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Growth-Supporting Activity for *Legionella pneumophila* in Tap Water Cultures and Implication of Hartmannellid Amoebae as Growth Factors

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Photosynthetic cyanobacteria, heterotrophic bacteria, free-living amoebae, and ciliated protozoa may support growth of *Legionella pneumophila*. Studies were done with two tap water cultures (WS1 and WS2) containing *L. pneumophila* and associated microbiota to characterize growth-supporting activity and assess the relative importance of the microbiota in supporting multiplication of *L. pneumophila*. The water cultures were incubated in the dark at 35°C. The growth-supporting factor(s) was separated from each culture by filtration through 1-μm-pore-size membrane filters. The retentate was then suspended in sterile tap water. Multiplication of *L. pneumophila* occurred when both the retentate suspension and the filtrate from either culture were inoculated into sterile tap water. *L. pneumophila* did not multiply in tap water inoculated with only the filtrate, even though filtration did not reduce the concentration of *L. pneumophila* or heterotrophic bacteria in either culture. Growth-supporting activity of the retentate suspension from WS1 was inactivated at 60°C but unaffected at 0, 25, and 45°C after 30-min incubations. Filtration experiments indicated that the growth-supporting factor(s) in WS1 was 2 to 5 μm in diameter. Ciliated protozoa were not detected in either culture. Hartmannellid amoebae were conclusively demonstrated in WS2 but not in WS1. *L. pneumophila* multiplied in tap water inoculated with the amoebae (10^3/ml) and the 1-μm filtrate of WS2. No multiplication occurred in tap water inoculated with the filtrate only. Growth-supporting activity for *L. pneumophila* may be present in plumbing systems; hartmannellid amoebae appear to be important determinants of multiplication of *L. pneumophila* in some tap water cultures.

Legionella pneumophila, both with and without associated respiratory disease, has been isolated from plumbing systems of hospitals, hotels, and homes (17, 18, 21, 27). Heavy contamination has frequently been found on the internal surfaces of faucet and shower assemblies (27). Hot-recirculating-water systems which contain hot-water tanks with centrally or peripherally located heating elements appear to be important sources of *L. pneumophila* (27). Temperatures at the bottom of these tanks are often 25 to 42°C, a range conducive for multiplication of *L. pneumophila* in tap water (23, 28). Methods to control contamination by *L. pneumophila* in plumbing systems include hyperchlorination (13) and flushing with heated water (3). An improved understanding of the factors that govern multiplication of *L. pneumophila* in potable-water systems may lead to refinements in control procedures.

Recently, a method to study multiplication of *L. pneumophila* and associated microbiota in tap water was developed (23). In this system, *L. pneumophila* multiplies in tap water in the dark under aerobic but not anaerobic conditions, at temperatures of 25 to 37°C, at pH values of 5.0 to 9.2, and in both stationary and shake cultures. The sources of nutrients for multiplication of *L. pneumophila* under these conditions are not completely understood.

The dependence of *L. pneumophila* on amino acids to meet its carbon and nitrogen requirements (19) suggests that other living microorganisms provide essential nutrients for growth of *L. pneumophila* in aquatic habitats. *Flavobacterium breve* and other heterotrophic bacteria support satellite growth of *L. pneumophila* on an L-cysteine-deficient medium (25, 26). However, these bacteria alone fail to support multiplication of *L. pneumophila* in tap water (26). Photosynthetic cyanobacteria support multiplication of *L. pneumophila* under conditions of illumination (20). However, since tap water in plumbing systems is shielded from light, it is likely that other microorganisms support growth of *L. pneumophila* in this environment.

Protozoa may be responsible for supporting multiplication of *L. pneumophila* in drinking-water systems. Rowbotham (11, 12) first showed that free-living amoebae of the genera *Acanthamoeba* and *Naegleria* can be infected with *L. pneumophila*. *L. pneumophila* may be used as a food source for free-living amoebae, although some amoebal cultures become chronically infected and support growth of *L. pneumophila* (22). Anand et al. (1) demonstrated intracellular multiplication of *L. pneumophila* in *Acanthamoeba palestinensis*. A recent report (8) showed that an isolate of *Acanthamoeba* spp. supported multiplication of *L. pneumophila* in autoclaved tap water and that free-living amoebae are frequently present in drinking water systems. These findings suggest that free-living amoebae may be responsible for *L. pneumophila* contamination in plumbing systems. Fields et al. (6, 7) have also shown that *L. pneumophila* multiplies within the ciliated protozoan *Tetrahymena pyriformis* in sterile tap water and that this capacity may be associated with the virulence of *L. pneumophila*. The occurrence of

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ciliated protozoa in plumbing systems is not well characterized. However, ciliated protozoa and *L. pneumophila* have been recovered from a cooling tower at the site of an outbreak of legionellosis, and these protozoa supported intracellular multiplication of *L. pneumophila* (2).

This study describes growth-supporting activity for *L. pneumophila* in two tap water cultures and demonstrates that the growth-supporting activity in one of the cultures was provided by hartmannellid amoebae.

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MATERIALS AND METHODS

Water cultures. Two water stock cultures, designated WS1 and WS2, were used as sources of naturally occurring *L. pneumophila* and associated microbiota. WS1 was derived from a hot-water tank of a gymnasium and had been used in previous studies (14, 15, 23, 26); WS2 was derived from a domestic hot-water tank. Direct fluorescent-antibody testing (4) showed that WS1 and WS2 contained *L. pneumophila* serogroups 1 and 5, respectively. The stock cultures were maintained in the laboratory by periodic passage into sterile tap water as previously described (23). Tap water was obtained from our laboratory and sterilized by filtration through cellulosic nitrate membranes (Micro Filtration Systems, Dublin, Calif.) having a pore size of 0.20 μm. Portions of the stock cultures that were used as inocula for experiments were obtained when the growth of *L. pneumophila* was in the late exponential to early stationary phase. Water cultures used in the experiments described below were prepared by inoculation of 1-ml portions of suspensions derived from the water stock cultures into sterile tap water to provide a final volume of 100 ml. The water cultures were incubated in sterile 250-ml polypropylene bottles (Nalgene Labware Div., Nalgene/Sybro Corp., Rochester, N.Y.) for 1 month at 35°C in the dark in a room air incubator. Viable counts of *L. pneumophila* and nonlegionella bacteria were obtained as previously described (23) by using differential glycine-vancomycin-polymyxin B agar (24, 27) that contained 1% (wt/vol) alpha-ketoglutarate (5) and unsupplemented buffered charcoal-yeast extract agar (28), respectively.

Membrane filtration experiments. Polycarbonate membrane filters (Nuclepore Corp., Pleasanton, Calif.) were used in attempts to remove the growth-supporting factor(s) from tap water cultures. In the initial experiment, a 20-ml portion of WS1 was sequentially filtered through membranes with pore sizes of 12, 5, and 1 μm. Each filtrate was inoculated into a separate sterile tap water sample. An unfiltered portion of WS1 was similarly inoculated into sterile tap water and served as a positive growth control for *L. pneumophila*. In a subsequent experiment, filtrates of WS1 were prepared by using membranes having 5-, 3-, 2-, and 1-μm pore sizes and similarly evaluated for multiplication of *L. pneumophila*. Filtered and retained material was designated according to the pore size of the membrane used (1-μm filtrate, 1-μm retentate, etc.).

Exploitable tap water system. A technique was developed to separate the growth-supporting factor(s) from water cultures. Two 5-ml portions of WS1 were filtered through separate 25-mm polycarbonate filters, each with a pore size of 1 μm. A funnel-type polysulfone filter holder (Gelman Sciences, Inc., Ann Arbor, Mich.) was used to facilitate separation and suspension of the growth-supporting factor(s) from the membrane filters. The two portions of 1-μm filtrate were combined, and 5 ml was used to suspend the retentate on one of the membranes. The suspension of retentate was inoculated into sterile tap water. Also, unfiltered WS1 and the 1-μm filtrate were inoculated into separate sterile tap water samples and served as positive and negative growth controls, respectively.

Temperature characterization. We measured the temperature stability of the growth-supporting factor(s) by preparing a suspension of 1-μm retentate from WS1 as described above and subjecting it to different temperatures. Portions (2 ml) of the suspension were placed into sterile glass test tubes and incubated for 30 min at room temperature (25°C) and at 60 and 100°C in water baths. After equilibration to room temperature, the suspensions were inoculated into sterile tap water. A 1-ml sample of the 1-μm filtrate was added to each culture to provide a source of viable *L. pneumophila* and other microorganisms. Positive and negative growth controls for *L. pneumophila* included sterile tap water samples inoculated with WS1 and the 1-μm filtrate, respectively.

A second temperature experiment was conducted to determine the lower temperature limit for stability of the growth-supporting factor(s). Portions (2 ml) of the retentate suspension were incubated for 30 min at 45°C in a water bath, at 0°C in an ice bath, and at −20 and −70°C in freezers. After equilibration to room temperature, the samples were inoculated into separate sterile tap water samples. After storage for 1 week at −20 and −70°C, equal portions of the retentate suspension were thawed at room temperature and inoculated into sterile tap water. Cultures initiated with the thawed suspension of retentate were also inoculated with the 1-μm filtrate from WS1.

Isolation of protozoa. The water stock cultures were examined for the presence of ciliated protozoa and free-living amoebae as previously described (7, 10). Before the samples were cultured for amoebae, 100- and 250-ml portions of WS1 and WS2, respectively, were filtered through 1.2-μm-pore-size membrane filters (Millipore Corp., Bedford, Mass.). The filters were cut in half with sterile scissors, and the pieces were inverted onto non-nutrient agar plates that had been seeded with heat-killed *Enterobacter aerogenes*. The cultures were incubated aerobically at 35°C and examined daily with an inverted microscope for 1 week for the presence of free-living amoebae. Amoebal isolates were submitted to a private consulting laboratory (Rescon Associates, Inc., Royal Oak, Md.) for identification. Identifications were based on characteristics given in the key to amoebae by Page (10).

Our early inexperience with free-living amoebae limited examination of *E. aerogenes*-seeded agar plates to detection of rapidly growing, relatively large amoebae which produced visible tracks in the bacterial lawn (e.g., *Acanthamoeba* spp. and *Naegleria* spp.). Later studies with plates inoculated with WS2 were closely examined for the smaller, more slowly growing hartmannellid amoebae.

Amoeba supplementation experiment. Hartmannellid amoebae recovered from WS2 were evaluated to determine whether they supported multiplication of naturally occurring *L. pneumophila*. The amoebal culture was maintained by transferring pieces of agar containing cysts and trophozoites to agar plates seeded with heat-killed *E. aerogenes*. After incubation for 18 days at 35°C, amoebae were harvested from the plates by addition of sterile tap water and scraping with a sterile bent-glass rod. The concentration of amoebae was determined with a hemacytometer, and the suspension was diluted with sterile tap water. The ratio of trophozoites to cysts in the suspension was 9.4:1. A negative-control

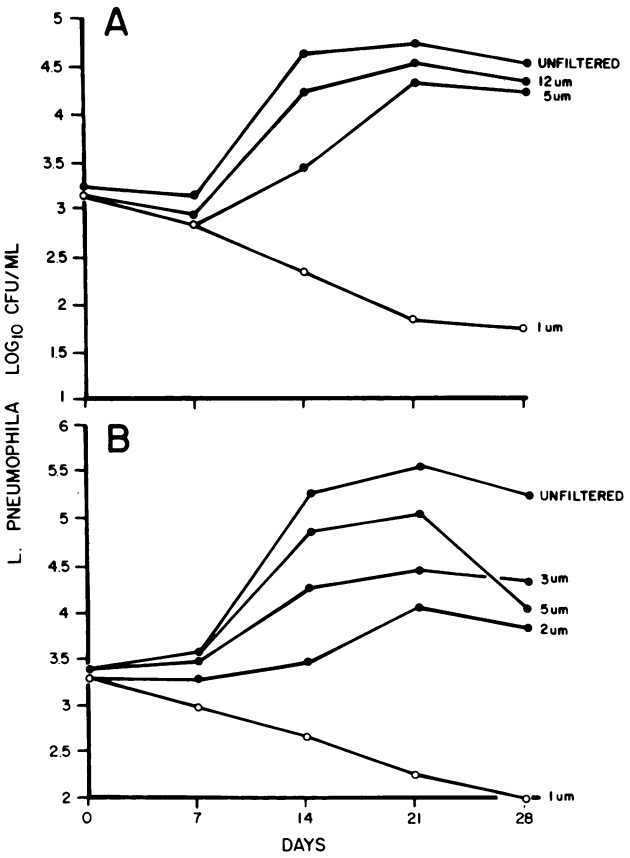

FIG. 1. Effect of filtration on growth-supporting activity for *L. pneumophila*. Panels A and B represent different experiments.

suspension containing no amoebae was similarly prepared from heat-killed *E. aerogenes*-seeded agar plates. The retentate suspension and 1-μm filtrate were prepared from WS2 as described above. The 1-μm filtrate was added to each of two samples of sterile tap water. These samples were inoculated with either the diluted amoebal suspension to yield 10^3 amoebae per ml or the negative-control amoebal suspension. Additional controls included sterile tap water samples inoculated with either WS2 or the retentate suspension (positive control) or the 1-μm filtrate of WS2 (negative control). In addition to these cultures, which were incubated in polypropylene bottles, a culture was incubated in a plastic tissue culture flask (Costar, Cambridge, Mass.). This flask was inoculated with the 1-μm filtrate, the amoebal suspension, and sterile tap water. This culture was included to compare growth of *L. pneumophila* in association with amoebae in different types of vessels.

RESULTS

Growth-supporting activity of WS1. Filtration experiments were performed to assess multiplication of *L. pneumophila* in tap water cultures from which growth-supporting activity for *L. pneumophila* may have been removed. The initial experiment showed that *L. pneumophila* multiplied in the tap water samples that had been inoculated either with the unfiltered control or with the 12- or 5-μm filtrate of WS1 (Fig. 1A). The rate of growth of *L. pneumophila* was highest in the sample inoculated with the unfiltered water culture, intermediate in the sample inoculated with the 12-μm filtrate, and lowest in the sample inoculated with the 5-μm filtrate. However, the yields of *L. pneumophila* growth were similar in these samples, i.e., 1.2 to 1.4 log CFU/ml. Multiplication of *L. pneumophila* was not detected in the sample inoculated with the 1-μm filtrate (Fig. 1A). The concentration of *L. pneumophila* in this sample gradually declined over the 28-day incubation period. The concentrations of *L. pneumophila* and of the nonlegionella bacteria present in WS1 were not appreciably affected by the sequential-filtration process (Table 1). These findings suggested that WS1 contained growth-supporting activity for *L. pneumophila* and that the factor(s) providing this activity was removed from WS1 by filtration through 1-μm-pore-size membranes. The inability of the 1-μm filtrate to support multiplication of *L. pneumophila* indicated that the heterotrophic bacteria in WS1 did not support growth of *L. pneumophila*.

TABLE 1. Concentrations of *L. pneumophila* and nonlegionella bacteria in water stock cultures and 1-μm filtrates

Bacterium	Log CFU/ml			
	WS1		WS2	
	Unfiltered	1-μm filtrate	Unfiltered	1-μm filtrate
L. pneumophila	5.1	4.9	5.8	5.7
Nonlegionella	6.0	6.1	6.2	6.2

(i) Size of growth factor(s). A subsequent filtration experiment confirmed the findings described above and more precisely characterized the size of the factor(s) responsible for growth-supporting activity in WS1 (Fig. 1B). A proportionate reduction in both the growth rate and the yield of *L. pneumophila* occurred as the pore sizes of the membranes used to prepare the filtrates decreased from 5 to 2 μm. Multiplication of *L. pneumophila* was not detected in the sample inoculated with the 1-μm filtrate. These results indicated that the factor(s) responsible for multiplication of *L. pneumophila* in WS1 had an approximate size of 2 to 5 μm.

(ii) Separation of activity. Filtration of WS1 with the 1-μm polycarbonate membrane filters permitted separation of growth-supporting activity from the water culture (Fig. 2). Growth-supporting activity was found in the water stock culture which served as a positive control (unfiltered inoculum) and in the retentate following suspension in the 1-μm filtrate. Growth-supporting activity was not detected in the 1-μm filtrate. Similar results were obtained after a subsequent experiment with WS2. In addition, we found that the 1-μm retentate could be suspended in sterile tap water rather than the 1-μm filtrate and retain growth-supporting activity.

(iii) Temperature stability of factor(s). Figure 3 shows the effect of temperature on the growth-supporting activity of the 1-μm retentate suspension from WS1. Growth-supporting activity was retained after exposures to −20, 0, and 45°C but abrogated after exposures to −70, 60, and 100°C. After the 30-min incubations at −20 and −70°C, we observed that the sample placed at −20°C was only partially frozen but that the sample placed at −70°C was completely frozen. Growth-supporting activity was eliminated when the retentate suspension was frozen for 1 week at −20°C (results not shown). Thus, growth-supporting activity appeared to be adversely affected by freeze-thaw processes and temperatures of 60°C or higher.

Effect of protozoa. Attempts were made to recover protozoa from the water stock cultures. Ciliated protozoa and free-living amoebae belonging to the genus *Acanthamoeba*

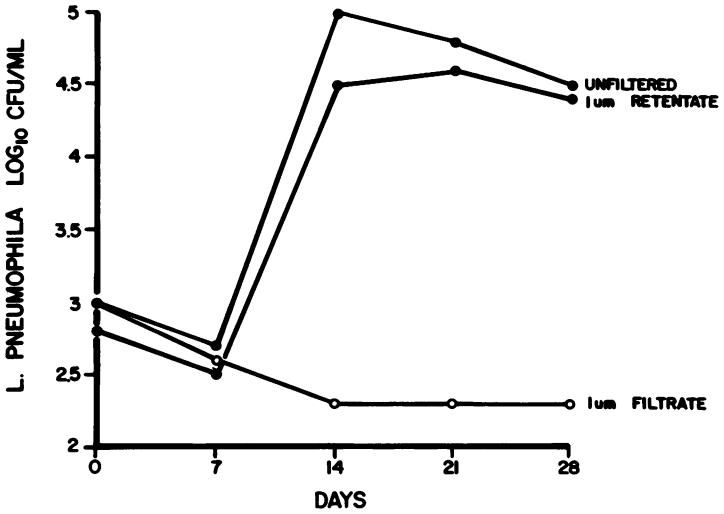

FIG. 2. Separation from WS1 of factor(s) supporting growth of *L. pneumophila*.

or *Naegleria* were not detected in either WS1 or WS2. However, unidentified hartmannellid amoebae were initially isolated by the laboratory at the Centers for Disease Control (B. S. Fields, G. N. Sanden, J. M. Barbaree, W. E. Morrill, R. M. Wadowsky, E. H. White, and J. C. Feeley, Curr. Microbiol., in press) from WS2. Subsequently, T. K. Sawyer, Rescon Associates, Inc., observed a variety of amoebal forms on the heat-killed *E. aerogenes*-seeded agar plates which had been inoculated with WS2 (personal communication). Two predominating forms were identified as *Hartmannella vermiformis* and *Hartmannella cantabrigiensis*. Relatively small numbers of a third form were also detected, but these could not be definitively identified. The latter form was characterized as a long amoeba, up to 57 μm in length, with some organisms having length-to-width ratios of 13:1. As described above, methods used on WS1 were unsuitable for detection of hartmannellid amoebae.

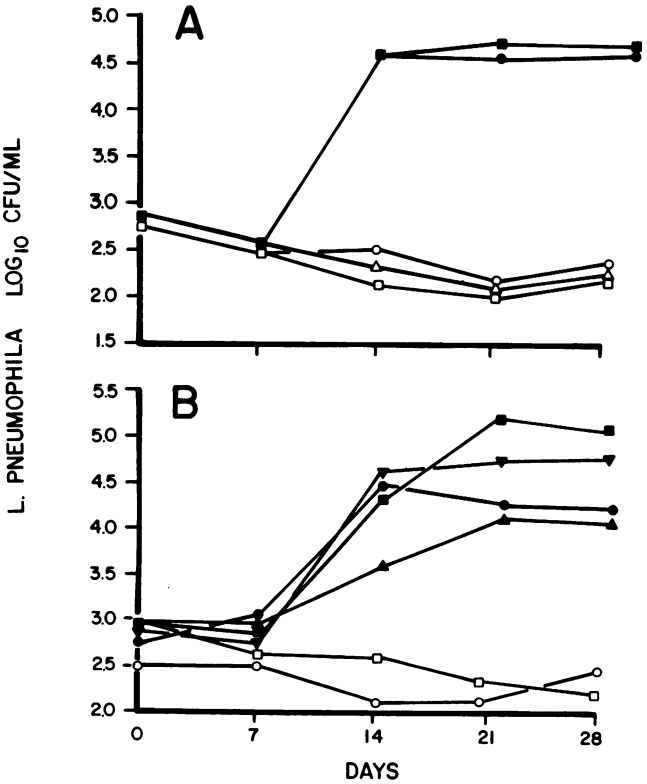

FIG. 3. Effect of temperature on growth-supporting activity in WS1 for *L. pneumophila*. Panels A and B represent different experiments. Symbols: ●, unfiltered; ○, 1-μm filtrate; (A) ■, 25°C; □, 60°C; △, 100°C; (B) ■, −20°C; ▼, 45°C; ▲, 0°C; □, −70°C.

Figure 4 shows the effect of the free-living amoebae on the multiplication of naturally occurring *L. pneumophila* in tap water. Naturally occurring *L. pneumophila* multiplied in tap water that had been inoculated both with the 1-μm filtrate of WS2 and with the amoebae, but growth was not detected in tap water inoculated with the 1-μm filtrate and the negative-control amoebal suspension. Multiplication of *L. pneumophila* in the presence of amoebae appeared slightly higher in the tissue culture flask than in the polypropylene bottle. The improved growth may have been related to the larger surface area provided at the bottom of the tissue culture flask. The control samples yielded predictable results; multiplication of *L. pneumophila* occurred in tap water samples inoculated with either WS2 or the retentate suspension of WS2 (positive control) but not in the sample inoculated with the 1-μm filtrate (negative control). After 1 month of incubation, amoebae were detected in the cultures which yielded multiplication of *L. pneumophila*; amoebae were not recovered from the culture in which there was no multiplication of *L. pneumophila*. The concentrations of *L. pneumophila* and the nonlegionella bacteria which were present in WS2 were unaffected by filtration through 1-μm-pore-size filters (Table 1). These results indicate that the free-living amoebae could replace the growth-supporting activity for *L. pneumophila* in WS2.

DISCUSSION

L. pneumophila multiplies in tap water in association with other microorganisms (28). Although cocultivation experiments show that cyanobacteria, ciliated protozoa, and free-living amoebae support growth of *L. pneumophila* (1, 2, 6, 7, 11, 12, 20), evidence suggesting that these microorganisms support multiplication of *L. pneumophila* in potable water

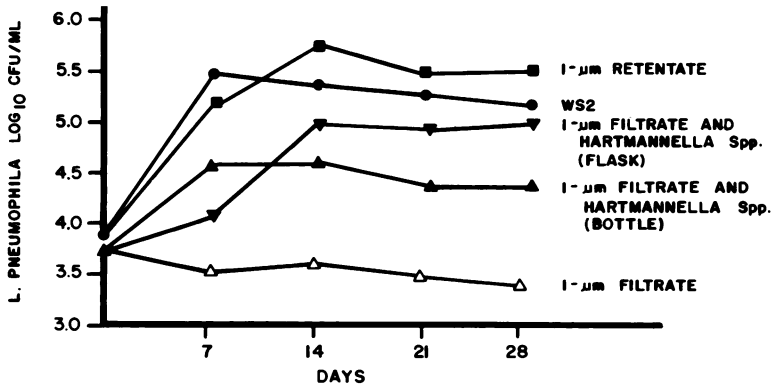

FIG. 4. Multiplication of naturally occurring *L. pneumophila* in association with *Hartmannella* spp.

and, therefore, are responsible for high levels of contamination in plumbing systems is limited.

Several of our findings provide evidence that hartmannellids may contribute to *L. pneumophila* contamination in plumbing systems by providing growth-supporting activity in potable-water environments. First, growth-supporting activity was detected in two tap water cultures. This activity could be removed from each culture by filtration through 1-μm-pore-size membrane filters. Membranes with this pore size are commonly used for recovery of amoebae from aquatic environments. Second, the factor(s) responsible for growth-supporting activity in one of the water cultures, WS1, was about 2 to 5 μm in diameter and inactivated by exposure to a temperature of 60°C. Although *Acanthamoeba* spp. and *Naegleria* spp. were not recovered from this culture, the presence or absence of *Hartmannella* spp. was not determined. These findings suggest that the factor(s) may be viable microorganisms that include hartmannellid amoebae. Third, free-living amoebae identified as *Hartmannella* spp. were recovered from WS2. The amoebae replaced growth-supporting activity in the culture. Fourth, growth-supporting activity of other microorganisms was excluded. Ciliated protozoa were not recovered from either tap water culture. Heterotrophic nonlegionella bacteria present in both water cultures readily passed through the 1-μm-pore-size membrane filters and did not support multiplication of *L. pneumophila* when the 1-μm filtrates were inoculated into sterile tap water. Incubation of the water cultures in the dark eliminated involvement of cyanobacteria as growth-supporting factors, since these bacteria require illumination to support growth of *L. pneumophila* (20).

Failure to detect amoebae in WS1 may have resulted from our early inexperience with the morphology of small free-living amoebae. When isolation was attempted from this water culture, we were looking mainly for typical *Acanthamoeba* spp. which form visible tracks in the lawn of *E. aerogenes*-seeded agar plates. These tracks were not formed by the hartmannellid amoebae recovered from WS2. In addition, growth of *Hartmannella* spp. on the plates was much slower than we had previously observed for *Acanthamoeba* spp. Unfortunately, WS1 is not available for further study.

The test samples used in the supplementation experiment contained *H. vermiformis*, *H. cantabrigiensis*, and possibly a rare unidentified form. It is unlikely that the unidentified form was inoculated into the test samples after dilution of the stock amoebal suspension, since very few of these forms were seen on the stock plate. Also, only hartmannellids were recovered from the amoeba-supplemented water cultures. We have not determined the relative importance of these amoebae in supporting multiplication of *L. pneumophila* in tap water. This evaluation must await the difficult and time-consuming process of purifying and cloning the amoebal isolates. These studies are currently in progress. A recent study has shown that unidentified hartmannellids recovered from WS2 support intracellular multiplication of *L. pneumophila* (Fields et al., submitted).

An application of our results would be the development of an in vitro tap water cultivation system consisting of amoebae, *L. pneumophila*, and non-*Legionellaceae* bacteria. Our current working hypothesis regarding the ecology of *L. pneumophila* in plumbing systems is that the non-*Legionellaceae* bacteria serve as food sources for free-living amoebae, which provide an intracellular environment for the multiplication of *L. pneumophila*. This sytem could be used to evaluate the use of disinfectants, or modification of the concentration of metals or pH (14, 16), for control of contamination by *L. pneumophila* in environments such as plumbing systems, cooling towers, and ice machines. It is likely that the intracellular milieu provided by amoebae for growth of *L. pneumophila* (1; Fields et al., submitted) protects *L. pneumophila* from these control measures. Kuchta et al. (9) reported that naturally occurring *L. pneumophila* organisms obtained from a tap water culture were much more resistant to chlorine than were agar-maintained stock cultures. It is possible that free-living amoebae or other microorganisms that were present in the water culture used in this study provided added protection to the naturally occurring *L. pneumophila*. Our finding that the growth-supporting factor(s) for *L. pneumophila* can be separated from tap water cultures should facilitate future studies comparing the activity of disinfectants and other control strategies on naturally occurring versus agar-maintained *L. pneumophila*.

Our study demonstrated growth-supporting activity for *L. pneumophila* in two tap water cultures derived from hot-water tanks. In addition, hartmannellid amoebae were shown to substitute for growth-supporting factor(s) in one of the cultures. Thus, hartmannellid amoebae may be important protozoans in the ecology of *L. pneumophila* in hot-water plumbing systems.

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