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Immune responses and early transcriptional profiles of murine macrophage following *Brucella abortus* internalization-defective mutant infection

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Immune responses and early transcriptional profiles of murine macrophage following *Brucella abortus* internalization-defective mutant infection

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Abstract

Immune responses and early transcriptional profiles of murine macrophage following *Brucella abortus* internalization-defective mutant infection

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Brucella abortus is the etiologic agent of bovine brucellosis and causes a chronic disease in humans known as undulant fever. In livestock, the disease is characterized by abortion and sterility. A characteristic of facultative intracellular bacteria is its ability to survive and even replicate within phagocytes. Because this bacterium is able to find a niche where they are inaccessible to circulating antibodies, their elimination requires the mechanism of cellular immunity, which

is very different from the mechanisms of defense against extracellular bacteria. Most important cytokine for cellular immunity is INF-γ produced from Th1 cell.

In the present study, an attempt was made to find out a novel antigen from the Korean field isolates by serological analysis and difference in IFN-γ production between the splenocytes of mice inoculated with outer membrane proteins (OMPs) of *B. abortus* and the whole live bacteria. Our results showed that the OMP-inoculated group showed more IFN-γ production than the whole bacteria-infected group, suggesting that OMPs are candidates for the induction of immune response.

The pathogenic mechanism of brucellosis is not fully understood except several virulence factors such as the VirB system and BvrR/BvrS two-component regulatory systems. In light of this, the present study was designed to reveal the bacterial factors which are related with host cellular internalization, a first step of the survival strategy of this bacterium. Moreover, the transcriptional responses in macrophage against bacterial infections including internalization-defective mutants were analyzed to understand the survival strategies of this bacterium in the host macrophages. To define these bacterial proteins, random insertion mutants of *B. abortus* were generated by the Tn5 transposome complexes. In all, 132 mutants were screened, cellular internalization-defective mutants were selected, and these genomic and envelope proteomic features were identified.

The transposon insertion sites were *ccmC*, *ppk* and BruAb2_0168 for the mutant C10, C29 and D7, respectively. Mutant C10 showed a deficiency in internalization without any changes in expression of the cell envelope proteins, however, mutant C29 showed a reduced expression of OMP25, and a mutant D7 also showed reduced expression of OMP25, OMP28 and Porin2b. These results suggest that OMP25 might not be an essential factor, but involved in host cellular internalization. The *ppk* gene and BruAb2_0168 locus which are associated to expression of OMP25, OMP28 and Porin2b as well as pleiotropic effects of *ccmC* gene were identified.

In the host cellular responses, compared to uninfected macrophage, both of the wild type and mutant-infected macrophage showed increased expression levels in proinflammatory cytokines, chemokines, apoptosis and G-protein coupled receptors (*Gpr84*, *Gpr109a* and *Adora2b*), while decreased in the genes related with small GTPase which mediates intracellular trafficking. Moreover, cytohesin 1 interacting protein (*Cytip*) and genes related to ubiquitination (*Arrdc3* and *Fbxo21*) were also decreased, suggesting the survival strategy of this bacterium. It was very difficult to clarify the alterations of host cellular transcription in response to infection between internalization-defective mutants and wild type. However, several novel gene changes related to the GPCR system (*Gpr84*, *Gpr109a* and *Adora2b*), ubiquitin-proteosome system (*Arrdc3* and *Fbxo21*), and

growth arrest and DNA damages (*Gadd45*) in response to *B. abortus* infection were found.

These findings suggested that a new diagnositic method should be developed using field isolates circulating in each country and provided a better understanding of the molecular mechanisms of the underlying host-pathogen interactions and need to be studied further.

Keyword: *Brucella abortus*, internalization, mutant, transcription, macrophage, gamma interferon, outer membrane protein, envelope protein

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List of Abbreviations

ACRONYM	FULL NAME
BCV	*Brucella* containing vacuole
BLAST	Basic local alignment search tool
cAMP	Cyclic adenosine monophosphate
CE	Cell envelope
CFU	Colony forming unit
CG	Cyclic β1,2-glucan
CXCR4	C-X-C chemokine receptor 4
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
DNA	Deoxy ribonucleic acid
EEA1	Early endosomal antigen 1
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPCR	G-protein coupled receptor
IFN-γ	Interferon-gamma
IL-1β	Interleukin 1-beta
LAMP-1	Lysosomal associated membrane protein-1
LC-ESI	Liquid chromatography-electrospray ionization
LPS	Lipopolysaccharide
ME	Mosaic end
MOI	Multiplicity of infection
MS	Mass spectrometry

ACRONYM	FULL NAME
NK cell	Natural killer cell
NVRQS	National Veterinary Research and Quarantine Service
OD	Optical density
OMP	Outer membrane protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
Ptl	Pertussis toxin liberation
RGS2	Regulator of G protein signaling 2
RILP	Rab7 interacting lysosomal protein
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPSS	Statistical package for social sciences software
TLR	Toll like receptor
TNF-α	Tumor necrosis factor-alpha
T4SS	Type 4 secretion system
2DE	2 dimensional gel electrophoresis

I. Literature Review

1. *Brucella*

The genus *Brucella* belongs to the family *Brucellaceae* within the order *Rhizobiales* of the class *Alphaproteobacteria*, which is one of the largest and most diverse groups within the phylum *Proteobacteria*. It is small, non-motile, non-encapsulated coccobacilli, which function as facultative intracellular parasites. It is a causative agent of brucellosis, which has a worldwide impact in terms of its epidemiology, human health risks and its impact on trade. In domestic ruminants, the disease is manifested mostly as abortion and infertility whereas undulant fever, osteoarthritis, endocarditis and several neurological disorders in human, and may leave severe sequelae if untreated.

2. Taxonomy and classification

Initially, two species in the genus *Brucella* was designated by Meyer and Shaw (1920). *B. melitensis*, the causative agent of Malta-fever and *B. abortus*, the etiological agent of bovine brucellosis characterized by abortion. Melitensis is

the Latin name for the island Malta, the region where the disease has been described for the first time. Abortus was designated after the discovery that the organism was responsible for abortion of cattle. Later names were given to the main source of isolation, preferred natural host. After that, the third *Brucella* species *B. suis*, was added in 1929 (Huddleson, 1929), followed by *B. ovis* in 1956 (Buddle, 1956), *B. neotomae* in 1957 (Stoenner and Lackman, 1957) and by *B. canis* in 1968 (Carmichael and Brunner, 1968). These species are today known as the classical *Brucella* species (Hogler *et al.*, 2012). Two species from marine mammal were isolated and named *B. ceti* and *B. pinnipedialis* whose preferred host was cetaceans and seals, respectively (Foster *et al.*, 2007). Then, *B. microti*, isolated form systemically diseased common voles (Scholz e*t al.*, 2008) and *B. inopinata* isolated from an inflamed breast implant of a 71-year-old patient in the USA with clinical signs of brucellosis were published as a novel species.

Although the members of the genus *Brucella* showed more than 94% of similarity with each other, they have different host preference and zoonotic potentials (Table I). Little is known about the genetic basis of *Brucella* virulence, and nothing on the basis of host preference.

Table I. Zoonotic potential and host preference of *Brucella* species

Species	Zoonotic potential	Host preference
Brucella melitensis	High	Sheep, goat
Brucella abortu	Moderate	Cattle
Brucella suis	Moderate	Pig
Brucella canis	Mild	Dog
Brucella ovis	Absent	Sheep
Brucella neotomae	Absent	Desert wood rat (*Neotomae lepida*)
Brucella ceti	Mild	Cetaceans
Brucella pinnipedialis	Mild	Seals
Brucella microti	Absent	Common voles (*Microtus arvalis*)
Brucella inopinata	Moderate	Human

Adapted from Xavier *et al.*, 2010

3. Pathogenic mechanisms

3-1. Overview

Unlike other pathogenic bacteria, brucellae do not produce exotoxins, cytolytic enzymes, and do not present antiphagocytic capsules, toxic lipopolysaccharides, thick cell walls, resistant forms, fimbriae or plasmids (Finlay & Falkow, 1989). Rather than producing classical virulence factors, brucellae can invade and replicate in host phagocytic cells of the reticuloendothelial system as well as in non-phagocytic cells such as trophoblast (Xavier *et al.*, 2010), thus avoiding the bactericidal effects of the host cell. Especially, brucellae have a predilection for reproductive tissues (Olsen *et al.*, 2010). And they can use various mechanisms to modify the host environment, to resist oxidative killing, and to modify their metabolism to survive (Olsen *et al.*, 2010). This ability involves a temporary fusion of the *Brucella*-containing vacuole with the lysosome, and subsequent exclusion of the lysosomal proteins (Xavier *et al.*, 2010). Finally, they associate with the endoplasmic reticulum (ER) membranes to form a replicative vacuole (Rambow-Larsen *et al.*, 2009). As a result, they may persist in the host and chronic infection becomes established (Corbel, 1997; Pizarro-Cerda´ *et al.*, 1998 & 2000).

3-2. Target cells and site

The major target cells for *Brucella* are macrophages, dendritic cells and trophoblasts especially in placenta. The symptom of brucellosis is characterized by persistent infectious in lymphoid tissue and inflammatory lesions in the reproductive tract of pregnant females (Xavier *et al.*, 2010). Localization in the reproductive or mammary gland is associated with the most severe pathology and capability to transmit infection. During pregnancy, hosts are more susceptible to *Brucella* infection however the mechanism for the increased susceptibility during pregnancy is not fully understood (Olsen *et al.*, 2010).

In order to reach its target cells, *Brucella* needs to cross mucosal barriers of the respiratory, genitourinary or digestive tract, where it undergoes phagocytosis by resident macrophages and dendritic cells (DCs). Subsequently, the bacteria are disseminated to lymphoid and reproductive organs (Anderson *et al.*, 1986; Ackermann *et al.*, 1988). Especially, for the bacteria invaded via the digestive tract, M cells are associated with epithelial transmigration of bacteria preferentially as well as the intra-epithelial phagocytes which may also transport *Brucella* from the intestinal lumen to the lamina propria (Ackermann *et al.*, 1988; Salcedo *et al.*, 2008; Paixão *et al.*, 2009).

Finally, *Brucella* reached to the uterus and fetus which are the most common clinical manifestation of brucellosis in experimental and natural hosts. And *Brucella* gain accessed via a hematogenous route, and the bacteria initially localize within erythrophagocytic trophoblasts of the placentome. As a result, adjacent chorioallantoic trophoblasts become infected to *Brucella*; result in an inflammatory resoponses (Olsen *et al.*, 2010). Eventually, infected trophoblasts may rupture and ulcerate the chorioallantoic membrane. In addition to the hematogenous route, fetuses may also become infected via ingestion of amniotic fluid containing *Brucella* (Olsen *et al.*, 2010). The endothelium is not infected with *Brucella* and remains largely intact except a diffuse submucosal inflammatory reaction.

Thus, for the most *Brucella* strains, the bacteremia is short-lasting, although they are infected and reach to the final destination via hematogenous route. This is the reason why the bacteria are not readily isolated from blood samples of infected individuals. While several virulence factors have been identified using *in vitro* models, many of these factors may have little or no *in vivo* relevance because of the complex microenvironment of the host.

3-3. Entry into host cells

In macrophages, *Brucella* is internalized by phagocytosis that requires a moderate recruitment of actin filaments. During the interaction of *Brucella* and receptors on the surface of the macrophage cell membrane, actin filaments are activated (Campbell *et al.*, 1994).

Opsonized and non-opsonized *Brucella* has different internalization mechanisms. Opsonization targets *Brucella* to specific receptors on the surface of macrophages (Fc receptor for bound IgG, complement receptors for C3b and C4b) whereas internalization of the non-opsonized smooth *Brucella* relies on the cytoskeleton of the host cell such as lectin and fibronectin receptors (Harmon *et al.*, 1988; Campbell *et al.*, 1994). Smooth strains of *Brucella* interact with cholesterol-rich lipid raft within the plasma membrane to facilitate contact with the host cell and mediate internalization by phagocytes as well as nonprofessional phagocytes. Lipid rafts are cholesterol-rich microdomains which contain glycosphingolipids, cholesterol, and glycosyl-phosphatidylinositol anchored protein (Cutler *et al.*, 2005). These rafts contribute to directing intracellular trafficking of bacteria via facilitating membrane-associated sorting events, such as the formation of multi-subunit membrane complexes, and signaling across membranes and membrane fusion (Kim *et al.*, 2004; Lapaque *et*

al., 2006) and they are found in plasma membranes as well as intracellular organelles, vesicles. O-polysaccharide chain on LPS of smooth *Brucella* appears to be a key molecule for interaction with lipid rafts on host cells but also prevents complement-mediated bacterial lysis and host cell apoptosis (Jiménez de Bagüés *et al.*, 2004).

Entry of rough strains of *Brucella* differs from smooth strains. The rough strains lack O-polysaccharide chain which makes interactions with lipid raft impossible. Instead, they are readily phagocytosed following either Toll-like receptor 4 (TLR4) or mannose receptor recognition of the LPS-deficient bacterial surface. As a result, rough strains demonstrate elevated invasions due to exposure of ligands and increased capability to adhere to macrophage (Jiménez de Bagüés *et al.*, 2004) however; they are rapidly targeted to the phagolysosomal compartment and finally unable to replicate (Olsen *et al.*, 2010).

Currently, opsonized *Brucella* or interferon-gamma activated macrophages leads to greater killing of internalized *Brucella* whereas non-opsonized *Brucella* can survive and replicate inside cells (Jones and Winter, 1992; Eze *et al.*, 2000).

3-4. Intracellular trafficking

Once *Brucella* has been internalized via non-opsonized, lipid raft mediated entry; they are trafficked through the vesicle sharing markers with an early endosome. This forms an early BCV (*Brucella* containing vacuole) around 5-10 min after infection, and transiently acquiring Rab5 and EEA-1 (Early endosomal antigen) (Fig. I) (Celli, 2005). Then, BCVs mature into acidic intermediate vacuoles that accumulate LAMP-1 (Lysosomal associated membrane protein-1), but not Rab7, avoiding interactions with late endosomes and fusion with lysosomes via LPS (Lipopolysaccharide) and cyclic β-glucan-depedent mechanisms (Celli, 2005). The intermediate BCVs interact with ER (Endoplasimc reticulum) exit sites. These interactions occur around 2-8 h post infection and require the VirB type IV secretion system (Celli, 2005). Consequently, an ER-derived organelle permissive for bacterial replication is generated at 12h post infection. The replicative BCVs exclude LAMP-1 and acquire various ER markers (calnexin, calreticulin, sec61β, PDI), as a result of membrane exchange with the ER (Celli, 2005). At this point, the pH of the *Brucella*-containing compartment increase to 5.5-6, becomes more permissive for replication.

However, vacuoles containing VirB-deficient *Brucella* cannot sustain interaction and fuse with lysosomes instead of the ER, result in generation phagolysosome and destroy of the bacteria.

Unlike the un-opsonized *Brucella*, opsonized *Brucella* has different intracellular lifestyle. Within minutes after internalization, similar to that observed in un-opsonized *Brucella*, opsonized *Brucella* are found within the early endosomal compartment defined by the presence of the small GTPase Rab5, early endosomal antigen 1 (EEA1) and the transferrin receptor (Gorvel and Moreno, 2002). The pH of *Brucella* containing vesicles changes from ~5.5 of the early endosome to pH < 4 by 2 hours post infection with the acquisition of the regulator Rab7 that recruits the late endosomal effectors LAMP-1, cathepsins (Bellaire *et al.*, 2005), vacuolar H^+-ATPase and rab7 interacting lysosomal protein (RILP) where the 60% of the bacteria are degraded (Ritchie, 2011).

Bacterial entrance, survival and replication have been intensively investigated in phagocytes, but these mechanisms are poorly characterized in trophoblasts, which represents an important gap in our understanding of the disease and transmission in the natural host species.

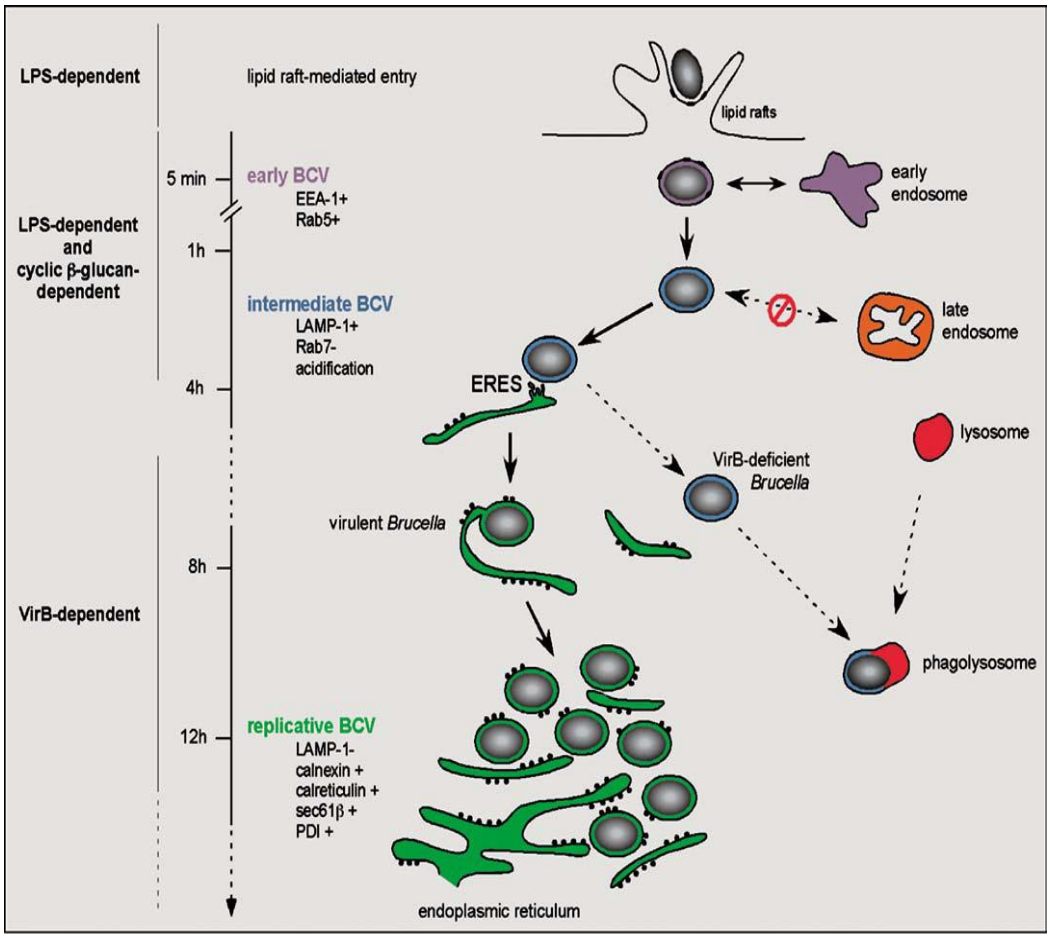


Fig I. Intracellular trafficking of *Brucella* in macrophage. Once internalized, *Brucella* is trafficked through a vesicle sharing markers with an early endosome. Later the *Brucella* is found in a compartment resembling a late endosome where a majority of the bacteria will be killed. The surviving bacteria will replicate in a vesicle sharing markers with the endoplasmic reticulum.

Adapted from Celli *et al.*, 2006

4. Virulence factors of *Brucella*

4-1. Type IV secretion system (VirB)

The type IV secretion system is homologous to conjugation machinery of bacterium and it is capable of transporting both DNA and proteins. The *Brucella* type IV secretion system (T4SS, VirB), encoded by the *virB1–virB12* genes, is required for intracellular growth of *Brucella* in phagocytic and non-phagocytic cells (Xavier *et al.*, 2010). It was first discovered in *Agrobacterium tumefaciens*, which uses this system to introduce the Ti plasmid and/or proteins into the host, and later on, it was recognized as virulence factor of pathogen such as *Bordetella pertussis*, *Legionella pneumophila* and *Helicobacter pylori* (Xavier *et al.*, 2010). In *Brucella*, T4SS is a major virulence determinant which has been shown to be essential for sustaining interaction and fusion events between BCVs and ER element (Celli *et al.*, 2006), since the *Brucella virB* mutant strain was not able to reach the endoplasmic reticulum. Translocation of effector proteins into the host cell or vacuolar membrane through T4SS likely to modulate host vesicular traffic, however, the molecules secreted by T4SS remained elusive (Marchesini *et al.*, 2011). Recently, de Jong and his colleague identified VceA and VceC, two members of the VjbR regulon that are translocated into macrophages by the

Brucella T4SS (de Jong *et al.*, 2008). They also suggested that T4SS of *Brucella* apparently does not secret DNA (de Jong *et al.*, 2008). Moreover, Marchesini *et al.* determined four proteins that are delivered to the eukaryotic cytoplasm upon infection, encoded by genes BAB1_1043, BAB1_2005, BAB1_1275 and BAB2_0123, require a functional T4SS for their delivery (Marchesini *et al.*, 2011). However, the precise route of translocation and translocation signals that are required for its transfer to the host cell are still remain elusive. As the T4SS are well conserved in the class alphaproteobacteria, such as *Bartonella* and *Rhizobium*, the information and relationship of T4SS to these bacteria might be useful to reveal the clue related with T4SS of brucellae.

4-2. BvrR-BvrS two component regulatory system

The BvrR-BvrS two component regulatory system is required for *Brucella* pathogenesis and is highly conserved within the group of α-Proteobacteria both in the gene sequence and the loci where they are found, including the ChvI-ChvG regulatory system in *Agrobacterium* which is important for interaction with the host plant (Rambow-Larsen *et al.*, 2009). Transposon insertion mutant of *Brucella* either in *bvrR* or *bvrS* showed deficiency in internalization and trafficking in cellular infection models as well as in mouse infection models (Rambow-Larsen *et al.*, 2009). These mutants were unable to inhibit lysosome fusion and replicate intracellularly (Rambow-Larsen *et al.*, 2009).

Mutation of BvrR/BvrS hinders the production of outer membrane protein such as Omp25 and Omp22, result in reduced resistance of *Brucella* to bactericidal polycations and increases its permeability to surfactants (Rambow-Larsen *et al.*, 2009). Furthermore, the recent results from proteome and transcriptome analysis show that the BvrR/BvrS system of *Brucella* is a master regulator capable of interacting with other regulators, such as ABC transporters, lipoproteins, stress proteins, chaperones, transcriptional regulators, metabolisms, cell envelopes and virulences (Fig. II).

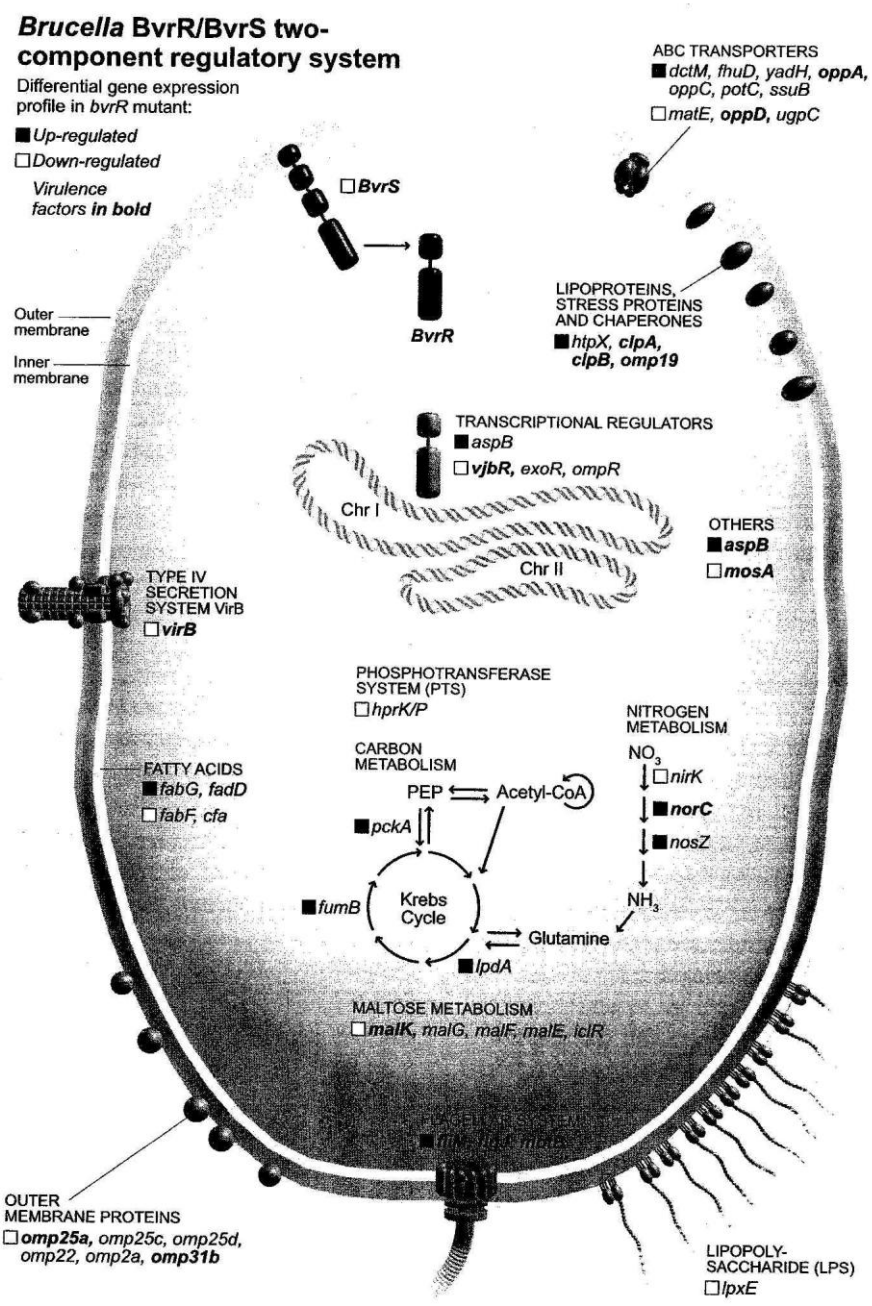


Fig II. The *Brucella* BvrR/BvrS two-component regulatory system.

Adapted from López-Goñi, 2012

4-3. Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) is a major component of the *Brucella* outer membrane (OM) which is essential for the functional and structural integrity as well as in the other Gram-negative bacteria (Fig III). Depending on the presence or absence of O-polysaccharide chains (O-PS), *Brucella* could be classified in smooth species which occurs as smooth LPS (S-LPS) and in rough species, such as *B. canis* and *B. ovis*, which occurs as rough LPS (R-LPS). As described previously, the O-PS carries the major antigenic determinants involved in the humoral responses. Moreover, the LPS are another virulence factor of *Brucella* that contributes to initial survival of bacteria in macrophages (Xavier *et al.*, 2010). As described previously, the S-LPS interact with lipid rafts of host cell and internalized by a way of non-opsonized pathway. The rough mutant derived from smooth strains generally invade host cells more efficiently than smooth strain but showed less ability to survive within the host cell suggesting the role of S-LPS in the early development of the *Brucella* containing phagosome and inhibition of phagosome-lysosome fusion (Xavier *et al.*, 2010). However, some rough strains are naturally virulent.

The genes involved in *Brucella* O-PS biosynthesis were identified by transposon mutagenesis (Table II).

Table II. Main genes involved in LPS biosynthesis in *B. suis* 1330. The genes identified were located in two major separate chromosomal loci *wbk* and *wbo*

Gene	**Locus**	**ORF in the genome of *B.suis* 1330**	**Role in biosynthesis of**	**Predicted function or characteristic**
wbkE	*wbk*	BR0540	O-PS	Mannosyltransferase
manA$_{o\text{-}Ag}$	*wbk*	BR0539	Uncertain	Mannose furnishing
manC$_{o\text{-}Ag}$	*wbk*	BR0538	Uncertain	Mannose furnishing
manB$_{o\text{-}Ag}$	*wbk*	BR0537	Uncertain	Phosphomannomutase
wbkA	*wbk*	BR0529	O-PS	Mannosyltransferase
gmd	*wbk*	BR0522	O-PS	GDP-mannose 4,6-dehydratase
per	*wbk*	BR0521	O-PS	Perosamine synthetase
wzm	*wbk*	BR0520	O-PS	ABC transporter
wzt	*wbk*	BR0519	O-PS	ABC transporter
wbkB	*wbk*	BR0518	Uncertain	Unknown
wbkC	*wbk*	BR0517	O-PS	Formylation
wbkF	*wbk*	BR0511	O-PS	Undecaprenyl-glycosyl transferase
wbkD	*wbk*	BR0510	O-PS	Epimerase/dehydratase
wboA	*wbo(GI-2)*	BR0981	O-PS	Mannosyltransferase
wboB	*wbo(GI-2)*	BR0982	O-PS	Mannosyltransferase
wa**		BR	Core	Glycosyltransferase
pgm			Core	Phosphoglucomutase
manBcore			Core	Phosphomannomutase

Adapted from Vizcaino and Cloeckaert, 2012

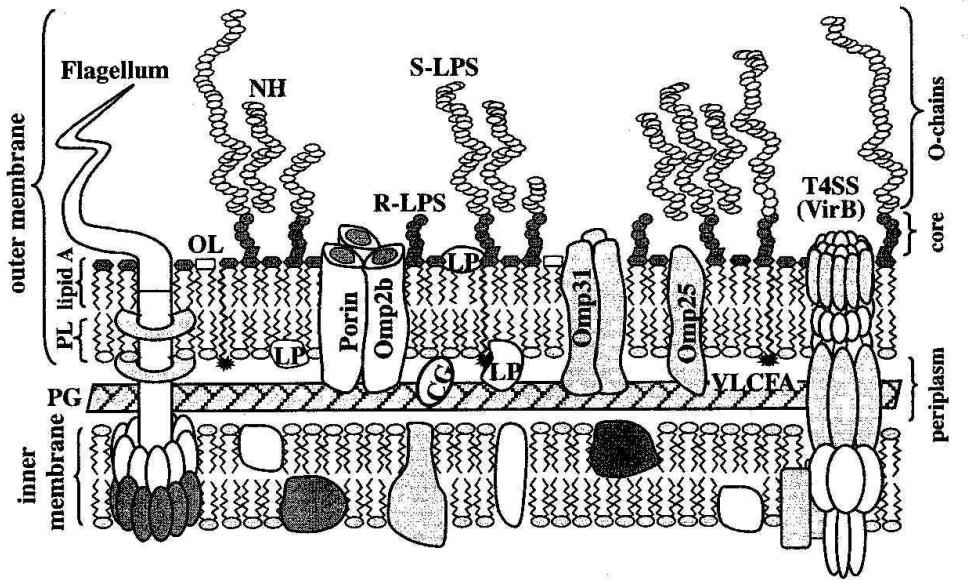


Fig III. Schematic representation of the outer membrane of a smooth *Brucella* strain. CG, cyclic glucan; LP, lipoprotein; NH, native hapten; OL, ornithine lipid; PL, phospholipid; PG, peptidoglycan; R-LPS, rough lipopolysaccharide; S-LPS, smooth lipopolysaccharide; T4SS, type IV secretion system; VLCFA, very long-chain fatty acids.

Adapted from Vizcaino and Cloeckaert, 2012

4-4. Cyclic β1,2-glucans

Glucans are component of bacterial periplasm play a role in osmoregulatory and cholesterol sequestering activity. Recent study showed that the cyclic β1,2-glucan (CG) plays important role in circumventing host cell defense (Arellano-Reynoso *et al.*, 2005). The CG was acted in lipid raft on host cell membrane and prevents the phagosome-lysosome fusions, although they did not affected in fusion between the endoplasmic reticulum and the *Brucella*-containing vacuole which is dependent on type IV secretion system.

The reason *Brucella* is such a powerful intracellular infectious bacterium is due to its ability it evade the host immune system.

4-5. Outer membrane protein (OMP)

With LPS, the outer membrane protein (OMP) of *Brucella* is located on the most external layer of the cell constituting an important barrier for survival in hostile environments and an accessible target for the interaction of bacterial pathogens with the host and the defense mechanisms of the immune system (Vizcaino and Cloeckaert, 2012).

The OMP of *Brucella* is classified by three groups. Group 1 OMPs, known as Omp89 or Omp1, were determined to be minor proteins of 88-94kDa. Minor proteins of 10kDa (Omp10), 16kDa (Omp16) and 19kDa (Omp19) were also included in group 1 OMPs (Vizcaino and Cloeckaert, 2012). Remaining two groups are major OMPs, the 36-38kDa and 25-27kDa OMPs of *B. abortus* were named group 2 and group 3 proteins, respectively (Vizcaino and Cloeckaert, 2012). Subsequently, group 2 OMPs, the 36-38kDa proteins were designated Omp2b after the gene *omp2b* of *B. abortus* was determined to be responsible for the synthesis of the 36-38kDa OMPs. The homologous gene *omp2a* was also identified close to *omp2b* in the genome (Vizcaino and Cloeckaert, 2012). For group 3 OMPs, the 25-27kDa and 31-34kDa OMPs were determined to be encoded by *omp25* and *omp 31*, respectively (Vizcaino and Cloeckaert, 2012).

Omp2b is a bacterial porin which could be advantageous for intracellular growth of *Brucella* spp. since the concentration of nutrients may be particularly low within the phagosome (Vizcaino and Cloeckaert, 2012). Omp2a is encoded by *omp2a* which share more than 85% sequence identity to *omp2b*, and they are oriented in opposite directions separated by 900bp in the genome. It shows some degree of antigenic variability among the *Brucella* strains as well as variable permeability which could be relevant for the interaction with the host and having a role in host tropism (Vizcaino and Cloeckaert, 2012).

Omp25, one of the major proteins in group3, plays important roles in intracellular replication and virulence of *Brucella spp*. although there are several studies with discrepant results. *B. abortus* Δomp25 mutant showed impaired replication inside late-gestational bovine chorionic trophoblasts and macrophage (Edmond et al., 2001). Moreover, deletion mutants of *B. abortus* 2308, *B. melitensis* 16M and *B. ovis* LSU99 were shown to be attenuated in the mouse model as determined by the splenic colonization along a 20-week period (Edmonds *et al.*, 2002) and member of the OMP25 family has previously been implicated in *Brucella ovis* internalization by host cells (Martín-Martín *et al.*, 2008). However, the splenic infection level reached in mice by another Δomp25 mutant of *B. abortus* 2308 was equivalent to that of the parental strain (Manterola *et al.*, 2007), and another study (Jubier-Maurin *et al.*, 2001) reported

that OMP25 was not required for internalization of *B. suis* by human macrophages.

Thus, the exact functions involved in the pathogenicity and host tropism characteristics of the OMPs remains to be elucidated.

Table III. Main genes involved in OMP biosynthesis in *B. suis* 1330

Gene	**ORF in the genome of *B.suis* 1330**	**Predicted function or characteristic**
omp2a	BR0637	Porin (experimental evidence); not synthesized in *Brucella* spp.
omp2b	BR0639	Porin (experimental evidence)
omp31	BRA0423	Omp25/Omp31 family. Porin
omp25	BR0701	Omp25/Omp31 family
omp31b	BR1622	Omp25/Omp31 family
omp25b	BR0971	Omp25/Omp31 family
omp25c	BR0119	Omp25/Omp31 family
omp25d	BR0118	Omp25/Omp31 family
omp22	BR1284	Omp25/Omp31 family
omp10	BRA0077	OM lipoprotein
omp16	BR1695	OM lipoprotein
omp19	BR1930	OM lipoprotein
bepC	BR0945	Efflux pump OMP component
ugpB	BRA0655	Adhesin/invasion SP41

Adapted from Vizcaino and Cloeckaert, 2012

II. General Introduction

Recently, infectious disease in animals including zoonotic disease induced social and economic disaster. Brucellosis, a term used to describe a disease caused by the members of the genus *Brucella* in animal and human, has been received attention in worldwide because of social and economic impact on animal and human. The genus *Brucella* is small Gram-negative facultative intracellular pathogens which can multiply within professional and nonprofessional phagocytes (Detilleux *et al.*, 1990a,b). Unlike the other intracellular bacteria, *Brucella* do not produce exotoxins, cytolytic enzymes and do not have antiphagocytic capsules, thick cell walls, resistant forms, fimbriae, or plasmids (Finlay and Falkow, 1997). For many years, the diagnosis of brucellosis depended on direct isolation and identification of the agent or serological approaches. However, culturing the zoonotic pathogen *Brucella* is hazardous and the serological tests targeting lipopolysaccharide (LPS) have several problems (Al Dahouk *et al.*, 2006) though the LPS from smooth *Brucella* strain induced strong immune responses (Al Dahouk *et al.*, 2003). The O-polysaccharide of LPS chain can elicit cross-reactivity with *Yersinia enterocolitica* O:9, *Salmonella urbana* group, *Vibrio cholera*, *Francisella tularensis*, *Escherichia coli* O:157 and *Stenotrophomonas maltophilia* resulting

in low specificity of the diagnosis (Caroff *et al.*, 1984). Moreover, anti-LPS antibodies may persist for more than a year after acute brucellosis (Almuneef and Memish, 2003) as well as the misdiagnosis of the rough strain *B. canis* and *B. ovis* infection. To overcome these problems, many researchers have tried to develop a new specific antigen with LPS free proteins (Cespedes *et al.*, 2000; Al Dahouk *et al.*, 2006). As protein expression profiles could be changed through endemic environments or growth conditions, the authors of several ongoing studies are making use of local field isolates (Cloeckaert *et al.*, 1995; Tcherneva *et al.*, 2000; Ridler *et al.*, 2005). For the elimination of the intracellular bacteria, cellular immunity is required, which is very different from the mechanisms of defense against extracellular bacteria. Among the cytokines for cellular immunity, the INF-γ produced from Th1 cell is the most important in macrophage activation and production of some antibody isotypes.

To control the brucellosis effectively, a new diagnostic method should be developed. This could be facilitated by understanding both the virulence of *B.abortus* on a genetic level and the host responses against the microorganism. For *Brucella*, internalization by macrophages occurs through a zipper-like mechanism via complement and Fc receptors, followed by opsonization, whereas un-opsonized brucellae also internalize via lectin or fibronectin receptors (Harmon *et al.*, 1988; Campbell *et al.*, 1994). In macropinocytosis, involving

lipid raft-associated molecules, a swimming internalization mechanism with generalized membrane ruffling may also be linked to its fate in macrophages (Watarai *et al.*, 2002). Although several virulence factors such as the VirB system and BvrR/BvrS two-component regulatory system have been discovered, the molecular mechanism of invasion by *Brucella* is not fully understood (Sieira *et al.*, 2000; Guzman-Verri *et al.*, 2002).

Thus, to gain more insight into these findings of antigenicity of local field isolates and pathogenic mechanisms of *B. abortus*, the first part of our study was focused on the expression profile and immune responses of outer membrane proteins (OMP) from local field isolates. The OMP expression profiles of field isolates to standard strain 1119-3 were compared. Moreover, the interferon (IFN)-γ production from mice inoculated with OMPs of *B. abortus* or whole live bacteria were evaluated. At the second chapter, as a part of finding virulence factors of *B. abortus*, random insertion mutants were generated and host cellular internalization-defective mutants were selected. Differences between cell envelope proteins of mutants and standard strain were analyzed. Finally, in continuation to our work of generation and envelope protein analysis of internalization-defective *B. abortus* mutants, the early transcriptional responses in macrophage infected with these mutants were investigated.

III. Main text

Chapter 1

Comparative study of gamma interferon production in mice immunized with outer membrane proteins and whole bacteria of *Brucella abortus*

Abstract

Brucella abortus is the intracellular bacteria that cause bovine brucellosis and a chronic human disease known as undulant fever. Interferon-γ plays critical roles in defending against intracellular bacterial infection. In this experiment, the difference in IFN-γ production, from the splenocyte of mice inoculated with outer membrane protein (OMP) of *B. abortus* and whole live bacteria were demonstrated. OMP inoculated group showed more IFN-γ production than bacteria infected group suggesting that OMPs could be one of candidates for the induction of immune response.

Key words: *Brucella abortus*, IFN-γ, outer membrane protein (OMP)

1.1. Introduction

Brucellosis is a zoonosis caused by bacteria of the genus *Brucella*. These bacteria are non-motile, non-spore-forming small Gram-negative rods. Facultative intracellular organisms that are very difficult to isolate, they have a long latent period that makes early diagnosis impossible, once infected. These microbes are able to grow in phagocytes, where they are inaccessible to bactericidal agents including circulating antibodies, and are very different from the mechanisms of defense against extracellular bacteria (Oliveira *et al.*, 1998).

Most widely used serological diagnostic methods are based on lipopolysaccharide (LPS), whole cell preparation and cell sonication extracts. LPS of the smooth *Brucella abortus* strains induces especially strong antigenic responses (Al Dahouk *et al.*, 2003); however, O-polysaccharide of LPS from *B. abortus* can cause a cross reaction with *Yersinia enterocolitica* O: 9, *Salmonella Urbana* group, *Vibrio cholera, and Francisella tularensis*, *Escherichia coli* O: 157 and *Stenotrophomonas ma lyophilic* (Caroff *et al.*, 1984; Nielsen *et al.*, 2004). In addition, diagnostic sensitivity could be low due to anti-LPS antibodies, which may persist long after acute brucellosis (Almuneef *et al.*, 2003). For these reasons, many researchers have tried to develop a new protective antigen with LPS free proteins (Cespede *et al.*, 2000; Al Dahouk *et al.*, 2006). As protein

expression profiles could be changed through endemic environments or the growth conditions, the authors of several ongoing studies are making use of local field isolates (Cloeckaert et al., 1995; Tcherneva et al., 2000; Ridler et al., 2005). In light of this, this study was performed to evaluate the interferon (IFN)-γ production from mice inoculated with outer membrane proteins (OMP) of *B. abortus* or whole live bacteria.

1.2. Materials and Methods

Bacterial strains

Four field isolates of *B.abortus* (1-7, 1-81, 2-55, and 3-60), *B.abortus* 1119-3, and a vaccine strain RB51 were obtained from Animal, Plant and Fisheries Quarantine and Inspection Agency and cultured for 48 h at 37 ℃ in Brucella broth (Difco Laboratories Inc., Detroit, MI, USA).

Extraction of Outer Membrane Proteins (OMP)

OMPs were extracted by the method described previously (Barenkamp *et al.*, 1981). Briefly, bacteria were harvested by centrifugation from 1L of bacterial culture. After two times washing with phosphate buffered saline (PBS, pH 7.0), cells were suspended in 30mL of 10mM HEPES buffer. And it was lysed with Sonic Dismembrator (Bandelin Electronic, Germany) at 60% power until it became clear. After sonication, supernatant were taken with centrifugation in 4,000 rpm to remove debris, and then pellets were harvested by ultracentrifugation in 20,000 rpm. The pellets were dissolved in 1mL of mixing buffer (2 % SDS, 10mM HEPES) and incubated for one hour at room temperature. Again, pellets were harvested by ultracentrifugation in 20,000 rpm and dissolved in 1mL of distilled water. Soluble supernatants were used as OMP antigen and concentration was measured by BCA™ protein assay kit (Thermo Scientific, Waltham, MA, USA).

SDS-PAGE and Immunoblotting

OMPs from each strain were separated by 12% SDS-PAGE (Soduim dodecyl sulfate-polyacrylamide gel electrophoresis). For checking their antigenicity, they were transferred to iBlot™ nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA) by iBlot™ gel transfer device (Invitrogen, Carlsbad, CA, USA).

Brucellosis positive bovine serum (1:400 in standard agglutination test), obtained from Animal, Plant and Fisheries Quarantine and Inspection Agency, was used as a first antibody and alkaline phosphatase conjugated anti-bovine IgG (SIGMA, St.Louis, MO, USA) was used as a secondary antibody at a 1:1000 dilution.

Infection of mice and preparation of splenocytes

ICR mice were purchased from ORIENT BIO Inc. (Korea). For OMPs inoculation groups, mice were immunized with 20 μg per 100 μL of OMPs by intraperitoneal injection. And for bacterial infection group, $2x10^4$ CFU per 200 μL of bacteria ($2x10^8$ CFU per 200 μL for the vaccine strain) was inoculated by intraperitoneal injection. After 1 day, 2 day and every 1 week until 5 weeks, mice were sacrificed; spleen was aseptically removed and homogenized and splenocytes were obtained by mincing the homogenates on a metal mesh. Erythrocytes were lysed with RBC lysis buffer (0.17 M NH4Cl), washed twice with Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA), and $4x10^6$ cells ml^{-1} of splenocytes were cultured in DMEM supplemented with 10 % fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) at 37 ℃, atmosphere of 5 % CO_2.

Gamma interferon induction and assay

Each group of splenocytes were stimulated with same strain's OMPs or bacteria which inoculated to the mice, concentration of 1 µg mL^{-1} for OMPs and $1x10^4$ CFU mL^{-1} for bacteria, respectively. Supernatants were taken after 12, 24, 48 and 72 hours. Concentration of interferon gamma was measured with Mouse INF-γ ELISA kit (eBioscience, San Diego, CA, USA) according to the protocol suggested by the manufacturer. The standard curve was prepared by serial dilutions of recombinant mouse INF-γ (2,000 pg mL^{-1}).

Statistical analysis

Statistical significance was determined by the Student *t* test using Statistical Package for Social Science (SPSS) software version 17.0. Differences were considered to be significant if a *p* value of < 0.05 was obtained.

1.3. Results

OMP and LPS profiles of each field isolates

OMP profiles of the *B. abortus* strains showed different protein expression patterns from each other, and antigenic reactivity with brucellosis-positive serum also showed different patterns among the strains (Fig 1.1). Every strain had major bands in 25 kDa and 36~38 kDa that differs in only amount of expression, and strain 1-81 and 2-55 showed very similar patterns. And also in antigenic reactivity with brucellosis positive serum, major bands in 36~38 kDa showed most strong reactivity except vaccine strain RB51. Likewise in OMP profiles, strain 1-81 and 2-55 showed same reactivity including 20 kDa proteins that differs from other strains. All strains used in this study were LPS positive smooth strains except RB51 and there were no significant differences in LPS patterns (data not shown).

Kinetics of IFN- γ production in mouse splenocyte and serum

The kinetics of IFN-γ production of each of the groups is shown in Fig 1.2. Most strains showed the highest IFN-γ concentration at 1 day post inoculation in

OMP-stimulated mice, and the concentration was significantly high compared with that in the bacteria-inoculated mice, except for the 1-81 inoculated mice, which showed a peak concentration at 4 week post inoculation. At 2 days post inoculation, IFN-γ production was very poor in all groups and only the 1119-3 and 2-55 OMP-stimulated mice showed significant production. Interestingly, in every strain except 1119-3, bacteria-inoculated mice showed their peak concentration at 2 weeks post inoculation. This result correlates with the IFN-γ concentration in serum shown in Fig 1.3, which started to increase at this point, indicating the strongest cellular immune responses against the bacterial infection.

From 1 week to 5 weeks post inoculation, except for 2 weeks and 4 weeks post inoculation in the 1119-3, 1-7, and 3-60 groups the OMP-stimulated group showed more IFN-γ production than did the bacteria-inoculated group, indicating that OMPs are more effective for producing IFN-γ. In both groups, there were no significant differences among the strains. Also there was no significant IFN-γ production in the negative control group.

1.4. Discussion

For immunity and protection against *B. abortus*, a host utilizes its innate and adaptive immune responses (Golding *et al.*, 2001). In innate immune responses, natural killer (NK) cells play a critical role by secreting IFN-γ, although NK cells do not play a role in innate immune responses against *B. abortus* infection in mice (Fernandes *et al.*, 1995). In adaptive immune responses, especially T cell-mediated immune response for intracellular bacteria, IFN-γ plays an important role in activating macrophages and in limiting *Brucella* infection both in vitro and in vivo (Jiang *et al.*, 1993). Because of the vital role of IFN-γ in the infection of intracellular bacteria, it is used in the diagnosis of intracellular bacterial infections such as tuberculosis (Pai *et al.*, 2004).

There is no previous report on polymorphism of *B. abortus* field isolates, except one on the polymorphism of *B. ovis* field isolates (Ridler *et al.*, 2005). Thus, this experiment was performed to analyze the IFN-γ production by spleen cells of mouse immunized by OMPs or bacteria. Our study results showed that IFN-γ production from splenocytes stimulated with OMP of each strain did not differ significantly among strains. This may have been due to the fact that the field isolates used in our experiment showed differences only in the minor OMPs and the amount of expression. Moreover, the OMP-inoculated mice showed higher production of IFN-γ compare to the bacteria-inoculated mice, indicating that OMPs are more effective than bacteria for inducing IFN-γ-mediated

immunity. In related research, subcellular fractions such as inner membrane protein and OMP of *B. ovis* induced cytokine production and delayed type hypersensitivity (Salas-Téllez *et al.*, 2005).

In summary, our experiment provides basic data for the development of an immunodiagnostic method for detecting brucellosis by measuring IFN-γ. Likewise, OMP could be a useful antigen for the eventual development of a vaccine based on the induction of cellular immune responses.

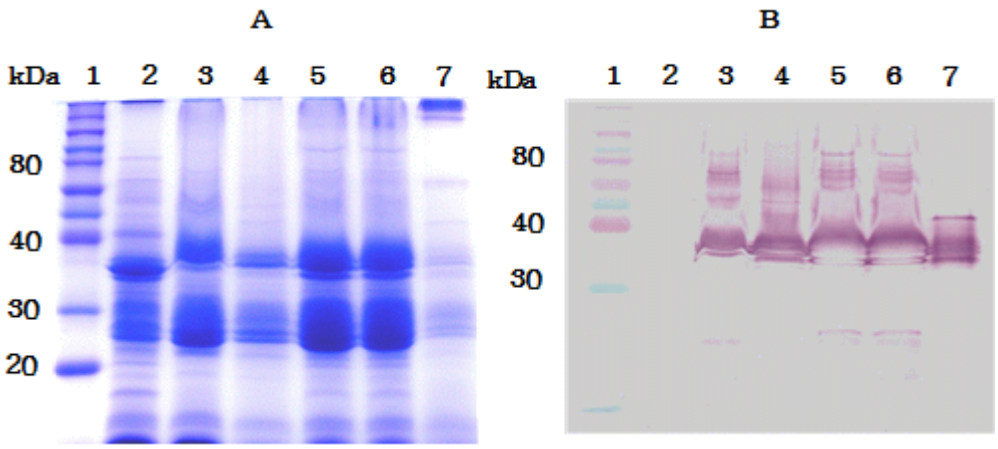
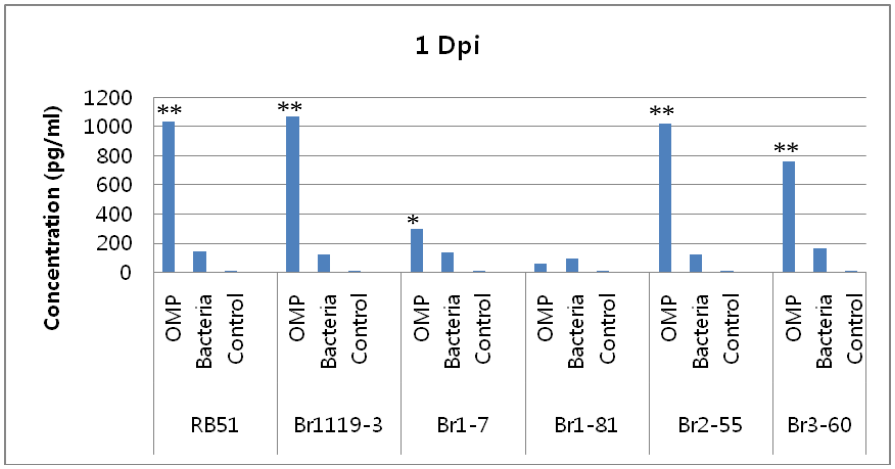


Fig 1.1. Electrophoretic and immunological analysis of outer membrane proteins extracted from *Brucella abortus* strains by SDS-PAGE (panel A) and Western blot (panel B). Lane 1, molecular weight marker (Invitrogen, USA) ; lane 2, *B.abortus* RB51 ; lane 3, *B.abortus* 1119-3 ; lane 4, *B.abortus* 1-7 ; lane 5, *B.abortus* 1-81 ; lane 6, *B.abortus* 2-55 ; lane 7, *B.abortus* 3-60.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Concentration (pg/ml)
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OMP
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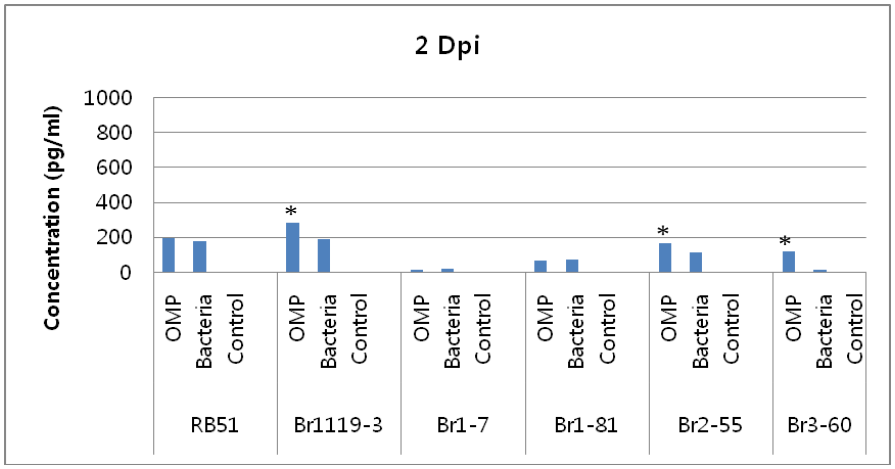
Br1119-3
Br1-7
Br1-81
Br2-55
Br3-60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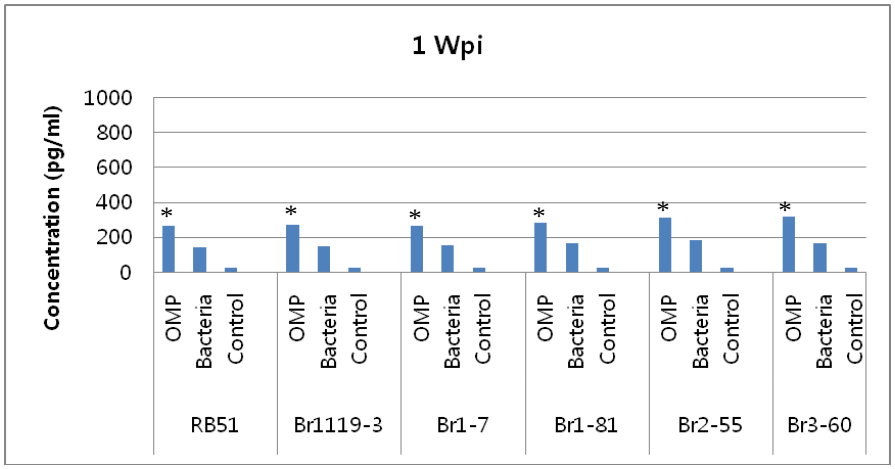
Br1-81
Br2-55
Br3-60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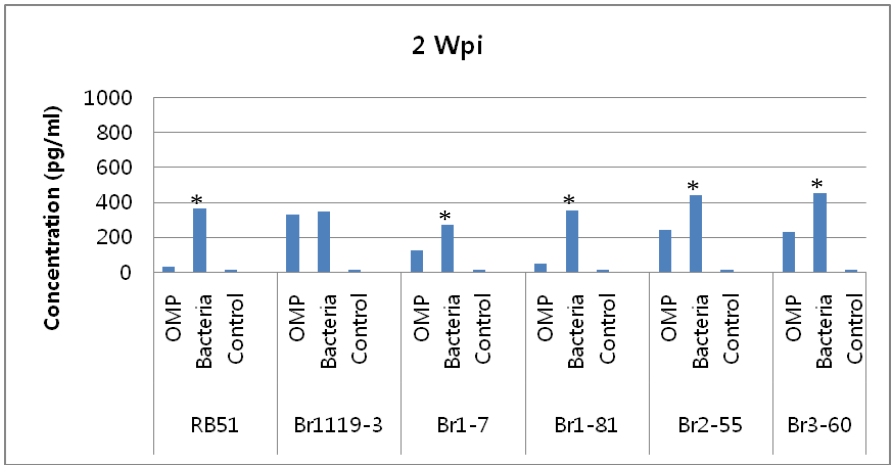
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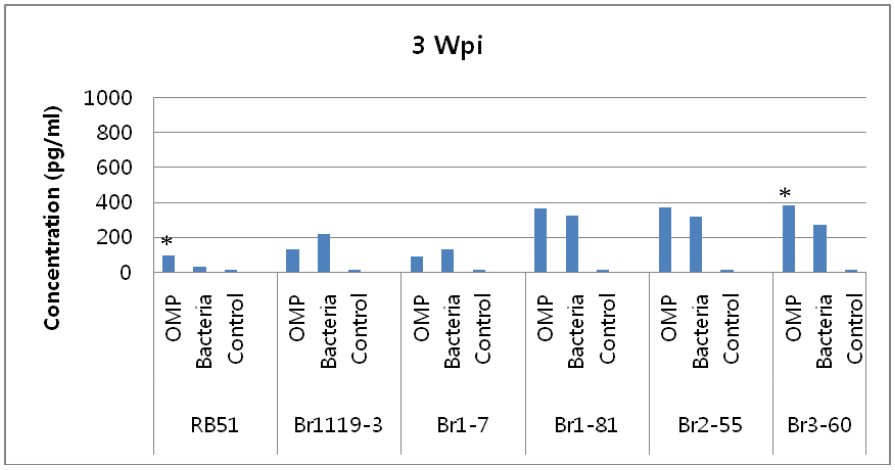
Br2-55
Br3-60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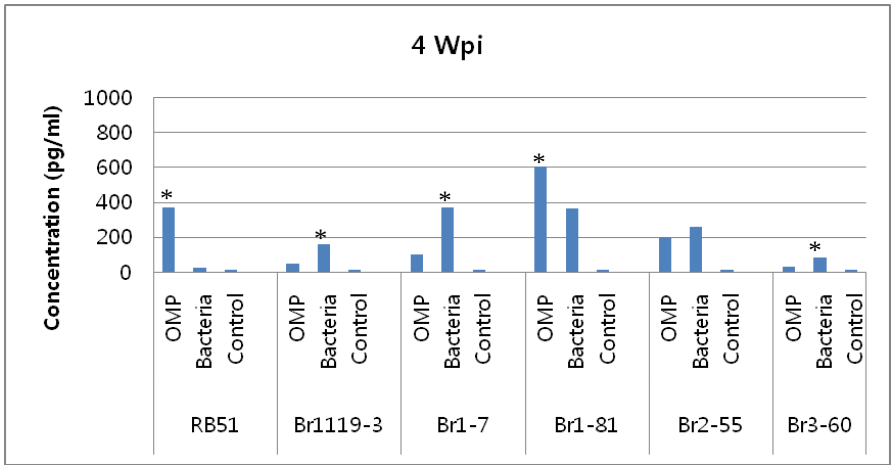
Br3-60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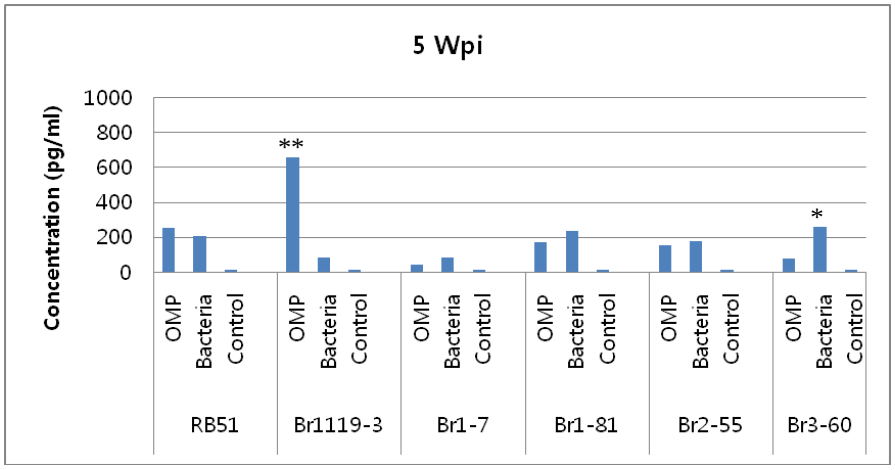


Fig 1.2. Induction of interferon (IFN)-γ in mouse splenocytes stimulated with OMP or bacteria after 48 hours. Dpi: Day post inoculation, Wpi: Week post inoculation, significantly different from other antigen stimulated group and control (*$p < 0.05$, **$p < 0.01$).

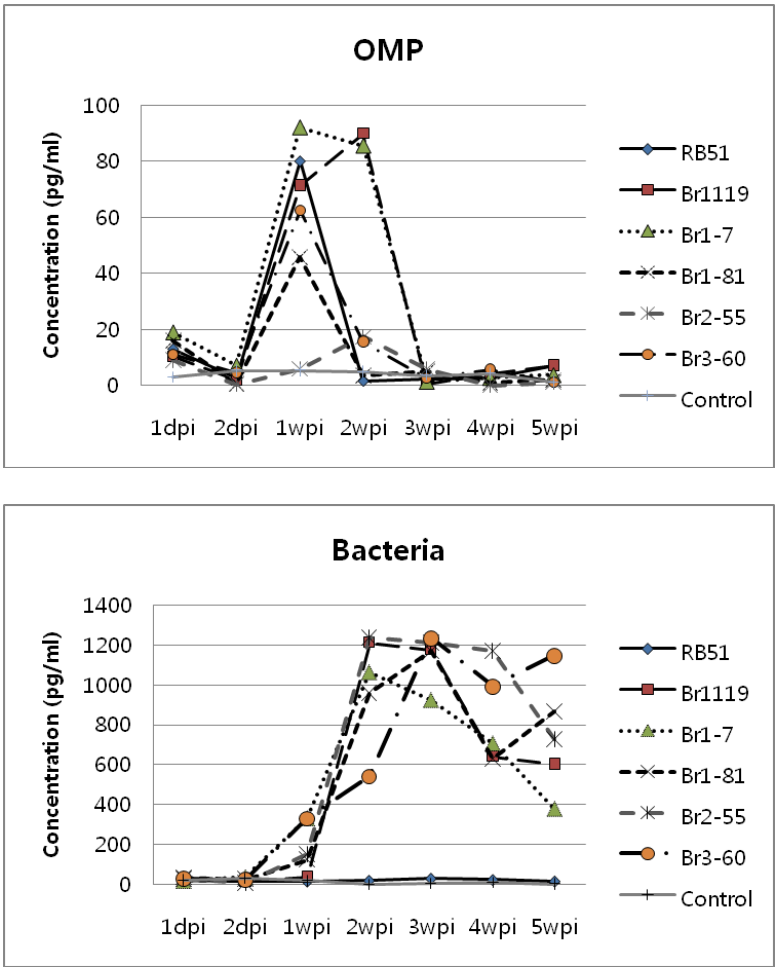


Fig 1.3. Kinetics interferon (IFN)-γ production in mouse serum after stimulated with OMP or bacteria. Dpi: Day post inoculation, Wpi: Week post inoculation. Standard error bars are not visible in the chart due to the scale.

Chapter 2

Generation and envelope protein analysis of internalization-defective *Brucella abortus* mutants in professional phagocytes RAW 264.7

Abstract

Brucella abortus is a facultative intracellular bacterium that replicates within a macrophage without producing any classical virulence factors. It can become internalized to cells by zipper-like and/or swimming internalization mechanisms. However, the bacterial proteins involved in internalization remain unclear. To define these bacterial proteins, random insertion mutants of *B. abortus* were generated by the Tn5 transposome complexes. In all, 132 mutants were screened, cellular internalization-defective mutants were selected, and these genomic and envelope proteomic features were identified. The transposon insertion sites were *ccmC*, *ppk* and BruAb2_0168 for the mutant C10, C29 and D7, respectively. Mutant C10 showed a deficiency in internalization without any changes in

expression of the cell envelope proteins; however, mutant C29 showed a reduced expression of OMP25, and a mutant D7 also showed reduced expression of OMP25, OMP28 and Porin2b. These results suggest OMP25 is not an essential factor, but might be involved in host cellular internalization. The *ppk* gene and BruAb2_0168 locus which are associated to expression of OMP25, OMP28 and Porin2b as well as pleiotropic effects of *ccmC* gene were identified.

Key words : *Brucella abortus*, internalization, defective mutants, OMP25

2.1. Introduction

Brucella abortus, a member of the Alphaproteobacteria family, causes the infectious disease brucellosis, affecting both humans and animals. The disease leads to spontaneous abortion and infertility in animals, resulting in serious economic losses and undulant fever, endocarditis, arthritis and osteomyelitis in humans (Boschiroli *et al.*, 2001; Franco *et al.*, 2007). *Brucella abortus* are small, non-motile, nonspore-forming Gram-negative rods that exist as intracellular organisms that are very difficult to isolate, and their long latent period makes early diagnosis impossible. Unlike other pathogenic bacteria, they do not produce exotoxins, cytolytic enzymes, antiphagocytic capsules, toxic lipopolysaccharides, thick cell walls, resistant forms, fimbriae or plasmids (Finlay & Falkow, 1989). Rather than producing classical virulence factors, they can invade and replicate in host professional and non-professional phagocytic cells, thus avoiding the bactericidal effects of the host cell. As a result, they may persist in the host and establish chronic infection (Corbel, 1997; Pizarro-Cerda´ *et al.*, 1998, 2000).

Several processes have been described for the internalization of intracellular pathogens. *Legionella pneumophila* internalizes via a macropinocytosis uptake pathway which is controlled by the Dot/Icm system and the mouse *Lgn1* locus

(Watarai *et al.*, 2001). Internalization of *Listeria* or *Yersinia* by professional and non-professional phagocytes occurs through a zipper-like mechanism in which bacterial surface proteins bind to host cell surface receptors (Isberg, 1996; Lecuit *et al.*, 1999), whereas *Salmonella* and *Shigella* invade via a trigger mechanism (Bourdet-Sicard *et al.*, 2000). For *Brucella*, internalization by macrophages occurs through a zipper-like mechanism via complement and Fc receptors, followed by opsonization, whereas un-opsonized brucellae internalize via lectin or fibronectin receptors (Harmon *et al.*, 1988; Campbell *et al.*, 1994). In macropinocytosis, involving lipid raft-associated molecules, a swimming internalization mechanism with generalized membrane ruffling may also be linked to its fate in macrophages (Watarai *et al.*, 2002). Although several virulence factors such as the VirB system and BvrR/BvrS two-component regulatory system have been discovered, the molecular mechanism of invasion by *Brucella* is not fully understood (Sieira *et al.*, 2000; Guzman-Verri *et al.*, 2002).

Transposon mutagenesis is frequently used as a genetic tool to characterize genes of unknown function (Handfield & Levesque, 1999). Of the various transposable elements used to characterize genes, the EZ-Tn5 system has a high transposition frequency (100-fold higher than those of phage Mu-based systems) and the highest degree of random insertions among the commercially available

transposition systems, including phage Mu (www.epibio.com). In light of this, random insertion mutants of *B. abortus* using the EZ-Tn5 system (Epicentre®) were generated and selected strains that were defective in cellular internalization. Of special interest were mutants defective for proteins located in the cell envelope (CE), which consists of the outer membrane, periplasmic space, and inner membrane. CE proteins have been shown to modify the host cell environment, mediate host cell–bacterial interaction, provide resistance to antibiotics and act as the first barriers to interaction with the host organism (Connolly *et al.*, 2006). In this study, differences between wild-type *B. abortus* and mutants were analyzed to identify proteins associated with invasion of macrophages.

2.2. Materials and Methods

Bacterial strains and media

The diagnostic reference strain *B. abortus* 1119-3 was kindly provided by the Animal, Plant and Fisheries Quarantine and Inspection Agency in Korea and was used for the transposon mutagenesis experiment. The strain was cultured in

brucella broth or agar (Difco Laboratories Inc., Detroit, MI, USA), *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, USA) was used to clone the transposon construction vector and was cultured in LB broth or agar (Duchefa Biochemie, Haarlem, Netherlands). Kanamycin (50 µg mL^{-1}) (Duchefa Biochemie, Haarlem, Netherlands) was used to select for *B. abortus*-containing transposon insertions or *E. coli* carrying the transposon construction vector.

Construction of the EZ-Tn5 transposon vector

To select for mutants with transposon insertions, the EZ-Tn5™ pMOD™-3 <R6Kγ*ori*/MCS> transposon construction vector (Epicentre® Biotechnologies, Madison, WI, USA) was modified. Briefly, the kanamycin-resistant gene (rKan) from pShuttle (Clontech, Palo Alto, CA, USA) was amplified via the polymerase chain reaction (PCR) using the primer pair Kan-SacI-F and Kan-XbaI-B (Table 2.1). The PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), digested with *Sac*I and *Xba*I, and then ligated with T4 ligase (Invitrogen, Carlsbad, CA, USA) to the *Sac*I and *Xba*I site in the multiple cloning sites located between the mosaic end (ME) sequence of the pMOD™-3 <R6Kγ*ori*/MCS> transposon construction vector. The GFPuv gene and *Lacz* promoter from pGFPuv (Clontech, Palo Alto, CA, USA) were

amplified and overlapped via SOEing PCR using primer sets to the SphI + *Lacz* promoter (FP-3 and BP-S) and GFPuv + SphI (FP-S and BP-2) (Table 2.1). The PCR product was purified and ligated to the *Sph*I site in the multiple cloning sites of the pMOD™-3 <R6Kcori/MCS> transposon construction vector (Fig. 2.1). The resulting pMOD™-3 vector carrying rKan, LacZ promoter, and the GFPuv gene, named pMOD-3 <Kan>, was transformed into chemically competent *E. coli* TOP10 cells (Invitrogen, Carlsbad, CA, USA) and inoculated on LB agar plates supplemented with ampicillin and kanamycin.

Transposome complex formation

For amplification of transposons containing ME sites, the pMOD-3 <Kan> transposon construction vector was subjected to PCR using the PCR-FP and PCR-RP primers (Table 2.1). The rKan-carrying transposon fragment was purified from agarose gel as described above, and the DNA concentration was quantified. Transposome formation was performed according to the manufacturer's instructions. Briefly, 2 µL of EZ-Tn5 transposon DNA [100 µg mL^{-1} in TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA)], 4 µL of EZ-Tn5 transposase (Epicentre® Biotechnologies), and 2 µL of 100% glycerol were

vortexed and incubated for 30 min at room temperature. The mixture was then stored at -20 ℃.

Electroporation of transposome into *B.abortus*

Electrocompetent *B. abortus* 1119-3 cells (50 μL) were mixed with 1 μL of transposome in a chilled 0.2-cm electroporation cuvette (Bio-Rad, Richmond, CA, USA). Electroporation was performed at 2.5 kV, 25 μF and 200 Ω using a Bio-Rad Gene Pulser II (Bio-Rad, Richmond, CA, USA). Electroporated bacteria were mixed with 1 mL of pre-warmed SOC medium (Invitrogen, Carlsbad, CA, USA), incubated on a 37 ℃ shaker for 6 h, and then plated onto brucella agar plates supplemented with kanamycin (50 μg mL^{-1}).

Selection of mutant strains defective in internalization

To screen for mutants defective in host cell internalization, the gentamicin protection assay was performed using the modified method described previously (Kim *et al.*, 2003). Briefly, RAW264.7 cells, a mouse leukemic monocyte macrophage cell line, were infected with *B. abortus* mutants at a multiplicity of infection (MOI) of 100. Infected cells were incubated at 37 ℃ and 5% CO_2 after

centrifugation for 10 min at 150 g. One hour post infection, the cells were washed twice with sterile phosphate-buffered saline (PBS) and incubated with fresh media supplemented with gentamicin (30 µg mL^{-1}). After 2 h incubation, infected cells were washed with PBS and lysed with 0.1 mL^{-1} of 0.01% Triton X-100 (Invitrogen, Carlsbad, CA, USA) in sterile water. The lysate (25 µL) was then plated onto brucella agar and incubated at 37 ℃ for 48 h. Samples with no growth were considered to be mutants with defects in cellular internalization and were selected for further analysis.

Southern blot analysis

Selected strains were cultured and embedded on an agarose block. Agarose blocks were incubated in lysis buffer (1% lauroyl sarcosine, 50 mM Tris, 50 mM EDTA, and 0.1 mg mL^{-1} proteinase K) at 55 ℃ overnight. Chromosomal DNA embedded in the agarose plug was digested with *Xba*I (no digestion site in the probe) and then separated via pulsed field gel electrophoresis (PFGE) in a 1% agarose gel. DNA fragments were transferred to a Hybond nylon membrane (GE Healthcare, UK) and fixed via UV cross linking. The rKan gene in the transposon was labeled using the BrightStar® Psoralen-Biotin labeling kit

(Ambion, Austin, TX, USA) and used as the DNA probe for Southern hybridization.

Sequencing of transposon insertion site

To determine the exact transposon insertion site, a protocol for sequencing randomly primed PCR products was used with slight modification (Shin *et al.*, 2006). Briefly, genomic DNA from the mutants was extracted using the Wizard® genomic DNA purification kit (Promega, Madison, WI, USA) and then subjected to standard PCR. For the first round of PCR, a primer specific for the rKan gene in the transposon (rKan-R) and a degenerate primer (RSBamHI and RSXbaI) (Table 2.1) were used to amplify the chromosomal sequence flanking the transposon insertion site. PCR was carried out in a total volume of 25 μL in 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.1 μM of rKan-R, 1.0 μM of RSBamHI and RSXbaI, and 0.75 U of *Taq* polymerase (Intron, Korea). First-round amplification was performed with an initial denaturing step at 94 ℃ for 5 min, followed by 40 cycles of denaturing at 94 ℃ for 1 min, 50 ℃ for 30 s, and 72 ℃ for 90 s, and a final extension step at 72 ℃ for 7 min. Second-round PCR was performed using 1 μL of the first-round amplification as the template, a nested

primer (SeqR) derived from the transposon, and the T7 primer (T7-F) present within the degenerative primer sequence (Table 2.1). Reactions were carried out in a total volume of 50 µL in 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.5 µM primers, and 0.75 U of *Taq* polymerase (Intron, Korea). The amplification was performed with a denaturing step at 95 ℃ for 5 min followed by 35 cycles of 94 ℃ for 30 s, 57 ℃ for 30 s, and 72 ℃ for 1 min, and a final extension step at 72 ℃ for 10 min. The product of the second amplification was gel-purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and was sequenced using an automatic sequencer and dye-termination sequencing system (Macrogen Co., Korea).

Bacterial CE isolation and total protein determination

The CEs of the selected mutants and wild-type strain were isolated as described previously (Connolly *et al.*, 2006), with slight modification. Briefly, cells from 250 mL cultures grown for 24 h were harvested via centrifugation at 8,000 g for 15 min at 4 ℃. The pellets were washed twice with PBS, resuspended in 100 mL of buffer (15 mM Tris–HCl pH 8.0, 0.45 M sucrose, 8 mM EDTA, and 0.4 mg mL^{-1} lysozyme), and incubated for 15 min at 4 ℃. The lysates from these cell pellets were then centrifuged at 8,000 g for 15 min, resuspended in 3 mL of 50

mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, and Xpert protease inhibitor cocktail solution (GenDepot, Barker, TX, USA), and chilled on ice. A Sonopuls instrument (Bandelin Electronic, Germany) was used to disrupt the cell membranes in the chilled lysates, followed by two rounds of centrifugation at 3,000 g for 15 min to remove intact cells. Ultracentrifugation at 100,000 g for 90 min was performed on the supernatants of these lysates. The pellets were then collected, resuspended in 50 mM Tris–HCl (pH 7.6), precipitated with trichloroacetic acid according to the manufacturer's instructions, and dissolved in resuspension buffer (7 M urea, 2 M thiourea, 1% C7BzO, and 40 mM Tris). The concentration of total CE protein was determined using the Bradford assay reagent (Pierce Biotechnology, Rockford, IL, USA).

Two-dimensional polyacrylamide gel electrophoresis (2-DE)

CE proteins (1 mg per strip) were separated using IEF on pH 3–7 nonlinear 24 cm IPG strips (GE Healthcare, UK). After 12 h of rehydration, the samples were focused using an Ettan IPGphor II Isoelectric Focusing System (GE Healthcare, UK) as follows: linear voltage increase over 8 h from 100 to 10,000 V and held at 10,000 V for another 8 h. IPG strips were equilibrated, and second-dimension electrophoresis was performed on an 8–18% gradient sodium dodecyl sulfate

(SDS) polyacrylamide gel with the Ettan DALTsix Large Vertical System (GE Healthcare, UK). The gels were stained with 0.25% Coomassie brilliant blue R250 (Bio-Rad, Richmond, CA, USA).

Analysis of protein expression

Protein spots from the wild-type strain and mutants were analyzed using IMAGEMASTER 2D PLATINUM software (GE Healthcare, UK). Spots with a greater than twofold change compared to the level of the wild-type strain were identified using liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) at the National Instrumentation Center for Environmental Management at Seoul National University. The MS/MS spectra were submitted to Mascot (Matrix Science) for database searching against the SwissProt. The search parameters consisted of a maximum of one missed cleavage by trypsin, a charge state of +2, an MS tolerance of ±1.2 Da, and an MS/MS tolerance of ±0.6 Da.

2.3. Results

Generation and isolation of cellular internalization-defective mutants

After electroporation of the transposome complex into *B. abortus* 1119-3, 132 rKan colonies were selected. PCR targeting the rKan gene within the transposon confirmed that all 132 mutants were carrying transposons. To select for cellular internalization-defective mutants, RAW 264.7 cells were infected with each mutant as described in Materials and methods. Of the 132 mutants, eight showed reduced internalization ability. However, the reductions in only four mutants, C10, C20, C29 and D7, were statistically significant ($p < 0.05$) compared to the level of the wild-type strain 1119-3 (Fig 2.2).

Selecting single insertion mutants with different insertion sites

Confirmation of random or single insertions of the transposon into the *B. abortus* genome was conducted using Southern blot hybridization of selected internalization-defective mutants. According to PFGE analysis, the mutants

showed similar patterns. However, Southern blot hybridization with the rKan gene probe showed differences in the insertion sites (Fig 2.3), with each mutant having a single insertion site in the transposon. Mutants C10, D6, and D7 had insertions in a 97, 132.3, and 138.9 kb *Xba*I fragment, respectively, and mutants C20, C21, C29, D5, and D9 had insertions in a 116.8 kb *Xba*I fragment.

Identification of transposon insertion sites

On the basis of the Southern blot hybridization results, four strains were selected and subjected to restriction site PCR for identification of the insertion site. Strains C10, D6, and D7 were selected, as was strain C29, which showed the greatest reduction in internalization among the mutants with insertions in the 116.8 kb *Xba*I fragment. DNA sequences from the nested PCR product were used to search the *B. abortus* biovar 1 complete genome using a BLASTN algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Transposon insertion sites of each mutant are shown in Table 2.2. Genes disrupted in the C10, C29, D6, and D7 mutants were identified as *ccmC*, *ppk*, BruAb1_1377, and BruAb2_0168, respectively. The proteins encoded by the disrupted genes in mutants C10, C29, D6, and D7 were identified as a heme exporter protein, polyphosphate kinase, a

transcriptional regulator in the TetR family and an outer membrane transporter, respectively.

CE maps of *B. abortus* 1119-3 and internalization mutants

To identify CE proteins that may be expressed differently in cells defective for internalization, CE proteome maps of *B. abortus* 1119-3 and each of the internalization-defective mutants were compared. A previous study of CE proteins in *B. abortus* (Handfield & Levesque, 1999) showed that most CE proteins function in an acidic pI range; therefore, isoelectric focusing was performed using a pH range of 3–7 and found similar protein expression patterns among the strains. Most of the protein spots occurred within a pH range of 4–5, with only a few proteins observed outside that range (Fig 2.4). However, proteins larger than 40 kDa showed horizontal streaking due to insolubility and therefore excluded those proteins from comparison.

Comparison and identification of differentially expressed proteins

CE proteome maps of each mutant were compared to the proteome map of *B. abortus* 1119-3 using IMAGEMASTER 2D PLATINUM software as described in Materials and methods. A total of 28 spots which showed a greater than twofold change were identified by LC-ESI-MS: five spots for mutant C10, six spots for mutant C29, seven spots for mutant D6, and 10 spots for mutant D7 (Fig 2.5). The spot number, locus, gene, protein description, molecular weight, theoretical pI, and fold-change of these proteins are presented in Table 2.3. Interestingly, internalization mutants C29 and D7 showed reduced expression levels of the 25-kDa outer membrane immunogenic protein (OMP25). Since the *B. abortus* genome contains three different ORFs that encode for different paralogs of OMP25, and SDS-resistant oligomerization is a characteristic of outer membrane proteins, our results showing a deviation between the theoretical and experimental pI and MW for OMP25 are consistent with previous proteomic analyses of the *B. abortus* CE (Vizcaı´no *et al.*, 1996; Connolly *et al.*, 2006).

2.4. Discussion

To understand the pathogenicity of *B. abortus*, several studies have generated random insertion mutants using transposable elements (Kim *et al.*, 2003). In this

study, *B. abortus* mutants using the Tn5-based EZ transposome system were generated. Similar to other studies using this system (Vidal *et al.*, 2009; Ito *et al.*, 2010), single insertion mutants which were stable over at least 10 sequential overnight cultures were obtained; however, the efficiency of this method was lower compared with those of other studies using different species. To improve the efficiency, factors such as growth phase and concentration of electrocompetent cells, electrical parameters, concentration of transposon DNA, and the volume of transposomes should be optimized for *B. abortus*.

In gram-negative bacteria, rough and smooth strains differ in their LPS composition, particularly the O-side chain, which suggests that the O-side chain is responsible for directing entry host cells through lipid rafts. Interestingly, rough mutants of both *B. abortus* and *Brucella melitensis* exhibit increased macrophage uptake relative to their smooth parent strain (Pei *et al.*, 2008). However, as none of our mutants was rough mutant (data not shown), the cellular envelope proteins were focused. To establish intracellular infection, extensive CE modulation is associated with virulence in *B. abortus* (Lamontagne *et al.*, 2007). In addition, the expression pattern of surface proteins has been shown to change remarkably for internalization mutants compared with that of the wild-type strain (Kim *et al.*, 2003). In light of this, mutants which had a deficiency in host cell internalization were generated and identified envelope

proteins which showed different expression levels.

For our internalization mutants, transposon insertion occurred within the *ccmC* and *ppk* genes or at the BruAb1_1377 and BruAb2_0168 loci. The *ccmC* gene encodes an integral membrane protein (CcmC) which binds heme c in the periplasm and transfers it to a membrane-anchored periplasmic heme chaperone (Thöny-Meyer, 1997). No modified expression levels of envelope proteins for the *ccmC* mutant C10 (both increased and decreased spots were identified as OMP25) was identified, although host cell internalization activity had been lost. Recently, several reports have described pleiotropic effects of *ccmC* mutations that cannot be explained only by the absence of c-type cytochrome synthesis. These effects include the production and/or uptake of siderophores (Cianciotto *et al.*, 2005), pyoverdine maturation, expression of certain quorum-sensing regulated traits in *Pseudomonas aeruginosa* (Baert *et al.*, 2008), and motility in *Sinorhizobium meliloti* (Yurgel *et al.*, 2007). Therefore, it is possible that *ccmC* mutations in *B. abortus* may also affect the expressions of quorum-sensing regulators. These regulators are known to control the expressions of both the type IV secretion system and the flagella apparatus (Delrue *et al.*, 2005), although the precise mechanism remains to be elucidated.

The *ppk* gene encodes for inorganic polyphosphate kinase (PPK), an enzyme which is responsible for the synthesis of inorganic phosphate from ATP. A

previous study using signature tagged mutants of *B. melitensis* identified the *ppk* gene as a virulence factor and suggested it may play an essential role during the initial steps of host colonization and may contribute to the virulence of *Brucella* through an energy-dependent mechanism (Henderson & Nataro, 2001). This hypothesis is consistent with our results showing that *ppk* mutant C29 was defective in the first step of host colonization. In addition, the OMP25 expression was decreased in mutant C29, suggesting that OMP25 may play an important role in host cell internalization.

In mutant D6, transposon insertion was located in locus BruAb1_1377, a putative transcriptional regulator belonging to the TetR family. The TetR family includes a common class of transcriptional regulators and has been identified in 115 genera of gram-positive bacteria, *Alpha-*, *Beta-* and *Gammaproteobacteria*, *Cyanobacteria*, and Archaea. These proteins are involved in the transcriptional control of multidrug efflux pumps, pathways for the biosynthesis of antibiotics, the response to osmotic stress and toxic chemicals, control of catabolic pathways, differentiation processes and pathogenicity (Ramos *et al.*, 2005). Mutant strain D6 showed reduced cellular internalization activity that was not statistically significant and expressed two weaker and one stronger spot, which were identified as OMP25. Considering that many signal transduction pathways and gene expression are mediated by the TetR family of transcriptional regulators, it

is difficult to explain why disruption of locus BruAb1_1377 did not affect cellular internalization.

In gram-negative bacteria, the outer membrane transporter system plays an important role in the transport of molecules across the double membrane envelope and in the secretion of proteinaceous virulence factors to the bacterial cell surface or the external environment (Desvaux *et al.*, 2004). In this study, mutant strain D7 showed a dramatic decrease in host cell internalization, and the transposon was inserted in locus BruAb2_0168 of chromosome II, a putative outer membrane transporter. In addition, outer membrane proteins including OMP25, OMP28, and 2b Porin showed decreases in the level of protein expression compared with that of the wild-type strain.

Five major protein secretion pathways have been characterized for gram-negative bacteria, and the type IV secretion system (T4SS) coded by the VirB operon is essential for intracellular survival and persistent infection of *Brucella* spp., as well as for triggering host innate immune responses (Roux *et al.*, 2007). Therefore, locus BruAb2_0168 was related to T4SS and that disruption of this locus would result in the loss of virulence. However, Bandara *et al.* (2005) showed that the outer membrane autotransporter (OmaA) of *Brucella suis* influences survival of BALB/c mice. The autotransporter (type V) secretion system is the simplest secretion apparatus and represents the largest family of

outer membrane porins in gram-negative bacteria that translocate proteins via a transmembrane pore formed by a self-encoded β-barrel structure (Desvaux *et al.*, 2004). Thus, mutation in locus BruAb2_0168 may be related to the autotransporter secretion system, and disruption of this system would result in reduced expressions of outer membrane proteins such as OMP25, OMP28 and 2b Porin.

In this study, the *ppk* gene and BruAb2_0168 locus associated with expressions of OMP25, OMP28, and Porin2b were identified. Although this study could not elucidate the signaling pathways affecting the expressions of these proteins, this results showed that *ppk* gene was related with the virulence of *B. abortus* via expression of OMP25, which has been revealed as an important outer membrane protein associated with virulence (Edmonds *et al.*, 2002) as well as an internalization factor of *Brucella ovis* in professional phagocytes (Martín-Martín *et al.*, 2008). Moreover, mutation in BruAb2_0168 locus was related to the expression of OMP25, OMP28 and Porin2b and found that the *ccmC* gene related to internalization without affecting OMP25. These finding may contribute to a better understanding of the molecular mechanisms underlying cellular internalization in *B. abortus*, as well as for defining the function of an unnamed locus of the *B. abortus* genome; however, this requires further study in the future.

Table 2.1. Oligonucleotides used in this study

Primer name	**Oligonucleotide sequence (5' to 3')**	**Purpose**	**References**
Kan-*SacI*-F	AGAGAGAGAGGAGCTCCTAGCGCTGAGGTCTG	Transposon construction	This study
Kan-*XbaI*-B	ACACACACTCTAGACTCTGCCAGTGTTACAAC	Transposon construction	This study
***SphI*+Lacz promoter FP-3**	AGAGGCATGCGCGCAACGCAAT	Transposon construction	This study
***SphI*+Lacz promoter BP-S**	CTCCTTTACTCATAGCTGTTT	Transposon construction	This study
Gfpuv+*SphI* FP-S	AGCTATGAGTAAAGGAGAAGA	Transposon construction	This study
Gfpuv+*SphI* BP-2	ACACGCATGCTTATTTGTAGAGCTCAT	Transposon construction	This study
PCRFP	ATTCAGGCTGCGCAACTGT	Amplication of transposon	Epicentre® Biotechnologies, USA
PCRRP	GTCAGTGAGCGAGGAAGCGGAAG	Amplication of transposon	Epicentre® Biotechnologies, USA
rKan-R	CTCTGCCAGTGTTACAAC	First round PCR	This study
RS*BamHI*	TAATACGACTCACTATAGGGATGCGGATCC	First round PCR	Shin *et al*. (2006)
RS*XbaI*	TAATACGACTCACTATAGGGATGCTCTAGA	First round PCR	Shin *et al*. (2006)
T7-F	TAATACGACTCACTATAGGG	Second round PCR	Shin *et al*. (2006)
Sq-R	GAGCCAATATGCGAGAACACCCGAGAA	Second round PCR	Epicentre® Biotechnologies, USA

Table 2.2. Disrupted genes and putative function of mutants used in this experiments. The sequences of insertion site were used to search the *Brucella abortus* biovar 1 complete genome using a BLASTN algorithm

Strain	Functional family	Disrupted genes or locus tag	Chromosome	Putative function
C10	Transport	*ccmC*	I	Heme exporter protein
C29	Oxidative metabolism	*ppk*	I	Polyphosphate kinase
D6	Transcriptional regulation	BruAb1_1377	I	TetR family transcriptional regulator
D7	Transport	BruAb2_0168	II	Outer membrane transporter

Table 2.3. List of proteins with reduced or increased expression level compare to *B.abortus* 1119-3 identified by 2-DE and ESI-LC-MS. Spot numbers refer to the numbers on the 2-DE gels shown in Fig. 2.5 (A)-(C)

Spot		Locus	Gene	Protein description	Mw(kDa) /pI(Theoretical)	Sequence coverage (%)	Location	Change fold
C10	1-1	BruAb1_720	*omp25*	25kDa outer-membrane immunogenic protein	23.137/8.58	66	OM	2.4
	1-2	BruAb1_720	*omp25*	25kDa outer-membrane immunogenic protein	23.137/8.58	54	OM	2.1
	1-3	BruAb1_720	*omp25*	25kDa outer-membrane immunogenic protein	23.137/8.58	46	OM	-2.2
	1-4	BruAb1_720	*omp25*	25kDa outer-membrane immunogenic protein	23.137/8.58	53	OM	-2.7
	1-5	BruAb1_1251	*rplA*	50S ribosomal protein L1	24.595/9.52	12	C	-7.5
C29	2-1	BruAb1_0474	*rplI*	50S ribosomal protein L9	20.952/4.86	60	C	-2.1
	2-2	BruAb1_720	*omp25*	25kDa outer-membrane immunogenic protein	23.137/8.58	53	OM	-2.9
	2-3	BruAb1_720	*omp25*	25kDa outer-membrane immunogenic protein	23.137/8.58	46	OM	-3.9
	2-4	BruAb1_720	*omp25*	25kDa outer-membrane immunogenic protein	23.137/8.58	50	OM	-8.3
	2-5	BruAb1_720	*omp25*	25kDa outer-membrane immunogenic protein	23.137/8.58	53	OM	-9.0
	2-6	BruAb1_1251	*rplA*	50S ribosomal protein L1	24.595/9.52	12	C	-9.4

Table 2.3. (*Continued*)

Spot		Locus	Gene	Protein description	Mw(kDa) /pI(Theoretical)	Sequence coverage (%)	Location	Change fold
D6	3-1	BruAb1_720	*omp25*	25kDa outer-membrane immunogenic protein	23.137/8.58	53	OM	3.4
	3-2	BruAb1_1251	*rplA*	50S ribosomal protein L1	24.595/9.52	41	C	-2.1
	3-3	BruAb1_720	*omp25*	25kDa outer-membrane immunogenic protein	23.137/8.58	53	OM	-2.5
	3-4	BruAb1_720	*omp25*	25kDa outer-membrane immunogenic protein	23.137/8.58	46	OM	-2.6
	3-5	BruAb1_1251	*rplA*	50S ribosomal protein L1	24.595/9.52	12	C	-4.7
	3-6	BruAb1_720	*omp25*	25kDa outer-membrane immunogenic protein	23.137/8.58	53	OM	-7.4
	3-7	BruAb1_720	*omp25*	25kDa outer-membrane immunogenic protein	23.137/8.58	50	OM	-8.3
D7	4-1	BruAb1_720	*omp25*	25kDa outer-membrane immunogenic protein	23.137/8.58	53	OM	-2.2
	4-2	BruAb1_0657	*omp2b*	Porin omp2b	38.699/4.58	40	OM	-2.3
	4-3	BruAb1_720	*omp25*	25kDa outer-membrane immunogenic protein	23.137/8.58	46	OM	-3.3
	4-4	BruAb1_1470	*omp28*	immunoreactive 28kDa outer membrane protein	26.537/5.66	57	OM	-3.4
	4-5	BruAb1_720	*omp25*	25kDa outer-membrane immunogenic protein	23.137/8.58	30	OM	-3.5
	4-6	BruAb1_1238	*rplC*	50S ribosomal protein L3	25.048/10.04	37	C	-3.9
	4-7	BruAb1_720	*omp25*	25kDa outer-membrane immunogenic protein	23.137/8.58	53	OM	-4.0
	4-8	BruAb1_1251	rplA	50S ribosomal protein L1	24.595/9.52	12	C	-4.7
	4-9	BruAb1_720	omp25	25kDa outer-membrane immunogenic protein	23.137/8.58	50	OM	-8.5
	4-10	BruAb1_720	omp25	25kDa outer-membrane immunogenic protein	23.137/8.58	53	OM	-16.5

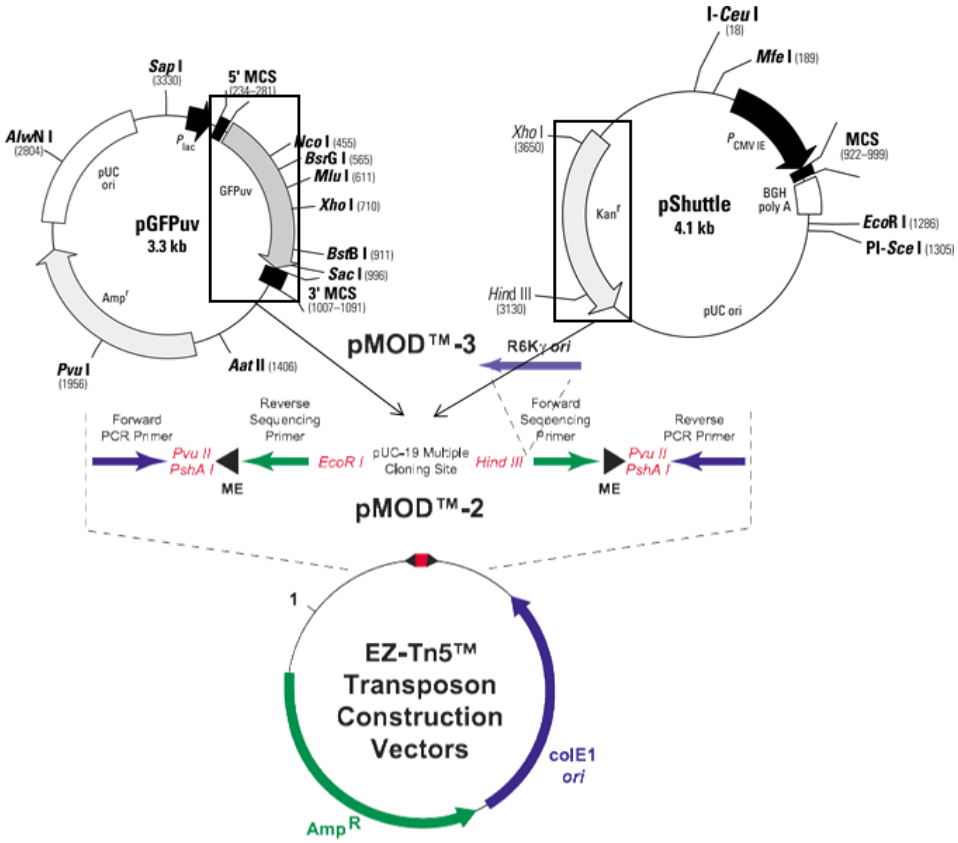


Fig 2.1. Schematic representation of vector harboring transposon. To select for mutants with transposon insertion, GFPuv gene from pGFPuv (left) and rKan gene from pShuttle (right) were cloned to multi cloning site located between the mosaic end (ME) sequences of pMOD™-3 transposon construction vectors (bottom). PCRFP and PCRRP were used to amplify the transposon and incubated with transposase to synthesize transposome complexes. The transposome complexes were electroporated to *B. abortus* 1119-3 and individual colonies from kanamycin supplemented plates were considered as a transposon insertion mutant.

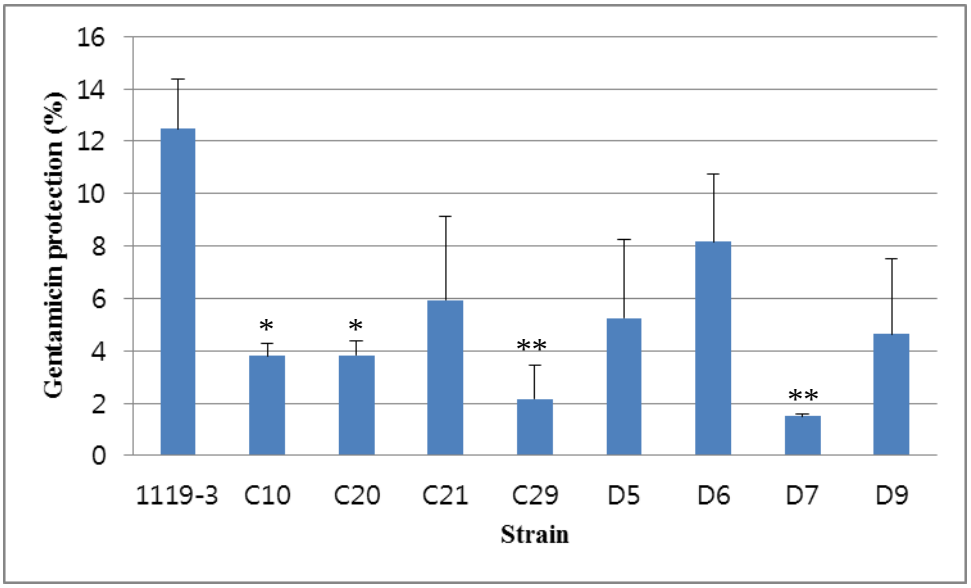


Fig 2.2. Bacterial internalization to RAW 264.7 cells. To select the internalization-defective strains, mutants were infected to RAW 264.7 cells with MOI of 100, and after 1 hour, gentamicin (30μg/ml) was added to kill the bacteria which could not internalized. After 2 hours of incubation, infected cells were lysed and the lysate was plated. Four strains showed reduced internalization ability compared to wild type strain. (*p<0.05, **p<0.01). The data are presented as the mean of triplicate results.

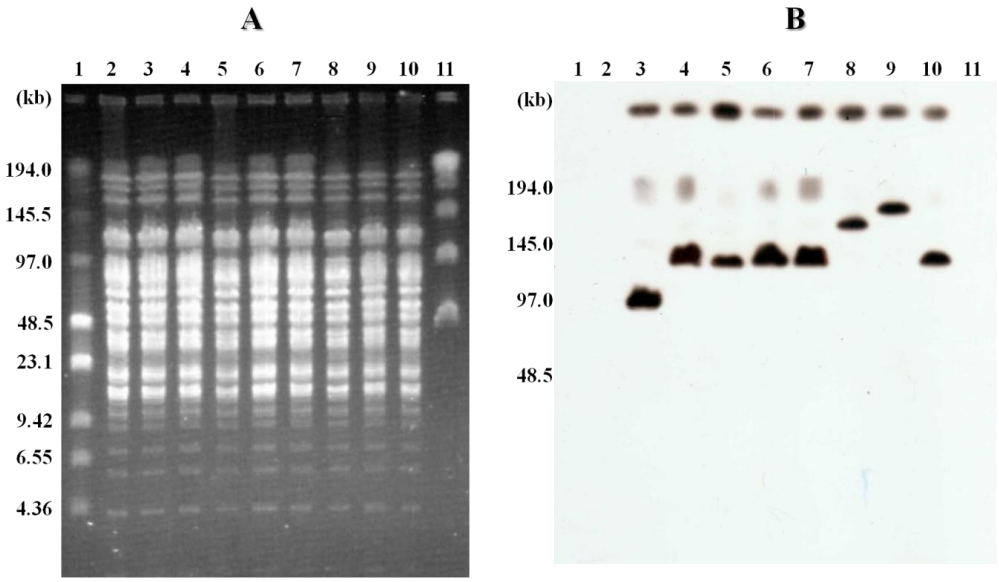


Fig 2.3. PFGE(A) and Southern blot(B) analysis of mutants. To confirm the insertion and analyze the copy number of inserted transposon, mutants genomic DNA embedded in agarose plug were digested with *Xba*I enzyme (no digestion site in probe). Samples were separated by pulsed field gel electrophoresis (6V/cm, included angle: 120℃, ramped pulse time 1-12sec, run time: 15 hours) and the gel was transferred to the nylon membrane, hybridized with a probe targeting rKan gene in the multi cloning site of transposon. (Lane 1 : Low range PFG marker, Lane 2 : 1119-3, Lane 3 : C10, Lane 4 : C20, Lane 5 : C21, Lane 6 : C29, Lane 7 : D5, Lane 8 : D6, Lane 9 : D7, Lane 10 : D9, Lane 11 : Lambda PFG marker)

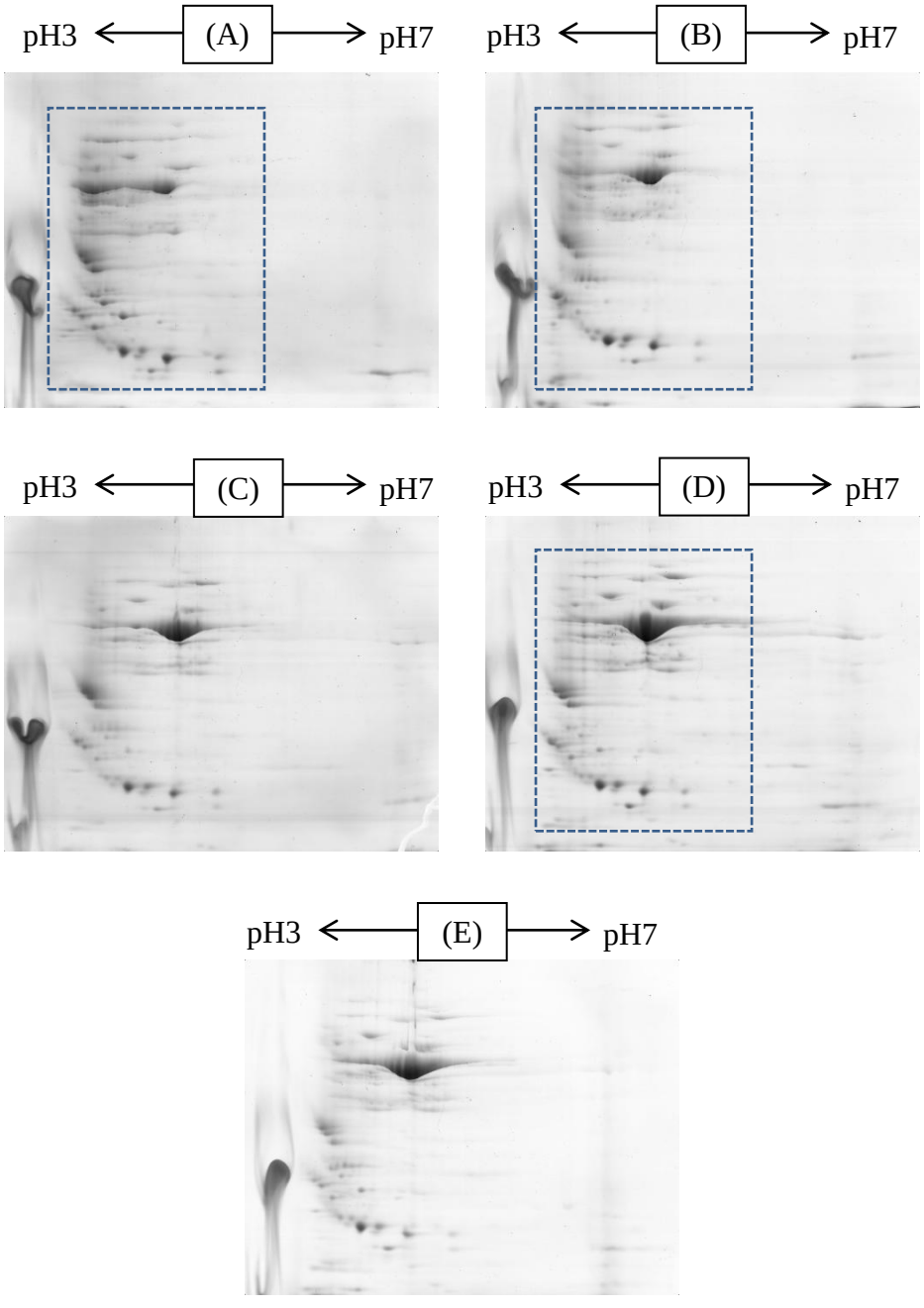


Fig 2.4. 2-DE analysis of CE proteins from *B.abortus* 1119-3 (A), mutant C10

(B), mutant C29 (C), mutant D6 (D), and mutant D7 (E). 1mg of CE proteins were separated by isoelectric focusing using a 24cm pH gradient strip (pH 3-7NL) in the first dimension and 8-18% gradient SDS-PAGE in the second dimension. Gels were stained with 0.25% Coomassie brilliant blue R250. Boxes with dotted line are enlarged and shown at Fig. 2.5.

(A) (B) (C)

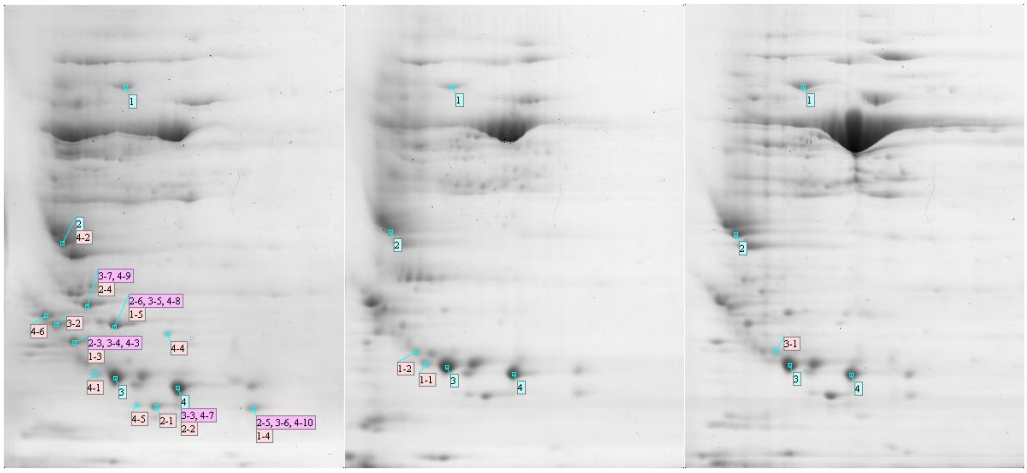


Fig 2.5. Comparison of spots with more than two fold changes to *B.abortus* 1119-3. Map of CE proteins from *B.abortus* 1119-3 (A), mutant C10 (B), and mutant D6 (C) are shown. Four spots designated with blue box were used as a landmark for spot matching. With reduced expression level, spots were excised and analyzed from *B. abortus* 1119-3 gel. 1- and 3- mean spot matching with mutant C10 and D6 respectively. For mutant C29 and D7, all spots were identified from gel (A) and designated as 2- and 4- respectively due to the absence of matching spots with increased expression level.

Chapter 3

Early transcriptional responses of internalization-defective *Brucella abortus* mutants in professional phagocyte RAW 264.7

Abstract

Brucella abortus is an intracellular zoonotic pathogen which causes undulant fever, endocarditis, arthritis and osteomyelitis in human and abortion and infertility in cattle. This bacterium is able to invade and replicate in host macrophage instead of getting removed by this defense mechanism. Therefore, understanding the interaction between virulence of the bacteria and the host cell is important to controlling brucellosis. Previously, internalization-defective mutants were generated and analyzed the envelope proteins. The present study was undertaken to evaluate the changes in early transcriptional responses between wild type and internalization-defective mutant infected mouse macrophage, RAW 264.7. Both of the wild type and mutant infected macrophage

showed increased expression levels in proinflammatory cytokines, chemokines, apoptosis and G-protein coupled receptors (Gpr84, Gpr109a and Adora2b), while the genes related with small GTPase which mediate intracellular trafficking was decreased. Moreover, cytohesin 1 interacting protein (Cytip) and genes related to ubiquitination (Arrdc3 and Fbxo21) were also decreased, suggesting the survival strategy of this bacterium. However, no significant changes in the mutant infected groups compared to the wild type infected group. In summary, it was very difficult to clarify the alterations in host cellular transcription in response to infection with internalization-defective mutants. However, several novel gene changes related to the GPCR system, ubiquitin-proteosome system, and growth arrest and DNA damages in response to *B. abortus* infection were found. These findings may contribute to a better understanding of the molecular mechanisms of the underlying host-pathogen interactions and need to be studied further.

Key words : *Brucella abortus*, infection, macrophage, response, transcription

3.1. Introduction

Brucella abortus is a zoonotic pathogen that causes undulant fever, endocarditis, arthritis and osteomyelitis in humans and abortion and infertility in cattle (Boschiroli *et al.*, 2001; Franco *et al.*, 2007). This bacterium is small, non-motile, non-spore-forming Gram-negative rods and facultative intracellular organisms that are very difficult to isolate and have a long latent period that makes early diagnosis after infection impossible. Instead of producing toxins or utilizing classical virulence factors, this bacterium is able to grow in phagocytes where they are inaccessible to the host humoral immune response. The brucellae also employ several intracellular survival strategies both in professional and nonprofessional phagocytic host cells (Pizarro-Cerdá *et al.*, 1998; Rittig *et al.*, 2001). Therefore, understanding the interaction between bacterial virulence and the host cell is important to controlling brucellosis.

Several studies have described the host cell responses to *Brucella* infection. Genes from macrophage RAW 264.7 demonstrated up-regulation of proinflammatory cytokines and antibacterial response-related chemokines (Oliveira *et al.*, 1998; Eskra *et al.*, 2003). In contrast, genes involved in cell cycling, apoptosis, and intracellular trafficking were decreased after four hours of *B. abortus* infection, suggesting the intracellular survival manner of this

bacterium (Eskra *et al.*, 2003). Moreover, microarray analysis of macrophages infected with three *Brucella* spp. revealed differentially expressed macrophage genes. Such studies discussed the host preference and virulence related to transcriptional responses elicited by this species (Covert *et al.*, 2009).

In light of the available information on *B. abortus* and host interactions, the transcriptional responses of macrophage RAW 264.7 infected with *B. abortus* mutants with defective internalization were analyzed. Previously, *B. abortus* mutants with defective host cellular internalization by Tn5 transposome complexes were generated. Their cell envelope (CE) proteins were analyzed regarding invasion of the macrophages that resulted in the ppk gene and BruAb2_0168 locus, which are associated with expression of the OMP25, OMP28 and Porin2b genes, as well as pleiotropic effects of the *ccmC* gene (Cha *et al.*, 2012). In the present study, the professional phagocyte RAW 264.7 was infected with the *B. abortus* mutants for four hours. Then compared the early transcriptional responses of the macrophage with those of uninfected macrophages and macrophages infected with a virulent strain to evaluate the potential entry mechanism of the bacteria and host cellular responses. Possible roles in the cellular responses for the different mutants of *B. abortus* are investigated.

3.2. Materials and Methods

Bacterial strains and cell line

The diagnostic reference strain *Brucella abortus* 1119-3 was provided by the Animal, Plant and Fisheries Quarantine and Inspection Agency in Korea. The internalization-defective mutant C10, C29, D6 and D7 were derived from our previous study (Cha *et al.*, 2012). Brucellae were cultured in Brucella broth or agar (Difco Laboratories Inc., Detroit, MI, USA), and Kanamycin (30ug/ml) was used when necessary. RAW264.7, a mouse leukemic monocyte macrophage cell line, was grown at 37℃ in a 5% CO_2 atmosphere in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA).

Macrophage infection and RNA preparation

RAW 264.7 cells were infected with each *Brucella* strain as described previously (Covert *et al.*, 2009). Briefly, RAW 264.7 cells were seeded (5×10^6 cells per flask) in T75 flasks one day before infection. Macrophages were

infected with 1 ml of a stationary phase culture of wild type and mutant *B. abortus* strains (MOI 1000:1). After 4 hours of incubation, cells were washed twice with PBS, and the RNA was extracted using the RNeasy mini Kit (Qiagen, Valencia, USA) according to the manufacturer's protocol. After processing with DNase digestion and clean-up procedures, RNA samples were quantified, aliquotted, and stored at -80℃ until use. For quality control, RNA purity and integrity were evaluated by denaturing the samples and performing gel electrophoresis, OD 260/280 ratio, and analyzed on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). To validate the microarray results, an independent experiment was conducted with the same conditions.

Labeling and purification

RNA amplification, labeling, array hybridization, and scanning were carried out by Macrogen Inc. (Seoul, Republic of Korea). Total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Ambion, Austin, TX, USA) to yield biotinylated cRNA according to the manufacturer's instructions. Briefly, 550 ng of total RNA was reverse-transcribed to cDNA using a T7 oligo (dT) primer. Second-strand cDNA was synthesized, transcribed *in vitro*, and labeled with biotin-NTP. After purification, the cRNA was quantified using the

ND-1000 Spectrophotometer (NanoDrop, Wilmington, USA).

Hybridization and data export

The labeled cRNA samples were hybridized to each mouse-6 expression bead array for 16-18 h at 58°C, according to the manufacturer's instructions (Illumina, Inc., San Diego, USA). Detection of the array signal was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) following the bead array manual. Arrays were scanned with an Illumina bead array Reader confocal scanner according to the manufacturer's instructions. Array data export processing and analysis were performed using Illumina BeadStudio v3.1.3 (Gene Expression Module v3.3.8).

Raw data preparation and statistical analysis

The quality of hybridization and overall chip performance were monitored by visual inspection of both internal quality control checks and the raw scanned data. Raw data were extracted using the software provided by the manufacturer (Illumina GenomeStudio v2009.2 (Gene Expression Module v1.5.4)). Array data were filtered by detection, p-value < 0.05, (similar to signal to noise) in at least

50% samples. A filtering criterion for data analysis was applied; a higher signal value was required to obtain a detection *p*-value < 0.05. A selected gene signal value was transformed by logarithm and normalized by the quantile method. The comparative analysis between the test sample and control sample was carried out using fold-change. Go-ontology analysis for a list of significant probes was performed using Protein Analysis Through Evolutionary Relationships (PANTHER) (http://www.pantherdb.org/panther/ontologies.jsp), text files containing Gene ID lists, and access numbers of illumina probe IDs. Gene Set Enrichment Analysis (GSEA) was performed to determine a priori if a defined set of genes showed a differential pattern for both biological processes and molecular function states. The one-tail Fisher Exact test was adopted to measure the gene-enrichment in annotation terms. All data analysis and visualization of differentially expressed genes were conducted using R 2.4.1 (www.r-project.org).

Validation of microarray results

To validate the microarray results, 3 samples with increased genes, 3 samples with decreased genes, and 6 randomly selected genes (Table 3.1) from infected macrophages with differential expression were submitted to quantitative real time RT-PCR. Total RNA from the macrophage, the remainder used for

microarray analysis, was reverse transcribed using the SuperScript® VILO™ cDNA synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RT-PCR reaction was performed with 1 µl of cDNA using the Rotor-Gene SYBR Green PCR kit (Qiagen, Hilden, Germany) and Rotor-Gene Q real time PCR cycler (Qiagen, Hilden, Germany). Amplification was done for 35 cycles at 95℃ for 15 sec followed by 45 sec at 60℃ with fluorescence detected during the extension phase. The expression level was determined by the $2^{-\Delta\Delta Ct}$ method (Livak *et al.*, 2001) using a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as a reference. The relative expression level was compared to a respective uninfected macrophage control to determine the expression-fold change of each gene.

3.3. Results

Microarray analysis of differentially expressed genes following infection

This study used microarrays to analyze early transcriptional responses of a mouse alveolar macrophage cell line infected with wild type or mutant *B.*

abortus using the Illumina Mouse WG-6 v2 Expression BeadChip which covers more than 45,000 transcripts. The threshold value for the microarray was chosen with an expression change of ≥1.5 or ≤1.5 –fold of both up- and down-regulated genes within a *p*-value of less than 0.05. Based on this criterion for selection, the 147, 115, 145, 157 and 152 genes were up-regulated and the 36, 21, 42, 64 and 57 genes were down-regulated in *B. abortus* 1119-3, C10, C29, D6 and D7 infected macrophages, respectively (Fig 3.1). Among the 30,854 genes analyzed, only the 183 (0.59%), 136 (0.44%), 187 (0.61%), 221 (0.72%) and 209 (0.68%) genes had altered expression levels in macrophages infected with the *B. abortus* strains 1119-3, C10, C29, D6 and D7, respectively.

The 20 most up-regulated and down-regulated genes are listed in Table 3.2 and 3.3. The *Cxcl2* gene, chemokine (C-X-C motif) ligand 2, was the most up-regulated gene with more than a 20-fold change in each experimental group. Additional genes that were strongly induced by *B. abortus* infection were related to immunity and defense (*Tnf*, *Nfkbiz*, *Ier3*, *Ccl2*, *Ccl7*, *Il1b*, *Cish* and *Nfkbia*), apoptosis (*Tnf*, *Phlda1*, *Il1b*, *Cish* and *Nfkbia*), signal transduction (*Cxcl2*, *Traf1*, *Gpr84*, *Gpr109a*, *Marcks11* and *Socs3*) and cell proliferation and differentiation (*Il1b*, *Nfkbia* and *Edn1*). These genes included *Irg1*, a previously described lipopolysaccharide (LPS)-inducible gene through a protein kinase C regulated pathway in macrophages (Chen *et al.*, 2003).

Unlike the up-regulated genes, there were no genes with decreases of more than a 3.0 fold change. The major genes down-regulated in the mouse macrophage cell line were related to signal transduction (*Cxcr4*, *5430435G22Rik*, *Tspan14* and *Fblim1*), developmental processes (*Enc1*), cell structure and motility (*Cxcr4*, *Enc1* and *Fblim1*), nucleic acid metabolism (*BC039093* and *Phf17*) and oncogenesis (*Fblim1* and *Phf17*). Most of the top 20 down-regulated genes belonged to the biological process unclassified group (*Cytip*, *Klhl6*, *Slc40a1*, *Tmem86a*, *Tmem51*, *Lhfpl2*, *Slc37a1*, *C130050O18Rik*, *AI595366*, *B930041F14Rik*, *LOC100045981*, *Arrdc3* and *Lzts2*). However, unlike in the up-regulated genes, signal transduction was involved in intracellular protein traffic and cell adhesion mediated signaling.

Gene enrichment and functional annotation analysis

To detect coordinated changes in pre-specified sets of related genes, gene enrichment and functional annotation were analyzed. Differentially expressed genes were categorized by biological process and molecular function state using the PANTHER classification database by means of Fisher's exact test.

As a result, 8 biological process categories (signal transduction, immunity and defense, apoptosis, cell proliferation and differentiation, developmental process,

cell cycle, cell structure and motility and oncogenesis) and 3 molecular function categories (signaling molecule, receptor and kinase) were found to be associated with early transcriptional changes following *B. abortus* infection (Fig 3.2 and 3.3).

Most of the up-regulated genes involved in the signal transduction category were cytokines (*Tnf, Il1a, Il1b, Ltb and Csf2*) and chemokines (*Cxcl2, Ccl2, Ccl7, Ccl3* and *Ccl9*), which are also related to host immune response and defense, similar to the previously reported transcriptional analysis of *B. melitensis* infected macrophages (Covert et al., 2009). Like the proinflammatory cytokines and chemokines, genes involved in the apoptosis category (*Fas, Traf1* and *Ripk2*) showed an increased transcription level as a response to an intracellular pathogen. This is a useful way for the host to eliminate infected cells, decreasing the likelihood of spread of the infection to neighboring cells and preventing pathogenicity. However, several genes involved in the inhibition of apoptosis (*Cish* and *Socs3*) were up-regulated as an effort to enhance bacterial survival in the host cell. In the signal transduction category, *Gpr84, Gpr109a* and *Adora2b* genes related to G-protein coupled receptors (GPCRs) were up-regulated following *B. abortus* infection.

In contrast to the up-regulated genes, genes down-regulated in the signal transduction category were *Rab40c, Rin2* and *5430435G22Rik*, small GTPases

which mediate intracellular trafficking of this bacterium without affecting internalization (Chaves-Olarte *et al.*, 2002). These were also down-regulated in macrophages infected with other *Brucella* spp. (Covert et al., 2009). Although more than 50% of the down-regulated genes were categorized as unclassified, cytohesin 1 interacting protein (*Cytip*), a membrane-bounded organelle that carries materials newly ingested by endocytosis and passes many of the materials to lysosomes for degradation, was down-regulated. In addition, genes related to ubiquitination were down-regulated (*Arrdc3* and *Fbxo21*), suggesting the survival strategy of this bacterium.

Differentially expressed genes in internalization-defective mutant infected cells compare to wild type infected cell

To analyze the genes with altered transcription between wild type and mutant infected macrophages, the median of the normalized hybridization signals of the wild type infected cells against the cells infected with the four other mutants were plotted (Fig 3.4). As shown in the graphs, most of the genes in each mutant infected group lie within a diagonal where expression is equivalent between the groups, indicating that the majority of genes are expressed at similar levels when compared to the wild type infected group. There was no up-regulated gene in the

mutant infected groups compared to the wild type infected group. There were 6 (*Il1b*, *Lcn2*, *Cxcl2*, *Edn1*, *Ccl2* and *Ccl7*) and 1 (*Rn18s*) down-regulated genes in the groups infected with mutants C10 and D7 compared to the wild type infected group, respectively. However, only two genes, *Cxcl2* (Chemokine C-X-C motif ligand 2) and *Ccl2* (Chemokine C-C motif ligand 2) in the C10 infected group were statistically significant ($p<0.05$). These genes are chemotactic for polymorphonuclear leukocytes and monocytes to the sites of infection.

Validation of microarray data

To confirm the microarray data, quantitative RT-PCR were performed with randomly selected genes. *Irg1*, *Fas* and *Ccl4* genes from the up-regulated gene group and *Rab40c*, *Rin2* and *Rab27a* genes from the down-regulated gene group were selected. The microarray data was validated because all genes tested by qRT-PCR showed more fluctuation (increased or decreased) than the microarray data, but in the same direction (Fig 3.5). Also, the significant changes in mutant infected cells compared to wild type infected cells were analyzed with qRT-PCR data from *Rab40c*, *Rin2* and *Rab27a*, which showed both more or less than 1.5-fold changes according to the infected strains with microarray data. However, no

significant changes were detected in the mutant infected groups compared to the wild type infected group, as shown in the microarray analysis data.

3.4. Discussion

Understanding the host-pathogen interaction is very important to reveal the mechanisms of the pathogen related to global host gene regulation during infection, genomics, and mechanisms of secretion of bacterial virulence factors, especially for intracellular bacteria. Microarray is a powerful tool that can increase our knowledge about the host-pathogen interaction by investigating host responses to the pathogen infection and vice versa (Kato-Maeda *et al.*, 2001; Leroy *et al.*, 2010). *B. abortus* is an intracellular pathogen that can survive and replicate within host macrophages. In light of this, to investigate the host-pathogen interaction of our internalization-defective *B. abortus* mutants described previously (Cha *et al.*, 2012), microarray analysis was performed with 30,854 murine genes following *B. abortus* infection of the RAW 264.7 macrophage. As four hours of infection was enough to elicit specific transcriptional responses in macrophages infected with different *Brucella* spp.

(Covert *et al.*, 2009), the macrophages following four hours of infection were analyzed.

The overall transcriptional profile was similar to the previous study of infected *B. abortus* strain 2308 (Eskra *et al.*, 2003), although different microarray chips were used covering more than 30,000 genes. However, some genes involved with the G protein coupled receptor (GPCR) showed an increased expression level compared to the non-infected group. The G protein-coupled receptor 84 (GPR84) is induced in monocytes and macrophages and functions as a specific receptor for medium-chain free fatty acids (FFAs) of C_9 to C_{14} length. It also amplifies LPS-stimulated IL-12 p40 production and is coupled to a pertussis toxin-sensitive $G_{i/o}$ pathway once activated (Wang *et al.*, 2006). The pertussis toxin is secreted by the pertussis toxin liberation (Ptl) type IV secretion system (T4SS) of *Bordetella pertussis* (Backert and Meyer, 2006) and results in accumulation of intracellular cyclic adenosine monophosphate (cAMP) (Carbonetti, 2010). This result suggests that the virB type IV secretion system (T4SS) of *B. abortus*, which is core virulence factor of this bacterium (Celli *et al.*, 2003) as well as a mediator for host innate immune response (Roux *et al.*, 2007), might secret some effector molecules that acts to increase intracellular cAMP for intracellular survival (Jimenez de Bagues *et al.*, 2005) via GPCR of the host cell. The other GPCR, GPR109A, is a member of the nicotinic acid receptor family of

GPCRs that reduces the level of intracellular cAMP following inhibition of lipolysis in adipocytes (Zhang *et al.*, 2005). Moreover, the effects of nicotinic acid on macrophages, spleen and probably adipocytes are mediated via an identical, unique G protein-coupled receptor (Lorenzen *et al.*, 2002). This suggests that *B. abortus* may utilize the GPCR system to prevent lipolytic processing within phagosomes in spite of cAMP reduction. As the previous study showed, the regulator of G protein signaling 2 (RGS2) expression was induced following *B. abortus* infection (Kim *et al.*, 2012). Several regulators of G protein signaling (*Arhgef3*, *Rassf4* and *Rgs16*) were found with increased expression levels, although the precise mechanism remains to be elucidated. Taken together, these alterations in the G protein mediated signaling system may result in increased survival of *B. abortus* within the macrophage.

Interestingly, *Cxcr4*, a gene coding chemokine (C-X-C motif) receptor 4 (CXCR4), was down-regulated, whereas other chemokine-mediated genes had been up-regulated. As the CXCR4 expression is reduced by inflammatory cytokines such as tumor necrosis factor-α (TNF- α) and interleukin-1β (IL-1β) (Gupta et al., 1998), considered as a consequence of up-regulation of *Tnf* and *Il1b*. However, a recent study showed that extracellular ubiquitin is a natural ligand of CXCR4 (Saini *et al.*, 2010), and also up-regulated (*Znrf1*, *Herpud1* and *Socs3*) and down-regulated (*Cxcr4*, *Fbxo21* and *Rab40c*) genes involved in the

ubiquitin-proteasome system were found. Although CXCR4 is a member of GPCR, which could affect the other signaling cascades, and a receptor for extracellular ubiquitin, it has been shown that cellular uptake of extracellular ubiquitin results in its covalent conjugation to intracellular proteins of the target cell (Gupta *et al.*, 1998; Daino *et al.*, 2000). In light of this, the exact function of remaining genes is not fully understood, yet the host cell utilizes the ubiquitin-proteasome system in an effort to clear this pathogen and controlling this system is a bacterial survival strategy might be speculated.

Gadd45 is a growth arrest and DNA damage gene and includes *Gadd45a*, *Gadd45b* and *Gadd45g*. A previous study found that *Gadd45a* was induced in response to DNA damage and function to inhibit the growth of damaged cells in *Brucella* infected macrophages (Eskra *et al.*, 2003; Covert *et al.*, 2009). In addition, increased expression of *Gadd45b* was observed, indicating the regulatory roles of activated macrophages against *Brucella* infection (Covert *et al.*, 2009) as well as anti-apoptotic activity since *Gadd45a*- and *Gadd45b*-deficient mice were sensitized to genotoxic-stress-induced apoptosis (Gupta *et al.*, 2005). In this study, increased expression of both *Gadd45a* and *Gadd45b* were observed; however, the expression level of *Gadd45g* gene was decreased. Gadd45a, Gadd45b and Gadd45g serve similar, but not identical, functions along different apoptotic and growth inhibitory pathways (Selvakumaran *et al.*, 1994;

Vairapandi *et al.*, 2002) and Gadd45g acts as a positive mediator of apoptosis in response to genetic and environmental stress (Zerbini *et al.*, 2006; Wang *et al.*, 2007). This suggests *Gadd45g* was down-regulated to protect against apoptosis, though the outcomes of Gadd45 function are determined by the stress stimulus encountered, cell type, and interactions with other proteins (Gupta *et al.*, 2005).

In spite of these novel genes identified with altered expression levels compared to uninfected macrophages, no genes that changed in a different direction. Only two genes (*Cxcl2* and *Ccl2*) in the mutant C10 infected group were slightly decreased in the same direction as wild type infected macrophages. As our mutants did not show a fully defective internalization phenotype or complete deletions in bacterial cellular envelope components (Cha *et al.*, 2012), assuming that very small amount of bacteria could elicit a response in the host cell. However, considering both *Salmonella typhimurium* infected macrophages and purified LPS inducted macrophages showed similar changes in gene expression (Rosenberger *et al.*, 2000) and all of mutants used in this study were smooth strains, concluding that an internalization deficiency in *B. abortus* would not affect transcriptional changes in macrophages if there was LPS contained. This is consistent with a previous study that showed few transcriptional changes in macrophages infected with different *Brucella* species including both smooth (*B. melitensis* and *B. neotomae*) and rough LPS strains (*B.ovis*) (Covert *et al.*, 2009).

In summary, it was very difficult to clarify the alterations in host cellular transcription in response to infection with internalization-defective mutants. Thus, this result concluded mutations within the *ccmC, ppk,* BruAb1_1377 and BruAb2_0168 loci would not affect the host cellular responses. However, several novel gene changes related to the GPCR system, ubiquitin-proteosome system, and growth arrest and DNA damages were found in response to *B. abortus* infection. Thus this result speculated about the virulence factors of this bacterium, including T4SS and its translocation of potential substrates. These findings may contribute to a better understanding the molecular mechanisms of the underlying host-pathogen interactions and need to be studied further.

Table 3.1. Primers used for qRT-PCR

Accession No.	Gene symbol (Description)	Forward primers (5'→3')	Reverse primers (5'→3')
NM_008392.1	Irg1 (Immunoresponsive gene 1)	CCTGTGCCTCGCTGCTCGAC	CGTGTCGAAGCTTGGCGGGT
NM_007987.2	Fas (TNF receptor superfamily member 6)	CCTGCGCCCCATGCACAGAA	TCTGGGTCAGGGTGCAGTTTGT
NM_013652.2	Ccl4 (Chemokine (C-C motif) ligand 4)	GCTCTGCGTGTCTGCCCTCTC	TGGTGCTGAGAACCCTGGAGCA
NM_139154.2	Rab40c (Rab40c, member RAS oncogene family)	GACGGCGCAGCTGAATCCCC	CCAGCTTGACACGCCGTCCA
NM_028724.4	Rin2 (Ras and Rab interactor 2)	TCTGCCCTGCCTCCTTGCGT	GCACTCCAGCTCCGAAGGCG
NM_023635.5	Rab27a (Rab27a, member RAS oncogene family)	AAAAGGCCAGTCGCACGGGG	TGTCCCTGCGGTGTTGCGTC
NM_008084.2	Gapdh (Glyceraldehyde-3-phosphate dehydrogenase)	CCCCAGCAAGGACACTGAGCAAG	TGGGGGTCTGGGATGGAAATTGTG

Table 3.2. The 20 most up-regulated genes in mouse macrophage cell line RAW 264.7 infected with each *B.abortus* compare to uninfected macrophage

Gene symbol	Description	1119-3		C10		C29		D6		D7	
		FC	P-value	FC	P-value	FC	P-value	FC	P-value	FC	P-value
Cxcl2	Chemokine (C-X-C motif) ligand 2	34.68	1.56E-193	21.51	1.54E-156	33.41	7.14E-220	43.82	5.67E-166	29.62	4.21E-205
Tnf	Tumor necrosis factor	17.47	4.45E-150	14.18	3.82E-133	21.83	1.58E-168	22.45	6.39E-120	18.35	3.46E-157
Nfkbiz	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	15.58	3.68E-118	11.51	1.42E-100	18.61	3.69E-151	17.88	3.63E-96	14.34	1.80E-128
Irg1	Immunoresponsive gene 1	12.29	2.47E-99	8.82	9.26E-80	10.29	4.04E-96	14.74	1.45E-83	11.29	1.04E-106
Ier3	Immediate early response 3	8.35	4.93E-82	6.36	1.01E-63	9.03	7.44E-87	9.04	1.02E-55	7.6	2.81E-78
Traf1	NOD-derived CD11c +ve dendritic cells cDNA, RIKEN full-length enriched library, clone:F630118K07 product:Tnf receptor-associated factor 1, full insert sequence	8.13	5.31E-56	5.68	3.78E-37	7.3	1.67E-58	9.22	1.21E-56	7.54	1.42E-66
Phlda1	Pleckstrin homology-like domain, family A, member 1	6.56	3.88E-53	5.04	8.66E-43	6.97	4.86E-67	7.27	3.88E-45	6.49	9.56E-62
Gpr84	G protein-coupled receptor 84	6.30	9.74E-59	5.36	2.85E-50	6.74	1.83E-64	7..45	1.48E-45	6.48	1.31E-67
Ccl2	Chemokine (C-C motif) ligand 2	6.16	4.74E-50	4.03	1.50E-31	5.2	2.46E-48	6.16	9.30E-38	5.14	3.57E-47
Ccl7	Chemokine (C-C motif) ligand 7	5.26	8.97E-32	3.48	2.07E-18	3.68	7.03E-21	5.91	5.23E-36	4.85	1.48E-39
Il1b	Interleukin 1 beta	4.89	2.78E-26	2.87	5.56E-13	3.63	2.88E-20	6.12	1.95E-37	4.29	6.18E-33
Gpr109a	Niacin receptor 1	4.45	2.13E-23	3.31	5.81E-17	3.73	4.66E-21	5.38	3.27E-32	4.43	8.81E-35
Cish	Cytokine inducible SH2-containing protein	4.43	4.39E-23	3.0	3.85E-14	3.83	6.24E-22	5.35	5.20E-32	4.21	2.63E-32
Marcksl1	MARCKS-like 1	4.32	2.26E-29	3.29	6.83E-19	4.27	1.75E-35	4.76	1.14E-27	4.27	2.45E-35

Table 3.2. (*Continued*)

Gene symbol	Description	1119-3		C10		C29		D6		D7	
		FC	P-value	FC	P-value	FC	P-value	FC	P-value	FC	P-value
Cd83	CD83 antigen	4.01	7.25E-26	3.31	4.48E-19	4.32	3.18E-36	4.47	1.92E-25	3.94	2.74E-31
Il4i1	Interleukin 4 induced 1	4.0	3.19E-20	3.11	4.16E-15	3.64	2.06E-20	4.56	4.58E-26	3.88	8.18E-29
Socs3	Suppressor of cytokine signaling 3	3.66	1.95E-20	2.45	3.55E-09	3.78	1.80E-26	4.93	6.06E-29	3.05	1.49E-19
Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	3.58	1.78E-22	3.34	2.00E-21	4.25	1.50E-36	4.46	2.12E-25	3.73	2.96E-29
Edn1	Endothelin 1	3.53	2.85E-16	2.28	8.95E-08	2.49	5.74E-10	3.51	1.20E-17	2.84	1.34E-15
Ehd1	EH-domain containing 1	3.45	8.17E-17	2.83	1.43E-12	3.22	2.12E-17	4.01	1.02E-21	3.5	1.30E-24

Table 3.3. The 20 most down-regulated in mouse macrophage cell line RAW 264.7 infected with each *B.abortus* compare to uninfected macrophage

Gene symbol	Description	1119-3		C10		C29		D6		D7	
		FC	P-value	FC	P-value	FC	P-value	FC	P-value	FC	P-value
Cytip	Cytohesin 1 interacting protein	-2.9	1.62E-11	-2.16	1.08E-06	-2.35	1.19E-08	-2.82	6.46E-12	-2.53	3.53E-13
Cxcr4	Chemokine (C-X-C motif) receptor 4	-2.17	6.43E-06	-1.81	8.36E-04	-2.13	9.94E-07	-2.63	2.59E-10	-2.12	4.46E-07
Klhl6	Kelch-like 6 (Drosophila)	-2.1	5.71E-07	-1.73	3.39E-04	-1.73	8.32E-05	-2.15	2.06E-06	-1.91	1.06E-06
Enc1	Ectodermal-neural cortex 1	-2.01	1.01E-04	-1.77	1.71E-03	-1.9	1.03E-04	-1.98	5.11E-05	-1.98	9.61E-07
Slc40a1	Solute carrier family 40 (iron-regulated transporter), member 1	-1.95	5.21E-06	-1.83	2.61E-05	-1.8	1.17E-05	-2.31	1.15E-07	-1.92	6.60E-07
Tmem86a	Transmembrane protein 86A	-1.85	1.23E-03	-1.51	>0.05	-1.79	7.58E-04	-2.02	2.43E-05	-1.79	4.44E-04
BC039093	cDNA sequence BC039093	-1.84	9.07E-04	-1.54	>0.05	-1.7	2.06E-03	-1.92	1.33E-04	-1.73	1.73E-04
5430435G22Rik	RIKEN cDNA 5430435G22 gene	-1.81	2.26E-03	-1.57	0.04	-1.74	1.90E-03	-1.93	1.25E-04	-1.68	5.34E-04
Tmem51	Transmembrane protein 51	-1.81	3.69E-04	-1.57	0.01	-1.65	8.54E-04	-2.08	9.03E-06	-1.86	4.84E-06
Lhfpl2	Lipoma HMGIC fusion partner-like 2	-1.78	1.60E-04	-1.52	0.02	-1.8	8.18E-06	-1.96	6.97E-05	-1.82	6.49E-06
Slc37a1	10 days neonate skin cDNA, RIKEN full-length enriched library, clone:4732478E01 product:solute carrier family 37 (glycerol-3-phosphate transporter), member 1, full insert sequence	-1.78	3.92E-03	-1.57	0.04	-1.61	0.02	-1.8	1.16E-03	-1.73	2.98E-04
C130050O18Rik	RIKEN cDNA C130050O18 gene	-1.78	3.88E-03	-1.62	0.02	-1.85	2.48E-04	-1.94	1.12E-04	-1.81	1.05E-04
AI595366	Leucine rich repeat containing 14B	-1.77	3.95E-03	-1.51	>0.05	-1.59	0.02	-1.85	4.90E-04	-1.63	0.01
B930041F14Rik	RIKEN cDNA B930041F14 gene	-1.75	4.00E-03	-1.54	>0.05	-1.67	3.77E-03	-1.87	3.39E-04	-1.74	1.36E-04

Table 3.3 (*Continued*)

Gene symbol	Description	1119-3		C10		C29		D6		D7	
		FC	P-value	FC	P-value	FC	P-value	FC	P-value	FC	P-value
LOC100045981	Similar to synaptotagmin XI	-1.74	5.45E-03	-1.58	0.04	-1.65	6.70E-03	-1.97	5.60E-05	-1.85	1.29E-05
Arrdc3	Arrestin domain containing 3	-1.74	6.50E-03	-1.6	0.03	-1.71	2.72E-03	-1.95	9.29E-05	-1.55	0.02
Tspan14	Tetraspanin 14	-1.73	1.60E-03	-1.43	>0.05	-1.45	0.04	-1.72	4.53E-03	-1.52	0.01
Lzts2	Leucine zipper, putative tumor suppressor 2	-1.72	8.22E-03	-1.63	0.02	-1.74	1.82E-03	-1.87	3.22E-04	-1.76	9.06E-04
Fblim1	Filamin binding LIM protein 1	-1.72	2.29E-03	-1.53	0.04	-1.49	0.02	-1.61	0.02	-1.56	5.47E-03
Phf17	PHD finger protein 17	-1.7	0.01	-1.59	0.03	-1.62	0.01	-1.72	4.73E-03	-1.55	8.58E-03

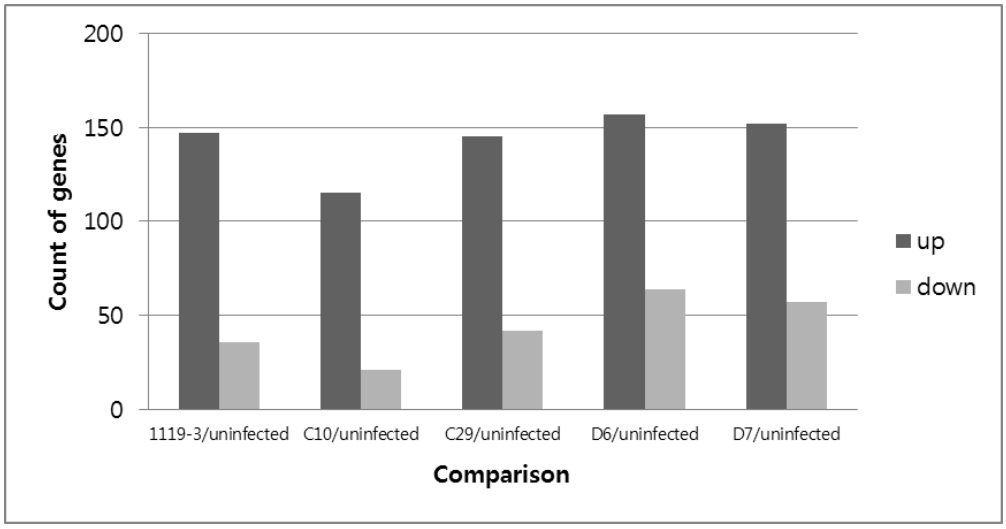
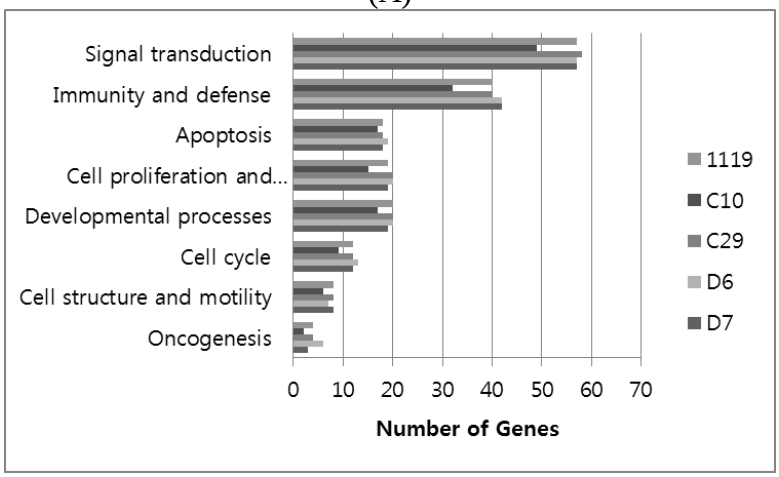


Fig 3.1. Count of genes with up and down regulated compare to uninfected macrophage.

(A)



(B)

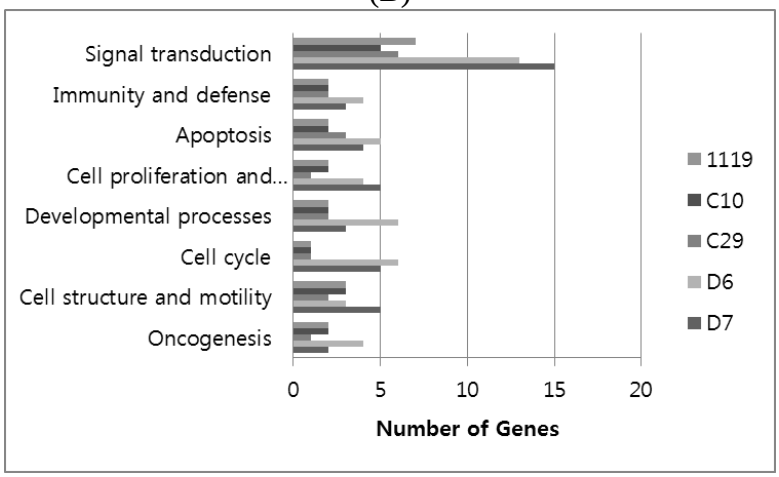
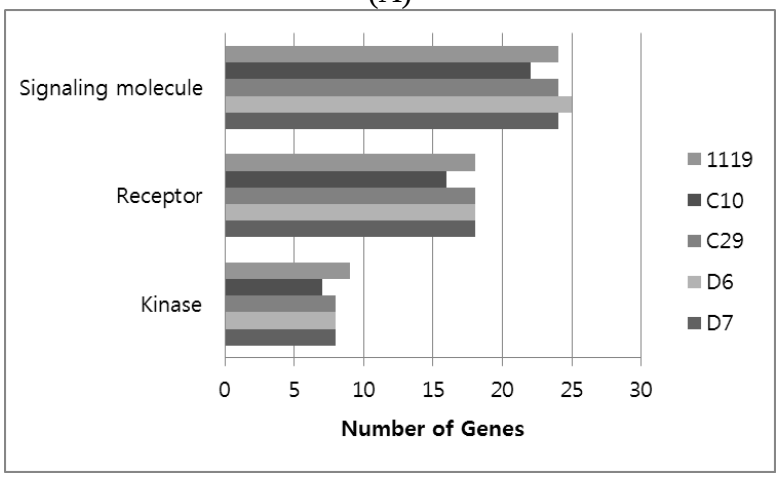


Fig 3.2. Categorization by biological process of genes with significantly regulated transcripts in RAW 264.7 macrophage at 4h post infection of each *B.abortus* strains. Up-regulated transcripts (A) and down-regulated transcripts (B).

(A)



(B)

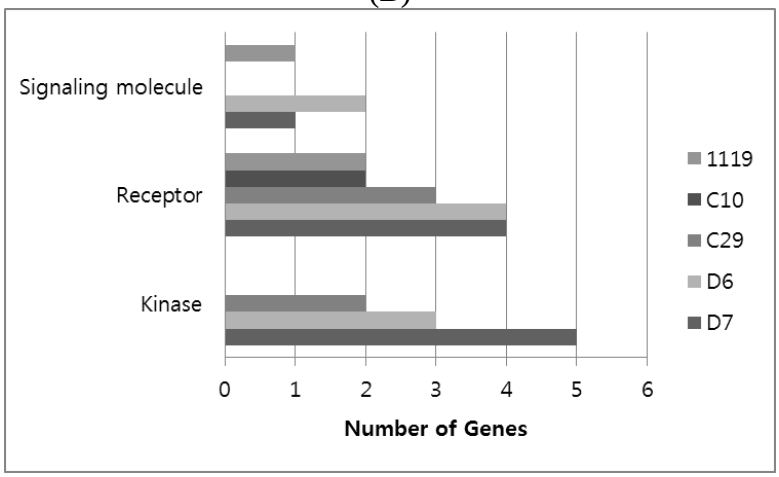


Fig 3.3. Categorization by molecular function of genes with significantly regulated transcripts in RAW 264.7 macrophage at 4h post infection of each *B.abortus* strains. Up-regulated transcripts (A) and down-regulated transcripts (B).

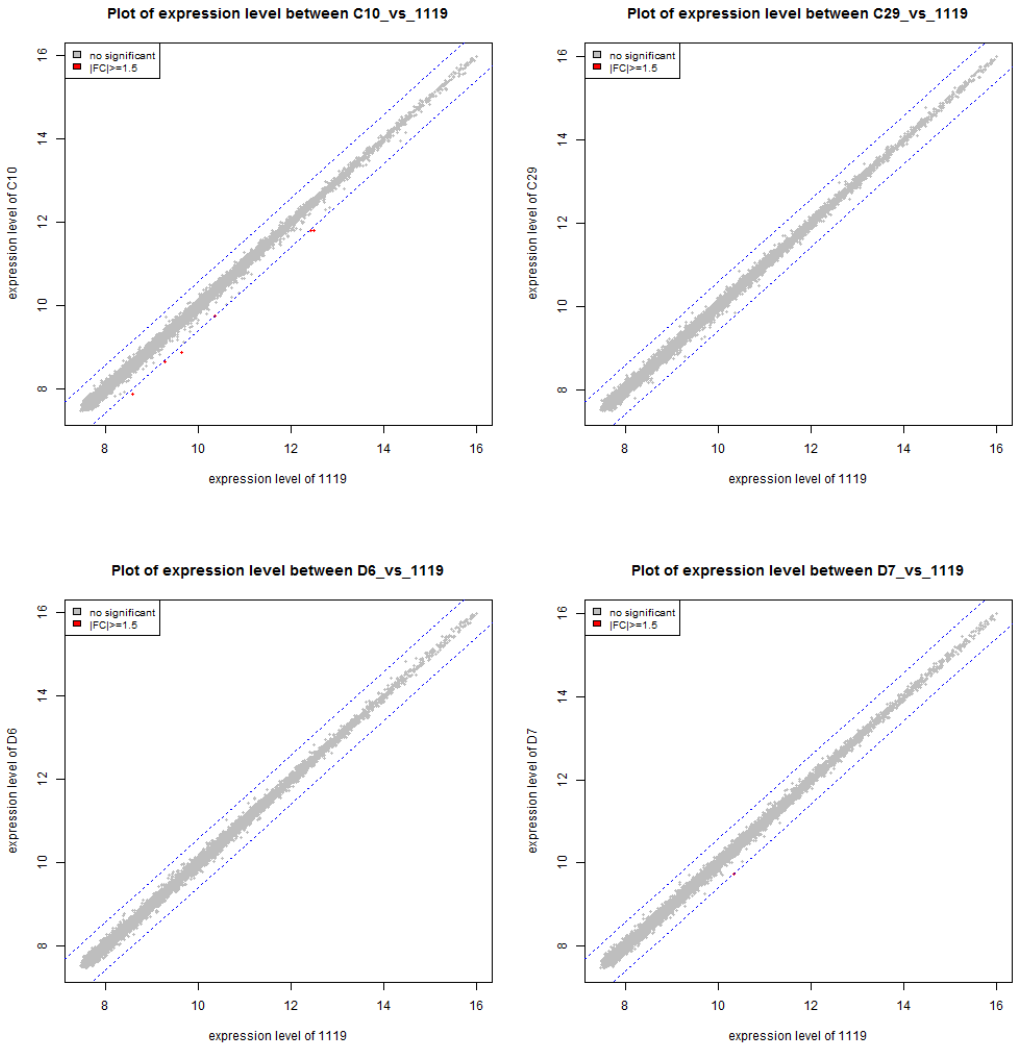
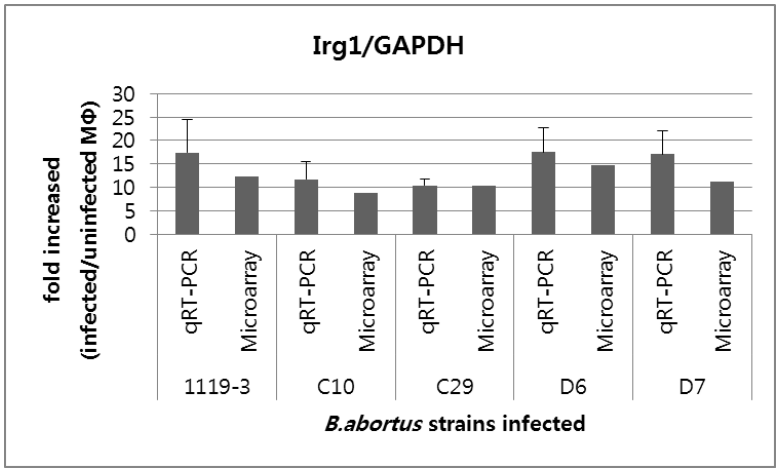
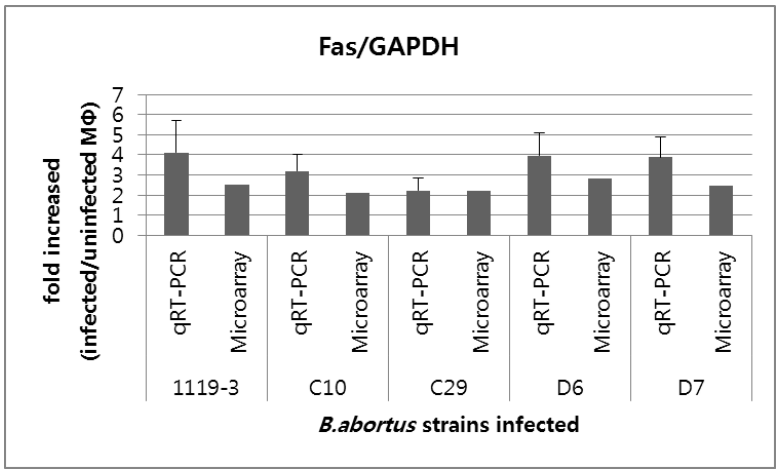
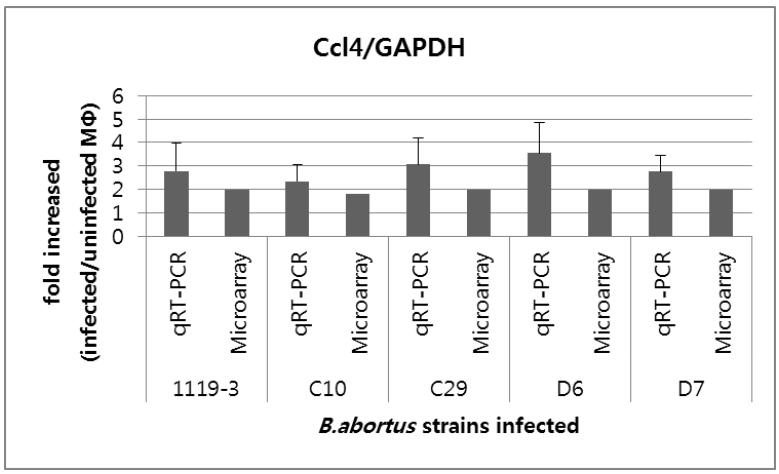
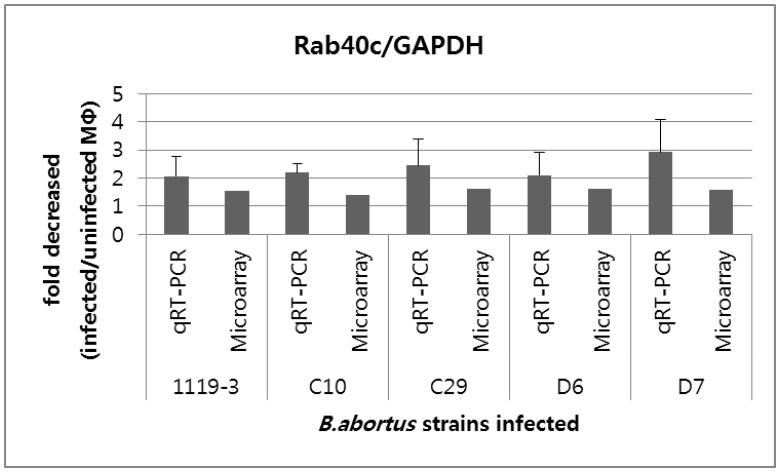


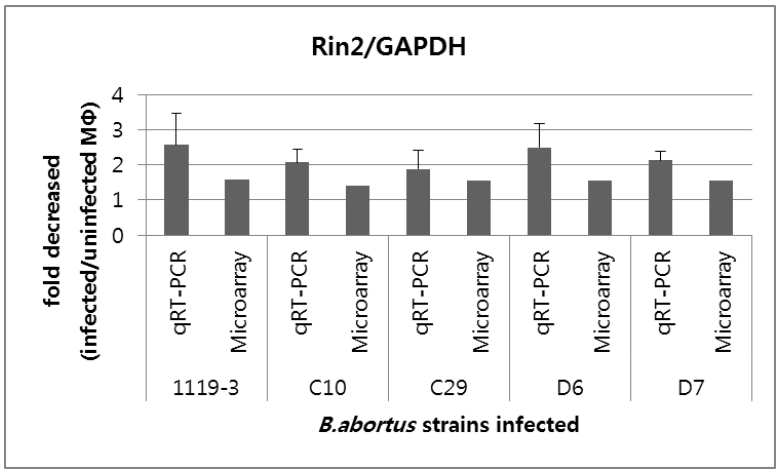
Fig 3.4. Plots of the expression level between wild type infected cell versus mutant infected cell. Red dots indicate an expression level change of ≥1.5 or ≤1.5 fold of both up- and down- regulated genes. Expression level was calculated by base 2 logarithm of normalized hybridization signals from each sample.


Irg1/GAPDH
fold increased
(infected/uninfected MΦ)
30
25
20
15
10
5
0
qRT-PCR
Microarray
1119-3
C10
C29
D6
D7
B.abortus strains infected


Fas/GAPDH
fold increased
(infected/uninfected MΦ)
7
6
5
4
3
2
1
0
qRT-PCR
Microarray
1119-3
C10
C29
D6
D7
B.abortus strains infected


Ccl4/GAPDH
fold increased
(infected/uninfected MΦ)
6
5
4
3
2
1
0
qRT-PCR
Microarray
1119-3
C10
C29
D6
D7
B.abortus strains infected


Rab40c/GAPDH
fold decreased
(infected/uninfected MΦ)
5
4
3
2
1
0
qRT-PCR
Microarray
1119-3
C10
C29
D6
D7
B.abortus strains infected


Rin2/GAPDH
fold decreased
(infected/uninfected MΦ)
4
3
2
1
0
qRT-PCR
Microarray
1119-3
C10
C29
D6
D7
B.abortus strains infected

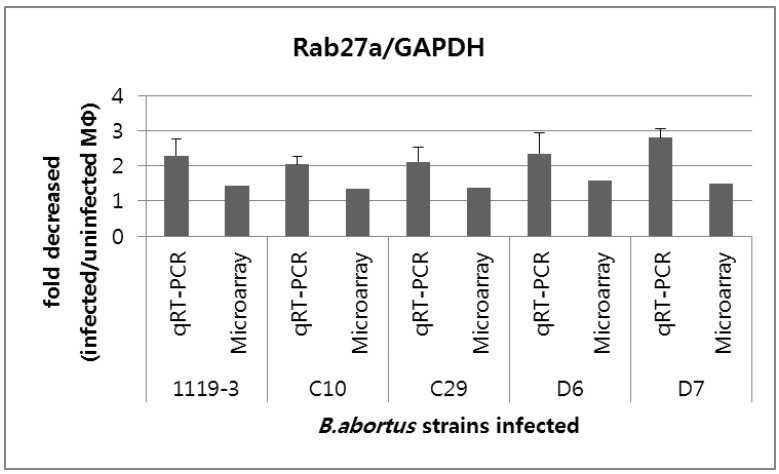

Rab27a/GAPDH
fold decreased
(infected/uninfected MΦ)
4
3
2
1
0
qRT-PCR
Microarray
1119-3
C10
C29
D6
D7
B.abortus strains infected

Fig 3.5. Validation of microarray data by quantitative RT-PCR. The relative expression level was normalized by *Gapdh* expression level and relative to uninfected cells. RT-PCR data were averaged from three independent RNA isolation and presented as mean relative expression with an error bar which represents SEM.

General Conclusions

Up to date, it is still far from complete understanding pathogenesis for *Brcuella* infection. To develop effective diagnostic methods, it is very important to understand the virulence factors and the complete pathogenic mechanisms of *Brucella* infection. Also, understanding host responses including immune responses are very important. To provide fundamental information on these questions, the outer membrane protein expression profiles of *B. abortus* from Korean field isolates and immune responses against these OMPs were analyzed. Moreover, host cell internalization-defective mutants were generated and their profiles of envelope proteins were analyzed. Finally, the transcriptional responses of macrophages infected with these mutants were analyzed.

1. OMPs of *B. abortus* from Korean field isolates showed different expression patterns from as well as antigenic reactivity with brucellosis-positive sera. Moreover, these OMPs were more effective than the whole live bacteria in induction of cellular immune responses.

2. OMP25 is not an essential factor for host cellular internalization, however it might be involved in host cellular internalization. Also, the

locus BruAb2_0168 was associated to the expression of OMP25, OMP28 and Porin2b. The pleiotropic effects of *ccmC* gene were investigated.

3. It was very difficult to clarify the alterations in host cellular transcription in response to infection with internalization-defective mutants. However, several novel gene changes related to the GPCR system, ubiquitin-proteosome system, growth arrest, and DNA damages were found in response to *B. abortus* infection.

Results from the Korean field isolates of *B.abortus* suggested that a new diagnostic method should be developed using their own field isolates in each country. Also, the results from the internalization-defective mutants suggested that these findings may contribute to a better understanding of the molecular mechanisms of underlying host-pathogen interactions and need to be studied further.

국 문 초 록

세포내 침입능 결손 *Brucella abortus* 감염에 대한 숙주세포의 면역 및 초기 전사반응

차 승 빈

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수의학과 수의 미생물학 전공

Brucella abortus 는 소 브루셀라병의 원인체로서 인체에서는 파상열(undulant fever) 로 알려진 만성 질병을 유발한다. 가축에서 이 질병은 유산과 불임의 형태로 나타난다. 통성(facultative) 세포내 세균의 특징은 대식구(phagocytes) 내에서도 생존하고 심지어 증식을 할 수 있는 능력이다. 세포내 기생세균들은 순환하는 항체가 접근할수 없는 적절한 위치를 찾을 수 있기 때문에, 이 세균의 제거를 위해서는 세포외 기생세균에 적용되는 방어기작과는 다른 세포면역의 메커니즘이

필요하다. 이러한 세포성 면역에서 가장 중요한 cytokine 은 Th1 세포에서 생성되는 INF-γ이다.

본 연구에서는 혈청학적 분석을 통하여 국내 분리주에서 특이하게 작용하는 항원을 발견하고자 하였으며, 진단적인 목적을 위해 *B. abortus* 의 외막 단백질 (OMPs)과 살아있는 세균을 접종시킨 마우스의 비장세포에서 생성하는 IFN-γ의 차이를 규명하였다. 세균 감염 그룹보다 OMP 접종 그룹에서 더 많은 IFN-γ를 생성하는 것을 확인하였으며, 이를 통하여 OMPs가 면역반응을 잘 유도하는 것으로 추정되었다.

브루셀라병의 예방 및 관리를 위해서는 증상을 보이지 않는 보균체까지 감지할 수 있는 새로운 진단기법이 필요하며, 이를 위해서는 발병 메커니즘을 이해하는 게 가장 중요하다. 하지만, 브루셀라병의 발병 메커니즘은 VirB 시스템과 BvrR/BvrS 조절시스템과 같은 몇 가지 발병 인자들을 제외하고는 제대로 밝혀져 있지 않은 실정이다. 이러한 점에 기인하여, 본 연구는 브루셀라균의 생존 전략의 첫 단계인 숙주 세포내 침입능과 관련된 세균측 인자를 밝히기 위해 노력하였다. 또한, 숙주 macrophages 내에서 브루셀라균의 생존전략을

이해하기 위해서, 숙주 세포내 침입능 결여 돌연변이주를 포함한 브루셀라균 감염에 따른 macrophage 내에서의 전사반응을 분석하였다.

숙주 세포내 침입능에 관여하는 브루셀라균의 단백질을 관찰하기 위하여, Tn5 transposome 복합체를 사용하여 *B. abortus* 에 삽입하여 변이주를 제작하였다. 총 132 개의 변이주가 제작되었고, 세포내 침입능이 결여된 변이주를 선별하였다. 또한 이들의 유전자좌 및 막단백질의 특성을 연구하였다. Transposon C10, C29, D7 의 삽입 사이트는 각각 *ccmC*, *ppk*, BruAb2_0168 였다. C10 균주에서는 세포내 침입능이 저하되었지만 세포 외막 단백질의 발현에는 아무런 변화를 볼 수 없었다. 하지만, 세포내 침입능이 저하된 다른 변이주 C29 는 OMP25 발현의 감소를 볼 수 있었고, D7 역시 OMP25, OMP28 과 Porin2b 의 발현량이 감소된 것을 관찰할 수 있었다. 이는 숙주세포 내 침입에 있어 OMP25 는 결정적인 요소가 아니라, 연관만 되어 있음을 보여준다. 본 실험에서는 OMP25, OMP28, Porin2b 의 발현에 관련이 있는 *ppk* gene 과 BruAb2_0168 을 발견하였고, 또한 *ccmC* gene 의 다면발현성 효과에 대해서도 확인하였다.

감염에 따른 숙주세포의 반응에서는, wild type 과 돌연변이주에 감염된 macrophage 모두 proinflammatory cytokines, chemokines,

apoptosis 의 발현을 증가시켰고, G-protein coupled receptors (*Gpr84, Gpr109a, Adora2b*)는 증가한 반면 세포간 trafficking 을 조정하는 작은 GTPase 와 관련된 유전자들은 감소하였다. 또한, cytohesin 1 과 상호작용을 하는 단백질 (*Cytip*)과 ubiquitination 연관 유전자들 (*Arrdc3* and *Fbxo21*)은 감소하였고, 이는 이 세균의 생존전략임을 시사한다. 하지만, wild type 감염 그룹과 돌연변이주 감염 그룹간에 주목할 만한 차이점은 발견하지 못하였다. 하지만, *B. abortus* 감염에 의해 GPCR 시스템, ubiquitin-proteosome 시스템 그리고 성장 억제 및 DNA 손상과 관련된 유전자가 변화하는 것을 새롭게 확인하였다.

본 연구를 통해 새로운 진단기법 개발은 국내 분리주를 이용하여 개발되어야할 필요성을 제시하였으며, 또한 이러한 발견은 기본적인 숙주-병원체 관계의 분자적 메커니즘을 더 잘 이해하는 데 기여할 것으로 보이며, 앞으로 더 많은 연구가 진행되어야 할 것이다.

키워드 : 브루셀라, 세포내 침입능, 변이주, 대식구, 전사체, 감마 인터페론, 세포외막 단백질

학번 : 2007-21793

References

Ackermann MR, Cheville NF, Deyoe BL. Bovine ileal dome lymphoepithelial cells: endocytosis and transport of *Brucella abortus* strain 19. Vet Pathol. 1988. 25(1):28-35.

Al Dahouk S, Nöckler K, Scholz HC, Tomaso H, Bogumil R, Neubauer H. Immunoproteomic characterization of *Brucella abortus* 1119-3 preparations used for the serodiagnosis of *Brucella* infections. J Immunol Methods. 2006. 309(1-2):34-47.

Al Dahouk S, Tomaso H, Nöckler K, Neubauer H, Frangoulidis D. Laboratory-based diagnosis of brucellosis--a review of the literature. Part II: serological tests for brucellosis. Clin Lab. 2003. 49(11-12):577-589.

Almuneef M, Memish ZA. Prevalence of *Brucella* antibodies after acute brucellosis. J Chemother. 2003. 15:148-151.

Anderson TD, Meador VP, Cheville NF. Pathogenesis of placentitis in the goat inoculated with *Brucella abortus*. I. Gross and histologic lesions. Vet Pathol. 1986. 23(3):219-226.

Arellano-Reynoso B, Lapaque N, Salcedo S, Briones G, Ciocchini AE, Ugalde R, Moreno E, Moriyón I, Gorvel JP. Cyclic beta-1,2-glucan is a *Brucella*

virulence factor required for intracellular survival. Nat Immunol. 2005. 6(6):618-25.

Backert S, Meyer TF: Type IV secretion systems and their effectors in bacterial pathogenesis. Curr Opin Microbiol. 2006. 9(2):207-217.

Baert B, Baysse C, Matthijs S, Cornelis P. Multiple phenotypic alterations caused by a c-type cytochrome maturation *ccmC* gene mutation in *Pseudomonas aeruginosa*. Microbiology. 2008. 154:127-138.

Bandara AB, Sriranganathan N, Schurig GG, Boyle SM. Putative outer membrane autotransporter protein influences survival of *Brucella suis* in BALB/c mice. *Vet Microbiol.* 2005. 109:95-104.

Barenkamp SJ, Munson RS jr, Granoff DM. Subtyping isolates of *Haemophilus influenzae* type b by outer-membrane protein profiles. J Infect Dis. 1981. 143: 668-676.

Bellaire BH, Roop RM, II, Cardelli JA. Opsonized virulent *Brucella abortus* replicates within nonacidic, endoplasmic reticulum-negative, LAMP-1-positive phagosomes in human monocytes. Infect Immun. 2005. 73(6):3702-3713.

Boschiroli ML, Foulongne V, O'Callaghan D: Brucellosis: a worldwide

zoonosis.Curr Opin Microbiol. 2001. 4(1):58-64.

Bourdet-Sicard R, Egile C, Sansonetti PJ & Tran Van Nhieu G. Diversion of cytoskeletal processes by *Shigella* during invasion of epithelial cells. Microbes Infect. 2000. 2:813-819.

Buchanan TM, Hendricks SL, Patton CM, Feldman RA. Brucellosis in the united states, 1960-1972; An abattoir-associated disease. Part III. Epidemiology and evidence for acquired immunity. Medicine (Baltimore) 1974, 53, 427-439.

Campbell GA, Adams LG, Sowa BA. Mechanisms of binding of *Brucella abortus* to mononuclear phagocytes from cows naturally resistant or susceptible to brucellosis. Vet Immunol Immunopathol. 1994. 41:295-306.

Carbonetti NH: Pertussis toxin and adenylate cyclase toxin: key virulence factors of *Bordetella pertussis* and cell biology tools. Future Microbiol. 2010. 5(3):455-469.

Caroff M, Bundle DR, Perry MB. Structure of the O-chain of the phenol-phase soluble cellular lipopolysaccharide of *Yersinia enterocolitica* serotype O:9. Eur J Biochem. 1984. 139:195-200.

Caroff M, Bundle DR, Perry MB, Cherwonogrodzky JW, Duncan JR. Antigenic S-type lipopolysaccharide of *Brucella abortus* 1119-3. Infect Immun. 1984.

46(2):384-388.

Celli J. Surviving inside a macrophage: the many ways of *Brucella*. Res Microbiol. 2006. 157(2):93-98.

Celli J, de Chastellier C, Franchini DM, Pizarro-Cerda J, Moreno E, Gorvel JP. *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J Exp Med. 2003. 198(4):545-556.

Cespedes S, Andrews E, Folch H, Oñate A. Identification and partial characterization of a new protective antigen of *Brucella abortus*. J Med Microbiol. 2000. 49:165-170.

Cha SB, Rayamajhi N, Lee WJ, Shin MK, Jung MH, Shin SW, Kim JW, Yoo HS. Generation and envelope protein analysis of internalization defective *Brucella abortus* mutants in professional phagocytes, RAW 264.7. FEMS Immunol Med Microbiol. 2012. 64(2):244-254.

Chaves-Olarte E, Guzmán-Verri C, Méresse S, Desjardins M, Pizarro-Cerdá J, Badilla J, Gorvel JP, Moreno E. Activation of Rho and Rab GTPases dissociates *Brucella abortus* internalization from intracellular trafficking. Cell Microbiol. 2002. 4(10):663-676.

Chen B, Zhang D, Pollard JW. Progesterone regulation of the mammalian ortholog of methylcitrate dehydratase (immune response gene 1) in the uterine epithelium during implantation through the protein kinase C pathway. Mol Endocrinol. 2003. 17(11):2340-2354.

Cianciotto NP, Cornelis P, Baysse C. Impact of the bacterial type I cytochrome c maturation system on different biological processes. Mol Microbiol. 2005. 56:1408-1415.

Cloeckaert A, Verger JM, Grayon M, Grépinet O. Restriction site polymorphism of the genes encoding the major 25 kDa and 36 kDa outer-membrane proteins of *Brucella*. Microbiology. 1995. 141:2111-2121.

Connolly JP, Comerci D, Alefantis TG, Walz A, Quan M, Chafin R, Grewal P, Mujer CV, Ugalde RA, DelVecchio VG. Proteomic analysis of *Brucella abortus* cell envelope and identification of immunogenic candidate proteins for vaccine development. Proteomics. 2006. 6:3767-3780.

Corbel MJ. Brucellosis: an overview. Emerg Infect Dis. 1997. 3:213-221.

Covert J, Mathison AJ, Eskra L, Banai M, Splitter G. *Brucella melitensis*, *B. neotomae* and *B. ovis* elicit common and distinctive macrophage defense transcriptional responses. Exp Biol Med (Maywood). 2009. 234(12):1450-

1467.

Cutler SJ, Whatmore AM, Commander NJ. Brucellosis--new aspects of an old disease. J Appl Microbiol. 2005. 98(6):1270-1281.

Daino H, Matsumura I, Takada K, Odajima J, Tanaka H, Ueda S, Shibayama H, Ikeda H, Hibi M, Machii T, Hirano T, Kanakura Y. Induction of apoptosis by extracellular ubiquitin in human hematopoietic cells: possible involvement of STAT3 degradation by proteasome pathway in interleukin 6-dependent hematopoietic cells. Blood. 2000. 95(8):2577-2585.

de Jong MF, Sun YH, den Hartigh AB, van Dijl JM, Tsolis RM. Identification of VceA and VceC, two members of the VjbR regulon that are translocated into macrophages by the *Brucella* type IV secretion system. Mol Microbiol. 2008. 70(6):1378-1396.

Delrue RM, Deschamps C, Léonard S, Nijskens C, Danese I, Schaus JM, Bonnot S, Ferooz J, Tibor A, De Bolle X, Letesson JJ. A quorum-sensing regulator controls expression of both the type IV secretion system and the flagellar apparatus of *Brucella melitensis*. Cell Microbiol. 2005. 7:1151-1161.

Desvaux M, Parham NJ, Henderson IR. The autotransporter secretion system. Res Microbiol. 2004. 155:53-60.

Detilleux PG, Deyoe BL, Cheville NF. Penetration and intracellular growth of *Brucella abortus* in nonphagocytic cells in vitro. Infect Immun. 1990. 58(7):2320-2328.

Detilleux PG, Deyoe BL, Cheville NF. Entry and intracellular localization of *Brucella* spp. in Vero cells: fluorescence and electron microscopy. Vet Pathol. 1990. 27(5):317-328.

Edmonds MD, Cloeckaert A, Booth NJ, Fulton WT, Hagius SD, Walker JV, Elzer PH. Attenuation of a *Brucella abortus* mutant lacking a major 25 kDa outer membrane protein in cattle. Am J Vet Res. 2001. 62(9):1461-1466.

Edmonds MD, Cloeckaert A, Elzer PH. *Brucella* species lacking the major outer membrane protein Omp25 are attenuated in mice and protect against *Brucella melitensis* and *Brucella ovis*. Vet Microbiol. 2002. 88:205-221.

Eskra L, Mathison A, Splitter G. Microarray analysis of mRNA levels from RAW264.7 macrophages infected with *Brucella abortus*. Infect Immun. 2003. 71(3):1125-1133.

Eze MO, Yuan L, Crawford RM, Paranavitana CM, Hadfield TL, Bhattacharjee AK, Warren RL, Hoover DL. Effects of opsonization and gamma interferon on growth of *Brucella melitensis* 16M in mouse peritoneal macrophages in

vitro. Infect Immun. 2000. 68(1):257-263.

Fernandes DM, Benson R, Baldwin CL. Lack of a role for natural killer cells in early control of *Brucella abortus* 2308 infections in mice. Infect Immun. 1995. 10:4029-4033.

Finlay BB, Falkow S. Common themes in microbial pathogenicity. Microbiol Rev. 1989. 53:210-230.

Finlay BB, Falkow S. Common themes in microbial pathogenicity revisited. Microbiol Mol Biol Rev. 1997. 61(2):136-169.

Foster G, Osterman BS, Godfroid J, Jacques I, Cloeckaert A. *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. Int J Syst Evol Microbiol. 2007. 57:2688-2693.

Franco MP, Mulder M, Gilman RH, Smits HL. Human brucellosis. Lancet Infect Dis. 2007. 7(12):775-786.

Golding B, Scott DE, Scharf O, Huang LY, Zaitseva M, Lapham C, Eller N, Golding H. Immunty and protection against *Brucella abortus*. Microbes Infect. 2001. 3:43-48. Review.

Gorvel JP, Moreno E. *Brucella* intracellular life: from invasion to intracellular

replication. Vet Micro. 2002. 90(1-4):281-297.

Gupta M, Gupta SK, Balliet AG, Hollander MC, Fornace AJ, Hoffman B, Liebermann DA. Hematopoietic cells from Gadd45a- and Gadd45b-deficient mice are sensitized to genotoxic-stress-induced apoptosis. Oncogene. 2005. 24(48):7170-7179.

Gupta SK, Lysko PG, Pillarisetti K, Ohlstein E, Stadel JM. Chemokine receptors in human endothelial cells. Functional expression of CXCR4 and its transcriptional regulation by inflammatory cytokines. J Biol Chem. 1998. 273(7):4282-4287.

Guzman-Verri C, Manterola L, Sola-Landa A, Parra A, Cloeckaert A, Garin J, Gorvel JP, Moriyon I, Moreno E, Lopez-Goni I. The two-component system BvrR/BvrS essential for *Brucella abortus* virulence regulates the expression of outer membrane proteins with counterparts in members of the *Rhizobiaceae*. Proc Natl Acad Sci U S A. 2002. 99:12375-12380.

Handfield M, Levesque RC. Strategies for isolation of in vivo expressed genes from bacteria. FEMS Microbiol Rev. 1999. 23:69-91.

Harada N. Characteristics of a diagnostic method for tuberculosis infection based on whole blood interferon-gamma assay. Kekkaku. 2006. 81, 681-686.

Review. Japanese.

Henderson IR, Nataro JP. Virulence functions of autotransporter proteins. Infect Immun. 2001. 69:1231-1243.

Scholz HC. *et al.*. *Brucella* : Relationship to other alphaproteobacteria, current taxonomy and the emergence of new species. In *Brucella*, Molecular Microbiology and Genomics, edited by Ignacio L. and David O. Caister Academic Press. 2002. Chapter 1, 1-12.

Isberg RR. Uptake of enteropathogenic *Yersinia* by mammalian cells. Curr Top Microbiol Immunol. 1996. 209:1-24. Review.

Ito M, Kim YG, Tsuji H, Kiwaki M, Nomoto K, Tanaka R, Okada N, Danbara H. A practical random mutagenesis system for probiotic *Lactobacillus casei* using Tn5 transposition complexes. J Appl Microbiol. 2010. 109:657-666.

Jiang X, Baldwin CL. Effects of cytokines on intracellular growth of *Brucella abortus*. Infect Immun 1993. 61:124-134.

Jiménez de Bagüés MP, Terraza A, Gross A, Dornand J. Different responses of macrophages to smooth and rough *Brucella* spp.: relationship to virulence. Infect Immun. 2004. 72(4):2429-2433.

Jiménez de Bagüés MP, Dudal S, Dornand J, Gross A. Cellular bioterrorism: how

Brucella corrupts macrophage physiology to promote invasion and proliferation. Clin Immunol. 2005. 114(3):227-238.

Jones SM, Winter AJ. Survival of virulent and attenuated strains of *Brucella abortus* in normal and gamma interferon-activated murine peritoneal macrophages. Infect Immun. 1992. 60(7):3011-3014.

Jubier-Maurin V, Boigegrain RA, Cloeckaert A, Gross A, Alvarez-Martinez MT, Terraza A, Liautard J, Köhler S, Rouot B, Dornand J, Liautard JP. Major outer membrane protein Omp25 of *Brucella suis* is involved in inhibition of tumor necrosis factor alpha production during infection of human macrophages. Infect Immun. 2001. 69(8):4823-4830.

Kato-Maeda M, Gao Q, Small PM. Microarray analysis of pathogens and their interaction with hosts. Cell Microbiol. 2001. 3(11):713-719.

Kim DH, Lim JJ, Lee JJ, Kim DG, Lee HJ, Min W, Kim KD, Chang HH, Endale M, Rhee MH, Watarai M, Kim S. RGS2-mediated intracellular Ca^{2+} level plays a key role in the intracellular replication of *Brucella abortus* within phagocytes. J Infect Dis. 2012. 205(3):445-452.

Kim S, Watarai M, Kondo Y, Erdenebaatar J, Makino S, Shirahata T. Isolation and characterization of mini-Tn5Km2 insertion mutants of *Brucella abortus*

deficient in internalization and intracellular growth in HeLa cells. Infect Immun. 2003. 71:3020-3027.

Kim S, Watarai M, Suzuki H, Makino S, Kodama T, Shirahata T. Lipid raft microdomains mediate class A scavenger receptor-dependent infection of *Brucella abortus*. Microb Pathog. 2004. 37(1):11-19.

Lamontagne J, Butler H, Chaves-Olarte E, Hunter J, Schirm M, Paquet C, Tian M, Kearney P, Hamaidi L, Chelsky D, Moriyón I, Moreno E, Paramithiotis E. Extensive cell envelope modulation is associated with virulence in *Brucella abortus*. J Proteome Res. 2007. 6:1519-1529.

Lapaque N, Forquet F, de Chastellier C, Mishal Z, Jolly G, Moreno E, Moriyon I, Heuser JE, He HT, Gorvel JP. Characterization of *Brucella abortus* lipopolysaccharide macrodomains as mega rafts. Cell Microbiol. 2006. 8(2):197-206.

Lecuit M, Dramsi S, Gottardi C, Fedor-Chaiken M, Gumbiner B, Cossart P. A single amino acid in E-cadherin responsible for host specificity towards the human pathogen *Listeria monocytogenes*. EMBO J. 1999. 18:3956-3963.

Leroy Q, Raoult D. Review of microarray studies for host-intracellular pathogen interactions. J Microbiol Methods. 2010. 81(2):81-95.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001. 25(4):402-408.

López-Goñi I. (2012). The two-component system BvrR/BvrS : a master regulator of *Brucella* virulence. In *Brucella*, Molecular Microbiology and Genomics, edited by Ignacio L. and David O. Caister Academic Press, Chapter 10, 201-209.

Lorenzen A, Stannek C, Burmeister A, Kalvinsh I, Schwabe U. G protein-coupled receptor for nicotinic acid in mouse macrophages. Biochem Pharmacol. 2002. 64(4):645-648.

Marchesini MI, Herrmann CK, Salcedo SP, Gorvel JP, Comerci DJ. In search of *Brucella abortus* type IV secretion substrates: screening and identification of four proteins translocated into host cells through VirB system. Cell Microbiol. 2011. 13(8):1261-74.

Mariana N. Xavier, Tatiane A. Paixão, Andréas B. den Hartigh, Renée M. Tsolis and Renato L. Santos. Pathogenesis of *Brucella* spp. The Open Veterinary Science Journal. 2010. 4:109-118.

Martín-Martín AI, Caro-Hernández P, Orduña A, Vizcaíno N, Fernández-Lago L. Importance of the Omp25/Omp31 family in the internalization and

intracellular replication of virulent *B. ovis* in murine macrophages and HeLa cells. Microbes Infect. 2008. 10:706-710.

Nielsen K, Smith P, Yu W, Nicoletti P, Jungersen G, Stack J, Godfroid J. Serological relationship between cattle exposed to *Brucella abortus*, *Yersinia enterocolitica* O:9 and *Escherichia coli* O157:H7. Vet Microbiol. 2004. 100:25-30.

Oliveira SC, Harms JS, Rech EL, Rodarte RS, Bocca AL, Goes AM, Splitter GA. The role of T-cell subsets and cytokines in the regulation of intracellular bacterial infection. Braz J Med Biol Res. 1998. 31(1):77-84

Olsen SC., Bellaire BH, Roop II RM, Thoen CO. *Brucella*. In Pathogenesis of Bacterial Infections in Animals, Fourth Edition, edited by C. L. Gyles [et al.]. Wiley-Blackwell, Chapter 22, 429-438.

Paixão TA, Roux CM, den Hartigh AB, Sankaran-Walters S, Dandekar S, Santos RL, Tsolis RM. Establishment of systemic *Brucella melitensis* infection through the digestive tract requires urease, the type IV secretion system, and lipopolysaccharide O antigen. Infect Immun. 2009. 77(10):4197-4208.

Pizarro-Cerdá J, Méresse S, Parton RG, van der Goot G, Sola-Landa A, Lopez-Goñi I, Moreno E, Gorvel JP. *Brucella abortus* transits through the

autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. Infect Immun. 1998. 66:5711-5724.

Pizarro-Cerdá J, Moreno E, Sanguedolce V, Mege JL, Gorvel JP. Virulent *Brucella abortus* prevents lysosome fusion and is distributed within autophagosome-like compartments. Infect Immun. 1998. 66(5):2387-2392.

Pizarro-Cerdá J, Moreno E, Gorvel JP. Invasion and intracellular trafficking of *Brucella abortus* in nonphagocytic cells. Microbes Infect. 2000. 2:829-835.

Rambow-Larsen AA, Petersen EM, Gourley CR, Splitter GA. *Brucella* regulators: self-control in a hostile environment. Trends Microbiol. 2009. 17(8):371-377.

Ramos JL, Martínez-Bueno M, Molina-Henares AJ, Terán W, Watanabe K, Zhang X, Gallegos MT, Brennan R, Tobes R. The TetR family of transcriptional repressors. Microbiol Mol Biol Rev. 2005. 69:326-356.

Ridler AL, Leyland MJ, Fenwick SG, West DM. Demonstration of polymorphism among *Brucella ovis* field isolates by pulsed-field gel electrophoresis. Vet Microbiol. 2005. 108:69-74.

Ritchie, Jennifer Ann, "*Brucella abortus* intracellular survival and intercellular trafficking" (2011). Graduate Theses and Dissertations. Paper 10162.

http://lib.dr.iastate.edu/etd/10162.

Rittig MG, Alvarez-Martinez MT, Porte F, Liautard JP, Rouot B. Intracellular survival of *Brucella* spp. in human monocytes involves conventional uptake but special phagosomes. Infect Immun. 2001. 69(6):3995-4006.

Rosenberger CM, Scott MG, Gold MR, Hancock RE, Finlay BB. *Salmonella typhimurium* infection and lipopolysaccharide stimulation induce similar changes in macrophage gene expression. J Immunol. 2000. 164(11):5894-5904.

Roux CM, Rolán HG, Santos RL, Beremand PD, Thomas TL, Adams LG, Tsolis RM. *Brucella* requires a functional Type IV secretion system to elicit innate immune responses in mice. Cell Microbiol. 2007. 9(7):1851-1869.

Saini V, Marchese A, Majetschak M. CXC chemokine receptor 4 is a cell surface receptor for extracellular ubiquitin. J Biol Chem. 2010. 285(20):15566-15576.

Salas-Téllez E, Núñez del Arco A, Tenorio V, Díaz-Aparicio E, de la Garza M, Suárez-Güemes F. Subcellular fractions of *Brucella ovis* distinctively induce the production of interleukin-2, interleukin-4, and interferon-gamma in mice. Can J Vet Res. 2005. 69:53-57.

Salcedo SP, Marchesini MI, Lelouard H, Fugier E, Jolly G, Balor S, Muller A,

Lapaque N, Demaria O, Alexopoulou L, Comerci DJ, Ugalde RA, Pierre P, Gorvel JP. *Brucella* control of dendritic cell maturation is dependent on the TIR-containing protein Btp1. PLoS Pathog. 2008. 8;4(2):e21.

Scholz HC, Hubalek Z, Sedlácek I, Vergnaud G, Tomaso H, Al Dahouk S, Melzer F, Kämpfer P, Neubauer H, Cloeckaert A, Maquart M, Zygmunt MS, Whatmore AM, Falsen E, Bahn P, Göllner C, Pfeffer M, Huber B, Busse HJ, Nöckler K. *Brucella microti* sp. nov., isolated from the common vole *Microtus arvalis*. Int J Syst Evol Microbiol. 2008. 58:375-382.

Scholz HC, Nöckler K, Göllner C, Bahn P, Vergnaud G, Tomaso H, Al Dahouk S, Kämpfer P, Cloeckaert A, Maquart M, Zygmunt MS, Whatmore AM, Pfeffer M, Huber B, Busse HJ, De BK. *Brucella inopinata* sp. nov., isolated from a breast implant infection. Int J Syst Evol Microbiol. 2010. 60:801-808.

Selvakumaran M, Lin HK, Sjin RT, Reed JC, Liebermann DA, Hoffman B. The novel primary response gene MyD118 and the proto-oncogenes myb, myc, and bcl-2 modulate transforming growth factor beta 1-induced apoptosis of myeloid leukemia cells. Mol Cell Biol. 1994. 14(4):2352-2360.

Shin SJ, Wu CW, Steinberg H, Talaat AM. Identification of novel virulence determinants in *Mycobacterium paratuberculosis* by screening a library of insertional mutants. Infect Immun. 2006. 74:3825-3833.

Sieira R, Comerci DJ, Sánchez DO, Ugalde RA. A homologue of an operon required for DNA transfer in *Agrobacterium* is required in *Brucella abortus* for virulence and intracellular multiplication. J Bacteriol. 2000. 182:4849-4855.

Tcherneva E, Rijpens N, Jersek B, Herman LM. Differentiation of *Brucella* species by random amplified polymorphic DNA analysis. J Appl Microbiol 2000. 88:69-80.

Thöny-Meyer L. Biogenesis of respiratory cytochromes in bacteria. Microbiol Mol Biol Rev. 1997. 61:337-376.

Vairapandi M, Balliet AG, Hoffman B, Liebermann DA. GADD45b and GADD45g are cdc2/cyclinB1 kinase inhibitors with a role in S and G2/M cell cycle checkpoints induced by genotoxic stress. J Cell Physiol 2002. 192(3):327-338.

Vidal JE, Chen J, Li J, McClane BA. Use of an EZ-Tn5-based random mutagenesis system to identify a novel toxin regulatory locus in *Clostridium perfringens* strain 13. PLoS One. 2009. 4:e6232.

Vizcaino N, Cloeckaert A. (2012). Biology and genetics of the *Brucella* outer membrane. Molecular Microbiology and Genomics, edited by Ignacio L. and

David O. Caister Academic Press, Chapter 7, 133-161.

Vizcaíno N, Cloeckaert A, Zygmunt MS, Dubray G. Cloning, nucleotide sequence, and expression of the *Brucella melitensis* omp31 gene coding for an immunogenic major outer membrane protein. Infect Immun. 2009. 64:3744-3751.

Wang H, Yadav JS. Global gene expression changes underlying Stachybotrys chartarum toxin-induced apoptosis in murine alveolar macrophages: evidence of multiple signal transduction pathways. Apoptosis. 2007. 12(3):535-548.

Wang J, Wu X, Simonavicius N, Tian H, Ling L. Medium-chain fatty acids as ligands for orphan G protein-coupled receptor GPR84. J Biol Chem 2006, 281(45):34457-34464.

Watarai M, Derre I, Kirby J, Growney JD, Dietrich WF, Isberg RR. *Legionella pneumophila* is internalized by a macropinocytotic uptake pathway controlled by the Dot/Icm system and the mouse Lgn1 locus. J Exp Med. 2001. 194:1081-1096.

Watarai M, Makino S, Fujii Y, Okamoto K, Shirahata T. Modulation of *Brucella*-induced macropinocytosis by lipid rafts mediates intracellular replication. Cell Microbiol. 2002. 4:341-355.

Yurgel SN, Berrocal J, Wilson C, Kahn ML. Pleiotropic effects of mutations that alter the *Sinorhizobium meliloti* cytochrome c respiratory system. Microbiology. 2007. 153:399-410.

Zhang Y, Schmidt RJ, Foxworthy P, Emkey R, Oler JK, Large TH, Wang H, Su EW, Mosior MK, Eacho PI, Cao G. Niacin mediates lipolysis in adipose tissue through its G-protein coupled receptor HM74A. Biochem Biophys Res Commun. 2005. 334(2):729-732.

Zerbini LF, Czibere A, Wang Y, Correa RG, Otu H, Joseph M, Takayasu Y, Silver M, Gu X, Ruchusatsawat K, Li L, Sarkar D, Zhou JR, Fisher PB, Libermann TA. A novel pathway involving melanoma differentiation associated gene-7/interleukin-24 mediates nonsteroidal anti-inflammatory drug-induced apoptosis and growth arrest of cancer cells. Cancer Res. 2006. 66(24):11922-11931.