number of pellets expelled by ciliates per culture
Time after incubation (day)
926
JR32 (Wildtype)
JR32 (Mutant)

FIG. 10

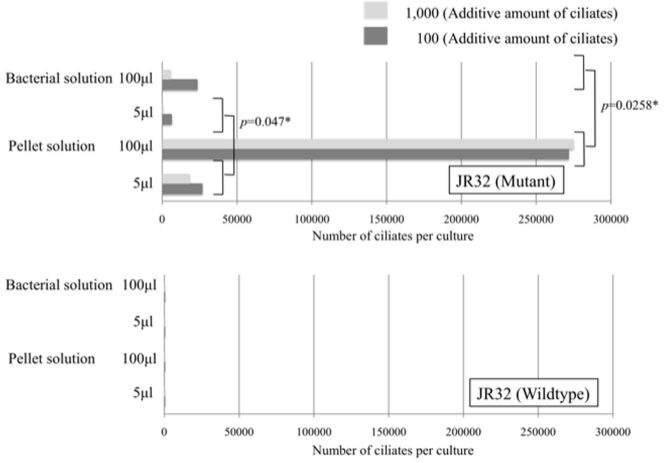

1,000 (Additive amount of ciliates)
100 (Additive amount of ciliates)
Bacterial solution 100μl
5μl
Pellet solution 100μl
5μl
p=0.047*
p=0.0258*
JR32 (Mutant)
0
50000
100000
150000
200000
250000
300000
Number of ciliates per culture
Bacterial solution 100μl
5μl
Pellet solution 100μl
5μl
JR32 (Wildtype)
0
50000
100000
150000
200000
250000
300000
Number of ciliates per culture

FIG. S1

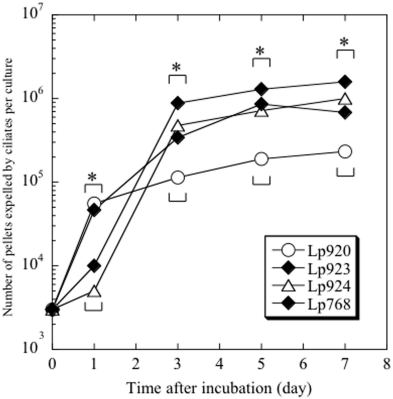
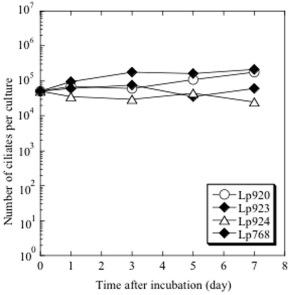

Number of pellets expelled by ciliates per culture
Time after incubation (day)
Lp920
Lp923
Lp924
Lp768
*
*
*
*

FIG. S2


Number of ciliates per culture
Time after incubation (day)
Lp920
Lp923
Lp924
Lp768