Roles of *Brucella abortus* SpoT in morphological differentiation and intramacrophagic replication

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The essential mechanisms and virulence factors enabling *Brucella* species to survive and replicate inside host macrophages are not fully understood. The authors previously reported that a putative guanosine 5′-diphosphate 3′-diphosphate (ppGpp) mutant (*spoT* mutant) of *Brucella abortus* failed to replicate in HeLa cells. The present study showed that the pattern of surface proteins and morphological change of the *spoT* mutant were different from *B. abortus* wild-type. *B. abortus* wild-type changed its morphology upon treatment with ppGpp synthetase I activation inhibitor. In various tests under stress conditions, including nutrient starvation, nitric oxide resistance, acid resistance and antibiotic resistance, the *spoT* mutant had a lower stress resistance than *B. abortus* wild-type. Although the *spoT* mutant has the same smooth phenotype and LPS profile as *B. abortus* wild-type, it had a higher rate of adherence to macrophages but lower internalization and intracellular replication within macrophages. The *spoT* mutant did not co-localize with either late endosomes or lysosomes and was almost cleared from the spleens of mice after 10 days, without splenomegaly. RT-PCR was used to detect *spoT* mRNA from around 10⁶ cells incubated in low-pH enriched medium; it showed that the expression of *spoT* increased after 30 min incubation. The data suggest that SpoT does not contribute to intracellular trafficking of *B. abortus*, but contributes to the maintenance of bacterial morphology and the physiological adaptation required for intracellular replication.

INTRODUCTION

Brucellosis is a major bacterial zoonosis and an important cause of a serious debilitating disease in humans and abortion and sterility in domestic animals. This disease is caused by species of the genus *Brucella*, classified in the α₂ subdivision of *Proteobacteria*, small Gram-negative and facultative intracellular pathogens that can multiply within professional and non-professional phagocytes (Delrue et al., 2001; Detileux et al., 1990). The genus *Brucella* consists of seven species according to antigenic variation and primary host: *B. melitensis* (sheep and goats), *B. suis* (hogs), *B. abortus* (cattle), *B. ovis* (sheep), *B. canis* (dogs), *B. neotomae* (wood rats) and *B. maris* (marine mammals) (Ko & Splitter, 2003). In contrast to other intracellular pathogens, *Brucella* species do not produce exotoxins, antiphagocytic capsules, thick cell walls, resistance forms or fimbriae and do not show antigenic variation (Finlay & Falkow, 1997).

A key aspect of the virulence of *Brucella* species is their ability to proliferate within professional and non-professional phagocytic host cells, therefore successfully bypassing the bactericidal effects of phagocytes. Their virulence and chronic infections are thought to be due to their ability to avoid the killing mechanisms within host cells (Comerci et al., 2001; Ugalde, 1999). Neither virulence factors that allow intracellular survival of *Brucella* species nor the specific factors that induce such virulence have been elucidated. *B. abortus* is internalized by macrophages by swimming on the cell surface with generalized membrane ruffling for several minutes, a process termed ‘swimming internalization’, after which the bacteria are enclosed by macropinosomes (Watarai et al., 2002a).

Recently we showed that Hsp60, a member of the GroEL family of chaperonins, of *B. abortus* is secreted on the bacterial surface through a type IV system and can interact with the cellular prion protein (PrP<sup>C</sup>) on macrophages (Watarai et al., 2003). Studies with non-professional phagocytes have shown that *B. abortus* invades host cells and is contained within early endosome-like vacuoles. These
vacuoles rapidly fuse with early autophagosomes that acquire vacuolar H\(^+\)-ATPase and lysosome-associated membrane proteins (LAMPs), mature into late autophagosomes, inhibit fusion with lysosomes and finally become replicating vacuoles, normally associated with the endoplasmic reticulum (Comerci et al., 2001; Pizarro-Cerda et al., 1998a, b). The genetic basis of B. abortus virulence is still poorly understood.

Bacteria control many complex regulatory networks, particularly changes in their environment, by inducing the stringent response. This is elicited by adverse nutritional conditions, such as amino acid starvation, which induce a global metabolic shift that results in physiological adaptation to the new environment. Specifically, nutrient stress, in the form of an uncharged tRNA molecule at the ribosome acceptor site, triggers the ribosome-associated RelA protein to catalyse the formation of guanosine pentaphosphate (pppGpp) from GTP using ATP as the phosphate donor. pppGpp is subsequently hydrolysed to guanosine tetraphosphate (pppGpp) which is thought to associate directly with RNA polymerase holoenzyme and alter its specificity for various promoters (Wells & Long, 2003). In Escherichia coli, ppGpp is hydrolysed by SpoT, an enzyme that is highly homologous to RelA, which has limited ability to synthesize pppGpp (Metzger et al., 1989). Although most Gram-negative bacteria have two (p)ppGpp synthetase enzymes, RelA and SpoT, a number of alpha proteobacteria, like Sinorhizobium meliloti, have a single enzyme with a dual function (Mittenhuber, 2001; Wells & Long, 2003). Borrelia burgdorferi possesses a spoT orthologue (BB0198), but no relA gene (Fraser et al., 1997). Borr. burgdorferi SpoT is a bifunctional enzyme and is responsible for the synthesis and degradation of ppGpp (Concepcion & Nelson, 2003). Recent studies have reported that the stringent response is associated with morphological changes and physiological adjustment. High levels of ppGpp cause a morphological change in Mycobacterium smegmatis (Ojha et al., 2000). ppGpp acts as a second messenger to co-ordinate virulence expression as an adaptive response to amino acid starvation in Legionella pneumophila (Hammer & Swanson, 1999). Mycobacterium tuberculosis mutants unable to produce (p)ppGpp are markedly impaired in their ability to survive long-term starvation and anaerobic conditions (Primm et al., 2000).

Köhler et al. (2002b) reported that B. suis 1330 was mutagenized by mini-Tn5Km2 transposon, and the spoT gene mutant was not able to replicate within human macrophage-like THP-1 cells. A preliminary characterization of a B. abortus spoT (stringent response) mutant showed that it has reduced intracellular growth within HeLa cells (Kim et al., 2003). In this study, we examined the virulence of the B. abortus spoT mutant in mice and its intracellular replication in macrophages. We also performed a detailed characterization of the spoT mutant, including morphological changes and physiological adjustment under conditions of stress.

**METHODS**

**Bacterial strains, growth conditions and media.** All B. abortus strains were from smooth virulent B. abortus biovar 1 strain 544 (ATCC 23448). The isogenic mutant of B. abortus 544 spoT::Tn5Km2 has been described by Kim et al. (2003). B. abortus strains were maintained as frozen glycerol stocks and were cultured in Brucella broth (Becton Dickinson) or Brucella broth containing 1·5 % agar. Kanamycin (30 mg ml\(^{-1}\)) and ampicillin (100 mg ml\(^{-1}\)) were used when necessary. pSKspoT(spoT\(^{+}\)) was constructed by cloning a PCR fragment into KpnI/SacI-cleaved pBRR1MCS-4 (Kovach et al., 1995). The 2596 bp KpnI–SacI PCR fragment spanned a site 265 nt upstream of the 5′-end of spoT to a position 49 nt downstream from the 3′-end (DelVecchio et al., 2002). It was amplified using the primers 5′-ACT GCT GGT ACC CAT TGA AGG CTT CGC-3′ (KpnI site underlined) and 5′-TCC CGC GAG CTC GAA GAG CAC ATC-3′ (SacI site underlined).

**Isolation and analysis of LPS.** Bacterial LPS was prepared by a modification of the method of Moreno et al. (1981). To isolate crude LPS from B. abortus wild-type, the spoT mutant and the complemented strain, bacteria were grown in 500 ml Brucella broth at 37 °C for 18 h. Bacteria collected by centrifugation were dried with acetone and were extracted with hot phenol/water and the phases were separated by centrifugation at 8000 r.p.m. for 30 min at 4 °C. Crude LPS in the aqueous phase was precipitated by addition of methanol, suspended in distilled water, dialysed against water, and lyophilized. LPS samples were treated with nuclease and Pronase (Sigma) and were purified further by dialysis as described previously (Moreno et al., 1981). Following separation of extracted LPS in SDS-polyacrylamide gel with a 15% separating gel containing 4 M urea, LPS heterogeneity was visualized by silver staining (Tsai & Frasch, 1982). To determine bacterial phenotype, the absence of the O side chain was verified by demonstrating uptake of crystal violet by the colonies (White & Wilson, 1951).

**Morphology.** A modified method described previously was used to examine bacterial morphology (Ojha et al., 2000; Steel et al., 2004). B. abortus wild-type, the spoT mutant and the complemented strain were cultured in Brucella broth at 37 °C for 24 h to ensure full growth. Cultured bacteria were heat-fixed on glass slides and Gram stained. Precultured bacteria (2–3 × 10\(^{5}\) c.f.u.) were also cultured in 5 ml Brucella broth containing 0, 0.5, 1, 2.5, 5, 10, 25 and 50 mg ml\(^{-1}\) chloramphenicol, an inhibitor of ppGpp synthetase I activation. The sub-bacteriostatic and sublethal concentrations of chloramphenicol were 1 and 25 mg ml\(^{-1}\), respectively. Bacterial suspensions were incubated in a shaker at 37 °C for 5, 5, 10, 25 and 50 mg ml\(^{-1}\) chloramphenicol, an inhibitor of ppGpp synthetase I activation. The sub-bacteriostatic and sublethal concentrations of chloramphenicol were 1 and 25 mg ml\(^{-1}\), respectively. Bacterial suspensions were incubated in a shaker at 37 °C for 9 h; the resulting cultured bacteria were heat-fixed, Gram stained and observed by phase-contrast or bright-field microscopy (Olympus; magnification ×1000).

Bacterial morphology within macrophages was assessed by using a modified method of the modification of the method of Kim et al. (2003). Infected macrophages were fixed in 4% periodate/lysine/parafomaldehyde/sucrose for 1 h at 37 °C. All antibody-probing steps were for 1 h at 37 °C. The samples were washed three times in PBS for 5 min and permeabilized at ~20 °C in methanol for 10 s. After incubating three times for 5 min with blocking buffer (2% goat serum in PBS), the samples were stained with anti-B. abortus polyclonal rabbit serum and FITC-conjugated goat anti-rabbit IgG in blocking buffer, placed in mounting medium and visualized by fluorescence microscopy.

**Measurement of MIC.** MICs were measured by using a modified method described previously (Greenway & England, 1999; Mortensen et al., 1986). Antibiotics or biocides were serially diluted twofold in test tubes containing 2 ml final volume Brucella broth, such that the final antibiotic or biocide concentration was reduced.
from 400 to 0.01 μg ml\(^{-1}\). Each tube was inoculated with \(B.\) abortus wild-type, the spo\(T\) mutant and the complemented strain (all three previously grown in Brucella broth) to the same OD\(_{600}\). The tubes were incubated at 37°C for 24 h and OD\(_{600}\) of each culture was measured. The MIC of each antimicrobial agent was the concentration that just prevented growth.

**Cell culture.** Bone-marrow-derived macrophages from female BALB/c mice were prepared as described previously (Kim *et al.*, 2003). After culturing in L-cell-conditioned medium, the macrophages were replated for use by placing in PBS on ice for 5–10 min, harvesting by centrifugation and resuspending in RPMI-1640 (Sigma) containing 10% fetal bovine serum (FBS). The macrophages were seeded at 2–3 \(\times\) 10\(^5\) in each well in 24-well tissue culture plates 1 day before infection.

**Efficiency of bacterial uptake and growth within macrophages.** Bacterial infection and intracellular survival assays were done by using a modification of the method of Kim *et al.* (2003). \(B.\) abortus strains were deposited onto mouse bone-marrow-derived macrophages grown on 24-well microtitre plates filled with RPMI-1640 with 10% FBS at room temperature. To measure bacterial uptake efficiency after 0 and 30 min incubation at 37°C, macrophages were washed once with RPMI-1640 and incubated with RPMI-1640 containing gentamicin (30 μg ml\(^{-1}\)) for 30 min. Macrophages were washed three times with PBS and lysed with distilled water. The c.f.u. value was determined by serial dilutions on Brucella plates. To measure intracellular growth efficiency, the infected macrophages were incubated at 37°C for 30 min, washed once with RPMI-1640 and incubated with RPMI-1640 containing gentamicin (30 μg ml\(^{-1}\)) for 2, 24 and 48 h. Cell washing, lysis and plating procedures were the same as for the bacterial uptake efficiency assay. The percentage protection was determined by dividing the number of bacteria surviving by the number in the infectious inoculum.

**LAMP-1 staining.** This was done by the method of Kim *et al.* (2003). Bacterial infection, fixation and permeabilization were performed as described above (Morphology). After incubating three times for 5 min with blocking buffer (2% goat serum in PBS), the samples were stained with anti-LAMP-1 rat monoclonal antibody 1D4B diluted 1:100 in blocking buffer. After washing three times for 5 min in blocking buffer, the samples were stained with Texas red-goat anti-rat IgG. They were then stained with anti-\(B.\) abortus polyclonal rabbit serum and FITC-conjugated goat anti-rabbit IgG in blocking buffer, placed in mounting medium and visualized by fluorescence microscopy. One hundred bacteria within macrophages were selected randomly and LAMP-1 acquisition was determined.

**Bacterial adherence.** Bacterial adherence was measured using the method of Kim *et al.* (2003). Prior to bacterial infection, macrophages were incubated with RPMI-1640 containing cytochalasin D (500 μg ml\(^{-1}\)) for 40 min at 37°C, and bacterial infection, fixation, staining and microscopy were performed as described above for bacterial detection. One hundred macrophages were selected randomly and adherent bacteria were counted.

**Labelling bacterial surface proteins.** To examine the modification of outer membrane proteins of mutants, each 1 ml of cultured bacteria was harvested by centrifugation at 5000 r.p.m., washed twice and suspended in 200 μl PBS. Ten microlitres 1% Sulfo-NHS-Biotin (Pierce) was added, then the sample was placed in ice for 2 min and washed once with PBS. Each sample was mixed with 2 x SDS-gel loading buffer and incubated at 100°C for 5 min before loading. After electrophoresis, the fractioned proteins were electro-transferred onto nitrocellulose membranes (Schleicher & Schuell) and stained with Western blotting reagents (Amersham Pharmacia Biotech).

**Comparative growth of the mutants under stress conditions.** Bacterial growth in minimal or rich medium was performed by using a modification of the method of Kim *et al.* (2004). Cells (2–3 \(\times\) 10\(^9\) c.f.u.) of \(B.\) abortus wild-type, the spo\(T\) mutant and the complemented strain were pelleted at 5000 r.p.m. for 15 min, resuspended to 2–3 \(\times\) 10\(^7\) c.f.u. in RPMI-1640 with or without 10% FBS or Brucella broth, and incubated at 37°C for 0, 6, 24 and 48 h with shaking. Equal portions were withdrawn from each culture, serially diluted with PBS, and spread on the surface of Brucella plates to count the number of viable cells at intervals of up to 48 h.

Acid resistance was measured by using a modification of the method of Porte *et al.* (1999). Bacteria (2–3 \(\times\) 10\(^7\) c.f.u.) were resuspended in PBS at pH 4, which was adjusted by adding 1 M HCl, incubated for 0 and 60 min at 37°C, and equal aliquots of each sample were spread on the surface of Brucella plates. The percentage survival was calculated by dividing the number of bacteria surviving under stress conditions by the number surviving before the stress conditions.

To assess the relationship between bacterial survival and phagosome acidification in macrophages, experiments were performed as described previously (Porte *et al.*, 1999). Bafilomycin A1 (100 nM, Sigma), a vacuolar-pH-neutralizing reagent, was added to macrophages at 0 or 1 h post-infection and the number of viable bacteria was determined after 3 and 7 h. Infected, untreated cells were also analysed. \(B.\) abortus was tested for possible toxic effects of bafilomycin A1 by exposure in Brucella broth for 8 h followed by viability assays. Macrophage viability was verified by trypan blue dye exclusion assay.

Nitric oxide (NO)-mediated bacterial killing was determined as previously described for \(B.\) suis (Gross *et al.*, 1998). A total of 2–3 \(\times\) 10\(^6\) viable \(B.\) abortus cells were suspended in 1 ml RPMI-1640 containing either 0.1 or 0.01 mM 3-morpholinosydnonimine hydrochloride (sin-1) (Sigma) as a source of peroxynitrite (ONOO\(^{-}\)). ONOO\(^{-}\) is generated by the reaction of \(\mathrm{O}_2^-\) with NO and plays an important role in both prokaryotic and eukaryotic cytostasis and cytotoxicity (Miller & Britigan, 1997). Bacterial culture in RPMI-1640 without sin-1 was also analysed. The bacterial suspension was incubated at 37°C for 8 h. After 0, 4 and 8 h of incubation, the numbers of viable bacteria in the medium supplemented with sin-1 and in controls were evaluated by c.f.u. determination.

**Virulence in mice.** Virulence was determined by quantifying bacterial survival in the spleen after 10 days. Six-week-old female BALB/c mice were infected intraperitoneally with approximately 10\(^6\) c.f.u. \(B.\) abortus in 0.1 ml saline. Groups of five mice were infected with each strain. At 10 days post-infection, the mice were sacrificed by decapitation and their spleens were removed, weighed and homogenized in saline. Tissue homogenates were serially diluted with PBS and plated on Brucella agar to count the number of c.f.u. in each spleen.

**RNA isolation and RT-PCR.** \(B.\) abortus has a stress response to acid pH stress (Teixeira-gomes *et al.*, 2000) and \(B.\) burgdorferi shows spo\(T\) expression during serum starvation (Concepcion & Nelson, 2003). To demonstrate spo\(T\) expression during pH stress or serum starvation, \(B.\) abortus wild-type and the spo\(T\) mutant were cultured in Brucella broth at 37°C for 24 h. Bacteria (2–3 \(\times\) 10\(^7\) c.f.u.) were resuspended in 1 ml Brucella broth, low-pH Brucella broth (pH 4.5) or RPMI-1640 and incubated at 37°C. Aliquots of 0-1 ml were taken at 0, 10 and 30 min after the start of incubation. Total RNA was isolated by using an RNA Purification Kit (Qiagen). Purified RNA samples were stored at −75°C until use. RNA was quantified by absorption at 260 nm by using an Ultraspec 4000 spectrophotometer (Amersham Pharmacia Biotech). RT-PCR was carried out by using a Superscript II kit (Invitrogen). The RT reactions were performed using primers SpoTR1 (5′-TCG TGG CAG GGC CAA CAA CCC GCA-3′) and SpoTR2 (5′-ACA
TCG GTC GAG GGC AGT TCC CCA-3'). Prior to reverse transcription, total RNA was treated with DNase (Toyobo) to eliminate residual DNA contamination. Total RNA was quantified and RT reactions were carried out for equal amounts of RNA (1 μg) from each sample.

RESULTS

Effects of disruption of the spoT gene in vitro and in vivo

To compare the morphology of B. abortus wild-type, the spoT mutant and the complemented strain, bacteria were Gram-stained and examined by light microscopy at × 1000 magnification. The spoT mutant had abnormal branching and swelling forms compared to B. abortus wild-type and the complemented strain (Fig. 1a–c). After sublethal chloramphenicol treatment (25 μg ml⁻¹), B. abortus wild-type showed abnormal branching and swelling forms compared to untreated B. abortus wild-type (Fig. 1a, d). B. abortus wild-type treated with sub-bacteriostatic chloramphenicol (1 μg ml⁻¹) showed the same morphology (data not shown). Wild-type and mutant bacteria within macrophages showed the same morphology as cultured cells (Fig. 1e, f).

The bacterial LPS phenotype was determined by assessing electrophoresis with silver staining. The spoT mutant had the same LPS profile as B. abortus wild-type and the complemented strain (data not shown). The bacterial phenotype was further verified by demonstrating the uptake of crystal violet by bacterial colonies (White & Wilson, 1951); all the strains showed the smooth phenotype (data not shown).

To determine the growth patterns in minimum or rich medium, B. abortus wild-type, the spoT mutant and the complemented strain were cultured in RPMI-1640, RPMI-1640 containing 10% FBS or Brucella broth. B. abortus wild-type and the complemented strain showed almost the same growth yield but the spoT mutant grew in neither RPMI-1640 nor RPMI-1640 containing 10% FBS (Fig. 2a, b). In RPMI-1640, growth of B. abortus wild-type and the complemented strain increased until 24 h and then entered stationary phase until 48 h (Fig. 2a). In RPMI-1640 containing 10% FBS, growth of B. abortus wild-type and the complemented strain increased until 48 h (Fig. 2b). The spoT mutant showed decreased growth until 48 h in RPMI-1640 and stationary growth until 48 h in RPMI-1640 containing 10% FBS (Fig. 2a, b). This was probably because the spoT or relA mutant could not adapt to the nutrient-starved stress conditions for bacterial survival (Concepcion & Nelson, 2003; Hammer & Swanson, 1999), suggesting that the B. abortus spoT mutant may need a more complex nutrient in addition to RPMI-1640 containing 10% FBS. In Brucella broth, we found that B. abortus wild-type, the spoT mutant and the complemented strain showed the same growth pattern, including lag phase, generation time and decreasing cell density at the transition into the stationary phase, by using standard semi-logarithmic plots of growth curves (data not shown). The three strains had a similar colony size on Brucella plate medium (data not shown).

Fig. 1. Morphology of B. abortus wild-type (a), spoT mutant (b), complemented strain (c) and chloramphenicol-treated (25 μg ml⁻¹) wild-type strain (d) observed by light microscopy (× 1000). Morphology of B. abortus within macrophages, wild-type (e) and spoT mutant (f), observed by fluorescence microscopy (× 1000).
We examined the internalization and intracellular replication of *Brucella* strains in mouse bone-marrow-derived macrophages. The results showed that the *spoT* mutant had the same ability to internalize as *B. abortus* wild-type and the complemented strain at 0 min, but a lower rate of internalization at 30 min (Fig. 2c). In the case of bacterial replication within macrophages, *spoT* showed a lower rate of intracellular replication than *B. abortus* wild-type and the complemented strain (Fig. 2d).

To investigate whether adherence of *B. abortus* wild-type, the *spoT* mutant and the complemented strain to macrophages contributes to bacterial internalization ability, the initial adherence of the strains to macrophages was analysed. *B. abortus* wild-type, the *spoT* mutant and the complemented strain showed 48 ± 4, 196 ± 38 and 56 ± 14 bacteria per 100 macrophages, respectively. Adherence of the *spoT* mutant was four times higher than that of *B. abortus* wild-type and the complemented strain.

Biotin-labelled bacterial surface proteins of the three strains were analysed by immunoblotting. The protein band pattern of the *spoT* mutant changed markedly more than *B. abortus* wild-type and the complemented strain (Fig. 3). Phagosomes containing virulent *B. abortus* are reluctant to fuse with lysosomes, whereas those with dead *B. abortus* colocalize with endocytic compartments in the early stage of infection in macrophages (Arenas et al., 2000). To test the ability of *B. abortus* to target properly within macrophages early in infection, interaction of the mutants with the endocytic pathway was quantified by immunofluorescence localization of LAMP-1, a membrane protein of late endosomes and lysosomes (Chen et al., 1988). From these
Interestingly, the MICs of the wild-type and the complemented strain did not differ for the RNA synthesis inhibitor streptomycin, the DNA gyrase inhibitor ciprofloxacin or polymyxin B, which disrupts the structure and permeability of the plasma membrane.

**NO- and pH-mediated bacterial killing**

NO has an important role in macrophage-mediated killing of brucellae and also in direct bactericidal activity, via NO radicals (Gross et al., 1998). The ability of NO-generating agents to kill viable *B. abortus* was examined. The three strains were cultured in RPMI-1640 for 4 or 8 h in the presence of different concentrations of sin-1, a generator of NO radicals. In RPMI-1640 or RPMI-1640 containing 0.01 mM sin-1, *B. abortus* wild-type and the complemented strain survived and proliferated slightly but the *spoT* mutant showed a stationary phase or decreased growth. (Table 1). In RPMI-1640 containing 0.1 mM sin-1, all strains showed decreased growth (Table 1).

*Brucella* species can survive but not multiply at pH 4 (Köhler et al., 2002a; Kulakov et al., 1997). To assess acid resistance in macrophages, 100 nM bafilomycin A1, a vacuolar-pH-neutralizing reagent, was added to macrophages at 0 or 1 h post-infection, and viable intracellular bacteria were enumerated following recovery at 4 and 8 h post-infection. At both time points the number of viable *B. abortus* wild-type and complemented strain in macrophages was about twofold higher than in bafilomycin A1-treated macrophages (Table 1). In contrast, the number of viable *spoT* mutants in macrophages was similar or twofold lower than in bafilomycin A1-treated macrophages (Table 1). Bafilomycin A1 has been described as a specific inhibitor of vacuolar ATPases (Porte et al., 1999), but it had no toxic effect on *B. abortus* in Brucella broth containing 100 nM bafilomycin A1 for 8 h (data not shown).

Porte et al. (1999) reported that early acidification of phagosomes is important for replication of *B. suis* within macrophages and the pH inside phagosomes containing live bacteria varies between 4.2 and 4.5 and remains at this level until at least 5 h post-infection. To assess acid resistance *in vitro*, the three strains were suspended in PBS at pH 4 and incubated for 60 min at 37 °C. The survival rates of *B. abortus* wild-type and the complemented strain were 51 ± 8% and 46 ± 7%, respectively. However, the survival rate of the *spoT* mutant was only 2%.

The pH of the vacuoles containing live *B. suis* is rapidly acidified to a mean value of 4.5 after 1 h infection. After treatment with bafilomycin A1, the intraphagosomal pH fluctuated between 6.5 and 7, and this neutralization resulted in enhanced reduction of viable intracellular *B. suis*, suggesting that phagosomes with *B. suis* acidify rapidly after infection, and that this early acidification is essential for replication of the bacteria within the macrophage (Porte et al., 1999). These data indicate that the observed enhanced decrease in survival and replication of *B. abortus* wild-type and the complemented strain may be linked to the loss of...
acidification in the compartment containing bacteria. In contrast, the spoT mutant might have a lower resistance to early acidification, thereby showing an increased survival within early acidification inhibited macrophages.

**Reduced virulence of the B. abortus the spoT mutant in mice**

To ascertain whether the defective internalization and intracellular replication within macrophages of the spoT mutant correlates with an inability to establish infection in the host, we experimentally infected mice with *B. abortus* wild-type, the *spoT* mutant and the complemented strain. The numbers of viable bacteria recovered from the spleens of mice infected with the wild-type strain and the complemented strain at 10 days after infection were \(6 \times 2 \times 10^8 \pm 0.3 \times 10^4\) and \(5 \times 8 \times 10^5 \pm 0.4 \times 10^4\) c.f.u. per spleen, respectively. Fewer bacteria \((2 \times 10^5 \pm 0.6 \times 10^5\) c.f.u. per spleen) were recovered from the spleens of the *spoT* mutant-infected mice. *B. abortus* wild-type and the complemented strain induced splenomegaly as a consequence of an inflammatory response, but the *spoT* mutant induced a lesser response compared to *B. abortus* wild-type strain.

**RT-PCR analysis of spoT expression during stress response**

To establish whether RT-PCR amplification can detect *spoT* mRNA from around 10⁶ cells incubated under conditions known to induce stress response (Porte et al., 1999; Teixeiragomes et al., 2000), RNA extracted from *B. abortus* wild-type and the *spoT* mutant cells incubated in enriched medium (Brucella broth and Brucella broth at pH 4.5) or minimal medium (RPMI-1640) for 0–30 min was used as a template for RT-PCR. The primers SpoTR1 and SpoTR2 were designed to detect a 587 bp fragment. *spoT* expression was not detected in the *spoT* mutant (Fig. 4). In *B. abortus* wild-type, *spoT* expression was detected at 0, 10 and 30 min in both Brucella broth and RPMI-1640, and increased expression was detected after 30 min incubation with acid stress in Brucella broth at pH 4.5 (Fig. 4). Incubation in low-pH medium resulted in increased *spoT* expression consistent with acid stress, suggesting that *spoT* expression of *B. abortus* may have been affected more significantly by acid stress than other factors. This experiment was repeated twice with identical results.

**DISCUSSION**

A number of recent studies have reported the virulence genes of *Brucella* (Drazek et al., 1995; Endley et al., 2001; Eskra et al., 2001; Foulongne et al., 1999). The VirB type IV secretion system of *Brucella* has been identified recently (O‘Callaghan et al., 1999; Sieira et al., 2000). This operon is composed of 13 ORFs that share homology with other bacterial type IV secretion systems involved in the intracellular trafficking of pathogens. Mutants affected in these ORFs were not able to replicate and survive within phagocytes (Sieira et al., 2000; Watarai et al., 2002b, 2003). Thus, the VirB proteins of *B. abortus* are thought to be constituents of the secretion apparatus. However, the mechanism of virulence of *Brucella* species is not yet fully understood. In this study, we have examined the role of the *B. abortus spoT* gene in intramacrophagic replication. Primm et al. (2000) reported that all Gram-negative bacteria have separate *relA* and *spoT* genes encoding ppGpp synthetase and hydrodase, respectively. From the GenBank database, however, *B. suis* and *M. tuberculosis* appear to have just one *relA/spoT* homologue (Wells & Long, 2002; Cole et al., 1998). The *spoT* gene encodes a bifunctional enzyme capable of degrading and synthesizing (p)pGpp. It is thought that RelA, or ppGpp synthetase I, is associated with

**Table 1. Bacterial survival under NO- and pH-mediated killing conditions**

Results are the mean of three determinations ± SD, expressed as a percentage of the number of viable bacteria at 0 h, considered to be 100 %. Data from the SpoT− mutant were statistically different compared to wild-type and the SpoT+ complemented strain: ***, *P<0.001 at 8 h incubation; *, *P<0.01 at 4 h incubation.

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<tr>
<th>Incubation time (h):</th>
<th>Wild-type</th>
<th>SpoT−</th>
<th>SpoT+</th>
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<td></td>
<td>4</td>
<td>8</td>
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<tr>
<td>NO-mediated killing†</td>
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<tr>
<td>RPMI 1640</td>
<td>104 ± 8</td>
<td>122 ± 9</td>
<td>101 ± 5</td>
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<tr>
<td>RPMI 1640 + 0.01 mM sin-1**</td>
<td>92 ± 8</td>
<td>102 ± 9</td>
<td>86 ± 7</td>
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<td>RPMI 1640 + 0.1 mM sin-1</td>
<td>90 ± 6</td>
<td>64 ± 5</td>
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<td>pH-mediated killing‡</td>
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<tr>
<td>Macrophage</td>
<td>72 ± 4</td>
<td>51 ± 6</td>
<td>34 ± 8</td>
</tr>
<tr>
<td>Macrophage + bafilomycin A₁ (0 h)*</td>
<td>41 ± 7</td>
<td>12 ± 3</td>
<td>70 ± 8</td>
</tr>
<tr>
<td>Macrophage + bafilomycin A₁ (1 h)</td>
<td>37 ± 6</td>
<td>18 ± 4</td>
<td>39 ± 7</td>
</tr>
</tbody>
</table>

†Bacteria were incubated in RPMI-1640 with or without sin-1, as a source of peroxynitrite.
‡After infection, macrophages were treated with 100 nM bafilomycin A₁, a vacuolar-pH-neutralizing agent, at 0 h or 1 h post-infection.
ribosomes and is activated by binding uncharged tRNA to the ribosome upon depletion of amino acids or nutrient starvation, designated the stringent response (Mitchison, 1992). The stringent response is a broad transcriptional programme that mediates prokaryotic adaptation to survival under conditions of starvation (Prim et al., 1992). The stringent response is a broad transcriptional programme that mediates prokaryotic adaptation to survival under conditions of starvation (Prim et al., 1992). Thus, the relA and spoT genes may have important roles for bacterial survival under various conditions.

We found that the B. abortus spoT mutant has abnormal branching and swelling forms and higher antibiotic sensitivity than wild-type. In M. smegmatis, carbon starvation results in activation of RelA, increased ppGpp concentration and morphological change from elongated rods to coccii (Ojha et al., 2000). Bacterial morphology is controlled by various factors such as peptidoglycan synthesis, penicillin-binding proteins (PBPs), FtsZ and ppGpp. Peptidoglycan synthesis is necessary not only for the maintenance of cell morphology but also for survival. The final steps of peptidoglycan synthesis are catalysed by PBPs (Waxman & Strorninger, 1983). PBPs are membrane-bound proteins that are present in all peptidoglycan-containing bacteria and covalently bind β-lactam antibiotics. These proteins are involved in the final steps of murein sacculus synthesis and perform specific functions related to elongation, septation and maintenance of cell shape. Inactivation of PBPs, especially PBP2, results in inhibited division and β-lactam antibiotic resistance, and the former is relieved by FtsZ overexpression or increasing the ppGpp pool in E. coli (Vinella et al., 1993). The inhibition of peptidoglycan synthesis and development of penicillin tolerance are both directly associated with accumulation of ppGpp (Rodionov & Ishiguro, 1995). Filamentation of Pseudomonas aeruginosa is caused by a response to the change in chloramphenicol concentration (Steel et al., 2004). Chloramphenicol is a highly potent inhibitor of bacterial protein biosynthesis. In E. coli, ppGpp accumulation is prevented by treatment with chloramphenicol, an inhibitor of ppGpp synthetase I activation (Rodionov & Ishiguro, 1995). Although we were not able to establish the relationship between chloramphenicol concentration and ppGpp synthetase inhibition, sublethal and sub-bacteriostatic chloramphenicol were shown to induce morphological change, suggesting that ppGpp synthetase of B. abortus is inhibited by both sublethal and sub-bacteriostatic chloramphenicol. These results also suggest that the morphological change of the B. abortus spoT mutant may be affected by inhibition of ppGpp synthesis, and ppGpp may activate the transcription of one or more genes including β-lactamase production, and affect an as yet uncharacterized regulatory mechanism related to cell morphology.

Greenway & England (1999) reported that E. coli able to synthesise ppGpp, either in a RelA- or SpoT-dependent manner, possesses greater resistance to antimicrobial compounds compared with E. coli mutants that cannot produce ppGpp. E. coli mutants showed a higher sensitivity, as demonstrated by MIC values, for β-lactam antibiotics, polymyxin, gentamicin, 1,2-benzisothiazolin-3-one (BIT) and bronopol. In this study, a B. abortus spoT mutant showed higher sensitivity for several antibiotics, indicating that B. abortus spoT can influence the cell’s resistance to antimicrobial agents. Narayshkina et al. (2001) and Barker et al. (2001) reported that the binding sites of rifampicin and ppGpp are RNA polymerase subunit β and β’. Jin et al. (1988) reported that some of the E. coli mutants that show a stringent response in the continued absence of ppGpp have been obtained as rifampicin-resistant isolates, and have been
shown to harbour a mutation in the RNA polymerase β-subunit. *B. abortus* wild-type and spoT mutant, however, show the same MIC value for rifampicin, suggesting that the interaction site of ppGpp and RNA polymerase may differ from that of rifampicin in *B. abortus*.

The relA or spoT mutants of *Borrelia burgdorferi* (Concepcion & Nelson, 2003), *E. coli* (Yang & Edward, 2003), *S. meliloti* (Wells & Long, 2003), *L. pneumophila* (Hammer & Swanson, 1999) and *M. tuberculosis* (Primm et al., 2000) have a lower resistance than their parent strains under stress conditions. In the case of *Listeria monocytogenes*, the relA mutant is defective in growth after attachment to an inert surface, cannot synthesize (p)ppGpp in response to nutritional starvation and is avirulent in murine models of infection. *B. abortus* mutants with rough LPS are more adhesive and enter Vero cells in greater numbers than the wild-type (Detileux et al., 1990). The question is raised why the *B. abortus* spoT mutant showed the same LPS profiles but a fourfold higher adherence to macrophages than wild-type. In many cases, adherence is mediated by one or more adhesins that can act simultaneously or in distinct steps during the infection process (Finlay & Falkow, 1997). Adhesins in the form of pili or outer membrane proteins may have important roles for bacterial adherence and mediate direct or indirect binding to host cells. For example, FimZ of *Salmonella enterica* serovar Typhimurium (Clegg & Hughes, 2002), FbpA of *Listeria monocytogenes* (Dramsi et al., 2004), FbsA of *Streptococcus agalactiae* (Schubert et al., 2004), FnBPs of *Staphylococcus aureus* (Brouillette et al., 2003), glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus suis* (Brassard et al., 2004), type IV pili of Gram-negative bacteria (Winther-Larsen & Koomey, 2002), and GspB and Hsa of *Streptococcus gordonii* (Bensing et al., 2004) are related to adherence to host cells. Analysis of the genome sequence of *Brucella* has revealed various genes encoding putative adhesion and invasion mechanisms involved in attachment and actin recruitment in cells, yet no fimbrial or non-fimbrial adhesins has been described in the genus *Brucella* (DelVecchio et al., 2002). As shown in this study, the surface proteins of the spoT mutant are different from wild-type. The alteration of the surface proteins of brucelae, not fimbriae but outer membrane proteins, may affect adherence to macrophages.

Several relA or spoT homologues have been cloned and characterized (Avarbock et al., 1999; Flärdh et al., 1994; Martinez-Costa et al., 1998; Mechold et al., 1996; Ostling et al., 1996; van der Biezen et al., 2000; Wendrich & Marahiel, 1997; Xiao et al., 1991). The induction of relA or spoT homologues is affected by various factors. For example, relA and spoT homologues of *Bacillus stearothermophilus*, *Bacillus subtilis*, *Streptococcus pyogenes* and *Streptococcus rattus* are induced only under conditions of amino acid depletion (Concepcion & Nelson, 2003). The stringent responses of *E. coli* or other bacteria are activated by starvation for amino acids, glucose and fatty acids (Primm et al., 2000). In contrast, the relA or spoT homologues of *M. smegmatis*, *M. tuberculosis* and *Streptococcus equisimilis* only respond to carbon starvation (Concepcion & Nelson, 2003). Porte et al. (1999) reported that the stress response of *Brucella* spp. is induced by low pH. Phagosomes with *B. suis* acidify rapidly after infection and early acidification is essential for replication within macrophages. The pH inside phagosomes containing live bacteria varies between 4·2 and 4·5 and remains at this level until at least 5 h post-infection. The pH of the vacuoles containing live *B. suis* is rapidly acidified to a mean value of 4·5 ± 1 h after infection. If the early acidification of phagosomes is abolished, *B. suis* does not replicate within macrophages. Moreover, at least 15 proteins are induced in *B. melitensis* incubated with low-pH medium for 30 min (Teixeira-gomes et al., 2000). From our RT-PCR findings, the spoT gene was induced by low pH (4·5) rather than nutrient starvation. Our results suggest that resistance of phagosomes to early acidification and low pH is controlled by ppGpp, and induction of the *B. abortus* spoT gene is important for bacterial survival and replication within macrophages. We could not demonstrate expression of the spoT gene in various nutrient-starvation conditions due to the complex nutritional requirements for *B. abortus* growth. In addition to low pH, some conditions or nutritional factors, such as amino acid-, carbon-, vitamin-, fatty acid- or nitrogen-starved stringent responses, may induce the *B. abortus* spoT gene. Further experiments may be needed to fully understand the role of SpoT.

This study showed that the spoT mutant did not affect intracellular traffic, such as phagosome–lysosome fusion, after uptake by macrophages and was avirulent in mice. Furthermore, the spoT mutant showed a higher sensitivity to various stress conditions than the parental strain, suggesting that spoT may control the stringent response under stress conditions. *Brucella* spp. are facultative intracellular pathogens that can survive and multiply in professional and non-professional phagocytes. To replicate within phagocytes, *Brucella* spp. must have not only regulatory systems but also adaptive systems for avoiding intracellular killing and persisting under intracellular stress conditions. Thus, SpoT does not contribute to intracellular trafficking of *B. abortus*, but contributes to the use of nutrients or to adaptation of stress responses for intracellular growth and proliferation.

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**REFERENCES**


Roles of Brucella abortus SpoT


