Genes Related to Intracellular Survival of *Brucella abortus* in THP-1 Macrophage Cells^S

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Brucella abortus can survive and replicate within host macrophages, and great efforts have been made to demonstrate the genes involved in pathogenicity, such as internalization, in *Brucella* research. Here, intracellular responses were compared between THP-1 macrophage cells stimulated with *B. abortus* wild-type and four mutants (C1, C10, C27, and C32) using microarray to demonstrate the role of genes related to intracellular survival and replication. These mutants were generated by deleting genes encoding *BAB_RS13225* (4-hydrobenzoate 3-monooxygenase, PHBH), *BAB_RS00455* (heme exporter protein cytochrome C, CcmC), *BAB_RS03675* (exopolyphosphatase, PPX), and *BAB_RS13225* (peptidase M24). The results showed that mutants C1 and C10 induced significant suppression of survival levels and cytokine expression relative to wild-type in the THP-1 macrophage cells. These findings suggest that the *BAB_RS13225* and *BAB_RS00455* genes play important roles in survival within human macrophages. Conversely, mutants C27 and C32 induced significantly higher survival level than wild-type in the cells inhibiting cellular signal transduction. It is assumed that the *BAB_RS03675* and *BAB_RS13225* genes play a role in cellular resistance to *B. abortus*. Therefore, the disrupted genes are involved in *B. abortus* intracellular growth, and especially in its survival, and they could be effective targets for understanding the intracellular bacterium, *B. abortus*.

Keywords: *B. abortus*, mutants, THP-1 macrophage cells, intracellular survival and replication, gene expression

Introduction

Brucella abortus (*B. abortus*) is a zoonotic pathogen and one of the causative agents of brucellosis, which causes life-threatening symptoms and has socio-economic impacts on humans and animals [1]. *Brucella* spp. infections in humans usually occur through ingestion of unpasteurized milk or other cattle products and inhalation of contaminated air [2, 3]. *Brucella abortus* is an aerobic, non-motile, non-spore-forming and gram-negative bacterium [4]. Notably, *B. abortus* does not possess pathogenic factors such as plasmids, exotoxins, cell lysates, or capsules, which are used to recognize infection [5, 6]. Global challenges to *B. abortus* research include rapid detection and early treatment

because it can survive and replicate through its ability to modulate host cell functions within the host cells [5, 7]. During *B. abortus* infection, macrophages, dendritic cells and trophoblasts are mainly targeted to replicate and survive in the host [8]. Particularly, macrophages have been shown to be significant in host defences and elimination of *Brucella* infection producing pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α), IL-6 and interferon- γ (IFN- γ) [9–11]. THP-1 macrophages have been widely applied for research in *Brucella* spp. studies such as cell internalization, intracellular growth and gene expression during the bacterial infection [12–14]. However, because of its ability to evade the immune system and survive in the macrophages, *B. abortus* leads to

long-lasting illness. The lack of knowledge about genetic expression in *B. abortus* is one of the major obstacles in investigating the pathogenic mechanism of this organism.

Over the years, many studies have identified gene functions via generation of a single gene mutation [15–18]. Previously, we constructed *B. abortus* mutants using transposons [17], and the characteristics, including growth rate, biochemical test, and biovar of mutants were analyzed [19]. In this study, four *B. abortus* mutants (C1, C10, C27, C32) were inoculated into differentiated THP-1 macrophage cells to find out the genes related to *B. abortus* internalization in the macrophages. The mutated genes of the selected mutants were revealed as *BAB_RS13225* (4-hydroxybenzoate 3-monooxygenase, PHBH), *BAB_RS00455* (heme exporter protein cytochrome C, CcmC), *BAB_RS03675* (exopolyphosphatase, PPX), and *BAB_RS13225* (Peptidase M24) through genomic DNA sequencing. Additionally, transcriptomic analysis was conducted to observe cellular immune responses of host cells after mutants were infected. The PHBH catalyzes oxidative and reductive reactions between 4-hydroxybenzoate and 3,4-dihydroxybenzoate [20], with the latter also being known as protocatechuic acid (PCA), which mediates entry into the Krebs cycle [21]. CcmC is a well-known protein supporting mitochondrial life during cellular emergency [22], and PPX participates in the regulation of various cellular processes, such as cell growth, proliferation and intracellular survival in several Gram-negative bacteria [23–25]. Peptidase M24 plays a role in transcription initiation within the organism [26]. The intracellular survival of these four mutants and gene expression of mutant-infected THP-1 macrophage cells showed significant alteration compared to that of wild-type. The research presented herein may contribute to understanding these gene functions related to the intracellular survival of *B. abortus*.

Materials and Methods

Bacterial Strains and Cell Lines

The *Brucella abortus* 1119-3 strain as a wild-type strain was kindly provided by the Animal and Plant Quarantine Agency in Korea. Mutants of *B. abortus* were generated by electroporation using the EZ-Tn5™ Transposome complex (Epicentre R Biotechnologies, USA) [17]. Single mutation induced by insertion of the transposon was confirmed by PCR and Southern blot analyses, and high-throughput gDNA sequencing. The bacterium was cultured in *Brucella* broth and agar (Difco, USA), and kanamycin (30 µg/ml) was added into the media. The THP-1 human leukaemic monocyte cell line from Korea Cell Line Bank

(KCLB, Korea) was grown at 37°C in humidified air under 5% CO₂ in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and antibiotic–antimycotic solution (Sigma, USA). THP-1 cells were differentiated into macrophages by stimulation with phorbol-12-myristate-13-acetate (PMA; Sigma, USA) (50 ng/ml) for 72 h, washed with RPMI 1640 medium and incubated in 5% FBS-RPMI 1640 medium without antibiotics for 24 h before the experiments. All procedures were approved by the Seoul National University Institutional Biosafety Committee (SNUIBC-R160314-1-1).

Selection of *B. abortus* Mutants

Prior to starting the experiment, four mutants were selected based on growth rate and cellular invasion into the THP-1 macrophage cells, since bacterial growth rate and initial invasion ability could be crucial to survive and replicate within the cells. The characteristics of all mutants involving growth rate, biochemical test, biovar test and expression of pathogenic factors such as VirB8 were identified [19]. The growth rate of all of mutants was measured according to optical density versus CFU, and mutants were divided into four groups based on the growth rate. In the previous study, mutants of Group 1, Group 2, and Group 3 showed increased growth rate, similar growth rate, and decreased growth rate compared to wild-type, respectively. However, mutants of group 4 were mostly defective in growth.

Then, the invasion ability of the mutants into the THP-1 macrophage cells was identified using a gentamicin protection assay as previously described [27]. Briefly, *B. abortus* strains were inoculated into differentiated THP-1 macrophages (2×10^5 cells/ml) at a multiplicity of infection (MOI) of 100:1. Following incubation of the cells for 1 h at 37°C in a 5% CO₂ atmosphere, the cells were washed with PBS and treated with gentamicin for 2 h. The cells were then lysed with lysis buffer (diluted water with 0.01% Triton X-100). Next, 25 µl of cell lysate was used for inoculation, and the CFU of each *B. abortus* strain was counted following incubation at 37°C for 48 h. The level of bacterial invasion was presented as the relative percentage compared to that of the wild-type when the level of wild-type was regarded as 100%. Clustering analysis was conducted using R 3.3 (R Foundation for Statistical Computing, Austria), and five cluster groups were divided according to invasion ability for classification of mutants. The mutants of Cluster 2 and Cluster 5 showed decreased and increased invasion rate compared to wild-type, respectively. In addition, mutants of Cluster 3 and Cluster 4 showed similar invasion rate to wild-type. However, mutants of Cluster 1 proved to be mostly defective in invasion ability. All experiments were carried out three times.

Intracellular Growth, Survival and Replication of *B. abortus* Mutants within the THP-1 Macrophage Cells

Using the selected four mutants, intracellular bacterial growth and survival and replication in the THP-1 macrophage cells were investigated at different times post-infection (p.i.): 2, 6, 12, and 24 h. All experiments were expressed as CFU and carried out three times.

Genomic DNA Library Construction and Sequencing

The transposon insertion site was identified using genomic DNA sequencing [28]. Defective loci of *B. abortus* mutants were analyzed by NICEM at Seoul National University, Korea. Briefly, genomic DNA libraries were constructed using the NEXTflex Rapid DNA-seq Kit (Bioo Scientific, USA). DNA shearing was performed using a Q-Sonica 800 sonicator (Qsonica, LLC, USA). Shearing was conducted according to the instrument guide for an ~500 bp span size. Fragmented DNA was prepared by the following enzymatic reaction: samples were blunt-end repaired, 3' adenylated, and ligated with multiplex compatible adapters to construct an Illumina-compatible DNA library. Size selection was then conducted using Agencourt AMPure XP SPRI beads to retain ~300–600 bp fragments. Next, PCR enrichment was employed to selectively amplify fragments containing DNA with adapters on both ends (Bioo Scientific, USA). Library validation was conducted using a Bioanalyzer, after which quantification was conducted using a Picogreen dsDNA HS Assay Kit (Thermo Fisher, USA) and the KAPA qPCR kit (Kapa Biosystems, USA) for the library. Equimolar amounts of each library were pooled at 10 nM for sequencing, after which high-throughput sequencing was conducted using the Illumina HiSeq 2500 Genetic Analysis System at the Genome Analysis Center of NICEM. The run mode was 250 cycles of Rapid Paired-End.

Production of Cytokines in THP-1 Macrophage Cells

The differentiated THP-1 macrophage cells (2×10^5 cells/ml) were stimulated with *B. abortus* wild-type and mutants at an MOI of 100:1. The culture supernatants were collected at 24 h after stimulation. Amounts of TNF- α , interleukin 1-beta (IL-1 β), IL-6 and IFN- γ were measured by Ready-SET-Go ELISA Kit (Thermo Fisher, USA) according to the manufacturer's instructions.

Bacterial Infection of THP-1 Macrophage Cells and Microarray Hybridization

After differentiation of THP-1 macrophage cells, 2×10^7 cells/ml of *B. abortus* wild-type and selected mutants suspended in PBS were added into the cell cultures (2×10^5 cells/ml) at an MOI of 100:1 and incubated at 37°C under 5% CO₂ for 24 h. Total RNA was then extracted from the infected cells using an RNeasy Mini Kit (Qiagen, Germany) as described by the manufacturer after washing three times with DPBS (Gibco, USA). The purity and integrity of RNA were determined by denaturing gel electrophoresis, while the OD260/OD280 ratio was calculated with an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). After quality control for microarray analysis, RNA amplification, labelling, array hybridization, and scanning were conducted by MacroGen Inc. (Korea). Total RNA was amplified and purified using a TargetAmp-Nano Labeling Kit for the Illumina Expression BeadChip (Epicentre, USA) to synthesize biotinylated cRNA according to the manufacturer's instructions. Briefly, 200 ng of total RNA was reverse transcribed to cDNA using a T7 oligo (dT) primer, after which second-strand cDNA was synthesized. The in

vitro transcription reaction produces biotin-cRNA by incorporating TargetAmp-Nano UTP/Biotin-UTP into the RNA transcripts. Following purification of the RNA transcripts labelled with Biotin-UTP, the cRNA was quantified using an ND-1000 spectrophotometer (NanoDrop, USA). Next, 750 ng of labelled cRNA samples were hybridized to each Human HT-12 v4.0 Expression BeadChip (Illumina, USA) for 17 h at 58°C, which covers more than 47,000 transcripts. Detection of the array signal was conducted by staining using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) according to the bead array manual. Arrays were scanned with an Illumina BeadArray Reader confocal scanner (Illumina, USA) according to the manufacturer's instructions.

Raw Data Preparation and Statistical Analysis

Raw microarray data were extracted using Illumina GenomeStudio v2011.1, Gene Expression Module v1.9.0, and array probes were log-transformed and then normalized by the quantile method. The statistical significance of the microarray expression data was determined using the local-pooled-error (LPE) test and fold change in which the null hypothesis was that no difference existed among groups. The false discovery rate (FDR) was controlled by adjusting the *p*-value using the Benjamini-Hochberg algorithm. Differences were considered significant if a $p \leq 0.05$ and a fold-change $> |2|$ were obtained. Gene-enrichment and functional annotation analysis for significant probes were performed using Gene Ontology (<http://geneontology.org/>), PANTHER (<http://pantherdb.org/about.jsp>) and the KEGG pathway (<http://kegg.jp>). In addition, Cytoscape (<http://www.cytoscape.org/>) and GeneMANIA (<http://genemania.org/>) were utilized for prediction of gene function. All datasets used in transcriptomic analysis by microarray are available at Gene Expression Omnibus (GEO) (<https://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE102260 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE102260>).

Verification of Microarray Results Using qRT-PCR

To verify the microarray results, seven genes (*CCL4L1*, *CXCL6*, *TNFAIP6*, *CCL8*, *ID3*, *IDO1*, *TNFSF10*) related to cytokines and immune defense and a house-keeping gene, β -actin, as a control, were selected and subjected to quantitative real-time RT-PCR. Briefly, cDNA was synthesized from 1 μ g of total RNA from each sample using a Quantitect Reverse Transcription Kit (Qiagen, Germany). Next, quantitative real-time RT-PCR was performed with 2 μ l of cDNA using the Rotor-Gene SYBR Green PCR Kit (Qiagen) and Rotor-Gene Q real-time PCR cyclers (Qiagen). Cycling parameters were as follows: 95°C for 5 min for one cycle followed by 45 cycles of 95°C for 15 sec and 60°C for 45 sec. Primers used in real-time PCR are shown in Table 1. The gene expression level was normalized using the $2^{-\Delta\Delta Ct}$ formula, and relative expression levels of the target genes were compared with the control to determine changes in the fold expression of each gene.

Table 1. Primers used for qRT-PCR.

Validation of microarray results	<i>CCL4L1</i>	F	CCT GAG CCT GGA TGC TTC TC	NM_001001435.2	Responses of chemokines
		R	GAG ACA GGA ACT GCG GAG AG		
	<i>CCL8</i>	F	GGC TGG AGA GCT ACA CAA GA	NM_005623.2	Responses of cytokines and chemokines
		R	TGA CCC ATC TCT CCT TGG GG		
	<i>CXCL6</i>	F	TGC GTT GCA CTT GTT TAC GC	NM_002993.2	Responses of cytokines and chemokines
		R	CGG GGA ACA CCT GCA GTT TA		
	<i>TNFAIP6</i>	F	TCT GTG CTG CTG GAT GGA TG	NM_007115.2	Responses of TNF alpha
		R	CTT TGC GTG TGG GTT GTA GC		
	<i>TNFSF10</i>	F	GGG ACC AGA GGA AGA AGC AAC	NM_003810.2	Responses of cytokines and TNF
		R	GTT GCT CAG GAA TGA ATG CCC		
	<i>ID3</i>	F	GGA GCG AAG GAC TGT GAA CT	NM_002167.2	Cellular growth, senescence, differentiation, apoptosis, angiogenesis
		R	TTA GAA CTT GGG GGT GGG GT		
	<i>IDO1</i>	F	GGG AAG CTT ATG ACG CCT GT	NM_002164.4	Limitation of the growth of intracellular pathogens by tryptophan deprivation
		R	CTG GCT TGC AGG AAT CAG GA		
	<i>β-actin</i>	F	GATCATTGCTCCTCCTGAGC	NM_001101.3	Cell motility, structure, and integrity
		R	ACTCCTGCTTGCTGATCCAC		

Statistics

Statistical significance of internalization was analyzed by Student's *t*-tests or repeated measures ANOVA using SPSS version 23.0 (USA). A $p < 0.05$ was considered to indicate significance.

Results

Characteristics of *B. abortus* Mutants

Among the twenty-one *B. abortus* mutants, C1, C10, C27, and C32 were selected based on the growth rate and invasion ability (Fig. 1). The other seven mutants belonging to Group 4 of the growth rate and Cluster 1 of the invasion ability were excluded from candidacy since they did not show significant differences in growth rate or notable

invasion ability compared to wild-type. In each cluster group, mutants that showed invasion ability close to wild-type were selected, since a large difference in invasion ability may dominantly affect the result of intracellular survival and replication and mask the effects of genetic expression due to the difference in initial numbers. Also, groups with different growth rates were considered for mutant selection. C10 and C27 were selected in the middle of Cluster 3 and 4, respectively. C1 was selected from the group of decreased invasion ability, Cluster 2. On the other hand, C32 was selected from the group of increased invasion ability, Cluster 5.

Identification of Transposon Insertion into the *B. abortus* Wild-Type

Insertion sites of transposon in the mutants (C1, C10, C27 and C32) were identified using genomic DNA sequencing referencing the *B. abortus* wild-type sequence (Fig. 2, Table 2). Genes disrupted by transposon mutagenesis included *BRUAB_RS13225* (4-hydrobenzoate 3-monooxygenase, PHBH), *BRUAB_RS00455* (heme exporter protein, CmcC), *BRUAB_RS03675* (exopolyphosphatase, PPX), and *BRUAB_RS08885* (peptidase M24), for mutants C1, C10, C27 and C32, respectively. The insertion site of mutant C1 is located at 593,728–594,899 bp, mutant C10 is at 102,857–103,645 bp, mutant C27 is located at 756,439–757,977 bp, and mutant C32 is located at 1,812,960–1,814,915 bp. The PHBH gene is located in chromosome II, while the other genes are in chromosome I.

Fig. 1. The invasion abilities of *B. abortus* mutants into the THP-1 macrophage cells.

Among the mutants, four mutants, C1, C10, C27, and C32 showing unique characteristics compared to other mutants were selected from each growth rate group.

Fig. 2. Transposon insertion site. Through genomic DNA sequencing, the insertion site of the transposon in the four mutants was analyzed. The disrupted gene of mutant C1 is located in chromosome II, while the other genes are in chromosome I.

Four Mutants Showed Altered Intracellular Survival and Replication Compared to the Wild-Type Strain in the THP-1 Macrophage Cells

To observe the difference in cellular internalization according to the mutation, intracellular growth of the wild-type and mutants C1, C10, C27, and C32 were analyzed at 2, 6, 12, and 24 h (Fig. 3). At 2 h p.i., mutants C27 and C32 showed significantly higher invasion, while the other mutants showed similar to or lower than that of the wild-type. Thereafter, the number of intracellular bacteria decreased overall at 6 h p.i. and increased again to initial levels at 12 h p.i., showing their intracellular survival. At 12 and 24 h p.i., mutants C27 and C32 increased dramatically and differed significantly compared to wild-type. The numbers of mutants C1 and C10 were similar to or lower than the wild-type strain throughout the infection. From the viewpoint of rate, the percentages of surviving bacteria were 32.5% (wild-type), 22.8% (C1), 30.5% (C10), 39.1% (C27), and 40.9% (C32). The results indicate that mutants C27 and C32 survived better than wild-type.

Cell-Mediated Immune Responses Induced by Mutants

Two *B. abortus* mutants, C27 and C32, induced the production of TNF- α , IL-1 β , and IL-6 in ELISA in THP-1 macrophage cells at 24 h after stimulation (Fig. 4). Production of IFN- γ was not detected in THP-1 macrophage cells stimulated with *B. abortus* wild-type or mutants (data not shown).

Differentially Expressed Genes in THP-1 Macrophage Cells Were Divided into Two Patterns Following Infection with Wild-Type and Mutants

Differentially expressed genes in THP-1 macrophage cells stimulated with *B. abortus* mutants were identified by comparison with *B. abortus* wild-type. The mutants C1, C10, C27, and C32 induced differential expression in 142, 137, 110, and 65 genes in the cells, respectively, when compared with those stimulated by wild-type *B. abortus*. The results of the genes showing an altered expression level of $> |2|$ in *B. abortus* mutant-infected THP-1 macrophage cells are provided in Tables S1–S4. When

Table 2. Information about the transposon insertion site.

Mutant	Disrupted genes	Functional family	Chromosome	Encoding proteins
C1	BRUAB_RS13225	Oxidoreductase	II	4-hydrobenzoate 3-monooxygenase (PHBH)
C10	BRUAB_RS00455	Transport	I	Heme exporter protein (CcmC)
C27	BRUAB_RS03675	Oxidative metabolism	I	Exopolyphosphatase (PPX)
C32	BRUAB_RS08885	Metallopeptidase	I	Peptidase M24

Fig. 3. The intracellular survival of *B. abortus* wild-type and mutants (C1, C10, C27, and C32) in the THP-1 macrophage cells.

Intracellular growth of four kinds of *B. abortus* compared to the wild-type strain. The high number and replication rate of mutants C27 and C32 were observed, and C1 and C10 showed similar to or lower than the wild-type strain. * $p < 0.05$, ** $p < 0.01$.

comparing gene expression in the mutant-infected cells with *B. abortus* wild-type-infected cells, C1 and C10, and C27 and C32 showed similar patterns in the induction of gene expression. Forty-three genes were commonly up-regulated in mutants C27 and C32, while six were down-regulated in those mutants. Conversely, one gene was up-regulated while 114 genes were commonly down-regulated in mutants C1 and C10.

To predict the putative cellular responses using the genes showing altered expression, gene set enrichment analysis was performed using the Protein Analysis Through Evolutionary Relationship (PANTHER) classification database. Transcriptomic changes were classified into molecular function and biological processes (Fig. 5). In molecular functions, mutants C1 and C10 altered the expression of several genes related to binding and catalytic activity

(Fig. 5A). However, mutants C27 and C32 up-regulated these genes while they down-regulated genes related to signal transducer activity. Also, only a few genes were up- or down-regulated in the other categories. Evaluation of biological processes revealed that mutants C27 and C32 showed highly altered expression of genes related to cellular processes, biological regulation, response to stimuli, metabolic processes and immune system processes (Fig. 5B). On the other hand, mutants C1- and C10-infected cells down-regulated these genes. These results suggest that C1- and C10-infected cells down-regulated the genes involved in cellular communication and response to invaders, while C27- and C32-infected cells up-regulated these genes compared to wild-type-infected cells.

Affected Pathways and Gene Networks following *B. abortus* Mutant Infection

To understand more specific cellular responses through activated or inactivated pathways and networks utilized by the genes, pathways were mapped using the Kyoto Encyclopedia of Genes and Genomics (KEGG) database, and gene network analysis was performed based on the Cytoscape and Gene MANIA databases.

The pathway analysis showed that mutants C1 and C10 almost always down-regulated pathways related to immune responses, while mutants C27 and C32 up-regulated the pathways (Table 3). Especially, cytokine-associated genes were differentially expressed between groups. Up-regulated pathways of mutants C1 and C10 and down-regulated pathways of mutants C27 and C32 were rarely observed. The expression of genes involved in cytokines is summarized in Table S5. These data were used to identify the different signals involved in the cytokine expression of cells infected with each mutant strain. The results showed that *B. abortus* mutant strains C1 and C10 down-regulated most cytokine-inducible genes, while mutant strains C27 and C32 up-regulated these genes.

Fig. 4. Production of cytokines in THP-1 macrophage cells after stimulation with *B. abortus* mutants.

The production of TNF- α , IL-1 β , and IL-6 was quantified using ELISA. The cytokines increased at 24 h p.i. with C27 and C32. * $p < 0.05$, ** $p < 0.01$.

Fig. 5. Categorization by molecular function and biological processes of differentially expressed genes during *B. abortus* infections. Genes that showed altered expression compared to those in the wild-type strain-infected cells were categorized by molecular function and biological function using the PANTHER database. (A) Genes differentially expressed during *B. abortus* mutant infection categorized by molecular functions. (B) Genes differentially expressed during *B. abortus* mutant infection categorized by biological processes.

As mentioned above, cells infected with C1 and C10, as well as with C27 and C32, showed similar gene expression in that almost all genes were commonly regulated. Therefore, the gene networks involved in the cellular inflammatory response and regulation of defense response in the cells infected with these mutants were analyzed (Fig. 6). The color of each node indicates its Fc value and the average change values of both strains of infected cells. Although genes of C1- and C10-infected cells down-regulated the expression of *CCL3* and *TNFAIP3*, inducing inhibition of *NFκB* and cell apoptosis, genes of C27- and C32-infected cells up-regulated the expression of *APOL3* or *IFIH1*.

Moreover, the *SLC11A1* gene, which is responsible for iron metabolism in inhibiting the iron uptake of the bacteria, was down-regulated in C27- and C32-infected cells.

Validation of Microarray Data

To confirm the microarray results, differentially expressed genes were identified by quantitative real-time polymerase chain reaction (qRT-PCR). Validation was performed based on these genes showing different expression levels in almost all mutants infected cells. The seven selected genes (*CCL4L1*, *CXCL6*, *TNFAIP6*, *CCL8*, *ID3*, *IDO1*, *TNFSF10*) evaluated by qRT-PCR showed similar expression patterns

Table 3. KEGG pathways in *B. abortus* mutants infected cells.

Pathways				
Mutant	Down-regulated	Count of genes	Up-regulated	Count of genes
C1	Cytokine-cytokine receptor interaction	22	-	
	Chemokine signaling pathway	20		
	Toll-like receptor signaling pathway	15		
	Cytokine-cytokine receptor interaction	22		
C10	Chemokine signaling pathway	22	-	
	NF-κB signaling pathway	18		
	TNF-signaling	16		
	Toll-like receptor signaling pathway	13		
C27	-			
C32	-			

with the microarray analysis (Fig. 7). As shown in below, qRT-PCR results revealed that almost all the genes in mutant C1- and C10- infected cells showed down-regulation while the genes in mutant C27- and C32- infected cells showed up-regulation.

Discussion

Brucella abortus is an intracellular pathogen with various strategies to persist during intracellular infection. They lead to chronic infection since the bacteria modulate host

Fig. 6. Gene network with differentially expressed genes in THP-1 macrophage cells infected with *B. abortus* mutants. The activated gene network in *B. abortus* mutant-infected cells was compared to that of wild-type strain-infected cells. (A) Inflammatory responses in C1- and C10-infected cells. (B) Defense responses in C1- and C10-infected cells. (C) Inflammatory responses in C27- and C32-infected cells. (D) Defense responses in C27- and C32-infected cells.

Fig. 7. Validation of the microarray experiment using real-time PCR.

The microarray experiment was confirmed by real-time PCR.

immune response including regulatory cytokines such as IL-10 [29, 30]. However, *B. abortus* does not have classical pathogenic factors such as plasmids, exotoxins, and capsules [5, 6]. Therefore, many researchers have attempted to identify the pathogenic factors and mechanism involved in chronic *B. abortus* infection [31–33]. Moreover, the role of bacterial genes or proteins in *B. abortus* infection has been identified through the single gene mutation [15, 17, 27, 32, 34–36]. Previous studies have shown that *B. abortus* expresses uncanonical virulence factors such as modulated LPS and bacterial proteins including PrpA and Btp1/TcpB to avoid host immune responses [37, 38].

Therefore, to identify *B. abortus* virulence factors, mutants were generated using transposon random insertion, and single insertions were certified in our previous study [17, 19]. Among the mutants, four mutants were selected to investigate genes related in intracellular survival which is one of most important properties of *B. abortus*. C1 and C10 showed lower invasion, and C27 and C32 showed higher invasion compared to wild-type in the THP-1 macrophage cells. Also, they have different growth rates from wild-type. The characteristics and growth rates of all the mutants were identified in our previous study, and C1, C10, and C27 showed no difference from wild-type in biochemical test, biovar test, and expression of pathogenic factors (phosphoglycerate kinase, cyclic β -1,2-glucans and T4SS) [19]. However, C32 showed a difference in succinate alkalization in the biochemical test. Using *B. abortus* wild-type and these mutants, intracellular survival and replication were observed up to 24 h in THP-1 macrophage cells. Additionally, host cellular responses were compared

between the cells infected with *B. abortus* strains at 24 h p.i. using microarray to investigate the difference between the wild-type and mutants that showed altered intracellular growth.

In the THP-1 macrophage cells, *B. abortus* strains were eliminated at 6 h p.i., and then surviving bacteria replicated their numbers until 24 h p.i. within the cells. Mutants C1 and C10 showed intracellular numbers lower than and similar to wild-type throughout the infection. On the other hand, mutant C27 and C32 showed higher numbers of survival and replication compared to wild-type. At 6 h p.i., C27 and C32 showed higher survival level compared to wild-type, C1 and C10, and they replicated more than others at 24 h p.i. Therefore, intracellular survival is considered important to intracellular number within the cells and host immune responses.

Transcriptomic analysis indicated that gene expression of infected cells was divided into two patterns: C1 and C10, and C27 and C32. The functional annotation analysis revealed that genes related to cellular binding, biological regulation, response to stimuli and immune system processes were expressed oppositely. C1 and C10 down-regulated these genes while C27 and C32 up-regulated. Also, C27 and C32 down-regulated genes related to signal transducer activity. This finding means that cellular cooperation involving antigen recognition and cellular protection against *B. abortus* mutants occurred differently.

Analysis of the KEGG pathway based on microarray analysis revealed that genes related to cytokine-cytokine receptor interaction in the THP-1 macrophage cells infected by the four mutants were differently regulated compared to wild-type-infected cells. The cytokines play key roles in inflammatory responses [39]. The production of cytokines, such as IFN- γ , IL-12, and IL-18, has been reported to be induced against the intracellular bacteria [40, 41]. The cytokine-cytokine receptor interaction pathway was down-regulated in THP-1 macrophage cells infected by mutants C1 and C10. On the other hand, the pathway was up-regulated in the cells infected by mutants C27 and C32. The ELISA results consistently showed that C27 and C32 induced TNF- α , IL-1 β , and IL-6 production, whereas C1 and C10 did not. However, IFN- γ was not measured by ELISA from the cells infected with wild-type and mutants. IFN- γ is important in overcoming *B. abortus* infection in macrophages [7]. Several studies have reported that IFN- γ was detected in the in vivo model but not in the in vitro [42, 43]. Additionally, J. Sanceau *et al.* reported that both IFN- γ and TNF- α are required to induce IL-6 in THP-1 macrophage cells [44]. Also, the microarray data show the

expression of mRNA in infected cells, which may be different from the actual production levels of cytokine at that time. Therefore, IFN- γ was not found but some response related to IFN- γ was thought to have occurred.

The disrupted genes of mutants C1 and C10 were *BAB_RS13225* and *BAB_RS00455*, respectively. They are encoding PHBH and CcmC. The PHBH catalyzes the chemical reaction of 4-hydroxybenzoate to form 3,4-dihydroxybenzoate (also known as protocatechuic acid) [20]. Protocatechuic acid, a product of the degradation, is ring-cleaved by 3,4-PCA to form an intermediate that enters the Krebs cycle [21, 45]. During cellular infection, *B. abortus* must overcome several obstacles, including intracellular pH, shortage of iron and high salt [46, 47]. To enable successful survival in macrophages, *B. abortus* must generate ATP from the Krebs cycle using the obtained 3,4-PCA, and the C1 strain may have failed to use this strategy because of the deficiency of the gene. The CcmC is an essential protein for heme delivery at the cellular membrane because it can directly attach to CcmE, which is responsible for direct heme transport to cytochrome C and induction of cytochrome C maturation [48, 49]. Cytochrome C is primarily known as an electron transfer protein in the respiratory chain and photosynthetic system [50]. Moreover, cytochrome C plays an important role in supporting mitochondrial life utilizing ATP synthesis during cellular emergency [22, 48, 51]. Deficiency of the CcmC-encoding gene in *B. abortus* may disturb heme delivery and cytochrome C maturation in the bacterium, and lower ATP synthesis and electron delivery occurred during macrophage infection.

Mutant C1- and C10-infected cells showed lower invasion and survival inducing inhibited cellular immune responses compared to wild-type. Gene network analysis showed that genes responsible for activating the NF κ B pathway were inhibited (Fig. 5A). The NF κ B pathway was shown to be the main mediator of TNF signalling in *B. abortus*-infected murine macrophages, and TNF in the infected macrophages is required for resistance against the bacterium [52]. Additionally, TNF- α is one of the key cytokines in resistance to *B. abortus* inducing sequential production of cytokines within the macrophages [53, 54]. Moreover, the significant genes related to cytokines were down-regulated in the cells infected by C1 (*CCL2*, *TNF*, and *IL1B*) and C10 (*CCL2*, *TNF*, *IL1B*, and *CD80*). These results suggest that C1 and C10 did not sufficiently induce cellular immune responses, and intracellular number as well as low production of cytokines may be proceeded in response to the mutants compared to wild-type. These findings

indicate that PHBH- and CcmC- encoding genes contribute to the intracellular survival of the bacteria.

The disrupted genes of mutants C27 and C32 were *BRUAB_RS03675* and *BRUAB_RS08885*, respectively. Additionally, they encode PPX and M24. The exopolyphosphatases (PPX) cut the terminal phosphate of linear poly (P) containing three or more phosphoanhydride bonds [55]. It is well known that the balance of poly (P) in bacteria is important because it participates in the regulation and balance of various cellular processes such as cell growth, proliferation, motility, sporulation, intracellular survival and virulence factors in several bacteria including *E. coli*, *M. tuberculosis*, and *Pseudomonas aeruginosa* [23–25]. Also, the increase of poly (P) in *Mycobacterium tuberculosis* has been shown to increase intracellular survival during macrophage infection leading to changes in cell wall permeability [56]. Mutant C27, in which the PPX-encoding gene is disrupted, may have failed to regulate these original processes and intracellular poly (P) accumulation may contribute to this phenomenon in *B. abortus*. Peptidase M24, which is present in both prokaryotes and eukaryotes, removes methionine and plays a role in transcription initiation and protein synthesis [26]. Also, they act only when the penultimate residue of nascent protein, especially prokaryotic cytoplasmic protein, is small and uncharged [57]. In *Brucella* spp. research, cytoplasmic proteins of the bacteria have been shown to be used as antigens [58, 59]. The deficiency of the gene may contribute to inhibition of bacterial antigens which are used to recognize infection in the host cells. C32 showed negative for succinate alkalization in the biochemical test, although even wild-type showed positive in our previous study [19]. The succinate is one of the important mediators of the Krebs cycle [60]. Inhibited synthesis of *B. abortus* antigen in C32 could lead to these different properties that may contribute to invasion and survival within the THP-1 macrophage cells.

Mutant C27- and C32-infected cells showed higher survival inducing higher cellular immune responses than the wild-type strain. The results of transcriptomic analysis also coincided with ELISA. The gene network showed that C27- and C32-infected cells recognized intracellular pathogens and activated interferons related to *IFIH1*, which inhibits bacterial replication [61]. Additionally, *APOL3* which is related to TNF induction [62] was up-regulated, but *KLF4*, which can act both as an activator and a repressor as transcription factor [63] was down regulated. Microarray data showed that cytokines related to antimicrobial responses were up-regulated in cells infected by C27 (*SLAMF7*, *CD80*, and *CCL2*) and C32 (*CCL2*). The

SLAMF7 gene is also known as CD2 Subset 1, and it is considered to be related to class I MHC-mediated antigen processing and presentation [64]. The expression of class I MHC was shown to be induced by IFN- γ , and *B. abortus* infection down-modulates that expression to evade immune surveillance [65]. The observed immune responses including expression of cytokine-related genes may have been activated because the number of bacteria increased within the cells, but not sufficiently for C27 and C32 growth restriction. Also, not only was the survival number of C27 and C32 higher, they down-regulated host cellular signal transduction as well. These findings indicate that deficiency of PPX- and peptidase M24-encoding genes could contribute to changes in cell walls by infection of C27, reduction of antigens of C32, and inhibition of cellular signalling, thereby inducing higher invasion and survival levels of the mutants compared to wild-type.

In conclusion, we showed that disruption of PHBH- (C1), CcmC- (C10), PPX- (C27), and peptidase M24- (C32) encoding genes altered the intracellular survival of *B. abortus* in THP-1 macrophage cells for the first time. Mutation of the *BRUAB_RS13225* (C1) and *BRUAB_RS00455* (C10) genes in *B. abortus* demote intracellular invasion and survival in the cells inducing inhibited immune responses compared to wild-type. Also, disruption of *BAB_RS03675* (C27) and *BRUAB_RS08885* (C32) of *B. abortus* inhibits signal transduction of macrophage cells promoting intracellular invasion and survival in the cells, thereby inducing high cytokine expression of THP-1 macrophage cells. Understanding these defective genes in each *B. abortus* mutant can be useful for investigating the pathogenicity of *Brucella* infection.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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