

## Temperature-Regulated Formation of Mycelial Mat-Like Biofilms by *Legionella pneumophila*

Zhenyu Piao,<sup>1</sup> Chun Chau Sze,<sup>2,\*</sup> Oksana Barysheva,<sup>1</sup> Ken-ichiro Iida,<sup>1</sup> and Shin-ichi Yoshida<sup>1</sup>

*Department of Bacteriology, Faculty of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan,<sup>1</sup>  
and School of Biological Sciences, Nanyang Technological University,  
60 Nanyang Drive, Singapore 637551, Singapore<sup>2</sup>*

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Fifty strains representing 38 species of the genus *Legionella* were examined for biofilm formation on glass, polystyrene, and polypropylene surfaces in static cultures at 25°C, 37°C, and 42°C. Strains of *Legionella pneumophila*, the most common causative agent of Legionnaires' disease, were found to have the highest ability to form biofilms among the test strains. The quantity, rate of formation, and adherence stability of *L. pneumophila* biofilms showed considerable dependence on both temperature and surface material. Glass and polystyrene surfaces gave between two- to sevenfold-higher yields of biofilms at 37°C or 42°C than at 25°C; conversely, polypropylene surface had between 2 to 16 times higher yields at 25°C than at 37°C or 42°C. On glass surfaces, the biofilms were formed faster but attached less stably at 37°C or 42°C than at 25°C. Both scanning electron microscopy and confocal laser scanning microscopy revealed that biofilms formed at 37°C or 42°C were mycelial mat like and were composed of filamentous cells, while at 25°C, cells were rod shaped. Planktonic cells outside of biofilms or in shaken liquid cultures were rod shaped. Notably, the filamentous cells were found to be multinucleate and lacking septa, but a *recA* null mutant of *L. pneumophila* was unaffected in its temperature-regulated filamentation within biofilms. Our data also showed that filamentous cells were able to rapidly give rise to a large number of short rods in a fresh liquid culture at 37°C. The possibility of this biofilm to represent a novel strategy by *L. pneumophila* to compete for proliferation among the environmental microbiota is discussed.

Legionnaires' disease is a form of pneumonia caused by bacteria of the genus *Legionella*, of which *Legionella pneumophila* is the type species. This infectious disease involves inhalation of aerosols of water containing *Legionella* into the lung. Within the lung, the bacteria are internalized by alveolar macrophages, replicate within the phagosomes, and eventually lyse the host macrophages (27). The genus *Legionella* currently contains as many as 48 species, and at least 20 species are known to be pathogenic to humans (22). Despite this, the general perception is that the species *L. pneumophila* is the main causative agent of Legionnaires' disease, due to the much higher incidence of the disease reported to be caused by *L. pneumophila* than by the other species of the genus (5). One or more of the following reasons could account for this higher incidence. First, lack of diagnostic reagents sufficiently discriminative to identify non-*L. pneumophila* species could lead to inaccurate clinical statistics (22). Second, *L. pneumophila* may be inherently more virulent than other species. However, only a few aspects of its pathogenicity e.g., cytopathogenicity and intracellular multiplication, have currently been shown to be enhanced, compared to non-*L. pneumophila* species (1, 23, 30, 42), providing no strong support for this hypothesis. Third, *L. pneumophila* may be easier to transmit than other *Legionella* species. However, transmission of *L. pneumophila* from humans to humans has not been reported, despite many cases of outbreaks in the recent few decades. Transmission to humans

has reportedly occurred only via mechanical means, such as air-conditioning units, showerheads, and sprinklers (27, 61); therefore, enhanced human-to-human transmissibility is an unlikely reason. Fourth and finally, *L. pneumophila* may have a higher chance than other *Legionella* species of coming into contact with humans. One speculation is that *L. pneumophila* has an innate ability to better survive in the water environment, especially man-made ones. Factors influencing survival of *L. pneumophila* have been studied previously (45), but its survival ability compared to that of other non-*L. pneumophila* species has not been explored.

The habitats for the genus *Legionella* are moist soil and aquatic environments, and outbreaks of legionellosis are often associated with contamination of man-made aquatic environments (for a review, see references 3, 22, and 27). Although *Legionella* bacteria do exist as free-living planktonic forms in the environment, they are more commonly found as intracellular parasites of protozoans such as *Acanthamoeba* spp., *Hartmannella* spp., and *Tetrahymena* spp. (3, 7) and as inhabitants of mixed-community biofilms (51, 62). Biofilms are assemblages of bacteria encased in extracellular polymeric matrices, attached to surfaces or phase boundaries. Being able to form such an adherent, hydrated, and structured community confers several advantages on bacteria for survival in harsh world, e.g., protection from harmful compounds and prevention of desiccation (for a review, see references 15 and 18). *Legionella* has been studied in the context of mixed-community biofilm experimental systems (9, 40, 52) or with the purpose of understanding the efficacy of biocides or biocidal treatments (26, 64, 65), but its ability to form biofilms per se has not been investigated.

\* Corresponding author. Mailing address: School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551, Singapore. Phone: 65-6316 2872. Fax: 65-6791 3856. E-mail: csze@ntu.edu.sg.

The initial motivation for this study was to address the question: has *L. pneumophila* better survival ability in terms of biofilm formation than other *Legionella* species? We were particularly interested in differences that may exist at temperatures such as 37°C and 42°C, often encountered in man-made environments, e.g., hot spring spas, in contrast to the more ambient temperature of 25°C. In the course of the investigation, we found that a novel form of biofilm composed of filamentous cells of *L. pneumophila* exists, which may represent another aspect of survival strategy by this organism. Here, we shall report on these findings.

#### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *Legionella* spp. were obtained from Gifu Type Culture Collection (GTC) (Table 1). Unless otherwise stated, all strains were grown in buffered yeast extract (BYE) broth (49) at 37°C with shaking or on buffered yeast extract charcoal agar (BYECA; BYE with 0.2% activated charcoal and 1.5% Bacto agar) (37) at 37°C for 3 days. Both types of media were supplemented with 0.4-g/liter of L-cysteine and 0.25-g/liter ferric pyrophosphate, and the pH was adjusted to 6.9 with 1 M potassium hydroxide. Kanamycin at 25 µg/ml was added where necessary.

**Biofilm formation and quantification.** *Legionella* strains were shaken at 37°C for 30 h and then diluted 1:10 to give a final optical density at 600 nm of 0.2 to 0.3. For general observation and quantification, biofilms were allowed to form from 2.2 ml of this diluted culture in round-bottom test tubes (diameter, 12 mm; height, 75 mm) of glass (Pyrex, Iwaki Glass, Japan), polystyrene (PS) (Falcon; Becton Dickinson), and polypropylene (PP) (Sarstedt, Germany), statically incubated in water vapor-saturated incubators set at 25°C, 37°C, or 42°C. For confocal laser scanning microscope (CLSM) observation, biofilms were allowed to form on Micro glass cover slides of 0.8 mm in thickness (Matsunami Glass Ind. Ltd., Japan), vertically immersed in the diluted culture in a thin-layer chromatography tank and similarly incubated at the indicated temperatures.

Quantification of biofilms were performed based on a protocol modified from that of O'Toole et al. (46). Briefly, a one-fifth volume of 0.25% crystal violet solution was added to the biofilm culture, and the biofilm was stained for 15 min. The biofilm was rinsed three times with deionized water, and the crystal violet stain was solubilized in 2 ml of 95% ethanol with shaking and then transferred as 200-µl aliquots (in triplicate) to polystyrene 96-well microtiter plates (Costar; Corning, Inc.). Absorbance at 600 nm was measured with a Wallac 1420 AR-VOSx multilabel plate reader (Perkin-Elmer Life Sciences, Japan).

**Plasmid construction, genetic manipulation, and *recA* mutant generation.** The plasmid pPZ1 carrying the green fluorescent protein (GFP) gene *gfp-mut3\** under the control of the LacI-repressible  $P_{A1/04/03}$  promoter (2) was constructed by standard recombinant DNA techniques (57). A NotI fragment from pJBA27 carrying  $P_{A1/04/03}$ -*gfp-mut3\** (2) was inserted into the NotI site of pKM330, an RSF1010-based broad-host-range vector derived from pVI397 (58), with the carbenicillin resistance marker replaced by a kanamycin resistance marker (a kind gift from V. Shingler). To visualize *L. pneumophila* directly under CLSM, pPZ1 was introduced via electroporation into the strain, using a Gene-Pulser (Bio-Rad Laboratories Pte. Ltd., Japan).

For the generation of the *recA* mutant, the region encoding the *L. pneumophila* *recA* gene was PCR amplified with primers 5'-CCAGATTGCTTACTCATCT C-3' and 5'-AGTTGTTC AAGCTCTGCAGC-3', ligated into pGEM-Easy Vector (Promega), and inserted at its internal BamHI site, a 1.7-kb kanamycin resistance cassette from pUT-miniTn5-Km (14). The interrupted *recA* gene was then transferred into the NotI site of pLAW344, which harbors the *sacB* gene that confers sucrose sensitivity and allows counterselection for homologous recombination by double crossover (33). The plasmid was introduced into *L. pneumophila* JR32 (56) and selected for kanamycin and sucrose resistance, and the recombinants were confirmed for allelic replacement via PCR and increased UV sensitivity, as would be expected of a *recA* mutant (17).

**Observation using CSLM, scanning electron microscope (SEM), and transmission electron microscope (TEM).** Glass slides with biofilms formed on both sides by *L. pneumophila* carrying pPZ1 were cleaned on one side with an alcohol swab and mounted on an Eclipse TE2000-U inverted microscope (Nikon) attached to a Radiance 2100 Laser Scanning system (Bio-Rad) to observe the biofilm attached on the intact side. Images were taken under an argon laser source with a dichroic mirror (excitation, 488 nm; emission, 515 nm) and processed by LaserSharp 2000 (Bio-Rad) software.

For EM observation, biofilms formed on the glass test tubes were transferred

TABLE 1. *Legionella* strains used in this study

Species (serogroup)	Strain (GTC <sup>a</sup> designation)	Numerical designation in Fig. 1
<i>L. pneumophila</i>		
<i>L. pneumophila</i> (1)	Philadelphia-1 (GTC 9134 <sup>b</sup> )	1
<i>L. pneumophila</i> (1)	Knoxville-1 (GTC 9135)	2
<i>L. pneumophila</i> (2)	Togus-1 (GTC 9136)	3
<i>L. pneumophila</i> (3)	Bloomington-2 (GTC 9137)	4
<i>L. pneumophila</i> (4)	Los Angeles-1 (GTC 9246 <sup>b</sup> )	5
<i>L. pneumophila</i> (5)	Dallas-1E (GTC 9139)	6
<i>L. pneumophila</i> (6)	Chicago-2 (GTC 9138)	7
<i>L. pneumophila</i> (7)	Chicago-8 (GTC 10064)	8
<i>L. pneumophila</i> (8)	Concord-3 (GTC 10065)	9
<i>L. pneumophila</i> (9)	IN-23-G <sub>1</sub> -C <sub>2</sub> (GTC 10738)	10
<i>L. pneumophila</i> (10)	Leiden-1 (GTC 11369)	11
<i>L. pneumophila</i> (11)	797-PA-H (GTC 11365)	12
<i>L. pneumophila</i> (5)	U8W (GTC 13567 <sup>b</sup> )	13
Non- <i>L. pneumophila</i> species		
<i>L. bozemanii</i> (1)	ATCC <sup>c</sup> 33217 (GTC 9140 <sup>b</sup> )	14
<i>L. micdadei</i> (NR <sup>d</sup> )	ATCC 33218 (GTC 9141 <sup>b</sup> )	15 <sup>e</sup>
<i>L. gormanii</i> (NR)	ATCC 33297 (GTC 9142 <sup>b</sup> )	16 <sup>f</sup>
<i>L. dumoffii</i> (NR)	ATCC 33343 (GTC 9244)	17 <sup>f</sup>
<i>L. longbeachae</i> (1)	ATCC 33462 (GTC 9245 <sup>b</sup> )	18 <sup>f</sup>
<i>L. dumoffii</i> (NR)	ATCC 33279 (GTC 9247 <sup>b</sup> )	19
<i>L. oakridgensis</i> (NR)	ATCC 33761 (GTC 10061 <sup>b</sup> )	20
<i>L. wadsworthii</i> (NR)	ATCC 33877 (GTC 10062 <sup>b</sup> )	21
<i>L. feeleii</i> (1)	ATCC 35072 (GTC 10063 <sup>b</sup> )	22 <sup>g</sup>
<i>L. longbeachae</i> (2)	ATCC 33484 (GTC 10066)	23
<i>L. sainthelensi</i> (1)	ATCC 35248 (GTC 10392 <sup>b</sup> )	24
<i>L. bozemanii</i> (2)	ATCC 35545 (GTC 10739)	25
<i>L. hackeliae</i> (1)	ATCC 35250 (GTC 10740 <sup>b</sup> )	26
<i>L. jamestowniensis</i> (NR)	ATCC 35298 (GTC 10741 <sup>b</sup> )	27 <sup>e</sup>
<i>L. rubrilucens</i> (NR)	ATCC 35304 (GTC 10743 <sup>b</sup> )	28
<i>L. feeleii</i> (2)	ATCC 35849 (GTC 10744)	29
<i>L. maceachernii</i> (NR)	ATCC 35300 (GTC 10745 <sup>b</sup> )	30
<i>L. spiritensis</i> (NR)	ATCC 35249 (GTC 11199 <sup>b</sup> )	31 <sup>g</sup>
<i>L. israelensis</i> (NR)	ATCC 43119 (GTC 11367 <sup>b</sup> )	32
<i>L. hackeliae</i> (2)	ATCC 35999 (GTC 11368)	33
<i>L. parisiensis</i> (NR)	ATCC 35299 (GTC 11745 <sup>b</sup> )	34
<i>L. erythra</i> (NR)	ATCC 35303 (GTC 11748 <sup>b</sup> )	35 <sup>g</sup>
<i>L. birminghamensis</i> (NR)	ATCC 43702 (GTC 11749 <sup>b</sup> )	36
<i>L. anisa</i> (NR)	ATCC 35292 (GTC 12075 <sup>b</sup> )	37 <sup>g</sup>
<i>L. cincinnatiensis</i> (NR)	ATCC 43753 (GTC 12201 <sup>b</sup> )	38
<i>L. quinlivanii</i> (NR)	ATCC 43830 (GTC 12648 <sup>b</sup> )	39
<i>L. moravica</i> (NR)	ATCC 43877 (GTC 12649 <sup>b</sup> )	40 <sup>g</sup>
<i>L. brunensis</i> (NR)	ATCC 43878 (GTC 12655 <sup>b</sup> )	41 <sup>e</sup>
<i>L. tusconensis</i> (NR)	ATCC 49180 (GTC 12656 <sup>b</sup> )	42
<i>L. jordani</i> (NR)	ATCC 33623 (GTC 12657 <sup>b</sup> )	43
<i>L. adelaidensis</i> (NR)	ATCC 49625 (GTC 13562 <sup>b</sup> )	44 <sup>e</sup>
<i>L. fairfieldensis</i> (NR)	ATCC 49588 (GTC 13563 <sup>b</sup> )	45
<i>L. gratiana</i> (NR)	ATCC 49413 (GTC 13564 <sup>b</sup> )	46 <sup>g</sup>
<i>L. lansingensis</i> (NR)	ATCC 49751 (GTC 13565 <sup>b</sup> )	47
<i>L. geestiana</i> (NR)	ATCC 49504 (GTC 13568 <sup>b</sup> )	48
<i>L. londiniensis</i> (NR)	ATCC 49505 (GTC 13635 <sup>b</sup> )	49
<i>L. nautarum</i> (NR)	ATCC 49506 (GTC 13636 <sup>b</sup> )	50 <sup>g</sup>
<i>L. worsliensis</i> (NR)	ATCC 49508 (GTC 13638 <sup>b</sup> )	51 <sup>g</sup>

<sup>a</sup> GTC, Gifu Type Culture Collection, Department of Microbiology, Gifu University School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan.

<sup>b</sup> Type strain.

<sup>c</sup> ATCC, American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209.

<sup>d</sup> NR, not reported.

<sup>e</sup> At 25°C, less-robust growth was observed on BYECA.

<sup>f</sup> At 37°C, less-robust growth was observed on BYECA.

<sup>g</sup> At 42°C, less-robust growth was observed on BYECA.

using a toothpick into glass petri dishes and washed briefly with deionized water. They were fixed with 2% glutaraldehyde for 2 to 3 h and then additionally with 1% OsO<sub>4</sub> in the case of TEM observation. Subsequent dehydration was performed step wise using 50%, 70%, 90%, 95%, 99.5%, and 100% ethanol. For SEM observation, the ethanol was replaced by isoamyl acetate and then liquid CO<sub>2</sub>, which was allowed to vaporize gradually. Following this, the samples were coated with 60% gold-40% palladium alloy powder. For TEM observation, dehydrated samples were embedded in Epon resin, ultrathin sectioned, and stained with uranyl acetate, followed by lead citrate. The samples were examined with or without using a scanning image-observing device attached to a JEOL JEM 2000EX electron microscope.

**Nucleoid staining of filamentous cells.** Filamentous cells from *L. pneumophila* biofilms were teased on glass slides to separate the strands and stained according to a modified version of HCl-Giemsa staining (50). Briefly, the preparation was fixed in OsO<sub>4</sub> vapor for 45 min, treated with 1 M hydrochloric acid at 60°C for 10 min, rinsed, and stained with Giemsa stain for 5 min, followed by phosphate buffer (pH 6.4) for 5 min. The air-dried preparation was observed under an oil immersion lens on a light microscope (Olympus).

**Monitoring distribution of filamentous and rod-shaped cells.** Biofilms formed on glass test tubes or colonies formed on agar were picked by toothpicks and spread extensively on glass slides for ease of examination of individual cells. For observation of cells in the medium surrounding biofilms, aliquots of the medium were taken with care to avoid disturbing the biofilms, centrifuged briefly to collect the cells in pellets, and then spread on glass slides. Cells from shaken culture were similarly collected and spread. Slides with these preparations were flame fixed, stained with Pfeiffer stain, and observed under oil immersion by a light microscope. Images in three different fields were processed with the Image-Pro 3D Suite (Media Cybernetics, Inc.) to quantify the number of pixel units for images of the filamentous and rod-shaped forms. The percentage of biovolume of filamentous cells was calculated as  $100 \times (\text{pixel units for filamentous cells} / \text{pixel units for all cells})$ .

**Growth of cells from biofilm and planktonic culture.** Biofilms cultured for 4 days in glass test tubes were collected with Pasteur pipettes and rinsed briefly in sterile deionized water. They were then transferred into a test tube containing 5 ml of AC buffer [4 mM MgSO<sub>4</sub>, 0.4 mM CaCl<sub>2</sub>, 3.4 mM sodium citrate, 2.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.5 mM KH<sub>2</sub>PO<sub>4</sub>, 20-mg/liter Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> · 6H<sub>2</sub>O, pH 6.5] and shaken vigorously for 1 h to physically separate the filaments. A microscopic check of this suspension revealed that 90% of the cells were untangled from each other. For the "planktonic control," a 30-h shaken culture in BYE was diluted 1:100 in 5 ml AC buffer and shaken similarly for 1 h. Aliquots (each, 4 ml) of the biofilm cell-derived and planktonic cell-derived suspensions were then transferred to conical flasks, each containing 66 ml of fresh BYE broth. Growth was monitored at 4-h intervals by counting the bacterial CFU on buffered yeast extract charcoal agar.

## RESULTS

**Biofilm formation by *Legionella* spp.** To assess the ability of *Legionella* spp. to form biofilms, particularly to compare *L. pneumophila* to non-*L. pneumophila* species, we utilized 13 strains of *L. pneumophila* and 38 strains of other *Legionella* spp. (Table 1). This gives a good representation of the genus *Legionella*. The experimental system is a static culture in the BYE broth medium, and three different materials (glass, PS, and PP) were chosen as surfaces for biofilm growth at three temperatures (25°C, 37°C, and 42°C). The growth of all test strains in BYE liquid and agar media at the experimental temperatures was checked to ensure that any inability to form a biofilm was not due to nonoptimal growth conditions. A preliminary experiment, in which biofilm formation was noted daily by eye over a period of 30 days, indicated that stable biofilms could be observed from day 11 at 25°C and from day 3 at 37°C or 42°C. The biofilms formed were therefore quantified by crystal violet staining on day 12 for the 25°C cultures and day 4 for the 37°C and 42°C cultures. The results are presented in Fig. 1. Statistical analysis of the data indicated that at 37°C and 42°C on glass and PS (Fig. 1A and B) and at 25°C on PP (Fig. 1C), *L. pneumophila* strains have significantly greater biofilm-forming capacity than all other *Legionella* spp.

**Quantity, speed of formation, and adherence stability of *L. pneumophila* biofilms.** In the above data, the quantity of the biofilms formed by *L. pneumophila* strains (Fig. 1, strains 1 to 13) was observed to be influenced by temperature. On glass and PS surfaces (Fig. 1A and B), biofilms were generally formed more extensively at the higher temperatures than at 25°C: biofilms at 37°C were two- to sevenfold higher (glass) and two- to fourfold higher (PS) in yield and at 42°C were

three- to fivefold higher (glass) and two- to fivefold higher (PS) in yield. On PP surfaces, the situation was reversed: at 25°C, 2- to 7-fold and 3- to 16-fold more biofilms were formed than at 37°C and 42°C, respectively.

The temperature dependence could also be observed in the speed of formation and the adherence stability of the biofilms of *L. pneumophila* strains. In three to five independent experiments looking at biofilm formation by the 13 *L. pneumophila* strains in glass test tubes, we documented (i) the number of days it took for the appearance of stable biofilms, as an indicator of the speed of biofilm formation; and (ii) the day at which the biofilms were observed to naturally detach from the walls of the test tubes, as an indicator of the adherence stability. Statistical analysis revealed that all *L. pneumophila* strains showed similar trends: at 25°C, biofilms were formed more slowly ( $12 \pm 1$  days) but remained stably attached throughout the 30-day period of observation. At 37°C and 42°C, biofilms were formed faster ( $3 \pm 1$  days), but adherence stability was lower ( $7 \pm 1$  days). Similar trends in the speed of biofilm formation were observed when biofilms were grown on PS or PP, but the difference in adherence stability was not as apparent (data not shown).

**Thickness and structure of *L. pneumophila* biofilms.** The temperature dependence may have biological significance related to possible survival advantages of *L. pneumophila* in man-made environments over non-*L. pneumophila* species. To look further into this, *L. pneumophila* strain Knoxville-1 was chosen as a representative of the 13 *L. pneumophila* strains for in-depth structural observation. We introduced pPZ1, which allows constitutive expression of the GFP into Knoxville-1 strain for visualization of its biofilms in the natural hydrated state by CLSM. The GFP-expressing Knoxville-1 strain showed biofilm formation properties at the three temperatures, similar to those of the wild-type Knoxville-1 strain (data not shown), indicating that the expression of GFP does not observably affect the gross phenomenon under study.

Biofilms were allowed to form on glass slides immersed in BYE broth and then observed directly under CLSM on the indicated days (chosen to arbitrarily represent an early and a mature stage of biofilm formation) to quantify the thickness (Table 2). Biofilms at 37°C and 42°C were found to be indeed thicker than that at 25°C by about twofold. In terms of structures, biofilms at 25°C (Fig. 2A) possessed features typical of biofilms reported to date, i.e., pillar- and mushroom-like structures and what seemed like water channels within (15). Biofilms at 37°C, however, showed an even and extensive mat of considerably greater cell density without the commonly observed water channel structures (Fig. 2B).

**Morphology of *L. pneumophila* cells in biofilms.** It was apparent that *L. pneumophila* biofilms at 37°C did not have the typical biofilm features (16) shown by most bacteria. At higher magnifications, cells within biofilm at 25°C showed the normal morphology of *L. pneumophila*, i.e., they were rod shaped (Fig. 2C). In contrast, the morphology of cells in biofilm at 37°C was filamentous (Fig. 2D). The meshwork of filaments resembled the mycelia of fungi; hence, we tentatively refer to it as a "mycelial mat-like biofilm." Filamentation of bacterial cells is sometimes associated with physiological abnormalities such as mutations or overexpression of certain proteins (8, 13). To check if the filamentation was an artifact due to *gfp* overex-



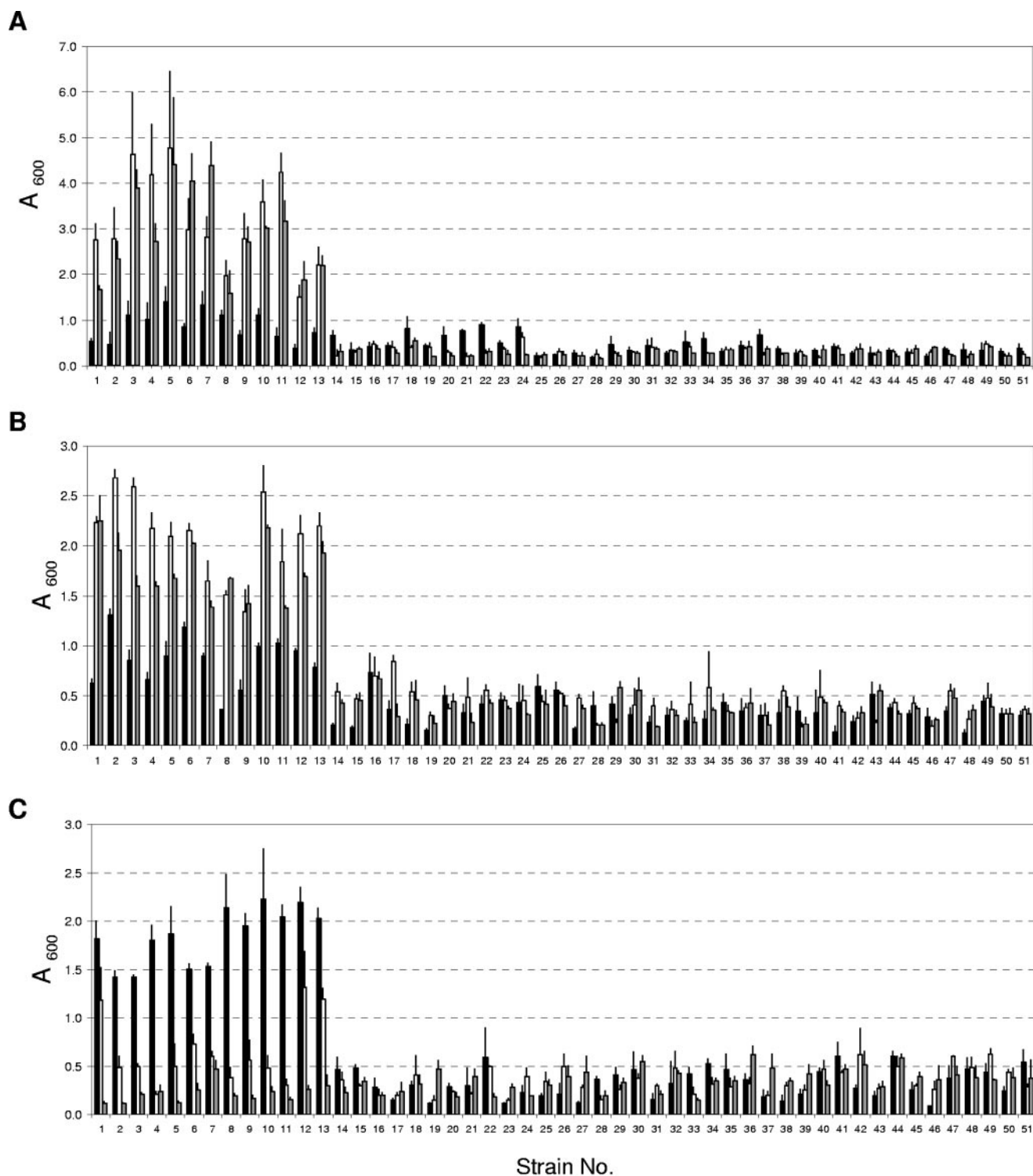


FIG. 1. Quantification of biofilms (as described in Materials and Methods) formed by *Legionella* spp. on surfaces of glass (A), polystyrene (B), and polypropylene (C) at incubation temperatures of 25°C (black bars), 37°C (white bars), and 42°C (gray bars). Strains or species are indicated as strain numbers and presented in Table 1. Values are presented as a means  $\pm$  standard deviation (SD) of three to six independent experiments. Statistical analysis was performed using Student's *t* test to compare the differences between groups, and *P* values of  $<0.05$  were considered statistically significant.

TABLE 2. Thickness of GFP-expressing *L. pneumophila* Knoxville-1 biofilm

Temperature (°C)	Day	Mean thickness <sup>a</sup> (μm)
25	9	20.4 ± 6.3
25	18	42.3 ± 10.9
37	3	37.5 ± 2.8
37	6	71.6 ± 4.4
42	3	40.3 ± 2.2
42	6	73.2 ± 3.5

<sup>a</sup> Values were obtained as means ± SD of six to eight measurements of thickness. Statistical analysis was performed using Student's *t* test to compare the differences between groups, and *P* values of <0.05 were considered statistically significant.

pression, we collected the biofilm formed by the wild-type Knoxville-1 strain at 37°C and prepared it for observation under the SEM. The electron micrograph (Fig. 2E) showed a filamentous meshwork similar to that seen by CSLM, ruling out the possibility of artifacts. Hence, the morphology of *L. pneumophila* cells in the biofilms formed on glass (and likewise on PS and PP surfaces) (data not shown) appears to be regulated by temperature.

**Distribution of rod-shaped and filamentous cells under different growth conditions.** The distribution of the morphological forms under differing growth conditions was next examined. We systematically assessed the relative contribution of filamentous cells to the total biovolume (i) within biofilms grown on glass, (ii) in the surrounding media, (iii) in colonies grown on agar, and (iv) in shaken cultures. At 25°C (Fig. 3, black bars), the static culture showed a consistent dominance by rod-shaped cells, both within the biofilm and outside (i.e., in the medium), throughout the period of observation. At 37°C and 42°C (Fig. 3, white and gray bars) the biofilms showed that close to 90% of the total biovolume consisted of filamentous cells as early as day 2 and continued to be dominated by the filamentous form until day 7. Upon detachment from the vessel walls (day 11 and day 13), these detached biofilms retained their mycelial characteristics. However, within the surrounding media of biofilms at 37°C and 42°C, rod-shaped cells were in the majority. Shaken cultures were predominantly rod shaped; it should be noted that at 37°C and 42°C, predominance of rod-shaped cells was maintained even up to day 3. Agar plate culture, on the other hand, showed a profile for distribution of forms similar to that observed in biofilms at the three temperatures.

**Filamentous cells are multinucleate and nonseptate.** The filamentous cells in *L. pneumophila*'s biofilm were stained for their nucleoids and found to be clearly multinucleate (Fig. 2F). This indicates that the filamentous form within the biofilm is one whereby DNA replication has proceeded in the absence of cell division. Indeed, observation by TEM showed the total lack of septa within the filamentous cells (Fig. 2G).

**Temperature-regulated filamentation in *L. pneumophila* is not RecA dependent.** The filaments formed as a result of non-septation, observed with *L. pneumophila* biofilms when cultured at higher temperatures, is reminiscent of the phenotype of the thermosensitive *recA* mutants of *Escherichia coli*. RecA in *E. coli* is a regulator involved in the SOS response induced

by DNA damage, and an abnormality in this regulator has been reported to result in filamentation at the elevated temperature of 40°C (10, 28). This is due to the control of RecA on factors involved in cell division, e.g., FtsZ (39) and SulA (20), both of which require well-regulated expression levels to ensure normal septation. A RecA homologue in *L. pneumophila* has been found to be able to functionally complement the *E. coli* counterpart and appears to be similarly regulated (17). The possible involvement of RecA in temperature-dependent filamentation by *L. pneumophila* cells was checked. An insertional null mutant of *recA* was generated in strain JR32, a *L. pneumophila* Philadelphia-1 derivative amenable to genetic manipulation (56) and observed to be able to form biofilms in a manner similar to that of the wild type, showing temperature-regulated morphological forms. Thus, the *recA* gene in *L. pneumophila* appears not to be responsible for the temperature-regulated filamentation phenomenon.

**Growth of cells from mycelial biofilm.** The fact that the filamentous form of *L. pneumophila* is multinucleate but nonseptate suggests that, given favorable conditions, the formation of septa may quickly give rise to more daughter cells than rod-shaped cells, since multiple DNA replication could be assumed to have already proceeded to completion during the filamentous state. To test this hypothesis, we mechanically "unraveled" a mature mycelial biofilm from a 37°C day 4 culture of Knoxville-1 strain by extensive shaking in a suitable buffer. We then followed the growth of its cells in fresh BYE medium shaken at 37°C over 36 h and compared it to that of the rod-shaped cells, seeded from a 30-h shaken culture (Fig. 4). The assumption is that one filamentous cell before septation will appear as one CFU. The rod-shaped cell culture continued in its lag phase until 12 h, but the culture from mycelial biofilm entered exponential phase by 8 h and mostly converted to rod-shaped cells. This shortened lag phase was remarkable, considering that cells from the biofilm were 4 days old and therefore expected to have an even longer lag phase than cells from a 30-h-old shaken culture. The growth rate (gradient at exponential phase) for the mycelial mat-like culture was not significantly higher than that for the rod-shaped cell culture during their respective exponential phases. Hence, the filamentous form of *L. pneumophila* seems to allow the organism to proliferate particularly rapidly in the initial stage, more than does the normal rod-shaped form.

## DISCUSSION

The ability to form biofilms provide a bacterium with survival advantages in the environment, e.g., anchorage at a location where growth is favorable, protection from desiccation, and resistance to biocides and detergents (15, 16, 18). Under the conditions used in this study, the 13 strains of *L. pneumophila* tested showed enhanced biofilm formation compared to other non-*L. pneumophila* strains: at 37°C and 42°C on glass and PS surfaces (Fig. 1A and B) and at 25°C on PP surfaces (Fig. 1C). To some extent, this may be due to the less-robust growth of a few non-*L. pneumophila* strains at specific temperatures (Table 1, footnotes *e* to *g*) in a medium optimized for cultivation of *L. pneumophila*, but these are minor exceptions. Within the genus *Legionella*, *L. pneumophila* is the species most often associated with human clinical cases (22). One

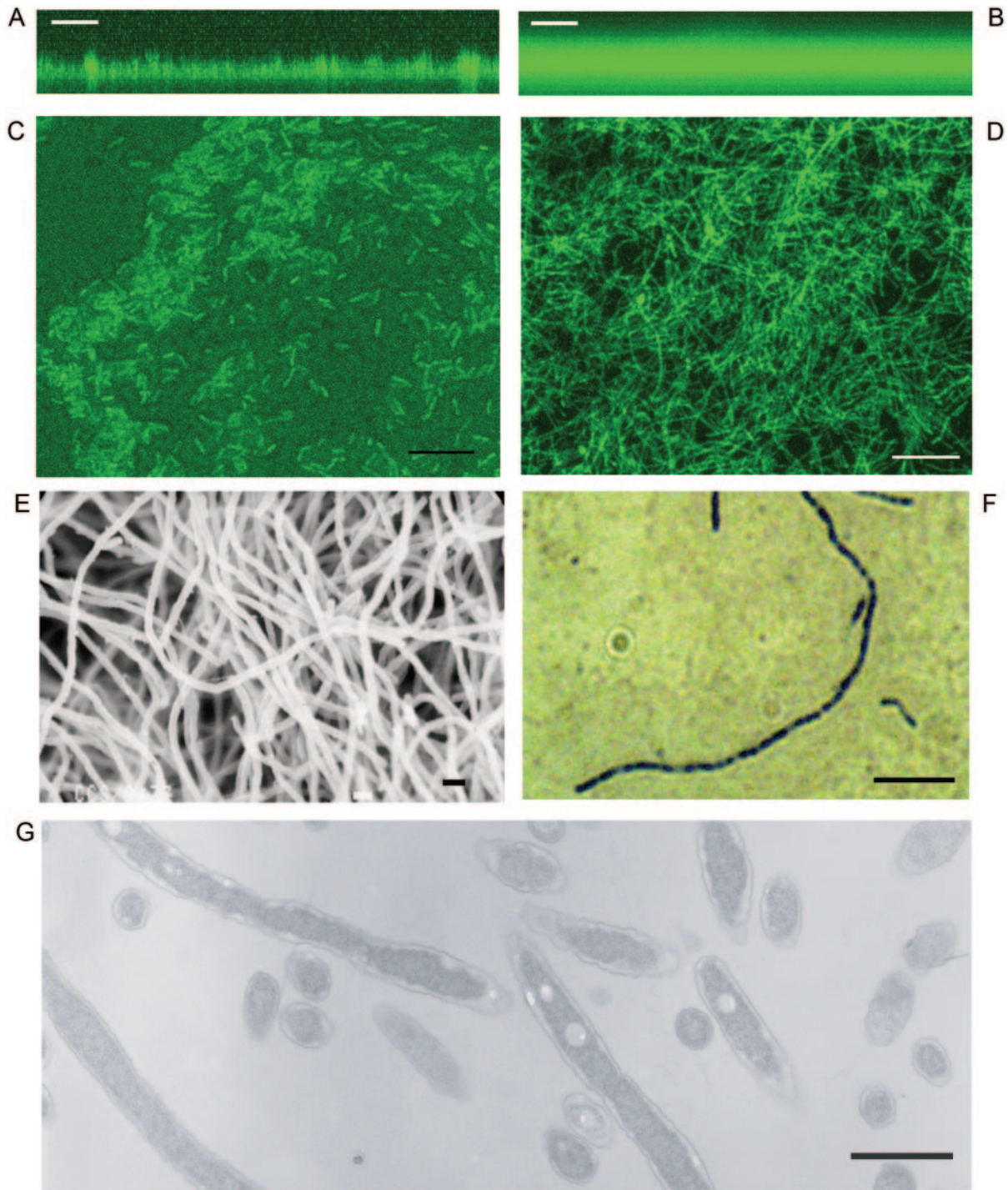


FIG. 2. Structure of *L. pneumophila* biofilms. The *L. pneumophila* Knoxville-1 strain expressing GFP was observed under CSLM (A to D). *x-z* plane projection of 25°C biofilm at day 18 (A) and 37°C biofilm at day 6 (B); *x-y* plane projection of 25°C biofilm at day 8 (C) and 37°C biofilm at day 4 (D). The *L. pneumophila* Knoxville-1 biofilm at 37°C on day 4 was observed under scanning electron microscope (E), and its filamentous cell stained with HCl-Giemsa was observed under the light microscope (F). (G) A section of the filamentous cells of *L. pneumophila* Philadelphia-1 strain was observed under the transmission electron microscope. Bars, 50  $\mu\text{m}$  (A and B), 10  $\mu\text{m}$  (C and D), 1  $\mu\text{m}$  (E), 5  $\mu\text{m}$  (F), and 1  $\mu\text{m}$  (G). Images were processed and compiled with Adobe Photoshop 7.0 software.

speculation is that it is better able to survive in man-made environments than non-*L. pneumophila* species and hence has a greater chance of crossing paths with the human population. Our finding that *L. pneumophila* is more proficient at biofilm

formation than other *Legionella* species supports this speculation. Since the temperatures 37°C and 42°C are often encountered in man-made aquatic environments, e.g., air-conditioning cooling towers in the summer and hot spring spas, the



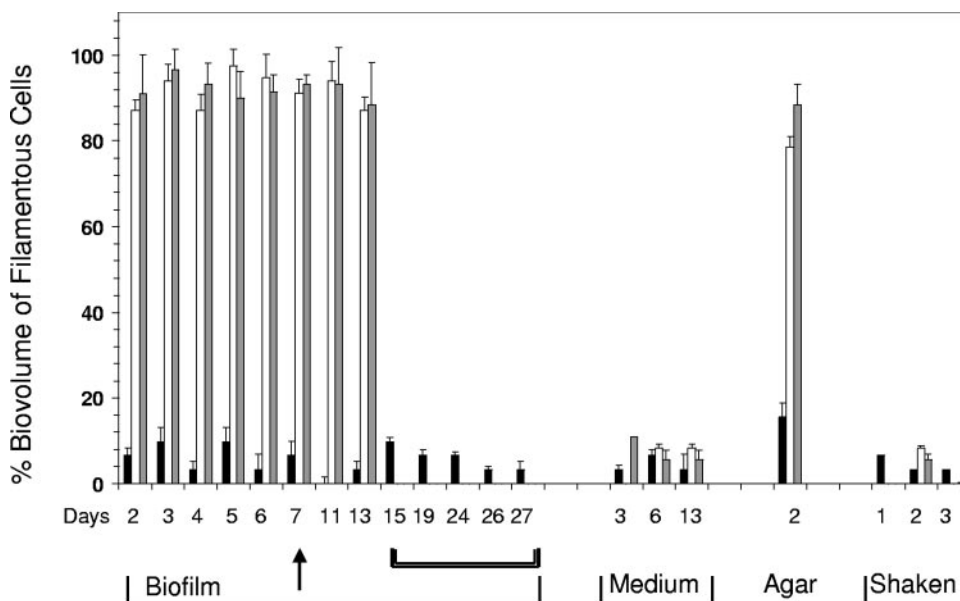


FIG. 3. Percentage of biovolumes represented by filamentous cells within various *L. pneumophila* Knoxville-1 cultures. Black bars represent growth at 25°C, white bars represent growth at 37°C, and gray bars represent growth at 42°C. The arrow indicates the point at which detachment of biofilms at 37°C and 42°C occurred. Double brackets indicate days when only 25°C biofilms were assessed. Values are presented as means ± SD of triplicate data. Statistical analysis was performed using Student's *t* test to compare the differences between groups, and *P* values of <0.05 were considered statistically significant.

enhanced ability by *L. pneumophila* to form biofilms on glass, PS, and possibly other surfaces would likely increase its chance of association with humans. On the other hand, the fact that biofilm formation is favored on PP at 25°C also suggests that the competitive edge conferred may be dependent on a combination of the temperature and the type of attachment surfaces.

It is interesting to note that the properties of *L. pneumophila* biofilms formed at 25°C differ considerably from those formed

at 37°C and 42°C. At higher temperatures, the biofilms are formed faster, thicker, and spread wider. Most interestingly, at higher temperatures, they are mycelial mat like in structure. Bacterial biofilms reported to date commonly show structures of polymeric matrices interspersed with water channels (32). For rod-shaped bacteria, e.g., *Pseudomonas* spp. and *Vibrio cholerae*, cells within biofilms remain rod shaped (60), with only a rare appearance of filaments (38). In rivers and on riverbeds, mixed-community biofilms may have filamentous components (bacteria or fungi) among rods and cocci (35), but biofilms composed primarily of extensive meshwork of filaments, to our knowledge, have only been reported for *Thiothrix* spp. in cases of biofouling (6) and *Methanosaeta* spp. in anaerobic sludge granules (29). Furthermore, although filamentation in rod-shaped bacteria with physiological abnormalities has been reported (13, 28, 48, 66), temperature-regulated filamentation in a biofilm context is highly unusual.

One question that may arise with this novel form of biofilm is, can we truly consider it a "biofilm"? The definition given by Donlan and Costerton (16) states that a biofilm is "a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription." For our mycelial mat-like structure, the criteria of attachment is certainly satisfied; furthermore, a thin layer of ruthenium red-stainable substance (possibly exopolysaccharide) (24) coats the filaments (data not shown). The filamentous cells have a growth profile (Fig. 4) that indicates an altered phenotype from the planktonic, rod-shaped form and are expected to have considerable modification in gene expression compared to expression in a binary fission mode. Our current analysis of

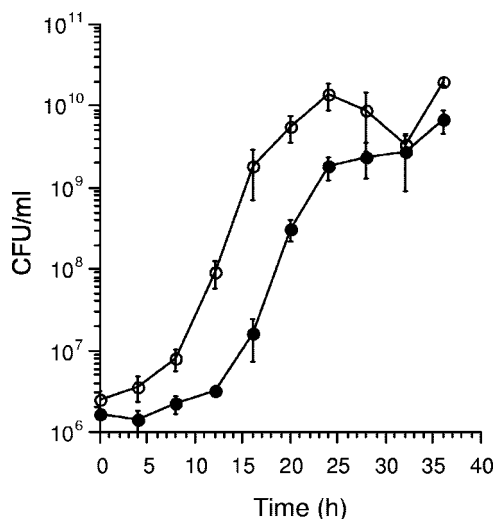


FIG. 4. Growth of *L. pneumophila* Knoxville-1 in BYE. The culture was shaken at 37°C and seeded from filamentous cells of a 37°C biofilm at day 4 (open circles) and rod-shaped planktonic cells of a 37°C culture shaken for 30 h (black circles). Data are means ± SD from three independent experiments.

the transcriptional profiles of the biofilm filamentous versus planktonic *L. pneumophila* cells also substantiates this point (Z. Piao, unpublished data). Thus, all the criteria seem to have been met for the mycelial mat-like structure to be referred to as a biofilm.

*L. pneumophila* have long been known to be pleomorphic (31), varying its morphology with physiological conditions (36, 43) or infectious cycle phases (61). Species of *Legionella* have also been reported to form varied microcolonies within Vero cells, some appearing filamentous, although the septation status has not been addressed (44). *L. pneumophila* also “differentiates” into a few extracellular or intracellular forms with varying degrees of infectivity for HeLa cells (25), which can be distinguished by the ultrastructural properties of their cell envelopes (21). In our study, the electron micrograph of the filamentous *L. pneumophila* cell (Fig. 2G) shows cell wall structure that is consistent with that of the extracellular stationary phase rod forms reported by Faulkner et al. (21), confirming its “extracellular” status.

Extensive filamentation by *L. pneumophila* has previously been described in conditions of physiological stress such as exposure to antibiotics (19, 59) and nutrient limitation (63). In other rod-shaped bacteria, filamentation by mutants of global regulator (28) or cell division genes (13, 48) and when exposed to high salt concentrations (66) has also been reported. We considered, by analogy with the phenotype of an *E. coli* mutant (10), that our filamentation phenomenon could be due to the regulatory action of the *recA* gene. The thermosensitive mutation *recA441* (formerly *tif-1*) of *E. coli* is known to convert cells to multinucleate, aseptate filaments at 40°C through the constitutive expression of the *sulA* gene that encodes an inhibitor of the cell division protein FtsZ (34). However, this possibility was ruled out for *L. pneumophila* because disruption of the *recA* homologue was found to be without effect on the phenotype. Another immediate possibility, suggested by analogy with *E. coli* *ftsZ* mutants (28, 47), is that *L. pneumophila* FtsZ protein might be thermosensitive. We have not yet been successful in substantiating this idea, but we intend to continue to dissect the mechanism of regulation in this novel form of biofilm. The recently completed *L. pneumophila* genome sequences of strains Philadelphia-1 (12) and Paris (11) ([http://genome3.cpmc.columbia.edu/~legion/seq\\_anno.html](http://genome3.cpmc.columbia.edu/~legion/seq_anno.html)) indicate that the organism possesses not only *recA* and *ftsZ* but also homologues of other *E. coli* genes, e.g., *ftsA* and *mreB*, involved in cell division and maintenance of cell morphology (48). We are currently looking into how these genes are regulated and correlated with the biofilm phenotype of *L. pneumophila*.

Filamentation is often a consequence of some stress-induced disruption of normal cell division functions. However, in rare cases, it serves as a strategy to counter specific types of stress. Elongation of cells by nutrient-deprived *Pseudomonas aeruginosa* has been proposed to be a strategic response to enlarge their nutrient collection surface without substantially changing the surface area-to-volume ratio (60). Temperature-regulated filamentation in *L. pneumophila* biofilm is unusual in that it is clearly not a simple case of cell division inhibition by sublethal heat stress, e.g., as seen with *Listeria monocytogenes* (53). Induction of filamentation at 37°C or 42°C was not observed for *L. pneumophila* cells outside of the biofilms or in a shaken liquid medium. Instead, only surface-associated cells, i.e.,

within biofilm or on agar, exhibited filamentation (Fig. 3). Some other signal(s) besides temperature, e.g., attachment to surfaces, is possibly involved, and it is conceivable that filamentation is a strategic regulated response rather than an accidental by-product of stress. Our growth experiment showed that cell proliferation of the biofilm filamentous form is faster at the initial stage than the planktonic rod-shaped form (Fig. 4). During this initial stage, the multinucleate filamentous cells have converted into rod-shaped forms, after which no difference in growth rate could be observed. The filament-turned-rod-shaped cells therefore do not differ in terms of growth rate from the normal rod-shaped cells. Presumably, the time and energy required by a filamentous cell to increase in number involve only the formation of cell septa, whereas binary fission involves DNA replication and biomass generation, in addition to septa formation. Hence, filamentation may represent a novel strategy utilized by *L. pneumophila* to rapidly increase its population.

It is worth noting that the experimental system of our study made use of a rich medium. In low-nutrient medium, very little formation of biofilm could be observed (C. C. Sze, unpublished data). In the environment, the bacterium more often encounters low-nutrient status, so one may wonder at the relevance of this particular study. However, it should not be forgotten that the environment is dynamic and that bacteria generally experience cycles of “feast and famine.” The survival of a bacterium, therefore, not only involves living through a famine but also hinges on how well it exploits the rare but valuable encounter of a feast. *L. pneumophila* is a slow grower compared to other bacteria such as *E. coli*, *P. aeruginosa*, and *V. cholerae* and may seem to be in danger of losing out to these fast growers in nature. It has, in part, made up for its shortcomings by finding a niche as an intracellular parasite of free-living protozoans (4, 41, 54, 55). By forming mycelial mat-like biofilms, *L. pneumophila* may have hit upon another strategy to compete with fast growers in the environment, which allows it to (i) anchor itself at a location transiently flooded with nutrients, (ii) increase rapidly in biomass while able to access the nutrients by eliminating the process of septum formation, and then (iii) proliferate and disperse in great numbers when conditions are appropriate. We intend to explore this phenomenon of filamentous biofilms further, from both genetic and ecological points of view.

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