Factors Influencing Survival of *Legionella pneumophila* Serotype 1 in Hot Spring Water and Tap Water

Akira Ohno,^{1*} Naoyuki Kato,² Koji Yamada,³ and Keizo Yamaguchi¹

Departments of Microbiology¹ and Chemistry,² Toho University School of Medicine, Tokyo, and Department of Chemistry, Faculty of Science, Toho University, Chiba,³ Japan

Received 10 October 2002/Accepted 13 February 2003

The factors involved in the survival of *Legionella pneumophila* in the microcosms of both hot spring water and tap water were studied by examining cultivability and metabolic activity. *L. pneumophila* could survive by maintaining metabolic activity but was noncultivable in all microcosms at 42°C, except for one microcosm with a pH of <2.0. Lower temperatures supported survival without loss of cultivability. The cultivability declined with increasing temperature, although metabolic activity was observed at temperatures of up to 45°C. The optimal range of pH for survival was between 6.0 and 8. The metabolic activity could be maintained for long periods even in microcosms with high concentrations of salt. The cultivability of organisms in the post-exponential phase in a tap water microcosm with a low inoculum size was more rapidly reduced than that of organisms in the exponential phase. In contrast, the loss of cultivability in microcosms of a high inoculum size was significant in the exponential phase. Random(ly) amplified polymorphic DNA analysis of microcosms where cultivability was lost but metabolic activity was retained showed no change compared to cells grown freshly, although an effect on the amplified DNA band pattern by production of stress proteins was expected. Resuscitation by the addition of *Acanthamoeba castellanii* to the microcosm in which cultivability was completely lost but metabolic activity was maintained was observed only in part of the cell population. Our results suggest that *L. pneumophila* cell populations can potentially survive as free organisms for long periods by maintaining metabolic activity but temporarily losing cultivability under strict environments and requiring resuscitation by ingestion by amoebas.

Legionella pneumophila, the organism that causes Legionnaires' disease and Pontiac fever, is a ubiquitous bacterium in natural or man-made aqueous environments and requires an intracellular environment of free-living amoebas for its replication (19). However, this pathogen is also known to survive as a free organism for long periods in low-nutrient environments under appropriate conditions (14, 15). Bacterial populations released in an aqueous environment are frequently exposed to stresses due to limitations and changes in nutrient availability, temperature, salinity, oxygen, and pH. To adapt to such a stressful environment, bacteria often enter a "temporarily noncultivable state," in which they regulate cell differentiation to adapt to such stresses and then resuscitate when environmental conditions become favorable for growth. This physical change is generally referred to as "viable but noncultivable," and some bacteria use this strategy (12). *L. pneumophila* cell populations that entered a noncultivable state in tap water were also reportedly resuscitated by injection into embryonated eggs (9) or by passage through *Acanthamoeba castellanii* (18).

Japan is an eminently volcanic country, and hot springs are widespread. Japanese people enjoy a culture of bathing in hot springs, and recently many hotels have been developed in hot spring resorts to capitalize on the Japanese passion for this activity. This tendency, however, has inevitably caused an exhaustion of sources of hot spring water, and therefore filtration

and circulation systems for bath water have been introduced in many facilities. The introduction of such systems has provided opportunities for *L. pneumophila* with free-living amoebas to propagate inside filters or distributing pipes, with outbreaks of Legionnaires' disease occurring that originated in hot spring water (21).

Hot spring water exhibits very diverse chemical components, pH levels, and temperatures compared to other natural freshwaters, and it is therefore important to clarify how *L. pneumophila* planktonic progeny that escape from lysed amoeba can survive in hot spring water. Furthermore, conventional methods for the detection of *L. pneumophila* in the natural environment include the cultivable cell count by a plating method. In the present study, the effects of inoculum size, growth phase, temperature, pH, and conductivity on long-term survival of *L. pneumophila* planktonic cells in hot spring and tap water from various sources were investigated, particularly in relation to a temporarily noncultivable state.

MATERIALS AND METHODS

Bacterial strains. *L. pneumophila* serogroup 1 Suzuki was used throughout the study. Cells were grown to an exponential phase (optical density at 630 nm = 0.15 to 0.3) or to a post-exponential phase (overnight growth) with vigorous aeration at 35°C in BYE broth [*N*-(2-acetamido)-2-aminoethanesulfonic acid (Sigma, St. Louis, Mo.)]-buffered yeast extract broth supplemented with 0.4 mg of L-cysteine and 0.135 mg of ferric nitrate ml⁻¹ (3). The change to the post-exponential phase was confirmed by shortening of the rod shape of the bacteria and the appearance of motility under the phase-contrast microscope.

Preparation of microcosms. Each cell of an exponential phase and a post-exponential phase were collected by centrifugation at 6,000 × *g* for 15 min. These samples were washed three times in sterilized water and adjusted to ca. 10¹⁰ CFU ml⁻¹ and 10⁸ CFU ml⁻¹. Hot spring water and tap water were used as microcosm samples. The 20 samples of hot spring water (samples A to T) were collected

* Corresponding author. Mailing address: Department of Microbiology, Toho University School of Medicine, 5-21-16 Omori-Nishi, Ota-Ku, Tokyo 143-8540, Japan. Phone: 81-3-3762-4151, ext. 2396-7. Fax: 81-3-5493-5415. E-mail: akira@med.toho-u.ac.jp.

TABLE 1. Chemical characteristics, pH values, and conductivities of hot spring waters used in this study

Sample	Chemical content	pH	Conductivity ($\mu\text{S}/\text{cm}$)
A	$\text{Na}-\text{CO}_3\cdot\text{HCO}_3$	9.76	128
B	$\text{Na}-\text{HCO}_3$	8.42	307
C	$\text{Na}-\text{SO}_4\cdot\text{Cl}$	8.68	3,580
D	$\text{Na}-\text{Cl}\cdot\text{SO}_4$	8.62	896
E	$\text{Na}-\text{SO}_4$	8.29	2,280
F	$\text{Ca}-\text{SO}_4$	8.70	2,790
G	$\text{Na}-\text{SO}_4\cdot\text{Cl}\cdot\text{HCO}_3$	9.55	656
H	$\text{Ca}\cdot\text{Na}-\text{Cl}$	7.95	18,550
I	$\text{Ca}\cdot\text{Na}-\text{Cl}$	7.67	26,800
J	$\text{Ca}\cdot\text{Na}-\text{SO}_4$	8.88	1,695
K	$\text{Ca}\cdot\text{Na}-\text{SO}_4$	8.86	1,174
L	$\text{Na}-\text{SO}_4\cdot\text{Cl}$	8.93	387
M	$\text{Ca}\cdot\text{Na}-\text{SO}_4\cdot\text{Cl}$	8.08	826
N	$\text{Ca}\cdot\text{Na}-\text{SO}_4$	8.72	1,286
O	$\text{Ca}\cdot\text{Na}-\text{SO}_4$	7.68	66
P	$\text{Na}-\text{HCO}_3$	6.97	220
Q	$\text{Na}\cdot\text{Ca}-\text{Cl}$	6.83	26,700
R	$\text{Na}-\text{Cl}\cdot\text{HCO}_3$	8.05	390
S	$\text{Na}-\text{Cl}$	9.17	937
T	$\text{Al}-\text{SO}_4\cdot\text{H}_2\text{S}$	1.6	8,000

from several areas of Japan and filtered through a 0.2- μm -pore-size filter. The chemical constituents, pH values, and electronic conductivities of these hot spring water samples are given in Table 1. Then, 1 ml of bacterial suspension with 10^8 CFU ml^{-1} adjusted to the exponential phase was added to 100 ml of each sample in a sterilized disposable 150-ml plastic flask, followed by incubation at 42°C. Hot spring water sample K, a sample of the typical hot spring type in Japan, was used to examine the effects of temperature (42, 45, 50, and 55°C at pH 8.0) and pH (2.0, 5.0, 6.0, 8.0, and 10.0 at 42°C) on bacterial survival. The pH was adjusted with 0.1 N hydrochloric acid and 0.1 N sodium hydroxide. Two kinds of hot spring water were used to study the effects of conductivity on survival. These hot spring waters were diluted at ratios of 1:10 and 1:100 with distilled water, respectively. The bacterial suspension (as described above) was also inoculated into these samples and incubated at 42°C. Tap water was obtained from a tap at the Toho University School of Medicine and autoclaved at 121°C for 30 min. Then 100 ml of tap water was added to each of 12 sterilized disposable 150-ml plastic flasks.

One-ml aliquots of bacterial cell suspension (inoculum size of 10^{10} and 10^8 CFU ml^{-1} of the exponential phase and post-exponential phase, respectively) were inoculated into each flask with tap water, respectively. These microcosms were maintained at 25, 35, and 42°C.

Cells and viability enumeration. Cultivable counts in microcosms were monitored periodically by calculating the average number of colonies on duplicate plates (BCYE α agar; *Legionella* agar base [Difco Laboratories, Detroit, Mich.] supplemented with 0.4 g of L-cysteine and 0.135 g of ferric nitrate liter $^{-1}$). Viable bacterial counts with metabolic activity were also performed by the Live/Dead BacLight bacterial viability kit (Molecular Probes, Inc., Eugene, Oreg.), a two-color fluorescent nucleic acid stain method. This kit is based on membrane integrity and utilizes mixtures of green fluorescence nucleic acid stain (SYTO 9) and red fluorescence nucleic acid stain (propidium iodide [PI]). SYTO 9 stains all cells with intact or damaged membranes in a population, whereas PI penetrates only cells with damaged membranes, fading the SYTO 9 stain green fluorescence when both dyes are present (2). Although there are no reports of BacLight stain for *Legionella* spp., heat-treated *L. pneumophila* cells stained fluorescent red, whereas intact cells stained fluorescent green. Therefore, the BacLight kit is considered useful for detecting viability of *Legionella* cells.

BacLight dye mixture (3 μl) was added into 1 ml of microcosm. The microcosm was mixed thoroughly and incubated at room temperature in the dark for 15 min. Stained bacteria were captured by microfiltration through 0.2- μm -pore-size black polycarbonate membrane filters (Toyo Roshi Kaisha). Filters were air dried and mounted with nonfluorescence immersion oil on glass microscope slides. Preparations were examined with the $\times 100$ oil immersion fluorescent objective lens of an Olympus BH2-RFC epifluorescence microscope equipped with a 100-W super-high-pressure mercury burner (USH-102D). The respective excitation and emission maxima for these dyes are ca. 480 and 500 nm for SYTO 9 stain and 490 and 635 nm for PI.

Total numbers and viable numbers of bacteria per milliliter were estimated from a count of at least 10 randomly chosen microscopic fields. An eyepiece micrometer disk was used to delineate a portion of the field for the actual counting. The numbers of green or red stained cells in the micrometer square (0.01 mm^2) at magnifying power $\times 1,000$ were counted and finally converted into numbers per milliliter. The sum of green and red stained cell numbers was estimated as the total bacterial number, and the number of green stained cells as the viable cell count.

In vivo resuscitation of *L. pneumophila* by *A. castellanii*. In vivo resuscitation of noncultivable *L. pneumophila* cells by phagocytosis of amoebae was performed as described previously (18). *A. castellanii* ATCC 30234 (American Type Culture Collection, Rockville, Md.) was cultured in 25- cm^2 tissue culture flasks (Corning Costar Corporation, Cambridge, Mass.) with 10 ml of PYG broth [2% proteose peptone no. 3, 0.1% yeast extract, 0.1 M glucose, 4 mM MgSO_4 , 0.4 M CaCl_2 , 0.1% sodium citrate, 0.05 M $\text{Fe}(\text{NH}_4)_2 \cdot 6\text{H}_2\text{O}$, 2.5 mM NaH_2PO_4 , 2.5 mM K_2HPO_4 ; pH 6.5] at 25°C for 10 days. Cultures of *A. castellanii* were transferred to a 15-ml polypropylene tube, centrifuged at 1,000 rpm for 10 min, washed twice with fresh PYG broth, and then adjusted to a titer of 10^5 cells ml^{-1} . Then, 1 ml of cell suspension was pipetted into each well of a 24-well tissue culture plate (Becton Dickinson Labware, Franklin Lakes, N.J.). After overnight incubation at 25°C, the medium was removed and washed three times with the *Acanthamoeba* buffer (AC buffer; PYG broth without proteose peptone, glucose, and yeast extract). The microcosms in which the cultivability was lost completely but metabolically active were used in the examination. The AC buffer in each well was replaced with a 1-ml aliquot from the microcosm and incubated at 35°C for 2 h. After 0, 24, and 48 h, the amoeba cells were harvested from the bottom of the wells by vigorous pipetting. The cell suspension was sonicated at a power of 7 W for 30 s by using a Branson Sonifier 150 (Branson Ultrasonic Corporation, Danbury, Conn.) to break up the amoeba cells. The amoeba cells were destroyed completely under the conditions of sonication, whereas almost 100% of *L. pneumophila* cells were still alive. A 100- μl aliquot from the suspension and the 10-fold serial dilute solutions were spread on a BCYE α agar plate and incubated at 35°C. The resuscitated bacterial number in 10^5 amoeba cells was calculated from the number of colonies grown on the plate.

RAPD analysis. Each 50- μl RAPD [random (ly) amplified polymorphic DNA] reaction mixture contained the following reagents: 5 μl of $10\times$ reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM MgCl_2 , and 0.001% gelatin), 5 μl of deoxynucleoside triphosphates (5 mM), 5.25 μl of sterile H_2O , 5.0 U of *Taq* DNA polymerase, 33.5 μl of template (15 $\mu\text{g}/\text{ml}$), and 1 μl of primer (5'-GTGGTGGTGGTGGTGGT-3'; 50 pmol/ μl) (Sigma Genosys, Tokyo, Japan). Thermal cycling was performed in a GeneAmp PCR system 2400 (Perkin-Elmer Cetus, Foster City, Calif.). The cycling profile was as follows: 1 cycle of 95°C for 5 min; 30 cycles of 95°C for 30 s, 35°C for 30 s, and 72°C for 30 s; and a final cycle of 72°C for 7 min. The RAPD products were electrophoresed on a 2% Nusieve 3:1 agarose gel (BMA, Rockland, Maine).

RESULTS

Survival in 20 hot spring water microcosms. After incubation for 1 month, rapid loss of *L. pneumophila* cultivability was evident in 19 kinds of hot spring waters; however, the metabolic activity was maintained during this incubation (Fig. 1). In hot spring water of pH 1.6, *L. pneumophila* organisms lost cultivability completely by 60 s after addition (data not shown).

Effects of temperature and pH on survival in hot spring water K microcosm. The loss of cultivability in microcosms of hot spring water was recognized at all temperatures except for 25°C, and the decreased ratio of cultivable cells and metabolically active cells depended on a rise in temperature (Fig. 2A). Metabolic activity was maintained up to 45°C but not at 50°C. Bacterial survival in a microcosm at 42°C was also influenced by pH (Fig. 2B). At pH 2.0, bacteria were rapidly killed. Colony-forming cells at pH 5.0 and 10.0 were not detected at days 14 and 21 after incubation, respectively; however, metabolic activity at pH 5.0 was maintained over the entire study period of 61 days (that at pH 10 was not examined because high pH influenced BacLight staining). Cultivability was slowly lost in the microcosms at pH 6.0 and 8.0 (particularly at pH 8.0).

FIG. 1. Survival of *L. pneumophila* in 19 kinds of hot spring waters collected from various locations in Japan. Results are shown after incubation for 1 month at 42°C. Symbols: ○, total bacterial cell numbers; □, viable cell numbers; ×; cultivable cell numbers.

Effect of conductivity on survival. Dilution of hot spring water by distilled water hastened the loss of cultivability with a decline in metabolic activity, particularly at high dilutions. Bacteria maintained in a microcosm of distilled water lost both cultivability and metabolic activity within 5 days of incubation at 42°C (Fig. 3).

Effects of inoculum size, temperature, and growth phase on survival in tap water microcosm. In the microcosms with an inoculum size of 10^6 CFU ml⁻¹ adjusted to the exponential phase, a marked decline of cultivability was recognized in the microcosm at 42°C, and no colony was detected by 63 days after incubation. On the other hand, the colony numbers formed at day 63 in microcosms at 35 and 25°C showed decreases of -1.89 and -1.27 log, respectively, compared to the initial inoculum size. The same tendency was observed in microcosms of post-exponential-phase organisms; however, the decrease in cultivable cells was more rapid in comparison to that seen in exponential-growth-phase microcosms. The number of cells that maintained metabolic activity was within at least -1 log of the initial value in all microcosm samples throughout the study period (Fig. 4A).

In contrast to the result with an inoculum size of 10^6 CFU ml⁻¹, cultivable cell numbers in microcosms of 10^8 CFU ml⁻¹ at 35 and 25°C did not decrease at all throughout the incubation period of 85 days. This result was obtained in both the exponential-phase and post-exponential-phase cultures. The microcosm at 42°C in exponential phase had completely lost colony-forming ability at day 85 of incubation. In the corresponding post-exponential-phase microcosm, however, ca. 10^5 cells maintained colony-forming ability at day 85. The finding that the exponential-phase microcosm lost cultivability more rapidly than the post-exponential-phase culture was contrary to the result obtained by using a microcosm with an inoculum size of 10^6 CFU ml⁻¹ (Fig. 4B).

Resuscitation by *A. castellanii* ATCC 302344. Each tap water microcosm at 42°C with inoculum sizes of 10^6 CFU ml⁻¹ of the

exponential and post-exponential phases showed complete loss of cultivability without loss of metabolic activity at days 61 and 41 of incubation, respectively. Complete loss of cultivability was confirmed by no colony formation on any plate when 1 ml of sample was added onto each of 10 plates of a BCYE α agar plate. These microcosm samples were used for the study of resuscitation. No colonies had formed by 2 h after the microcosm sample was added into well-attached *Acanthamoeba* spp. Colonies appeared after 24 and 48 h in both exponential-phase and post-exponential-phase cultures (Table 2).

RAPD. The RAPD profile in a microcosm with an inoculum size of 10^8 adjusted as an exponential phase at 93 days after incubation at 42°C was not different from that in a microcosm of freshly adjusted exponential-phase cells (Fig. 5).

DISCUSSION

L. pneumophila cell populations in 19 of 20 kinds of hot spring water microcosms maintained metabolic activity during incubation at 42°C, although the cultivability declined. Paszko-Kolva et al. (16) demonstrated that the survival of *L. pneumophila* in natural water was enhanced at lower temperatures. In the present study, *L. pneumophila* in microcosms with lower temperatures also exhibited longer potential survival without loss of cultivability, suggesting that this genus may have evolved to adapt to aqueous environments at low temperatures, although the preferred growth temperature in laboratory medium is ca. 36°C.

Kusnetsov et al. (13) reported that cell growth and metabolic activity decreased markedly in all strains of *L. pneumophila* at temperatures above 44 to 45°C; however, metabolic activity was still retained at 51.6°C, beyond the maximum temperature for cell growth. On the other hand, in our study, cultivability in hot spring water microcosms at 45 and 50°C was lost more rapidly than at 42°C. However, metabolic activity exhibited only a 1-log decline during incubation for 61 days in both microcosms at 42 and 45°C, whereas metabolic activity was lost completely at 50°C by day 8. The finding that the metabolic activity was maintained for long periods even at 45°C contrasted with the result of Kusnetsov et al. (13). On the other hand, Dennis et al. (5) showed that *L. pneumophila* SG1 had a decimal reduction time in water at 50°C, and there was little loss of viability at 46°C. The differences observed in these studies may due to variability between the metabolic activities among bacterial strains, which may reflect different physiological capacities for tolerating high temperatures. It should be noted, however, that *L. pneumophila* could survive planktonically, keeping the metabolic activity, although the cultivability was lost in the environment with a high temperature as in hot spring water.

Both the cultivability and the metabolic activity of *L. pneumophila* were rapidly lost in sterilized distilled water. Furthermore, dilution with sterilized distilled water of two hot spring water samples decreased the survival ability of *L. pneumophila* depending on the dilution rate.

It is not clear what factor caused this phenomenon; however, some trace minerals have been known to play an important role in bacterial metabolism (4). For example, Kim et al. (12a) reported that cells of *Leuconostoc mesenteroides* on starvation media showed a greater cultivability in the presence of trace

FIG. 2. Effect of temperature and pH on survival of *L. pneumophila* in microcosm of hot spring water sample K. (A) Effect of temperature: 25°C (a), 42°C (b), 45°C (c), and 50°C (d). Symbols: ○, total bacterial cell numbers; □, viable cell numbers; ×, cultivable cell numbers. (B) Effect of pH: pH 2 (a), pH 5 (b), pH 6 (c), pH 8 (d), and pH 10 (e). The results of BacLight staining at pH 2.0 and 10.0 are not shown because the staining was influenced by the low and high pH values, respectively. Symbols: ○; total bacterial cell numbers, □; viable cell numbers, ×; cultivable cell numbers.

minerals than without trace minerals. Although the effect of minerals on the survival of *L. pneumophila* in a starveling environment is not well understood, States et al. (17) showed that lower levels of certain minerals, such as iron, zinc, and

potassium are important factors in the survival and growth of *L. pneumophila* in tap water; in contrast, higher levels of minerals are toxic. Two of the hot spring water samples analyzed here also contained lower levels of minerals that were not

FIG. 3. Effect of conductivity on survival of *L. pneumophila* in both microcosms of hot spring waters A and M. (a to c) Mean dilution with distilled water: a, $\times 1$; b, $\times 10$; c, $\times 100$; row 1, hot spring sample A; row 2, hot spring sample M; row 3, distilled water. Symbols: $\circ$, total bacterial cell numbers; $\square$, viable cell numbers; $\times$, cultivable cell numbers.

present in distilled water (data not shown). We will study further the effect of trace minerals on the survival of *L. pneumophila*, although other factors may also have influence its survival.

Three kinds of hot spring water with high concentrations of salt were included in the present study. The effect of salt on the survival of *L. pneumophila* was studied by Heller et al. (8). These studies showed that *L. pneumophila* could survive in salt solutions up to 3% NaCl at lower temperatures but not at high temperatures. Our results indicated that *L. pneumophila* could maintain metabolic activity but was noncultivable in hot spring water containing salts at almost the same concentration as seawater salts.

pH is also an important factor for the survival of *L. pneumophila*. Katz and Hammel (11) demonstrated that *L. pneumophila* showed a 2-log decline after incubation for 1 month in tap water varying in pH from 4.0 to 7.0 but a 6-log decline at pH 8.0. In hot spring water microcosms adjusted artificially for pH from 2.0 to 10.0, the lowest pH killed *L. pneumophila* within 1 day of incubation at 42°C (*L. pneumophila* was also killed rapidly in hot spring water sample T with pH 1.6). A faster decline of cultivability was detected both at pH 5.0 and at pH 10.0 compared to that seen at pH 6.0 and pH 8.0; however, the metabolic activity at pH 5.0 was maintained. The best survival was evident in a hot water microcosm adjusted to pH 8.0, a

finding which was inconsistent with the result of Katz and Hammel (11). However, hot spring water at around pH 8.0 is predominant throughout Japan, and thus it may not be surprising that pH 8.0 best supported the survival of *L. pneumophila*.

Bacterial cells are known to communicate with each other in populations at a high cell density, a process that optimizes the population survival for adaptation to stressful environments (so-called quorum sensing) (10, 20). *L. pneumophila* in tap water microcosms with an inoculum size of 10^8 CFU ml^{-1} maintained cultivability much longer than those at lower densities (10^6 CFU ml^{-1}). Long-term survival with the inoculum size of 10^8 CFU ml^{-1} might reflect adaptation by quorum sensing. Furthermore, post-exponential-phase cultures of *L. pneumophila* express a number of traits that have been correlated with virulence (3). Hambleton et al. (6) reported that *L. pneumophila* cell populations in the exponential phase survived poorly in aerosols with a humidity of 65% compared to post-exponential-phase cells. Interestingly, in comparisons of the exponential and post-exponential phases, cultivability loss occurred faster in the post-exponential phase with an inoculum size of 10^6 CFU ml^{-1} , whereas the converse was found at 10^8 CFU ml^{-1} . Stress resistance is usually induced when bacteria enter the post-exponential phase (1). Our results with post-exponential-phase cells at 10^6 CFU ml^{-1} suggest that the temporary loss of cultivability is an effective strategy for survival in

FIG. 4. Effect of inoculum size, growth phase, and temperature on survival of *L. pneumophila* in a microcosm of tap water. (A) Inoculum size of 10^6 cells/ml; (B) inoculum size of 10^8 cells/ml. Temperatures: 42°C (a), 35°C (b), 25°C (c). Symbols: $\circ$, total bacterial cell numbers; $\square$, viable cell numbers; $\times$, Cultivable cell numbers. The same results were reproduced in the repeated experiment.

an aqueous environment. Various stress proteins bind to DNA and may block primer or polymerase-binding sites, thus causing changes of band patterns by prevention of amplification. However, RAPD analysis did not reveal any differences in band patterns between the cell population that lost cultivability completely but was active metabolically, on the one hand, and

a fresh culture cell population on the other. In our future studies, we will attempt to clarify the gene expression profile related to the survival strategy of *L. pneumophila* during long-term incubation in an aqueous environment and to the variation in aspects of survival identified in the present study (differences dependent on growth phase and cell density).

TABLE 2. Resuscitation of *L. pneumophila* by ingestion by *A. castellanii* ATCC 30334 in a microcosm that lost cultivability^a

Expt group and no.	No. of colonies detected at:		
	0 h	24 h	48 h
Group A ^a			
1	ND ^c	1 × 10 ^{1d}	1 × 10 ⁴
2	ND	4 × 10 ¹	ND
3	ND	ND	1 × 10 ⁴
Group B ^b			
1	ND	3.4 × 10 ³	5 × 10 ³
2	ND	2.6 × 10 ²	5 × 10 ¹
3	ND	1 × 10 ¹	3.8 × 10 ²

^a A tapwater microcosm of the exponential phase.^b A tapwater microcosm of the postexponential phase.^c ND, no colonies were detected.^d Colony-forming units per 10⁵ amoebas/well.

From a public health perspective, it is important to identify whether or not *L. pneumophila* that retains metabolic activity but is noncultivable in hot spring water environments can be resuscitated. Steinert et al. (18) reported that whole cells of *L. pneumophila* Philadelphia JR32 were noncultivable after 125 days of incubation at 10⁴ CFU ml⁻¹ in a sterile tap water microcosm at 20°C. Addition of *A. castellanii* ATCC 30234 to the cells induced colony-forming cells and increased cell density to 10⁷ CFU ml⁻¹ 3 days after the addition of amoebas. In our study, although *A. castellanii* ATCC 30234 was used, the resuscitated cells represented only a minor portion of the cell population. Furthermore, when two other serotype 1 strains were used, no resuscitation was observed (data not shown). Although Steinert et al. (18) did not address differences be-

tween strains, the process involved in the temporary loss of cultivability under various stresses is complex, and differences in loss of metabolic activity associated with loss of cultivability may be affected by the expression of specific stress-related genes (1). Interestingly, when *A. castellanii* was added into a hot spring water microcosm incubated at pH 5.0 for 2 months, which completely abolished cultivability, metabolic activity was maintained much longer than in the original microcosm without amoebas and, surprisingly, bacterial cell numbers in the microcosm increased up to 10-fold after incubation for a further 1 month. However, no colonies formed on a BCYE α plate, even when excess catalase was added into the culture as an activator for cells injured by toxic oxygen radical species (data not shown). Hay et al. (7) changed cysts to trophozoites after the uptake of *L. pneumophila* cells in mid-log phase by mature amoebal cysts and further changed trophozoites to cysts and then repeated this process. No bacteria were detected on BCYE α plates, although DNA amplification and hybridization permitted detection of *L. pneumophila* in amoebas. It is not clear why colonies did not form on BCYE α plates in our study, even though cells could multiply intracellularly in *A. castellanii*. Further investigation into this phenomenon is required as new problems of detection of *Legionella* in natural environments by using plating methods may develop.

ACKNOWLEDGMENTS

This work was supported by a Grant in Aid for Scientific Research (no. 13680634) (to N.K.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

We thank K. Tateda for providing *L. pneumophila* serogroup 1 Suzuki.

REFERENCES

1. Abu Kwaik, Y., and N. C. Engleberg. 1994. Cloning and molecular characterization of a *Legionella pneumophila* gene induced by intracellular infection and by various in vitro stress conditions. *Mol. Microbiol.* **13**:243–251.
2. Boulos, L., M. Prevost, B. Barbeau, J. Coallier, and R. Desjardins. 1999. LIVE/DEAD BacLight: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *J. Microbiol. Methods* **37**:77–86.
3. Byrne, B., and M. S. Swanson. 1998. Expression of *Legionella pneumophila* virulence traits in response to growth conditions. *Infect. Immun.* **66**:3029–3034.
4. Dawes, E. A. 1985. Growth and survival of bacteria, p. 67–187. In J. S. Poindexter and E. R. Leadbetter (ed.), *Bacteria in nature*, vol. 3. Plenum Press, Inc., New York, N.Y.
5. Dennis, P. J., D. Green, and B. P. Jones. 1984. A note on the temperature tolerance of *Legionella*. *J. Appl. Bacteriol.* **56**:349–350.
6. Hambleton, P., M. G. Broster, P. J. Dennis, R. Henstridge, R. Fitzgeorge, and J. W. Conlan. 1983. Survival of virulent *Legionella pneumophila* in aerosols. *J. Hyg.* **90**:451–460.
7. Hay, J., D. V. Seal, B. Billcliffe, and J. H. Freer. 1995. Non-culturable *Legionella pneumophila* associated with *Acanthamoeba castellanii*: detection of the bacterium using DNA amplification and hybridization. *J. Appl. Bacteriol.* **78**:61–65.
8. Heller, R., C. Holler, R. Sussmuth, and K. O. Gundermann. 1998. Effect of salt concentration and temperature on survival of *Legionella pneumophila*. *Lett. Appl. Microbiol.* **26**:64–68.
9. Hussong, D., R. R. Colwell, M. O'Brien, E. Wess, A. D. Pearson, R. M. Weiner, and W. D. Burge. 1987. Viable *Legionella pneumophila* not detectable by culture on agar media. *Bio/Technology* **5**:947–950.
10. Kaprelyants, A. S., and D. B. Kell. 1996. Do bacteria need to communicate with each other for growth? *Trends Microbiol.* **4**:237–242.
11. Katz, S. M., and J. M. Hammel. 1987. The effect of drying, heat, and pH on the survival of *Legionella pneumophila*. *Ann. Clin. Lab Sci.* **17**:150–156.
12. Kell, D. B., A. S. Kaprelyants, D. H. Weichert, C. R. Harwood, and M. R. Barer. 1998. Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie Leeuwenhoek* **73**:169–187.
- 12a. Kim, D. S., S. Thomas, and H. S. Fogler. 2000. Effect of pH and trace minerals on long-term starvation of *Leuconostoc mesenteroides*. *Appl. Environ. Microbiol.* **66**:976–981.

FIG. 5. RAPD analysis. Lanes: a, microcosm at 42°C, 85 days of incubation, 10⁸ CFU ml⁻¹, exponential-phase cells; b, freshly cultured *L. pneumophila*; c, size marker, 100-bp DNA ladder.

13. Kusnetsov, J. M., E. Ottoila, and P. J. Martikainen. 1996. Growth, respiration, and survival of *Legionella pneumophila* at high temperatures. J. Appl. Bacteriol. **81**:341–347.
14. Lee, J. V., and A. A. West. 1991. Survival and growth of *Legionella* species in the environment. Soc. Appl. Bacteriol. Symp. Ser. **20**:121S–129S.
15. Paszko-Kolva, C. 1992. Long-term survival of *Legionella pneumophila* serogroup 1 under low-nutrient conditions and associated morphological changes. FEMS Microbiol. Ecol. **102**:45–55.
16. Paszko-Kolva, C., M. Shahamat, and R. R. Colwell. 1993. Effect of temperature on survival of *Legionella pneumophila* in the aquatic environment. Microb. Releases **2**:73–79.
17. States, S. J., L. F. Conley, M. Ceraso, T. E. Stephenson, R. S. Wolford, R. M. Wadowsky, A. M. McNamara, and R. B. Yee. 1985. Effects of metals on *Legionella pneumophila* growth in drinking water plumbing systems. Appl. Environ. Microbiol. **50**:1149–1154.
18. Steinert, M., L. Emody, R. Amann, and J. Hacker. 1997. Resuscitation of viable but nonculturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii*. Appl. Environ. Microbiol. **63**:2047–2053.
19. Steinert, M., U. Hentschel, and J. Hacker. 2002. *Legionella pneumophila*: an aquatic microbe goes astray. FEMS Microbiol. Rev. **26**:149–162.
20. Swift, S., J. A. Downie, N. A. Whitehead, A. M. Barnard, G. P. Salmond, and P. Williams. 2001. Quorum sensing as a population-density-dependent determinant of bacterial physiology. Adv. Microb. Physiol. **45**:199–270.
21. Tominaga, M., Y. Aoki, S. Haraguchi, M. Fukuoka, S. Hayashi, M. Tamesada, E. Yabuuchi, and K. Nagasawa. 2001. Legionnaires' disease associated with habitual drinking of hot spring water. Intern. Med. **40**:1064–1067.