Cytotoxicity of *Brucella* smooth strains for macrophages is mediated by increased secretion of the type IV secretion system

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Some *Brucella* rough mutants cause cytotoxicity that resembles oncosis and necrosis in macrophages. This cytotoxicity requires the type IV secretion system (T4SS). In rough mutants, the cell-surface O antigen is shortened and the T4SS structure is thus exposed on the surface. Cytotoxicity effector proteins can therefore be more easily secreted. This enhanced secretion of effector proteins might cause the increased levels of cytotoxicity observed. However, whether this cytotoxicity is unique to the rough mutant and is mediated by overexpression of the T4SS has not been definitively determined. To test this, in the present study, a *virB* inactivation mutant (BM Δ virB) and an overexpression strain (BM-VIR) of a smooth *Brucella melitensis* strain (BM) were constructed and their cytotoxicity for macrophages and intracellular survival capability were analysed and compared. Cytotoxicity was detected in macrophages infected with higher concentrations of strains BM or BM-VIR, but not in those infected with BM Δ virB. The quorum sensing signal molecule *N*-dodecanoyl-DL-homoserine lactone (C₁₂-HSL), a molecule that can inhibit expression of *virB*, inhibited the cytotoxicity of BM and BM-VIR, but not of BM Δ virB. These results indicated that overexpression of *virB* is responsible for *Brucella* cytotoxicity in macrophages. Transcription analysis showed that *virB* is regulated in a cell-density-dependent manner both in *in vitro* culture and during macrophage infection. When compared with BM, BM-VIR showed a reduced survival capacity in macrophages and mice, but both strains demonstrated similar resistance to *in vitro* stress conditions designed to simulate intracellular environments. Taken together, the cytotoxicity of *Brucella* for macrophages is probably mediated by increased secretion of effector proteins that results from overexpression of *virB* or an increase in the number of bacterial cells. The observation that both inactivation and overexpression of *virB* are detrimental for *Brucella* intracellular survival also indicated that the expression of *virB* is tightly regulated in a cell-density-dependent manner.

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INTRODUCTION

Brucella spp. are Gram-negative intracellular pathogens, belonging to the class *Alphaproteobacteria*, which live in close association with eukaryotic hosts. Bacteria of the genus *Brucella* are the aetiological agents of brucellosis, a worldwide zoonosis which affects a broad range of mammals and causes great economic losses. According to conventional taxonomy, three *Brucella* species, *Brucella*

melitensis, *Brucella abortus* and *Brucella suis*, can infect humans, causing a chronic, debilitating disease with severe, and sometimes fatal outcomes.

Brucella is well adapted to an intracellular lifestyle, being able to invade and survive in both phagocytic and non-phagocytic cells. Internalization of *Brucella* occurs through lipid-raft-mediated macropinocytosis (Kim *et al.*, 2002; Watarai *et al.*, 2002). After being taken up into the phagosome, *Brucella* is packaged into vacuoles called *Brucella*-containing vacuoles, which move in host cells by a specific intracellular trafficking mechanism that avoids fusion with lysosomes. *Brucella*-containing vacuoles fuse

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Abbreviations: C₁₂-HSL, *N*-dodecanoyl-DL-homoserine lactone; LDH, lactate dehydrogenase; T4SS, type IV secretion system.

with the endoplasmic reticulum membrane and replicate in vesicles associated with the endoplasmic reticulum (Celli *et al.*, 2003, 2005; Gorvel & Moreno, 2002; Pizarro-Cerda *et al.*, 1998; Starr *et al.*, 2008). The type IV secretion system (T4SS), encoded by the *virB* operon, plays a central role in *Brucella* intracellular survival and replication (Foulongne *et al.*, 2000; Hong *et al.*, 2000; Lestrade *et al.*, 2000; O'Callaghan *et al.*, 1999). *virB* mutants are unable to persist within host cells and are therefore unable to cause chronic infection in mice (Alvarez-Martinez *et al.*, 2001; Boschioli *et al.*, 2002a, b; Commerci *et al.*, 2001; O'Callaghan *et al.*, 1999; Sieira *et al.*, 2000).

The function of the T4SS in bacterial intracellular survival has been shown to be mediated by effector proteins that are secreted into host cells. Other studies have suggested that the *virB* operon of *Brucella* mediates secretion of unknown effectors which are required for establishment of an intracellular compartment favourable for *Brucella* survival and replication (Boschioli *et al.*, 2002b). This is supported by data from co-infection experiments which show that wild-type strains of *B. abortus* or *B. suis* are able to rescue *virB* mutants in macrophages, and that this rescue requires bacterial protein synthesis (Nijskens *et al.*, 2008). de Jong *et al.* (2008) have recently demonstrated that two members of the *vjbR* regulon, VceA and VceC, are translocated into macrophages by the T4SS, providing the first direct evidence for the secretion of effector proteins by the *Brucella* T4SS.

The cytotoxicity of *Brucella* for macrophages was observed more than 40 years ago (Freeman & Rumack, 1964). It has been shown more recently that rough mutants are more cytotoxic to macrophages than smooth strains (Pei & Ficht, 2004), and these authors predicted that rough mutants secrete more toxin molecules into host cells. The cytotoxicity of rough mutants for macrophages resembles oncosis and necrosis, but not apoptosis, and requires bacterial protein synthesis (Pei & Ficht, 2004; Pei *et al.*, 2006). It is presumed that an O antigen expressed on the bacterial cell surface of smooth strains may affect effector secretion of T4SS. This has been observed for type III secretion systems in *Shigella* and *Yersinia* (Mota *et al.*, 2005; West *et al.*, 2005). In *Shigella*, shortening of the lipopolysaccharide molecule by O-antigen glucosylation enhances the secretion of effector proteins and the function of the type III secretion system. A minimal 'needle' length is required for efficient functioning of the *Yersinia enterocolitica* type III secretion system. In smooth *Brucella* strains, the T4SS apparatus might be partially shielded by the O antigen, and therefore the effector proteins are secreted in a tightly controlled way. In contrast, for the rough *Brucella* strains, the O antigen is lacking or is shortened, the T4SS structure is more exposed on the surface and the effector proteins can be easily secreted. The enhanced secretion of effector proteins might cause the increased cell death (Pei & Ficht, 2004; Pei *et al.*, 2006, 2008).

Although several reports have shown that the cytotoxicity of the rough mutant is mediated by the increased secretion

of effector proteins via the T4SS, there is no direct evidence to show that this cytotoxicity is a unique rough strain phenotype mediated by the T4SS. Based on previous studies, it can be predicted that the T4SS is tightly regulated in smooth strains during its intracellular survival in host cells. To test this in the present study, a *virB* inactivation mutant (BM Δ virB) and a *virB* overexpression strain (BM-VIR) of a smooth strain of *B. melitensis* were constructed. Their cytotoxicity for macrophages and transcription of *virB* was analysed and compared. The results showed that overexpression of *virB* in *B. melitensis* increased the cytotoxicity of smooth *Brucella* for macrophages, and in contrast, inactivation of *virB* prevented the cytotoxic effects. Transcription of *virB* is growth-phase-dependent *in vitro* and infection-stage-dependent during macrophage infection. These results indicate that the cytotoxicity of smooth strains is mediated by *virB*, and that expression of *virB* in smooth strains is tightly regulated during macrophage infection.

METHODS

Bacterial strains, plasmids, primers and reagents. The *virB* inactivation mutant BM Δ virB and *virB* overexpression strain BM-VIR were constructed as described below. *Brucella* strains were grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA). *Escherichia coli* DH5 α was grown in Luria-Bertani (LB) medium. When necessary, ampicillin (Ap; 100 μ g ml⁻¹) and kanamycin (Km; 50 μ g ml⁻¹) were added to the cultures. Plasmid pBBR1MCS-4, a broad-host-range plasmid capable of replicating in *Brucella*, was provided by Professor Kenneth M. Peterson (Kovach *et al.*, 1995). The primers used in this study are listed in Table 1. The CytoTox 96 non-radioactive cytotoxicity assay kit was purchased from Promega. The autoinducer *N*-dodecanoyl-DL-homoserine lactone (C₁₂-HSL) was obtained from Fluka.

Construction of the *virB* inactivation mutant BM Δ virB. BM Δ virB was constructed from *B. melitensis* 55009 (BM), a strain derived from 16M. The 1.7 kb *sacB* gene was released from pKOBEG-*SacB* by *Nde*I and cloned into the *Nde*I site of pUC19 to give pUC19-*SacB*. This allowed positive selection of double cross-over events by selection using sucrose resistance (Suc^R). A 504 bp region just upstream of *virB1* was deleted as follows. The N-terminal fragment was amplified with primers IVB-N-F and IVB-N-R, and the C-terminal fragment with IVB-C-F and IVB-C-R. The two PCR products were purified, equally mixed, and then amplified with primers IVB-N-F and IVB-C-R to generate IVB that was cloned into pUC19-*SacB* to generate pUCS*SacB*-IVB. Competent BM was transformed with pUCS*SacB*-IVB and the *virB* mutant BM Δ virB was isolated by its Ap^S Suc^R phenotype. The unmarked deletion mutant was further confirmed by PCR and DNA sequencing.

Construction of the *virB* overexpression strain BM-VIR. The *virB* overexpression strain was constructed as follows. A 1087 bp fragment located downstream of the *virB* operon was amplified with IVGT-F and IVGT-R and cloned into pUC19 to generate the targeting plasmid pUC19-IVT. This plasmid was then transformed into competent BM, resulting in targeted strain BM-IVT. Genomic DNA was extracted from BM-IVT and digested with *Eco*T22I, and then purified, self-ligated and transformed into DH5 α to rescue plasmid pUC19-IVGT. The rescued *virB* operon in pUC19-IVGT was confirmed by PCR with primers for *virB2*, *virB5* and *virB8*, and the PCR products were sequenced for sequence confirmation. The two termini of the rescued

Table 1. Primers used in this study

Primer	Sequence (5'–3')
IVB-N-F	CTGCGAAGCTTGCAAATTCCTGTCGGTTTCG
IVB-N-R	GAGGACAAGGAATGGCACCACGACGCAGGACGAAAGGAC
IVB-C-F	GTCCTTTCCGTCCTGCGTCGTGGTGCCATTCTTGTCTC
IVB-C-R	CGACCGGAATTCGAAGCCGCCCGTAAAGTTGC
IVGT-F	ACGTCGGATCCGAATTCACAGGCATTATCCGCTCGTC
IVGT-R	AGTCGAAGCTTTCTAGAAGCATAGCCAGTAGGTCCAG
<i>virB2</i> -F	ATGAAAACCGCTTCCCCCAGC
<i>virB2</i> -R	CCTAAGCAGGTAAGAGGC
<i>virB5</i> -F	ATGAAGAAGATAATTCTCAGC
<i>virB5</i> -R	ATAGGCGGCTTCCAGTGC
<i>virB8</i> -F	ATGTTTGGACGCAAACAATCTC
<i>virB8</i> -R	TTGCACCACTCCCATTCTG
<i>virB1</i> -RT-F	AAGCAATCACGACAGCACAG
<i>virB1</i> -RT-R	CGGCGTAGTAACAGGAGAATG
<i>virB8</i> -RT-F	GGGCTTTCGGCACCATTAC
<i>virB8</i> -RT-R	AGCGTGTACCAGTCGTAGG
16S rRNA-RT-F	CACTGGACCATTACTGACGC
16S rRNA-RT-R	ACTAAGGGCGAGGGTTTCG

fragment were confirmed by sequencing with sequencing primers of pUC19. The *virB* operon was released from pUC19 by digestion with *Kpn*I and *Pst*I, and subcloned into pBBR1MCS-4 to generate pBBR1-IVGT, which was electroporated into strain BM, resulting in the overexpression strain BM-VIR.

Reverse transcription PCR (RT-PCR). For transcriptional analysis of *virB*, strains BM, BMΔ*virB* and BM-VIR were cultured in TSB to exponential phase ($OD_{600}=1.0$) and then subjected to RNA isolation. Total RNA was isolated with Trizol agent (Invitrogen) as recommended by the manufacturer. RNA samples were then treated with DNase I (Promega) to remove contaminating genomic DNA. RNA quantity and quality were analysed by using an ND-1000 Spectrophotometer (Nanodrop Technologies). cDNA was generated from total RNA by using a random hexamer primer, following the protocol of Superscript II reverse transcriptase (Invitrogen). 16S rRNA, which is constantly transcribed in bacteria, was chosen as an internal control. Different cDNA samples were amplified with primers for 16S rRNA. The cDNA samples were normalized by differential dilutions according to the quantity of 16S rRNA products. Then, *virB1* and *virB8* were amplified from normalized cDNA samples with specific primers. The PCR products were analysed on a 1% agarose gel and visualized by ethidium bromide staining.

Real-time quantitative RT-PCR (qRT-PCR). The primers used for qRT-PCR are listed in Table 1. Samples were run in triplicate and amplified in a 20 µl reaction containing 10 µl 2×SYBR Green I Master Mix (TaKaRa), 100 nM forward/reverse primer and 1 µl cDNA sample. Moreover, no-template controls were used as recommended. qRT-PCR was performed by using the Roche LightCycler 2.0 system. The reaction conditions were as follows: denaturation at 95 °C for 15 s, and then 50 cycles of amplification (95 °C for 5 s, and 60 °C for 20 s). The relative transcriptional level was determined as described by Livak & Schmittgen (2001): relative fold change (treatment/control) = $2^{-\Delta\Delta C_T}$, where ΔC_T (gene of interest) = C_T (gene of interest) – C_T (reference gene of same sample), and $\Delta\Delta C_T$ (gene of interest) = ΔC_T (treatment) – ΔC_T (control). The 16S rRNA gene, the expression of which is relatively constant in bacteria, was used as the reference gene, and bacteria grown in TSB7.0 were used as control.

Macrophage infection and intracellular survival assay. Murine macrophage-like J774A.1 cells were seeded in 24-well plates and infected with *B. melitensis* at an m.o.i. of 200, as described by Pei & Ficht (2004). To synchronize the infection, the infected plates were centrifuged at 200 g for 5 min at room temperature. Following 60 min incubation at 37 °C in an atmosphere containing 5% (v/v) CO₂, the cells were washed three times with PBS to remove extracellular bacteria. To assess intracellular growth of the bacteria, the cells were incubated at 37 °C in fresh DMEM supplemented with 100 µg Ap ml⁻¹ plus 50 µg Km ml⁻¹ (to kill extracellular bacteria) for 1 h, then the concentration of antibiotics was reduced to 50 or 25 µg ml⁻¹, respectively (time zero). At different time points post-infection (p.i.), the supernatant was discarded and cells were lysed with 0.1% (v/v) Triton X-100. The number of c.f.u. was obtained by plating serial dilutions of the lysates on TSA plates. All survival assays were performed in triplicate wells, and the results represent the means from at least three separate experiments. For transcription analysis during macrophage infection, at different time points p.i., total RNA was isolated with Trizol agent. RT-PCR and qRT-PCR analyses were carried out as described above. RNA of uninfected J774A.1 cells was isolated and used as a negative control.

Macrophage cytotoxicity assay. Cells cultured in 24-well plates were infected with *B. melitensis* strains at an m.o.i. of 20, 200 or 2000 as described above. The culture supernatant was collected at various time points p.i., and the level of lactate dehydrogenase (LDH) was determined by the Promega CytoTox 96 non-radioactive cytotoxicity assay, according to the manufacturer's instructions with minor modifications (Pei & Ficht, 2004). Cytopathic cell death is expressed as a percentage of maximum LDH release, i.e. $100 \times (OD_{490} \text{ of infected cells} - OD_{490} \text{ of uninfected cells}) / (OD_{490} \text{ of lysed uninfected cells} - OD_{490} \text{ of uninfected cells})$. The maximum release was determined following dissolution of cell monolayers using 1% Triton X-100. The assays were performed in triplicate wells, and the data represent the mean ± SD from at least three separate experiments.

Autoinducer C₁₂-HSL treatment of *Brucella*-infected cells. Macrophages cultured in 24-well plates were infected with *B. melitensis* strains at an m.o.i. of 200 as described above. The infected cells were treated with fresh media containing various concentrations

of C₁₂-HSL at 1 h p.i., and cytotoxicity was determined at 24 h p.i. using the LDH release assay.

Mouse virulence assay. Female 6-week-old BALB/c mice were inoculated intraperitoneally with 0.2 ml PBS containing approximately 5×10^7 c.f.u. of *B. melitensis*. Infected mice were held in microisolator cages in biosafety level 3 facilities. Bacterial counts in the spleen or liver were determined for five mice of each group at 3, 5, 7, 14 and 28 days p.i. Mice were euthanized by CO₂ asphyxiation and the spleens and livers were collected aseptically. Spleens and livers from individual animals were homogenized with 1 ml 0.1% (v/v) Triton X-100 in PBS, 10-fold serially diluted, and plated on TSA. Plates were incubated at 37 °C and the c.f.u. was counted 3 days later. The c.f.u. per spleen or per liver was transformed exponentially to normalize the distribution. Results were expressed as the mean $\pm$ SD. All animal experiments were approved by the Academy of Military Medical Science Laboratory Animal Care and Use Committee and conducted in accordance with institutional guidelines.

In vitro environmental stress. *B. melitensis* strains were grown in TSB to the early exponential phase ($OD_{600}=0.6$) at 37 °C, and then subjected to different *in vitro* stress treatments. The *in vitro* stress conditions were designed to simulate intracellular environments and were chosen as described by Teixeira-Gomes *et al.* (2000) with minor modifications. To determine the effect of oxidative stress, bacteria grown to exponential phase were incubated in 440 mM H₂O₂ for 30 min. For low pH stress, bacteria were incubated at 37 °C in TSB medium at pH 2.5 for 5 min. For high-temperature stress, bacterial cultures were transferred to pre-warmed 50 °C tubes and incubated at 50 °C for 60 min. After the treatment, cells were diluted and plated on TSB agar to determine the number of viable bacteria. All the results represent the means from at least three separate experiments. The relative transcription of *virB* under these conditions was quantified by qRT-PCR.

Growth curve and transcription analysis of *virB* in different growth phases. BM and BM-VIR were cultured in TSB for 24 h, and then diluted with TSB to an OD_{600} of 0.05 and cultured in a rotary shaker (250 r.p.m.) at 37 °C. Aliquots of cultures were taken at intervals of 2 h and cell density (OD_{600}) was recorded. Transcription of *virB* at different growth stages was analysed by qRT-PCR.

Statistical analysis. Statistical significance was determined using Student's *t*-test; a *P*-value of <0.05 was considered significant, and a *P*-value of <0.01 was considered greatly significant.

RESULTS

Construction of the *virB* inactivation strain BM Δ virB and overexpression strain BM-VIR

Eleven sequential ORFs encoding type IV secretion machinery make up the *virB* operon, making *virB* inactivation possible by promoter deletion. To construct an unmarked *virB* deletion mutant, the counter-selection gene *sacB* was first cloned into pUC19 to give pUC19-*sacB*. This plasmid was then used to construct the *virB* mutant as described in Methods. The unmarked deletion mutant BM Δ virB, which resulted from two rounds of cross-over, was obtained by its Ap^S Suc^R phenotype (data not shown). To construct the *virB* overexpression strain, *virB* was first cloned by plasmid rescue as described in Methods. PCR and DNA sequencing of the internal *virB* genes and the two

termini showed that *virB* was correctly cloned (data not shown). *virB* was then subcloned into pBBR1MCS-4 to yield complementary plasmid pBBR-IVGT, and confirmed by PCR and sequencing. This plasmid was transformed into strain BM and the *virB* overexpression strain BM-VIR was generated. To confirm the two strains, transcription of *virB* genes was quantified by RT-PCR. As shown in Fig. 1, the abundance of *virB1* and *virB8* in strain BM-VIR was higher than that in strain BM, and no transcripts for these genes were detected in strain BM Δ virB. This indicated that *virB* genes were overexpressed in BM-VIR and inactivated in BM Δ virB.

Smooth strain BM is cytotoxic for macrophages when infected at a high m.o.i.

Cytotoxicity is classically evaluated by the quantification of plasma membrane damage. LDH is a stable cytosolic enzyme, present in all cell types, and rapidly released into the cell culture medium upon damage of the plasma membrane. LDH, therefore, is the most widely used marker in cytotoxicity studies. To test whether the smooth strain BM is cytotoxic for macrophages, J774A.1 cells were infected with BM at m.o.i.s of 20, 200 and 2000, and LDH release was analysed. As shown in Fig. 2(a), at 12 h p.i., for macrophages infected with BM at an m.o.i. of 20, only $3.29 \pm 1.11\%$ LDH was released; however, for those infected at m.o.i.s of 200 and 2000, LDH release increased to 33.53 ± 1.67 and $60.30 \pm 2.36\%$, respectively ($P < 0.01$). This indicated that, at higher m.o.i., more LDH was released. The LDH release at 24 h was higher than that at 12 h p.i. for all m.o.i.s. However, when the infected cells were cultured for 48 h p.i., all macrophages showed high levels of LDH release and no great differences between those infected at different m.o.i.s were observed ($P > 0.05$), implying that at this time point, most of the infected cells

Fig. 1. RT-PCR verification of *virB* transcription in strains BM, BM Δ virB and BM-VIR. RNA was isolated from strains BM, BM Δ virB and BM-VIR and then reverse-transcribed into cDNA. cDNA samples of the three strains were normalized by the transcription level of 16S rRNA. Transcription of *virB* genes (*virB1* and *virB8*) was detected in BM and BM-VIR, but not in BM Δ virB. The transcription level of *virB* genes of BM-VIR was much higher than in BM.

Fig. 2. T4SS-dependent cytotoxicity of smooth strain for macrophage. (a) J774A.1 cells were infected with strain BM at m.o.i.s of 20 (white bars), 200 (black bars) and 2000 (grey bars). (b) J774A.1 cells were infected with strains BM (white bars) and BMΔvirB (black bars) at m.o.i.s of 20, 200 and 2000. (c) J774A.1 cells were infected with strains BM (white bars) and BM-VIR (black bars) at m.o.i.s of 20, 200 and 2000. The supernatant was collected at the indicated time points, and the LDH released by the infected cells was detected by using the CytoTox 96 non-radioactive cytotoxicity assay. The data shown are the means $\pm$ SD of a representative experiment that was repeated twice.

were dead because of the long period of incubation. These results implied that the smooth strain is cytotoxic for macrophages at higher levels of infection.

Cytotoxicity of the smooth strain for macrophages is *virB*-dependent

Previous studies indicated that rough mutants are cytotoxic for macrophages, and this is presumed to be the result of exposure of the T4SS on the surface. Subsequent experiments have shown that cytotoxicity is T4SS-dependent. To test whether the cytotoxicity of the smooth strain for macrophages is also related to *virB* expression, macrophages were infected with strains BM and BMΔvirB, and the release of LDH was quantified. When infected at an m.o.i. of 200, at 12 h p.i. 33.53 % of LDH was released in BM-infected macrophages; however, for cells infected with BMΔvirB, only 1.47 % LDH was released, a reduction of about 23-fold. Even when infected with BMΔvirB at an m.o.i. of 2000, the macrophages released only very low levels of LDH. The high level of LDH

released in macrophages infected with both BM and BMΔvirB at 48 h p.i. further indicated that LDH release after this time is an effect of the long incubation period. As shown in Fig. 2(b), all macrophages infected with BMΔvirB released very low levels of LDH and, furthermore, there was no m.o.i.-dependent increase in LDH ($P > 0.05$), in contrast to strain BM. These results indicated that the cytotoxicity of the smooth strain for macrophages is dependent on expression of *virB*.

Overexpression of *virB* in *Brucella* is cytopathic for macrophages at low m.o.i.

The experiments above indicate that when infected at higher levels of m.o.i., macrophages release higher levels of LDH, and when *virB* is inactivated, the release of LDH is greatly reduced. We presumed that at high m.o.i., plenty of *Brucella* cells reside in the macrophages and, therefore, large quantities of effector proteins are secreted into the host cells through the T4SS, disrupting signal networks and leading to the cytotoxicity. This is supported by the

observation that the *virB* mutant did not induce a high level of LDH release, even at high m.o.i. To further test whether LDH release is related to overexpression of *virB*, the *virB* overexpression strain BM-VIR was used to infect macrophages. As shown in Fig. 2(c), even at the lowest m.o.i. of 20, macrophages infected with strain BM-VIR released 7.8 and 36.9% LDH at 12 and 24 h p.i., respectively, three and nine times higher than the levels released in macrophages infected with strain BM (3.2 and 4.2% at 12 and 24 h p.i., respectively) at the two time points ($P < 0.01$), indicating that LDH release is significantly increased in macrophages infected with strain BM-VIR. When infected at an m.o.i. of 200, the level of LDH release in macrophages infected with strain BM-VIR at 12 h p.i. was 68.6%, twice that of macrophages infected with strain BM (33.5%) ($P < 0.01$). At 24 h p.i., LDH release for BM-infected macrophages was 44.3%, but for cells infected with strain BM-VIR it reached the plateau level of over 85%, implying that overexpression of *virB* shortened the time required to reach the LDH release plateau. However, when macrophages were infected with strain BM-VIR at an m.o.i. of 2000, the level of LDH release (60.6%) was comparable to that of cells infected with strain BM (60.2%) at 12 h p.i. ($P > 0.05$). These data indicate that the cytotoxicity of smooth strains is induced by overexpression of *virB*. Based on these results, it can be reasoned that cytotoxicity of wild-type strains for macrophages at high levels of m.o.i. might be mediated by overexpression of *virB*, for the number of bacterial cells in a single macrophage cell is increased, and higher levels of effector proteins are secreted in this cell.

C₁₂-HSL inhibits *Brucella* cytotoxicity for macrophages in a *virB*-dependent manner

Quorum sensing is a cell-density-dependent gene expression regulation mechanism, mediated by acyl homoserine lactone (AHL) or auto-inducer 2 (AI-2) signal molecules. The AHL-type molecule C₁₂-HSL has been identified in supernatants of *Brucella* cultures (Delrue *et al.*, 2005; Taminiau *et al.*, 2002). This molecule has been shown to inhibit expression of the *virB* operon. Subsequent experiments indicated that the quorum sensing regulator *vjbR* regulates expression of *virB* (Taminiau *et al.*, 2002). To determine whether C₁₂-HSL downregulates expression of *virB* and accordingly inhibits *virB*-dependent cytotoxicity of *Brucella*, macrophages were infected with strains BM, BMΔ*virB* or BM-VIR at an m.o.i. of 200 and then treated with different concentrations of C₁₂-HSL. Since the survival and replication of *Brucella* are not affected by C₁₂-HSL treatment, even at the highest concentrations, C₁₂-HSL does not appear to be bactericidal or bacteriostatic to *Brucella*, and therefore cytotoxicity effects could be considered to be mediated by C₁₂-HSL. As shown in Fig. 3, when C₁₂-HSL was added into the media of cells infected with strain BM, the cytotoxicity for macrophages was inhibited in a dose-dependent manner: the higher the concentration of C₁₂-HSL, the greater the inhibition of

Fig. 3. Inhibition of cytotoxicity by C₁₂-HSL in a *virB*-dependent manner. J774.A1 macrophages were infected with strains BM, BMΔ*virB* and BM-VIR at an m.o.i. of 200. C₁₂-HSL was added to the culture media at various concentrations (μg ml⁻¹: white bars, 0; black bars, 0.126; dark grey bars, 0.63; light grey bars, 1.26) and the release of LDH was measured at 24 h p.i. The data shown are the means ± SD of a representative experiment that was repeated twice.

cytotoxicity ($P < 0.01$). When the concentration of C₁₂-HSL was increased to 1.26 μg ml⁻¹, the level of cytotoxicity was comparable to that of BMΔ*virB*-infected macrophages, indicating that cytotoxicity was completely inhibited. For the *virB* mutant BMΔ*virB*, no or only trace cytotoxicity was observed, and therefore no inhibition was observed. For strain BM-VIR, cytotoxicity was also inhibited by C₁₂-HSL in a dose-dependent manner ($P < 0.01$). However, even when treated with the highest concentration of C₁₂-HSL, the level of LDH release (53.3%) was higher than that of untreated strain BM (37.4%), indicating that cytotoxicity could not be completely inhibited in BM-VIR. These results indicated that C₁₂-HSL inhibits *Brucella* cytotoxicity for macrophages in a *virB*-dependent manner. For the *virB* overexpression strain, the cytotoxicity could not be completely inhibited, probably because of the increased level of transcription of *virB*.

B. melitensis virB overexpression strain exhibits reduced survival in macrophages and mice

Intracellular bacteria have developed various strategies to evade elimination by host cells. On one hand, intracellular bacteria attempt to adapt to the hostile environments in the host cell. On the other hand, a number of bacteria, including *Brucella*, are found to inhibit host-cell apoptosis and death, which presumably promotes bacterial survival and replication in host cells (Gross *et al.*, 2000). T4SS is an essential determinant for *Brucella* intracellular survival. The results described above indicate that overexpression of *virB* in *Brucella* is cytotoxic for macrophages, and we therefore speculated that such overexpression of *virB* might be detrimental for *Brucella* intracellular survival.

To evaluate the effects of overexpression of *virB* on *Brucella* intracellular survival, J774.A1 cells were infected with strains BM-VIR, BMΔ*virB* and BM. When macrophages were infected with strain BM, about 90% of the invading bacteria were killed by the macrophages within the first 4 h (Fig. 4a). Thereafter, an increase in intracellular bacteria

Fig. 4. Survival of *virB* overexpression strain in macrophages (a) and mice (b, c). (a) J774.A1 cells were infected with strains BM (◆), BMΔ*virB* (■) and BM-VIR (▲) at an m.o.i. of 200:1. At the indicated time points p.i., cells were lysed and bacteria were enumerated by plating serial dilutions on TSA plates. (b, c) Five BALB/c mice were injected intraperitoneally with 5×10^7 c.f.u. BM (◆), BMΔ*virB* (■) and BM-VIR (▲) per group. Infected mice were killed by CO₂ asphyxiation at 3, 5, 7, 14 and 28 days p.i. Spleens and livers were aseptically removed and homogenized with 1 ml PBS. Bacteria cell number was counted by plating serial dilutions on TSA plates.

was observed, resulting from replication of the surviving bacteria. For strains BM-VIR and BMΔ*virB*, there was an identical decrease in live bacteria in the first 4 h p.i. However, after this time point, the two strains seemed to have a different fate from strain BM. The number of intracellular live bacteria of strain BMΔ*virB* progressively

decreased ($P < 0.01$), indicating a loss of survival capability in macrophages. For strain BM-VIR, the number of intracellular live bacteria did not increase sharply as it did with strain BM, indicating that although strain BM-VIR could survive in macrophages, there was a decreased level of intracellular survival. To further analyse the virulence of the *virB* overexpression strain, we compared the survival of strains BM, BMΔ*virB* and BM-VIR in BALB/c mice. Strain BM-VIR showed similar kinetics of infection in the spleen and liver to strain BM, with maximum colonization at 5 days p.i., decreasing slowly thereafter until the end of the experiment at 28 days p.i. However, the maximum level of colonization of strain BM-VIR was much lower than that of strain BM (Fig. 4b, c) ($P < 0.01$). Therefore, although strain BM-VIR can survive in macrophages and mouse spleen and liver cells, it exhibits a decreased capability to survive.

Overexpression of *virB* does not affect survival of *Brucella* under stress conditions

Overexpression of *virB* in *Brucella* is cytopathic for macrophages and decreases the survival capability of *Brucella* in macrophages and mouse spleen and liver cells. The reason for this is presumably that overexpression of *virB* leads to increased secretion of effector proteins and excessive interference with the signal network of host cell. To survive in host cells, in addition to interfering with host cell signals, bacteria need to adapt to the intracellular environment. To test whether overexpression of *virB* affects *Brucella* adaptation to the intracellular environment, the survival of strains BM, BMΔ*virB*, and BM-VIR under various stress conditions simulating intracellular environments was analysed and compared. The *virB* mutant BMΔ*virB* showed reduced survival under the stress conditions tested compared with strain BM ($P < 0.01$), implying a role for *virB* in the ability of *Brucella* to resist these stresses. However, compared to strain BM, the survival of strain BM-VIR under these conditions was not very different (Fig. 5) ($P > 0.05$). This indicates that expression of *virB* is important for the ability of *Brucella* to adapt to stress conditions and thus probably the intracellular environment, but overexpression of *virB* has little effect on this ability. These data indirectly imply that the cytotoxicity of the *virB*-overexpressing strain is mediated by an increased level of effector proteins.

virB in strain BM-VIR is highly transcribed during macrophage infection and under stress conditions

The above experiments showed that the *virB*-overexpression strain is more cytotoxic for macrophages, and this effect is most probably mediated by increased expression of *virB*. To test whether the *virB* is overexpressed in strain BM-VIR during host-cell infection and under stress conditions, the relative levels of transcription of *virB* were analysed by qRT-PCR. During macrophage infection, the

Fig. 5. Overexpression of *virB* had little effect on *Brucella* survival under stress conditions. Strains BM (white bars), BMΔ*virB* (black bars) and BM-VIR (grey bars) were first cultured in TSB to exponential phase and then subjected to the appropriate stress conditions as described in the text. After treatment, the surviving bacteria were enumerated by plating serial dilutions onto TSA plates. The data shown are the means $\pm$ SD of a representative experiment that was repeated twice.

transcription profile of *virB* in strain BM-VIR was identical to that of strain BM, being activated immediately post-infection, peaking at 12 h p.i. and then downregulated. However, the level of transcription of *virB* in strain BM-VIR was much higher than that in strain BM ($P < 0.01$), being about fourfold higher at 0 h p.i. and 12 h p.i., and 10-fold higher by 24 h p.i. (Fig. 6). This elevated level of transcription is related to the increased cytotoxicity for macrophages, but inversely related to the decreased survival of strain BM-VIR in macrophages. In the *in vitro* stress study, *virB* in strain BM-VIR was transcribed at a higher level than in strain BM under all stress conditions tested ($P < 0.01$), indicating that *virB* was overexpressed under these conditions, but this has no bearing on its ability to survive under these conditions.

***virB* is transcribed in a cell-density-dependent manner both *in vitro* and during macrophage infection**

It is known that the number of *Brucella* cells in macrophages is maintained at a relatively low level. The experiments described above showed that with a higher m.o.i., higher cytotoxicity was observed. This implies the possibility of cell-density-dependent expression regulation of *virB*. Further evidence to support this possibility comes from the fact that *virB* is regulated by *vjbR*, a quorum sensing system which senses and responds to cell density. Therefore, we analysed the transcription of *virB* at different growth stages. Growth curves showed that strain BM-VIR had a similar growth curve to strain BM, but the growth rate was lower (Fig. 7a). However, transcription of *virB* in the two strains seemed to be very different. In strain BM, *virB* was transcribed at the highest level at low cell densities (early exponential phase), and as cell density increased, transcription decreased. However, for strain BM-VIR, transcription of *virB* increased as cell density increased

Fig. 6. Overexpression of *virB* in strain BM-VIR during macrophage infection (a) and under stress conditions (b). (a) Macrophage cells were infected with strains BM (◆), BMΔ*virB* (■) and BM-VIR (▲). At different times (0, 12, 24 and 48 h p.i.), RNA was isolated from the infection mixtures and reverse transcribed into cDNA. Transcription of *virB* genes was then quantified by qRT-PCR. (b) Strains BM (white bars), BMΔ*virB* (bars not visible) and BM-VIR (grey bars) were cultured in TSB to early exponential phase ($OD_{600} = 0.6$) at 37 °C and then subjected to different stress conditions. RNA was isolated and the transcription of *virB* was quantified by qRT-PCR.

from early to late exponential phase, and at stationary phase the transcription of *virB* was downregulated. At high cell density, transcription of *virB* in strain BM-VIR was much higher than that observed in strain BM ($P < 0.01$). Transcription of *virB* during macrophage infection peaked at 12 h p.i., and then decreased at 24–48 h p.i. Combined with the change in the number of intracellular bacteria over the infection period, it can be inferred that the transcription of *virB* during macrophage infection is cell-density-dependent.

DISCUSSION

The T4SS has been identified in many pathogenic bacteria, such as *Legionella pneumophila*, *A. tumefaciens*, *Helicobacter pylori* and *Bartonella* spp. These bacteria use the T4SS apparatus to interfere with host-cell function by secretion of effector proteins (Cascales & Christie, 2003). As an intracellular bacterial pathogen, *Brucella* needs to adapt to the intracellular environment and interfere with

Fig. 7. Growth-dependent transcription of *virB*. (a) Growth curve of strains BM (▲) and BM-VIR (■) in TSB. OD₆₀₀ was monitored at 2 h intervals. (b) Transcription of *virB8* during different growth phases. Strains BM (▲) and BM-VIR (■) were cultured in TSB, and then samples of each were grown to early exponential (EL), mid-exponential (ML), late exponential (LL) and stationary phases (SP).

host-cell function for survival and replication. The T4SS of *Brucella* has been shown to be an essential virulence determinant, which plays important roles in phagosome-lysosome fusion inhibition and replication vacuole formation (Celli *et al.*, 2003; O'Callaghan *et al.*, 1999). The T4SS seems to play different roles in different *Brucella* species. For smooth strains, T4SS is essential for intracellular survival and plays a very important role in the inhibition of host-cell apoptosis. However, for some rough strains, the T4SS induces cytotoxicity for macrophages, implying complex regulation of the T4SS in different *Brucella* backgrounds (Freeman & Rumack, 1964; Pei *et al.*, 2008).

Cytotoxicity in macrophages induced by rough *Brucella* mutants requires bacterial protein synthesis, and the T4SS is critical for this cytotoxicity. One explanation of the cytotoxicity of the rough mutant is assumed to be the increased secretion of toxin (Pei *et al.*, 2008). In the present study, we found for the first time that smooth strains also induce cytotoxicity in macrophages. The cytotoxicity of smooth strains for macrophages occurs at high levels of m.o.i. Inactivation of the T4SS eliminated cytotoxicity; in contrast, overexpression of the T4SS increased cytotoxicity even at low levels of m.o.i. These data indicate that the cytotoxicity of smooth strains for macrophages is also T4SS-dependent.

To determine whether smooth strains are cytotoxic for macrophages, J774A.1 cells were infected with smooth strain BM at different m.o.i.s. To our surprise, when macrophages were infected with a high m.o.i., cytotoxicity was observed. Furthermore, the cytotoxicity was positively correlated with m.o.i., implying that the increased numbers of bacteria in the host cells might be harmful to the host. The length of time post-infection seemed to affect host cells detrimentally; when the infection was prolonged to 48 h, all the macrophages infected at different m.o.i.s showed high levels of cytotoxicity (Fig. 2a). This provided further evidence that large numbers of bacteria are harmful for the host cell. Previous studies have also shown that optimal numbers of bacteria are required for *Brucella* to protect infected and neighbouring host cells, implying that bacteria cell numbers in the host are probably tightly regulated (Delrue *et al.*, 2005; Pei *et al.*, 2008).

Following the observation that smooth strains are cytotoxic for macrophages, we then tested whether this effect is related to the T4SS, as seen in the rough strains. As shown in Fig. 2(b), the *virB* mutant did not induce high levels of cytotoxicity in macrophages at any m.o.i. by 12 and 24 h p.i.; furthermore, there were no differences in the level of cytotoxicity for the three m.o.i.s. The cytotoxicity of strain BMΔ*virB* for macrophages at 48 h p.i. may result from a nutritionally deprived environment due to the long incubation time, as found with strain BM at a low m.o.i. of 20. Therefore, based on these results, it can be concluded that the cytotoxicity of the smooth strain is *virB*-dependent. Pei *et al.* (2006) found that the cytotoxicity of rough mutants of *Brucella ovis* for macrophages requires protein synthesis. Their subsequent study showed that this cytotoxicity is T4SS-dependent (Pei *et al.*, 2008). In our present study, we found that the smooth strain of *B. melitensis* is also cytotoxic for macrophages. This implies that cytotoxicity for macrophages is not unique to rough mutants, and that *virB*-dependent cytotoxicity might be a common phenomenon among different *Brucella* strains.

The cytotoxicity of the rough mutant for macrophages is predicted to result from the increased secretion of toxin into the host cell by the bacteria because of increased exposure of the T4SS in the rough mutant. Based on the mechanism of the T4SS, these toxins are probably effector proteins. Thus we speculated that the cytotoxicity of the smooth strain might also result from overexpression of *virB*. As our results show, when infected with *virB*-overexpressing strain BM-VIR, even at a low m.o.i. of 20 (at which little cytotoxicity of strain BM was observed), the infected macrophage exhibited a high degree of cytotoxicity. The time to reach the LDH release plateau was also shortened with strain BM-VIR. This indicated that overexpression of *virB* increases cytotoxicity of *Brucella* for macrophages. Both overexpression of *virB* in strain BM-VIR and a high m.o.i. of strain BM induced a high degree of cytotoxicity, the common characteristic of which is that *virB* is expressed at elevated levels. Thus the main cause of cytotoxicity of *Brucella* for macrophages is elevated

expression of *virB*, and most probably the increased secretion of effector proteins. This is confirmed by the result that C₁₂-HSL inhibits expression of *virB*, and also inhibits cytotoxicity of *Brucella* for macrophages (Fig. 3).

In *B. suis*, *virB* is regulated in a growth-phase-dependent manner, with *virB* transcription being reduced in late exponential phase. Delrue *et al.* (2005) observed that the level of *virB* mRNA in *B. melitensis* decreased significantly upon reaching stationary phase. In the present study, we found that transcription of *virB* is also growth-dependent in *B. melitensis* strain BM. Transcription of *virB* peaks at early exponential growth phase, and then as the cell density increases, transcription decreases (Fig. 7b). Addition of C₁₂-HSL inhibits *virB* transcription in *B. melitensis* and this is independent of growth stage. Inactivation of *vjbR* downregulates transcription of *virB*. The inhibition effect of C₁₂-HSL on transcription of *virB* is mediated by *vjbR*, indicating that *vjbR* regulates the expression of *virB*. The regulation of *virB* by *vjbR* appears to be correlated with intracellular survival, for increased bacterial numbers are detrimental for host cells. Interestingly, we found that *virB* also affects transcription of *vjbR* in a growth-phase-dependent manner. Therefore, *vjbR* and *virB* regulate each other, forming a positive regulation circuit (Wang *et al.*, 2009). Both *vjbR* and *virB* mutants could not replicate in host cells, nor cause chronic infection in mice. Thus the numbers of intracellular *Brucella* cells are tightly controlled and closely related to intracellular survival.

Inactivation of the T4SS in the smooth strain resulted in rapid clearance of the mutant by host cells. However, the elimination of T4SS expression in rough mutants resulted in enhanced replication and persistence, suggesting that the T4SS is not required for rough mutant intracellular trafficking and survival in macrophages. Therefore, it is possible that rough *Brucella* strains invade macrophages by a different pathway from smooth strains, which influences both the intracellular trafficking and the ultimate fate of rough organisms (Gorvel & Moreno, 2002; Jimenez de Bagues *et al.*, 2004; Pei *et al.*, 2006). A recent study showed that two proteins, VceA and VceC, were translocated into host cells by the *Brucella* T4SS (de Jong *et al.*, 2008). Translocation of VceC by *B. abortus* into J774A.1 cells resulted in lysis of the host cells, indicating that this protein mediates the cytotoxicity effect for macrophages. At an m.o.i. of 500, at 11 h p.i., only 30 % cytotoxicity for macrophages was observed. This indicated that there are other effector proteins that are involved in the cytotoxicity (de Jong *et al.*, 2008). In other bacteria, a large number of effector proteins involved in different functional categories are secreted via the T4SS. Therefore, VceC and VceA will not be the only effector proteins secreted by the T4SS of *Brucella*. It will be very interesting to construct a VceC deletion mutant and analyse the cytotoxicity of this mutant for macrophages.

The *virB* mutant could not replicate in macrophages and the *virB* overexpression strain was cytotoxic for macro-

phage, indicating that the expression of *virB* is tightly regulated. The *virB* mutant showed increased sensitivity to *in vitro* stress conditions, implying that *virB* plays important roles in the adaptation of *Brucella* to intracellular environments. With comparative proteomic methods, we found that inactivation of *virB* resulted in great changes in gene expression, including many involved in bacterial intracellular survival. The major membrane proteins, such as Omp25 and Omp31, were also downregulated in the *virB* mutant (Wang *et al.*, 2009). The downregulation of outer-membrane proteins may explain the increased sensitivity of the mutant to stress. We speculate that some of these differentially expressed proteins might be the effector proteins of the T4SS. At present, we are trying to identify these effector proteins.

In summary, this report provides evidence that smooth strains of *Brucella* are toxic for macrophages at a high infection m.o.i., and this cytotoxicity is also T4SS-dependent. Inactivation of the T4SS eliminated intracellular survival capability, and overexpression of the T4SS increased cytotoxicity but not sensitivity to intracellular stress conditions, indicating that cytotoxicity is mediated by increased secretion of effector proteins and that *virB* is tightly regulated during survival in macrophages. Transcription of *virB* both under stress conditions and during macrophage infection showed that *virB* is tightly regulated in a cell-density-dependent manner. Therefore, it is possible that the secretion of effector proteins is also regulated in this way. The identification of more effector proteins of the T4SS and research into their functions will provide more detailed answers.

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