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Comparative analysis of virulence traits between a *Legionella feeleii* strain implicated in Pontiac fever and a strain that caused Legionnaires' disease

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ABSTRACT

Legionella strains of the same species and serogroup are known to cause Legionnaires' disease (a potentially fatal atypical pneumonia) or Pontiac fever (a mild, flu-like disease), but the bacterial factors that define these dramatic differences in pathology have not been elucidated. To gain a better understanding of these factors, we compared the characteristics of *Legionella feeleii* strains that were isolated from either a sample of freshwater implicated in an outbreak of Pontiac fever (ATCC 35072, serogroup 1, LfPF), or a patient with Legionnaires' disease (ATCC 38549, serogroup 2, LfLD). Growth of LfPF and LfLD in BYE broth was slower than the positive control, *Legionella pneumophila* strain JR32. However, LfLD grew faster than LfPF at 42 °C. After *in vitro* infection to J774 murine or U937 human macrophage cell lines and A549 human lung epithelial cell line, LfLD showed a higher cell infection rate, stronger internalization by host cells, and greater cytotoxicity than that of LfPF. Large amounts of IL-6 and IL-8 were secreted by human host cells after infection with LfLD, but not with LfPF. LfLD possessed mono-polar flagellum while LfPF was unflagellated. When LfLD was cultured at 25, 30 and 37 °C, the bacteria had higher motility rate at lower temperatures. Based on our results, this is the first study that showed distinct characteristics between LfPF and LfLD, which may give important leads in elucidating differences in their virulence.

1. Introduction

Legionellae are gram-negative bacteria, which ubiquitously live in natural or artificial aquatic environments, such as lakes, streams, spa baths, fountains, and air-conditioned cooling systems [1]. The bacteria can cause human respiratory diseases, known as Pontiac fever (a mild, flu-like disease) or Legionnaires' disease (an atypical pneumonia). The illness can be transmitted by inhalation of aerosolized droplet water contaminated with the bacteria [2]. However, it cannot be transmitted from person to person. It had been reported that *Legionella pneumophila* [3], *Legionella micdadei* [4], *Legionella anisa* [5], *Legionella longbeachae* [6] and *Legionella feeleii* [7] can cause Pontiac fever, which is a self-limiting, flu-like disease without pneumonia [8]. It has a short incubation period, and short illness duration, usually within 48 h, and the causative bacteria have not been isolated yet from Pontiac fever patients. While the disease progresses acutely and shows a high attack rate of approximately 95%, the mortality rate is zero [9]. On the other hand, some *Legionella* species cause a severe community-acquired pneumonia (CAP), which is mainly due to *L. pneumophila* (91.5%), followed by *L. longbeachae* (3.9%), *Legionella bozemanii* (2.4%), *L. micdadei*, and *L. feeleii* [10,11]. CAP caused by *Legionella* has a fatality rate of approximately 10%, which can increase up to 27% if adequate therapy is not carried out [12]. Patients with Legionnaires' disease usually have fever, cough, chills, headache, and suffer from pneumonia. The underlying mechanisms of Pontiac fever or Legionnaires' disease, however, have not been elucidated.

Legionella is an intracellular pathogenic organism, which can proliferate in mammalian cells, such as macrophages or epithelial cells [2,13,14]. It has been demonstrated that flagella played a crucial role in enhancing bacterial infection capacity when human macrophage-like cell lines were infected with *L. pneumophila* [15].

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The innate immune system is the first defense line of humans that prevents them from being infected by microbial pathogens. Toll-like receptor (TLR) family plays an important role in recognizing pathogen-associated molecular patterns (PAMPs), such as bacterial flagellin, and mediates the secretion of cytokines in order to induce the immune response to eradicate the infectious agents [16].

In this study, we focused on the comparison of the virulence-related traits of *L. feeleii* ATCC 35072 (serogroup 1, hereinafter referred to as LfPF) and *L. feeleii* ATCC 35849 (serogroup 2, hereinafter referred to as LfLD). Until now there is no in-depth report on the comparison of the pathogenicity of *L. feeleii* strains. To understand the virulence-related traits of *Legionella* species causing Pontiac fever and pneumonia, we compared LfPF and LfLD in terms of their growth at high temperatures, their *in vitro* infection capacity, intracellular growth, cytotoxicity, and cytokine induction. In this paper, we report some distinct characteristics of LfPF and LfLD.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Both *L. feeleii* serogroup 1 (ATCC 35072) and *L. feeleii* serogroup 2 (ATCC 35849) were obtained from Gifu Type Culture Collection (GTC). ATCC 35072 strain was isolated from the water of an infection source of an outbreak of Pontiac fever in 1984 in Canada [7]. Since it is a mild and self-limiting disease, until now there is no clinical report on the isolation of the causative agent from the patients. ATCC 35849 strain was isolated from a patient with Legionnaires' disease in 1985 [17]. *L. pneumophila* serogroup 1 strain JR32 [18], a restriction-deficient derivative of Philadelphia-1 which was used as a positive control, was donated by Dr. Hiroshi Miyamoto (Saga University). All bacteria were cultured on buffered charcoal yeast extract (BCYE) agar plates or in buffered yeast extract (BYE) broth [19]. The pH was adjusted to 6.9 with 10 M potassium hydroxide.

2.2. Effect of temperature on bacterial growth in vitro

Legionella strains were harvested from BCYE agar plates after 2 days of incubation at 37 °C and suspended in BYE broth. The optical density of the culture was measured at 660 nm (OD_{660}) starting around 0.1, and the bacterial suspension was incubated at 37, 40, and 42 °C with shaking. The values of OD_{660} at different culture temperatures were recorded at 12 h intervals until 36 h.

2.3. Cell culture

J774, U937 and A549 cells were used in this study and cultured as described previously [14,20]. J774 macrophages (JCRB9108) were derived from murine macrophage-like cells. A549 cells (JCRB0076) were from human cancerous lung tissue and possess the characteristics of type II pneumocytes. Both of these cell lines were cultured in RPMI 1640 medium (SIGMA) with 10% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.). The cells were harvested by treating with trypsin and EDTA, and then cultured in chamber slides or 24-well plates. The human macrophage-like cells, U937 (JCRB9021), were cultured in suspension in RPMI 1640 with the addition of 10% fetal bovine serum and 2 mM glutamine. In order to transform U937 cells into adherent cells, they were differentiated with phorbol 12-myristate 13-acetate (PMA, final concentration of 10^{-8} M) (Sigma) in a test tube and cultured in a 24-well plate for 16 h before use [21]. All cell types were cultured in 75-cm^2 flasks (NUNC) and incubated at 37 °C or 40 °C with 5% CO_2.

2.4. Bacterial infection of cells and Gimenez staining

J774 macrophages and A549 epithelial cells were cultured using Millicell EZ slides (MILLIPORE). *Legionella* spp. were harvested from BCYE agar plates after 2 days of incubation. The bacteria were added to each well of cell monolayers to give a multiplicity of infection (MOI) of 10 or 100. J774 macrophages were infected for 1.5 h and A549 epithelial cells were infected for 2 h. After the *in vitro* co-culture, host cells were washed twice with 0.5 ml PBS to remove non-adherent bacterial cells. The cultures were treated with cell medium supplemented with 100 μg/ml gentamicin, incubated for another 1 h at 37 °C to kill the extracellular bacteria, and washed twice with PBS. At 12, 24, and 48 h post-infection, the infected cultures were stained by Gimenez staining [13,22]. Briefly, the cells were stained for 2 min with 1 ml carbol fuchsin mixed with 2.5 ml phosphate buffer (pH 7.45), then with 5% malachite green for another min, and washed gently with running water. After drying, slide glasses were observed under a light microscope. Three microscopic fields were evaluated for each post-infection time and in each microscopic field, more than 200 host cells were counted for each cell line and bacterial strain. The infected and non-infected host cells were counted in the same microscopic fields, and the percentage of the host cells infected with bacteria was calculated.

2.5. Intracellular growth assay

The experiments were carried out as described previously [14] but using both 37 and 40 °C of culture temperatures. Briefly, host cells (4×10^5/well) were cultured in 24-well tissue culture plates (Greiner CELLSTAR) overnight. At 37 °C, *Legionella* spp. were added to each well of the A549 cell monolayers to give a multiplicity of infection (MOI) of 100, and MOI of 10 for J774 and U937 macrophages. The infected A549 cells were incubated at 37 °C in 5% CO_2 for 2 h, while the macrophages were incubated for 1.5 h, and washed twice with PBS to remove non-adherent bacterial cells. The cultures were treated with 100 μg/ml gentamicin in RPMI 1640 medium for another 1 h to kill the bacteria that were not internalized into the host cells, and then washed twice with PBS to remove residual gentamicin. Fresh RPMI medium (0.5 ml) was added to the wells and the cells were cultured in a CO_2 incubator at 37 or 40 °C. At 0 h, 1 ml sterile distilled water was added to the wells, and after 4, 12, 24, 48 h of *in vitro* infection, the culture supernatants were transferred to test tubes and 0.5 ml sterile distilled water was added to the wells. Host cells were scraped by a rubber policeman from the bottom of the wells and were also transferred to the test tubes. After serial dilution in sterile distilled water, the lysates were plated on BCYE agar plates to determine the intracellular bacterial growth. CFUs at 0 h were considered as the time point when the bacteria were internalized into the host cells.

In order to obtain similar numbers of CFUs at 0 h, the host cells were infected with the bacteria at different MOIs at 40 °C. Similar to 37 °C, intracellular bacterial numbers were counted at 0, 24 and 48 h of *in vitro* infection.

2.6. Cytopathogenicity assay

Cytopathogenicity of *Legionella* strains was estimated by infecting J774 and U937 macrophages as previously described using Alamar Blue dye [20,23]. The macrophages were cultured overnight in 96-well microtiter plates (Falcon no. 3072; Becton Dickinson Labware, Oxnard, Calif.), at a density of 5×10^4/well, and infected with bacteria for 1.5 h at a MOI of 100. After *in vitro* phagocytosis, cells were treated with 100 μg/ml gentamicin in RPMI 1640 culture medium for 1 h, and washed twice with PBS to remove extracellular

bacteria. After 24 h of *in vitro* infection, the monolayers were treated with 10% Alamar Blue (Invitrogen) for 4 h. Alamar Blue is a redox potential indicator and its color changes from blue to red (reduced form) when glucose in the RPMI 1640 medium is oxidized by macrophages. *Legionella* cells are not involved in the process because they cannot oxidize glucose. The specific absorbance of oxidized Alamar blue was measured at 570 nm with a Bio-Rad SmartSpec 3000 spectrophotometer. The percentage of cell death was calculated by the following equation: [1- (value of absorbance of infected cells/value of absorbance of uninfected cells)] × 100.

2.7. Minimum inhibitory concentration (MIC) of gentamicin

MIC of gentamicin (Sigma) was determined by the microdilution method as described previously [24,25]. *Legionella* strains were harvested from BCYE agar plates after 2 days of incubation at 37 °C. One hundred microliters bacterial suspensions in BYE broth were prepared and mixed with 100 μl serially diluted antibiotic in 96-well plates. The inoculum size was approximately 10^5 CFU per well, and the MIC was defined as the minimum concentration of gentamicin solution that inhibited bacterial growth after culture at 37 °C for 3 days.

2.8. Cytokine assay

U937 macrophages and A549 epithelial cells were cultured in 24-well culture plates, at a density of 8×10^5/well and 3×10^5/well, respectively. The cells were infected with *Legionella* spp. at a MOI of 10 in U937 macrophages and 100 in A549 cells. After 24 and 48 h of infection, supernatants harvested from wells were centrifuged at 200× *g* for 5 min and 4400× *g* for 3 min prior to measurement. The levels of cytokines produced were measured by BD Cytometric Bead Array Cytokines Kit (BD Biosciences) according to the manufacturer's instruction.

2.9. Bacterial motility

Legionella strains were harvested from BCYE agar plates at 25 and 30 °C after 4 days of incubation or at 37 °C after 2 days of incubation. The bacteria were suspended in sterile distilled water, and 10 μl of each bacterial suspension was transferred to slide glasses. The bacteria were observed under a dark-field microscope and were qualitatively assessed as positive or negative for motility.

2.10. Identification of flagella expression

Legionella strains were harvested from BCYE agar plates at 25 and 30 °C after 4 days of incubation or at 37 °C after 2 days of incubation. The bacteria were suspended in 2% ammonium acetate to remove the medium, and stained by 2% sodium phosphotungstate. The samples were applied to copper Formvar-coated grids and observed under JEM-ARM 200F (JEOL) transmission electron microscope.

2.11. Statistical analyses

All experiments were performed at least three times. The two-tailed, unpaired *t*-test was performed to calculate *P* values. For the percentage of bacterial infection assay, all the data were analyzed by Wilcoxon test with the JMP12 software (SAS Institute Japan). Data were expressed as averages ± standard deviations. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Effects of temperatures on growth of L. feeleii strains in broth

L. feeleii ATCC 35072 (serogroup 1, LfPF), *L. feeleii* ATCC 35849 (serogroup 2, LfLD), and *L. pneumophila* JR32 were cultured at 37, 40, and 42 °C with shaking, and optical densities (OD) of the bacterial cultures were measured (Fig. 1). Growth of *L. feeleii* strains was significantly slower than that of *L. pneumophila* JR32 at all temperatures. The two strains of *L. feeleii* grew well at 37 and 40 °C, but not at 42 °C. The growth of LfPF was significantly ($P < 0.01$) slower than that of LfLD at 42 °C.

3.2. Bacterial infection of cells and Gimenez staining

The ability of *Legionella* to infect host cells at 37 °C was determined using J774 macrophages (Fig. 2A) or A549 cells (Fig. 2B) at a MOI of 10 or 100, respectively. After 12, 24 and 48 h infection, the infected host cells were stained with Gimenez stain and the percentage of host cells infected with bacteria was calculated by observing more than 200 host cells from 3 independent experiments under a light microscope at the indicated time points. *L. feeleii* LfPF and LfLD showed significantly different infection ability to host cells. LfLD was observed to have significantly stronger infection ability than that of LfPF and *L. pneumophila* JR32. LfPF showed the weakest infection ability among the bacterial strains used. The same phenomenon could be observed by Gimenez staining (Fig. 3). *L. feeleii* LfLD exhibited the strongest infection capacity in both epithelial cells (Fig. 3C) and macrophages (Fig. 3D).

3.3. Intracellular bacterial growth

Intracellular bacterial growth at 37 °C was observed after J774 (Fig. 4A) and U937 (Fig. 4C) macrophages or A549 epithelial cells (Fig. 4E) were infected with the different *Legionella* strains. CFUs at different times of infection represents internalization of bacteria by host cells (0 h), the initial killing ability of host cells (4 h), and intracellular growth (12 h and thereafter). The minimum inhibitory concentration (MIC) of gentamicin was 0.78 μg/ml for *L. feeleii* strains and 1.56 μg/ml for *L. pneumophila* JR32. Hence, gentamicin treatment (100 μg/ml) was thought to be effective in killing the bacteria that were not internalized and were present outside the host cells.

LfLD exhibited a stronger infection capacity at 37 °C, which was at least 10 times higher than the positive control, *L. pneumophila* JR32, while LfPF showed the weakest infection ability, using the

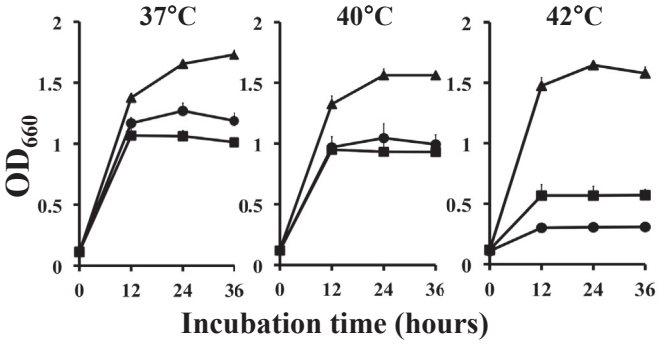

Fig. 1. Growth curves of LfPF (●), LfLD (■) and *Legionella pneumophila* strain JR32 (▲) in BYE broth according to temperatures (37, 40 and 42 °C). OD_{660} of the culture was measured at 12 h intervals until 36 h. All data represent independent experiments performed in triplicates.

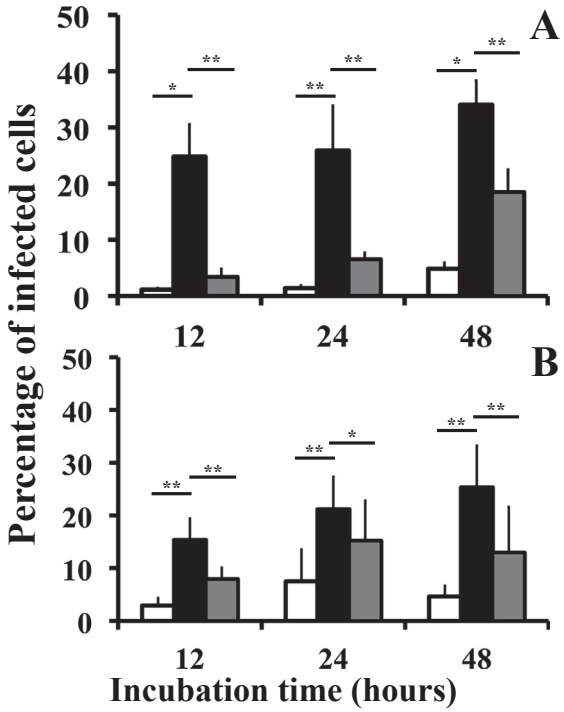

Fig. 2. The percentage of host cells infected with *L. feeleii* ATCC 35072 (LfPF, □), *L. feeleii* ATCC 35849 (LfLD, ■) and *L. pneumophila* JR32 (▩). J774 macrophages (A) or A549 (B) epithelial cells were infected at 37 °C, at a MOI of 10 or 100, respectively. After 12, 24 and 48 h infection, cultures of infected cells were stained by Gimenez stain. The percentage of bacterial infection was calculated based on the observations from 3 independent experiments at each time, and calculated as follows: (infected cells/total cells) × 100 (%) (**, $P < 0.01$; *, $P < 0.05$).

same MOI. Decrease in CFU (initial intracellular bacterial killing) at 4 h of infection was observed in all the bacterial strains (Fig. 4A and C).

After 4 h post-infection, LfPF and LfLD were able to initiate multiplication in J774 (Fig. 4A) and U937 (Fig. 4C) macrophages. In contrast, the intracellular growth of *L. pneumophila* JR32 became predominant at 48 h post-infection. After 24 and 48 h infection, all bacteria were found to be able to proliferate inside the host cells and the CFUs increased for about 10–100 times.

The infected macrophages or epithelial cells were then incubated at 40 °C for 48 h after *in vitro* infection (Fig. 4B, D, and F) to determine whether the bacteria could proliferate in host cells at higher temperature. The host cells were infected with the bacteria at different MOIs to obtain similar CFUs at 0 h. Intracellular growth was not observed in either of the strains after 48 h infection of macrophages (Fig. 4B and D). LfPF showed reduced numbers after 2 days infection of A549 cells (Fig. 4F), but LfLD could maintain the initial bacterial burden observed after internalization, which indicates that LfLD may be able to survive at high temperature (40 °C) during infection of human epithelial cells.

3.4. Cytotoxicity of *L. feeleii* infection in macrophages

The cytopathogenicity of LfPF and LfLD to J774 or U937 macrophages were examined at a MOI of 100 using the Alamar blue method. The cultures of infected cells were incubated at 37 °C, 5% CO_2 for 24 h, and the redox potential of the culture medium was measured. LfLD was cytotoxic to the host cells, as around 93% of J774 (Fig. 5A) or 70% of U937 (Fig. 5B) were killed after 24 h infection. LfLD showed the strongest cytopathogenicity among the bacterial strains used, followed by *L. pneumophila* JR32.

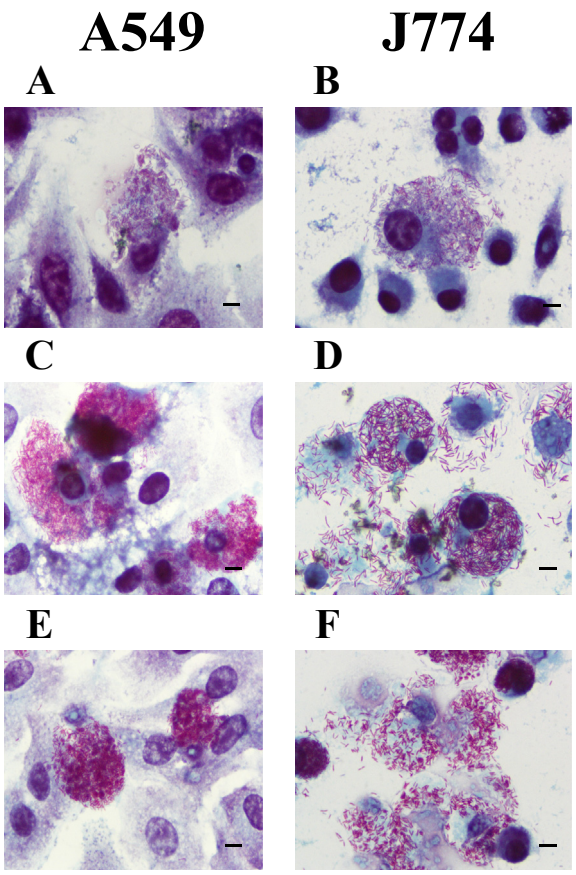

Fig. 3. Features of Gimenez-stained intracellular colonies of *Legionella*. A549 epithelial cells (A, C, E) and J774 macrophages (B, D, F) were infected with *L. feeleii* ATCC 35072 (LfPF; A, B), *L. feeleii* ATCC 35849 (LfLD; C, D), or *L. pneumophila* JR32 (E, F) at 37 °C, at a MOI of 100. At 24 h post-infection, cultures of infected cells were stained by Gimenez staining, and observed under light microscope. In A and B, only one infected host cell could be found in the microscopic field. Bars, 1 μm.

3.5. Stimulatory ability of *L. feeleii* for cytokine production

Cytokines produced by host cells infected with *Legionella* spp. were measured using Cytokine Kits after 24 and 48 h infection at a MOI of 10 for U937 macrophages or at a MOI of 100 for A549 epithelial cells. Secretion levels of IL-6, IL-8, and IL-10, which were triggered by infection with *L. feeleii* strains showed statistically significant differences in U937 macrophages (Fig. 6A) and A549 epithelial cells (Fig. 6B). The amounts of IL-6 or IL-8 elicited through the stimulation by LfLD were greater than that of LfPF, and even larger than the positive control *L. pneumophila* JR32. However, the expression of IL-10 (Fig. 6A) at the same time points had opposite results. LfLD was found to induce the least level of IL-10, which was even less than the negative control. There was no IL-10 detected in the supernatant of infected A549 cells (data not shown). Although IL-2, IL-4, IL-12p70, TNF-α, IFN-γ, and IL-1β were also measured simultaneously, they were not detected in any supernatants (data not shown).

3.6. Motility of *Legionella* strains

Motility of LfPF, LfLD, and *L. pneumophila* JR32 cultured in BCYE at 25, 30, or 37 °C was observed under a dark-field microscope. There were more motile LfLD cultured at 25 compared to those grown at 30 °C. However, motility of this strain could not be seen at 37 °C. LfPF did not express motility after being cultured at 25, 30, or

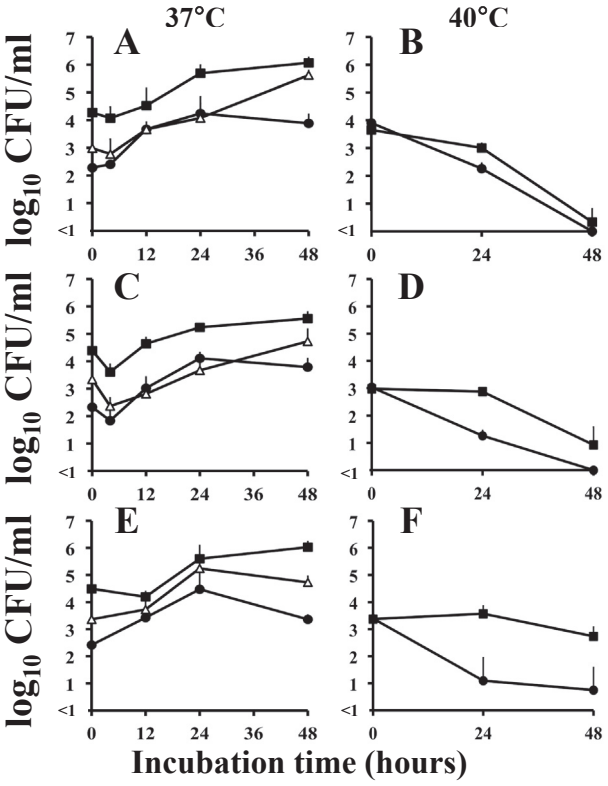

Fig. 4. Kinetics of intracellular *Legionella* growth and the effect of culture temperature. After *in vitro* phagocytosis, J774 (A, B), U937 (C, D) macrophages and A549 epithelial cells (E, F) were cultured at 37 °C (A, C, E) or at 40 °C (B, D, F). *L. feeleii* ATCC 35072 (LfPF, ●), *L. feeleii* ATCC 35849 (LfLD, ■), *L. pneumophila* JR32 (△) were used to infect J774 (A) or U937 (C) macrophages at a MOI of 10, or A549 cells (E) at a MOI of 100. At 40 °C, MOI was changed to obtain similar CFUs at 0 h of infection (B, D, F). All data are representative of independent infection experiments performed in triplicates.

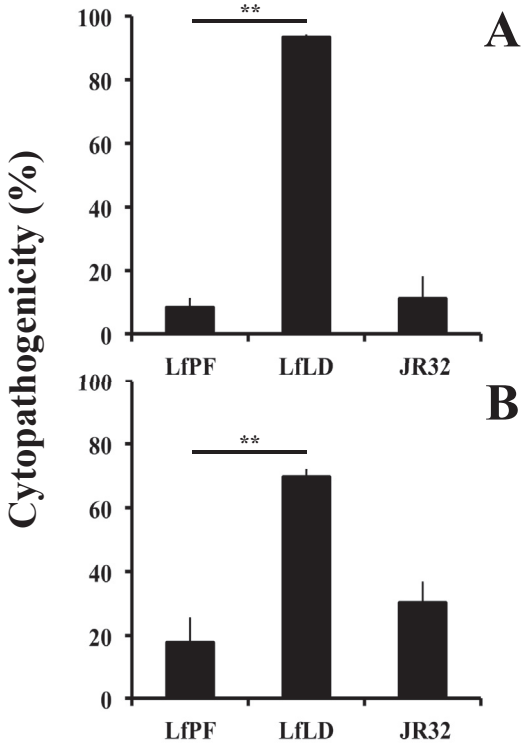

Fig. 5. Cytopathogenicity of *L. feeleii* ATCC 35072 (LfPF), *L. feeleii* ATCC 35849 (LfLD) and *L. pneumophila* JR32. J774 (A) or U937 (B) macrophages were infected with the bacteria at a MOI of 100 at 37 °C. The values indicated represent the percentage of dead host cells at 24 h-bacterial infection determined by 10% Alamar Blue assay. All data were derived from independent experiments performed at least three times (**, $P < 0.01$).

37 °C. Similar to LfLD, *L. pneumophila* JR32 also expressed bacterial motility at 25 and 30 °C, but not at 37 °C (Table 1).

3.7. Electron microscopy of flagella of Legionella strains

The presence of flagella of *Legionella* strains was determined by negative staining and observed by transmission electron microscopy. It was found that LfLD showed mono-polar flagellum at all temperatures (Fig. 7) while LfPF did not possess flagellum (Table 1). At 25 and 30 °C, the flagellum of LfLD showed a smooth wave morphology. However, at 37 °C, it expressed non-wavy flagellum. *L. pneumophila* JR32 showed smoothly waved mono-polar flagellum at each temperature (data not shown).

4. Discussion

We compared the characteristics of *L. feeleii* strains that were isolated from Pontiac fever-related environment (LfPF) and a patient with Legionnaires' disease (LfLD). The differences between the two strains were observed in terms of their growth in BYE broth at 42 °C, infection ability, internalization by host cells, cytotoxicity to host cells, and stimulatory ability for IL-6 and IL-8 production.

LfLD showed better resistance to higher temperature than LfPF, based on the culture results at 42 °C. This may be an advantage for LfLD in order to survive inside the bodies of pneumonia patients with high fever. It is considered that heat shock proteins (HSP) may play a role in protecting LfLD from the effects of high temperatures [26,27].

According to the results of bacterial infection assays, LfLD showed a stronger infection ability than LfPF, which was even stronger than the positive control, *L. pneumophila* JR32. Internalization was 100 times higher in LfLD than LfPF (Figs. 2 and 4, at 0 h), and we thought that flagella play an important role in bacterial infections. It has been shown that the *flaA* mutant of *L. pneumophila* was severely deficient in the infection of macrophages [28]. Heuner et al. reported that Western blot analysis for FlaA and flagellation in an electron microscope were positive in *L. feeleii* ATCC 35849 (LfLD), but negative in ATCC 35072 (LfPF) when cultured at 30 °C [29]. Based on the observations from transmission electron microscopy in our study, LfLD showed mono-polar flagellum after culturing the bacteria at 25, 30 and 37 °C while LfPF did not possess any flagellum. At 37 °C, the flagellum of LfLD showed a different morphology compared to other temperatures. In bacterial motility assays, LfLD showed motility in cultures at 25 and 30, but not at 37 °C. On the other hand, LfPF did not express motility at any temperature and were unflagellated. Based on these results, it is strongly suggested that the flagella of LfLD promoted internalization by host cells.

Legionella species are able to proliferate in host cells via sophisticated intracellular growth tactics [14,30,31]. The organism can even form a special morphology during infection in host cell cytosol [13,22]. The two strains of *L. feeleii* were observed to also exhibit a multiplication capacity at 37 °C during infection of J774, U937 macrophages or A549 epithelial cells, and the numbers of CFUs increased at least 10 times after 48 h of infection. Differences in intracellular bacterial CFUs at 24 and 48 h between LfPF and LfLD are a reflection of differences in initial internalization. LfPF could also proliferate in host cells and faster than LfLD (Fig. 4). Subsequently, J774, U937 or A549 cells were infected with the bacteria at

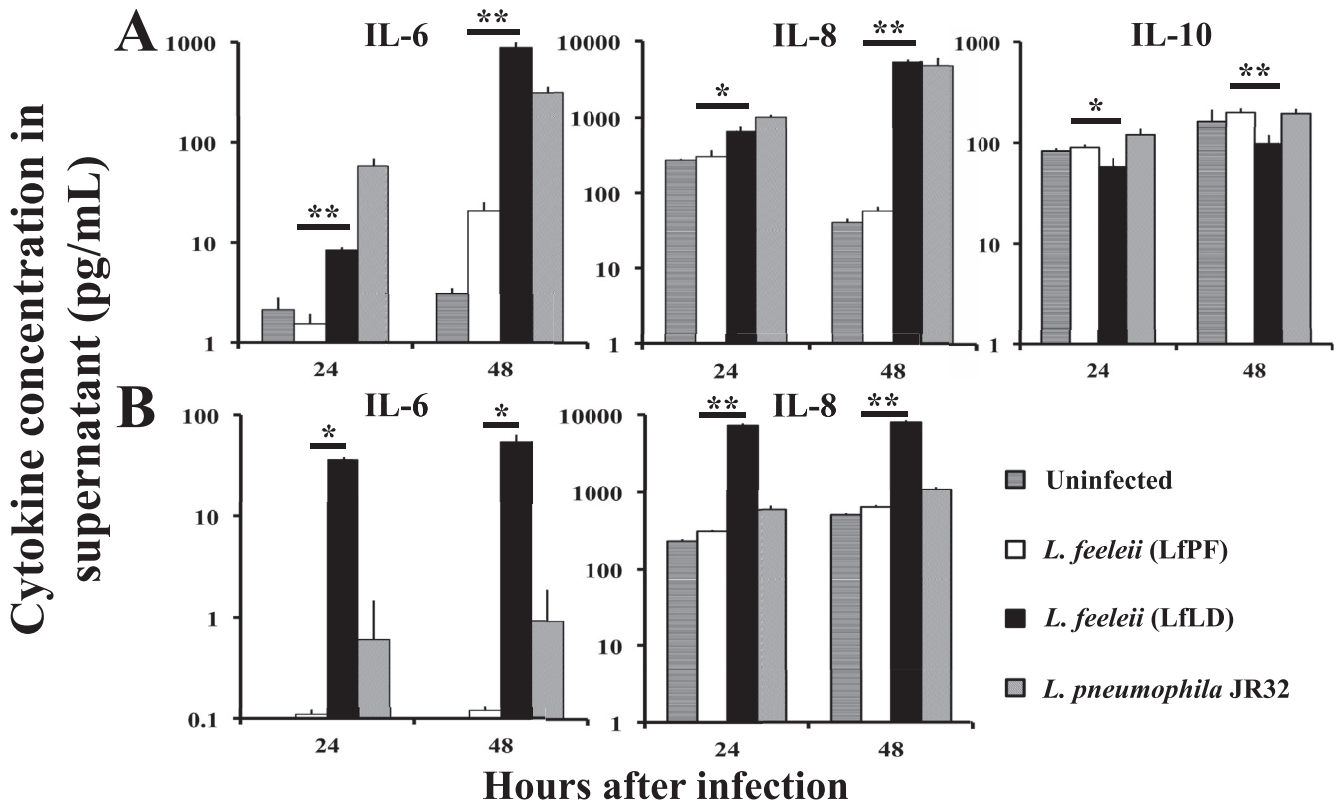

Fig. 6. Cytokines secreted from macrophages and epithelial cells infected with *Legionella*. U937 (A) or A549 (B) cells were either uninfected or infected with *L. feeleii* ATCC 35072, *L. feeleii* ATCC 35849, or *L. pneumophila* JR32 at a MOI of 10 (U937) or 100 (A549) at 37 °C. Data were derived from three independent experiments, represented as averages ± standard deviations (**, $P < 0.01$; *, $P < 0.05$).

different MOIs to obtain similar CFUs at time 0 and cultured at 40 °C for 48 h. The difference in resistance to 40 °C was clear inside U937 (Fig. 4D) and A549 cells (Fig. 4F). Although patients with Pontiac fever and Legionnaires' disease usually have high fever, which are approximately 40 °C [32], the former is self-limiting while pneumonia is a severe disease. That intracellular survival of LfLD was stronger than that of LfPF at 40 °C may be one of the reasons why LfLD causes severe disease in the human respiratory system.

Cytokines are important in cell signaling and are produced by a variety of cells, such as macrophages and epithelial cells [33,34]. The cytokines produced play a critical role in response to pathogen infection. It has been demonstrated that wild type *L. pneumophila* could induce greater IL-6 and IL-8 secretion from A549 epithelial cells than Δ*flaA* strain [33,35]. In our study, flagellated LfLD showed stronger cytopathogenic traits and stimulated greater IL-6 and IL-8 production than non-flagellated LfPF (Figs. 5 and 6). It is hypothesized that bacterial flagellin play an important role in this distinction. The cytokines can be triggered by bacterial flagellin that is recognized by Toll-like receptor 5 (TLR5), followed by the TLR5-MyD88-NF-κB [16,36]. Flagellin could also be recognized by inflammasome via NOD-like receptor protein NLRC4 to cause pyroptosis and proinflammatory cytokine secretion [37]. Therefore, when the organisms infect human macrophage-like or epithelial cell lines, bacterial virulence can be promoted when the flagellar expression is positive [15,38].

Table 1
Motility and flagellar expression of *Legionella* strains at different temperatures.

Bacterial strain	Temperature	Motility[a]	Flagellation[b]
LfPF	25 °C	(−)	(−)
	30 °C	(−)	(−)
	37 °C	(−)	(−)
LfLD	25 °C	(++)	(+)
	30 °C	(+)	(+)
	37 °C	(+)	(+)
L. pneumophila JR32	25 °C	(+)	(+)
	30 °C	(+)	(+)
	37 °C	(−)	(+)

[a] Determined by dark-field microscopy. Rate (%) of motile bacteria, ++: 10–90%, +:1–10%, −: 0%.
[b] Determined by transmission electron microscopy.

Interlukin-6 (IL-6) is secreted by human macrophages as an important mediator of fever and stimulates acute phase protein synthesis. The concentrations of IL-6 in the blood of patients who suffer community-acquired pneumonia (CAP) are positively associated with severity of the disease [39]. LfLD triggered larger amounts of IL-6 secretion than LfPF after 48 h infection in U937 macrophages. The highly induced IL-6 may explain severe pneumonia due to LfLD.

Interlukin-8 (IL-8), or CXCL8, is known as a neutrophil chemotactic factor, which can induce chemotaxis of target cells, directing them to migrate toward the infection site [40]. In our study, great amounts of IL-8 were induced in a time-dependent manner after 24 and 48 h infection of U937 macrophages or A549 cells by LfLD. This suggests that LfLD could exhibit a more potent pathogenicity than LfPF to human host cells. Hence, abundant IL-8 expression may indicate that LfLD triggers pulmonary inflammation.

In contrast to IL-6 and IL-8 production, IL-10 induction by LfLD was less than that of LfPF (Fig. 6C). IL-10 is an anti-inflammatory cytokine that inhibits the effector function of macrophages and expression of proinflammatory cytokines [41]. The reduction of IL-10 expression may be benign for augmenting proinflammatory cytokines response against LfLD infection, since the bacteria could

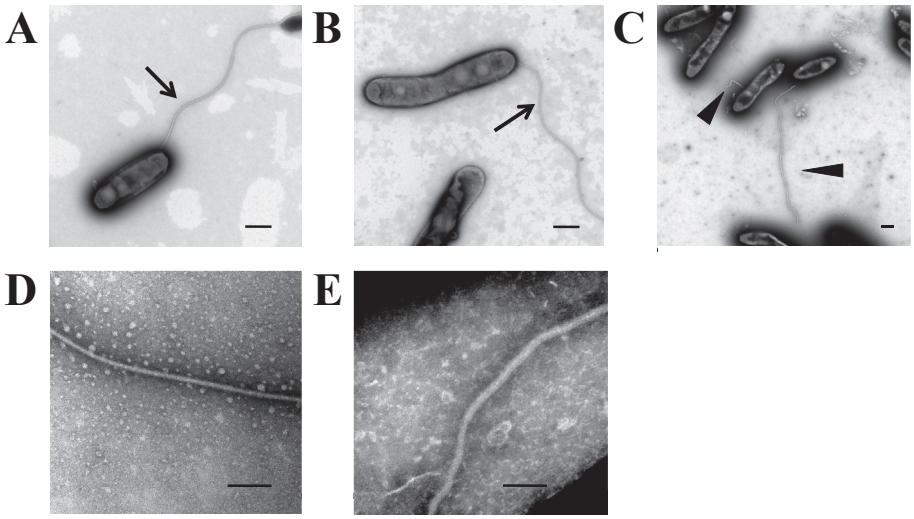

Fig. 7. Transmission electron micrographs of flagella produced by *L. feeleii* LfLD. The bacteria were grown at 25 (A, D), 30 (B) and 37 °C (C, E), negatively stained, and observed under JEM-ARM 200F (JEOL) transmission electron microscope. LfLD showed flagellum with smooth wave morphology (arrow) at 25 (A) and 30 °C (B), but it expressed a non-wavy shape (arrowhead) at 37 °C (C), bars, 0.5 μm. Panels D and E are higher magnification of the flagellum at 25 and 37 °C, bars, 0.1 μm.

cause severe disease. In the case of *L. pneumophila*, however, IL-10 could enhance bacterial growth after infecting human monocytes and alveolar macrophages, which indicates that the induced IL-10 may promote bacterial replication in human legionellosis [42]. Hence, inhibition of IL-10 secretion could help in controlling the pathogen and reducing the severity of illness [43].

The innate immune system plays a critical role in preventing infection in mammalian lungs. The alveolar epithelial cells and macrophages are the front defenders in the lung and important in sensing pathogenic infection and inducing cytokine secretion to cause inflammation. Once the pathogens succeed in evading the immunity defense, they will injure the pulmonary epithelial tissue. Hence, the pathogenesis of pneumonia is due to both host cell injury by bacterial virulence and its ability to induce host inflammation [44]. Proinflammatory cytokine production is a double-edged sword for the host. It works as a host defense or innate immunity when produced moderately. Conversely, it works as a pathogenic factor when produced massively. In the case of LfLD infection, it stimulates excess production of IL-6 and IL-8, and it may develop into pneumonia.

Although our study has to be proven using more *L. feeleii* strains in order to investigate the difference in the virulence-related traits, cases of *L. feeleii* infection are rare and strains available to the study are limited. Animal experiments are also necessary in order to elucidate the pathogenesis of the disease. We are currently performing experiments to produce *flaA* mutant of LfLD. Although all the experimental results and our conclusion are based on *in vitro* studies, this paper presents the first data that reports the differences in the characteristics of LfPF and LfLD. Taken together, our study showed that LfLD had stronger infection ability and cytopathic effects on macrophages than LfPF when host cells were infected.

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References

[1] H.J. Newton, D.K. Ang, I.R. van Driel, E.L. Hartland, Molecular pathogenesis of infections caused by *Legionella pneumophila*, Clin. Microbiol. Rev. 23 (2010) 274–298.
[2] R.R. Isberg, T.J. O'Connor, M. Heidtman, The *Legionella pneumophila* replication vacuole: making a cozy niche inside host cells, Nat. Rev. Microbiol. 7 (2009) 13–24.
[3] E.J. Mangione, R.S. Remis, K.A. Tait, H.B. Gee, G.W. Gorman, B.B. Wentworth, P.A. Baron, A.W. Hightower, J.M. Barbaree, C.V. Broome, An outbreak of Pontiac fever related to whirlpool use, Michigan 1982, JAMA 253 (1985) 535–539.
[4] R.J. Fallon, T.J. Rowbotham, Microbiological investigations into an outbreak of Pontiac fever due to *Legionella micdadei* associated with use of a whirlpool, J. Clin. Pathol. 43 (1990) 479–483.
[5] B.S. Fields, J.M. Barbaree, G.N. Sanden, W.E. Morrill, Virulence of a *Legionella anisa* strain associated with Pontiac fever: an evaluation using protozoan, cell culture, and guinea pig models, Infect. Immun. 58 (1990) 3139–3142.
[6] G.J. Cramp, D. Harte, N.M. Douglas, F. Graham, M. Schousboe, K. Sykes, An outbreak of Pontiac fever due to *Legionella longbeachae* serogroup 2 found in potting mix in a horticultural nursery in New Zealand, Epidemiol. Infect. 138 (2010) 15–20.
[7] L.A. Herwaldt, G.W. Gorman, T. McGrath, S. Toma, B. Brake, A.W. Hightower, J. Jones, A.L. Reingold, P.A. Boxer, P.W. Tang, C.W. Moss, H. Wikinson, D.J. Brenner, A.G. Steigerwalt, C.V. Broome, A new *Legionella* species, *Legionella feeleii* species nova, causes Pontiac fever in an automobile plant, Ann. Intern. Med. 100 (1984) 333–338.
[8] B.S. Fields, R.F. Benson, R.E. Besser, *Legionella* and Legionnaires' disease: 25 years of investigation, Clin. Microbiol. Rev. 15 (2002) 506–526.
[9] M.S. Swanson, B.K. Hammer, *Legionella pneumophila* pathogenesis: a fateful journey from amoebae to macrophages, Annu. Rev. Microbiol. 54 (2000) 567–613.
[10] M. Palusińska-Szysz, M. Cendrowska-Pinkosz, Pathogenicity of the family Legionellaceae, Arch. Immunol. Ther. Exp. Warsz 57 (2009) 279–290.
[11] R.R. Muder, V.L. Yu, Infection due to *Legionella* species other than *L. pneumophila*, Clin. Infect. Dis. 35 (2002) 990–998.
[12] R. Fiumefreddo, R. Zaborsky, J. Haeuptle, M. Christ-Crain, A. Trampuz, I. Steffen, R. Frei, B. Müller, P. Schuetz, Clinical predictors for *Legionella* in patients presenting with community-acquired pneumonia to the emergency department, BMC Pulm. Med. 9 (2009) 1471–2466.
[13] Y. Takekawa, M. Saito, C. Wang, T. Qin, M. Ogawa, T. Kanemaru, S. Yoshida, Characteristic morphology of intracellular microcolonies of *Legionella oakridgensis* OR-10, Can. J. Microbiol. 58 (2012) 179–183.
[14] H. Ohnishi, Y. Mizunoe, A. Takade, Y. Tanaka, H. Miyamoto, M. Harada, S. Yoshida, *Legionella dumoffii* DjlA, a member of the DnaJ family, is required for intracellular growth, Infect. Immun. 72 (2004) 3592–3603.
[15] K. Heuner, M. Steinert, The flagellum of *Legionella pneumophila* and its link to the expression of the virulent phenotype, Int. J. Med. Microbiol. 293 (2003) 133–143.
[16] O. Takeuchi, S. Akira, Pattern recognition receptors and inflammation, Cell 140 (2010) 805–820.
[17] W.L. Thacker, H.W. Wilkinson, B.B. Plikaytis, A.G. Steigerwalt, W.R. Mayberry, C.W. Moss, D.J. Brenner, Second serogroup of *Legionella feeleii* strains isolated from humans, J. Clin. Microbiol. 22 (1985) 1–4.

[18] A. Marra, H.A. Shuman, Isolation of a *Legionella pneumophila* restriction mutant with increased ability to act as a recipient in heterospecific matings, J. Bacteriol. 171 (1989) 2238–2240.
[19] J.D. Ristroph, K.W. Hedlund, R.G. Allen, Liquid medium for growth of *Legionella pneumophila*, J. Clin. Microbiol. 11 (1980) 19–21.
[20] O.A. Alli, S. Zink, N.K. von Lackum, Y. Abu-Kwaik, Comparative assessment of virulence traits in *Legionella* spp, Microbiology 149 (2003) 631–641.
[21] E. Pearlman, A.H. Jiwa, N.C. Engleberg, B.I. Eisenstein, Growth of *Legionella pneumophila* in a human macrophage-like (U937) cell line, Microb. Pathog. 5 (1988) 87–95.
[22] M. Ogawa, A. Takade, H. Miyamoto, H. Taniguchi, S. Yoshida, Morphological variety of intracellular microcolonies of *Legionella* species in Vero cells, Microbiol. Immunol. 45 (2001) 557–562.
[23] N.N. Whitfield, B.G. Byrne, M.S. Swanson, Mouse macrophages are permissive to motile *Legionella* species that fail to trigger pyroptosis, Infect. Immun. 78 (2010) 423–432.
[24] S. Yoshida, Y. Mizuguchi, Antibiotic susceptibility of *Legionella pneumophia* Philadelphia-1 in cultured guinea-pig peritoneal macrophages, J. Gen. Microbiol. 130 (1984) 901–906.
[25] F. Higa, N. Kusano, M. Tateyama, T. Shinzato, N. Arakaki, K. Kawakami, A. Saito, Simplified quantitative assay system for measuring activities of drugs against intracellular *Legionella pneumophila*, J. Clin. Microbiol. 36 (1998) 1392–1398.
[26] Y. Abu Kwaik, B.I. Eisenstein, N.C. Engleberg, Phenotypic modulation by *Legionella pneumophila* upon infection of macrophages, Infect. Immun. 61 (1993) 1320–1329.
[27] R.C. Fernandez, S.M. Logan, S.H. Lee, P.S. Hoffman, Elevated levels of *Legionella pneumophila* stress protein Hsp60 early in infection of human monocytes and L929 cells correlate with virulence, Infect. Immun. 64 (1996) 1968–1976.
[28] C. Dietrich, K. Heuner, B.C. Brand, J. Hacker, M. Steinert, Flagellum of *Legionella pneumophila* positively affects the early phase of infection of eukaryotic host cells, Infect. Immun. 69 (2001) 2116–2122.
[29] K. Heuner, L. Bender-Beck, B.C. Brand, P.C. Lück, K.H. Mann, R. Marre, M. Ott, J. Hacker, Cloning and genetic characterization of the flagellum subunit gene (*flaA*) of *Legionella pneumophila* serogroup 1, Infect. Immun. 63 (1995) 2499–2507.
[30] K. Maruta, H. Miyamoto, T. Hamada, M. Ogawa, H. Taniguchi, S. Yoshida, Entry and intracellular growth of *Legionella dumoffii* in alveolar epithelial cells, Am. J. Respir. Crit. Care Med. 157 (1998) 1967–1974.
[31] K. Maruta, M. Ogawa, H. Miyamoto, K. Izu, S.I. Yoshida, Entry and intracellular localization of *Legionella dumoffii* in Vero cells, Microb. Pathog. 24 (1998) 65–73.
[32] D.W. Fraser, D.C. Deubner, D.L. Hill, D.K. Gilliam, Nonpneumonic, short-incubation-period Legionellosis (Pontiac fever) in men who cleaned a steam turbine condenser, Science 205 (1979) 690–691.
[33] B. Schmeck, P.D. N'Guessan, M. Ollomang, J. Lorenz, J. Zahlten, B. Opitz, A. Flieger, N. Suttorp, S. Hippenstiel, *Legionella pneumophila*-induced NF-kappaB- and MAPK-dependent cytokine release by lung epithelial cells, Eur. Respir. J. 29 (2007) 25–33.
[34] K. McCoy-Simandle, C.R. Stewart, J. Dao, S. DebRoy, O. Rossier, P.J. Bryce, N.P. Cianciotto, *Legionella pneumophila* type II secretion dampens the cytokine response of infected macrophages and epithelia, Infect. Immun. 79 (2011) 1984–1997.
[35] J. Lorenz, J. Zahlten, I. Pollok, J. Lippmann, S. Scharf, P.D. N'Guessan, B. Opitz, A. Flieger, N. Suttorp, S. Hippenstiel, B. Schmeck, *Legionella pneumophila*-induced IκBζ-dependent expression of interleukin-6 in lung epithelium, Eur. Respir. J. 37 (2011) 648–657.
[36] T.R. Hawn, A. Verbon, K.D. Lettinga, L.P. Zhao, S.S. Li, R.J. Laws, S.J. Skerrett, B. Beutler, L. Schroeder, A. Nachman, A. Ozinsky, K.D. Smith, A. Aderem, A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to Legionnaires' disease, J. Exp. Med. 198 (2003) 1563–1572.
[37] Y. Zhao, J. Yang, J. Shi, Y.N. Gong, Q. Lu, H. Xu, L. Liu, F. Shao, The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus, Nature 477 (2011) 596–600.
[38] A.B. Molofsky, L.M. Shetron-Rama, M.S. Swanson, Components of the *Legionella pneumophila* flagellar regulon contribute to multiple virulence traits, including lysosome avoidance and macrophage death, Infect. Immun. 73 (2005) 5720–5734.
[39] G. Antunes, S.A. Evans, J.L. Lordan, Systemic cytokine levels in community-acquired pneumonia and their association with disease severity, Eur. Respir. J. 20 (2002) 990–995.
[40] N. Mukaida, A. Harada, K. Matsushima, Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions, Cytokine Growth Factor Rev. 9 (1998) 9–23.
[41] M. Saraiva, A. O'Garra, The regulation of IL-10 production by immune cells, Nat. Rev. Immunol. 10 (2010) 170–181.
[42] D.R. Park, S.J. Skerrett, IL-10 enhances the growth of *Legionella pneumophila* in human mononuclear phagocytes and reverses the protective effect of IFN-gamma: differential responses of blood monocytes and alveolar macrophages, J. Immunol. 157 (1996) 2528–2538.
[43] K.N. Couper, D.G. Blount, E.M. Riley, IL-10: the master regulator of immunity to infection, J. Immunol. 180 (2008) 5771–5777.
[44] N.A. Eisele, D.M. Anderson, Host defense and the airway epithelium: frontline responses that protect against bacterial invasion and pneumonia, J. Pathog. 2011 (2011) 249802.