Influence of Temperature on Growth of *Legionella pneumophila* Biofilm Determined by Precise Temperature Gradient Incubator

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Received 27 December 2005/Accepted 22 March 2006

Bacterial growth is influenced by several different culture conditions. Temperature is one of an essential component which regulates bacterial growth and their morphology. The influence of temperature on the length of bacteria was investigated in broth and on agar in a temperature range from 30.0°C to 47.0°C in 0.5°C steps using a newly developed temperature gradient incubator. The incubator is able to reach a set temperature within 2 h and maintain temperature as accurate as ±0.1°C of the set temperature. Three *Legionella pneumophila* serotype 1 strains were incubated for 48 h in BCYE-a agar at various temperatures ranging from 30.0°C to 48.0°C and length of bacteria grown at each temperature was microscopically measured. Ability of bacteria to multiply at a given temperature was also determined. *L. pneumophila* serotype 1 strains ATCC 33152, a clinical isolate Okinawa 02-001 were going to elongate to longer than 100 µm when cultured higher than at 39.5°C and at 41.5°C, respectively. Each strain was unable to multiply when cultured higher than at 44.2°C (ATCC 33152) or at 44.0°C (Okinawa 02-001). Those data would provide insights for establishing regulations in terms of maintaining hot water temperature in a facility where a circulating hot water supply-system is available and contamination with *Legionella* spp. is likely to happen.

[Key words: *Legionella pneumophila*, biofilm, temperature gradient incubator, cell elongation]

Bacteria of the genus *Legionella* are gram-negative, aerobic, and rod-shaped with one or more polar flagella (1, 2). *Legionella pneumophila* is considered to be an important etiologic microbial agent of lower respiratory tract infections in the US or in Western Europe, accounting for 2% to 8% of all cases of community-acquired pneumonia, second only in importance to *Streptococcus pneumonia* (3). The number of *Legionella* spp. associated with pneumonia was rarely reported in Japan until April 1999, when a new Law Concerning the Prevention of Infectious Diseases and Patients with Infectious Diseases (New Law on Infectious Diseases) was enacted. The law classified *Legionella* pneumonia as a nationally notifiable infectious disease, with mandatory reporting of every case of *Legionella* pneumonia to the Ministry of Public Health and Welfare via a prefectural health center. The number of reported *Legionella* pneumonia cases has since increased dramatically: 53 cases in 1999, 151 cases in 2000, and 83 cases in 2001. In 2002, an outbreak of legionellosis occurred at a public bathhouse in Miyazaki, Japan. In the outbreak, 32 cases were laboratory confirmed as *Legionella* pneumonia and 263 cases were suspect. *Legionella* spp. are commonly present in natural and man made aquatic environments (4–6). The bacteria proliferate in stagnant warm water, as found in cooling towers, certain plumbing systems and evaporative condensers of large air-conditioning systems. Outbreaks of legionellosis have occurred when people have inhaled aerosols from water sources contaminated with *Legionella* bacteria (7). In these conditions, the bacterial count would reach 1010–1012 cfu ml−1, much higher than that seen in the natural environment. In circulating water systems, legionellae are most frequently detected in biofilms in plumbing fixtures (5, 8–10). Biofilm has a potential role as a vehicle to convey large numbers of embedded bacteria, causing pneumonia when aerosol droplets containing fragments of biofilm are inhaled by humans (11). In flowing plumbing systems, temperature gradients are formed over a solid wall due to the temperature difference between hot water and the ambient environment. As scale (such as silica compounds), debris, biofilm or sediment adhere to the pipe wall, radial temperature gradients are formed inside the biofilm. Various disinfection measures to prevent legionellosis have been used (12–15). Legionellae are sensitive to temperature (16–21) and chlorine. Legionellae grow poorly at temperatures below 20°C or above 50°C and are easily killed at temperatures above 60°C. In hospital or public water systems Legionellae can be killed by raising the temperature of the hot water supply to 70°C or 80°C (22, 23). In Japan, government regulations recommend an operating temperature ranging from 50°C to 60°C. Legionellae are also susceptible to chlorine- and bromine-containing compounds, ozone, heavy metal ions, and ultraviolet light (21,
Hyperchlorination (sodium and calcium hypochlorite, chlorine dioxide), thermal eradication (superheating and flushing) and copper/silver ionization are widely used in hospital or public water systems. However, these measures are not always efficient because of biofilms and their heterogeneous features. No previous studies (11, 26, 27) on morphological changes of \( L. pneumophila \) by cultivation temperature clarified the quantitative relationship between cultivation temperature and length of the strain. This paper shows the length of three strains of \( L. pneumophila \) (a type strain ATCC 33152, two clinical isolates; Okinawa 02-001 and Nagasaki 80-045) at temperature ranging from 30.0°C to 47.0°C at intervals of 0.5°C determined by newly developed precise temperature gradient incubator. Viability of bacteria at particular temperature was also determined. Those data obtained from a setting with precise temperature regulating module would provide insights for introducing a guideline for regulating hot water temperature in a plumbing pipes of hot water supply systems.

**MATERIALS AND METHODS**

**Design and assembly of the temperature gradient incubator apparatus (TGI)** A bacterial incubator with an accurate temperature gradient control was fabricated for this study (Figs. 1 and 2). Ten aluminum blocks (80 × 80 × 25 mm) with cylindrical chambers (17 mm diameter and 85 mm depth) holding a glass test tube for incubating \( L. pneumophila \) were placed side by side in a row to give 10 different culture temperatures. Heat sources were applied to the aluminum blocks by two constant temperature bath circulators (Thermo NESLAB RTE-7; Thermo Electron Corporation, Newington, NH, USA) set at different temperatures using water jackets placed at both ends of the row of aluminum blocks. The water jackets were made of brass, and contained a pectinated brass sheet to provide a higher heat transfer rate. Each aluminum block was separated by a silicon sheet that allowed a constant stepped temperature gradient to be attained. The module of aluminum blocks with water jackets was wrapped with fiberglass fabric to minimize heat loss. Temperatures outside and inside a test tube wall were measured by standard thermocouples (Chromel-Alumel sheath type, 1 mm diameter, THERMIC TMB-KS10/316L-10; Yamato Industry, Tokyo) and a standard thermometer (no. 20907; Japan Measuring Instrument Industrial Incorporated Company, Tokyo), respectively, and compared. Temperatures measured by the standard thermocouples were monitored by a digital multimeter (Keithley model 2001 TCSCAN, model 2001; Keithley Instruments, Cleveland, OH, USA) and recorded in a personal computer.

**Bacterial strains and inoculation** Three strains of \( L. pneumophila \) serogroup 1 were employed; a type strain and two clinical isolates. A type strain of \( L. pneumophila \) serogroup 1, Okinawa 02-001 was obtained from the American Type Culture Collection (ATCC). Two clinical isolates of \( L. pneumophila \) serogroup 1, Okinawa 02-001 (isolated in 2002, male patient) and Nagasaki 80-045 (isolated in 1980, 02-001 (isolated in 2002, male patient) and Nagasaki 80-045 were placed side by side in a row to give 10 different culture temperatures. Heat sources were applied to the aluminum blocks by two constant temperature bath circulators (Thermo NESLAB RTE-7; Thermo Electron Corporation, Newington, NH, USA) set at different temperatures using water jackets placed at both ends of the row of aluminum blocks. The water jackets were made of brass, and contained a pectinated brass sheet to provide a higher heat transfer rate. Each aluminum block was separated by a silicon sheet that allowed a constant stepped temperature gradient to be attained. The module of aluminum blocks with water jackets was wrapped with fiberglass fabric to minimize heat loss. Temperatures outside and inside a test tube wall were measured by standard thermocouples (Chromel-Alumel sheath type, 1 mm diameter, THERMIC TMB-KS10/316L-10; Yamato Industry, Tokyo) and a standard thermometer (no. 20907; Japan Measuring Instrument Industrial Incorporated Company, Tokyo), respectively, and compared. Temperatures measured by the standard thermocouples were monitored by a digital multimeter (Keithley model 2001 TCSCAN, model 2001; Keithley Instruments, Cleveland, OH, USA) and recorded in a personal computer.

The culture was then diluted with sterile distilled water to an optical density at 660 nm (OD\(_{660}\)) of 0.6. To start the experiments, a 500 µl aliquot of suspensions was transferred into a test glass tube containing 4.5 mL of fresh BCYE-α broth. The final concentration in the test tube reached approximately 5 × 10\(^4\) cfu mL\(^{-1}\). To measure the length of \( L. pneumophila \) biofilm grown on the glass wall in broth, ten identical glass tubes containing broth samples were installed in the temperature gradient incubator operating at 10 different temperatures simultaneously. Sterile slide glass plates, 13 mm wide and 76 mm long, were placed in each test tube. For analysis by scanning electron microscopy (Hitachi, Tokyo), a sterile cover glass (10 mm square) was used instead of a slide glass. The \( L. pneumophila \) biofilm adhered to the glass test tube wall and to the slide glass plate. The slide glass plate was readily removed from the test tube without loosening the biofilm. The effect of cultivation temperature on bacterial length was verified on BCYE-α agar. The vials were thawed at room temperature, and the contents were streaked onto buffered charcoal-yeast extract agar (Kanto Kagaku, Tokyo) supplemented with 0.1% α-ketoglutarate (Sigma-Aldrich, St. Louis, MO, USA) (BCYE-α). The plates were incubated for 48 to 72 h at 37.0°C. Growth was harvested from plates with a platinum loop and placed into sterile distilled water in a tube. The tube was mixed in a Vortex mixer.
strains grown at temperatures ranging from 30.0°C to 47.0°C was measured and evaluated. For the analysis of cell multiplication at different temperatures, three strains were cultured at a temperature ranging from 41.0°C to 45.0°C for 72 h. Two separate experiments with temperature windows of 41.0°C to 43.0°C and 43.0°C to 45.0°C were performed with the TGI apparatus so as to achieve a serial step-wise culture temperature ranging from 41.0°C to 45.0°C at intervals of 0.2°C. Even one colony of *L. pneumophila* resulted in a positive finding for cell multiplication. The experiments were repeated 2 to 3 times.

**Scanning electron microscopy**  For scanning electron microscopy, the biofilm grown on a cover glass was fixed with 3% glutaraldehyde and stained with 1% osmium tetroxide and washed in 0.1 mM phosphate buffer at pH 6.9 for 2 h and then dehydrated through an alcohol series. Samples were mounted on 20 mm scanning electron microscopy specimen stubs with high-conductivity silver paint. Specimens were coated with a 20 nm layer of gold in a vacuum coating unit and 10 were examined in a Hitachi scanning electron microscopy operated at 10 kV accelerating voltage.

**Bacterial staining and measurement of bacterial length**  Biofilms grown on glass slides in broth and colonies on agar cubes were harvested with a platinum loop and placed into 2 ml volumes of sterile distilled water in a vial. The vial was mixed in a Vortex mixer. We verified that the length of bacteria was not affected by mixing (data not shown). The sample was stained conventionally on a slide glass by the Gimenez (Muto Pure Chemicals, Tokyo) method and observed with a microscope (Nikon TE2000; Nikon, Tokyo) or with a CCD camera (Sony DXC-390, Sony, Tokyo) and recorded in a personal computer. After Gimenez staining, photographs including bacterial images were taken and recorded in a personal computer and processed with image processing software (Adobe Photoshop). Approximately 200 well-isolated bacterial images were randomly chosen from photos and the length of bacteria was measured with Scion Image software (Beta 4.02 for Windows XP; Scion Corporation Frederick, Maryland, MD, USA) by tracing the image on the computer display with a mouse/pen device.

**RESULTS**

**Status of culture temperatures generated by the temperature gradient incubator (TGI) apparatus**  A stepped temperature gradient with 10 different temperatures in the range of 30.0°C (test tube no. 1) to 47.0°C in 2.0°C (test tube no. 10) steps was achieved when the constant temperature bath circulators were set at 30.0°C and 50.0°C in the TGI apparatus. Each test tube reached a stable temperature within 2 h and was able to maintain this temperature throughout the experiment with a temperature fluctuation of ±0.1°C. The temperature in each test tube and the maximum and minimum temperatures were recorded in the personal computer.

**Influence of culture temperature on the length of *L. pneumophila***  Three strains were cultured in broth and on agar at 10 different temperatures for 48 h in the TGI apparatus. As no significant difference between two clinical isolates was observed, the results were only shown for a type strain ATCC 33152 and Okinawa 02-001. The results for ATCC type strain 33152 and a clinical isolate; Okinawa 02-001 strain incubated on agar and on broth are shown in Fig. 3. The average length of the strain ATCC 33152 exceeded 50 µm when cultured at temperatures ranging from 39.0°C to 44.0°C. The bacteria had the longest average length of approximately 115 µm when cultured at 42.5°C. The average length of the clinical isolate Okinawa 02-001 exceeded 50 µm when cultured at temperatures ranging from 41.0°C to 43.0°C, with the greatest average length of approximately 70 µm at 42.5°C. Figure 3 shows the different bacterial lengths formed on agar or in broth media of the Okinawa 02-001 clinical isolate and the ATCC33152 strain. The average length of the strains ATCC 33152 and Okinawa 02-001 cultured in test tubes was longer than when cultured on agar at temperatures ranging from 35.0°C to 43.0°C. A significant difference of bacterial length was recognized at 38.5°C for Okinawa 02-001 and at 43.0°C for ATCC 33152.

**Influence of culture temperature on cell multiplication of *L. pneumophila**  All 36 ATCC 33152 cultures tested were able to multiply when cultured on BCYE-α agar at temperatures ranging from 41.0°C to 43.1°C. Thirteen out of 22 (59.1%) cultures multiplied when cultured at temperatures higher than 44.1°C (Fig. 4a). Dead cells were determined by plate method on which the strain did not form a colony at 37°C any longer. All 36 Okinawa 02-001 cultures tested were able to multiply when cultured on BCYE-α agar at temperatures ranging from 41.0°C to 43.1°C. Fifteen out of 22 (68.2%) cultures multiplied when cultured at the temperature ranging from 43.1°C to 44.1°C. Eight out of nine (88.9%) Okinawa 02-001 cultures did not multiply when cultured at the temperature higher than 44.1°C (Fig. 4b). The results of Nagasaki had no meaningful difference to Okinawa.

**Growth of *L. pneumophila*** biofilm on glass walls at different temperatures  The clinical isolate strain Okinawa
02-001 was incubated in BCYE-α broth in the glass tube at 42.0°C for 72 h. Figure 5 shows *L. pneumophila* biofilm growth on the glass wall. The field of view of the photographs covers the glass tube wall at the air-liquid interface. Three pictures show 0 h (Fig. 5a), 24 h (Fig. 5b), 48 h (Fig. 5c) after inoculation. The level of the air-liquid interface in the broth fell due to evaporation of the medium. We speculated that the suspended cells would agglomerate on the surface of the broth (Fig. 5a). Thick biofilm developed on the glass wall above the air-liquid interface and granular biofilm adhered to the glass wall below the air-liquid interface after 48 h of incubation. The biofilm sloughed off the glass wall surface and sank to the bottom of the tube after 72 h of incubation.

Figures 6 and 7 show the typical results of biofilm formed on the glass wall surface at 35.0°C and 42.0°C, respectively. At 35.0°C the biofilm adherent to the glass wall surface did not grow closely together or in large amounts. The biofilm was granulated but was not easy to detach from the wall; in other words, it was sticky. We could not observe any difference between the three strains at 35.0°C. Three locations in the biofilm were magnified: above the air-liquid interface (Fig. 6c), near the air-liquid interface (Fig. 6d), and below the air-liquid interface (Fig. 6e). Figures 6f and 6g show magnified images of Fig. 6e (two different locations). The biofilm exhibited a typical short rod morphology that did not change much with the location in the biofilm. The cell was 0.2–0.3 µm in diameter and 2–4 µm long. Above the air interface, the isolated cells rested on the slide glass wall as shown in Fig. 6c. Cells embedded in a sheet of amorphous material on the glass surface appeared near or below the air-liquid interface as shown in Fig. 6d and 6e. Figure 6f shows the magnified image of the sheet of amorphous material. Figure 6g shows three strands connecting the cells to one another. At 42.0°C the biofilm of *L. pneumophila* developed a heterogeneous structure with three distinct layers. The lower layer was granulated biofilm approximately 0.2–0.5 mm in diameter, and lay approximately 2 mm below the air-liquid interface. The middle layer was a thick biofilm and 2–3 mm in height. The upper layer consisted of surface pellicles 1–3 mm in height. Although the thickness of the three layers was different in all three strains, no significant differ-
FIG. 7. The L. pneumophila strain (clinical isolate; Okinawa 02-001) was cultured for 72 h. Typical results of biofilm formed on the glass wall surface at 42.0°C (a) and magnified images (b, c). Scanning electron micrographs were obtained for entire image (d), near the air interface (e), in the middle of the sediment (f), and at the liquid interface (g).

ence in three-layered structure were recognized at 42.0°C. At 45.0°C no biofilm developed in the three strains. Figures 7b and 7c show the middle biofilm developed on the glass wall of Okinawa 02-001 strain at 42.0°C. The air-liquid interface seemed to be located in the center of the biofilm. The upper edge of the biofilm was adjacent to the thin upper biofilm and to the surrounding air in a glass tube. The lower edge was attached to the granulated layer and was soaked in BCYE-α broth. The shape of the border at the upper edge was straight, but the lower edge was a complex semicircular shape. The degree of border complexity was expressed as the ratio of the boundary lengths of the liquid side to the air side, and was found to be 3.6. Figures 7d–g show a typical image of the middle biofilm of L. pneumophila formed at 42.0°C obtained by a scanning electron micrograph of the Okinawa 02-001 strain. Three locations in the biofilm were magnified: near the air interface (Fig. 7e), in the middle of the biofilm (Fig. 7f), and above the liquid (Fig. 7g). The biofilm exhibited a typical filamentous morphology that did not change much with the location in the biofilm. The density of L. pneumophila was greatest near the air interface, and lowest in the middle of the biofilm.

**DISCUSSION**

The average length of type 1 strain ATCC 33152, a clinical isolate Okinawa 02-001 was greatest at a temperature of 37.5°C and 39.0°C, respectively. These results indicate that the clinical isolates had a potential to retain their length within a range of 1–10 µm at higher culture temperatures than did the type strain. Pine *et al.* (28) observed marked changes in cell surface-to-volume ratio, shape, and size which could be related to temperature, nutrients, and aeration conditions for both the bacillary and filamentous forms of L. pneumophila. They noted a large mass of filaments or chain of bacilli in the logarithmic phase breaking into shorter filaments and ultimately forming single and double cigar-shaped cells at 30.0°C and 37.0°C incubated on semisynthetic medium (28). Berg *et al.* (29) investigated the morphological changes of L. pneumophila in continuous culture. Filamentous forms approximately 100 µm long were seen at 37.0°C and 44.0°C. In the present study, most of the filamentous forms approximately 100 µm long were observed over 39.0–41.0°C for all three strains incubated on BCYE-α agar and in broth, but the Okinawa strain incubated at 37.5°C in broth was an exception. There have been many reports that bacterial elongation was closely related to the function of constitutional enzymes for bacterial binary fission (30).

The metabolic activity associated with cell division is likely to be affected by culture temperature. Werner *et al.* (31) suggested that when *Pseudomonas aeruginosa* is exposed to the antibiotic carbenicillin, cell division is blocked and growing cells form filaments. The constitutional enzymes of the two clinical isolates used in the study probably kept their functional integrity at higher culture temperature (39.0°C) than ATCC 33152 (37.0°C). The average length of type 1 strain ATCC 33152, clinical isolate Okinawa 02-001 exceeded 50 µm when cultured at temperatures from 39.5°C to 44.0°C (range, 4.5°C) and from 41.0°C to 43.0°C (range, 2.0°C), respectively. Since L. pneumophila strains are likely to form biofilm structures when bacterial length exceeds 50 µm, the culture temperature that allows two clinical isolates to form biofilm is probably higher than that of the type strain.

The heterogeneous structure of L. pneumophila biofilm formed on glass walls in BCYE-α broth was observed and analyzed by scanning electron microscopy. Microscale gradients in concentration of nutrients, oxygen and metabolic products were assumed to be formed along the glass wall. The environment above an air-liquid interface is oxygen-rich but poor in nutrients, and below the air-liquid interface it is oxygen-poor but rich in nutrients. How does L. pneumophila transport oxygen and nutrients into biofilm? Porous structures are known to be liquid carriers by capillary effect. The filamentous form of L. pneumophila biofilm resembles this pattern. Nutrients are absorbed by capillary effect from the bottom of the biofilm and transported into the upper region and accumulated inside the biofilm. The accumulation of nutrients in the biofilm allows L. pneumophila to survive. The rounded shape of the bottom of the biofilm increases the absorption surface area to 3.6 times that of the flat upper surface of the biofilm. This configuration is well adapted to absorbing more nutrient into the biofilm. The layer of dense population of L. pneumophila at the upper surface seems to prevent the evaporation of nutrients and external contamination. Thus, the heterogeneous structures of the L. pneumophila biofilm formed on the glass wall were found to be unique.

In terms of bacterial multiplication, culture temperature was categorized into three ranges: range I (<43.1°C), a culture temperature in which almost all bacterial clones tested multiplied; range II (43.1°C to 44.1°C), a culture temperature in which 9.1% to 40.9% of bacterial clones tested did not multiply; and range III (>44.1°C) in which most (more than 88.9%) bacterial clones tested did not multiply. Kusnetsov *et al.* (20) demonstrated that the culture temperature at which
no cell multiplication of strain ATCC 33152 was observed was 48.4°C when the bacteria was grown in BCYE-α broth without charcoal for 69 h. This difference in critical temperature was probably due to: (i) agar being used rather than broth, (ii) more accurate temperature control with the TGI apparatus used in our experiment. It could be extrapolated that when the water temperature in a recirculating water supply system is kept higher than at 44.1°C, more than 88.9% of wild strains would not survive, and that when the temperature is kept higher than at 44.5°C, no strains would survive. However, this extrapolation would not apply to bacteria embedded in a biofilm structure, because the biofilm would protect the bacteria from unfavorable growth conditions, and a much higher temperature would be needed to kill any bacteria embedded in the biofilm. In this work, bacteria were grown in a rich medium (BCYE-α) whereas the real-world system of a hot water distribution system is expected to be oligotrophic, in which the nutrient concentrations are perhaps two orders of magnitude smaller. In the next future work, we would like to verify the heterogeneous structure of Legionella biofilm by using biofilm flow cell system, in which oligotrophic environment can be demonstrated.

ACKNOWLEDGMENTS

This research was sponsored in part by a grant-in-aid (nos. 15500337 and 17500324) for Science Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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