Study on the Relationship between *Legionella pneumophila* and Host *Paramecium*

（レジオネラニューモフィラと宿主ゾウリムシとの関係に関する研究）

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PREFACE

Legionella pneumophila is known as a human pathogen and is ubiquitous in natural or artificial aquatic environments. This bacterium replicates within alveolar macrophages after infection to human by inhalation of a *Legionella* containing aerosol (Cunha *et al*., 2016). The intracellular replication is thought to be a major factor of its pathogenicity. Besides *L. pneumophila,* more than 50 *Legionella* spp. with over 70 distinct serogroups have been described, and at least 25 spp. were associated with human disease (Diederen, 2008, Parte, 2018). Although these *Legionella* spp. have the same virulence factors like a type IVB Dot/Icm secretion system with *L. pneumophila* (Burstein *et al*., 2016), most human infections are caused by *L. pneumophila*, which is responsible for approximately 90% of the identified clinical cases (Cunha *et al*., 2016, Yu *et al.*, 2002, Ameura-Maekawa *et al*., 2018). However, the ratio of *L. pneumophila* in environmental isolates is approximately from 20% to 40% (Doleans *et al*., 2004, Harrison *et al*., 2007, Den Boer *et al*., 2008). The reason why *L. pneumophila* mainly causes human Legionnaires' disease is unclear, and little is known about the ecological factors influencing the distribution of *Legionella* spp. in the environment.

Many studies have been revealed the virulence traits of *L. pneumophila* using

clinical strains (Andrews *et al*., 1998, Al-Khodor *et al*., 2009, Fuche *et al*., 2014). In contrast, little is known about environmental strains, and many biological characteristics and environmental behaviors are still largely unknown. For this reason, the investigation of environmental strains can lead to obtain new insights different from what have been revealed using clinical strains. The ecology of *L. pneumophila* in the environment may be influenced by several factors as water temperature, disinfectants, microbial competitor, biofilm and protists. Protistan hosts are thought to have important roles in the ecology of *L. pneumophila* (Al-Quadan *et al*., 2012, Declerck *et al*., 2007, Rowbotham, 1980). Although many studies on the interaction between *L. pneumophila* and amoebae have been reported, the relationship between *L. pneumophila* and other protists in natural environment are still unclear. To control *L. pneumophila*, it is important to understand its ecology in the environment. Therefore, I examined environmental strains from following two aspects:

1. Isolation of a plasmid from environmental strains of *L. pneumophila* and construction of a new shuttle vector using this plasmid (chapter 1)
2. Analysis of the relationship between *L. pneumophila* and *Paramecium*, which was reported recently as a candidate of natural host of *L. pneumophila* (chapter 2)

CHAPTER 1

Characterization of the cryptic plasmid pOfk55 from *Legionella pneumophila* and construction of a pOfk55-derived shuttle vector

INTRODUCTION

Legionella pneumophila is a facultative intracellular Gram-negative bacterium, and the major causative agent of Legionnaires' disease and Pontiac fever (Cunha *et al*., 2016). The infectious mechanisms of *L. pneumophila* have been investigated, and regulation of virulence, invasion, intracellular multiplication and cytotoxicity have been revealed (Molmeret *et al*., 2004). Plasmid vectors are thought to be a beneficial genetic tool to reveal these infectious mechanisms. Several plasmid vectors of *L. pneumophila* have been constructed and used for multipurpose genetic manipulation (Andrews *et al*., 1998; Engleberg *et al*., 1988; Watarai *et al*., 2001). For example, pMS8, pSR47s, pAM239, have been used as cloning vector, suicide vector and expression vector. These plasmids may prove useful for the genetic analysis of pathogenicity genes. However, no vector has been constructed based on a plasmid originally isolated from *L. pneumophila*. It has been reported that some environmental isolates and some clinical strains have plasmids (Knudson and Mikesell, 1980; Brown *et al*., 1982; Lévesque *et al*., 2014).Whether the function of plasmids is related to the virulence is not clear but it has been reported that the plasmid analysis is useful in the study on the epidemiology, and plasmid carriage confers resistance to UV light. Moreover, some plasmids have been reported as a conjugative plasmid (Mintz *et al*., 1992; Maher *et al*., 1983; Tully.,

1991). On the other hand, these plasmids are not appropriate for use as vectors in genetic studies due to their large size. The small cryptic plasmids found in many bacteria have often been used to develop various genetic tools such as complementation and expression vectors. They do not appear to confer any advantage on their host but they are typically maintained with high stability (Park *et al*., 2015, Seubert *et al*., 2003). However, small cryptic plasmids have not been reported previously in *L. pneumophila*.

In the previous study, environmental strains of *L. pneumophila* from environmental water were isolated (Tachibana *et al*., 2013). In the present study, I identified a small cryptic plasmid designated as pOfk55 in one of these environmental strains, Ofk308. Using pOfk55, I constructed a new shuttle vector, pNT562, for *L. pneumophila–Escherichia coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions

All of the bacterial strains used in this study are listed in Table 1. *L. pneumophila* strains were cultured at 37°C in either AYE or on AYE containing activated charcoal (1.5 mg / ml) and 1.5% agar (BCYE). A base solution for AYE was prepared as follows. Ten grams of N-(2-acetamido)-2-aminoethanesulfonic acid (Sigma-Aldrich, St Louis, MO, USA) and 2.6 g of Potassium Hydroxide (Wako, Tokyo, Japan) were dissolved in 1 liter of distilled water. The solution was adjusted to pH 6.9 with 1 N H_2SO_4 (Wako). Then, ten grams of yeast extract (Becton Dickinson, Franklin Lakes, NJ, USA) and 1 gram of α-ketoglutaric acid (Sigma-Aldrich) were added and sterilized. After sterilization, 10 ml each of filter-sterilized solutions of L-cysteine (37 mg/ml; Wako) and iron (III) phosphate hydrate (22.5 mg/ml; Wako) were added. When *L. pneumophila* Lp02 was cultured, 100 μg/ml of thymidine (Wako) was supplemented. *Escherichia coli* DH5α was cultured in either Lysogeny Broth (LB; Nacalai Tesuque, Kyoto, Japan) or on LB containing 1.5% agar. Media were supplemented with kanamycin (30 μg/ml; Wako) and streptomycin (50 μg/ml; Sigma-Aldrich) as required.

DNA manipulation

All of the plasmids and primers used in this study are listed in Tables 2 and 3. Plasmid DNA from *E. coli* DH5α and *L. pneumophila* was prepared using a QIAGEN Plasmid Mini Kit (QIAGEN, Hilden, Germany). Restriction enzymes (Takara, Tokyo, Japan; or New England Biolabs Inc, Ipswich, MA, USA) were used according to the manufacturers' protocols. A 2.4 kbp transposon region including a kanamycin-resistance gene (Km^R) marker was obtained from pUTmini-Tn5Km by PCR using the primers XhoIF/XhoIR, HaeIIF/HaeIIR, HincIIF/HincIIR, and BamHIF/EcoRIR. These amplicons were inserted into several restriction sites in pOfk55 using an In-Fusion HD Cloning Kit (Takara) (see **Characterization of pOfk55** in **RESULTS AND DISCUSSION**). The recombinant plasmid was introduced into *L. pneumophila* Lp02 by electroporation as described below. A 1.0 kbp Km^R marker including its promoter region and a 0.8 kbp origin of replication were obtained from pUTmini-Tn5Km and pBluescript SK (+), respectively, by PCR using the primers KmF/KmR and oriF/oriR. These amplicons were fused with a fragment from 2383 nucleotides (nt) to 1599 nt in pOfk55 to construct pNT561 using an In-Fusion HD Cloning Kit (see **Construction of the pOfk55-derived shuttle vector pNT562** in **RESULTS AND DISCUSSION**). The modified plasmid was introduced into *E. coli* DH5α competent cells by the heat-shock

method, as described below. Multiple cloning sites (MCS) from pBluescript SK (+) as well as the tac promoter (Ptac) region and *lacI* gene from pAM239-GFP were amplified using the primers mcsF/mcsR and ptacF/ptacR. These amplicons were fused with pNT561 obtained from *E. coli* DH5α using an In-Fusion HD Cloning Kit. The recombinant plasmid, pNT562, was introduced into *E. coli* DH5α. A 0.7 kbp *gfpmut3* gene was obtained from pAM239-GFP by PCR using the primers gfpF/gfpR. The amplicon digested by *Hind*III and *Spe*I was ligated with pNT562 obtained from *E. coli* DH5α using a DNA Ligation Kit (Takara), and the ligation mixture was introduced into *E. coli* DH5α. *L. pneumophila* Lp02 carrying pNT563 was prepared by introducing pNT563 obtained from *E. coli* DH5α.

DNA sequence analysis

pOfk55 was digested by *Xho*I and ligated with pBluescript SK (+) using a DNA Ligation Kit, and the ligation mixture was introduced into *E. coli* DH5α competent cells by the heat-shock method, as described below. The pOfk55 fragment in pBluescript SK (+) was sequenced using the universal primers T7/T3. DNA sequencing was performed using a Big Dye Terminator v3.1 Cycle Sequencing Kit and an ABI3031 Genetic Analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA). DNA sequences and

amino acid sequences were analyzed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the GenBank database. DNA structures were predicted using the mfold program (http://unafold.rna.albany.edu/?q=mfold/dna-folding-form). Inverted repeats were predicted using the oligonucleotides repeats finder (http://wwwmgs.bionet.nsc.ru/mgs/programs/oligorep/InpForm.htm).

Transformation of *L. pneumophila* and *E. coli* DH5α

Electroporation was performed to transform *L. pneumophila* and *E. coli* DH5α with a Gene Pulser Electroporator (Bio-Rad Laboratories) in 10% glycerol solution at 2.5 kV/25 μF. After electroporation, the cells were resuspended in 1 ml of AYE or LB, incubated for 1 h or 30 min at 37°C, and spread onto BCYE or LB agar containing kanamycin. The heat shock method was also used to transform *E. coli* DH5α. Bacterial cells were cultured overnight at 37°C and collected by centrifugation at 3000 rpm for 10 min at 4°C. To make *E. coli* DH5α competent, the bacterial pellet was resuspended in 30 mM $CaCl_2$ and mixed by vortexing. The bacteria were harvested by centrifugation and resuspended in 75 mM $CaCl_2$. This suspension and the plasmid DNA were mixed, kept on ice for 30 min, and then kept at 42°C for 1 min. Cells were resuspended in 1 ml of LB, incubated for 30 min at 37°C, and spread onto LB agar containing kanamycin.

Conjugation

Conjugation was performed as described previously (Qin *et al*., 2009). Briefly, 0.1 ml of AYE containing 1×10^8 donor cells and AYE containing 4×10^8 recipient *L. pneumophila* Lp02 cells were mixed and incubated for 24 h at 37°C. Recombinants were selected using BCYE supplemented with streptomycin and kanamycin. In addition, the total *L. pneumophila* Lp02 counts were obtained by spreading on BCYE supplemented with streptomycin, which was incubated at 37°C for 5 days. The conjugation frequency was calculated based on the CFU on BCYE containing streptomycin and kanamycin divided by the CFU on BCYE containing streptomycin.

Plasmid copy number analysis

The relative copy number of pNT562 was assessed by quantitative real-time PCR (qPCR) using an Applied Biosystems StepOne Real-Time PCR System with Power SYBER green PCR Master Mix (TOYOBO, Osaka, Japan) according to the manufacturers' protocols. Amplification of a 102 bp segment was performed using the primers pOfkF/pOfkR based on the putative *rep* gene sequence of pNT562. A 115 bp segment of the single-copy *mip* gene was used as a reference. The relative copy number was calculated based on three identical experiments using the formula: $N_{relative} = 1^{-DCT}$,

where DCT denotes the difference between the threshold cycle number for the *mip* gene reaction and that for the putative *rep* gene.

Plasmid stability analysis

Plasmid stability analysis was performed as described previously (Park *et al*., 2015). Briefly, *L. pneumophila* Lp02 harboring pNT562 was grown for 24 h in AYE with kanamycin at 37°C. The culture obtained was used to inoculate (1%, v/v) fresh AYE without kanamycin and the inoculated culture was incubated for 24 h at 37°C. Daily inoculation was repeated for up to 1 week. Each day, part of the culture was serially diluted using phosphate-buffered saline. Diluted samples were spread onto BCYE with or without kanamycin, before incubating at 37°C for 4 days. The percentage of cells harboring pNT562 was calculated by dividing the CFU on BCYE with kanamycin by the CFU on BCYE and multiplying by 100.

Fluorescence microscopy to detect green fluorescent protein (GFP) expression

L. pneumophila Lp02 harboring pNT563 and *E. coli* DH5α harboring pNT563 were cultured on kanamycin containing agar plates. Colonies were picked up and suspended in five ml of liquid media with or without 0.1 mM isopropyl β-D-1-

thiogalactopyranoside (IPTG), then incubated for 24 h at 37°C. Fluorescent images were obtained using a FluoView FV100 confocal laser scanning microscope (Olympus, Tokyo, Japan).

RESULTS AND DISCUSSION

Detection of a small cryptic plasmid in *L. pneumophila* Ofk308

In a previous study, five *L. pneumophila* environmental strains were isolated and draft genome sequences were determined for these strains (Watanabe *et al*., 2015). It has been reported that several environmentally isolated *L. pneumophila* strains have plasmids (Brown *et al*., 1982). Then, I tried to extract a plasmid from these environmental strains and I found that two strains, i.e., Ofk308 and Bnt314, each contained a plasmid (Fig. 1). The sizes of these plasmids appeared to be relatively small compared with those of other plasmids in *L. pneumophila*. To determine the sequence of the *L. pneumophila* Ofk308-derived plasmid designated as pOfk55, plasmid DNA was digested using *Xho*I and ligated with the pBluescript SK (+) vector cut with the same enzyme. Two fragments were cloned and sequenced. The sequenced internal regions were analyzed by BLAST and both regions shared homology with part of lpofk_contig000055 (accession number (AC): NZ_BBUH01000055). Several unique restriction sites were found in lpofk_contig000055 (*Bam*HI, *Eco*RI, *Hae*II, *Hinc*II, *Pac*I, *Sac*I, and *Xho*I). Indeed, only one fragment was observed when pOfk55 was cut by these restriction enzymes except for *Xho*I and the size of these fragments were the same as the size of lpofk_contig000055 (2,583bp) (data not shown). Moreover, it was thought

that two *Xho*I sites existed on pOfk55 because two fragments were observed when it was cut by *Xho*I. I found that lpofk_contig000055 could be further digested by *Xho*I if a thymine base was added to the end of the sequence. Therefore, I determined that the sequence of lpofk_contig000055 with a thymine base at the end was the sequence of the pOfk55 (Fig. 2; AC: LC215275).

Characterization of pOfk55

The size of pOfk55 was 2584 bp and the GC content was 37.3%. The GC content of chromosomal DNA in most *L. pneumophila* strains ranges from 38.1% to 38.5% (NCBI; https://www.ncbi.nlm.nih.gov/genome/genomes/416). The host strain *L. pneumophila* Ofk308 had a GC content of 38.2%, thereby suggesting that pOfk55 could have originated from *L. pneumophila.* It has been reported that some *Legionella* spp. contain several plasmids (Gomez-Valero *et al*., 2014), but a small cryptic plasmid similar to pOfk55 has never been reported previously. Similar sequences to pOfk55 were found only in the draft genome sequence of *L. pneumophila* Bnt314 (AC: BBUG00000000) (data not shown). This strain was isolated from a region close to that where *L. pneumophila* Ofk308 was isolated (Tachibana *et al*., 2013) and the sequence type of Ofk308 and Bnt314 is 1288 according to the protocol Version 5.0 developed by

the European Working Group for Legionella Infections (Gaia *et al*., 2005; Ratzow *et al*., 2007). Thus, the function of pOfk55 might only be beneficial to this sequence type.

Three putative ORFs with the same orientation were located at 1015–1602, 1715–2059, and 2297–2533 nt (Fig. 2). *orf1* potentially encodes a protein of 195 amino acids, and the putative protein shared 39% and 37% sequence identity with a putative plasmid replication protein RepL (AC: CDW96533.1) and a firmicute plasmid replication family protein (AC:KGD42882.1), respectively. *orf2* and *orf3* potentially encodes proteins of 114 amino acids and 78 amino acids (Fig. 3). The functions of these putative proteins are not clear because they shared no significant homology with other proteins in the database.

The sequence of pOfk55 had no homologues so it was difficult to estimate the possible replication mechanism. However, two long inverted repeats (IRs) were located upstream of *orf1* (Fig. 2), where IR-1 and IR-2 comprised 186 bp and 93 bp. The ΔG values for potential stem-loop formation by IR-1 and IR-2 were –301.86 kcal/mol and –151.06 kcal/mol, respectively. Stem-loop flanking regions upstream of a *rep* gene have been reported in both pOM1 and pBGR1 (Hefford *et al*., 1997; Seubert *et al*., 2003).

These plasmids are known to employ a rolling-circle replication (RCR) mechanism. The size of natural RCR plasmids is relatively small (from 846 bp to 30 kbp) and these plasmids have a medium copy number (10–30 per chromosome equivalent) (Ruiz-Masó *et al*., 2015). Thus, I investigated the homology between pOfk55 and RCR plasmids. *orf1* in pOfk55 shared low homology with the *rep* genes in pOM1 and pBGR1 (23% and 21%, respectively). In addition, there was no similarity between IR-1 and flanking sequences upstream of the *rep* genes in pOM1 and pBGR. The size of pOfk55 was small like RCR plasmids, but the copy number was lower (3.2 ± 0.8 per chromosome equivalent). Thus, further studies are needed to uncover whether pOfk55 replicates via an RCR mechanism.

To determine the region that is required for replication and investigate the functions of the encoded proteins, I attempted to insert the Km^R marker in several restriction sites in pOfk55, and the recombinant plasmids were transformed into *L. pneumophila* Lp02 and *E. coli* DH5α. I successfully inserted the marker into *Xho*I, *Hae*II, and *Hinc*II sites located at 2583, 2382, and 1848 nt, respectively, thereby obtaining pNT527, pNT528, and pNT529 (the upper right of Fig. 4), but I failed to insert *Bam*HI/*Eco*RI and *Xho*I sites at 1102/1172 and 554 nt. I found that pNT527,

pNT528, and pNT529 replicated in *L. pneumophila* Lp02 but not in *E. coli* DH5α. Thus, the replication mechanism of pOfk55 did not work in *E. coli* DH5α, and the IR-1 region and *orf1* were required for replication. Next, I investigated the conjugation frequencies to elucidate the mobilization of pOfk55, which showed that pNT527, pNT528, and pNT529 had the same capacity to transfer from the donor strain (Ofk308) to the recipient strain (Lp02) (Table 4). These results suggest that pNT527, pNT528, and pNT529 are conjugative and *orf2* and *orf3* are not necessary for transfer. Conjugative plasmids of *L. pneumophila* have been reported (López de Felipe., 1993; Segal and Shuman, 1998; Meyer, 2009). In addition, conjugative chromosome regions known as episomes of *L. pneumophila* have been identified (Glöckner, 2008). I compared pOfk55 and oriT regions of these plasmids and episomes but could not find homology. Further study is needed to identify the oriT region of pOfk55. *L. pneumophila* Ofk308*ΔdotH* could also transfer pNT527 in the same frequency, but *L. pneumophila* Philadelphia-1 failed to transfer. DotH is a component of the type IVB secretion system (T4BSS), which is known to be a DNA delivery system among bacteria (Christie, 2001; Sexton and Vogel, 2002). These results suggest that pOfk55 can be transferred by conjugation in a T4BSS-independent manner. *L. pneumophila* possesses several types of T4SSs (Gomez-Valero *et al*., 2014) and a type IVA secretion system (T4ASS) of several *L.*

pneumophila strains differs from each other (Gomez *et al*., 2011). From draft genome sequences of Ofk308, I found that Ofk308 had an F-type T4ASS on lpofk_contig000070 (AC: NZ_BBUH01000070.1) which shared more than 99% homology in the DNA sequence level with a T4ASS of *Legionella pneumophila* Lens plasmid pLPL (AC: NC_006366.1) but this F-type T4ASS had very low homology with an F-type T4ASS of *L. pneumophila* Philadelphia-1 even in amino acid sequence level (data not shown). Hence, the transfer mechanism in pOfk55 might be dependent on the F-type T4ASS of Ofk308.

Construction of the pOfk55-derived shuttle vector pNT562

I constructed a *L. pneumophila–E. coli* shuttle vector, pNT562 (AC: LC215276), as shown in the middle of Fig. 4. All of the replication functions of pOfk55 may be assigned to a 1.6 kbp *Xho*I-*Pac*I fragment containing the most region of an IR and *orf1* (putative *rep* gene). First, the *Xho*I-*Pac*I fragments from pOfk55, the Km^R from pUTmini-Tn5Km, and the origin of replication from pBluescript SK (+) was connected together by In-Fusion cloning to obtain pNT561. Therefore, pNT561 is a high-copy number *E. coli* cloning vector that confers kanamycin resistance. To construct a useful expression shuttle-vector, MCS from pBluescript SK (+) as well as Ptac region and *lacI*

gene from pAM239-GFP were inserted to obtain pNT562. As expected, this vector could replicate in all *L. pneumophila* strains. The transformation efficiencies are shown in Table 5. The transformation efficiency for *L. pneumophila* strains ranged from 1.6×10^1 to 1.0×10^5 CFU/ng. The efficiencies were almost the same in all of the strains, except for *L. pneumophila* Philadelphia-1. The low efficiency in *L. pneumophila* Philadelphia-1 is probably due to a restriction activity of this strain (Marra *et al*., 1989). The transformation efficiency of pNT562 was relatively high compared with that of pAM239-GFP in *L. pneumophila* Lp02 (Fig. 5), which might be attributable to the original replication mechanism in pOfk55.

Copy number and stability of pNT562

The relative copy number per chromosome equivalent was analyzed for pNT562 in *L. pneumophila* Lp02 by qPCR and it was estimated as 5.7 ± 1.0 copies. The stability of pNT562 was also assessed under non-selective conditions in *L. pneumophila* Lp02 (Fig. 6), which showed that 73.6% of cells maintained the plasmid after 1 week of daily culturing in AYE without kanamycin selection. The rate of cells that lost pNT562 increased rapidly after the 5th day. The stability of pNT562 was higher than that of pAM239-GFP but there was no statistical difference (data not shown). It has been

reported that ColE1 plasmids replicate within *L. pneumophila*, but these plasmids are unstable without antibiotic selection. (Engleberg *et al*., 1988). Indeed, the plasmid constructed by removing the putative *rep* gene from pNT562 named pNT564 could replicate within *L. pneumophila* Lp02 (Fig. 4). However, as expected, more than 90% of the cells lost this plasmid after culturing for 24 h without kanamycin selection (data not shown). These results suggest that pNT562 is a useful vector with high stability.

Usefulness of pNT562 as an expression vector

To test the potential utility of pNT562 as an expression vector for *L. pneumophila*, I assessed the expression of the *gfpmut3* gene, which was amplified from pAM239-GFP using the primers gfpF2/gfpR2. The forward primer gfpF2 includes a sequence that has been reported as an effective SD region for GFP (Miller and Lindow, 1997). The amplicon was then cloned into pNT562 to yield pNT563 (Fig. 4). pNT563 was introduced into *L. pneumophila* Lp02 and *E. coli* DH5α. The strains harboring pNT563 were cultured with or without and IPTG. Both strains successfully expressed GFP, as shown in Fig. 7. Therefore, it is considered that pNT562 is a useful expression vector for *L. pneumophila.*

TABLES AND FIGURES

Table 1. Bacterial strains used in this study.

Strain	Characteristics	Source or reference
Legionella pneumophila		
Philadelphia-1	Isolated from human lung	GTC 00296 (ATCC 33216)
Philadelphia-1 pNT527	Philadelphia-1 carring pNT527	This study
Knoxville-1	Isolated from human lung	GTC 00745 (ATCC 33153)
Togus-1	Isolated from human lung	GTC 00746 (ATCC 33154)
Lp02	Philidelphia-1 *rpslL hsdR*$^{-}$ *thyA*$^{-}$	Berger and Isberg (1993)
Ofk308	Isolated from environmental water	Tachibana *et al*. (2013)
Ofk308 pNT527	Ofk308 carring pNT527	This study
Ofk308 pNT528	Ofk308 carring pNT528	This study
Ofk308 pNT529	Ofk308 carring pNT529	This study
Ofk308 *ΔdotH*	*dotH*-deletion mutant of Ofk308	Watanabe *et al*. (2016)
Ofk308 *ΔdotH* pNT527	Ofk308 *ΔdotH* carring pNT527	This study
Bnt314	Isolated from environmental water	Tachibana *et al*. (2013)
Twr292	Isolated from environmental water	Tachibana *et al*. (2013)
Ymg289	Isolated from environmental water	Tachibana *et al*. (2013)
Ymt294	Isolated from environmental water	Tachibana *et al*. (2013)
Esherichia coli DH5α	*Φ80lacZΔM15, Δ(lacZYA-argF)U169, recA1, endA1, hsdR17, supE44, thi-1, gyrA96, relA1*	Takara, Tokyo, Japan

Table 2. Plasmids used in this study.

Plasmids	Characteristics	Reference
pBluescrip SK (+)	Standard cloning vector (phagemid excised from lambda ZAP)	Merck, Darmstadt, German
pUTmini-Tn5Km	pUT vector containing mini-Tn5 carring Km resistance gene	BioMedal, Seville, Andalucía, Spain
pAM239-GFP	pMMB-derived vector encording GFP	Watarai *et al*. (2001)
pOfk55	*L. pneumophila* Ofk308 cryptic plasmid, 2.6kb	This study
pNT527	pOfk55 carrying Km^R in the XhoI site, 4.9kbp	This study
pNT528	pOfk56 carrying Km^R in the HaeII site, 4.9kbp	This study
pNT529	pOfk57 carrying Km^R in the HincII site, 4.9kbp	This study
pNT561	XhoI-PacI fragment from pOfk55, pUC ori from pBlue scrip, Km^R, 3.4kbp	This study
pNT562	*E.coli-L.pneumophila* shuttle vector derived from pNT561, MCS, Ptac, LacI, 5.1kbp	This study
pNT563	pNT562 carring *gfpmut3* in MCS, 5.8kbp	This study
pNT564	pNT562 removed Rep, Ptac, LacI, 2.9 kbp	This study

Table 3. Primers used in this study.

Primer	Sequence	Target region
XhoIF	CGCCATGGGGCTCGAGCTGTCTCTTGATCAGATCTGGC	Km^R marker of
XhoIR	GTAAGCCACCCTCGAGCTGACTCTTATACACAAGTTCGGC	pUTmini-Tn5Km
HaeIIF	TTTTGATTCAAGCGCTCTGTCTCTTGATCAGATCTGGC	Km^R marker of
HaeIIR	TCTTTTTAAAAGCGCTCTGACTCTTATACACAAGTTCGGC	pUTmini-Tn5Km
HincIIF	TTTTGAAATTATGTTAACCTGTCTCTTGATCAGATCTGGC	Km^R marker of
HincIIR	ATATTTATGTTTGTTAACCTGACTCTTATACACAAGTTCGGC	pUTmini-Tn5Km
BamHIF	GATAAAAATAGGATCCCTGTCTCTTGATCAGATCTGGC	Km^R marker of
BamHIR	TTTACGACGTGAATTCCTGACTCTTATACACAAGTTCGGC	pUTmini-Tn5Km
KmF	TTAATTAAGGGCTTACATGACGATAGCTAG	Km^R marker of
KmR	GTAAGCCACCCTCGAGTCAGAAGAACTCGTCAAGAAGG	pUTmini-Tn5Km
oriF	TAAGCCACCCTCGAGGCCGCGTTGCTGGCGT	Replication origin of
oriR	GTTCTTCTGACTCGATAACTGTCAGACCAAGTTTAC	pBluescript SK (+)
mcsF	CAAATAATATTTAATTAACGACTCACTATAGGGCGAATTG	Mulutiple cloning sites
mcsR	ATGTAAGCCCTTAATAACAAAAGCTGGAGCTCC	of pBluescript SK (+)
ptacF	CAAATAATATTTAATTAACGCCAGCAAGACGTAGC	Ptac and *lacI* of
ptacR	TAGTGAGTCGTTAATTAATGTTTCCTGTGTGAAATTG	pAM239-GFP
gfpF	CGAATTAAGCTTACACAGGAAACAGCATGCGTAAAGGAGAAGAAC	*gfpmut3* of pAM239-
gfpR	CGAATTACTAGTTTATTTGTATAGTTCATCCATG	GFP
T3	CAATTAACCCTCACTAAA	Fragment of pOfk55 in
T7	TAATACGACTCACTATAGGG	pBluescript SK (+)
RTmipF	GCAACGTTCCAGGTTTCACA	*mip* gene of *L.*
RTmipF2	CCAGCAACTTTTCAGGTTTCACA	*pneumophila*
RTmipR	TACGTGGGCCATATGCAAGA	chromosome
pOfkF	CTCTTCGGCTCCTACCCAAG	Putative *rep* gene of
pOfkR	TTGAAACCAACAGCTCAAAGGG	pOfk55

Table 4. Conjugation frequency of Lp02 with each donor strains.

Donor strains	Conjugation frequency
L. pneumophila	
Ofk308 pNT527	5.1×10^{-6}
Ofk308 pNT528	5.0×10^{-6}
Ofk308 pNT529	3.2×10^{-6}
Ofk308*ΔdotH* pNT527	4.2×10^{-6}
Philadelphia-1 pNT527	3.1×10^{-8}

Data represent averages based on three identical experiments.

Table 5. Transformation efficiency of pNT562.

Strains	Transfomants per ng of pNT562
L. pneumophila	
Philadelphia-1	1.6×10^{1}
Togus-1	6.4×10^{3}
Knoxville-1	2.4×10^{4}
Lp02	1.0×10^{5}
Bnt314	2.1×10^{4}
Ofk308	6.7×10^{4}
Twr292	6.0×10^{3}
Ymg289	1.1×10^{3}
Ymt294	1.3×10^{4}
E. coli DH5α	1.7×10^{2}

Data represent averages based on three identical experiments.

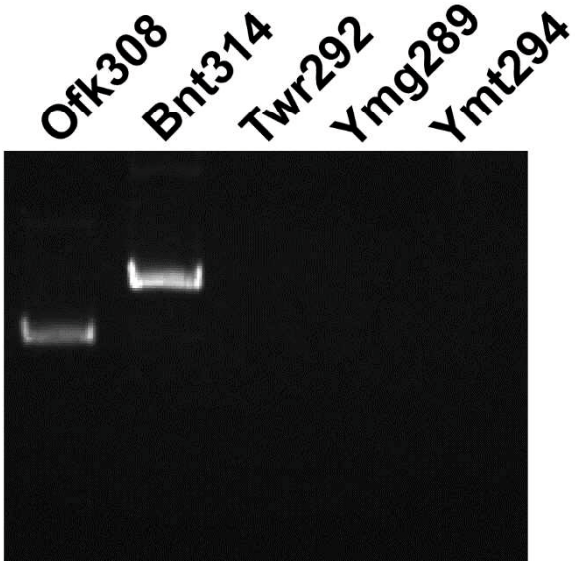


Figure 1. Extraction of plasmids from environmental *L. pneumophila*.

Plasmids were isolated from Ofk308, Bnt314, Twr292, Ymg289, and Ymt294.

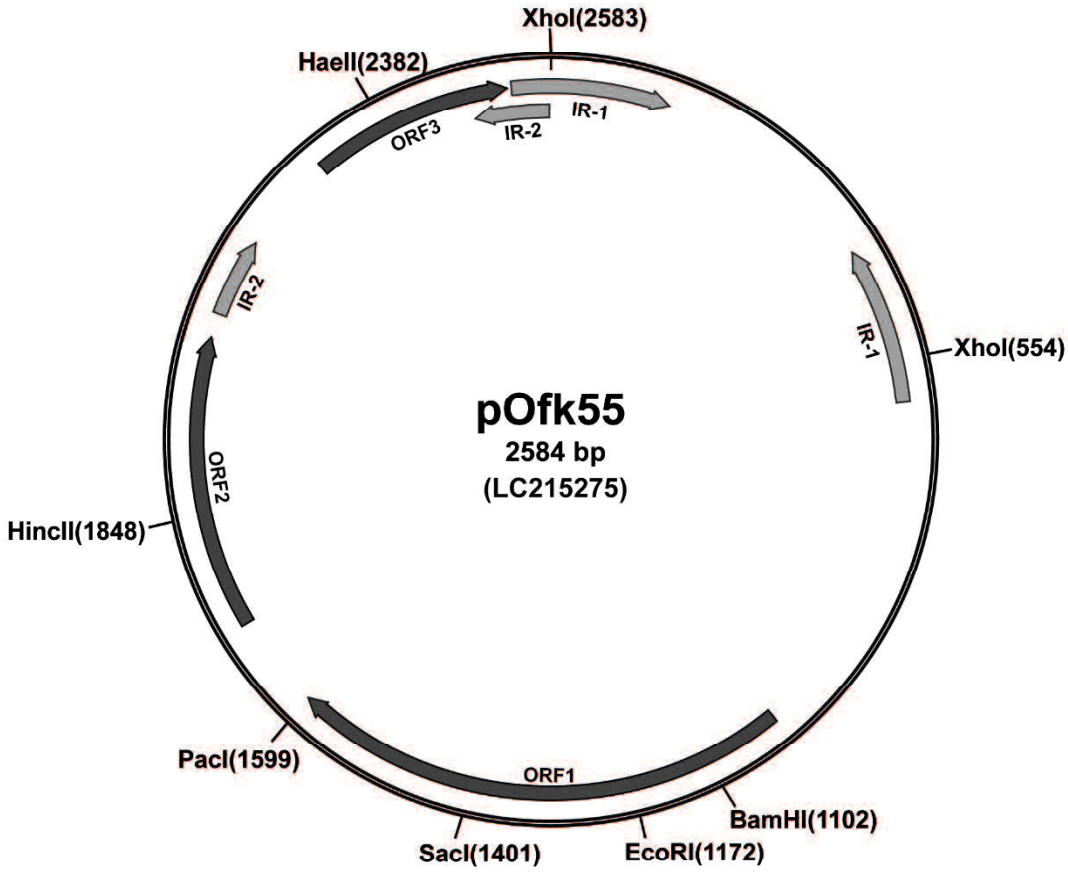


Figure 2. Physical and genetic map of the cryptic plasmid pOfk55.

Black arrows indicate the directions and lengths of ORFs. Gray arrows indicate inverted repeats. The restriction sites used in this study are also shown. IR; inverted repeat.

A

orf1

```
ATGAAAAAAG AACCAATCCC AGGAAAAAGG AAAAATGTAT TGGCGTACCC AGAAAACCCA
M  K  K  E   P  I  P   G  K  R   K  N  V  L   A  Y  P   E  N  P

TTTTGGCAAA AAACAGAGAT AAAAATAGGA TCCAAAATGG TTAAAGTTTC TGGAGGAAAA
F  W  Q  K   T  E  I   K  I  G   S  K  M  V   K  V  S   G  G  K

CACATAAATA TTGAAGGAGA AAGTATTTCT CATAGTGGAA TTCACGTCGT AAAAGAAATT
H  I  N  I   E  G  E   S  I  S   H  S  G  I   H  V  V   K  E  I

GATGAAAATG AGTTTGTAAA AATTTACACA AAGAACATAA AAGCAATTTT CGATTTGAAA
D  E  N  E   F  V  K   I  Y  T   K  N  I  K   A  I  F   D  L  K

CCAACAGCTC AAAGGGTATT ACAATATTTG ATAACTGAAT TACAAAAAAC ACCAAACGCC
P  T  A  Q   R  V  L   Q  Y  L   I  T  E  L   Q  K  T   P  N  A

GACGCAGTTT ATTTGGCTTG GGTAGGAGCC GAAGAGTACT TTTCAGAAAA CCATATAAAA
D  A  V  Y   L  A  W   V  G  A   E  E  Y  F   S  E  N   H  I  K

TCATCAAGAG CATCTTTTCA CAGAGCTCTC AGTGAATTAA TTCAAAAAGG TTTTTTGGCC
S  S  R  A   S  F  H   R  A  L   S  E  L  I   Q  K  G   F  L  A

GAGAGCACCA AGCCAAATAT GTTTTGGTTT AATCCAAATC TATTTTTTAA CGGCAATAGA
E  S  T  K   P  N  M   F  W  F   N  P  N  L   F  F  N   G  N  R

TTAACTTTTA TTCATGAATA TCGAAAGAAA ACCATCCAAG AAATAGGAAA AGAGGAAAAT
L  T  F  I   H  E  Y   R  K  K   T  I  Q  E   I  G  K   E  E  N

CTAGTTCAAT CTGATATCGA TAAACAAATA CAAATAATAT TTAATTAA
L  V  Q  S   D  I  D   K  Q  I   Q  I  I  F   N  *
```

orf2

```
ATGCAAATAT ATACTCATGA TGAATTAGAA AATCCATCCC TAGAAGCCGA AGAGTTTTTA
M  Q  I  Y   T  H  D   E  L  E   N  P  S  L   E  A  E   E  F  L

TCAAATTTCA ATATTTATGT TGATGTAATT GCTGTTTTTA ATGGACCAGT TCTCGCTCAT
S  N  F  N   I  Y  V   D  V  I   A  V  F  N   G  P  V   L  A  H

TTTGAAATTA TGTTAACAAA CATAAATATT CCTCAAAATT CTATTGCTTC AGTCCTAATT
F  E  I  M   L  T  N   I  N  I   P  Q  N  S   I  A  S   V  L  I

ATTGAAACCA TGACACAACT AGATAACTAT TTGCTTGAAT TGAAATTAGA AAACGTAAAA
I  E  T  M   T  Q  L   D  N  Y   L  L  E  L   K  L  E   N  V  K

AACAATAGAT TTCTCAAGTG TGACTTCAAT CAAATTTTTT ACTCTACACA CAAAAACCTT
N  N  R  F   L  K  C   D  F  N   Q  I  F  Y   S  T  H   K  N  L

GTCTACTATT GCTCGAATCA AGTGAAAGAA AACAACTCTG AATAG
V  Y  Y  C   S  N  Q   V  K  E   N  N  S  E   *
```

orf3

```
ATGTTTTTGT TTCATAGTTT CATTTGTGCT GCAAAAATCA AAGGAAAAAA AATGAAAATA
M  F  L  F   H  S  F   I  C  A   A  K  I  K   G  K  K   M  K  I

ATAAGCTGGA TTTTTGATTC AAGCGCTTTT AAAAAGAAAC AAAAAATAAA TGAAATTAAA
I  S  W  I   F  D  S   S  A  F   K  K  K  Q   K  I  N   E  I  K

AAAAAATTAA ATGAAATAAT TATTCTTGAT CGATTCGAAA CAAAAGGGTT TGTCAATAAG
K  K  L  N   E  I  I   I  L  D   R  F  E  T   K  G  F   V  N  K

GCACAAGAAA TGTCATCGCC TAACGGCTCA GGAATTGCGT CTAACGACGC GATTTAG
A  Q  E  M   S  S  P   N  G  S   G  I  A  S   N  D  A   I  *
```

B

```
ORF1        MKKEPIPGKRKNVLAYPENPFWQKTEIKIGSKMVKVSGGKHI--NIEGESISHSGIHVVKEIDENEFVKIYTKNIKAIFDL
CDW96533.1 MSDLPSINLRNLDYSPTVNPLVESQALTIKRRLVRAGRGEDLINSRTGEIVGVSAIHQIEERDDAEFVKVFAAGVSAAYGL
KGD42882.1                  MNPLLEPRKITIKRRYVDSGVSRDLMDA-DGVVQAATVIRNIEEKDDAEFVKVFAAGVAASYDL

            KPTAQRVLQYLITELQKTPN----ADAVYLAWVGAEEYFSENHIKSSRASFHRALSELIQKGFLAESTKPNMFWFNPNLFF
            NRTAQRVFQAVLDEYQKTPMRGGYADSVELFWF--DGGLSGRDIGMSEKTFQRGLKELLAKGFIAAKTASS-FWVNPALFF
            SRTGQRVFQAVLDEYQRTPMQGGFADAVYLSWFG--EGLSGRDIGMSDKTFQRGLRELLAKAFLAPKL-PNVYWVNPALFF

            NGNRLTFIHEYRKKTIQEIGKEENLVQSDIDKQIQIIFN
            KGDRVMFIKEYRRRT---TSAEASTLEHQSQLNLDT
            KGDRVMFVREYRRRSSQQTKAEQVADDRQGSLV
```

Figure 3. (A) Amino acid sequences of *orf1*, *orf2* and *orf3*. (B) Multiple alignment of ORF1, a putative plasmid replication protein RepL (AC: CDW96533.1) and a firmicute plasmid replication family protein (AC: KGD42882.1).

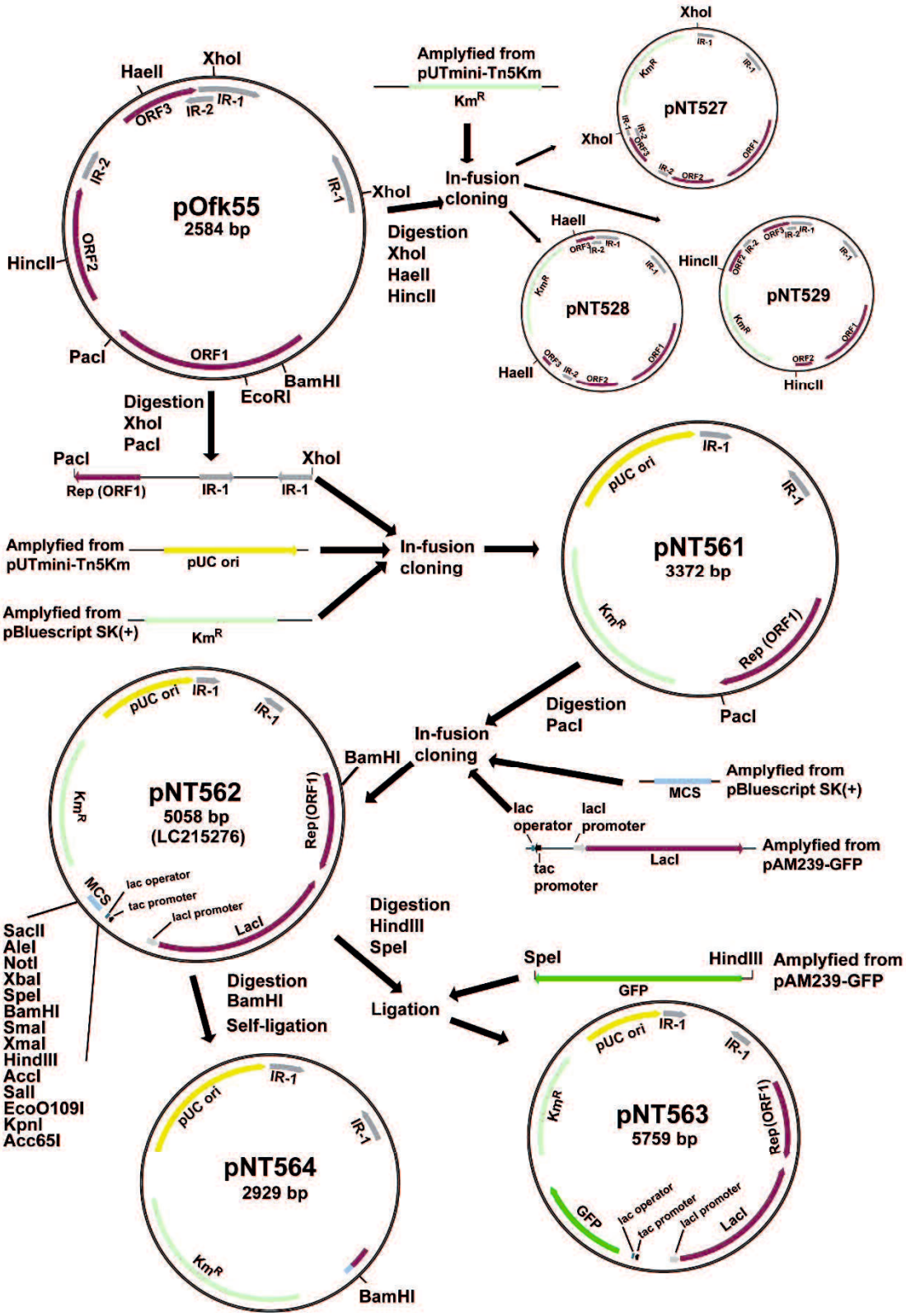

pOfk55
2584 bp
HaeII
XhoI
HincII
PacI
BamHI
EcoRI
ORF1
ORF2
ORF3
IR-1
IR-2
Digestion
XhoI
HaeII
HincII
Amplyfied from
pUTmini-Tn5Km
KmR
In-fusion
cloning
pNT527
pNT528
pNT529
Digestion
XhoI
PacI
Rep (ORF1)
Amplyfied from
pUTmini-Tn5Km
pUC ori
Amplyfied from
pBluescript SK(+)
pNT561
3372 bp
pNT562
5058 bp
(LC215276)
Digestion
PacI
BamHI
MCS
lac operator
tac promoter
lacI promoter
LacI
Amplyfied from
pBluescript SK(+)
Amplyfied from
pAM239-GFP
SacII
AleI
NotI
XbaI
SpeI
BamHI
SmaI
XmaI
HindIII
AccI
SalI
EcoO109I
KpnI
Acc65I
Digestion
HindIII
SpeI
Digestion
BamHI
Self-ligation
Ligation
GFP
pNT563
5759 bp
pNT564
2929 bp

Figure 4. Construction of pOfk55-derived vectors.

Colored arrows on the maps indicate genes and other features. Several restriction sites are indicated. IR; inverted repeat. MCS; multiple cloning sites.

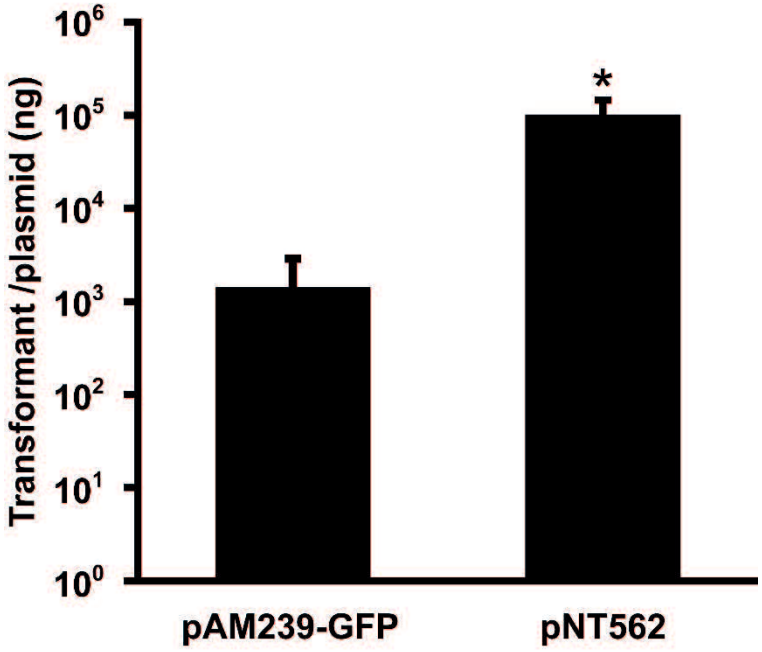


Figure 5. Comparison of the transformation efficiency of pAM239-GFP and pNT562 in *L. pneumophila* Lp02.

Data represent the averages based on three identical experiments and error bars represent standard deviations. Statistical analyses were performed using Student's t-test. The statistically significant difference is indicated by an asterisk (*$P < 0.01$).

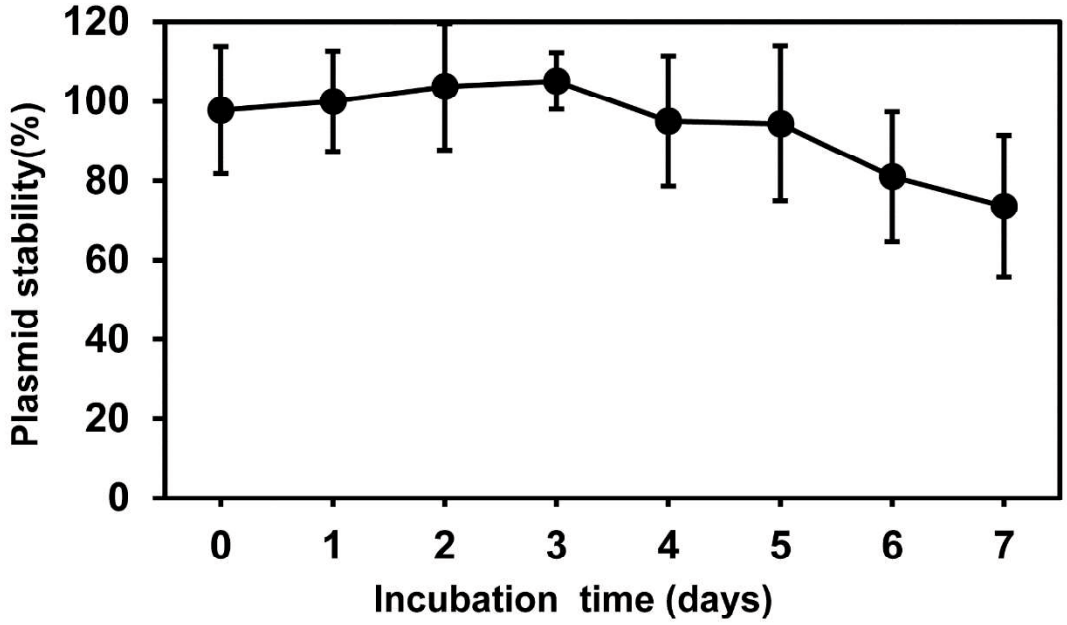


Figure 6. Stability of pNT562 in *L. pneumophila* Lp02 without antibiotic selection.

The percentage was calculated by dividing kanamycin resistant bacteria with kanamycin non-resistant bacteria. Data represent the averages based on three identical experiments and error bars represent standard deviations.

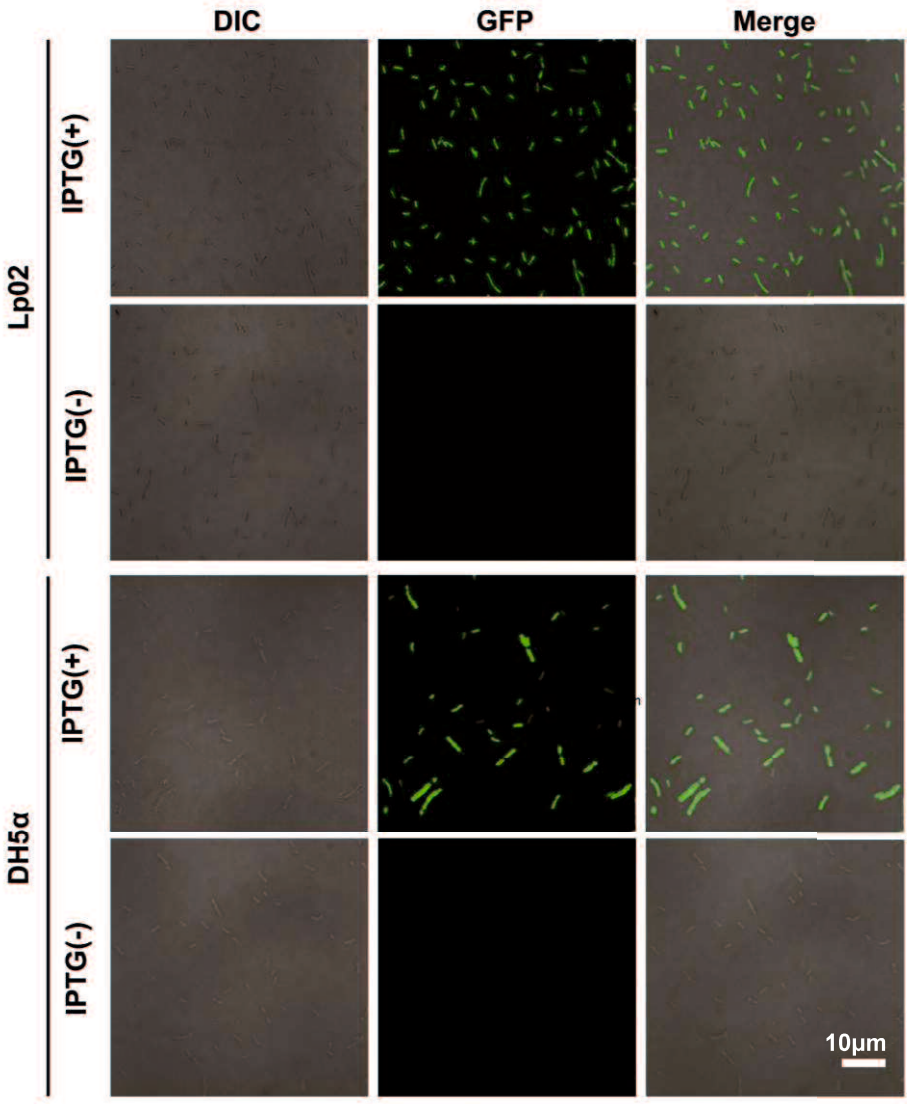


Figure 7. GFP expression in *L. pneumophila* Lp02 and DH5α harboring pNT563 with or without IPTG.

CHAPTER 2

Crucial role of *Legionella pneumophila* TolC in the inhibition of cellular trafficking in the protistan host *Paramecium tetraurelia*

INTRODUCTION

Legionella pneumophila is a facultative intracellular gram-negative bacterium that is the major causative agent of Legionnaires' disease (Cunha *et al*., 2016), which is a severe pneumonia and a mild flu-like illness called Pontiac fever. The infection of humans occurs by inhalation of a *Legionella*-containing aerosol. Once *L. pneumophila* enters the lung, it invades alveolar macrophages and replicates within *Legionella*-containing vacuoles (LCVs) derived from host phagosomes (Isberg *et al*., 2009) that serve as a suitable niche for bacterial replication.

L. pneumophila normally inhabits natural or artificial aquatic environments where it can survive for long periods as free-living forms as well as in biofilms (Fliermans *et al*., 1981; Paszko-Kolva *et al.*, 1992; Declerck *et al*., 2007). Further *L. pneumophila* can survive in free-living protists. Amoebae are protistan hosts of *L. pneumophila* in bodies of fresh water (Rowbotham, 1980), and evidence indicates that *Tetrahymena* serves as its host (Fields *et al*., 1984). In the environment, the association of *L. pneumophila* and protists leads to the replication and spread of this bacterium as well as to the development of antibiotic resistance (Winiecka-Krusnell and Linder, 1999; Barker *et al*., 1995). Thus, from a public health perspective, their association can

enhance the risk of *L. pneumophila* infection for humans.

Many bacteria other than *L. pneumophila* are resistant to protists (Greub and Raoult, 2004), including human pathogenic intracellular bacteria such as *Coxiella burnetii* and *Listeria monocytogenes* as well as nonpathogenic bacteria. Protistan hosts are thought to represent a primary evolutionary factor for the acquisition and maintenance of virulence toward humans (Al-Quadan *et al*., 2012). Because of the similarities of the digestive process between macrophages and protists, certain mechanisms to resist protist digestion help bacteria to survive in macrophages. Thus, association between bacteria and protists can cause the emergence of new pathogenic bacteria, because nonpathogenic bacteria may acquire a pathogenic phenotype within protistan hosts. To control current and future pathogenic bacteria as well as *L. pneumophila*, it is extremely to understand and identify the association between environmental bacteria and protistan hosts.

Paramecium spp. are ciliates that exist widely in freshwater environments and are appreciated as model organisms for the analysis of cellular and molecular biology, including phagocytosis and exocytosis (Steinman *et al*., 1983; Plattner, 2010). Further,

Paramecium spp. are used to study endosymbiosis, because they possess several endosymbionts (Görtz and Fokin, 2009), and symbionts of *Paramecium* spp. affect the host's phenotype. For example, *Paramecium caudatum* can acquire resistance to salinity (Duncan *et al*., 2010) and heat-shock (Hori and Fujishima, 2003; Fujishima *et al*., 2005) if infected with *Holospora* spp. Previously, it has been reported that *Paramecium* spp. are candidates of a natural host of *L. pneumophila* (Watanabe *et al*., 2016). *L. pneumophila* have resistance to *Paramecium* digestion and several *L. pneumophila* strains show cytotoxicity toward *Paramecium* spp. *Paramecium* spp. may increase the risk of *L. pneumophila* infection for humans as well as other protistan hosts such as amoebae and *Tetrahymena*. However, it is still unclear whether *L. pneumophila* establish endosymbiosis with *Paramecium* spp. in the environment.

Type I secretion systems (T1SS) are widespread in pathogenic gram-negative bacteria such as *Escherichia coli, Vibrio cholerae*, and *Bordetella pertussis* (Thomas *et al*., 2014). This secretion machinery comprises three components that transport substrates to the exterior in one step across both lipid bilayers. The first reported protein secreted through T1SS is the hemolysin A (HlyA), which is produced by certain *E. coli* strains (Goebel and Hedgpeth, 1982). HlyA is a member of the repeats-in-toxin (RTX)

family that is the most extensively studied. Further, *L. pneumophila* employ T1SS, and RtxA serves as its substrate in *L. pneumophila.* The association of T1SS and RtxA with virulence of *L. pneumophila* such as invasion, intracellular growth, and pore-forming activities toward amoebae has been revealed (Fuche *et al*., 2015). In addition, TolC, the outer membrane protein of T1SS, plays roles in virulence and multidrug resistance (Ferhat *et al*., 2009). TolC is a trimeric membrane protein and forms a long channel that protrudes deeply into the periplasm. TolC is composed of a short β-barrel (outer membrane) and a long α-helical (periplasmic) structure (Koronakis *et al*., 2000). TolC forms the T1SS by interacting with an ATP-binding cassette transporter and a membrane fusion protein that resides in the inner membrane. TolC couples with numerous inner membrane or periplasmic proteins and forms efflux systems such as the AcrAB-TolC efflux pump (Symmons *et al*., 2015). These efflux systems transport diverse molecules such as virulence-associated proteins, antibiotics, and detergents (Zgurskaya *et al*., 2011). However, I are unaware of published studies on the role of TolC in the relationship between *L. pneumophila* and *Paramecium* spp.

In the present study, I focused on the role of TolC in the association between *L. pneumophila* and *Paramecium* spp. I found that TolC was essential for *L. pneumophila*

to remain within *Paramecium* cells. My results suggest that *L. pneumophila* employs a TolC-dependent mechanism to survive within *Paramecium* spp. in the environment.

MATERIALS AND METHODS

Bacterial strains and culture conditions

All bacterial strains used in this study are listed in Table 1. *L. pneumophila* strains were cultured at 37°C on N-(2-acetamido)-2-aminoethanesulfonic acid-buffered charcoal yeast extract agar (BCYE) or in the same medium without agar and charcoal (AYE) prepared as described in chapter 1. *E. coli* strains were cultured in Lysogeny Broth (LB; Nacalai Tesuque, Kyoto, Japan) or on LB containing 1.5% agar (Wako, Tokyo, Japan). Media were supplemented with chloramphenicol (10 µg/mL, Wako), kanamycin (30 µg/mL, Wako), and ampicillin (250 µg/mL, Wako) as required.

P. tetraurelia and culture conditions

P. tetraurelia st110-1a (ID: PT041001A) was provided by the Symbiosis Laboratory, Yamaguchi University with support, in part, from the NBRP. Culture and maintenance were previously described (Fujishima *et al*., 1990). Briefly, the culture medium used was 2.5% (w/v) fresh lettuce juice in Dryl's solution (Dryl, 1959) inoculated with a nonpathogenic strain of *Klebsiella pneumoniae* the day before use. The cultivation was performed at 25°C. Cells at the stationary phase of growth (20–24 h after the last feeding) were used for the experiments.

Construction of deletion mutants and complementary strains

Each deletion mutant was constructed using the homologous-recombination method. Briefly, two PCR fragments were cloned into SalI/NotI or BamHI-cleaved pSR47s (Andrews *et al*., 1998) using an In-Fusion HD Cloning Kit (Takara, Tokyo, Japan). Fragment 1 was a 1,500 bp or 2,000 bp fragment spanning a site located upstream of the 5' end of each target gene. Fragment 2 was a 1,500 bp or 2,000 bp fragment spanning a site located downstream of the 3' end of each target gene. These fragments were amplified using PCR. Each plasmid was introduced into *E. coli* DH5α λ pir and subsequently transferred into Ofk308 using electroporation with a Gene Pulser electroporator (Bio-Rad Laboratories, CA, USA) in 10% glycerol at 2.5 kV/25 μF. Isolation of in-frame deletion mutants by positive selection for sucrose resistance has been described (Andrews *et al*., 1998). The *tolC* complementary strain was constructed by cloning a PCR fragment of *tolC* into PstI/EcoRI-cleaved pAM239-GFP (Green Fluorescence Protein) using a DNA Ligation Kit (Takara). This *tolC*-inserted plasmid, pAM239-TolC, was introduced into *E.coli* DH5α and subsequently transferred into *L. pneumophila* using electroporation. GFP or TolC expression in *L. pneumophila* was induced by adding isopropyl-β-D-thiogalactopyranoside (1 mM, Wako) to AYE.

All primers and plasmids used in this work are listed in Tables 1 and 2. Plasmid DNA from *E. coli* DH5α was prepared using a QIAGEN Plasmid Mini Kit (QIAGEN, Hilden, Germany). Restriction enzymes (Takara) were used according to the manufacturers' protocols.

Cytotoxicity assay and determination of the bacterial loads in *P. tetraurelia*

Cytotoxicity assays and determination of bacterial loads were performed as previously described (Watanabe *et al*., 2016). Briefly, *Legionella pneumophila* Ofk308 (Ofk308) or each mutant strain was added to *P. tetraurelia* in 1.5 mL tubes and then incubated at 25°C. After incubation, viable *P. tetraurelia* were counted using microscopy. To determine the bacterial load, *P. tetraurelia* was infected with each strain of *L. pneumophila* at multiplicity of infection [MOI] = 20,000. After incubation at 25°C, *P. tetraurelia* was washed five times with 5 mL of fresh lettuce juice in Dryl's solution through a 15-μm pore nylon mesh to remove extracellular bacteria. Samples were further treated at 50°C for 30 min to purge the *K. pneumoniae* fed to *P. tetraurelia*. Colony-forming units were determined using serial dilution on BCYE. In infections with *E. coli* JM109, incubation at 50°C was omitted, and LB containing ampicillin (250 μg/mL) was used.

Fluorescence microscopy

GFP-expressing bacteria were added to *P. tetraurelia* at MOI = 20,000, which were then incubated at 25°C for 30 min to 48 h. *P. tetraurelia* was fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Images of fluorescence were obtained using a FluoView FV100 confocal laser scanning microscope (Olympus, Tokyo, Japan).

When LysoTracker (Life Technologies, CA, USA) was used, *P. tetraurelia* was fixed 30 min after infection. After fixation, samples were washed twice with PBS and then incubated with LysoTracker (50 nM) for 30 min. LysoTracker-positive LCVs were counted using microscopy, and the data are shown as an average of three fields.
When a Texas Red-conjugated dextran (TRDx, Thermo Fisher Scientific, MA, USA) was used, samples were washed 1 h after infection to remove extracellular bacteria using a nylon mesh as described above. TRDx (50 μg/mL) was added to *P. tetraurelia*. At each sampling time, *P. tetraurelia* was fixed and washed twice with PBS. The number of TRDx -containing vacuoles in individual *P. tetraurelia* was counted and expressed as the average of 30 *P. tetraurelia* cells.

Observations of individual *P. tetraurelia* under $NiCl_2$-induced paralysis

P. tetraurelia was infected with *L. pneumophila* at MOI = 20,000 and then incubated at 25°C for 1 h. After washing as described above, $NiCl_2$ (2 mM) was added and the cells were incubated for 10 min at room temperature. After incubation, the *P. tetraurelia* cells were collected and transferred into new media supplemented with 0.2 mM $NiCl_2$. Samples were immediately transferred to a 48-well plate. An IX75 inverted fluorescence microscope was used to observe and photograph the cells at 5 min intervals. The number of LCV-containing *P. tetraurelia* in 50 cells was counted at 0 min and 40 min.

Statistical analyses

Statistical analyses were performed using the Tukey–Kramer test or the Student's t-test. Statistically significant differences between groups were accepted at $P < 0.05$ or $P < 0.01$. Data are presented as the average of three identical experiments, and the error bars shown in the figures represent standard deviations.

RESULTS

L. pneumophila Ofk308 exhibits cytotoxicity toward *P. tetraurelia*

Paramecium spp. feed on bacteria and bacteria uptaken by *Paramecium* are normally digested. On the other hand, Ofk308 showed cytotoxicity toward several *Paramecium* strains (Watanabe *et al.*, 2016). However, the bacterial properties in *Paramecium* was not evaluated. Then, I evaluated intracellular localization of Ofk308. As a result of infection assays in which GFP-expressing Ofk308 was added to 93 strains of *Paramecium* spp. (MOI = 10,000), the intracellular localization of Ofk308 was clearly observed in *P. tetraurelia* strains (data not shown). Therefore, I decided to use *P. tetraurelia* st110-1a as a model to analyze the relationship between Ofk308 and *Paramecium* spp. in the present study.

Ofk308 exhibits cytotoxicity toward several *Paramecium* strains in an MOI-dependent manner (Watanabe *et al*., 2016). Therefore, the cytotoxicity of Ofk308 toward *P. tetraurelia* was assessed. *P. tetraurelia* was infected with Ofk308 at different MOIs, and viable *P. tetraurelia* were counted 48 h after infection. At MOI ≤5,000, the number of viable *P. tetraurelia* was the same as that of uninfected *P. tetraurelia.* However, at MOI = 10,000, the number of viable *P. tetraurelia* tended to decrease, and

at MOI ≥20,000, the number of viable *P. tetraurelia* decreased significantly compared with uninfected *P. tetraurelia* (Fig. 1). These results suggest that Ofk308 is cytotoxic to *P. tetraurelia* in an MOI-dependent manner.

TolC plays a crucial role in the cytotoxicity of *L. pneumophila* Ofk308

Type IV secretion systems of *L. pneumophila* play a major role in its replication within mammalian macrophages and amoebae (Al-Quadan *et al*., 2012). However, the lack of Type IV secretion systems does not affect the cytotoxicity of Ofk308 toward *P. caudatum* (Watanabe *et al*., 2016). Therefore, I focused on T1SS, which is a virulence factor of *L. pneumophila* to amoebae (Fuche *et al*., 2015). For this purpose, I constructed deletion mutants of *tolC*, *lssBD,* and *rtxA*. TolC is an outer membrane component of T1SS. LssBD serve as inner membrane and periplasmic components of T1SS, and RtxA is a substrate of T1SS (Fuche *et al*., 2015). Using these strains, I examined the cytotoxicity toward *P. tetraurelia* at MOI = 20,000. The *tolC*-deletion mutant lost its cytotoxicity toward *P. tetraurelia*, and its complementary strain recovered cytotoxicity comparable to that of the parental strain Ofk308 (Fig. 2). However, the number of viable *P. tetraurelia* decreased to the same degree as Ofk308 when the *lssBD*- and *rtxA*-deletion mutants were infected. These results indicate that the

cytotoxicity of Ofk308 is dependent on TolC but not on T1SS.

The cytotoxicity of *L. pneumophila* Ofk308 is independent on the number of bacteria within *P. tetraurelia*

To investigate how Ofk308 exhibits cytotoxicity toward *P. tetraurelia* through a TolC dependent mechanism, I compared bacterial numbers in *P. tetraurelia* using Ofk308, the *tolC*-deletion mutant, and *E. coli* as a control. From 2 h to 48 h after infection at MOI = 20,000, the numbers of *E. coli* decreased but the numbers of Ofk308 and those of the *tolC*-deletion mutant were unchanged (Fig. 3). Comparable results were observed at lower MOIs (data not shown). In cells infected with Ofk308, the shape of *P. tetraurelia* changed unnaturally, although damage was not observed in cells infected with the *tolC*-deletion mutant. These results suggest that the number of bacteria in *P. tetraurelia* is not associated with cytotoxicity and that TolC is not involved in the mechanism of resistance against the digestion of *P. tetraurelia* in Ofk308.

***L. pneumophila* Ofk308 inhibits the phagocytic activity of *P. tetraurelia* through a TolC-dependent mechanism**

Next, I focused on phagocytic activity of *P. tetraurelia* after mixing with Ofk308

because I hypothesized that the inhibition of phagocytic activity could cause cytotoxicity. *P. tetraurelia* exhibits high phagocytic activity (Plattner and Kissmehl, 2003), and *L. pneumophila* modulates host phagosomes to survive within them (Isberg *et al*., 2009). In infection of *P. caudatum*, Ofk308 inhibits phagosome-lysosome fusion (PL-fusion) (Watanabe *et al*., 2016). Therefore, I used a LysoTracker to evaluate the maturation of *P. tetraurelia* phagosomes containing Ofk308. I found that there was no difference between Ofk308 and the *tolC*-deletion mutant. 97.1% of LCVs were LysoTracker positive in cells infected with Ofk308 and 97.4% of LCVs were LysoTracker positive in cells infected with the *tolC*-deletion mutant (Fig. 4).

Next, to assess the formation of phagosomes, TRDx-uptake assay was performed (according to the schedule shown in Fig. 5A). Dextran-containing vacuoles (DCVs) represent vacuoles that formed after adding TRDx. In cells infected with Ofk308, 5 min, 10 min, and 15 min after adding dextran, the average numbers of DCVs per cell were 2.5, 4.1, and 4.2, respectively. In contrast, in cells infected with the *tolC*-deletion mutant, the average numbers of DCVs were 5.8, 7.0, and 8.3, respectively (Fig. 5B and C). There were significant differences between Ofk308 and the *tolC*-deletion mutant. These results suggest that Ofk308 inhibits phagosome formation by *P. tetraurelia*

through a TolC-dependent mechanism.

L. pneumophila Ofk308 remains within *P. tetraurelia* through a TolC-dependent mechanism

*S*urviving in free-living protists is beneficial for *L. pneumophila*. *L. pneumophila* must inhibit the exocytosis of *Paramecium* to remain within them, because *Paramecium* exhibits high exocytic activity (Plattner and Kissmehl, 2003). I assumed that Ofk308 terminates the exocytic activities of *P. tetraurelia.* However, because of high mobility of *P. tetraurelia*, evaluation of the exocytic activities in individual *P. tetraurelia* is difficult. Therefore, the digestion vacuolar cycles of individual *P. tetraurelia* was observed under $NiCl_2$-induced paralysis (according to the schedule shown in Fig. 6A). By treated with $NiCl_2$, *P. tetraurelia* stopped swimming but cytoplasmic streaming was observed. 93.6% of *P. tetraurelia* possessed Ofk308-containing vacuoles for at least 40 min (Fig. 6B and C). In contrast, the *tolC*-deletion mutant-containing vacuoles were gradually excreted. Finally, most *tolC*-deletion mutants were excreted until 40 min and only 18.0% of *P. tetraurelia* possessed LCVs. These results suggest that *P. tetraurelia* failed to excrete undigested Ofk308 and that Ofk308 remained within cells through a TolC-dependent mechanism.

DISCUSSION

In the environment, *L. pneumophila* replicates within a protistan host. Previous reports reveal the mechanisms of infection and replication in protists such as amoeba and *Tetrahymena* (Richards *et al*., 2013; Al-Khodor *et al*., 2009). However, in these reports, the temperatures used for most infection procedures ranged from 30°C to 37°C, because these temperatures are appropriate for the culture of *L. pneumophila* and amoebae. Thus, it is possible that there are other mechanisms, which function at lower temperatures. In the present study, I used *P. tetraurelia* as a model protistan host of *L. pneumophila*. *Paramecium* spp. are candidates of natural host of *L. pneumophila* (Watanabe *et al*., 2016). *Paramecium* spp. are widely present in fresh water environments but their resistance to high temperature is lower compared with amoeba or *Tetrahymena* (Hori and Fujishima, 2003, Thatcher and Gorovsky, 1993). Thus, all infection assays in the present work were performed at 25°C, which is typical for *Paramecium* spp. culture conditions. Although the cytotoxicity of *L. pneumophila* toward amoeba decreases at lower temperature (Ohno *et al*., 2008), cytotoxicity of Ofk308 toward *P. tetraurelia* were clearly observed at 25°C in an MOI-dependent manner (Fig. 1). The association of *L. pneumophila* and *Paramecium* spp. can lead to the replication and spread of *L. pneumophila* in natural aquatic environments. For this

reason, my assay likely reflects the natural environmental conditions that are conducive to the survival of *L. pneumophila* and can be helpful to reveal the ecology of *L. pneumophila* in the environment.

The Dot/Icm system of *L. pneumophila* contributes to intracellular survival and replication in amoeba (Richards *et al.*, 2013). However, in a previous report, the Dot/Icm system had no effect on cytotoxicity toward *P. caudatum* (Watanabe *et al*., 2016). Therefore, I focused on another secretion system, T1SS. Many gram-negative pathogenic bacteria such as *E. coli* and *B. pertussis* employ T1SS, whose association with bacterial virulence is established (Goebel and Hedgpeth, 1982; Glaser *et al*., 1988). The role of T1SS in intracellular bacteria has been investigated as well. Several T1SS substrates are present in *Orientia tsutsugamushi* and *Ehrlichia chaffeensis* (Wakeeletal *et al.*, 2011; VieBrock *et al*., 2015). T1SS in *L. pneumophila* was investigated, and the roles of T1SS in pore-forming activity and intracellular replication is established (Fuche *et al*., 2015). In the present study, I evaluated the effects of T1SS on cytotoxicity toward *P. tetraurelia* using a deletion-mutant of T1SS. As a result, in infection with the *tolC*-deletion mutant, decreased cytotoxicity was observed (Fig. 2). However, other components of T1SS, including the T1SS substrate RtxA, did not affect cytotoxicity.

These results suggest that TolC of *L. pneumophila* possesses another function separate from T1SS and that this function may be important in cytotoxicity of *L. pneumophila* toward *P. tetraurelia*. TolC forms several multidrug efflux pumps (Symmons *et al*., 2015), and TolC of *L. pneumophila* contributes to multidrug-resistance (Ferhat *et al*., 2009). Multidrug efflux pumps are associated with the virulence of *Salmonella enterica* serovar Typhimurium (Buckley *et al*., 2006; Nishino *et al*., 2006). Therefore, an efflux pump composed of TolC may provide an important function in cytotoxicity of *L. pneumophila* toward *P. tetraurelia*. Further, *Rickettsia typhi* may employ another TolC-dependent secretion mechanism (Kaur *et al*., 2012). In this model, an ankyrin repeat-containing protein translocates to the periplasm via the Sec translocon. This protein is secreted via TolC. How TolC recognizes this protein in the periplasm is unknown, and it is unclear if *L. pneumophila* employs a similar secretion mechanism. Further study is therefore required to determine the function of TolC in *L. pneumophila*.

Legionella-endosymbiosis modulating factor A (LefA) regulates the relationship between *L. pneumophila* and *Paramecium* spp. (Watanabe *et al*., 2016). LefA is associated with intracellular replication and inhibition of PL-fusions in *P. caudatum*. In mammalian macrophages or amoebae, *L. pneumophila* modifies host phagosomes and

avoids PL-fusions (Richards *et al*., 2013, Xu and Luo, 2013). Therefore, I examined the effects of TolC on intracellular replication and PL-fusions of *P. tetraurelia* with Ofk308. As a result, the same levels of intracellular localization and PL-fusions were observed in infections with Ofk308 and the *tolC*-deletion mutant (Fig. 3 and Fig. 4**)**. These results indicate that Ofk308 avoids *P. tetraurelia* digestion independently of TolC. The mechanism of the resistance to digestion is unclear, but temperature may serve as a key factor, because all experiments were performed at 25°C.

Paramecium spp. possess high phagocytic and exocytic activities (Plattner and Kissmehl, 2003). In contrast, *Chlorella*, which is known as an endosymbiont of *P. bursaria*, remains for at least 72 h (Kodama and Fujishima, 2005). The symbiotic relationship between *P. bursaria* and *Chlorella* was well investigated. Then, endosymbionts of *Paramecium* may inhibit phagocytic and exocytic activities. I show here that TolC played a role in the inhibition of the excretion of LCVs (Fig. 6). This inhibition follows that *L. pneumophila* remains within the host *Paramecium*. Therefore, TolC may represent a crucial factor required for Ofk308 to remain within *P. tetraurelia* and to establish symbiosis.

High concentrations of Ofk308 exhibited cytotoxicity toward *P. tetraurelia* through a TolC dependent mechanism (Fig. 1 and 2). In infections with *P. caudatum,* significant intracellular replication of Ofk308 occurs and causes the death of the host *P. caudatum* (Watanabe *et al*., 2016). Thus, intracellular replication can represent a major cause of death. However, in the present study, such intracellular replication of Ofk308 was not observed in *P. tetraurelia* (Fig. 2). Therefore, I reasoned that the inhibition of phagosome formation can cause the death of *P. tetraurelia*. After infection with *P. tetraurelia*, Ofk308 inhibited new phagosome formation through a TolC-dependent mechanism (Fig. 5). In *Paramecium* spp., the membrane used to form new phagosomes is provided by recruitment of cytoplasmic discoidal vesicles that originate, in part, at the cytoproct (Guerrier *et al*., 2017; Steinman *et al*., 1983). Thus, excess Ofk308 remaining within *P. tetraurelia* cells can stop the recycling of the phagosome membrane to form new phagosomes. As a result, *P. tetraurelia* may starve and subsequently die because of poor nutrition. Further, the inhibition of phagosome formation can result in inhibition of invasion of other microbes to *Paramecium*. In natural condition, *L. pneumophila* may inhibit phagocytic activities by TolC dependent mechanism to occupy host *Paramecium*.

TABLES AND FIGURES

Table 1. Bacterial strains and plasmids used in this study

Strain	Characteristics	Source or reference
Legionella pneumophila		
Ofk308	Isolated from environmental water	Tachibana *et al*. (2013)
Ofk308 *ΔtolC*	*tolC*-deletion mutant of Ofk308	This study
Ofk308 *ΔtolC/tolC*	Ofk308 *ΔtolC* carrying pAM239-TolC	This study
Escherichia coli		
DH5α	*Φ80lacZΔM15, Δ(lacZYA-argF)U169, recA1, endA1, hsdR17, supE44, thi-1, gyrA96, relA1*	Takara
DH5α λ pir	DH5α (λ pir) *tet*::Mu *recA*	Takara
JM109	*recA1, endA1, gyrA96, thi-1, hsdR17, e14-(mcrA-), supE44, relA1, Δ (lac-proAB)*	Takara
Plasmids		
pAM239-GFP	pMMB-derived vector encoding GFP, CmR	Watarai *et al*. (2001)
pAcGFP	pUC19-derived vector encoding AcGFP1, AmpR	Takara
pSR47s	*ori* R6K *ori* TRP4 *sacB* , KmR	Andrews *et al*. (1997)
pAM239-TolC	pAM239 vector expressing TolC, CmR	This study

Table 2. Primers used in this study.

Primer	Sequence	Target region
tolCuF	ACCGCGGTGGCGGCCGCTGCGAGTGGCAATTGC	upstream of the 5'
toCuR	CGAATTGGATCCAAATTTAGGTTTTCTTATGTC	end of *tolC*
tolCdF	ACCTAAATTTGGATCCTCGAGCTTTCCCTAGAAAAC	downstream of the
tolCdR	ATCCTCTAGAGTCGCAAAATGCTCTGGTGTTCC	3' end of *tolC*
lssBDuF	ATCCTCTAGAGTCGACTTACCAGATTGCTGATGC	upstream of the 5'
lssBDuR	TTGCTTAATGATTGTCTTCTCGGAGTATTC	end of *lssB*
lssBDdF	ACAATCATTAAGCAATCAGCACTTAGAGAG	downstream of the
lssBDdR	ACCGCGGTGGCGGCCGCGTGATTCCAGCGAATTAG	3' end of *lssD*
rtxAuF	ATCCTCTAGAGTCGACCAAGCGATAAGGTAATAATTG	upstream of the 5'
rtxAuR	GTTCATCGTTCTGTCCTCAAAGTTTACTATT	end of *rtxA*
rtxAdF	GACAGAACGATGAACCCATTACATTGGTG	downstream of the
rtxAdR	TAGAACTAGTGGATCCGCAGAAGAGCGTATGCCA	3' end of *rtxA*
tolCcF	CGAATTGAATTCGTTTTCTAGGGAAAGCTCG	*tolC*
tolCcR	CGAATTCTGCAGTGTGAATGAATCTTTTCC	

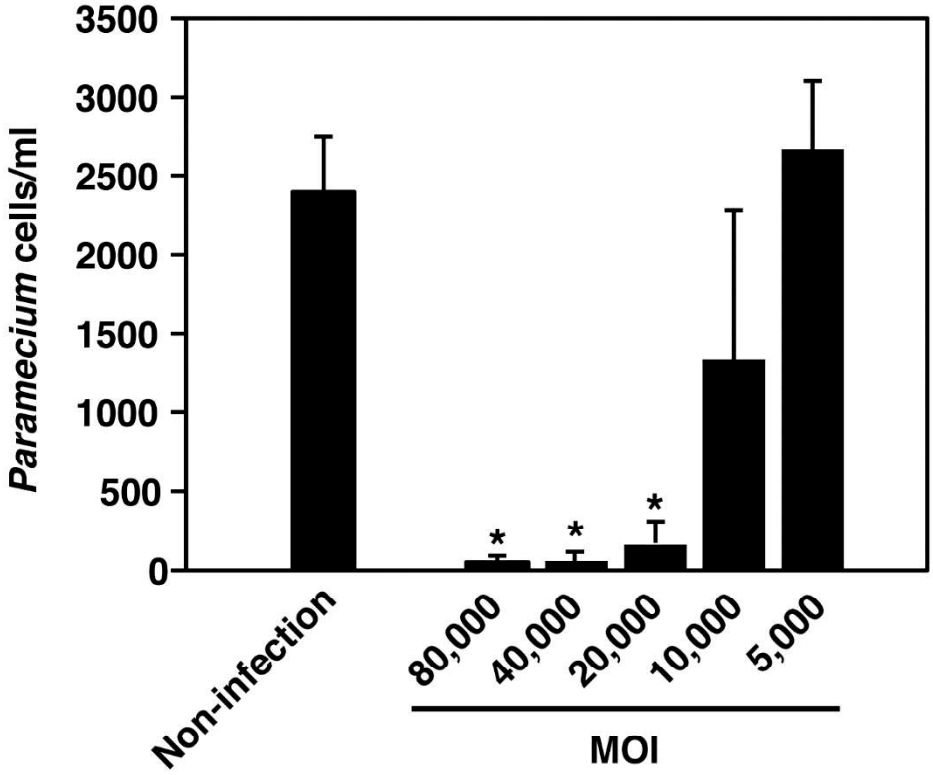


Figure 1. *L. pneumophila* Ofk308 exhibits cytotoxicity toward *P. tetraurelia* in an MOI-dependent manner.

The number of *P. tetraurelia* 48 h after infection. *P. tetraurelia* was infected with Ofk308 at MOIs = 5,000, 10,000, 20,000 40,000, and 80,000. Data are expressed as the average of three identical experiments, and the error bars represent standard deviations. Asterisks indicate statistically significant differences compared with Non-infection (*$P < 0.05$).

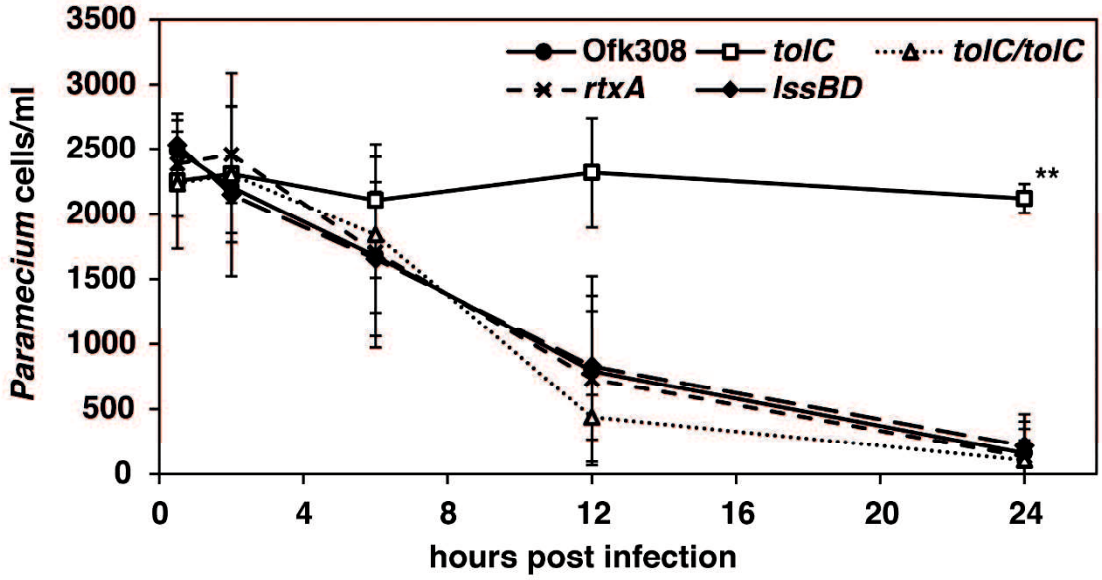
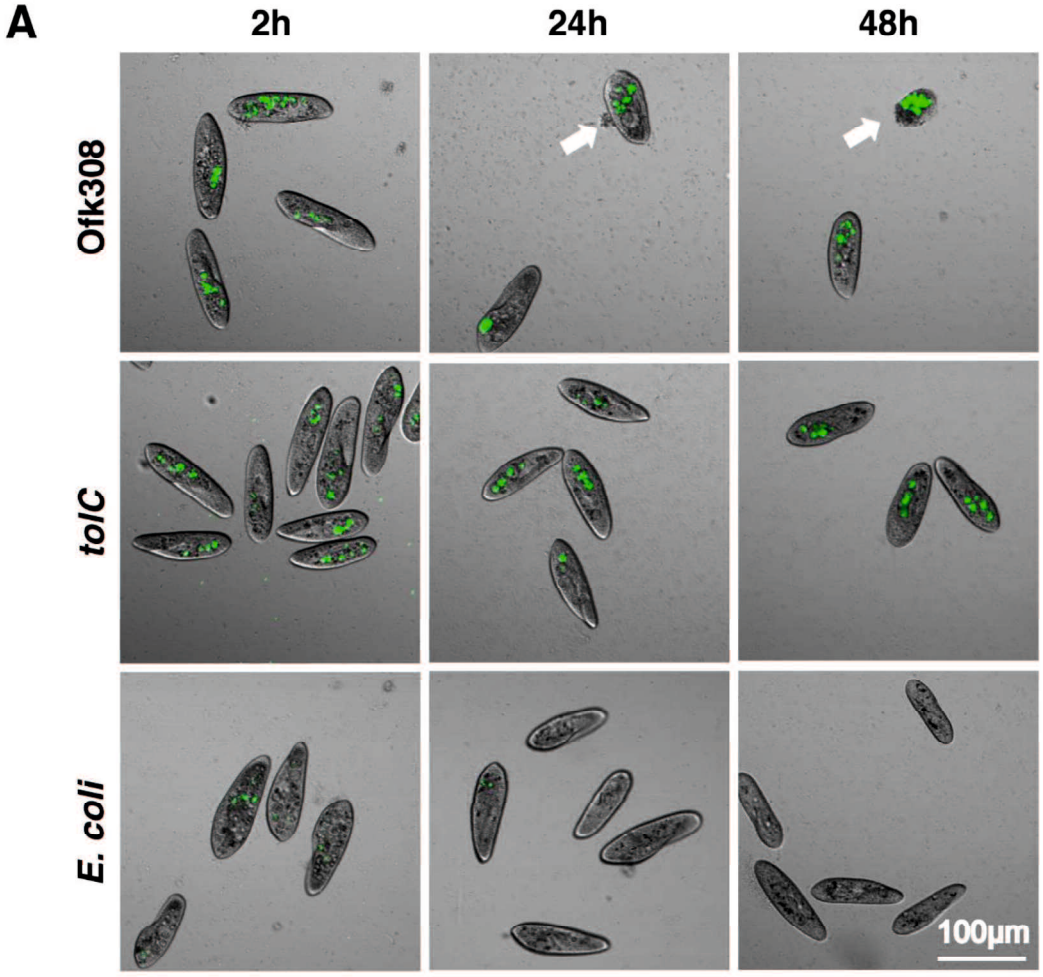


Figure 2. The *tolC*-deletion mutant of *L. pneumophila* Ofk308 is defective for cytotoxicity.

P. tetraurelia was infected with Ofk308, the *tolC*-deletion mutant (*tolC*), the lssBD-deletion mutant (*lssBD*), the rtxA-deletion mutant (*rtxA*), or the *tolC*-complementary strain (*tolC/tolC*) at MOI = 20,000. Data are presented as the average of three identical experiments, and the error bars represent standard deviations. Asterisks indicate statistically significant differences compared with Ofk308 (**$P < 0.01$).


A
2h
24h
48h
Ofk308
tolC
E. coli
100μm

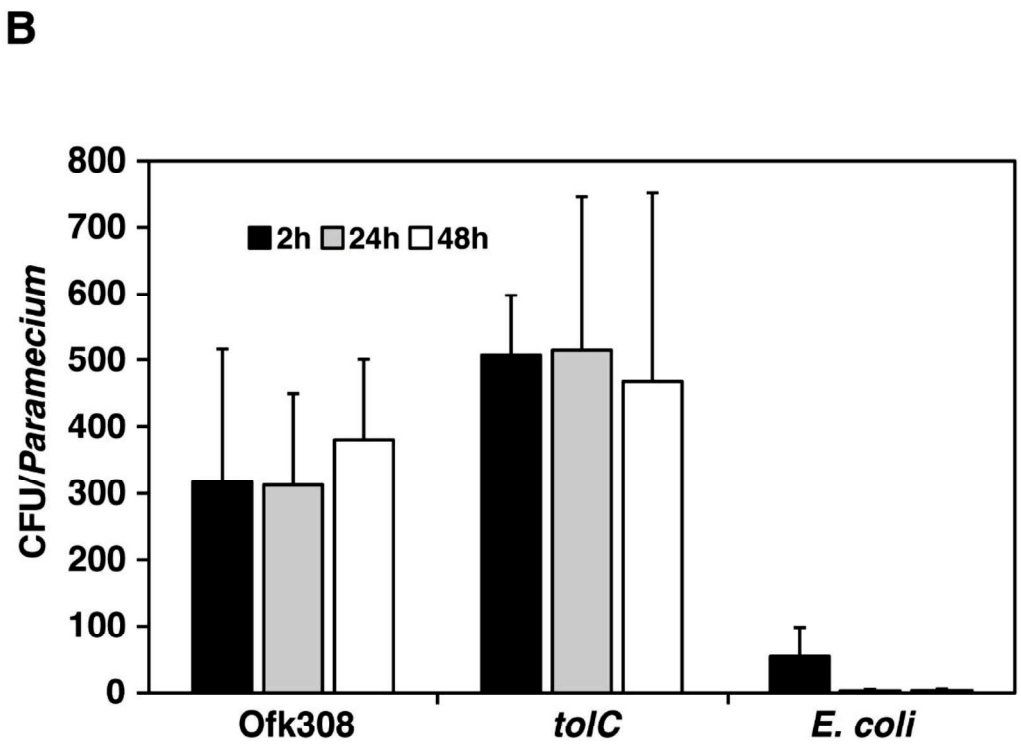

B
800
700
600
500
400
300
200
100
0
CFU/Paramecium
2h 24h 48h
Ofk308
tolC
E. coli

Figure 3. The number of *L. pneumophila* in *P. tetraurelia* is constant.

(A) GFP-expressing bacteria, Ofk308, the *tolC*-deletion mutant (*tolC*), and *E. coli* JM109 in *P. tetraurelia* 2 h, 24 h, and 48 h after infection at MOI = 20,000. Arrows point to unnaturally-shaped *P. tetraurelia*. (B) Number of bacteria per *P. tetraurelia.* Data are expressed as the average of three identical experiments, and the error bars represent standard deviations.

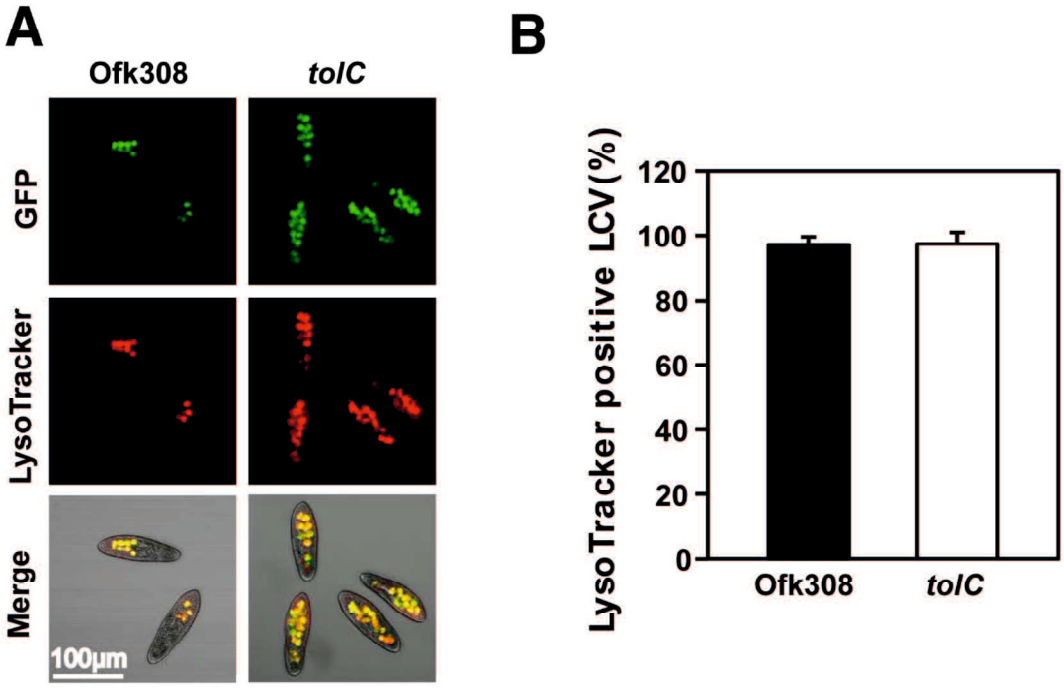


Figure 4. The maturation of host LCVs containing *L. pneumophila* Ofk308 is not inhibited.

(A) LCV maturation 30 min after infection was evaluated using LysoTracker. Ofk308 or the *tolC*-deletion mutant (*tolC*) was added to *P. tetraurelia* at MOI = 20,000. (B) The percentage of LysoTracker-positive LCVs. Data are expressed as the average of three identical experiments, and the error bars represent standard deviations.

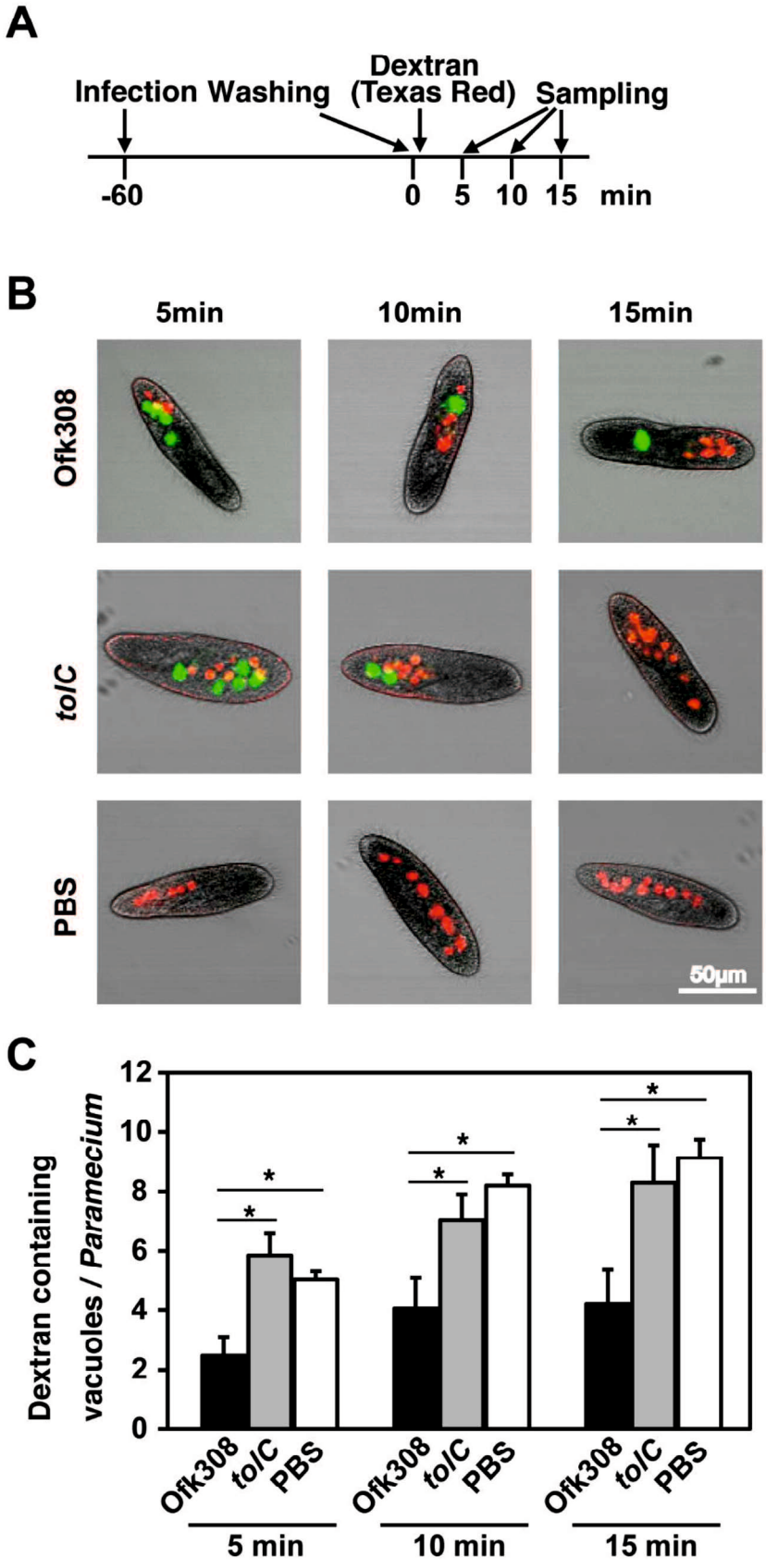

A
Infection
Washing
Dextran
(Texas Red)
Sampling
-60
0
5
10
15
min
B
5min
10min
15min
Ofk308
tolC
PBS
50µm
C
Dextran containing
vacuoles / Paramecium
12
10
8
6
4
2
0
Ofk308
tolC
PBS
5 min
10 min
15 min

Figure 5. *L. pneumophila* Ofk308 inhibits phagosome formation by *P. tetraurelia* through a TolC-dependent mechanism.

(A) Bacteria and TRDx were added to *P. tetraurelia* according to this schedule. The number of DCVs was counted at each indicated time point. (B) DCVs in *P. tetraurelia* infected with Ofk308 and *tolC* at each indicated time point. DCVs are red, and *Legionella*-containing vacuoles are green. (C) The number of DCVs in individual *P. tetraurelia.* Data are expressed as the average of three identical experiments, and error bars represent standard deviations. Asterisks indicate statistically significant differences (*$P < 0.05$).

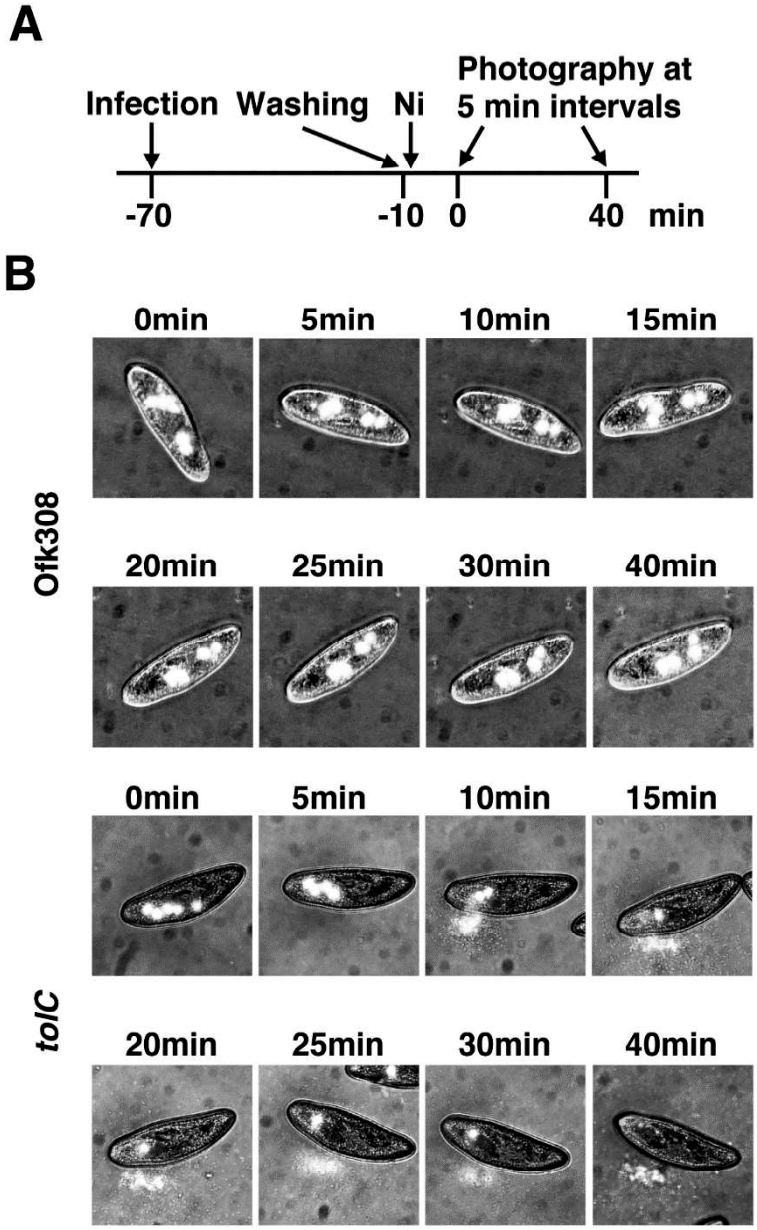

A
Infection
Washing
Ni
Photography at
5 min intervals
-70
-10
0
40
min
B
Ofk308
0min
5min
10min
15min
20min
25min
30min
40min
tolC
0min
5min
10min
15min
20min
25min
30min
40min
C
LCV containing Paramecium (%)
120
100
80
60
40
20
0
**
Ofk308
tolC
0 min
Ofk308
tolC
40 min

Figure 6. *L. pneumophila* Ofk308 inhibits excretion of LCVs from *P. tetraurelia* through a TolC-dependent mechanism.

(A) Images of individual *P. tetraurelia* under $NiCl_2$-induced paralysis were taken according to this schedule. (B) LCVs in individual *P. tetraurelia* at each indicated time point. LCVs are white. (C) The percentages of LCV-containing *P. tetraurelia* at 0 min and 40 min. Data are expressed as the average of three identical experiments, and the error bars represent standard deviations. Asterisks indicate statistically significant differences (**$P < 0.01$).

CONCLUSION

Legionnaires' disease was first recognized in 1976, when an outbreak of pneumonia occurred in Philadelphia (Fraser *et al*., 1977). Since this outbreak, the pathogenesis of this disease have been clarified. Artificial aquatic environments are known to be reservoir, and amoebae are the host of *L. pneumophila* in such environments. Further, the virulence mechanisms like Dot/Icm secretion system have been revealed. Despite these knowledges, the incidence of this disease are rising (Ameura-Maekawa *et al*., 2018, CDC, 2018), and the studies on the ecology of *L. pneumophila* in the environment are insufficient. Therefore, I focused on environmental strains.

In chapter 1, I characterized a small cryptic plasmid pOfk55 from *L. pneumophila* Ofk308, which I used to construct pNT562 as a shuttle vector for *L. pneumophila-E. coli*. Because this vector is originated from *L. pneumophila*, it can be a more useful tool for the genetic analysis of *L. pneumophila* compared with other vectors. Actually, the stability and transformation efficiency of this plasmid were higher, and the *gfpmut3* gene was expressed successfully in *L. pneumophila* and *E. coli* DH5α using this plasmid. Further, this new finding based on environmental strains implies that the analysis of environmental strain is helpful to obtain new aspects of *L. pneumophila*.

In chapter 2, I focused on the relationship between *L. pneumophila* and

Paramecium in the environment and found TolC as an important factor of *L. pneumophila* to remain in *Paramecium* as a host. Because of the high mobility and high cell division rate of *Paramecium* spp*.,* remaining within *Paramecium* cells would beneficial for *L. pneumophila* to expand its habitat, and the association between *L. pneumophila* and *Paramecium* spp. in the environment can enhance the risk of infection to human. It is still unclear whether *Paramecium* serves as a host of *L. pneumophila* in natural environments. However, *Paramecium* can uptake *L. pneumophila*, and *L. pneumophila* can remain/glow in *Paramecium*. Moreover, their habitats are overlapping. These facts suggest that *L. pneumophila* lives together with *Paramecium* and establish various relationships such as symbiosis and parasitism. In this study, cytotoxicity of *L. pneumophila* toward *Paramecium* was observed only at a high MOI. Although, in natural condition, such a high MOI would not occur, some *L. pneumophila* strains exponentially replicate in *Paramecium* spp. (Watanabe *et al*., 2015). Therefore, the infection assay at a high MOI can represent the interaction between *Paramecium* and *L. pneumophila* after replication in host cells.

To control Legionaries' disease, understanding the ecology of *L. pneumophila* in the environment including the association with protists can be a clue. My work promises to facilitate further studies focused on the ecology of *L. pneumophila* in the environment.

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