Original Article

Legionella dumoffii Tex-KL Mutated in an Operon Homologous to *traC-traD* is Defective in Epithelial Cell Invasion*

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Abstract

Objective To understand the mechanism of invasion by *Legionella dumoffii.*

Methods The *L. dumoffii* strain Tex-KL was mutated using the Tn903 derivative, Tn903dIIlacZ. After screening 799 transposon insertion mutants, we isolated one defective mutant. We then constructed the gene-disrupted mutant, KL16, and studied its invasion of and intracellular growth in HeLa and A549 cells, and in A/J mice survival experiments. The structure of *traC-traD* operon was analyzed by RT-PCR.

Results The transposon insertion was in a gene homologous to *Salmonella typhi traC*, which is required for the assembly of F pilin into the mature F pilus structure and for conjugal DNA transmission. Results from RT-PCR suggested that the *traC-traD* region formed an operon. We found that when the *traC* gene was disrupted, invasion and intracellular growth of *L. dumoffii* Tex-KL were impaired in human epithelial cells. When mice were infected by intranasal inoculation with a *traC* deficient mutant, their survival significantly increased when compared to mice infected with the wild-type strain.

Conclusion Our results indicated that the *traC-traD* operon is required for the invasion and intracellular growth abilities of *L. dumoffii* Tex-KL in epithelial cells.

Key words: *L. dumoffii* Tex-KL; *traC-traD* operon; Invasion ability; Epithelial cells

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INTRODUCTION

Legionellae, the causative agents of Legionnaire's disease, are facultative intracellular Gram-negative bacteria[1]. To date, there are over 50 species in the genus *Legionella*, among which the most common human pathogen is *Legionella pneumophila*[2-3]. Legionnaire's disease develops mainly through the aspiration of aerosolized water contaminated by the bacteria[4]. *L. pneumophila*-containing phagosomes are not acidified and do not fuse with lysosomes because they are internalized by alveolar macrophages[5-7]. Instead, *L. pneumophila* begins to

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multiply in this unique niche after the mitochondria, smooth vesicles, and rough endoplasmic reticula are recruited near *L. pneumophila*-containing vacuoles[7]. The *Legionella* Dot/Icm type IV protein secretion system controls this altered endocytic pathway[8-9]. The *dot/icm* genes are essential for the intracellular growth of *L. pneumophila*[10-11]. The IcmX protein, one of the Dot/Icm proteins, has been detected by immunoblotting in several other species of *Legionella*[12], but the contributions of the Dot/Icm loci to the pathogenesis of other species have yet to be investigated.

Legionella dumoffii (synonym *Fluoribacter dumoffii*) was first isolated in 1979 as an atypical *Legionella*-like organism from cooling-tower water[13], and later from a postmortem lung specimen[14]. It was later classified as a new species and named *L. dumoffii*[15]. Members of the *Legionella* species are capable of growing within epithelial cells after being transmitted to humans[4]. *L. dumoffii* is the fourth or fifth most common pathogen that causes Legionnaires' disease[16-17]. Some of the proteins or factors that may promote *L. pneumophila* pathogenesis, such as flagella, catalase, and gelatinase, are also present in *L. dumoffii*, while several putative virulence factors, such as lipase, oxidase, and a zinc metalloprotease, are absent[18-19].

L. dumoffii is capable of invading and replicating within Vero cells and the human type II alveolar epithelial cell line A549 *in vitro* more effectively than *L. pneumophila*[20-21]. The *L. dumoffii* strain Tex-KL undergoes the replication phase in the cytosol after disruption of the parasitosome membrane, which is different from *L. pneumophila*-a model organism commonly used to examine interactions with eukaryotic cells[21]. To understand the molecular mechanisms of *L. dumoffii* invasion, we used transposon mutagenesis to isolate mutants that were defective in the invasion of HeLa and A549 cells. We isolated one clone with attenuated virulence in mammalian cells by screening 799 derivatives with Tn903dIIlacZ insertions. The transposon insertion was in a gene encoding a homolog of the *Salmonella typhi* TraC. We have previously reported the discovery and complete nucleotide sequence of a 66-kb plasmid, pLD-TEX-KL (pLD) harbored by the *L. dumoffii* strain TEX-KL[22]. Open reading frames (ORFs) were assigned as putative transfer conjugation proteins and the genes were located within a 25 kb region of this plasmid, referred to as the transfer (tra) region. Although a TraC homolog is present in *L. pneumophila*, the role of this gene in pathogenesis has yet to be determined. Here, we analyzed the genetic structure of the *L. dumoffii traC* and the downstream genes, and investigated the role of the *traC* homolog in adherence, invasion, and intracellular growth in HeLa and A549 cells. We report that the *L. dumoffii* Tex-KL *traC-traD* operon is important for the invasion of epithelial cells.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media

Bacterial strains and plasmids used in this work are described in Table 1. The *Legionella* strains and their derivatives were grown on buffered charcoal-yeast extract (BCYE) agar plates or in buffered yeast extract (BYE) broth, which lacked agar. *Escherichia coli* DH5α was used for cloning. Antibiotics were used as required at the following concentrations: for *L. dumoffii*, kanamycin (KM), 30 μg mL^{-1}; chloramphenicol (CM), 5 or 20 μg mL^{-1}; for *E. coli*, KM, 30 μg mL^{-1}; ampicillin (AMP), 50 μg mL^{-1}; CM, 20 μg mL^{-1}.

DNA Manipulation

All restriction enzymes used in this study were purchased from Toyobo (Japan). The oligonucleotide primers for PCR amplification are listed in Table 2, and PCR amplification was performed using Ex-*Taq* polymerase (Takara Bio., Japan). Plasmid DNA and chromosomal DNA were isolated using the Wizard Plus Mini Prep kit (Promega, USA) and the Genomic Prep Cells and Tissue DNA Isolation kit (Amersham Pharmacia Biotech), respectively. For the preparation of pLD DNA, whole DNA extracted from *Legionella* cells by an alkaline lysis method was subjected to CsCl-ethidium bromide density gradient centrifugation, as described previously[27]. Purification of DNA fragments from agarose gels for subcloning or labeling was carried out with the GFX PCR DNA and Gel Band Purification kit (Amersham Pharmacia Biotech). Standard procedures were employed in all other *in vitro* experiments using DNA[27].

Transposon Mutagenesis and Mutant Library Construction

L. dumoffii was mutated with the Tn903 derivative Tn903dIIlacZ, as described previously[25]. Tn903dIIlacZ confers kanamycin (KM) resistance (Km^r) and chloramphenicol (CM) sensitivity (Cm^s), and contains a 5'-truncated *lacZ* gene. Briefly, after electroporation of plasmid pLAW330, which

contained Tn903dIIlacZ, *L. dumoffii* Tex-KL were incubated in BYE broth for 5 h at 37 °C and plated onto BCYE agar supplemented with KM. Km^r transformants showing ß-galactosidase activity were identified as blue colonies after the plates were overlaid with 0.8% agar containing 0.6 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-Gal). KM resistant and CM sensitive colonies were saved as Tn903dIIlacZ insertion mutants of *L. dumoffii*. In addition, we constructed a mutant of TEX-KL in which the *traC* gene was disrupted by insertion of a mini-Tn5/Km cassette.

Table 1. Bacterial Strains and Plasmids Used in This Work

Strain or Plasmid	Genotype and/or Relevant Information	Source or Reference
L. dumoffii		
L. dumoffii Tex-KL		ATCC33343
KL15	Tex-KL *traC*::Tn*903*dIIl*acZ*	This study
KL16	Tex-KL *traC*::Km^r	This study
KLc	Tex-KL plasmid cured	This study
KL17	Tex-KL (pMMB207*traC*)	This study
L. pneumophila		
AM511	Strr Res- Mod+ derivative of strain Philadelphia-1	[23]
E. coli		
DH5α	*supE*44 Δ*lac*U169 (Φ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	[24]
Plasmids		
pGEM-T Easy	Ap^r	Promega
pLAW344	*oriT* (RK2) *oriR* (ColE1) *sacB* Cm^r Ap^r	[25]
pUT-mini-Tn5 Km	Km^r	[26]
pQT011	pGEM-T Easy Ω*traC+*	This study
pQT011Km	pQT201 Ω*traC*::Km^r	This study
pQT011LAW	pLAW344 Ω*traC*::Km^r	This study
pMMB207	RSF1010, derivative RSF1010 derivative, *lncQ lacIq* Cm^r P*tac oriT*	[27]

Note. Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance.

Table 2. PCR Primers Used in This Work

Primer	Sequence (5'-3')[a]	Description[b]
1F	TGAAACGGTGAATGAAGCCG	For *traC* -disrupted mutant, forward
1R	AAACTCGCAATCGCTTTGGC	For *traC* -disrupted mutant, reverse
2F	GCTCTAGAGCGAAGATAAGAGTCAGCACGC (*Xba*I)	For *traC* + promoter, forward
2R	GGGGTACCCCGCTCCACCTGAGATTCAGTC (*Kpn*I)	For *traC* + terminator, reverse
RT1	GAAGATAAGAGTCAGCACGC	For *traB-traC* RT-PCR, forward
RT2	TCGTAGTTTTGCGAAAGCCG	For *traB-traC* RT-PCR, reverse
RT3	ATTGAATCCTTTGGGGAGGC	For *traC-orf4* and *traC-traD* RT-PCR, forward
RT4	ATGTGCCATTTGGCCAGAAG	For *traC-orf4* RT-PCR, reverse
RT5	AAAAGACAGGCAGAATGCCC	For *traC-traD* RT-PCR, reverse
3F	TTGCAAACCCTCACTGATCC	For km^r cassette probe, forward
3R	CTGCCATCACGAGATTTCGA	For km^r cassette probe, reverse
4F	TTTCGCAAAACTACGAGCCC	For *traC* probe, forward
4R	AGAGCGTGTTGAAATCAGGC	For *traC* probe, reverse
lacZ	CCCAGTCACGACGTTG	For sequencing of the chromosomal junction of Tn903dIIl*lacZ* insertion
Km^r	AATTTAATCGCGGCCTCGAG	

Note. [a]Underlined sequences are restriction site adapters. The restriction enzymes are indicated in parentheses. [b]These primers were designed based on the sequence of *L. dumoffii* Tex-KL plasmid, pLD-TEX-KL (GenBank accession number AB297474.1).

Determination of the Tn903dIIlacZ-insertion Position Sites within the Genome of L. dumoffii Mutants

Genomic DNA from the *L. dumoffii* mutant, KL15, was digested with *Hin*dIII and *Bam*HI and ligated into similarly cut pBR322. The ligation mixture was used to transform *E. coli* DH5α competent cells (Takara Bio), and the transformation mixture was plated onto Luria-Bertani agar plates containing KM and AMP. Plasmid DNA was extracted, and the regions flanking Tn903dIIlacZ were sequenced with the *lacZ* primer and the Km^r primer, corresponding to the 5' and 3' ends of Tn903dIIlacZ, respectively.

Construction of Gene-disrupted Mutants

Construction of gene-disrupted mutants was carried out by inserting a Km^r cassette, which contained mini-*Tn*5 Km and was excised from pUT-mini-*Tn*5Km[26], into the target gene using the following steps: (i) a DNA fragment containing the *traC* coding region was amplified by PCR with primers 1F and 1R (Table 2); (ii) this PCR product was ligated into pGEM-T-Easy vector (Promega) using the TA-cloning method to produce pQT011; (iii) a *Sma*I-excised Km^r cassette was inserted into the *Eco*RV site of pQT011. The resulting plasmid was named pQT011Km; (iv) pQT011Km was digested with *Not*I, and a fragment carrying the disrupted *traC* region was cloned into the *Not*I site of allelic exchange vector, pLAW344, to produce pQT011LAW[25]; (v) this plasmid was introduced into *L. dumoffii* Tex-KL cells by electroporation, and transformants were selected on BCYE agar plates supplemented with KM[28]; (vi) finally, Km^r transformants were grown and subjected to selection for the Km^r cassette, and the loss of the integrated pLAW344 was confirmed by culturing on BCYE agar containing KM and 2% (w/v) sucrose. The *traC*-disruption of this newly generated strain, KL16, was verified by PCR and Southern hybridization (data not shown) using the PCR primers 1F and 1R and a Southern hybridization probe that was amplified with primers 3F and 3R.

Plasmid Curing

L. dumoffii Tex-KL was cured of pLD by growing cells in BYE broth supplemented with 50 μg mL^{-1} novobiocin for 24 h at 37 °C with shaking and 10-fold dilutions were plated onto BCYE agar plates. After incubation, colonies that grew on the plates were tested for the absence of the plasmid DNA by banding in CsCl-ethidium bromide density gradients[29]. The resulting strain was named *L. dumoffii* KLc.

Southern Hybridization

Chromosomal DNA from *L. dumoffii* was digested with *Hin*dIII, resolved on a 0.7% agarose gel in Tris-borate-EDTA buffer (TBE buffer), and blotted onto a nylon membrane. PCR-amplified *L. dumoffii traC* probes (4F and 4R) were prepared by random-primed labeling with digoxigenin-11-dUTP (Roche Diagnostics, Switzerland). The methods for prehybridization and hybridization, and the washing conditions, were as described previously[30]. Colorimetric detection of hybridized DNA was performed using the digoxigenin system (Roche Diagnostics).

Reverse Transcript PCR (RT-PCR)

Total RNA was isolated from test strains by standard procedure[31]. The isolated RNA was treated with DNase I (Takara Bio.) and stored at -80 °C until use. RT-PCR was performed using the SuperScript III First-strand Synthesis System (Invitrogen) according to the manufacturer's instructions, with 2 μg of total RNA in a final volume of 20 μL. The resulting cDNA was subjected to amplification with primers RT1, RT2, RT3, RT4, and RT5 (Table 2). The DNA size markers used were the 100 bp DNA Ladder and the λ/*Hin*dIII DNA Ladder (Toyobo).

Cell Culture

HeLa (JCRB9004) and A549 (JCRB0076) cell lines were donated by the Health Science Research Resources Bank (Osaka, Japan). A549 cells were established from a human alveolar epithelial carcinoma and have characteristics of well-differentiated type II pneumocytes. HeLa and A549 cells were cultured in RPMI 1640 medium (Gibco, NY, USA), supplemented with 10% fetal bovine serum (Gibco) at 37 °C with 5% CO_2.

Adhesion and Invasion Assays

A suspension of *L. dumoffii* culture in RPMI 1640 medium was added to A549 or HeLa monolayers at multiplicities of infection (MOI) of 10 to 100 and was further cultured for 1 h. The monolayers were washed twice with PBS to remove nonadherent bacteria. For the adhesion assay, the cells were detached from the well by addition of 0.1% Triton X-100 and sonicated with a Bioruptor UCD-200T

(Cosmo Bio, Tokyo, Japan) at 130 W for 24 s. A series of 10-fold dilutions of the sonicated solution was plated on BCYE plates, and the number of bacteria was counted. For the invasion assay, after washing twice with PBS, 0.5 mL of culture medium supplemented with 100 μg mL^{-1} of gentamicin (Wako, Osaka, Japan) was added to each well and incubated for 1 h to kill extracellular bacteria. The cells were washed again with PBS, and the number of intracellular bacteria was determined as described above. For both assays, each dilution was tested in triplicate in separate wells.

Intracellular Growth Assay

L. dumoffii strains were grown in BYE broth to early stationary phase. Approximately 2×10^9 bacteria were pelleted, resuspended, and diluted (1:1000) in RPMI 1640 tissue culture medium. The bacteria were then added to A549 or HeLa cells (2×10^5 per well) in 24-well dishes at an MOI of approximately 10. The infected cells were incubated at 37 °C under 5 % CO_2 for 1.5 h and washed three times with phosphate-buffered saline (PBS) to remove extracellular bacteria. To measure bacterial internalization, 1 mL of sterile, distilled H_2O was added to wells to release intracellular bacteria from the host cells, and the numbers of CFU were determined by plating dilutions on BCYE agar. Fresh tissue culture medium (0.5 mL) was added to each of the remaining wells. At 24-h intervals, the intracellular and extracellular bacteria in each well were combined, and the total CFU were determined by plating the dilutions onto BCYE agar.

Gimenez-staining

The cells were stained with 1 mL carbol fuchsin mixed with 2.5 mL phosphate buffer (pH 7.45) for 2 min, and with 5% malachite green for 1 min, and then were washed gently with running water. The cells were allowed to dry on glass slides and then were observed.

Mice Survival Experiments

To measure the differences in the virulence of the *L. dumoffii* strains, 6-week-old male A/J mice (SLC, Shizuoka, Japan) were infected by intranasal inoculation with a 40 μL suspension of wild-type *L. dumoffii* (approximately 1.4×10^9 cells) or with *L. dumoffii* KL15 (approximately 1.1×10^9 cells). Mice were returned to their cages and monitored daily for death. All animal experiments were reviewed and approved by the Ethics Committee on Animal Experiment at the Faculty of Medical Sciences, Kyushu University. The experiments were carried out under the conditions stipulated in the Guideline for Animal Experiments of Kyushu University and The Law (No. 105) and Notification (No. 6) of the Government of Japan.

RESULTS

Isolation of Intracellular Growth Mutants

L. dumoffii Tex-KL was mutagenized with Tn903dIIlacZ as described previously[25,32]. Plasmid pLAW330, which contained Tn903dIIlacZ, was introduced into *L. dumoffii*, and 799 Km^r Cm^s strains of *L. dumoffii* Tex-KL with ß-galactosidase activity were isolated. The 799 mutants were individually infected into HeLa cells, and were visualized by Gimenez-staining for their ability to replicate in cells. One strain with weak intracellular replication was named KL15 and chosen for further analysis.

Identification of the Tn903dIIlacZ Insertion Site

We cloned the *Hin*dIII fragment containing the Tn903dIIlacZ insert and the flanking sequences from the mutant KL15. Using a primer located within Tn903dIIlacZ, we partially sequenced and analyzed the DNA to identify gene(s) responsible for intracellular multiplication. Sequence homology searches were performed using the GenBank database. The insertion was within a gene homologous to *Salmonella typhi traC* (85% nucleotide sequence identity), which has not been previously characterized in *L. dumoffii*; Tn903dIIlacZ was inserted after the 1613^{rd} bp of *traC*.

Isolation of a traC Mutant by Allelic Exchange

To demonstrate that the effect on human cell infection was not caused by presence Tn903dIIlacZ itself, we constructed an additional *traC* mutant (*traC*::mini-Tn5) by insertion of a Km^r cassette into the *traC* gene; this mutant was named KL16. Then, we completely digested the genomic DNAs of KL15, KL16 and KLc (the *L. dumoffii* Tex-KL plasmid cured strain) with *Hin*dIII and the performed a Southern blot using a *L. dumoffii traC* probe (Figure 1A). The results indicated that the Km^r cassette was correctly integrated into the mutant strain, KL16. We detected the *traC* gene was on the plasmid (pLD-TEX-KL) of *L. dumoffii* Tex-KL (Figure 1B), but not in DNA from the plasmid cured strain, KLc.

traC does not Affect Adhesion of L. dumoffii to A549 and HeLa Cells

First, adhesion of KL15 and KL16 to A549 and HeLa cells was examined (data not shown). The rates of bacterial adhesion to A549 and HeLa cells were not significantly different among the wild-type, KL15, and KL16 strains. Thus, the *traC* gene does not affect the adhesion of *L. dumoffii* Tex-KL to A549 and HeLa cells.

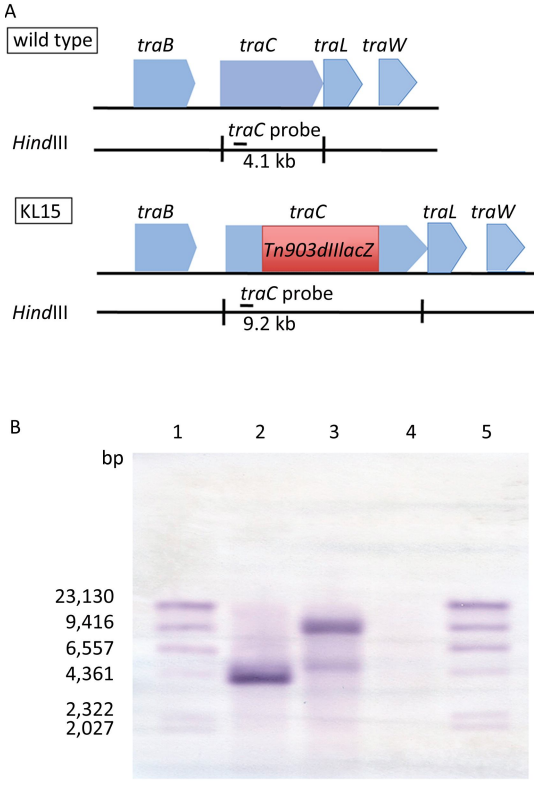

Figure 1. Confirmation of Tn903dIIlacZ insertion into *traC* by Southern hybridization. (A) Schematic drawing of the genomic organization of the *traC* cluster. The *Hind*III restriction site and probe location are indicated. (B) Southern hybridization analysis of the *L. dumoffii traC* gene in wild-type and in strains KL15 and KLc. Total DNA was digested with *Hind*III. Lanes 1 and 5, *Hind*III markers; Lane 2, wild-type strain (ATCC 33152); lane 3, *traC*::mini-Tn5 strain (KL16); lane 4, KLc strain. The DNA probe was the *traC* gene randomly labeled with digoxigenin-dUTP and the digoxigenin-dUTP hybrid DNA in the Southern membrane was detected using an enzyme immunoassay kit.

Invasion Ability of L. dumoffii Tex-KL Depends on traC

The ability of *L. dumoffii* Tex-KL to invade A549 and HeLa cells was examined. The invasion ability of KL15 into A549 cells was 90 % lower than that of the wild-type (Figure 2A), and it was 85 % lower in HeLa cells (Figure 2B). The invasion ability of KL16 was 90 % lower than that of the wild-type in A549 cells (Figure 2C) and 77 % lower in HeLa cells (Figure 2D). These results suggest that the *traC* gene product is involved in the invasion of *L. dumoffii* into epithelial cells.

Mutants in traC are Defective in Epithelial Cell Intracellular Growth

We examined the wild-type, KL15, and KL16 strains for their capacity to replicate within A549 and HeLa cells. Numbers of bacterial CFUs were determined over 3 d. Within epithelial cells, the wild-type strain increased approximately 1000 fold over the 3 d period, while there was a 100 fold increase in the number of intracellular KL15 over 3 d (Figure 3A, Figure 3C). The same proliferative defect

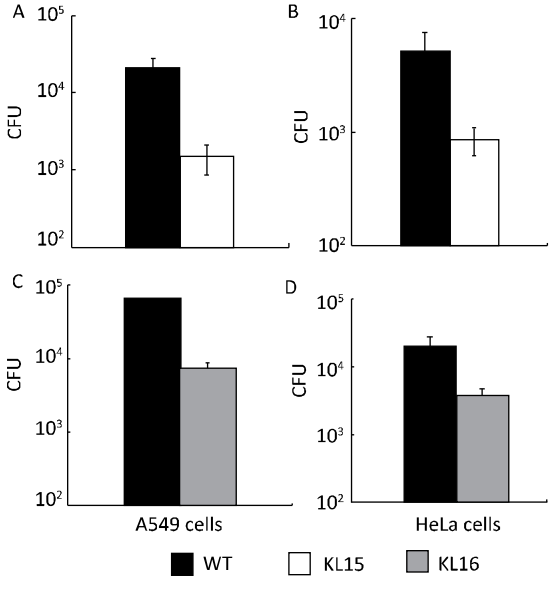

Figure 2. *L. dumoffii* Tex-KL invasion into A549 (A and C) and HeLa (B and D) cells. The formation of colonies (CFU/mL) was determined at the times indicated, in triplicate, in at least three independent experiments. Error bars indicate the standard deviations determined from samples taken from one experiment. The black, white and gray columns indicate wild-type (WT), KL15, and KL16, respectively.

was seen for KL16 in both A549 and HeLa cells (Figure 3B, Figure 3D).

Micrographs of Gimenez-stained HeLa Cells

We examined *L. dumoffii*-HeLa cell interactions by Gimenez staining. Despite the same multiplicity of infertion (MOI), microcolonies of wild-type *L. dumoffii* were detected intracellularly in most of the HeLa cells 3 d after *in vitro* phagocytosis, while KL15 was detected only in a few of the cells (Figure 4).

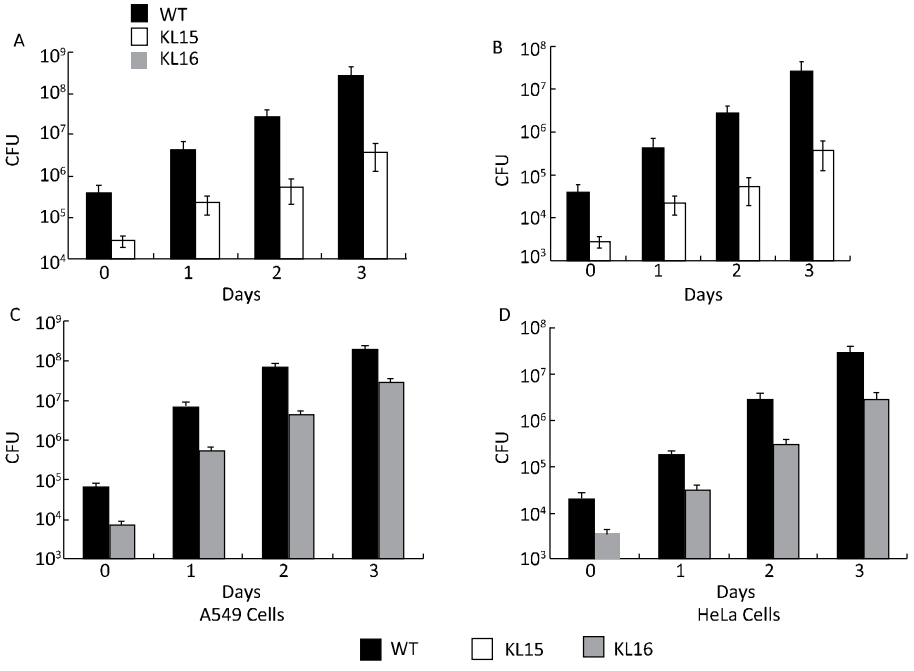

Figure 3. Intracellular growth of *L. dumoffii* Tex-KL within A549 (A and C) and HeLa (B and D) cells. The formation of colonies (CFU/mL) was determined at the times indicated, in triplicate, in at least three independent experiments. Error bars indicate the standard deviations determined from samples taken from one experiment. The black, white and gray columns indicate wild-type, KL15, and KL16, respectively.

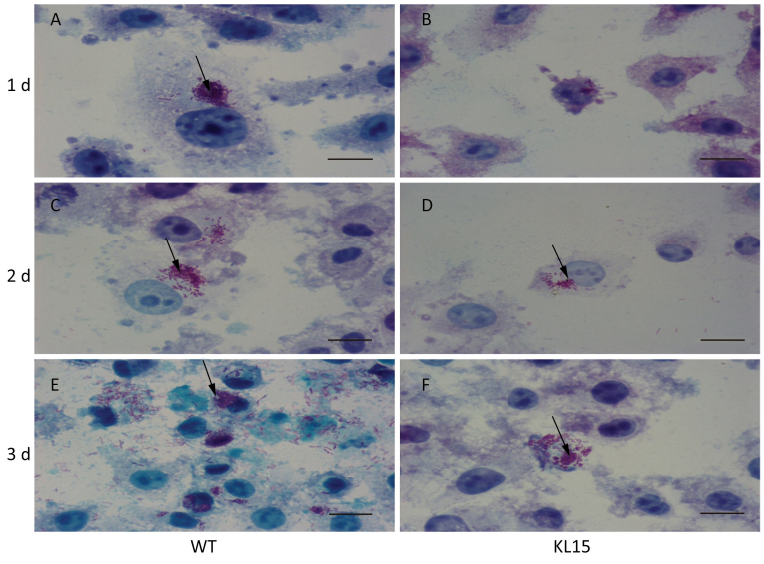

Figure 4. Micrographs of Gimenez-stained monolayers of HeLa cells from 1 d to 3 d after infection by *L. dumoffii* Tex-KL wild-type (A-C) or KL15 (D-F). Bar=25 μm. 100 × oil objective was used. WT, wild-type.

Role of traC in L. dumoffii Infection of A/J Mice

Wild-type and KL15 were tested for their ability to cause disease in mice after intranasal infection. The KL15-infected group survived significantly longer than the wild-type-infected group (P<0.05). After intranasal instillation of 1×10^9 bacterial cells in 40 µL, 90% of wild-type-infected mice died by d 6 of infection, while 80% of KL15-infected mice survived (Figure 5). Thus, the *traC* gene is involved in the virulence of *L. dumoffii* TEX-KL in the A/J mouse model.

Analysis of the traC-traD Operon by RT-PCR

To analyze the transcriptional unit from *traC* to *traD*, a series of primers were designed based on the *traC* cluster sequence to detect transcripts of *traB-traC*, *traC-orf4*, and *traC-traD* (Table 2; Figure 6A). RT-PCR was performed using total RNA extracted from bacteria grown in BYE medium to late exponential phase. cDNA, which was synthesized by reverse transcription from the total RNA, was used as the template in subsequent PCR. Transcripts for *traB-traC* (564 bp) and *traC-orf4* (13,703 bp) were not detected (Figure 6B, Figure 6C). Transcripts for *traC-traD* (10,746 bp) were detected (Figure 6D). These results clearly indicated that the genes from *traC* to *traD* were transcribed polycistronically (as an operon).

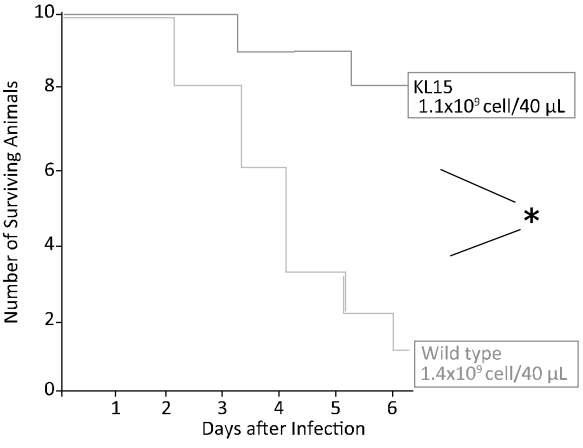

Figure 5. The survival rate of mice infected intranasally with *L. dumoffii* Tex-KL and KL15 at dose of approximately 1×10^9 bacteria in 40 µL. Each group consisted of 10 mice. *Indicates a significant difference (P<0.05).

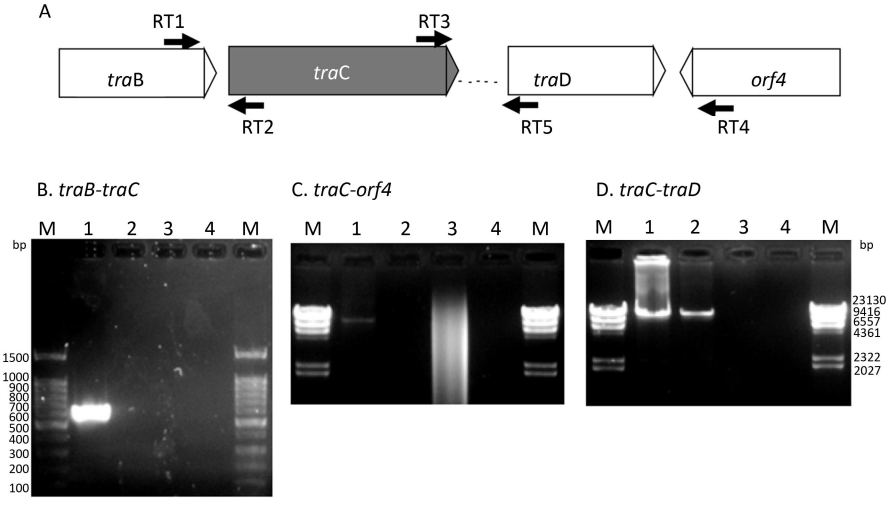

Figure 6. RT-PCR results of *traC-traD* operon analysis. (A) Primer design based on wild-type sequence. (B) Transcription of the *traB-traC* region in wild-type *L. dumoffii* (using primers RT1 and RT2-see Table 2). M, 100 bp DNA marker; lane 1, RT-PCR product and genomic DNA from wild-type as template (positive control); lane 2, the same as lane 1, but with cDNA from wild-type as the template; lane 3, the same as lane 2, but with total RNA from wild-type as template (negative control); lane 4, the same as lane 3, but with distilled water as template (blank). (C) Transcription of the *traC-orf4* region in wild-type *L. dumoffii* (using primers RT3 and RT4). M, λ/*Hin*dIII DNA ladder; Lanes 1-4, the same as the corresponding lanes in (B). (D) Demonstration of the *traC-traD* gene transcript (using primers RT3 and RT5). M, λ/*Hin*dIII DNA ladder; Lanes 1-4, the same as the corresponding lanes in (C) serving as references.

DISCUSSION

In this study, a *L. dumoffii* mutant defective in intracellular growth was shown to have a transposon insertion in a gene with nucleotide sequence similarity to *S. typhi traC* (85%). We found that when *traC* was disrupted, *L. dumoffii* Tex-KL invasion and intracellular growth were impaired in human epithelial cells. When mice were infected by intranasal inoculation with a *traC*-disrupted mutant, their survival significantly increased when compared to mice infected with the wild-type strain. Genes from *traC* to *traD* were transcribed as one unit (i.e. an operon).

In a previous study, Ohnishi et al. used the mutants obtained from transposon mutagenesis to act on J774 macrophages, A549 human alveolar epithelial cells, and on Acanthamoeba culbertsoni, and indicated that DjlA is required for intracellular growth, organelle trafficking, and resistance to environmental stress[33]. Here we determined the function of traC-traD by using mutants that were obtained during the same series of experiments using transposon mutagenesis. The gene products encoded by *traC-traD* may have a role in cellular invasion by the *L. dumoffii* strain Tex-KL. TraC is one of the proteins encoded by the F transfer region of the F conjugative plasmid, which is required for the assembly of F pilin into the mature F pilus structure and for conjugal DNA transmission[34-35]. F pili are filamentous appendages that help to establish physical contact between donor and recipient cells during conjugation. Recent research suggests that TraC localizes at the cell exterior, e.g., as a pilus component, allowing transfer of the F plasmid to the receptor strain and incorporation into its plasmid transfer machinery[36]. The different Tra proteins likely have specialized functions in assembly and/or stabilization of the membrane-bound plasmid transfer complex and conjugative pili. TraC is partially functional in a well-defined heterologous system, indicating a role in pilus assembly[37]. The pilus of bacteria is related to virulence. We propose that the *traC-traD* operon structure encodes a pilus homolog. If the pilus genes are disrupted, the virulence of the strain may be weakened. It has been reported that the pilus structure of *Salmonella typhi* plays an essential role in invasion into epithelial cells[38].

Type IV secretion systems (T4SS) of gram-negative bacteria are mainly involved in the spread of plasmids. T4SS constitute complex conjugation machineries composed of a pilus and a mating channel through which the DNA transfer intermediate is translocated. The T4SS genes involved in these kinds of processes are generally carried by conjugative plasmids, such as RP4 and pKM101[39]. Over recent years, the adaptation in some T4SS bacteria for delivering virulence factors into eukaryotic cells has been described[40-41]. These T4SS are divided into two subgroups according to their similarities to the *Agrobacterium tumefaciens* VirB system (type IVA) or to the *L. pneumophila dot/icm* system (type IVB). It is well established that some components of T4SS involved in conjugation share structural similarity, display considerable sequence homology, and, consequently, have identical protein functions[40,42]. For instance, *traH*, *traI*, *traJ*, and *traK* from the *traHIJKCLMN* operon, which are found on the plasmid R64[43-44], code for proteins similar to those encoded by the *dot/icm* genes of the *L. pneumophila* type IVB secretion system. In our previous study, we found that the *dot/icm* secretion systems of *L. dumoffii* and *L. pneumophila* both contain 26 genes[45].

The *traC-traD* operon does not appear to play a direct role in invasion and intracellular trafficking, but it might contribute to the folding or transportation of proteins. The Dot/Icm proteins, for example, play an important role in invasion and intracellular growth. Most of the Dot/Icm proteins are located in the bacterial membrane and may associate to form the type IV secretion apparatus. Our data suggest that the *traC-traD* operon functions similarly to the type IVB secretion system to transfer materials, and may play an important role during invasion and intracellular growth in epithelial cells. The specific substrates with which the *traC-traD* operon proteins interact need to be identified in order to understand the intracellular survival mechanism of *Legionella* species. By comparing gene sequences, we found that *L. dumoffii* Tex-KL has a more complete transfer region than *L. pneumophila*. Between *traC* and *traD*, *L. dumoffii* Tex-KL contains three genes that are absent from the corresponding region of *L. pneumophila* (*trbC*, *orf3 traD*, etc.). We propose that, because of the lack of these genes, the invasiveness of *L. pneumophila* is lower than that of *L. dumoffii* Tex-KL. To analyze *traC* function in detail, we attempted to recover the invasion ability by introducing a plasmid that constitutively expressed *traC* into KL16 (*traC*::Km^r); however, invasion ability was not recovered. Because *traC* is the first gene of the

operon, *traC* disruption may result in decreased expression of the downstream genes. We hypothesize that the decrease in invasiveness of the mutant strains is not directly caused by mutation of *traC*, but through a polar effect on the *traC-traD* operon.

In conclusion, we demonstrate the role of the *L. dumoffii* Tex-KL *traC-traD* operon during invasion of and intracellular growth in epithelial cells. The precise mechanism of *traC-traD* involvement remains to be determined.

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