# Expression and regulation of the *ery* operon of *Brucella melitensis* in human trophoblast cells

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Received April 28, 2015; Accepted May 18, 2016

DOI: 10.3892/etm.2016.3688

**Abstract.** Brucellosis is primarily a disease of domestic animals in which the bacteria localizes to fetal tissues such as embryonic trophoblast cells and fluids containing erythritol, which stimulates *Brucella spp.* growth. The utilization of erythritol is a characteristic of the genus *Brucella*. The *ery* operon contains four genes (*eryA*, *eryB*, *eryC* and *eryD*) for the utilization of erythritol, and plays a major role in the survival and multiplication of *Brucella spp.* The objective of the present study was to conduct a preliminary characterization of differential genes expression of the *ery* operon at several time points after *Brucella* infected embryonic trophoblast cells (HPT-8 cells). The result showed that the *ery* operon expression was higher in HPT-8 cells compared with the medium. The relative expression of *eryA*, *eryB* and *eryC* peaked at 2 h post-infection in HPT-8 cells, and *eryD* expression peaked at 3 h post-infection. The expression of *eryA*, *eryB* and *eryC* may be inhibited by increased *eryD* expression. However, the expression of the *ery* operon was stable in the presence of erythritol in cells. 2308Δ*ery* and 027Δ*ery* mutants of the *ery* operon were successfully constructed by homologous recombination, which were attenuated in RAW 264.7 murine macrophages. The characterization of the *ery* operon genes and their expression profiles in response to *Brucella* infection further contributes to our understanding of the molecular mechanisms of infection and the pathogenesis of brucellosis.

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*Key words:* *Brucella melitensis*, *ery* operon, reverse transcription-quantitative polymerase chain reaction, HPT-8 cells

## Introduction

The Gram-negative bacteria *Brucella* causes Brucellosis, a zoonotic disease that is widely disseminated throughout the world (1). In humans, Brucellosis causes undulant fever, arthritis and myocarditis (2). *Brucella spp.* are able to survive and multiply inside the placenta and fetus of pregnant mammals, causing abortion during pregnancy (3). The interactions between *Brucella* and their hosts are extremely complex, as these facultative intracellular parasites are able to adapt to the harsh environment of host cells, which include oxidative damage, nitrosative damage, acidic pH, antimicrobial peptides and nutrient deprivation (4,5). *Brucella spp.* are able to achieve this by regulating gene expression differently when growing *in vitro* or *in vivo*. However, the mechanisms underlying this survival and multiplication within host cells require further characterization.

*Brucella spp.* have a tropism for cells containing erythritol, such as embryo-trophoblasts found in the placenta (6), and erythritol has a growth-promoting effect on some *Brucella* strains. Therefore, *Brucella spp.* are able to colonize and reproduce in embryo trophoblast cells, which can cause placentitis and result in abortion (7). Furthermore, the virulence of *Brucella spp.* is correlated with erythritol metabolism (8), and the *Brucella*-encoded catabolic erythritol pathways are required for intracellular survival (9). Erythritol usage relies on the *ery* operon, which consists of the genes *eryA*, *eryB*, *eryC* and *eryD* (10). The *eryA* gene encodes a 519 amino acids (AA) putative erythritol kinase (10). The *eryB* gene encodes an erythritol phosphate dehydrogenase (10). The *eryC* gene product has been assigned as a D-erythrulose-1-phosphate dehydrogenase, and the *eryD* gene encodes a regulator of *ery* operon expression (10-12). Although *ery* operon expression is correlated with erythritol metabolism, growth conditions can regulate gene expression (10). To understand *ery* operon regulation in *Brucella melitensis* during infection, we examined gene expression at several timepoints following growth in HPT-8 trophoblast cells. The results help to characterize the mechanisms required for *Brucella spp.* pathogenesis.

## Materials and methods

*Bacterial strains, plasmids and growth conditions*. Table I lists the strains and constructed plasmids used in this study. *Brucella abortus* 2308 was obtained from the Chinese Center of Disease Prevention and Control (CDC; Beijing, China). *B. melitensis* 027 strain was isolated from Xinjiang, China, and was identified by the CDC. *Brucella* strains were cultured in tryptic soy agar (TSA) or tryptic soy broth (TSB; Sigma-Aldrich, St. Louis, MO, USA). Plates were incubated at a temperature of 37˚C in an atmosphere enriched with 5% $CO_2$. *Escherichia coli* strain JM109 (Promega Corporation, Madison, WI, USA) was grown in Luria-Bertani (LB) media. The culture media were supplemented with 50 $\mu$g/ml ampicillin (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA). The plasmid pMD18-T Simple Vector was purchased from Takara Bio, Inc. (Otsu, Japan). The standard curves were constructed using pMD18-T Simple Vector.

*Cells*. Murine macrophages (RAW 264.7) and human trophoblasts (HPT-8) were used in this study. HPT-8 cells and RAW 264.7 murine macrophage were purchased from the Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China).

*Construction of 2308Δery and 027Δery*. Deletion of the *ery* operon in *B. abortus* 2308 and *B. melitensis* 027 (2308Δ*ery* and 027Δ*ery*) was performed as previously described (13).

*Growth curve of 2308Δery and 027Δery*. To monitor the growth of *B. abortus* 2308, *B. melitensis* 027, 2308Δ*ery* and 027Δ*ery*, cells were cultured in TSB to an optical density at 600 nm (OD600) of 0.6, then diluted with TSB to an OD600 of 0.05 and cultured in rotary shaker (100.62 x g) at 37˚C for 48 h. Aliquots of cultures were collected at 4-h intervals, and bacterial growth was measured at OD600.

*Erythritol sensitivity of 2308Δery and 027Δery*. To detect erythritol sensitivity in 2308Δ*ery* and 027Δ*ery*, stationary phase pre-cultures of *B. abortus* 2308, *B. melitensis* 027, 2308Δ*ery* and 027Δ*ery* were diluted in TSB containing 20 mM erythritol (Sigma-Aldrich), and grown for 48 h. The bacterial growth was measured at an OD600.

*Evaluation of 2308Δery and 027Δery attenuation in RAW 264.7 murine macrophages*. RAW 264.7 murine macrophages were used to assess the intracellular survival of *B. abortus* 2308, *B. melitensis* 027, 2308Δ*ery* and 027Δ*ery*. RAW 264.7 cells were infected as previously described (14). Briefly, $5x10^5$ cells/well were cultured in 24-well plates for 16 h at 37˚C and infected with *Brucella* at a multiplicity of infection (MOI) of 100. Culture plates were centrifuged for 5 min at 350 x g at room temperature and placed in an incubator at 37˚C with 5% $CO_2$ atmosphere. At 45 min post-infection, the cells were washed twice with media and then incubated with Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Rockville, MD, USA) containing 50 $\mu$g/ml gentamicin (Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h to kill extracellular bacteria. The media was then replaced with DMEM containing 25 $\mu$g/ml gentamicin (incubation point 0 min). At 4, 12, 24 and 48 h post-infection, the number of colony-forming units (CFU) was obtained by plating serial dilutions of the lysates on TSA plates. All assays were performed in triplicate and repeated at least three times.

*HPT-8 cells invasion assay*. HPT-8 cells were infected by *B. abortus* 2308 and *B. melitensis* 027 in media with or without erythritol (20 mM). HPT-8 cells were grown at 37˚C in a 5% $CO_2$ atmosphere in DMEM containing 20% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Cells were seeded ($1x10^6$) in 12-well culture dishes 24 h prior to each infection assay. HPT-8 cells were infected at a MOI of 100 bacteria per cell as previously described (14,15). Culture plates were centrifuged for 5 min at 350 x g at room temperature. Post-infection, cells were grown in the presence of erythritol at a concentration of 1% as a nutritional supplement or at 20 mM for induction of the *ery* operon and placed in an incubator at 37˚C with 5% $CO_2$ atmosphere. Cells were washed three times with phosphate-buffered saline (PBS) and monolayers of cells were further incubated with culture media supplemented with 50 $\mu$g/ml gentamicin for 1 h to kill extracellular bacteria. The cells were washed with DMEM containing 10% fetal bovine serum to remove gentamicin, then the cells were lysed with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.).

*RNA isolation and reverse transcription*. Total RNA (1 $\mu$g) from HPT-8 cells at 0 min (bacterial culture), 20 min, 1 h, 2 h, 3 h, 4 h and 12 h post-infection was isolated (Qiagen RNeasy Mini-kits; Qiagen, Hilden, Germany) and cDNA was generated using random hexamer primers and MMLV-RT according to the manufacturer's recommendations (Gibco; Thermo Fisher Scientific, Inc.). Genomic DNA was removed using a DNase RT kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The 10 $\mu$l reaction mixture system containing 1 $\mu$g RNA, 1U DNase, 1 $\mu$l DNase buffer and ddH2O was added to 10 $\mu$l. The mixture system was mixed and allowed to rest for 10 min at room temperature. A total of 1 $\mu$l 25 mM EDTA was added and incubated for 10 min at 65˚C. The DNA polymerase was obtained from Invitrogen (Thermo Fisher Scientific, Inc.). cDNA was stored at -80˚C and used as a template for reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

*Oligonucleotide primers*. TaqMan primers for 16S rRNA (housekeeping gene), *eryA*, *eryB*, *eryC* and *eryD* genes were designed using Primer Express 5.0 software (Applied Biosystems, Palo Alto, CA, USA) according to sequences in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (Table II).

*Construction of recombinant plasmids*. *Ery* operon (*eryA*, *eryB*, *eryC* and *eryD*) and 16S rRNA open reading frames were amplified by PCR with specific primers (see Table II; Premier Biosoft, Palo Alto, CA, USA) from the *B. abortus* 2308 genome. The amplified DNA fragments and pMD18-T simple vectors were ligated overnight at 16˚C using T4 DNA ligase (Takara Bio, Inc.). The ligation reaction was transformed into *E. coli* JM109, and insert-containing plasmids were identified by restriction analysis or PCR. Positive recombinant plasmids were sequenced to confirm the correct construction.

Table I. Bacterial strains and plasmids used in this study.

| Name | Description | Source |
|---|---|---|
| Bacteria strain | | |
| *Brucella abortus* 2308 | Wild-type, virulent strain | China CDC |
| *Brucella melitensis* 027 | Biotype 3, virulent strain (China), identified by China CDC | Present study |
| 2308Δ*ery* | Δ*ery* promoter mutant of strain 2308 | Present study |
| 027Δ*ery* | Δ*ery* promoter mutant of strain 027 | Present study |
| *Escherichia coli* JM109 | *end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 ($r_k$-, $m_k$+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *laq*IqZΔM15] | Promega |
| Plasmid | | |
| pMD18-T simple vector | Broad-host range vector; $Amp^r$ | Takara |
| pMD18-*ery*A | pMD18-T containing 87 bp fragment amplified with *eryA*-RT-S and *eryA*-RT-A including a fraction of *eryA* | Present study |
| pMD18-*ery*B | pMD18-T containing 104 bp fragment amplified with *eryB*-RT-S and *eryB*-RT-A including a fraction of *eryB* | Present study |
| pMD18-*ery*C | pMD18-T containing 118 bp fragment amplified with *eryC*-RT-S and *eryC*-RT-A including a fraction of *eryC* | Present study |
| pMD18-*ery*D | pMD18-T containing 120 bp fragment amplified with *eryD*-RT-S and *eryD*-RT-A including a fraction of *eryD* | Present study |
| pMD18-16S rRNA | pMD18-T containing 88 bp fragment amplified with 16s-RNA-S and 16s-RNA-A including a fraction of 16S rRNA | Present study |

*Transcriptional analysis of ery operon genes by RT-qPCR*. The concentration and purity of recombinant plasmids (dilution, x100) were measured using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Inc.) and used to compute target gene copy numbers. A three-step, 45-cycle RT-qPCR method was conducted using a LightCycler® 480 System (Roche Diagnostics, Basel, Switzerland). A linear standard curve was created with the LightCycler® 480 System Software and serial dilutions of recombinant plasmids containing the genes encoding 16S rRNA, *eryA*, *eryB*, *eryC* and *eryD*. RT-qPCR was conducted with the following reaction conditions: 5 min at 95˚C, followed by 45 cycles at 58˚C for 30 sec and 72˚C for 30 sec. A negative control (no cDNA) and RT control (no reverse transcription) were used in the experiments. All assays were performed in triplicate and repeated at least three times. The expression levels of the target genes were calculated by comparison with cycle threshold (Ct) and determined at 0 min, 20 min, 1 h, 2 h, 3 h, 4 h and 12 h post-infection. 16S rRNA expression was used as a reference value to compare the relative expression levels at the various time points. Target genes were amplified in triplicate using the LightCycler® 480 System, and the data were presented as the mean of each triplicate with standard deviations (SDs). All assays were repeated a minimum of three times.

*Statistical analysis*. The data were analyzed using Student's t-test and expressed as the mean value ± SD. The differences between groups were analyzed by analysis of variance using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Table II. Primer sequences used in this study.

| Primer | Sequence |
|---|---|
| 16S rRNA-RT-sense | GCGGCTCACTGGTCCATTAC |
| 16S rRNA-RT-anti-sense | CGTTTACGGCGTGGACTACC |
| *eryA*-RT-sense | CGCACACGCCAGTATGATGA |
| *eryA*-RT-anti-sense | CGACCCGTCGATGATTTCAG |
| *eryB*-RT-sense | GAGATTGCCAATGCCGATTA |
| *eryB*-RT-anti-sense | GCACCATAGAGCCGTCCATA |
| *eryC*-RT-sense | GCTTTCGCTCAACACCAATC |
| *eryC*-RT-anti-sense | CATGGGTAAGCTGGAGGTCA |
| *eryD*-RT-sense | CGTGGAAAACGCCGATATGA |
| *eryD*-RT-anti-sense | GTCCGTTCGTCGGTGATGAG |

## Results

*ery operon was successfully deleted in B. abortus 2308 and B. melitensis 027*. The *ery* operon deletion was confirmed by PCR in the 2308Δ*ery* and 027Δ*ery* clones (Fig. 1A). Bacteriological analysis and typing of the mutant showed that deletion of the *ery* gene was stable after passage in culture media (Fig. 1B).

*Growth curve of 2308Δery and 027Δery*. To test whether deletion of the *ery* operon affected the growth of 2308 or 027, we measured the bacterial growth in nutrient-replete (TSB 7.0) media. When cultured in normal TSB media, 2308Δ*ery* and 027Δ*ery* displayed a similar lag phase and reached the stationary phase at a similar optical density compared with 2308 and 027 (Fig. 2A).

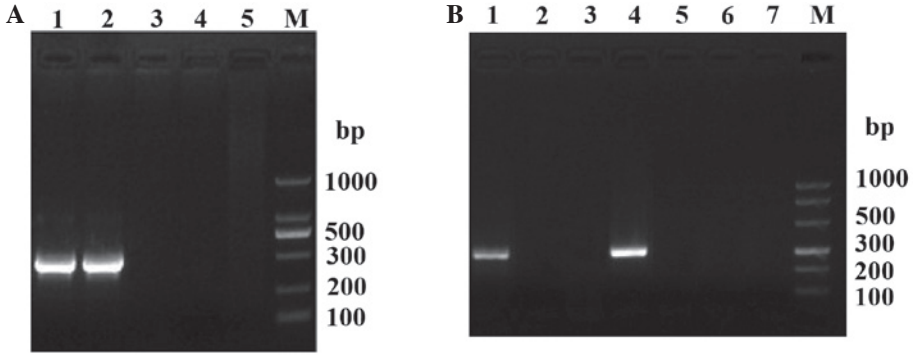


Figure 1. Identification of construction of the 2308Δ*ery* and 027Δ*ery*. (A) Polymerase chain reaction identification of 2308Δ*ery* and 027Δ*ery*. Lanes: 1, the strain 2308; 2, the strain 027; 3, 2308Δ*ery* mutant strain; 4, 027Δ*ery* mutant strain; 5, negative control; M, DNA marker. (B) Genetic stability detection of 2308Δ*ery* and 027Δ*ery*. Lanes: 1, the strain 2308; 2 and 3, 2308Δ*ery* mutant strain; 4, the strain 027; 5 and 6, 027Δ*ery* mutant strain; 7, negative control; M, DNA marker.

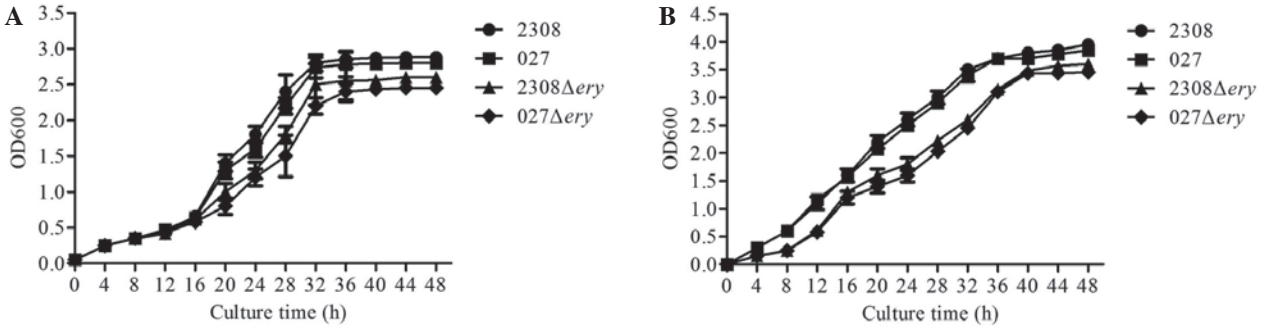


Figure 2. Growth curves of *Brucella* strains in (A) nutrient-replete (TSB 7.0) media (A) or containing (B) erythritol (20 mM). The OD600 value was measured to determine growth.

*Erythritol growth response in 2308Δery and 027Δery*. To test whether the 2308Δ*ery* and 027Δ*ery* strains were sensitive to erythritol when grown in broth containing erythritol (20 mM), we measured the bacterial growth. The results showed that the virulent strains 2308 and 027 grew well in broth containing erythritol; however, the 2308Δ*ery* and 027Δ*ery* mutants grew at a slower rate (Fig. 2B). These results indicated that 2308Δ*ery* and 027Δ*ery* do not respond to erythritol, and that the virulent strains 2308 and 027 may utilize erythritol for growth.

*2308Δery and 027Δery are attenuated and experience reduced survival in macrophages*. To assess whether the *ery* operon influences virulence, RAW 264.7 macrophages were infected with 2308Δ*ery*, 027Δ*ery*, 2308 and 027. The surviving bacteria were enumerated, and there was no difference in the number of surviving bacteria 4 h post-infection (Fig. 3; P>0.05). This indicated that deletion of the *ery* operon does not affect macrophage invasion. However, at 12 h post-infection, there was a 1.50-log decrease (P<0.05) in the number of 2308Δ*ery* compared to 2308, and there was a 1.55-log decrease (P<0.05) in the number of 027Δ*ery* compared to 027 (Fig. 3). At 24 h post-infection there was a 2.70-log decrease in the number of 2308Δ*ery* compared to 2308 (Fig. 3; P<0.01); and there was a 2.70-log decrease in the number of 027Δ*ery* compared to 027 (Fig. 3; P<0.01). At 48 h post-infection, there was a 4.10-log decrease in the number of 2308Δ*ery* compared to 2308 (Fig. 3; P<0.01); and there was a 4.00-log decrease in the number of 027Δ*ery* compared to 027 (Fig. 3; P<0.01). Therefore, 2308Δ*ery* and 027Δ*ery* mutants had a replication defect in RAW264.7 macrophages.

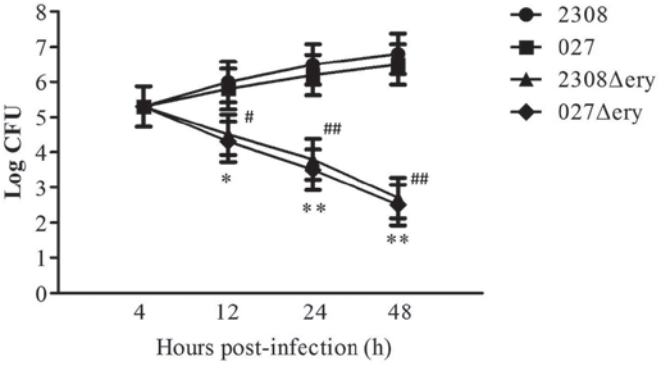


Figure 3. Survival of bacterial strains 2308Δ*ery*, 027Δ*ery*, 2308 and 027 in RAW 264.7 macrophages. Monolayers of macrophages were infected with *Brucella* at 4, 12, 24 and 48 h post-infection, the macrophages were lysed and supernatants were diluted for colony-forming factor enumeration. Significant differences in replication are indicated as follows: Significant differences between 2308Δ*ery* and 2308 are indicated by $^{*}$P<0.05 and $^{**}$P<0.01. Significant differences between 027Δ*ery* and 027 are indicated as follows $^{\#}$P<0.05 and $^{\#\#}$P<0.01.

*Preparation of standard curves of different genes by RT-qPCR assay*. The equations for the linear regression line for the standard curves of different genes generated by RT-qPCR assay and the corresponding $R^2$ value are as follows: 16S rRNA, y=0.238x+11.041, $R^2$=0.986; *eryA*, y=-0.267x+12.217, $R^2$=0.986; *eryB*, y=-0.266x+11.081, $R^2$=0.983; *eryC*, y=-0.216x+10.165, $R^2$=0.977; and *eryD*, y=-0.297x+11.414, $R^2$=0.995. Based on the slope rates of these regression lines,

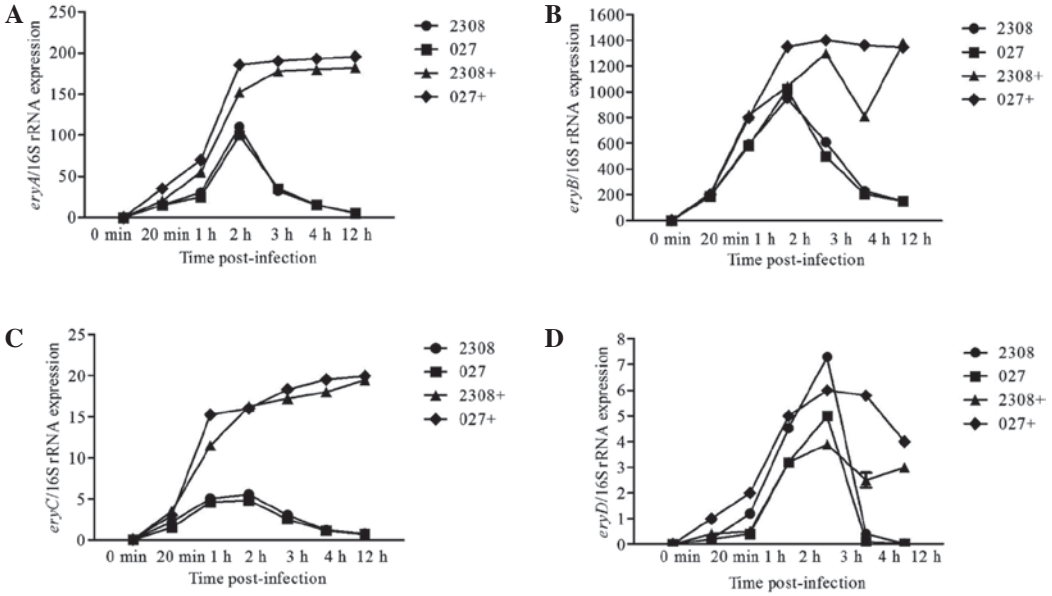


Figure 4. Relative expression level of *eryA* (A), *eryB* (B), *eryC* (C) and *eryD* (D) of *Brucella abortus* 2308 and *Brucella melitensis* 027 in media with or without erythritol (20 mM) at several time points. Growth of *B. abortus* 2308 and *B. melitensis* 027 grown in media without erythritol. In addition, expression in *B. abortus* 2308+ and *B. melitensis* 027+ were followed in the presence of erythritol (20 mM). The relative expression of *eryA*, *eryB* and *eryC* peaked at 2 h post-infection in HPT-8 cells, and that *eryD* expression peaks at 3 h post-infection. Data points represent means of triplicate reactions, and error bars indicate standard deviation. The data set is representative of three independent experiments using the same sample.

the levels of amplification efficiency ($E=10^{-a}-1$) were 72.98% for 16S rRNA, 84.93% for *eryA*, 84.50% for *eryB*, 64.44% for *eryC*, 98.15% for *eryD*. Melt curve (unpublished) analysis of amplification products indicated that there was a single peak with a Tm of 86.8˚C for 16S rRNA, 86.2˚C for *eryA*, 84.6˚C for *eryB*, 90.06˚C for *eryC* and 91.75˚C for *eryD*.

*Ery operon gene expression following infection:* We analyzed gene expression at 0 min, 20 min, 1 h, 2 h, 3 h, 4 h and 12 h post-infection. The relative expression levels of *eryA*, *eryB*, *eryC* and *eryD* were significantly higher in *Brucella* grown with medium containing erythritol. Furthermore, the relative expression levels of *eryA*, *eryB* and *eryC* were highest ($P<0.01$) at 2 h post-infection (Fig. 4A-C), but were highest ($P<0.01$) at 3 h post-infection for *eryD* ($P<0.05$) (Fig. 4D). Gene expression levels were calculated based on the comparison to the expression of 16S rRNA. Based on these calculations, *eryB* was the most highly expressed gene followed by *eryA*, *eryC* and *eryD* (Fig. 5).

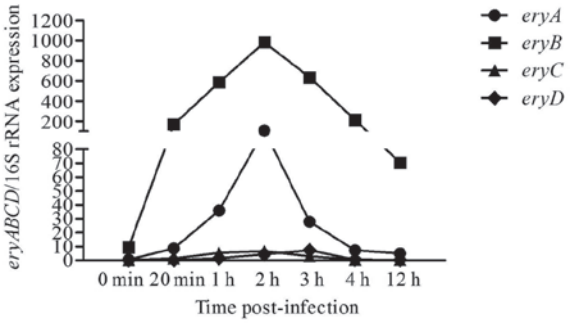


Figure 5. Comparison of the relative expression levels of *ery* operon, including *eryA*, *eryB*, *eryC* and *eryD*, in *Brucella melitensis* strain 2308 at different time points. Each value in the figure represents the mean of triplicate experiments ± standard deviation.

## Discussion

The majority of *Brucella spp*. utilize erythritol to promote growth (except *B. abortus* S19). Metabolism and usage of erythritol are regulated by the 7.7-kb *ery* operon, which consists of four genes *eryA*, *eryB*, *eryC* and *eryD* (EryA, 519 AA; EryB, 502 AA; EryC, 309 AA and EryD, 316 AA) (16-18). The functions of these four proteases are similar to xylulose kinase (*E. coli xylB*), glycerol-3-phosphate dehydrogenase (*E. coli glpD*), hydrogenase (*Alcaligenes hydrogenophilus hupL*), operon regulators (*Rhodobacter sphaeroides smoC* and *Klebsiella pneumoniae dalR*) (10). In addition, the promoter of the *ery* operon also includes an integration host factor binding site. This study demonstrates that the expression of the *ery* operon in HPT-8 cells is higher than in culture medium, and suggests that the *B. melitensis* 027 *ery* operon is induced by erythritol within HPT-8 cells. Furthermore, the expression of the *eryD* gene may repress the expression of the other genes in the operon. This is the first demonstration of the expression and regulation of the *ery* operon of *B. melitensis* in human trophoblast cells.

*Brucella* is detected in non-professional phagocytes 30 min post-infection (19). Therefore, the infection time used in this study was 20 min, at which time *Brucella* were detectable within host cells. Once entry has been established a brucellosome is formed, a process that lasts 2-3 h. To study the expression of the *ery* operon during the initial stage of infection, we measured *ery* operon expression at 20 min, 1 h, 2 h, 3 h and 4 h post-infection in HPT-8 cells. In addition, the expression of the *ery* operon was measured at 12 h post-infection and was markedly decreased, which may be due to changes in the intracellular environment induced by *Brucella*. Notably, HPT-8

cells easily detached from the culture flask after infection. In general, longer infection times correlated with higher levels of detachment. In this study, we used expression of the 16S rRNA gene as a reference to eliminate determine genes expression levels at 20 min, 1 h, 2 h, 3 h, 4 h and 12 h post-infection.

Numerous genes from pathogen bacteria, such as *Brucella* and *Mycobacterium tuberculosis*, are regulated by environment signals *in vitro* (20). Mariani *et al* (17) investigated the expression of 14 genes in *Mycobacterium tuberculosis* H37Rv in both medium and macrophages. Five of these genes were expressed in media and macrophages, four genes were expressed in media, and the remaining five genes were only expressed in macrophages. In this study, we characterized the expression of the *ery* operon in media and at different timepoints following infection.

All four genes of the *ery* operon are essential for optimal *Brucella* virulence. The function of the genes in the *ery* operon control erythritol catabolism, which has been postulated to increase virulence in the host environment (21). Cells infected with *Brucella* were grown in liquid media with or without erythritol at a concentration of 20 mM. During the early phase of infection without erythritol, the expression levels of *eryA-C* are highest at 2 h post-infection; *eryD* is highest at 3 h post-infection. Therefore, as *eryD* expression increased the expression of *eryA-C* declined (Fig. 4). When erythritol was present in the media, expression was similar to the above-mentioned results. However, the expression levels of *eryA-C* peaked at 2 h then remained constant, whereas *eryD* peaked at 3 h and then declined (Fig. 5). Two explanations may account for this phenomenon. First, EryD protein expression may inhibit transcription of the *ery* operon (7). A second explanation may involve acidification of the brucellosome at 3 h post-infection, as multiplication in the acidified-brucellosome may represent a starvation condition due to decreased *ery* operon expression induced by erythritol.

Trophoblastic cells contain high levels of erythritols and are targeted for infection by *Brucella*. It has been previously reported that *Brucella* preferentially utilizes the carbon source erythritol. This study demonstrates that the expression of the *ery* operon is significantly lower in media compared with trophoplastic cells.

Therefore, the present results indicate that the expression of the *ery* operon of *B. melitensis* 027 differs when grown in media or HPT-8 cells, and that this expression is regulated by environmental signals. Specifically, the *ery* operon is induced by erythritol after entry into HPT-8 cells. However, a number of questions remain to be answered before we can understand the role of the *Ery* system in *Brucella* virulence.

## Acknowledgements

The present study was supported by grants from the International Science and Technology Cooperation Project of China (grant nos. 2013BC005 and 2015DFR31110), the Outstanding Youth Project of Shihezi University (grant no. 2012ZRKXJQ02), the National Natural Science Foundation of China (grant nos. 31460650 and 31260596), and the University Key Research Project of Henan Province (grant no. 16A230013).

## References

1. Bercovich Z: The use of skin delayed-type hypersensitivity as an adjunct test to diagnose brucellosis in cattle: A review. Vet Q 22: 123-130, 2000.
2. Pappas G, Papadimitriou P, Akritidis N, Christou L and Tsianos EV: The new global map of human brucellosis. Lancet Infect Dis 6: 91-99, 2006.
3. Boschiroli ML, Foulongne V and O'Callaghan D: Brucellosis: A worldwide zoonosis. Curr Opin Microbiol 4: 58-64, 2001.
4. Köhler S, Michaux-Charachon S, Porte F, Ramuz M and Liautard JP: What is the nature of the replicative niche of a stealthy bug named *Brucella*? Trends Microbiol 11: 215-219, 2003.
5. Roop RM II, Gee JM, Robertson GT, Richardson JM, Ng WL and Winkler ME: *Brucella* stationary-phase gene expression and virulence. Ann Rev Microbiol 57: 57-76, 2003.
6. Anderson JD and Smith H: The metabolism of erythritol by *Brucella abortus*. J Gen Microbiol 38: 109-124, 1965.
7. Meyer ME: Metabolic characterization of the genus *Brucella* VI. Growth stimulation by i-erythritol compared with strain virulence for guinea pigs. J Bacteriol 93: 996-1000, 1967.
8. Keppie J, Williams AE, Witt K and Smith H: The role of erythritol in the tissue localization of the *Brucellae*. Br J Exp Pathol 46: 104-108, 1965.
9. Delrue RM, Lestrate P, Tibor A, Letesson JJ and De Bolle X: *Brucella* pathogenesis, genes identified from random large-scale screens. FEMS Microbiol Lett 231: 1-12, 2004.
10. Sangari FJ, Agüero J and García-Lobo JM: The genes for erythritol catabolism are organized as an inducible operon in *Brucella abortus*. Microbiology 146: 487-495, 2000.
11. Burkhardt S, Jiménez de Bagüés MP, Liautard JP and Köhler S: Analysis of the behavior of eryC mutants of *Brucella suis* attenuated in macrophages. Infect Immun 73: 6782-6790, 2005.
12. Eoh H, Jeon BY, Kim Z, Kim SC and Cho SN: Expression and validation of D-erythrulose 1-phosphate dehydrogenase from *Brucella abortus*: A diagnostic reagent for bovine brucellosis. J Vet Diagn Invest 22: 524-530, 2010.
13. Zhang J, Yin S, Guo F, Meng R, Chen C, Zhang H, Li Z, Fu Q, Shi H, Hu S, *et al*: A potent *Brucella abortus* 2308 Δery live vaccine allows for the differentiation between natural and vaccinated infection. J Microbiol 52: 681-688, 2014.
14. Hernández-Castro R, Verdugo-Rodríguez A, Puente JL and Suárez-Güemes F: The BMEI0216 gene of *Brucella melitensis* is required for internalization in HeLa cells. Microb Pathog 44: 28-33, 2008.
15. Pizarro-Cerdá J, Méresse S, Parton RG, van der Goot G, Sola-Landa A, Lopez-Goñi I, Moreno E and Gorvel JP: *Brucella abortus* transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. Infect Immun 66: 5711-5724, 1998.
16. Lillo AM, Tetzlaff CN, Sangari FJ and Cane DE: Functional expression and characterization of EryA, the erythritol kinase of *Brucella abortus* and enzymatic synthesis of l-Erythritol-4-phosphate. Bioorg Med Chem Lett 13: 737-739, 2003.
17. Mariani F, Cappelli G, Riccardi G and Colizzi V: *Mycobacterium tuberculosis* H37Rv comparative gene-expression analysis in synthetic medium and human macrophage. Gene 253: 281-291, 2000.
18. Smith H, Williams AE, Pearce JH, Keppie J, Harris-smith PW, Fitz-George RB and Witt K: Foetal erythritol: A cause of the localization of *Brucella abortus* in bovine contagious abortion. Nature 193: 47-49, 1962.
19. Watanabe K, Tachibana M, Tanaka S, Furuoka H, Horiuchi M, Suzuki H and Watarai M: Heat shock cognate protein 70 contributes to *Brucella* invasion into trophoblast giant cells that cause infectious abortion. BMC Microbiol 8: 212, 2008.
20. Wang Y, Chen Z, Qiao F, Zhong Z, Xu J, Wang Z, Du X, Qu Q, Yuan J, Jia L, *et al*: The type IV secretion system affects the expression of Omp25/Omp31 and the outer membrane properties of *Brucella melitensis*. FEMS Microbiol Lett 303: 92-100, 2010.
21. Dizon-Townson DS, Lu J, Morgan TK and Ward KJ: Genetic expression by fetal chorionic villi during the first trimester of human gestation. Am J Obstet Gynecol 183: 706-711, 2000.