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Ciliates Expel Environmental *Legionella*-Laden Pellets To Stockpile Food

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When *Tetrahymena* ciliates are cultured with *Legionella pneumophila*, the ciliates expel bacteria packaged in free spherical pellets. Why the ciliates expel these pellets remains unclear. Hence, we determined the optimal conditions for pellet expulsion and assessed whether pellet expulsion contributes to the maintenance of growth and the survival of ciliates. When incubated with environmental *L. pneumophila*, the ciliates expelled the pellets maximally 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 assessing 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 what was seen 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 days postinfection 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 coculturing with amoebae, depending on Dot/Icm expression. Thus, while ciliates expel pellet-packaged environmental *L. pneumophila* for stockpiling food, the pellets packaging the human isolate are harmful to ciliate survival, which may be of clinical significance.

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 *Acanthamoeba* 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, the interaction between *L. pneumophila* and protozoa such as amoebae has been investigated with a view toward controlling the dissemination of the bacteria or preventing infection (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).

Along with amoebae, ciliated *Tetrahymena* protozoa, 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 the multiplication of *L. pneumophila* at temperatures around 35°C, lower temperatures of 20 to 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 to 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 into the outer environment (4, 18, 27). However, whether the pellets contribute to the maintenance of the growth and survival of the ciliates themselves still remains unknown.

In the present study, we therefore determined the optimal conditions for the expulsion of the pellets from ciliates cocultured 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

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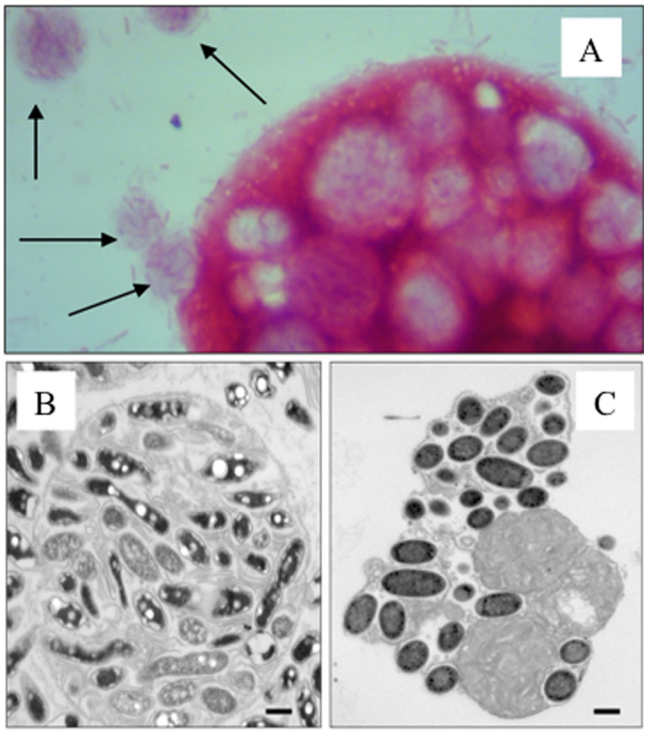

FIG 1 Morphology of expelled pellets. Bacteria (*E. coli* or environmental *L. pneumophila* Lp926) were mixed with ciliates (TP) at an MOI of 10,000 and cultured for up to 2 day at 30°C in a normal atmosphere. (A) Gimenez staining of expelled pellets from ciliates at 2 days after the incubation. Arrows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

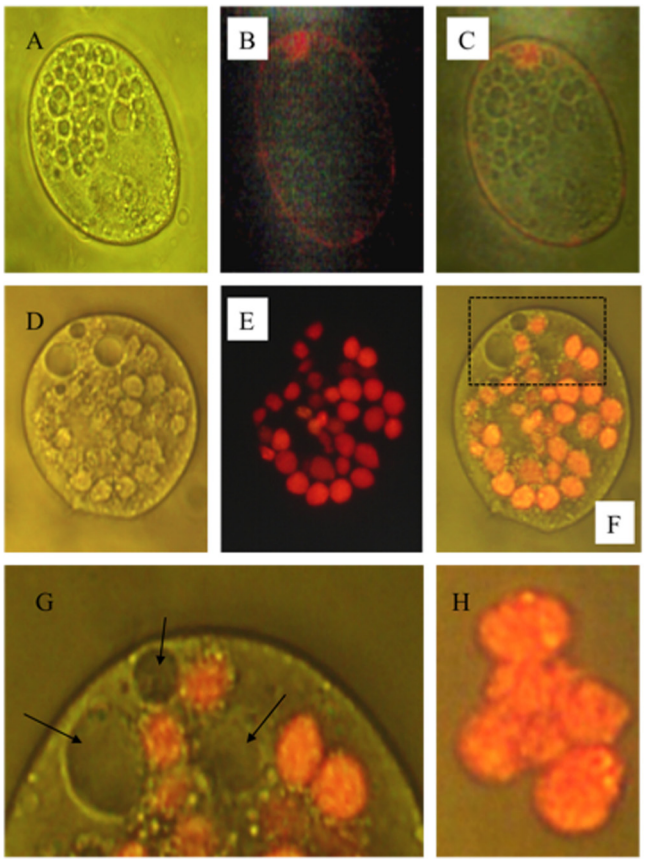

FIG 2 Selective accumulation of plasma membrane lipid relocated from the surface of ciliates to pellets laden with bacteria. The cultures of ciliates (TP) with environmental *L. pneumophila* (Lp926) (at an MOI of 10,000) were incubated with FM4-64FX dye for 24 h at 30°C in a normal atmosphere. (A to C) Representative images showing lipid located on the surface of ciliates immediately after incubation with bacteria. (A) Phase-contrast image. (B) Green color, fluorescent image. (C) Merge. Magnification, ×200. (D to G) Representative images showing lipid relocated on pellets laden with bacteria at 8 h after incubation. (D) Phase-contrast image. (E) Orange color, fluorescent image. (F) Merge. (G) Enlarged view of the square in F. Arrows indicate 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.

1-derived strain JR32 [wild type] [39]; and the null mutant [Dot/Icm translocation-defective mutant] of JR32 with mutations in the *dot/icm* genes encoding a type IV secretion system required for intracellular growth [mutant] [26]) was cultured on buffered charcoal yeast extract (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 a showerhead (Lp768 strain) or several cooling towers (the other strains, i.e., Lp920, Lp923, Lp924, and 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 included *Tetrahymena thermophila* inbred strain B [TIB; gift from Toshiro Sugai of Ibaraki University, Japan], *Tetrahymena thermophila* SB021 [TSB; gift from Tetuya Yomo of Osaka University, Japan], *Tetrahymena pyriformis* [TP; gift from Seiji Sonobe of Hyogo University, Japan], and *Tetrahymena* sp. [TS; gift from Yuji Tukii of Hosei University, Japan]; free-living amoebae included *Acanthamoeba castellani* [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]) 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 hemocytometers. The bacteria (1 ml; 10^5 to 10^9 CFU/ml) were equally mixed with ciliates (1 ml; 10^5 cells/ml) at multiplicities of infection (MOI) of 1 to 10,000 in a 24-well plate and then cultured for up to 7 days at 4, 15, or 30°C under a normal atmosphere. As a control, bacteria (1 ml; 10^5 to 10^9 CFU/ml) were also incubated without ciliates (1 ml; PAS alone). At several time points, samples were collected, and bacterial CFU (see "Assessment of bacterial culturability and viability" below), protozoa, and pellet numbers were monitored.

Morphological analysis of pellets. Either *E. coli* (clinical isolate) or *L. pneumophila* (Lp926) (1 ml; 10^9 CFU/ml) was equally mixed with ciliates (TP) (1 ml; 10^5 cells/ml) at an MOI of 10,000 in a 24-well plate and cultured for 2 days at 30°C under a normal atmosphere. After centrifugation (100 × *g* for 5 min), the morphology of pellets in the sediment was assessed by Gimenez staining (Nikken Biomedical Laboratory, Kyoto, Japan) and transmission electron microscopy (TEM). Gimenez staining was

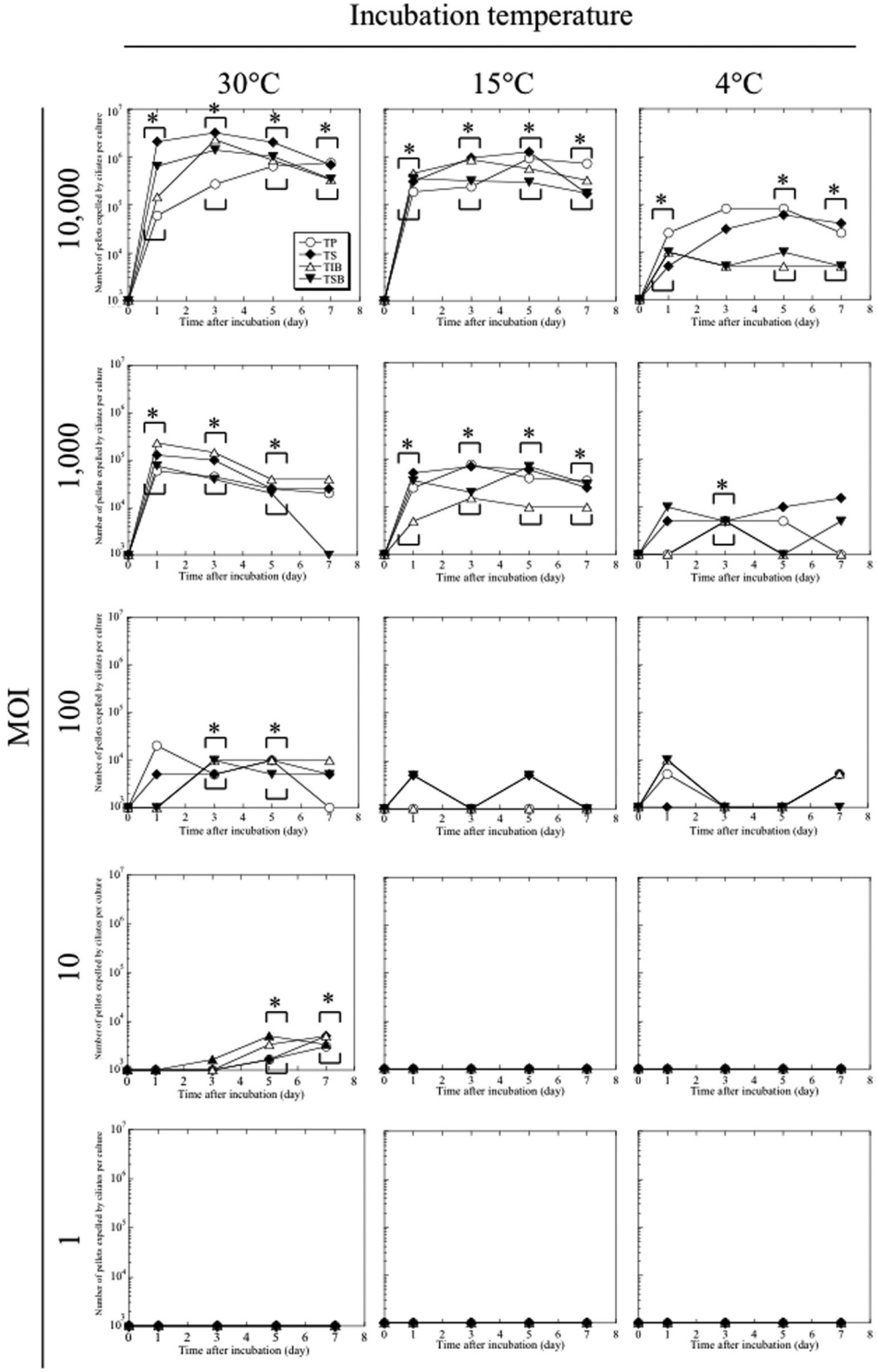

FIG 3 Changes in the number of pellets expelled from ciliates with environmental *L. pneumophila* under various conditions. *L. pneumophila* (Lp926) was mixed with ciliates (TP, TS, TIB, TSB) at an MOI of 1 to 10,000 and cultured for up to 7 days at 4, 15, or 30°C in a normal atmosphere. Each plot shows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

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 phosphate-buffered saline (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).

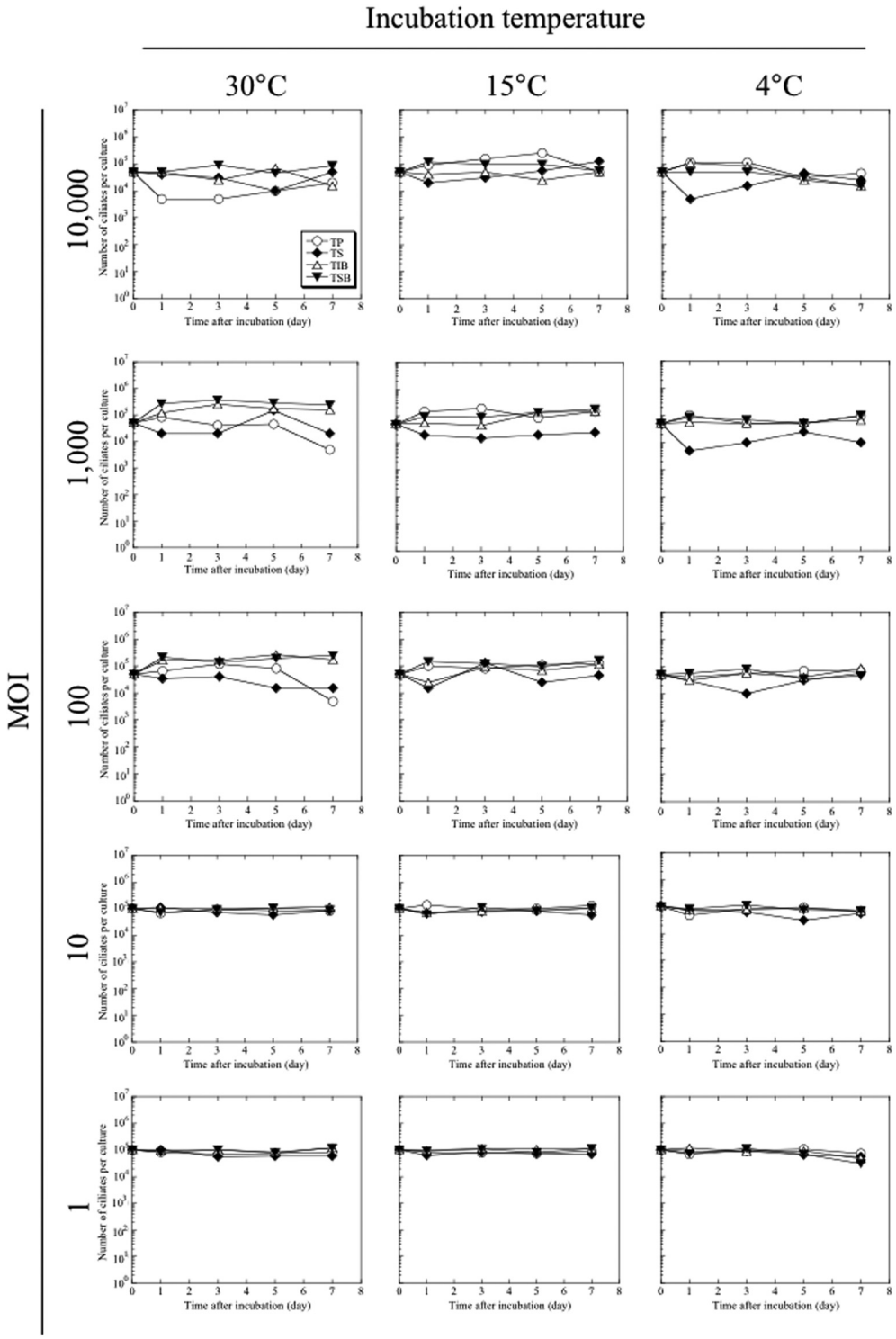

FIG 4 Changes in the number of ciliates when cultured with environmental *L. pneumophila* under various conditions. See the Fig. 3 legend for further specifics.

Assessment of lipid accumulation. To clarify the relocation of membrane lipid to the pellets, the bacteria (Lp926) (1 ml; 10^9 CFU/ml) were equally mixed with ciliates (TP) (1 ml; 10^5 cells/ml) in the presence of FM4-64FX dye (final concentration, 5 μg/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 24 h 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

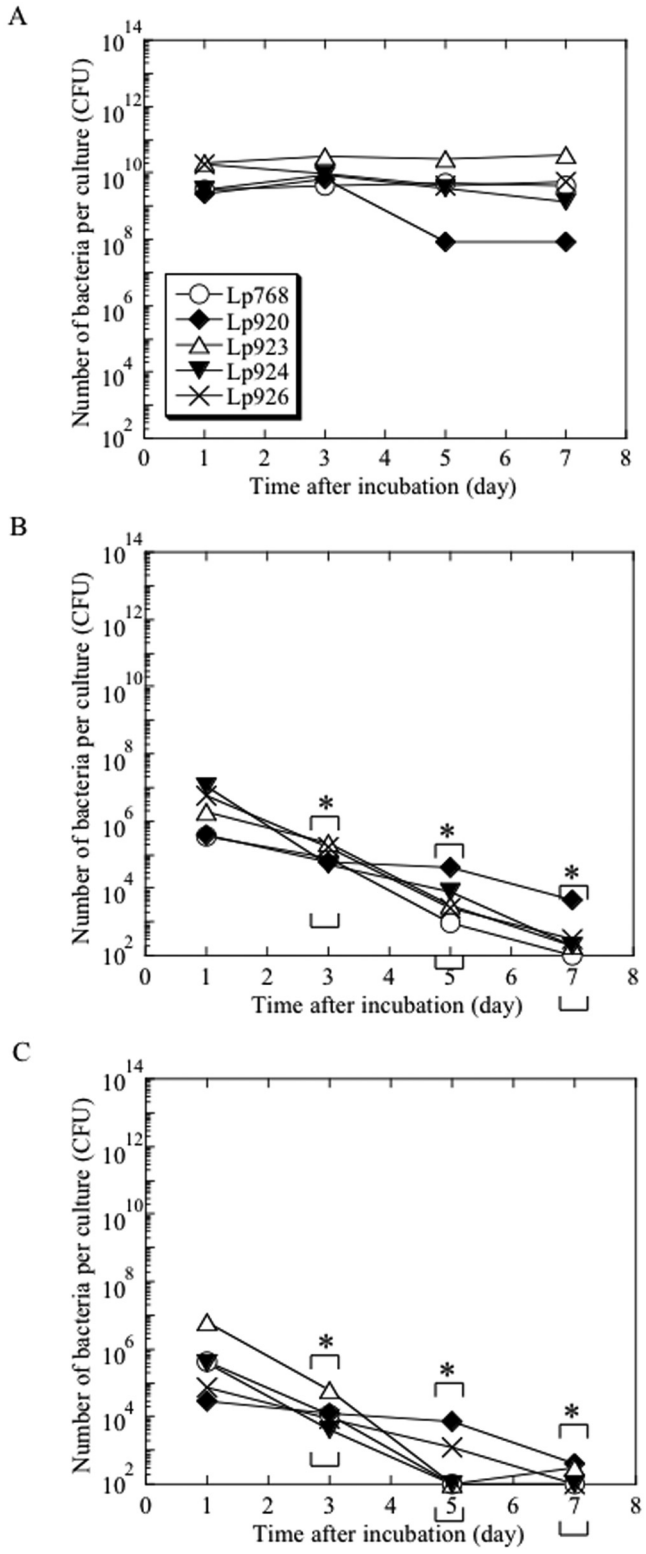

FIG 5 Changes in the culturability of environmental *L. pneumophila* in cultures without ciliates (A) or with ciliates (supernatant of pellet solution [B] or whole pellet solution including sediment [C]). *L. pneumophila* (Lp768, Lp920, Lp923, Lp924, and Lp926) was mixed without or with ciliates (TP) 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 performed by centrifugation (see "Enrichment of pellets" in Materials and Methods). Each plot shows an average number of CFU obtained from at least two experiments. The average value of plots at the same time point in parentheses was compared with the value immediately after incubation. *, $P < 0.05$ versus each value immediately after incubation.

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 (pellet solution), and the concentration was adjusted by counting on hemocytometers (see "Induction of pellets" above) and then used for the experiment below. As a control, the whole culture solution of the bacteria without ciliates (bacterial solution) was also treated in a similar way and used for the experiment below.

Assessment of bacterial culturability and viability. The number of *L. pneumophila* cells in culture with or without ciliates was assessed by a 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 number of stained, expelled pellets was 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 coculturing with amoebae. Resuscitation of *L. pneumophila* by coculturing with amoebae was performed according to the method previously described (39). In brief, the above-mentioned samples (100 μl of the pellet solution or bacterial solution) were added to axenic cultures of amoebae adjusted to 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 whether *L. pneumophila* (Lp920, Lp923, Lp924, Lp926, Lp768, JR32 wild type, JR32 Dot/Icm mutant) could grow in amoebae alone 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 the survival of ciliates, the number of ciliates newly cultured with enriched pellets was compared with that without pellets (see "Induction of pellets" above). The ciliates were adjusted to a concentration of 1 to 1,000 cells mixed with different amounts of pellet solution, from 1 μl (containing approximately 1,000 pellets) to 100 μl (containing approximately 100,000 pellets), and cultured in PAS (1 ml) on a 24-well plate for 3 days. As a control, the bacterial solution (1 to 100 μl), i.e., an equivalent amount of the pellet solution obtained from the culture of bacteria alone without ciliates (See "Enrichment of pellets" above), was also mixed with the ciliates and cultured.

Statistical analysis. The influence of the pellets on the survival of ciliates was analyzed by Fisher's exact test (two-way analysis of variance [ANOVA]; Statview, Abacus Concepts Inc., Piscataway, NJ). Comparison of bacterial or ciliate numbers was also performed with an unpaired *t* test (Statview, Abacus Concepts Inc.). A *P* value of <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 *L. pneumophila* was mixed with *E. coli*,

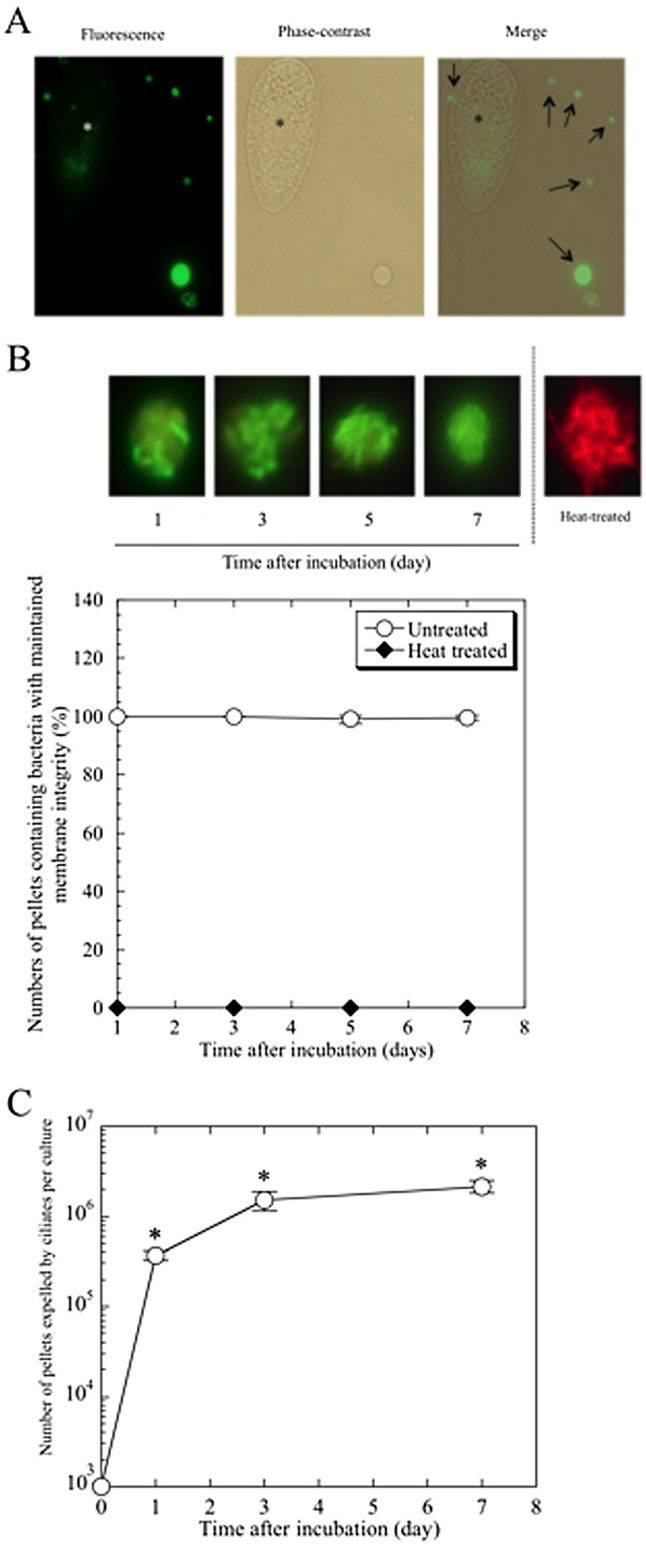

FIG 6 Representative images and change in the number of pellets showing viability of environmental *L. pneumophila* in pellets. *L. pneumophila* (Lp926) was mixed without or with ciliates (TP) at an MOI of 10,000 and cultured for up to 7 days at 30°C in a normal atmosphere. Bacterial membrane integrity 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. Top images, representative pellets with laden *L. pneumophila* with stable bacterial membrane integrity (green color) and heat-treated pellets laden with bacteria (80°C, 20 min) (red color). Magnification, ×1,000. The change indicates the percentage of pellets showing viability of environmental *L. pneumophila* in pellets with or without the heat treatment. The data shown represent the means ± standard deviations (SD) obtained from at least three independent experiments performed in triplicate. The numbers of stained, expelled pellets were estimated under a fluorescence microscope (see "Assessment of bacterial culturability and viability" in Materials and Methods). (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 data immediately (0 day) after incubation.

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 which is specific for plasma membrane lipid, FM1-43FX, could accumulate in pellets laden with *L. pneumophila* (Lp926). As expected, the fluorescent dye accumulated rapidly and surrounded the plasma membrane of the ciliates at 30 min or earlier after addition (Fig. 2A to C, orange color). At 8 h after incubation, the dye rapidly moved selectively to nascent pellets laden with bacteria (Fig. 2D to G); the arrows show representative vesicles not laden with bacteria and without fluorescence dye, suggesting that the rapid accumulation of membrane lipid is specific to vesicles harboring *L. pneumophila*. At 24 h, the bacterium-containing pellets that were formed in ciliates and had a fluorescence signal were expelled (Fig. 2H).

Optimal conditions for production of pellets. To determine the optimal conditions for the production of pellets, we monitored the numbers of pellets and ciliates under different culture conditions and temperatures 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 to 10^6 per culture. Thus, the production of pellets dramatically changed depending on MOI and culture temperature. With the use of different strains (*L. pneumophila* Lp768, Lp920, Lp923, and Lp924 strains), no difference in pellet production levels was observed (see Fig. S1 in the supplemental material).

Role of pellet expulsion in ciliate survival or replication in a coculture system. To assess whether pellet production in conjunction with the culturing of environmental *L. pneumophila* (Lp926) 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 differences in MOI or culture temperatures. Moreover, when we used different envi-

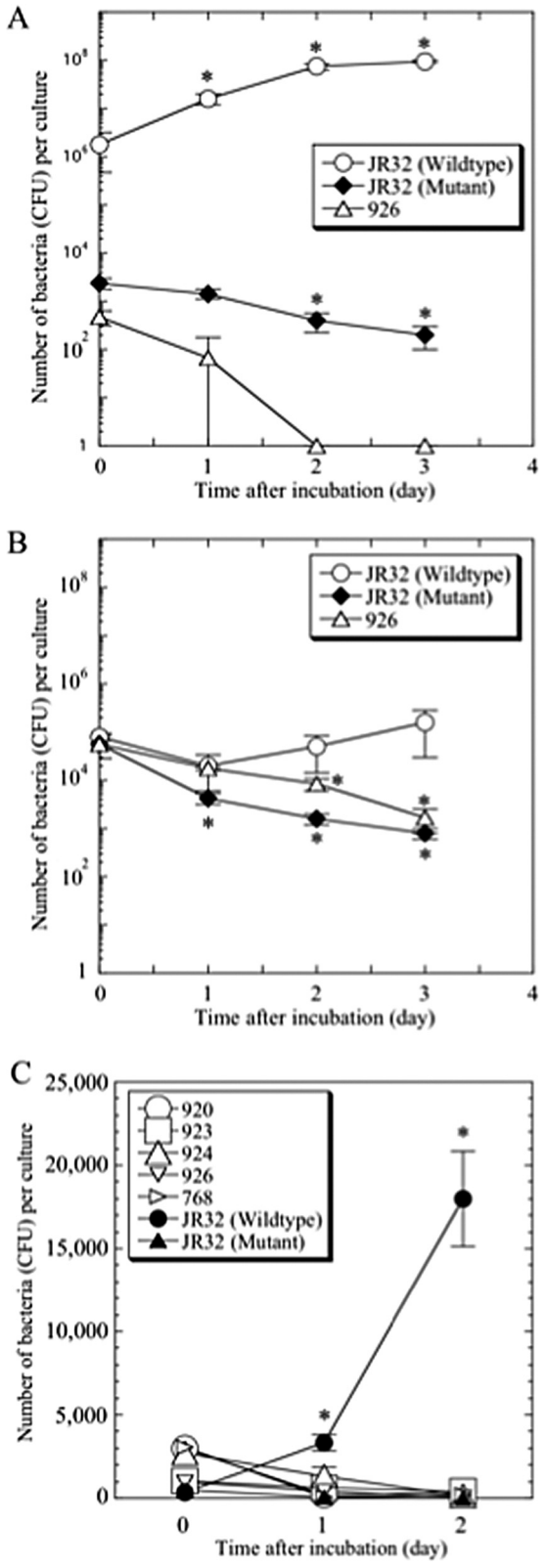

FIG 7 Resuscitation of *L. pneumophila* strains (JR32, mutant, Lp926) by coculturing with amoebae. (A and B) Pellet solution (A) or bacterial solution (B) was added in axenic cultures of amoebae adjusted at 10^5 cells in 1 ml of PYG or PAS prepared in 24-well plates and then incubated for up to 72 h.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

ronmental strains (*L. pneumophila* Lp768, Lp920, Lp923, and Lp924), no difference in ciliate numbers was found (see Fig. S2 in the supplemental material). The results suggest that the expulsion of pellets laden with environmental *L. pneumophila* may be required for the normal maintenance of growth and the survival of ciliates.

Survival of bacteria in membrane-wrapped pellets expelled by ciliates. We assessed whether the environmental *L. pneumophila* cells packaged in the pellets expelled into the culture supernatant were still viable, 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 bacterial membrane integrity in the non-heat-treated 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 was seen with the quantity of the pellets (Fig. 6B and C). Since it is well known that *Acanthamoeba* 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 cocultured with amoebae. As a result, the human isolate JR32, packed in the pellets, was resuscitated only by amoeba coculturing, depending on Dot/Icm expression (Fig. 7A and B), indicating that the human isolate was still alive in the egested pellets. Meanwhile, the environmental strains failed to proliferate in the test amoebae when they were cocultured with amoebae alone (Fig. 7C).

Effect of pellets in supporting growth and survival of ciliates. To assess the possible beneficial effect of the expulsion of pellets from ciliates cocultured with environmental *L. pneumophila* on the maintenance of ciliate growth and survival, the 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 were added to the cultures (pellet solution) compared to the growth with the addition of the simple bacterial culture (bacterial solution), although this effect was seen only with the addition of the pellets to low numbers of ciliates (Fig. 8). These 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 cocultured with either human isolate JR32 or its null mutant with mutations in *dot/icm* genes. We finally assessed whether the expulsion

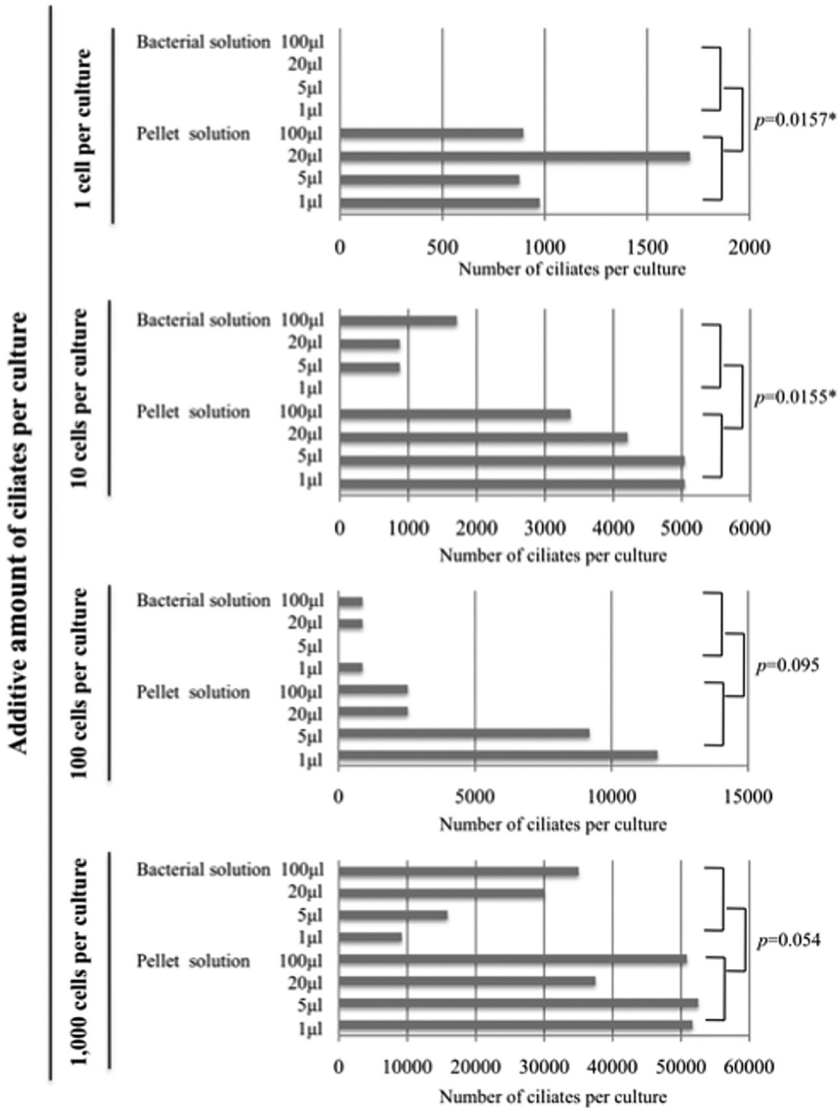

FIG 8 Effect of pellets expelled from ciliates incubated with environmental *L. pneumophila* (Lp926) on supporting the growth and survival of ciliates. The number of ciliates newly cultured with enriched pellets was compared with that without pellets. Each bar shows an average obtained from at least 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.

of pellets from ciliates cocultured with the human isolate *L. pneumophila* JR32 or its null mutant with mutations in *dot/icm* genes, which encode a type IV secretion system required for intracellular growth, can support ciliate survival. As expected, the pellet productions of human isolate JR32 and its null mutant occurred similarly, with maximum pellet production occurring 1 day postinfection (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 days after incubation (Fig. 9C). Furthermore, while the pellet of the null mutant worked for stockpiling food, the pellet of JR32 completely killed ciliates in coculture (Fig. 10). Thus, it appears that the pellets packaged in the human isolate are harmful for ciliate 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 rapidly and freely grow in 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 delivers 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

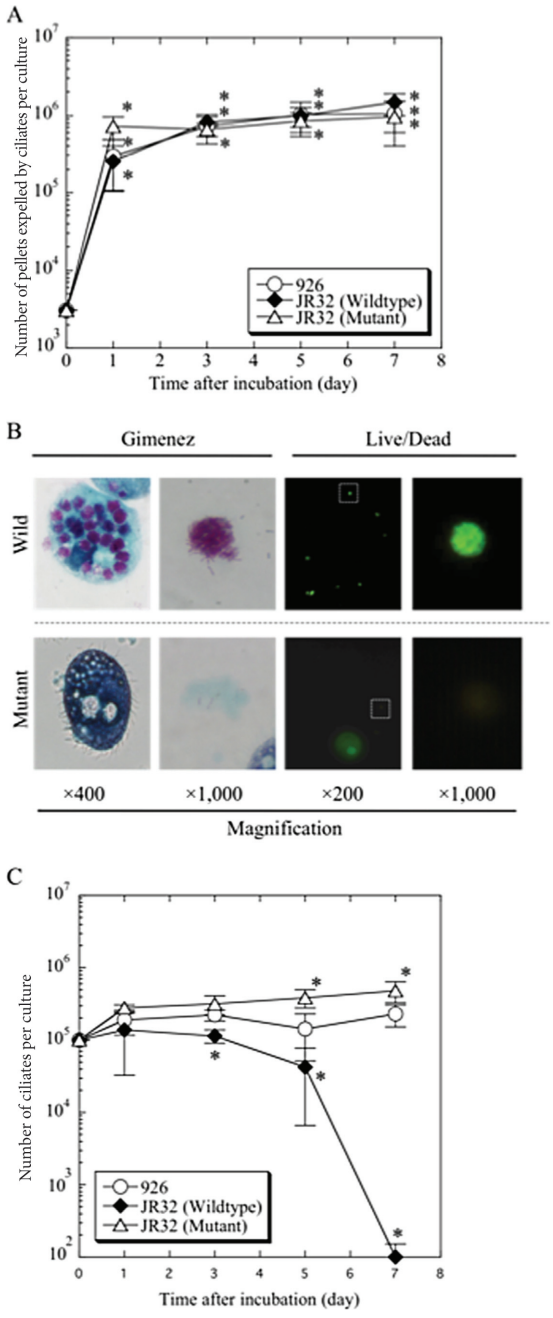

FIG 9 Characterization of pellets expelled from ciliates exposed with either human isolate JR32 or its null mutant with mutations in *dot*/*icm* genes. *L. pneumophila* (JR32, the mutant, Lp926) was mixed with ciliates (TP) at an MOI of 10,000, and cultured for up to 7 days at 30°C in a normal atmosphere. (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 data immediately (0 day) after incubation. (B) Gimenez and LIVE/DEAD representative images of expelled pellets from ciliates incubated with either JR32 (wild type) or the 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 data immediately (0 day) after incubation.

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 with *E. coli*, indicating that this packing and expulsion might be required for the 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, the expulsion of pellets provides an advantage for the feeding or survival of ciliates in a natural environment that lacks sufficient nutrients. To assess this hypothesis, we examined whether the 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 temperatures, 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 bacterium-laden pellets but not to vesicles without bacteria. This suggests that the mechanism for handling the bacteria properly may also be required for the rapid accumulation of lipid in the pellets.

As expected, the addition of pellets packed with environmental *L. pneumophila* to ciliate culture supported ciliate growth and survival, suggesting that ciliates briefly expel the pellets to ward off *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 bacterium-laden pellets may be crucial for maintaining ecosystems, and ciliates might need to use environmental *Legionella* as a food source, even though *Legionella* can have a strong cytotoxic effect against ciliates. The enhancement of ciliate growth occurred only when pellets were added to low numbers of ciliates. Although the exact reason remains unknown, it seems that culturing of high numbers of ciliates with bacteria (not pellets) resulted in the formation of new pellets that were able to support ciliate survival.

Contrary to our expectation, while the pellet of the mutant *Legionella* with mutations in *dot*/*icm* genes definitely worked for stockpiling food as well as the environmental *Legionella* used for this study, the pellet of a human JR32 wild-type isolate completely

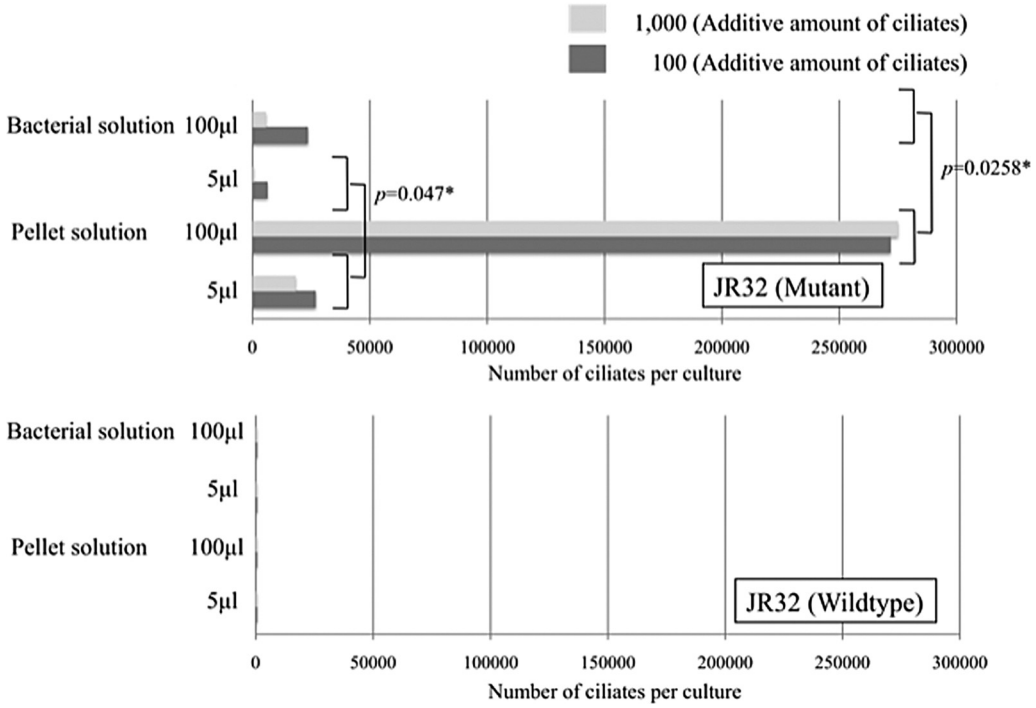

FIG 10 Effect of pellets expelled from ciliates incubated with human *L. pneumophila* isolates (JR32 [wild type] and the mutant) on supporting the growth and survival of ciliates. The number of ciliates newly cultured with enriched pellets was compared with that without pellets. Each bar shows an average obtained from at least two experiments. The influence of the pellets on the 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.

killed ciliates in coculture, suggesting that these pellets were inherently different. Unfortunately, at the present time, it is not clear how the JR32 wild-type 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 different cohorts of type IV effectors and that many effectors exhibit host-specific tropism (7, 8, 9, 10, 33, 38), the primary distinction likely consists of strain-specific differences in the effector molecules harbored by JR32 and the environmental strains via the coevolution of different *L. pneumophila* strains with their various protozoan hosts. In addition, although the environmental strains failed to proliferate in the test amoebae when they were cocultured with amoebae alone, the possibility that the environmental-strain pellets may be resuscitated by other host amoebae that are actually permissive for their replication cannot be ruled out. Thus, whether the environmental bacteria used for this study remain alive in the egested pellets needs to be clarified by using other suitable amoeba strains.

In conclusion, we demonstrated that while ciliates expel pellet-packaged environmental *L. pneumophila* for stockpiling food, the pellets packaged in the human isolate (JR32) are harmful to ciliate survival and are able to be resuscitated by amoeba coculturing; this may be of 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 between microorganisms that are present in natural environments but also a hint for the development of infection control of *L. pneumophila* as a human pathogen.

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