Microbial Pathogenesis 89 (2015) 79–86

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

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

Changle Wang [a, *], Mitsumasa Saito [c], Tamami Tanaka [b], Kazunobu Amako [a], Shin-ichi Yoshida [a]

[a] Department of Bacteriology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan
[b] Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan
[c] Department of Microbiology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu 807-8555, Japan

ARTICLE INFO

Article history:
Received 16 March 2015
Received in revised form
30 August 2015
Accepted 10 September 2015
Available online 18 September 2015

Keywords:
Legionella feeleii
Pontiac fever
Legionnaires' disease
Bacterial infection
Host cell immune response

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].

* Corresponding author.
E-mail address: wcl1986@bact.med.kyushu-u.ac.jp (C. Wang).

http://dx.doi.org/10.1016/j.micpath.2015.09.004
0882-4010/

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