Structural and Transcriptional Analysis of Gene Clusters for a Type IV Secretion System in *Orientia tsutsugamushi*

Jeong - Eun Goo

Department of Medicine
Graduate School
Cheju National University

June, 2006

Structural and Transcriptional Analysis of Gene Clusters for a Type IV Secretion System in *Orientia tsutsugamushi*

Jeong - Eun Goo

Department of Medicine
Graduate School
Cheju National University

June, 2006

*Orientia tsutsugamushi*에서 Type IV Secretion System 유전자군의 구조와 전사 분석

지도교수 고 영 상

구 정 은

이 논문을 의학 석사학위 논문으로 제출함

2006 년 6 월

구정은의 의학 석사학위 논문을 인준함

심사위원장 _____ 印

위 원 _____ 印

위 원 _____ 印

제주대학교 대학원

2006 년 6 월

Structural and Transcriptional Analysis of Gene Clusters for a Type IV Secretion System in *Orientia tsutsugamushi*

Jeong - Eun Goo

(Supervised by Professor Young - Sang Koh)

A thesis submitted in partial fulfillment of
the requirement for the degree of
Master of Science
in medicine

Date Approved:

Department of Medicine
Graduate School
Cheju National University
June, 2006

ABSTRACT

Orientia tsutsugamushi, causative agent of scrub typhus, is an obligate intracellular bacterium. *O. tsutsugamushi* usually infects vascular endothelial cells, macrophages, polymorphonuclear leukocytes (PMN), and lymphocytes in patients or experimental animals. *O. tsutsugamushi* escapes from the phagosome into the cytoplasm, and then freely replicates in the host cytoplasm. Type IV secretion systems are ancestrally related to the bacterial conjugal system and are thought to function to deliver effector molecules produced by parasitic or symbiotic bacteria into eukaryotic target cells. Genomic sequence data indicate that 11 genes (*virB3, B4, B6, B8, B9, B10, B11,* and *virD4*) encoding products that are similar to components of the bacterial type IV secretion system (T4SS) are located on separate loci of the *Orientia* genome. Five genes (*virB8, B9, B10, B11*, and *virD4*) were polycistronically transcribed. Several *vir* genes (*virB4, virB10* and *virD4*) for T4SS were expressed in *O. tsutsugamushi* during host infection in cell culture (L929 fibroblast and J774A.1 macrophage) and murine host (C3H/HeN). Transcripts for *virB4* and *virB10* gene were temporally expressed. In contrast, *ts1* gene for 56-kDa outer membrane protein was constitutively expressed during infection. Temporal regulation of bacterial *vir* gene expression may be associated with host type I interferon response.

Key word : *Orientia tsutsugamushi*, type IV secretion system,
virB/D genes, temporal regulation, host cytokine response

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

Ⅰ. INTRODUCTION

1. *Orientia tsutsugamushi*

Orientia tsutsugamushi, an obligate intracellular bacterium, is the causative agent of scrub typhus (tsutsugamushi disease). This bacterium is transmitted by the bite of the larval stage of certain trombiculid mites of chiggers. The disease in characterized by fever, rash, eschar, pneumonitis, meningitis, and disseminated intravascular coagulation, often leading to multiple organ failure (Chi *et al.,* 1997; Watt *et al.,* 1994). *O. tsutsugamushi* usually infects endothelial cells, macrophages, polymorphonuclear leukocytes (PMN), and lymphocytes in patients or in experimental animals (Murata *et al.*, 1985; Ng *et al.*, 1985; Rikihisa *et al.*, 1979; S. *et al.*, 1996; D. S. Walsh *et al.*, 2001; Moron *et al.*, 2001).

After attachment to the host cell plasma membrane, *O. tsutsugamushi* induces its own uptake through a process called induced phagocytosis in case of nonprofessional phagocyte (Rikihisa *et al.*, 1979; Urakami *et al.*, 1983; Urakami *et al.*, 1984). Subsequently, *O. tsutsugamushi* escapes from the phagosome into the cytoplasm where the bacterium can replicate (Rikihisa *et al.*, 1979; Kawamura *et al.*, 1995). *O. tsutsugamushi* moves along the microtubules to the microtubules organizing center, replicating in the perinuclear region (Kim *et al.*, 2001). The virulence factors related to the phagosomal membrane lysis have not been identified. As *O. tsutsugamushi* multiple in the cytoplasm of host cells, some organisms move to the cell surface, acquire a host - membrane coat derived from the host cell's plasma membrane, and bud from the cell surface. Membrane - enclosed bacterium appeared to be phagocytozed by other cells.

2. Type IV Secretion System

Pathogenic bacteria employ many devices to subvert the defenses of host cells. Among these, some secrete virulence factors to the host cell environment, more craftily, directly into the interior of the host cell. A pathogenic bacterium is evolved specialized multi-component secretion systems for this task. Recently, type IV secretion systems (T4SS) have been shown to be specifically required for virulence in many human pathogens including *Bartonella henselae* (cat-scratch disease), *Helicobacter pylori* (gastric ulcers) (Covacci *et al.* 1997), *Legionella pneumophila* (Legionnaire's disease) (Vogel *et al.* 1998), *Ehrlichia chaffeensis*, *Anaplasma phagocytophila* (Norio *et al.* 2002), *Wolbachia* spp.(Shinji *et al.* 2000), and *Rickettsia prowazekii* (epidemic typhus) (Andersson *et al.* 1998). The T4SS is ancestrally related to the bacterial conjugal system and is thought to deliver effector macromolecules produced by parasitic or symbiotic bacteria into eukaryotic target cells.

T4SS are promiscuous macromolecular transporters of Gram-negative bacteria that mediate intercellular transfer of various substrates between bacteria or bacteria and eukaryotic cells (Winans *et al.* 1996; Censini *et al.* 1996; Vogel *et al.* 1998). Each of these systems exports distinct protein or protein-DNA substrates to affect a myriad of changes in host cell physiology during infection. This subgroup of T4SS is exemplified by the *Agrobacterium tumefaciens* (Christie *et al.* 1997) VirB system involved in delivering the oncogenic T-DNA into plant cells, the *Helicobacter pylori* Cag system mediating transfer of the CagA protein into infected gastric epithelial cells (Tanaka *et al.* 2003), and the *Bordetella pertussis* Ptl system mediating export of the multicomponent pertussis toxin. In contrast to the pathogens described above, *L. pneumophila* is

facultative intracellular pathogens, the infection cycles of which depend on type IV secretion after internalization into the host cell. During infection, *L. pneumophila* uses this transfer system to inject effector proteins into the phagosome, to control biogenesis of the replicative vacuole and to modulate the activity of host factors involved in vesicle traffic (Conover *et al*. 2003; Nagal *et al.* 2001; Eric *et al*. 2003).

The T4SS have been classified into two groups. One group consists of genes orthologs to the *virB* and *virD* genes of *A. tumefaciens*. The T4SS in most bacteria, including *L. pneumophila lvh*, belong to this group. Another group contains the *dot - icm* genes of *L. pneumophila* and *coIIb* genes of *Shigella flexneri.* The Lvh system of *L. pneumophila* functions as a DNA conjugation system, while the *dot - icm* system is required for the bacterial virulence. In former group, VirB/D T4SS of the *A. tumefaciens,* the subcellular locations and topologies of the VirB mating pore formation (Mpf) proteins have been predicted (Lavigne *et al.*, 2006). For this, the VirB proteins can be divided into three classes (Eric *et al.*, 2003). First, the putative channel components include the inner - membrane proteins VirB6, VirB8 and VirB10 (Judd *et al.*, 2005; Kumar *et al.,* 2000), and the outer - membrane proteins VirB3, VirB7, VirB9(Suleyman *et al.*, 2003). Second, energetic components include two ATPases, VirB4 and VirB11, probably provide energy to drive substrate transfer. Finally, the pilus component, VirB2, assembles as the T - pilus in association with VirB5 and the VirB7 lipoprotein (Jones *et al.*, 1996). The VirD4 protein act as coupling protein (CP), recruits DNA substrates to the T4SS machine, then, through the VirB10 contact, the CP coordinates passage of the substrates through the Mpf protein channel (Cascales, *et al*, 2005; Liosa *et al*, 2003; Peter, 2004.). With the increasing availability of complete bacterial genome

sequences, the number of putative T4SS family members is expanding rapidly, suggesting that macromolecular transfer by these systems is a wide - spread phenomenon in nature.

3. Type IV Secretion System in *Orientia tsutsugamushi*

The virulence factors related to the phagosomal membrane lysis have not been identified. In addition, I don't know whether *O. tsutsugamushi* control host factors for its survival. If the *O. tsutsugamushi* change host cell physiology during infection or regulate host signal just like *L. pneumophila,* perhaps *O. tsutsugamushi* use T4SS to transfer effector molecules into host cytoplasm.

The genome sequences of *O. tsutsugamushi* are almost identified, that allow studying of T4SS for *O. tsutsugamushi*. In the present study, I characterized 15 *vir* genes (1 *virB3*, 2 *virB4*, 5 *virB6*, 2 *virB8*, 2 *virB9*, 1 *virB10*, 1 *virB11*, and 1 *virD4*) in *O. tsutsugamushi* and compared those with the *vir* genes of other bacteria. In addition, transcriptional analysis of these genes was performed in cell culture and experimentally infected mice. This is the first report of the type IV secretion system in the *O. tsutsugamushi.*

Ⅱ. MATERIALS AND METHODS

1. Orientiae

The prototype strain, *O. tsutsugamushi* Boryong was propagated in monolayers of L-929 cells. L929 cells proliferate in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, NY, USA), and antibiotic-antimycotic. The cells were infected by orientiae inoculum, and infected cells were incubated for 2 hr in a humidified 5% CO_2 atmosphere at 37℃. When more than 90% of the cells were infected, as determined by an indirect immunofluorescent-antibody technique (Chang *et al.*, 1990), cells were collected, centrifuged at 9,940 × g for 20 min. Pellet was diluted with media, then homogenized with a glass Dounce homogenizer (Wheaton Inc., NJ, USA), and centrifuged at 500 × g for 5 min. The supernatant was recovered and stored in liquid nitrogen until use. The infectivity titer of the inoculums was determined with modification that five-fold serially diluted oriential sample were inoculated onto L929 cell layers on 24-well tissue culture plates. After 3 days of incubation at 34℃, the cells were collected, fixed, and indirect immunofluorescent antibody assay was performed. The ratio of infected cells to the counted number of cells was determined microscopically, and infected-cell counting units (ICU) of the oriential sample were calculated as follows (Tamura *et al.,* 1981): ICU=(total number of cells used in infection) × (percentage of infected cells) × (dilution rate of the orientiae suspension)/100. A total of 2×10^7 to 1.2×10^8 ICU/ml of *O. tsutsugamushi* was used.

2. Mice

Female C3H/HeN mice that are susceptible to *O. tsutsugamushi*, purchased from SLC Inc. (Japan) were kept in the animal facility located in the Cheju National University College of medicine. Utmost precautions were taken so the mice remained free from infection from environmental pathogens. Animal procedures were performed according to the guidelines of the Laboratory Animal Care Committee of Cheju National University College of Medicine. All mice used were 7 to 10 weeks old. The *O. tsutsugamushi* inoculums (5×10^5 ICU /mouse) were injected into the peritoneal cavity of C3H/HeN mice. After 11 days, when bacterium replication is maximum, the *O. tsutsugamushi*-infected mice were sacrificed by cervical dislocation. The mouse was pined to the cutting board, skin was cut to expose the entire peritoneal cavity. With the bevel side of the needle up, the needle was inserted along the mid-line and injected PBS into the peritoneal cavity. And then, the PBS was aspirated into the syringe slowly. The collected cell solution was centrifuged at 6,000 × g, 4℃, for 10 min. The pellets were stored at –70℃ until use.

3. Indirect immunofluorescent-antibody (IFA) staining

Slide was fixed with acetone. The slide was incubated with diluted primary antibody (human), and then incubated for 30 min at 37°C. Slide was washed 3 times in 1 × PBS for 5min by shaking. After the slide was dried, it was stained with FITC-labeled antibody to human IgG, and incubated in the dark at 37°C for 30 min. The slide was washed 3 times with PBS for 5 min by shaking. The dried slide was incubated in Evans Blue (Sigma, MO, USA) for 1 min, and then washed two times in PBS for 1min by shaking. Finally, the slide was mounted each cover slip using Mounting Media (90% glycerol,

10% PBS).

4. Synchronous infection

J774A.1 cells were seeded onto 6-well plates. For synchronous infections, J774A.1 cells that infected with *O. tsutsugamushi* (50 ICU/cell) were centrifuged at 500 × g for 5 min immediately. The cells were incubated for 2 hr, then media were exchanged. Cells and culture supernatant were recovered at 0, 1, 3, 6, 12, 24, and 48 hr after infection of *O. tsutsugamushi*. The medium was stored at -70℃ after centrifugation. For removal of any bacterial inoculum, cells were subjected to PBS washing, and collected into centrifuge tubes. Tubes were centrifuged at 400 × g for 3 min. The pellet was frozen in liquid nitrogen, then stored at -70℃ until use. The same volume of *O. tsutsugamushi* inoculums was centrifuged at 6,000 × g for 3 min, and then pellet was frozen as above.

5. Asynchronous infection

J774A.1 cells were seeded onto 6-well plates. The cells were infected with *O. tsutsugamushi* by shaking. Infected cells were harvested at 0, 1, 3, 6, 12, 24, and 48 hr after infection with *O. tsutsugamushi*. Tubes were centrifuged at 400 × g for 3 min. The pellet was frozen in liquid nitrogen, then stored at -70℃ until use for RNA preparation.

6. Reverse transcription (RT)-PCR

Total RNA was prepared from *O. tsutsugamushi*-infected cells by using SV Total RNA Isolation System (Promega, WI, USA). After RQ1 DNase (Promega, WI, USA) treatment, RNA was reverse transcribed using Reverse Transcription System (Promega, WI, USA) with random primers

(for bacterial RNA) or Oligo dT primers (for eukaryotic RNA) at 42℃ for 50 min. The cDNA were subjected to PCR amplification in a 25 ㎕ reaction mixture (50 mM KCl, 10 mM Tris - HCl, 0.1% Triton X - 100, 1.9 mM $MgCl_2$, 200 ㎛M dNTP mixture, 1 ㎛M of each primer, and 0.625 U of *Taq* DNA polymerase). The sequences of the primers and amplicon sizes are shown in Table 1 & 2. PCR conditions were 35 to 40 cycles consisting of 1 min of denaturation at 95℃, 1 min of annealing at each temperature (Table 1 & 2), and 1 min of extension at 72℃. The PCR products (10 ㎕ samples) were electrophoresed in a 1.5% agarose gel containing 0.5 ㎍ of ethidium bromide per ml. A 100 - bp DNA ladder (Promega, WI, USA) was used as molecular size markers. The band intensities shown in autoradiography were digitized by scanning the images and analyzed using Gel Doc 2000 Gel Documentation System and Quantity One software (Bio - Rad, CA, USA).

7. Sequence analysis

A database search was carried out with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/), (Altschul *et al.* 1997). Multiple alignments were done using the CLUSTAL V method in the DNASTAR program (DNASTAR, WI, USA). Phylogenetic analysis was performed with the DNASTAR program (DNASTAR, WI, USA). GenBank accession numbers of published Vir protein sequences used for the phylogenetic analysis are as follows: *R. prowazekii,* AJ235269; *R. conorii*, AE006914; *R. felis*, CP000053; *R. typhi*, AE017197; *Wolbachia* sp. *wMel*, AE017196; *E. chaffeensis*, CP000236; *An. phagocytophila*, CP000235; *L. pneumophila*, CR628337; *Bo. pertussis*, BX470249; *H. pylori*, AE000511; *Ag. tumefaciens*, AE008688; *Ba. henselae*, BX897699.

Table 1. The sequence of primers for bacterial RNA and the expected size of the RT-PCR products.

Target	Amplicon size(bp)		Primer sequence	Annealing Temp.
virB4	501	F	GCTCTAGACATTAGATATGGCGACCAGT	50℃
		R	GCTCTAGATGATAAAAATCTCTGCACCTC	
virB8	419	F	GCTCTAGAGAAAGTTGAACAGCAAAAGC	〃
		R	GCTCTAGAGCGTTAGCGCAATATTTTAT	
virB8 - virB9	556	F	GCTCTAGAGCGTTAGCGCAATATTTTAT	〃
		R	GCTCTAGAACTTGATTGAAATCCAGCAT	
virB9	284	F	GCTCTAGAAGTATTGCATGCTGGATTTC	〃
		R	GCTCTAGACTTTATTGCACGCTTTCTTT	
virB9 - virB10	512	F	GCTCTAGATAGAAGTAGGGCGTGAACAT	〃
		R	GCTCTAGACTTTTGGCAGTTTATTGGAC	
virB10 (1)	457	F	GCTCTAGAAGTCCAATAAACTGCCAAAA	〃
		R	GCTCTAGAATCATCATTCTGCCATAAGC	
virB10 (2)	585	F	GCTCTAGAATATTTATTGGGAAGGGGAA	〃
		R	GCTCTAGAGTCTCAATTTCCATGCATTT	
virB10 - virB11	567	F	GCTCTAGATAGCGTTACAGCAACAGATG	〃
		R	GCTCTAGACGGTTAATCATCACCTCATT	
virB11 (1)	485	F	GCTCTAGAAATGAGGTGATGATTAACCG	〃
		R	GCTCTAGATGCGGAACTTCTAATAAAGC	
virB11 (2)	492	F	GCTCTAGAAGCATGCAATAAAGTCCAAA	〃
		R	GCTCTAGAATCTAATTCCACGATCACCA	
virB11 - virD4	390	F	GCTCTAGATGGTGATCGTGGAATTAGAT	〃
		R	GCTCTAGATGGTTGCCAACTCTTAATTT	
virD4 (1)	427	F	GCTCTAGAAAATTAAGAGTTGGCAACCA	〃
		R	GCTCTAGAAGGTTAGCAATTTTCTGCAC	
virD4 (2)	513	F	GCTCTAGAAGGTAAAGGGGTAGGTTTTG	〃
		R	GCTCTAGATCAAGCCCAGAATTCATAGT	
virD4 (3)	542	F	GCTCTAGAAAACATTTGGTGAAGTGGTC	〃
		R	GCTCTAGATGAATTCATTCCTGCTTCTT	
virD4 (4)	521	F	GCTCTAGAAATGGAACAATTTAAAACCG	〃
		R	GCTCTAGACTTATTTTTGATTATTATCTACGTT	
rpoB	247	F	CTCCAGAGGAGAAGTTATTAAGAG	〃
		R	AGCTCCTTTACCCTAACAATAGTA	
ts1	519	F	CCAGGATTTAGAGCAGAG	42℃
		R	CGCTAGGTTTATTAGCAT	

Table 2. The sequence of primers for eukaryotic RNA and the expected size of the RT-PCR products.

Target	Amplicon size(bp)		sequences	Annealing temperature
MIP - 1α	357	F	GGTCTCCACCACTGCCCTTGC	55℃
		R	GGTGGCAGGAATGTTCGGCTC	
MIP - 2	536	F	AGTTTGCCTTGACCCTGAAGCC	〃
		R	CCATGAAAGCCATCCGACTGCA	
MCP - 1	582	F	TCTCTTCCTCCACCACCATGCAG	〃
		R	GGAAAATGGATCCACACCTTGC	
RANTES	215	F	CCTCACCATCATCCTCACTGCA	〃
		R	TCTTCTCTGGGTTGGCACACAC	
IP - 10	436	F	CCTATCCTGCCCACGTGTTGAG	〃
		R	GGCGTCGCACCTCCACATAGCT	
TNF - α	340	F	GCGACGTGGAACTGGCAGAAG	〃
		R	TCCATGCCGTTGGCCAGGAGG	
IFN - β	296	F	GCACTGGGTGGAATGAGACTATTG	〃
		R	TTCTGAGGCATCAACTGACAGGTC	
β - actin	349	F	TGGAATCCTGTGGGATCCATGAAAC	〃
		R	TAAAACGCAGCTCAGTAACAGTCCG	

Ⅲ. RESULT

1. Gene organization of *virB* and *virD* clusters in genome of *O. tsutsugamushi*

O. tsutsugamushi has 15 *vir* genes for a Type IV secretion system (Figure 1). Of 15 *vir* ORFs, eleven are arranged into two separate loci. One contains *virB3*, *virB4*, *virB6*, and *virB6*-like coding sequences (CDSs) forming a 15 kb gene cluster (OTBSv289_383–387). The other contains *virB8*, *virB9*, *virB10*, *virB11*, and *virD4* that form part of a 5.4 kb cluster (OTBSv289_570–573). The gene organizations of the *virB* and *virD* clusters of *O. tsutsugamushi* were similar to the corresponding clusters of the T4SS in other Gram-negative bacteria. Through the genome sequences of *O. tsutsugamushi,* the T4SS genome sequences were searched to encoding products that are similar to components of other bacterial T4SS (Appendix 1). In addition to the two major *vir* clusters, a paralog of *virB4* (OTBSv289_1278), *virB8* (OTBSv289_812) and *virB9* (OTBSv289_811) are also present in the *Orientia* genome.

2. Protein encoded by *virB* and *virD* genes of *O. tsutsugamushi*

Comparison with the database available for Gram-negative bacteria showed that the VirB and VirD orthologous proteins of *O. tsutsugamushi* had highest amino acid identities with those of *Rickettsia* spp.(Table 3). Between *O. tsutsugamushi* and *Rickettsia* spp., three orthologs of VirB4, Vir B11, and VirD4 are more conserved (66.3 to 72.4%) than the remaining orthologs. To analyze the relationship between VirB and VirD orthologs from *O. tsutsugamushi* and several other bacteria, I constructed

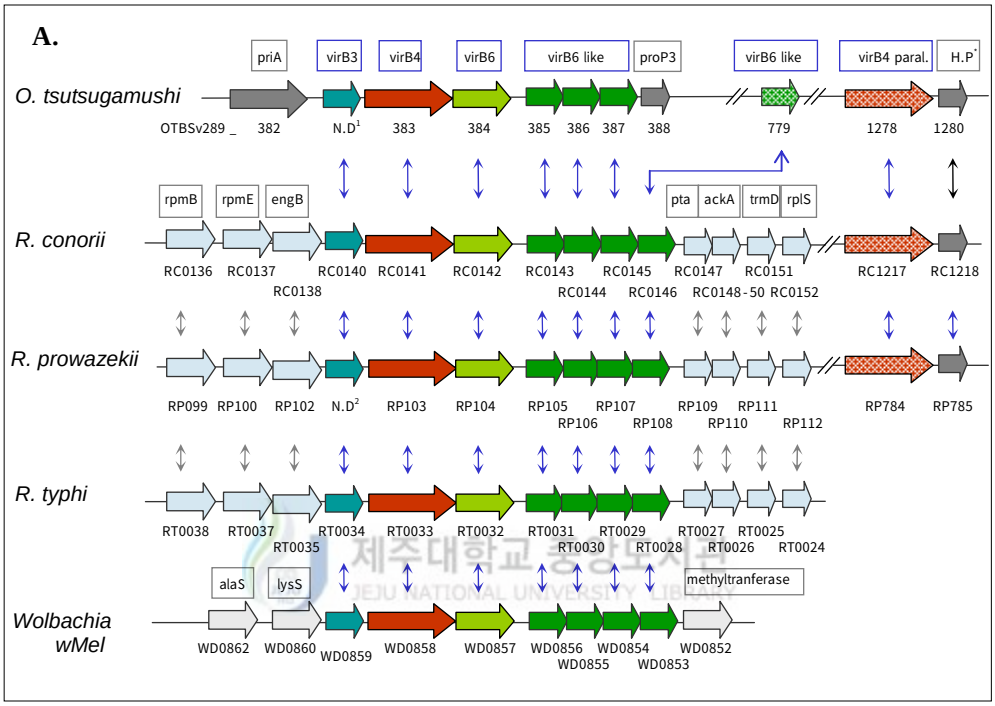


Figure 1. Genomic organization of gene clusters for a Type Ⅳ Secretion System in *O. tsutsugamushi*. *Vir* orthologs of selected gram-negative bacteria were compared with those of *O. tsutsugamushi*. **(A)** *virB3*, *-B4* and *-B6* cluster. N.D[1], not determined in the genome sequencing database (Genotech). The *virB3* gene of *O. tsutsugamushi* was found between OTBSv289_382 and OTBSv289_383 from the genome sequence; N.D[2], The *virB3* gene of *R. prowazekii* was found between RP102 and RP103 from the genome sequence; H.P*, hypothetical protein.

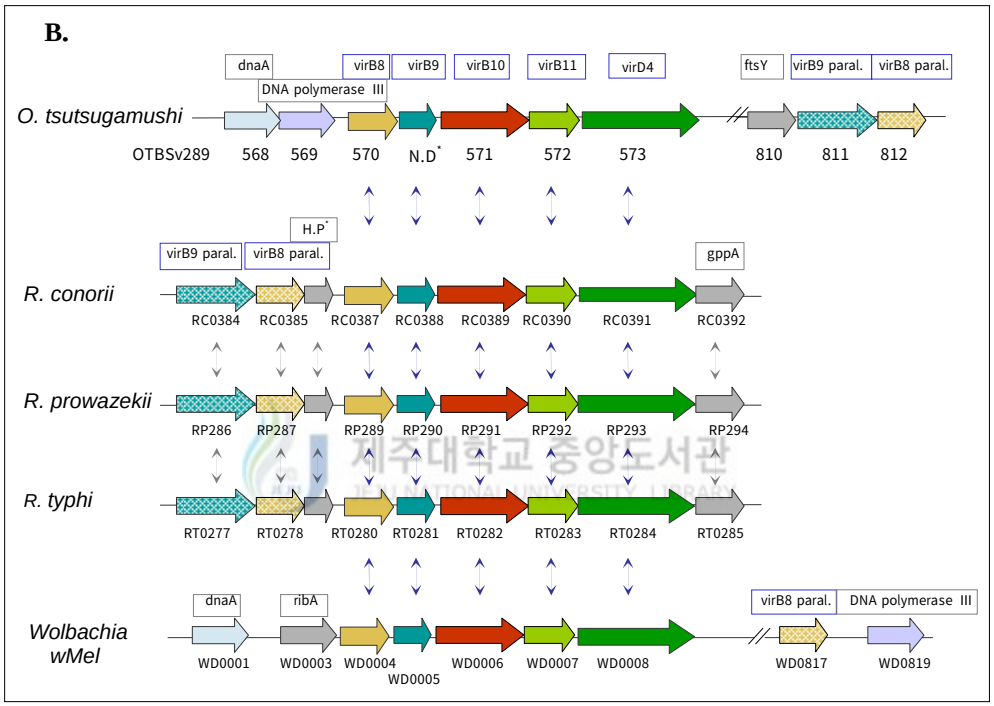


Figure 1 continued. (B) *virB8, -B9, -B10, -B11* and *–D4* cluster. N.D*, not determined *virB9* gene of *O. tsutsugamushi* was found between OTBSv289_570 and OTBSv289_571 from the genome sequence; H.P*, hypothetical protein.

Table 3. Identities of amino acid (aa) sequences of *O. tsutsugamushi vir* gene products to those of other bacteria
% aa identity (gene, aa no.)

protein	*O.tsutsugamushi*	*R. conorii*	*R. prowazekii*	*R. typhi*	*R. felis*	*Wolbachia wMel.*	*An. phagocytophila*	*E. chaffeensis*
VirB3	100 (N.D[1], 102)	47.4 (RC0140, 95)	46.3 (N.D[3] , 95)	47.4 (RT0034, 98)	47.4 (RF0087, 95)	38.8 (WD0859, 98)	40.2 (APH_0372, 97)	40.2 (ECH_0494, 97)
VirB4	100 (OTBSv289_383, 805)	66.3 (RC0141, 805)	66.5 (RP103, 805)	66.4 (RT0033, 805)	66.7 (RF0088, 805)	58.6 (WD0858, 801)	56.7 (APH_0373, 801)	58 (ECH_0495, 800)
	20.6 (OTBSv289_1278,817)	18.4 (RC1217, 805)	20.1 (RP784, 810)	20.1 (RT0771, 810)	18.3 (RF1250, 810)	22.8 (WD1173, 788)	18.7 (APH_1129, 777)	20.2 (ECH_1041, 791)
VirB6	100 (OTBSv289_384, 815)	29.9 (RC0142, 993)	29.8 (RP104, 1124)	29.6 (RT0032, 1135)	30.6 (RF0089, 1138)	12.1 (WD0857, 851)	12.1 (APH_0374, 846)	12 (ECH_0496, 826)
VirB6 like	100 (OTBSv289_385, 1290)	28.3 (RC0143, 661)	27.4 (RP105, 672)	27.3 (RT0031, 674)	16.7 (RF0090, 672)	14.1 (WD0856, 795)	13.7 (APH_0375, 928)	12 (ECH_0497, 922)
VirB6 like	100 (OTBSv289_386, 1091)	27.4 (RC0144, 966)	28.5 (RP106, 971)	27.8 (RT0030, 971)	11.3 (RF0091, 977)	15.6 (WD0855, 992)	14.2 (APH_0376, 1477)	14.5 (ECH_0498, 1468)
VirB6 like	100 (OTBSv289_387, 855)	17.5 (RC0145, 891)	17.5 (RP107, 888)	17.1 (RT0029, 886)	11.2 (RF0092, 890)	10.6 (WD0854, 1242)	11.6 (APH_0377, 2360)	11.6 (ECH_0499, 2768
VirB6 like	100 (OTBSv289_779, 738)	33.2 (RC0146, 1153)	36.7 (RP108, 1155)	37.3 (RT0028, 1154)	11.0 (RF0093, 1155)	13.3 (WD0853, 210)		
VirB8	100 (OTBSv289_570, 226)	42.0 (RC0387, 243)	40.7 (RP289, 202)	40.3 (RT0280, 243)	42.0 (RF0465, 243)	30.1 (WD0004, 226)	28.3 (APH_1406, 238)	27.4 (ECH_0044, 237)
	13.3 (OTBSv289_812, 233)	13.7 (RC0385, 232)	13.7 (RP287, 247)	13.7 (RT0278, 232)	15.0 (RF0463, 232)	13.2 (WD0817, 220)		13.3 (ECH_0579, 229)
VirB9	100 (N.D[2] , 145)	55.9 (RC0388, 157)	56.6 (RP290, 157)	56.6 (RT0281, 158)	56.6 (RF0466, 158)	28.3 (WD0005, 264)	27.6 (APH_1405, 281)	31 (ECH_0043, 273)
	29.0 (OTBSv289_811, 253)	33.1 (RC0384, 250)	31.7 (RP286, 250)	33.1 (RT0277, 250)	33.1 (RF0462, 250)		29.7 (APH_0081, 271)	31 (ECH_0219, 270)
VirB10	100 (OTBSv289_571, 509)	29.9 (RC0389, 482)	28.0 (RP291, 483)	28.8 (RT0282, 483)	29.7 (RF0467, 481)	21.0 (WD0006, 486)	17.7 (APH_1404, 434)	18.3 (ECH_0042, 447)
VirB11	100 (OTBSv289_572, 329)	69.3 (RC0390, 334)	67.2 (RP292, 334)	67.5 (RT0283, 334)	68.4 (RF0468, 334)	63.8 (WD0007, 330)	60.5 (APH_1403. 332)	66.6 (ECH_0041, 332)
VirD4	100 (OTBSv289_573, 593)	72.4 (RC0391, 591)	72.3 (RP293, 591)	72.4 (RT0284, 591)	71.7 (RF0469, 591)	63.6 (WD0008, 648)	62.9 (APH_1402, 740)	63.7 (ECH_0040, 714)

N.D[1] , not determined. The VirB3 gene of *O. tsutsugamushi* was found between OTBSv289_382 and OTBSv289_383 from the genome sequence. N.D[2] , The VirB9 locus of *O. tsutsugamushi* was found between OTBSv289_570 and OTBSv289_571 from the genome sequence. N.D[3] , The VirB3 locus of *R. prowazekii* was found between RP102 and RP103 (113551..113835) from the genome sequence.

phylogenetic trees based on the deduced amino acid sequences. The alignments were generated by the CLUSTAL V method in the MegAlign program (DNASTAR)(Appendix 2). These phylogenetic tree shown that proteins for T4SS of *O. tsutsugamushi* are similar with those of *Rickettsia* spp. than *Anaplasmaceae.*

3. Transcription of genes in the *virB* and *virD* cluster of *O. tsutsugamushi*

I utilize RT - PCR to analyze the expression of the clustered virB and virD genes in *O. tsutsugamushi* cultivated in J774A.1 cells (Figure 2). Without reverse transcriptase, no amplicon was detected in RT - PCR analysis indicating the absence of contamination of genomic DNA in the RNA preparation. As shown in Figure 2B, transcript of *virB8*, *- B9*, *- B10*, *- B11*, and *virD4* within one of the *virB - D4* cluster was detected, indicating the polycistronic transcription of these genes. As a result, I guess transcriptional promoters are located upstream of the *virB8* gene.

4. Transcriptional analysis of the *virB* and *virD4* gene *in vitro* and *in vivo*

I examined by RT - PCR whether *O. tsutsugamushi* expresses the virB gene in L929 fibroblast, J774A.1 macrophage cell lines and experimentally infected mice (Figure 3 & 4). Transcripts of *O. tsutsugamushi virB4, virB10, virD4* and *rpoB* were detected in infected L929 fibroblast and J774A.1 macrophage cell lines (Figure 3). Without reverse transcriptase, no amplicon was detected in RT - PCR analysis using any of the primer pairs, indicating the absence of contamination of genomic DNA in the RNA preparation. Inability to detect these products when using RNA from uninfected cells indicates that orientia - derived RNA served as the template for these products. To examine oriential multiplication, IFA stain

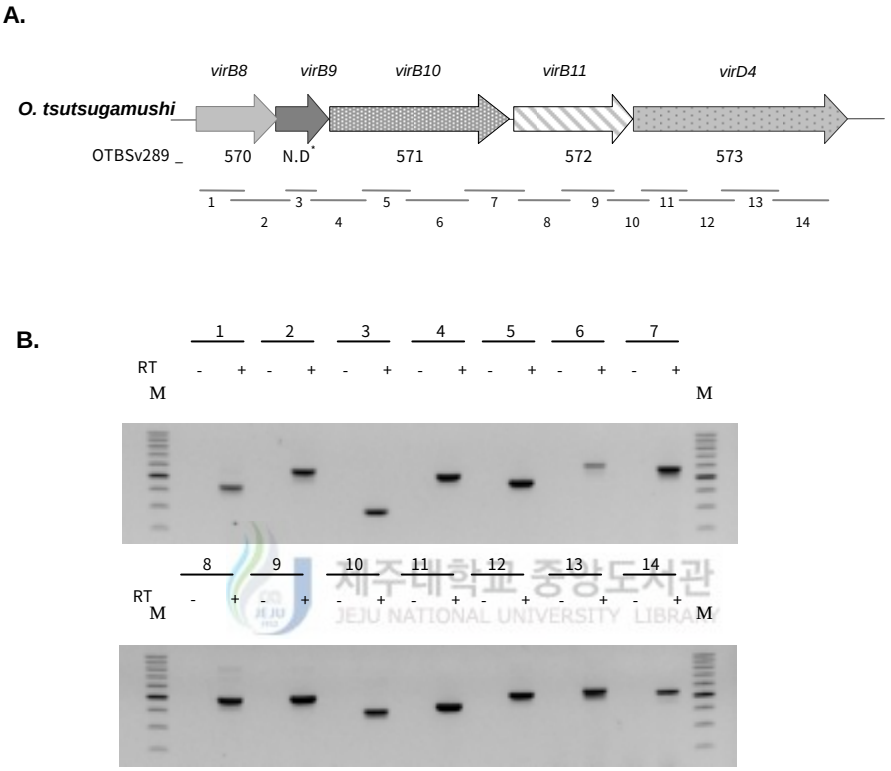


Figure 2. RT-PCR analysis of *virB8* - *virD4* cluster. (A) Diagrams of *virB8 – virD4* clusters and RT-PCR products. **(B)** RT-PCR analysis was performed using RNA isolated from O. *tsutsugamushi* infected J774A.1. RT+, with reverse transcriptase; RT-, without reverse transcriptase; M, 100bp size marker.

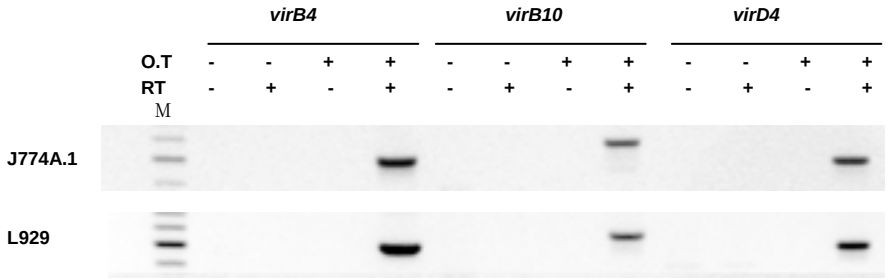


Figure 3. Transcriptional analysis of the *virB* and *virD4* genes *in vitro*.

RT-PCR analysis was performed using RNA isolated from *O. tsutsugamushi* infected J774A.1 & L929 cells. Reactions were carried out using primers specific for *virB4*, *virB10* or *virD4*. O.T-, RNA isolated from uninfected cells; O.T+, RNA isolated from infected cells; RT-, without reverse transcriptase; RT+, with reverse transcriptase; M, 100bp marker.

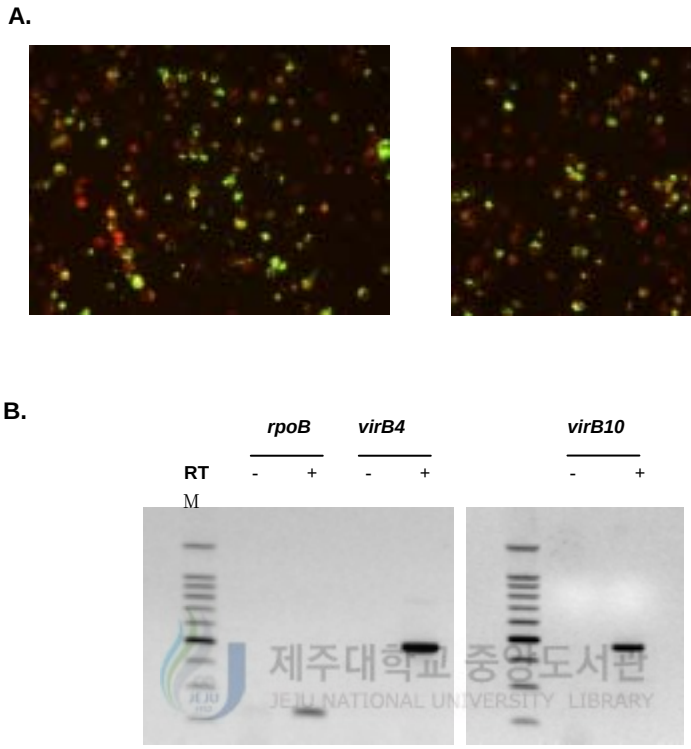


Figure 4. Transcriptional analysis of the *virB* and *virD4* gene *in vivo*. (A) Indirect immunofluorescent staining of peritoneal macrophages recovered from C3H/HeN mice after injection with *O. tsutsugamushi* strain Boryong. **(B)** RT-PCR analysis of *virB/-D* transcript in peritoneal macrophages recovered from C3H/HeN mice that infected with *O. tsutsugamushi*. Reactions were carried out using primers specific for *virB4*, *virB10* or *rpoB*. RT-, without reverse transcriptase; RT+, with reverse transcriptase; M, 100bp size marker; *rpoB*, *O. tsutsugamushi* RNA polymerase beta subunit gene.

was performed with peritoneal macrophage that isolated from infected mice. The result of IFA shown that almost all of the cells were was infected with orientiae. Transcripts of *O. tsutsugamushi virB4, virB10* and *rpoB* were detected on days 11 after injection of *O. tsutsugamushi* in experimentally infected mouse (Figure 4). Consequently, mRNAs for T4SS genes of *O. tsutsugamushi* are expressed during infection process in mice. Without reverse transcriptase, no amplicon was detected in RT - PCR analysis.

5. Transcriptional analysis of *virB* and *virD4* genes during infection process

It is possible that a T4SS of *O. tsutsugamushi* plays an important role in virulence and intracellular growth in host cytosol. If this is in the case, transcription of *vir* genes in *O. tsutsugamushi* may be expressed differently during infection process. To investigate temporal regulation of the *vir* genes during infection process, total RNA was prepared from J774.A.1 cells at specific times after synchronous infection by *O. tsutsugamushi*. The bacterial inoculums had amount of *vir* RNA. The *virB4* RNA level was decreased by 1 hr, after 1 hr it began to increase till 24 hr. The *virB4* RNA level was decreased at 48 hr. The *virB10* & *virD4* RNA level were similar to *virB4* until 24 hr, but these genes continue to rise till 48 hr (Figure 5). On the other hand, level of *ts1* RNA for 56 kDa outer membrane protein gene was not changed. The expression of host cytokine mRNA was analyzed too (Figure 6). Peak expression of IP - 10, MIP - 2, and MCP - 1 was observed between 1 and 3 hr after infection. And peak expression of RANTES, IFN - β, MIP - 1α and TNF - α was observed between 6 and 12 hr after infection. Infected cells express little IFN - β and RANTES between 1 and 3 hr, on the other hand, other chemokine already expressed between 1 and 3 hr.

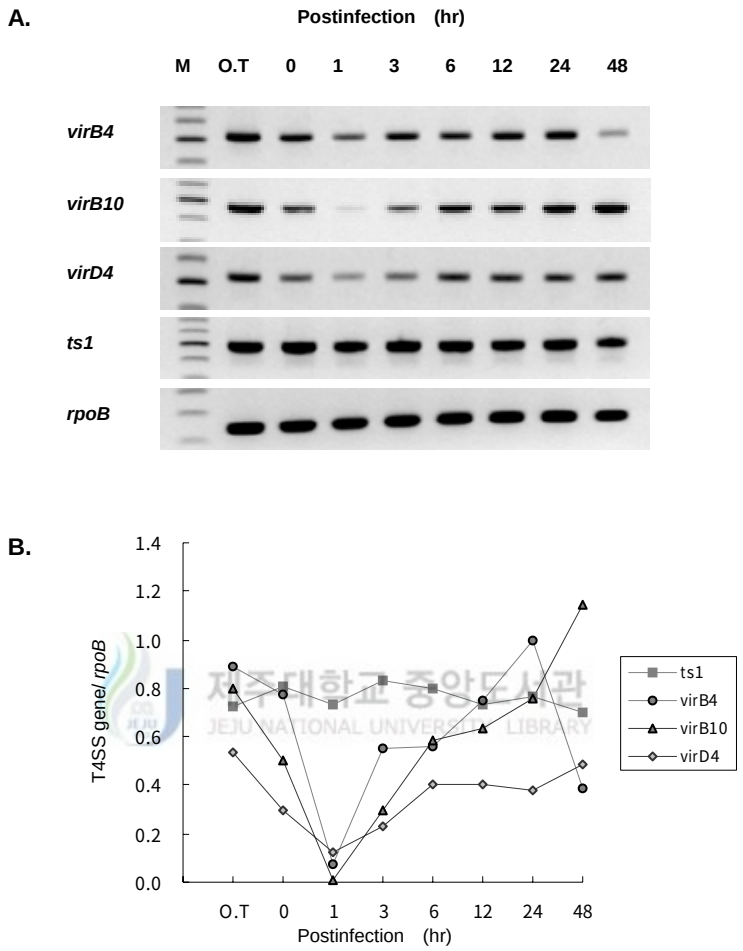


Figure 6. RT-PCR Analysis of *virB* and -*D4* genes during infection process.

(A) RT-PCR analysis was performed using RNA isolated 0, 1, 3, 6, 12, 24 and 48hr after infection of macrophage J774A.1 cells by *O. tsutsugamushi* strain Boryong, and isolated from same amount of bacterial inoculums. The levels of *rpoB* transcripts were also determined to normalize the transcripts of *vir* genes. **(B)** The band densities were determined, and mRNA expression level for *vir* genes was normalized with respect to the intensities of the bands of *rpoB*. *rpoB*, RNA polymerase beta subunit; *ts1*, 56-kDa outer membrane protein gene.

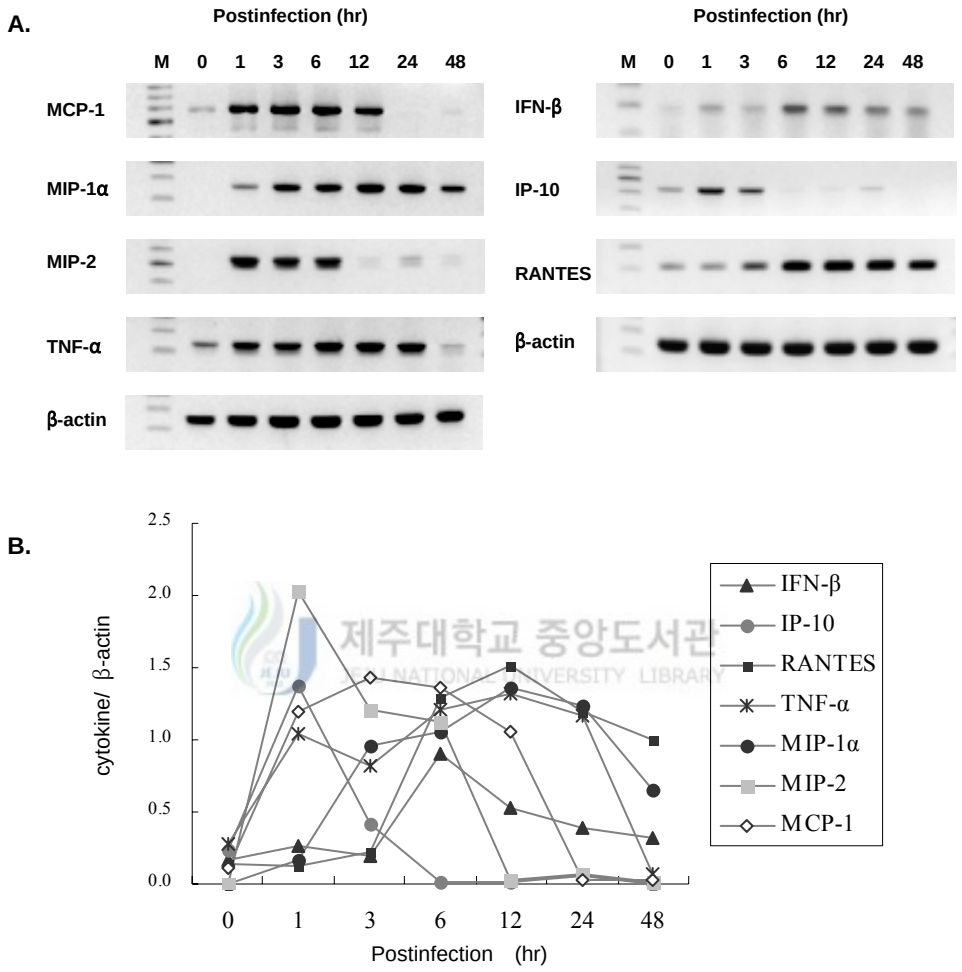


Figure 7. Determination of chemokine and cytokine mRNA induction in macrophage J774A.1 infected with *O. tsutsugamushi*. (A) Kinetics of *O. tsutsugamushi*-stimulated chemokine and cytokine mRNA levels induced by the infection of *O. tsutsugamushi*, analyzed by RT-PCR at each time point. M, 100bp size marker. (**B)** Normalized expression level of each chemokine and cytokine mRNA determined with respect to the intensities of the bands of β-actin.

IV. DISCUSSION

In the present study, the genes for a T4SS in *O. tsutsugamushi* were characterized, and their transcriptional analysis was performed. Unlike the single locus of *virB* and *virD* genes in extracellular or facultative intracellular bacteria, the *virB* and *virD* genes of *O. tsutsugamushi* were found in two separate loci like in *Rickettsia* spp. (Figure 1). Gene clusters for a type IV secretion system (T4SS) in *Orientia* are located on separate contiguous 65 kb and 23 kb regions of the chromosome. This may allow for an independent transcriptional control of separate loci. As shown in Table 3, there have been 15 *vir* genes (1 *virB3*, 2 *virB4*, 5 *virB6*, 2 *virB8*, 2 *virB9*, 1 *virB10*, 1 *virB11*, and 1 *virD4*) identified in *O. tsutsugamushi.* According to this analysis, the pilus components composed of VirB2, VirB5, and VirB7, do not exist in T4SS of *O. tsutsugamushi*. Table 3 shows that *O. tsutsugamushi* had higher identities with *Rickettsia* spp. of obligate intracellular bacterial species,. Between *O. tsutsugamushi* and *Rickettsia* spp., three orthologs of VirB4, - B11, and - D4 are more conserved (66.3 to 72.4%) than the remaining orthologs. Among the VirB and VirD orthologs, the VirB6 proteins of *O. tsutsugamushi, Rickettsia* spp, and *Wolbachia* were significantly larger than those of *A. tumefaciens, Bartonella henselae,* and *L. pneumophila* (295 to 346 amino acids), and retained the VirB6 - honologous region. Because the VirB6 protein is involved in assembling the conjugal pore at the inner membrane and may interact with the effector molecules to be delivered, the diversity of VirB6 proteins suggest that a type of effector molecules is different from those of other bacteria (Ohashi *et al.*, 2002).

In *O. tsutsugamushi, virB8, virB9, virB10, virB11,*and *virD4* were polycistronically transcribed (Figure 2). A remarkable result is that these genes were transcribed not only in cell cultures but also in the peritoneal macrophage of experimentally infected mice (Figures 3 & 4). Therefore, The T4SS is expected to have a significant role in tsutsugamushi disease.

The expression of *virB/D* was temporal regulated during infection process (Figure 4). On the other hand, RNA expression of *ts1* for 56-kDa outer membrane protein genes was not changed. Transcripts for *vir* genes were regulated differently during infection process. In view of the results so far achieved, I guess the time of secrete antigen (effector molecules) by T4SS may be determined by infection process, too. Similarly, the host cytokine response was analyzed. Figure 6 show that gene expression of IFN-β and RANTES occurs lately than other cytokines. Time of peak expression coincides with those of *vir* genes. These results support hypothesis that temporal regulation of *vir* gene expression may be associated with host IFN-β and RANTES responses.

O. tsutsugamushi is the causative agent of scrub typhus. These agents enter host cell, and replicate in cytosol similarly other intracellular bacterium. *O. tsutsugamushi* induce chemokine and cytokine production, significantly contribute to inflammation observed in tsutsugamushi disease (Koh *et al.*, 2004). Some bacteria can survival in the host cell using the T4SS to change host environment. It is supposed the T4SS of *O. tsutsugamushi* have evolved in intracellular bacteria to modulate eukaryotic cells for their intracellular survival. Temporal regulation in *vir* gene expression for T4SS of *O. tsutsugamushi* is associated with type I IFN induction. Further study is required for explanation of the roles of the T4SS

in intracellular survival of *O. tsutsugamushi* and identification of effector molecules.

V. APPENDICES

Appendix 1. Open Reading Frame (ORF) of *vir* gene. The VirB/D protein sequences for a T4SS in *O. tsutsugamushi* are shown. The list of genetic information are shown as follow: gene number, name, locus in chromosome, and reading frame.

OTBSv289_ (virB3) 510798..511103 [1]
MLGKLEADPLFLGLTRPPMIFGVSLPYALLNIMLSTMYLTVASNFYVVPVSLVVHGVGYLLC
FKEPRFMEIYLMRAQKFNKCPNRLYYGANSYGIYLNNEIF

OTBSv289_383 (virB4) 511096..513513 [1]
MKFFKTKVAREYYSKREVHVAKFIPYAYHWNKSTIITKKNELIKVIKISGFAFETADDQDLD
IRKRLRNLLFKGMASGSLNLYFHIIRRRKQLASAMDEGDIDPTAGRAKDFVTYVDNEWKKKY
SDFQSFVNDIYITILYKPDVEGGEILKYFYNKLLQKSDKNAWMQSMNEMYANLDEMVSRVVT
TFSDYDAQILKVKNEPNGVFCEILEFLATIVNCGSSMPVLLPRGSIDSYIPTHRLFFGDRSI
EARGAGQRRYAGIVSIQEYGPKTSAGVLDSFLQLPFELIISQSFQFSSRTAAINKMQLQQNR
MIQTEDKAVSQIAEISQALDMATSGEIGFGEHHLTVLCIADSLKALENALSIASVEIANTGM
QPVREKVNLEAAYWAQLPGNIEYAVRKSVINTLNLAGFASMHNYLPGKAKGNHWGDSVTVLD
TSSGTPFYFNFHVRDVGHTLLIGPTGAGKTVLMNFLCAQAQKFYPRTFFFDKDRGAEIFIRA
LDGKYTVINPFQQCNFNPLQLPDTNENRNFLVEWIKTLVTSNGESISADDMHYITLAVEGNY
KLNPSDRYLSNIVAFLGIGGPDTLAGRIAIWHGTGAKAGIFDNIHDNMDLQSGRVFGFEMGE
LLKDPVSLAPVLLYLFHRINLSLDGSPTMIVLDEAWALIDNPVFAPKIKDWLKVLRKLNTFV
IFATQSVEDASKSSISDTLIQQTATQIFLPNLKATDIYRTAFMLSEREFSIIKSTDPGSRYF
LIKQGIGSVVAKLNLAGMNNIIGVLSGRVETVILLDQLRSEYGDDSRKWLPKFYKHLETAK

OTBSv289_384 (virB6) 513513..515960 [3]
MQWLYQLAKIVILISVFALQIEAIKAIPAIDMINMGDIIPDEVKDVADDIKDGVKKVVDTLS
NITCETRGVNNLLFKDEFSHTCTPAPFFSLATSSILGVGTYLPMVLKLNMTNAELFGEQFPG
GQCLKKNRADPADPRISFALCNNVKLMVAAATAIGGTVVNAAVAIASGDNVWEAVAKAWHIK
AQDIFEVYKDKEVGYSHHFVDINLAGSPYIPYKIVRDKDKICVAAWTLLGGYLNVGCKYISE
PCSSSIYSNFLNNSSSVSDISKPVCHTGNGNKESKELSDKNRLVECGNMSGCYADAVENSKT
LLPITGPIVKCFVQMARKILGESTVCKLNAKGDEVITNASDVNMISKFSRHMRRAVAAFMCL
YIIFWGYNILLSPNEISRKDVINTVLKIVLVTYFSIGISTGDDKKLINGVQSWGMELLRGDV
MAKVAAWVMTKSSNEEGKKVSGLCNFQPSDYDSSKHGADARQLLALWDSIDCRLTHYLGINA
IKDYVRQKIQAVKGSGGDPLGNSIPPYYYLLVPALWSGNMTLLGLVLFYPLLVISIAAYMVN
AYIVCIIGILILGLLAPIFVPMVLFEYTKSYFHSWLKLVISFVLQPMVVIVFMMMMFHVYEY
GFYTDCAYDYRLVKDSSGNVSRMRKMFFINVNDKNLYDGDADQKIARCKKSLGWMLNNPLFA
VINTAKTVAKDVISVDNVGSEDASENHQFSDTVEVDQGLFFKTTRSIFQLVKDLIMALLVAC
LTLYLIYHLSDSLSQFVSSLVSGINLSGMTVNPKQMHDTLGVLAKKGGGKASSAMKGGGDGG
QSMRSGSLK

OTBSv289_385 (virB6 like1) 515982..519854 [3]

MIKHLLYNFRIVINNHSILKAFKEAIYSILLLVIALPSMAYAEKNCCVRQNNKKRCCTYDQR
FCYAYVQSKCIQKEELRYQNPDVLQEKVKLLESSAKQAWNNAQDHRFSSNLEKFNAVTNASA
QTREIISLSEQLKKALVNEGKARQAAFTESSKTGKDKINRKMSEDFQQLIEERKAALKLAQE
SGDKASSQYKIIKASARDLVVVAGMDFWNALLLYKKIDEELDSKVRNGADKKSILIAKKKVL
YEIEKVLKAFVEGRDMYNKFVAWVIGYSDVTEEKENKAIEFLLQNLSYSTVRRSASFETTLK
AVIEVYKNGDFINELKLQNESVKTDIAKIEHSIQVAEDEMKRKQEEEEAKKLLEFQNKVKKA
KEEVDAAKDKFKEKQTQAREAHDQASASGDLEDKIEALNKKKKEYEAAKKYKEALDKAIDIG
NNNPDLVRERDSLGNDIDNLSQSIDQMTDDVNRDKEIYKQQFEEDKNSSVSANCDFSQYRSI
SPESSVFTLASFVYQNINFKCGNVCNTADKGFNLCLKVKKSDLCRDCIPIYIGPQSEFKSIS
ELFDTSGVKKGRRDPILVTENAVKNIGFKVEHTDKISCLKIKTSYGDIPIVCKNISADIDLI
PQARSNNRICSLSTTKSRVPFNFSGRAIGCLKEALDKMFYADSITHDQVSSASILKPLSSFQ
RGMTVTVKAALMLYIIFFGIKMIFVERFFSLERLVTGVLQILIVMYFSVGLGPMTNKDGKIS
YNNGMQDHFLPFLSSVTSELAHMVFSSVGSDSAVGGTKLCYFDPNKDYTPDARYYALWDAID
CRIGYYLGFRLLHNYTVDRSKPSLGGKLVGSSIGTSANDDITKHLSNIKDDHALKKDDTFLV
FPTLWGLLLSGEIIFFLVMLLFVIIFLTLFWRFMAVFITSLVNLYVMCYISPIFIPMVLFEK
TKSMFNNWLHLTLSFALQPAIVAAFIALFVTLMDSITYKTCQFARYDYQQGNKILSTFELRV
PDRYIQDHSLFDDAYNSDAAKICESAGYRLVEYFNGKNWHQRIFLFFKAFVVYPEPDFLPTM
IVVLLFCGIFYYFFQEAHRFAAVITSGITAVNMELPSLRIPSFRNKSTRTKGGKDDKEEESS
DKFSSRGGIDKTATDKISSRSNIESSAQDKISTGLKSNGISGIEHGLKKDHDAIRAAKKFDI
SNNDEVIGADKFCTSGNKQTDKLSSENIKPRDTSVVSAEDKFSTSFKLKDHKDIVQSEIDKK
LESNVSDKISNNQTIETRKSNIESDALSKKIDANDLNEIKKGSDDKKNI

OTBSv289_386 (virB6 like2) 519908..523183 [2]
MQSKIIKFAIIAVCIAVVSFILMLFGALRSGDDCWYRYNADGQDLDVIRLTQNVRASGQYDS
LRLGGIVDPSKPCGLWIKSDFYVSVHEENGQLKPVNIDFLIDGTVSLCQAYLPKNALTCKLK
YNDGKECNISDINKFFNGEKSDCIEDCEDNTDDSGNLIPIPRILDPGDGLPVILKANVGEWR
NLAQVSAGDEIEVKIGRNQNFSHGGVENTEIGYAYSRPEFDWFNDTSLDWQKGTNDTFGIIK
RTKQVRADCQEGKKNYSPLCGRYSPWGEGDNYIKQCDECKGCTCEDCGMKPKCPGSKPVCPA
PECEMAGSHGWFSKHFAVFNSELGKDKCFYTGTVKDIFGGKDCPEKYLLDCKRAVLADGLPE
EYRDDGTRTVPATLTEQDRNDIKSLGRIACSNRLDEQLVKNRQKVYSWRSATYATDLKYRFS
SVQDKDSILGNKCKSDFPSGKDLATGCYGITKVNQDLIIYKDQISETLFSGPRYLQYMIRPD
INIESMKNSVGGYVLYLKQNSCVRKSGVAFDDSKFHGRGKILYAIVPAGVDVNKLKSHELDV
YTTGSLDVKVEDLVGKAKISSSQSGYIWFKILNDEKDYKASVGEYKITMSTIVPRGKFISKV
LNPIIRIFENKIANASKTMFKNITCYQQNDKSRCTNFFNYIRAMLTLYIMGCGFAFMLGAVN
FTAKELVIRIVKVIIVSGLINEGTFNFFNAYLYDLIMNFSKQLMVNVSGYEYDQNIGTFLFL
DELMSKIFFNKTFYVQLLSLLSMGLMGVVYYIIIFVPIILIIISLLKTIMVYLVSTTAVALL
VGLAPLFLSFMLFNRTWYLFDNWVRMLFRYLIEPVILMAGITILTQLFIVYLDFVLGYSVCW
KCAIVFKLPKIDALGGVLGSYGGQELFCINWFGPWGYDNRADNNIGLAMNYIIALVIIAYCM
YSYIDFSSLMVDILTAGGAGGIAPSVGHVAPQVWDTAVQWTEKAVMKVASIGKKGSFAVRKG
IGKGISHAKSKYSSSQTEKSKDKDNVVNRRTNLENKSLNDKRGSAIRGKNSNVENVKPNQNT
YLRVNNQEIDLNSPKPSHNESSVRRHDLNKKNFENND

OTBSv289_387 (virB6 like3) 523167..525743 [3]
LRIMTKILKILITKNKSLMTYYINILILLSYIFVSTSIQASNELLQAGEQLPTSNSLQWMDK
YPNDNNTGCTNVSPTWLSVADVKWIDIQANSNNNWIDSGIMTKAGKSISVEIPKISQNFRKL
QKRYLVLHRVDPRFPVIGKTFIIELGTNNKPISRLHNFENGKLLNYQNENSSNFQNGSTNFT
NAINLQKKFFNGADTKISVKAGDIIDIALISSVDFFNKLQLKGGTEKSGFTGELYPRWDDYK
YYKPYGIYTVSLKSNVGVVDNALLVTGQTSDKKALSKLLVGVKSIDSISSFEKCNLNNLSKS
QSCIMQSGIGMEIKLDQEVLKSKFDKFLVGYNVGNANSSAFLDKPIEGMYHIVAKSDGDLSF

STPLFNNNVKYRTNGLQDVEYSSILSSCSTLNEFEQKYLNSILSSKQEIIQGIVVDKTLVGR
YLMYITIDRHNIVSDEYDGDIEYIISNTTPNLSTKGTTLPRNGINIVTPESAKLWFRFVKTN
NEQFNLKVTVPPDSEGKIKVAGFFYDNIYIPIKQKVEKFSKLFYFGLAKNAALKKVFSILAI
LYITLYGIYFLLGVVKVTAYDLLIRCSKIIVIAALFNESSQYIFYDTLFPMFDGGINSLISY
AVKTTASDVDNPFKFFDLVVSRYIDVNFLKIILIEIVNIHNGLTILGILTLWSIMRFIIIMV
KVCMELLMSMIAIAILVGLAPMFIIFILFDRTAEIFKRWLTALLLNTLMPVIMIIFILIINE
LMLVAVEAAFPEIRICWGTLFDIELNLDLSAIGLPTAFSIPLMAVPFYNVVFVGGNLFNAMD
LGNSFAGSLAGVFLLYNLVLLAGTLVGSQVFTKGINRKGMSYVKSIMMQLLS

OTBSv289_570 (virB8) 764100..764780 [3]
MFFWKNKKKVEQQKQNKPAIKSWFNDHYETVLVQRNIAVLLSFICIILIFIAVVAVMKISLA
RNFEPFVIQIEEKTGATRVVNPVGIEVLNTEKALAQYFIKLYISARETYNPVDFEIKARQTI
KLLSNNNVYWSYLNFINRKENDPRLIYGQRETTTLKVRSWSELPDRRFIVRIAIHESISGKV
FNKIVVVQYQYVPMELSPEEFDINPVGFQIIGYRIDDDYS

OTBSv289_ (virB9) 764764..765198 [2]
MMIIVRIFLILNLLFSSVVYSSYFEEEFPTTADDRIKTYIYNPSEVYLLVLHAGFQSSIEFA
KNEEIRSIFFGDNYAWEVTYPLPNRIFIKSLEKNVRTNMTIITNKRTYEFDIVSKELEVGRE
HDLVYLIRFYYPQKKACNKEK

OTBSv289_571 (virB10) 765201..766730 [3]
MQNINNDSNQDPQGQYDHSEDSKVVGPEVHNEFSKVASSAKRSVLLTIVIVVISIVILYFVI
SSIFIDESHKEESIAVPVPVDVIKPNIELSGDIVIPELPTIPTLITPELPEIKVPDQAKLNN
LNNDNSSKDEVVQSNKLPKESKLIKPPVLPLTEQGEYNNAKQDAKIPSLDLSAQTKDRAARL
QEKRTSTNIVLINKSPPSPTREEMEQIRNFTDAGELRYLLGRGTVIDAVIISAVNSDFIGEI
VAMVSKDVYSQEGKTVLIPRGSKIFGYPSVLDNAAYGRMMITWSEVQIAGSQYKLNLSAPTI
DRLGKIGVTGKVNNKNLARIMHAVLVSAVNIGAAFTLDKVVALQQQMANTTSGLTGTLIKNA
MNINNDSVKSDSQKITEICGMSRSLITDKSSSVYTTINKKCMEIETSIIANVDDKQKLSSLI
NTIINISNSTANNSNSNTQKATDTALNDVVNAIKKIIPSQEFNRTITLNQGQNIKIYVNKDY
LFPTKAVSGVKVL

OTBSv289_572 (virB11) 766730..767719 [2]
MKNNIALETYLTPFKLIFDEAGVNEVMINRPGEVWVEKKGSYRQELMKDLDFSHLAALARLI
AQSTEQVISEEKPLLSATLPNGYRVQVVFPPACEVGSIGIAIRKVSSLNMTLEDYNKTGAFD
YTAIDEVIDENDKILSEYLINKQFKNFLQHAIKSKKNIIISGGTSSGKTTFTNSALLEVPHT
ERLITVEDAREISIPNHPNRLHLLASKGNQGRAKVTTQDLIEACLRLRPDRIIIGELRGAEA
FSFLRAINTGHPGSISTLHADTTAMALEQLKLMVMQAGLGMPPDEIKNYINAVIDIVVQLKR
GDRGIRYISEIYFKKMRKK

OTBSv289_573 (virD4) OTBSv289_573 767719..769491 [1]
MNENAFLTKIRNLVIKIMVHCIGVILVVFIIKFFILLFTVKHQNLIDVLLTFNVSIVYQYLI
WIINSWSLIDFSSYDYLKLKLVFSFIVPPVITIVLYIKNFEKIKSWQPYKLKEKVYGDARWG
DQDDIKRAGLRSKHGTLLGVDKGGYYVADGFQHALLFAPTGSGKGVGFVIPNLLFWEHSVIV
HDIKLENYELTSGWRAQQGQNVYVWEPANPDGITHCYNPIDWVSSKPGQMVDDVQKIANLIM
PEKDFWNNEARSLFLGVVLYLIADNTKAKTFGEVVRTMRSNDVVYNLAVVLDTMGAVIHPVA
YMNIAAFLQKADKERSGVISTMNSGLELWANPLIDAATASSDFNILEIKRKKTTIYVGLTPD
NINRLQKLMQVFYQQSTEFLSRKIPDVKEEPYGVMFLLDEFPTLGKMEQFKTGIAYFRGYRV
RLFLIIQDTQQLKGTYEEAGMNSFLSNSTYRITFAANNYETANLISQLCGNKTVKQASHSQP
RFFDLNPATRTMNISEVQRALLLPQEVILLPRDEQIILIESFPPIRSKKVKYYEDKFFTTRL

LPPSFVPTQTPYVPPDYNATADDSNNVDNNQK

OTBSv289_1278 (virB4) paralog 1685153..1687606 [2]
MGFIHKFLHSRQIDAKQFDSNAQNFIPITCHYNDETLLTKNGELIQILQIKGINSEYTNKDL
LGIRAAIRNAIKNHINNNNFAVWIHVVRRCDNIDDPTPYSQALPNFIHNVWSKKNYWQDKFV
STLYISIVHHGCNLNIRNLDSFLNSLSFKTLDNFYDKFIASALKNLSMAINNIITELQRYNP
CKLSIKIDNNKVYSDLLNFYKKIIQLNTGLIEVKDVDCAVQLATHKYSFGGDKIEVIHQQEK
KFASILGIKEYQDIKDELVSKCLHAPVEFIATEIFYFADQVKAINSYSQQSKIISASRDSEL
ALFKGIDPIVNSDNTLPQFCHQQISIMIMADSIDLLESRVAYISQRLVKQGIIHVREDINLA
QTFWSQLPGNFNYIRRVSYNIIDNVAALAALHHYPVGMQHSRWGRSVTIFRTECGTPYFMNF
HDESAKGHTCIIGGAQSGRATVTNFLLSEASKYSPTILYLTNRNNAQVFIKGIEGTWYNGDL
PFNPLSYLESSDDLLELFKIITGSYFNNLSDSEIECLTELAIAVMDMPPESRSLIKIIKDFN
FKDYSAGASVKKRIKLFISDDKCSKIFNQSSTLELQDNNVTAINLADYTDYQFNQSYTPQVG
EKPEIYHNKLQTLHCLRSGIIFSLIKKFSLLDSSEPKILVVDNMPDLIVEQVFMNLIKQITE
QLSENNGIILSVVQVNQNDQFYYSDFWKKWLKFNNTKIILPTNIRNLPIAEICNLSTSEAKK
FNTISPNSRLFMVKQDNNYVIVELNLEGFPAIRKLLSAEEEDLNLFHKIMQKIGDDAPSAWL
SDVYDQFQNSE

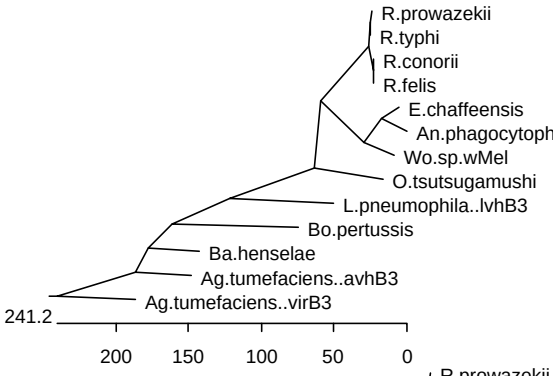
OTBSv289_779 (virB6 like4) paralog 1040543..1042759 [2]
MILHNFSEFNTRNLNIMNQQNTLFLKFLVGIILFCSVISYSIAECIDADDFGFPIIAISSRY
DTKQLTGQKDNQVAPWIDSKLLVNGKPLVVMVKHWNYHEYDNDISHLSAWSAWYGTNKNKHT
LASITKRFPECRFRNNKTFSDSYDDNDDIPVINPPCLFKHGIGLYALIAKPGVDPNANVHSQ
SYGIPKKTINFHVGQNYLSSLNSTELDSGFLDTTPDGNIVKTGGYFHKYQDQEAEQYVGGRL
YFKILDRFYDDNNGQYKIIIKSGVGDEKDSPETFLINIVKEMLFGNKKNQNKNGIIQNLFIN
ILKNPSYKIVVNLTLILFIAFSGLAFLIGNINMTAHELVLRTVKILVISVLLNSDTAWKFFY
DYLFFIFVDGPQFIIKTINEATAIGPGSSSILGLMIAPHTLKKLFSILFVDWGGFIYIICYL
ILLYYIFIISFKATVLYLNALILVGIGIIVGPVFLCFVLFQFTKPIFENWIKQLTIYALQPV
ILFAGIAFVGMFIRHEIYASLGFRVCEVPFPPIANTLIKIISGDSSKKQSLLNLWFPAQVLK
KTLLFSQKCANIPVPEDHIVYRDQKPWQCSDGISGNSKQLITSTDPSNEKHCSAYECKANRY
VELPFLDPNINKDRYRIRNFFAGNFVQWDSLLLLAACVFLLSMFNDNAIALANYISSGGDRS
SASEKSTTAIVSTVTPPSPTTFIPAAFSKIGQQRTKNSNSHSNSNSRSGINTGPKK

OTBSv289_812 (virB8) paralog 1095016..1094315 [2]
MLMDNSEIAVDIKNSKYFQDAYRWYKAKYLYPFLHRSVVFIILALLLLLIVLSIINVYSLLP
VTQSLKYIISVDEKFNAAAKITPADQVLKNPLQSIVKILAESYVVKRESYNYNQLASQFQYI
KNRSTRGIFRQFVNYMSLQNYQSPVLRYQKTAKRKIKILSSVFLNNDELAVYFHSCAIDDKG
NMVEDMKWKANIIFETDQVNLNAASGSIFKFFVTDYKITLLQNNNEK

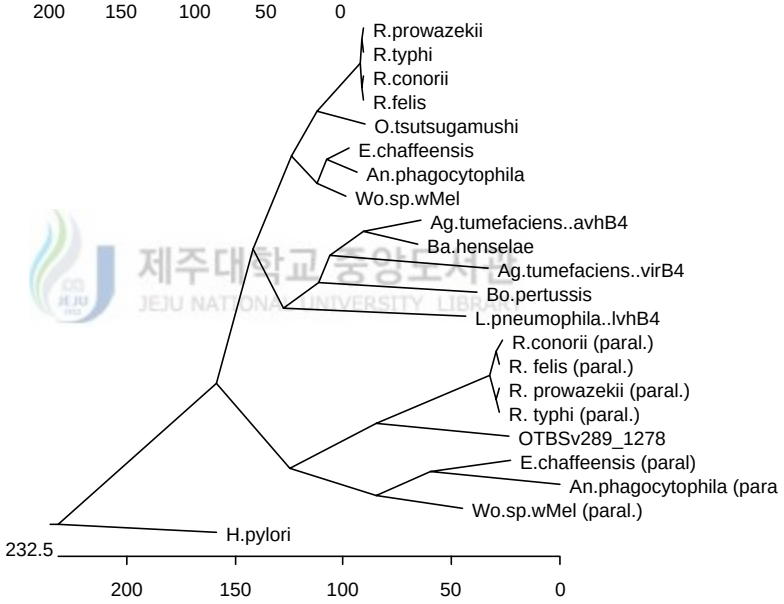
OTBSv289_811 (virB8) paralog 1094325..1093564 [1]
MRNSNNQISFFIILLTIIMLPFRAMAIREPKPTAIDSRIRVMTYSPNDVFKFTGYYNYQGCI
ELDKGEEIVNLSMGDPNAWFIQEFGNRIFIKPIKDDATTNMLLITNKRTYFFELHAEEVQNI
NDSNMIFNVKFLYPDNEKSNSTNLVSLTSEVDLSHPENYNFYYTISGHQEIAPIKIFDDGDF
TYIYFRDKNVVMPTVYIVDDRKKESLVNFRISTKNPNLMIVEQVSLRLSLRLGKKVVYVFNE
ALLSK

Appendix 2. Phylograms of VirB and VirD proteins in *O. tsutsugamushi* and several other bacteria

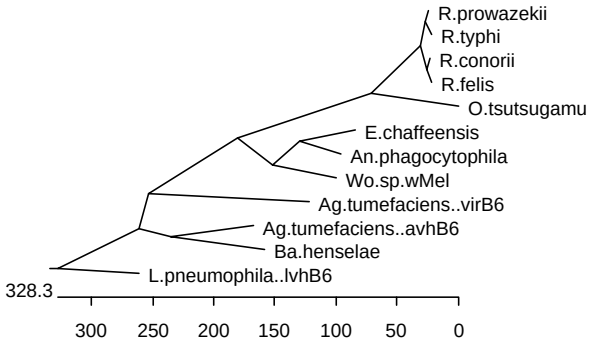
VirB3



VirB4

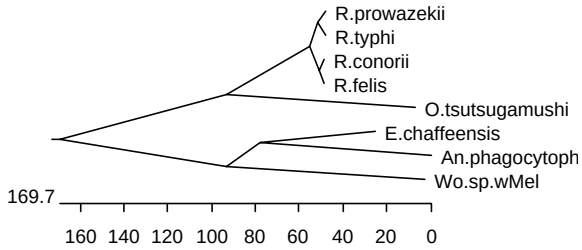


VirB6

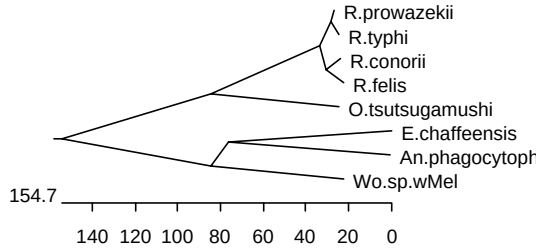


Appendix 2. continued.

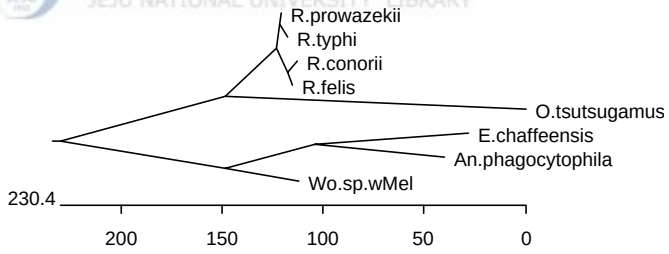
VirB6 like-1



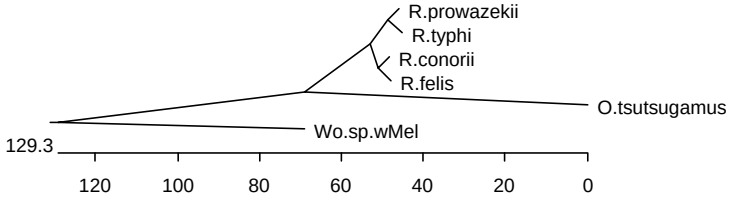
VirB6 like-2



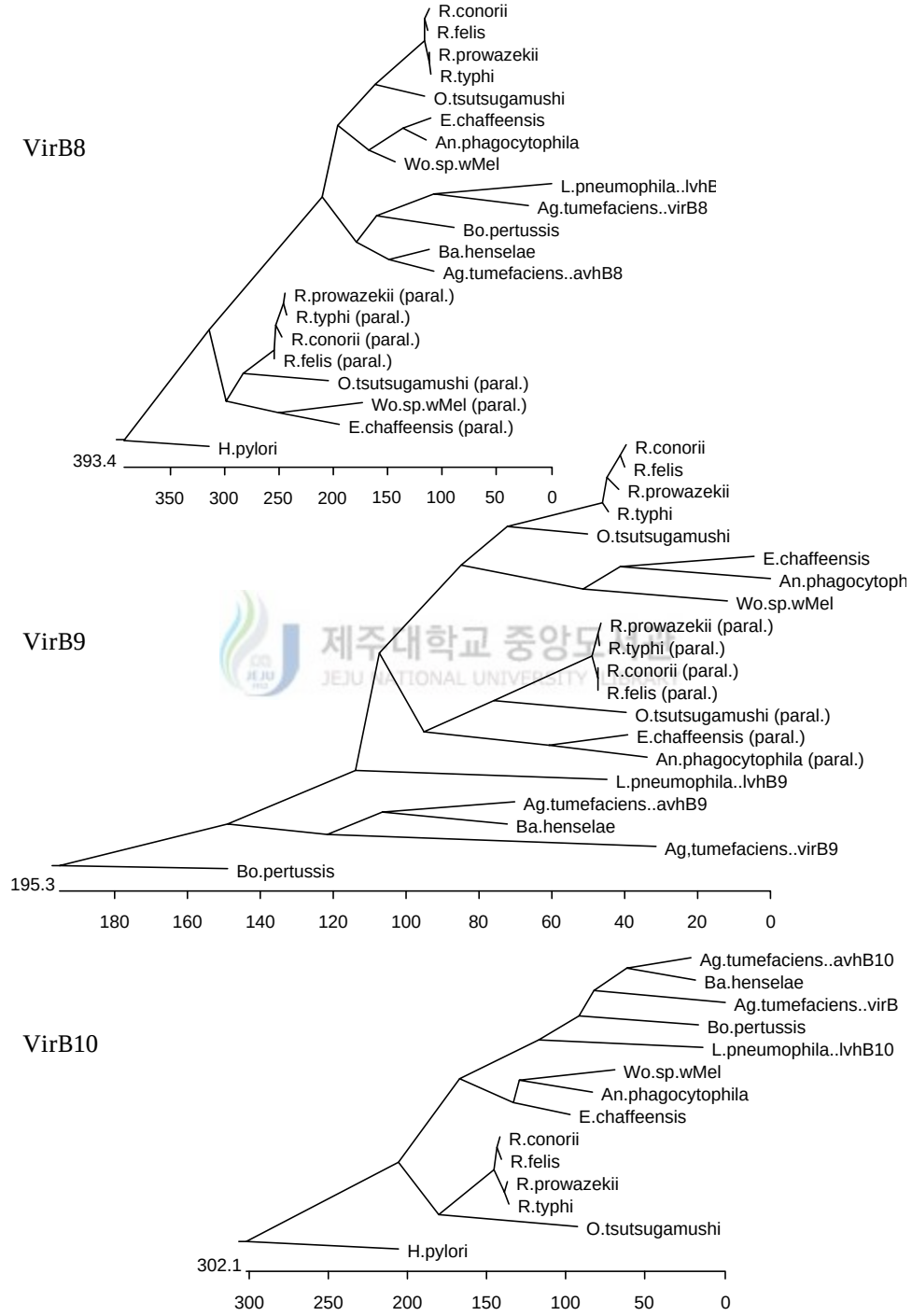
VirB6 like-3



VirB6 like-4

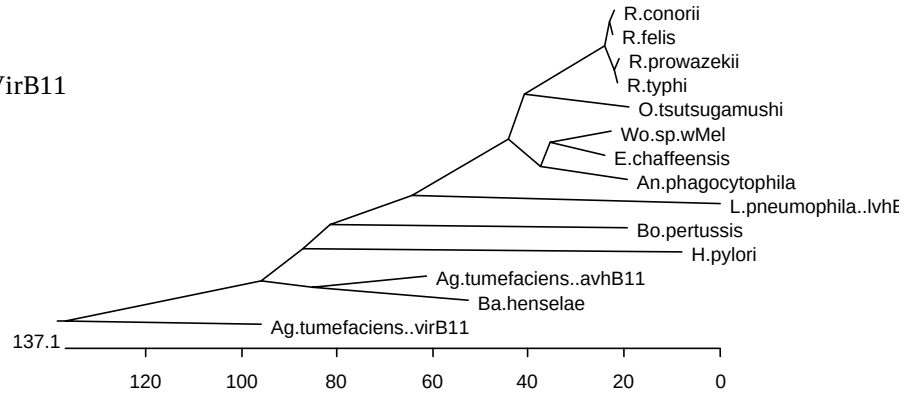


Appendix 2. continued.

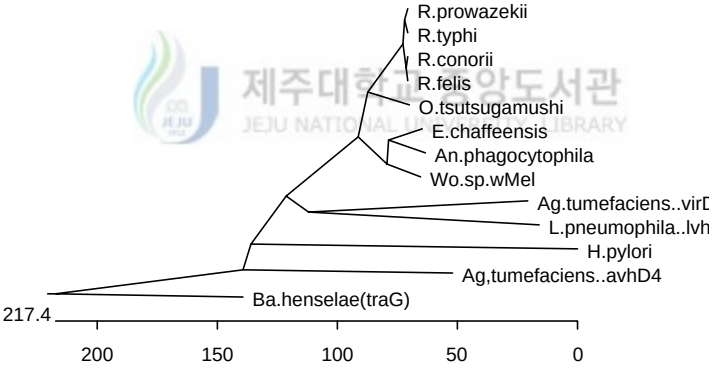


Appendix 2. continued.

VirB11



VirD4



VI. REFERENCES

Altschul, Stephen F., Thomas l. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402

A. L. Jones, E. M. Lai, K. Shirasu and C. I. Kado. 1996. VirB2 is a processed pilin-like protein encoded by the Agrobacterium tumefaciens Ti plasmid. 178: 5706-5711

Andersson, S. G., A. Zomorodipour, J. O. Andersson, T. Sicheritz-Ponten, U. C. Alsmark, R. M. Podowski, A. K. Naslund, A. S. Eriksson, H. H. Winkler, and C. G. Kurland. 1998. The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. Nature 396: 133-140

Cascales, E., Atmakuri, K., Liu, Z., Binns, A. N., and Christie, P. J. 2005. Agrobacterium tumefaciens oncogenic suppressors inhibit T-DNA and VirE2 protein substrate binding to the VirD4 coupling protein. 58: 565-579

Censini, S., Lange C., Xiang, Z., Crabtree, J. E., Ghiara, P., Borodovsky, M., Rappuoli, R., Covacci, A. 1996. Cag, a pathogenicity island of *Helicobacter pylori,* encodes type I-specific and disease-associated virulence factors. Proc. Natl Acad. Sci. USA 93: 14648-14653

Chang, W.H., J.S. Kang, W.K. Lee, M.S. Choi, and J.H. Lee. 1990. Serological classification by monoclonal antibodies of *Rickettsia tsutsugamushi* isolated in Korea. J. Clin. Microbiol. 28: 685-688

Chi, W.C., J.J. Huang, J.M. Sung, R.R. Lan, W.C. Ko, and F.F. Chen. 1997. scrub typhus associated with multiorgan failure :a case report. Scand. J. Infect. Dis. 29: 634-635

Christie, P. J. 1997. The *Agrobacterium tumefaciens* T-complex transport apparatus: a paradigm for a new family of multifunctional transporters in eubacteria. J. Bacteriol. 179: 3085-3094

Conover, G. M., Derre, I., Vogel, J. P., and Isberg, R. R. 2003. The *Legionella pneumophila* LidA protein: a translocated substrate of the Dot/Icm system associated with maintenance of bacterial integrity. Mol. Microbiol. 48: 305-321

Covacci, A., S. Falkow, D. E. Berg, and R. Rappuoli. 1997. Did the ingeritance of a pathogenicity island modify the virulence of *Helicobacter pylori*?.

Trends Microbiol. 5: 205-208
D. S. Walsh, K. S. Myint, P. Kantipong, K. Jongsakul, and G. Watt. 2001. *Orientia tsutsugamushi* in peripheral white blood cells of patients with acute scrub typhus. 65: 899 - 901
Daniel, B. Stetson, and Rusian, Medzhitov. 2006. Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. Immunity. 24: 93-103
Ewing, E. P. Jr, Takeuchi, A., Shirai, A., and Osterman, J. V. 1978. Experimental infection of mouse peritoneal mesothelium with scrub typhus rickettsiae: an ultrastructural study. Infec. Immun. 19: 1068-1075
Ewing, E. P. Jr, Takeuchi, A., Shirai, A., and Osterman, J.V. 1978. Experimental infection of mouse peritoneal mesothelium with scrub typhus rickettsiae: an ultrastructural study. Infec. Immun. 19: 1068-1075
Jianxiong Li, Sharon G. Wolf, Michael Elbaum, and Tzvi Tzfira. 2005. Exploring cargo transport mechanics in the type IV secretion systems. 13: 295-298
Judd, P. K., Kumar, R. B., and Das, A. 2005. The type IV secretion apparatus protein VirB6 of *Agrobacterium tumefaciens* localizes to a cell pole. 55: 115-124
Kawamura, A. Jr, Tanaka, H., and Tamura, A. 1995. Tsutsugamushi disease. University of Tokyo Press, Tokyo.
Koh, Y. S., Yun, J. H., Seong, S. Y., Choi, M. S., and Kim, I. S. 2004. Chemokine and cytokine production during *Orientia tsutsugamushi* infection in mice. Microbial Pathogenesis. 36: 51-57
Kumar, R. B., Xie, Y. H., and Das, A. 2000. Subcellular localization of the Agrobacterium tumefaciens T-DNA transport pore proteins: VirB8 is essential for the assembly of the transport pore. 36: 608-17
Lavigne, J.P., Botella, E., and O'Callaghan, D. 2006. Les systèmes de sécrétions de type IV et leurs effecteurs Type IV secretion system and their effectors: an update. Pathologie Biologie. Epub ahead of print
Liosa, M., Zunzunegui, S., and de la Cruz, F. 2003. Conjugative coupling proteins interact with cognate and heterologous VirB10-like proteins while exhibiting specificity for cognate relaxosomes. Proc. Natl Acad. Sci. USA. 100: 10465-10470
Moron, C. G., Popov, V. L., Feng, H. M., Wear, D., and Walker, D. H. 2001. Identification of the target cells of *Orientia tsutsugamushi* in human cases of scrub typhus. Mod Pathol. 14: 752-759

Murata, M., K. Sudo, K. Suzuki, Y. Aotama, S. Nogami, H. Tanaka, and A. Kawamura, Jr. 1985. Proliferating sites of *Rickettsia tsutsugamushi* in mice by different routes of inoculation evidenced with immunofluorescence. Jpn. J. Exp. Med. 55: 193-199

Nagai, H., Kagan, H. C., Zhu, X., Kahn, R. A., and Roy, C. R. 2002. A bacterial guanine nucleotide exchange factor activates ARF on *Legionella* phagosomes. Science. 295: 679-682

Nagal, H., and Roy, C. R. 2001. The DotA protein from *Legionella pneumophila* is secreted by a novel process that requires the Dot/Icm transporter. EMBO J. 20: 5962-5970

Ng, F. K., S. C. Oaks, Jr., M. Lee, M. G. Groves, and G. E. Lewis, Jr. 1985. A scanning and transmission electron microscopic examination of *Rickettsia tsutsugamushi*-infected human endothelial, MRC-5, and L-929 cells. Jpn. J. Med. Sci. Biol. 38: 125-139

Norio, O., Ning, Z., Quan, L., and Yasuko, R. 2002. Characterization and Transcriptional Analysis of Gene Clusters for a Type IV Secretion Machinery in Human Granulocytic and Monocytic Ehrlichilsis Agents. Infection and Immunity 70: 2128-2138

Peter J. Christie. 2004. Type IV secretion: the Agrobacterium VirB/D4 and related conjugation systems. Biochimica et Biophysica Acta 1694: 219–234

Rikihisa, Y. and S. Ito. 1979. Intracellular localization of *Rickettsia tsutsugamushi* in polymorphonuclear leukocytes. J. Exp. Med. 150: 703-708

Schaffer, Alejandro A., L. Aravind, Thomas L. Madden, Sergei Shavirin, John L. Spouge, Yuri l. Wolf, Eugene V. Koonin, and Stephen F. Altschul. 2001. Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. Nucleic Acids Res. 29: 2994-3005.

S. Kasuya, I. Nagano, T. Ikeda, C. Goto, K. Shimokawa, and Y. Takahashi. 1996. Apoptosis of lymphocytes in mice induced by infection with Rickettsia tsutsugamushi. Infect. Immun. 64: 3937-3941.

S. W. Kim, K. S. Ihn, S. H. Han, S. Y. Seong, I. S. Kim, and M. S. Choi, 2001. Microtubule- and Dynein-mediated movement of *Orientia tsutsugamushi* to the microtubule organizing center. Infec. Immun. 69: 494-500

Shinji, M., Tetsuhiko, S., and Hajime, I. 2000. Genes for the Type IV Secretion System in an Intracellular Symbiont, *Wolbachia*, a causative

agent of various sexual alterations in arthropods. Journal of Bacteriology 182: 6529-6531

Suleyman Felek, Haibin Huang, and Yasuko Rikihisa. 2003. Sequence and expression analysis of *virB9* of the Type IV secretion system of *Ehrlichia canis* strains in ticks, dogs, and cultured cells. Infec. Immun. 71: 6063-6067

Tamura, A., and H. Urakami. 1981. Easy method for infectivity titration of *Rickettsia tsutsugamushi* by infected cell counting. Nippon Saikingaku Zasshi. 36: 783-785

Tanaka, J., Suzuki, T., Mimuro, H., and Sasakawa, C. 2003. Structural definition on the surface of *Helicobacter pylori* type IV secretion apparatus. Cell. Microbiol. 5: 395-404

Tsuruhara, T., Urakami, H., and Tamura, A. 1982. Surface morphology of Rickettsia tsutsugamushi-infected mouse fibroblasts. Acta Virol. 26: 506-511

Urakami, H., Tsuruhara, T., and Tamura, A. 1983. Penetration of Rickettsia tsutsugamushi into cultured mouse fibroblasts (L cells): an electron microscopic observation. Microbiol. Immunol. 27: 251-263

Urakami, H., Tsuruhara, T., and Tamura, A. 1984. Electron microscopic studies on intracellular multiplication of *Rickettsia tsutsugamushi* in L cells. Microbiol.Immunol. 28: 1191-1201

Vogel, J. P., H. L. Andrews, S. K. Wong, and R. R. Isberg. 1998. Conjugative transfer by the virulence system of *Legionella pneumophila.* Science 279: 873-876

Watt, G., and Strickman, D. 1994. Life-threatening scrub typhus in a traveler returning from Thailand. Clin. Infect. Dis. 18: 624-626

Winans, S. C., Burns, D. L., and Christie, P.J. 1996. Adaptation of a conjugal transfer system for the export of pathogenic macromolecules. Trends Microbiol. 4 : 64-68

Ⅶ. 적 요

쯔쯔가무시병의 원인균인 *Orientia tsutsugamushi*는 절대 세포내 기생세균이다. Orientiae는 환자와 실험동물의 혈관 내피세포, 대식세포, 다형핵 백혈구. 림프구 등 다양한 세포에 감염한다. 포식작용에 의하여 식포 내로 들어간 orientiae는 세포질로 빠져나와 분열한다. Type Ⅳ Secretion System은 세균의 접합계와 유연관계가 높으며, 세균에 의하여 생성된 작용 분자를 표적세포로 전달하는 역할을 한다. Orientiae의 유전체 염기서열을 살펴본 결과 두개의 다른 유전자 좌위에 T4SS의 구성성분과 유사한 단백질을 암호화하는 11개의 유전자(*virB3*, *virB4*, *virB6*, *virB8*, *virB9*, *virB10*, *virB11*, *virD4*)가 존재함을 밝혔다. 이 중 *virB8*, *-B9*, *-B10*, *-B11*, *-D4* 는 하나의 mRNA로 전사됨을 밝혔다. *O. tsutsugamushi*에서 몇몇의 *vir* 유전자들이 L929 섬유아세포와 J774A.1 대식세포에서 발현되었고, 실험동물 C3H/HeN 생쥐에서 발현됨을 밝혔다. *virB4*와 *virB10* 유전자의 mRNA는 감염과정에 따라 다르게 발현됨을 보았다. 반면, 56-kDa 외막 단백질 유전자인 *ts1*은 감염과정 동안 동일하게 발현되었다. T4SS 유전자들의 발현이 감염과정 동안 다르게 조절되는 것은 아마도 숙주의 제 Ⅰ형 인터페론 반응과 상관관계가 있을 것이다.

감사의 글

대학원 생활동안 참 많은 것을 배웠습니다. 새로운 나를 만들기 위한 과정이었고, 이제 막 시작점을 지났다고 생각합니다. 아직은 출발선에 서 있지만, 앞으로 더욱 열심히 하여 많은 분들의 도움에 보답하겠다 다짐합니다

저에게 있어 2년간의 대학원 생활이란 제 인생의 큰 전환점을 준 고마운 시간들이었습니다. 어릴 적부터 관심 있던 분야라 막연히 공부하고 싶다는 생각만으로 찾아왔던 저에게 배움의 기회를 주신 고영상 교수님께 진심으로 감사의 말씀을 올립니다. 또한 논문의 부족한 부분을 점검해 주신 박덕배 교수님, 이근화 교수님께도 감사 드립니다. 그리고 대학원 과정동안 늘 관심과 애정 어린 조언을 해주신 현진원 교수님, 김수영 교수님, 홍성철 교수님, 배종면 교수님, 정영배 교수님, 강현욱 교수님, 이봉희 교수님, 은수용 교수님, 조수현 교수님, 유은숙 교수님, 강희경 교수님께도 감사의 말씀 올립니다.

실험의 기초도 모르는 저에게 많은 것을 가르쳐 주신 윤지현 선생님, 늘 격려해주시고 아낌 없는 조언을 주신 희경 선생님, 영미 선생님, 원종 오빠, 변경희 선생님, 안춘산 선생님, 정일이, 경진이, 양영자 선생님, 근희 언니를 비롯한 많은 실험실 선생님들, 대학원 행정업무를 도와주신 이정희 선생님과 정명선 선생님께 감사 드립니다. 마음의 벗이 되어준 재희, 혜자 언니, 경아, 우리 진영이, 윤정, 지영, 은희 선생님, 지윤, 보경... 앞으로도 늘 지금처럼 변함없이 지낼 수 있기를 바라고, 지면으로나마 감사의 마음을 전합니다. 2년간 연락이 뜸했던 나를 이해해준 내 친구 민경, 양선, 은실, 힘들 때마다 위로해주느라 애써준 동안 오빠, 행우 오빠, 형훈 오빠.. 고마워요.

끝으로 부족한 딸을 이끌어주신 하느님께 감사 드리며, 말없이 믿고 지켜봐 주신 부모님과 윤호, 창호 오빠, 막둥이 정미에게 끝없는 고마움과 사랑을 전합니다. 처음과 같이 이제와 항상 영원히 행복하기를 기도합니다.