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Production of the Type IV Secretion System Differs among *Brucella* Species as Revealed with VirB5- and VirB8-Specific Antisera

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Expression of the *virB* operon, encoding the type IV secretion system required for *Brucella suis* virulence, occurred in the acidic phagocytic vacuoles of macrophages and could be induced in minimal medium at acidic pH values. To analyze the production of VirB proteins, polyclonal antisera against *B. suis* VirB5 and VirB8 were generated. Western blot analysis revealed that VirB5 and VirB8 were detected after 3 h in acidic minimal medium and that the amounts increased after prolonged incubation. Unlike what occurs in the related organism *Agrobacterium tumefaciens*, the periplasmic sugar binding protein ChvE did not contribute to VirB protein production, and *B. suis* from which *chvE* was deleted was fully virulent in a mouse model. Comparative analyses of various *Brucella* species revealed that in all of them VirB protein production increased under acidic conditions. However, in rich medium at neutral pH, *Brucella canis* and *B. suis*, as well as the *Brucella abortus*- and *Brucella melitensis*-derived vaccine strains S19, RB51, and Rev.1, produced no VirB proteins or only small amounts of VirB proteins, whereas the parental *B. abortus* and *B. melitensis* strains constitutively produced VirB5 and VirB8. Thus, the vaccine strains were still able to induce *virB* expression under acidic conditions, but the VirB protein production was markedly different from that in the wild-type strains at pH 7. Taken together, the data indicate that VirB protein production and probably expression of the *virB* operon are not uniformly regulated in different *Brucella* species. Since VirB proteins were shown to modulate *Brucella* phagocytosis and intracellular trafficking, the differential regulation of the production of these proteins reported here may provide a clue to explain their role(s) during the infection process.

Bacteria belonging to the genus *Brucella* are gram-negative facultative intracellular pathogens of various wild and domestic mammals, and they also cause severe zoonotic infections in humans. Traditionally, three major species are distinguished by their preferences for certain animal hosts; *Brucella abortus* has a preference for cattle, *Brucella melitensis* has a preference for caprines, and *Brucella suis* has a preference for hogs. Whereas *B. abortus* is the livestock pathogen with the greatest economic impact, *B. melitensis* and *B. suis* account for most clinical cases in humans (15, 42).

In an attempt to unravel *Brucella* virulence factors by transposon mutagenesis, the crucial role of an operon similar to the *virB* operon of *Agrobacterium tumefaciens* encoding a type IV secretion system (T4SS) was revealed (35). The importance of the *virB* operon for *Brucella* virulence was further confirmed by signature-tagged mutagenesis both in vitro in a human macrophage infection model (24) and in vivo with mice (26). Further studies indicated that a complete *Brucella virB* operon was required for wild-type virulence in mice (47) or in macrophage-like cells (52, 53). In nonphagocytic HeLa cells, the absence of some functional VirB proteins (B2, B4, and B9) did not affect bacterial entry or prevention of the phagolysosomal fusion (17). However, integrity of the *virB* operon was required for *Brucella* to reach the proper niche and to replicate in HeLa cells (13, 47).

Of the gene products deduced from the 12 open reading frames of the *B. suis virB* operon, the first 11 proteins exhibit significant sequence similarity to the VirB proteins of the *A. tumefaciens* T4SS and also with Tra proteins required for the transfer of broad-host-range plasmids from the IncP, -N, and -W incompatibility groups (6, 12, 16). *A. tumefaciens*- and plasmid-encoded systems presumably form a multicomponent pore, which spans both bacterial membranes and allows transport of a single-stranded DNA-protein complex into a recipient plant or bacterial cell. The sequence similarity of the *Brucella* VirB proteins to the proteins of the T4SS of *A. tumefaciens* does not necessarily indicate that *Brucella* transfers DNA through its VirB-like complex, because T4SS from *Bordetella pertussis* (14) and *Helicobacter pylori* (36) are known to translocate proteins. It could be speculated that T4SS present in other intracellular pathogens, such as *Rickettsia prowazekii* (2) and *Bartonella henselae* (45), may have similar functions in intracellular survival, although effector proteins have not been described yet for these bacteria.

The virulence regulon of *A. tumefaciens* is induced in response to chemical signals at the plant wound site by a two-component system composed of the sensor VirA and the tran-

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TABLE 1. Bacterial strains and plasmids used in this study

Strains or plasmid	Relevant genotype and/or description	Reference or source[a]
E. coli BL21 (λDE3)	F^- *hsdS gal1* λDE3	49
B. suis strains		
1330	Biotype 1; ATCC 23444^T	ATCC
1330 Δ*chvE*	*B. suis* 1330 Δ*chvE*::*kan*	1
1330 *omp25*	*B. suis* 1330 *omp25*::*kan*	28
1330 *nikA*	*B. suis* 1330 *nikA*::*kan*	28
1330 *virB5*	*B. suis virB5*::*kan*	8
1330 *virB12*	*B. suis virB12*::*kan*	35
B. melitensis strains		
16M	Biotype 1; ATCC 23456^T	ATCC
Rev.1	Vaccine strain	Jiménez de Bagüés
B. abortus strains		
544	Biotype 1; ATCC 23448^T	ATCC
2308	Biotype 1; wild type, smooth, virulent	Jiménez de Bagüés
S19	Vaccine strain, smooth	Jiménez de Bagüés
RB51	Vaccine strain, rough	Jiménez de Bagüés
B. canis	ATCC 23365^T	ATCC
B. ovis	Reo 198	Jiménez de Bagüés
Plasmid pT7-H_6-TrxFus	Amp^r, *trxA* fusion plasmid for T7 promotor-controlled overproduction	30

[a] ATCC, American Type Culture Collection; Jiménez de Bagüés, M. P. Jiménez de Bagüés.

scription factor VirG (11). Plant signals, including low pH and phenolic compounds, such as acetosyringone, induce virulence gene expression, which is potentiated by monosaccharides (5, 10). Sugars like galactose and arabinose bind to the periplasmic multiple sugar binding protein ChvE, which is encoded by an operon composed of *chvE*, *gguA*, and *gguB*; the latter two genes encode sugar transporter proteins. Upon sugar binding to ChvE, the complex potentiates the response to phenolic molecules (38, 46). In *B. suis*, we recently cloned a *chvE* operon similar to that of *A. tumefaciens*, which is specifically required for D-(+)-galactose utilization (1). The *B. suis* operon was dispensable for intracellular survival and multiplication of the bacteria in J774 macrophage-like cells (1).

Despite the high similarity between the *virB* and *chvE* operons of *A. tumefaciens* and *B. suis*, our previous attempts to identify a *Brucella* ortholog of the VirA-VirG two-component system by DNA-DNA hybridization failed. These results are in accord with recent analyses of the *B. suis* (37) and *B. melitensis* (18) genomes, which failed to detect orthologs of the *A. tumefaciens virA* or *virG* genes. However, there is evidence for regulated expression of the *B. suis virB* operon. Use of promoter *virB*::*gfp* reporter gene fusions and analysis of the *virB* mRNAs showed that the *B. suis virB* operon is expressed intracellularly in macrophages, and this effect could be mimicked in vitro in minimal medium (MM) at an acidic pH (8). This finding is in agreement with the requirement of an acidified phagosome for survival and multiplication of *B. suis* in macrophages (3, 39). However, use of *lacZ* reporter gene fusions showed that the *virB* operon of *B. abortus* 2308 is expressed during the stationary phase without a requirement for acidic induction conditions (47).

In order to investigate the regulation of *virB* operon expression in various *Brucella* wild-type and attenuated strains, we raised specific antisera against the VirB5 and VirB8 proteins. These new tools allowed determination of VirB protein production for the first time. Analysis of different *Brucella* species revealed marked differences in VirB protein production under various growth conditions, suggesting that the VirB proteins play different roles in infection of the different hosts.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The characteristics of the bacterial strains and plasmids used are described in Table 1. *Escherichia coli* strains were routinely grown at 37°C in Luria-Bertani medium, whereas *B. suis* and other *Brucella* strains were grown in tryptic soy (TS) broth. TS broth was supplemented with kanamycin (50 μg ml^{-1}) to grow *Brucella* mutants. *Brucella* strains were grown for 20 h at 37°C in TS broth with or without antibiotics to the stationary phase (optical density at 600 nm, 1.5 to 1.8). Four volumes of phosphate-buffered saline (PBS) was added before centrifugation at 2,500 × *g* for 25 min, and the pellets were resuspended in 4 volumes of PBS, resulting in optical density values around 0.5. For interspecies comparisons, the optical density at 600 nm was adjusted at this step to 0.5. Two-milliliter aliquots of the various bacterial suspensions were sedimented by centrifugation for 2 min at 13,000 × *g*, and the pellets were then resuspended in 0.5 ml of MM at pH 4.5 or 7 as indicated below. The Eppendorf tubes were incubated horizontally with shaking at 37°C for various times. MM, derived from MMA (4), was composed of 7.56 mM $(NH_4)_2SO_4$, 33 mM KH_2PO_4, 60.3 mM K_2HPO_4, 1.7 mM sodium citrate supplemented with 1 mM $MgSO_4$, 10 mM glucose (or other carbon sources as indicated below), 0.1% yeast extract, 2 μg of vitamin B_6 per ml, 2 μg of vitamin B_1 per ml, and 0.0005 μg of biotin per ml, as previously described (1). The pH was adjusted with 2 M citric acid. The eukaryotic cell culture medium, RPMI 1640, was obtained from Gibco (Life Technologies, Cergy Pontoise, France).

Production of the VirB5 and VirB8 recombinant proteins and antiserum preparation. DNA fragments encoding *B. suis* VirB5 and VirB8 without signal peptides were amplified by PCR with the following primers derived from the *B. suis* 1330 sequence (GenBank accession no. AF 141604): the oligonucleotides used for amplification of *virB5* were T7B5suis5′ (CAGGGTACCCGCGCACG CGCAGCTCC) and T7B5suis3′ (GAGCTGCAGCTAATAGGCGGCTTCCA GTGC), and the oligonucleotides used for amplification of *virB8* were T7B8suis5′ (CAGGGTACCCCGCGTCAACGCACAGAC) and T7B8suis3′ (GAGCTGCAGCTATTGCACCACTCCCATTTCTGG). PCR fragments were cleaved with *Acc*65I/*Pst*I and ligated into a similarly cleaved pT7-H_6-TrxFus vector (30). Hexahistidyl-thioredoxin fusion proteins were overproduced in BL21 by isopropyl-β-D-thiogalactopyranoside (IPTG) induction of the T7 promoter and were purified by immobilized metal affinity chromatography performed as recommended by the manufacturer (Amersham Bioscience, Saclay, France). Polyclonal antisera were raised in New Zealand White rabbits, and VirB5 antisera were further purified by affinity chromatography as described previously (41)

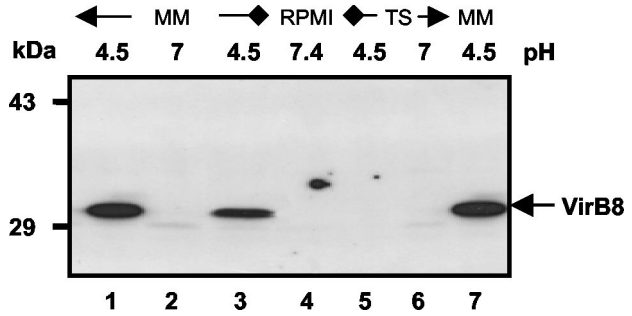

FIG. 1. In vitro induction of *B. suis* VirB8 in different media. *B. suis* wild-type strain 1330 was grown in TS broth to the stationary phase and washed with PBS by centrifugation, and the bacteria were resuspended in different media as follows. Further cultivation was carried out at 37°C for 5 h in MM at pH 7.0 (lane 2) or at pH 4.5 (lanes 1, 3, and 7), in TS medium, or in RPMI 1640. Lane 3 contained lysates from a *B. suis* culture grown in MM containing D-(+)-galactose instead of D-(+)-glucose. VirB8 production in cells was evaluated by SDS–15% PAGE of cell lysates, followed by Western blotting with VirB8-specific antiserum and chemoluminescent detection.

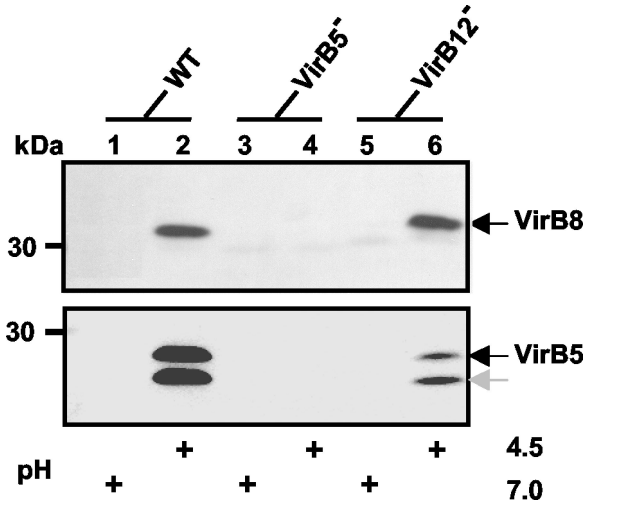

FIG. 2. Comparison of production of VirB8 in acidic MM by the *B. suis* wild type (WT) and by *virB5*- and *virB12*-disrupted mutants. Bacteria grown in TS broth to the stationary phase were washed with PBS and resuspended in MM at pH 7.0 or 4.5. Further incubation was carried out at 37°C for 6 h prior to evaluation of VirB8 (upper panel) and VirB5 (lower panel) production by Western blotting.

Western blot analysis. After cell cultivation for various times, bacteria were sedimented, and the pellets were resuspended in Laemmli sample buffer and heated to 100°C for 5 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 15% (wt/vol) acrylamide separating gels (31). The proteins were transferred onto Immobilon polyvinylidene difluoride membranes (Millipore, Saint Quentin-Yvelines, France) by using a semidry transfer procedure, and the membranes were stained with Coomassie blue for detection of reference proteins. Immunodetection of proteins in total cell lysates was performed with polyclonal VirB8 antiserum (1/5,000), VirB5 antiserum (1/3,000), or affinity-purified anti-VirB5 serum (1/5,000). Horseradish peroxidase-conjugated goat anti-rabbit antibodies (Jackson Immunoresearch Laboratories Inc.; obtained from Immunotech, Marseilles, France) were used in combination with the ECL system (Amersham Bioscience Saclay, France) to develop the chemiluminescence for visualization on Kodak X-AR film (Sigma-Aldrich, Saint-Quentin Falavier, France). The molecular weight markers were obtained from Sigma or Amersham Bioscience.

Infection of mice, preparation of spleens, and bacterial counts. Eight-week-old female BALB/c mice obtained from IFFA Credo were challenged intraperitoneally with 5×10^4 CFU of either wild-type *B. suis* 1330 or the *chv*E null mutant as described previously (22). Brucellae were grown on TS agar (Life Technologies) supplemented with 0.1% (wt/vol) yeast extract (Difco, Detroit, Mich.) (TSA-YE). Viable counts were determined retrospectively by enumeration on TSA-YE plates. Five infected mice for each *B. suis* strain were sacrificed on days 1, 7, 14, 21, 35, and 56 by cervical dislocation. Spleens were harvested, weighed, and frozen at −20°C. After homogenization in a buffered saline solution, bacterial counts were determined on TSA-YE plates. Normalization and statistical analysis of the results were carried out as previously reported (9, 22).

RESULTS

Both acidic conditions and MM are required for in vitro production of *B. suis* VirB8. Polyclonal rabbit antisera were raised against *B. suis* VirB5 and VirB8 hexahistidyl-thioredoxin fusion proteins. The sera were tested by Western blotting by using lysates of *B. suis* cultivated in MM at either pH 7.0 or pH 4.5, because previous studies had shown that there was increased *virB* mRNA in acidic MM (8). After 5 h of cultivation in acidic MM containing 10 mM glucose (Fig. 1, lanes 1 and 7) or galactose (lane 3), the VirB8 antiserum detected a protein with an apparent molecular mass that was consistent with the predicted molecular mass, 31.5 kDa. In contrast, when bacteria were grown at neutral pH in MM, RPMI 1640, or TS medium (Fig. 1, lanes 2, 4, and 6), the VirB8 antiserum did not detect any protein. Furthermore, acidification of TS medium (lane 5) or of RPMI 1640 (data not shown) to pH 4.5 was not sufficient to trigger VirB8 production in this time. To determine the optimal pH for in vitro VirB8 production by *B. suis*, the bacteria were incubated in MM adjusted to pH values ranging from 4.0 to 7.0. The maximal amount of VirB8 detected after 6 h of incubation was obtained at pH values of 4.5 to 5.5 (data not shown). At pH 4.0, VirB8 was not detected, although the presence of *virB* mRNA was reported previously (8). This suggests that biosynthesis and the stability of the protein were impaired in MM at pH values below 4.5, while transcription was still operative at pH 4.0.

***B. suis virB5* and *virB8* genes are expressed as part of the same operon.** In contrast to the easily detectable amount of VirB8 in the *B. suis* wild type at pH 4.5 (Fig. 2, lane 2), no VirB8-specific immunostaining was detected in lysates from the *virB5*::*kan* mutant (Fig. 2, lane 4). Insertion of the kanamycin cassette into *virB5* obviously exerted a polar effect towards the downstream genes, thus confirming that *virB8* was part of the *virB* operon. Accordingly, knockout of the 12th gene of the *virB* operon should not have affected expression of the downstream *virB8* gene, and analysis of the *virB12*::*kan* insertion mutant confirmed this assumption (Fig. 2, lane 6). Parallel observations were made with the affinity-purified VirB5 antiserum (Fig. 2, lower panel). However, in this case, two proteins were detected, and they had apparent molecular masses of 28 and 26 kDa, which are slightly greater than the molecular mass expected for the full-length protein or the mature form after removal of the signal peptide (35). The lower-molecular-mass form of VirB5 may be a degradation product, as suggested for the *A. tumefaciens* VirB5 ortholog (44). Together, use of VirB8- and VirB5-specific antisera demonstrated for the first time that *B. suis* VirB proteins are produced in acidic MM, and the results further supported the operon structure predicted previously.

Kinetics of *B. suis* VirB5 and VirB8 protein production in

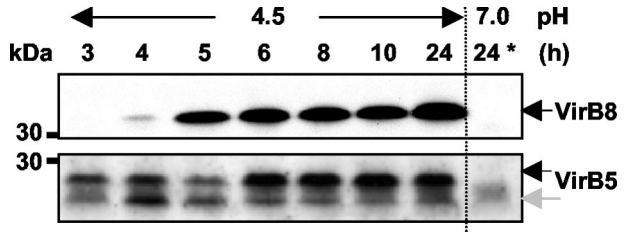

FIG. 3. Kinetics of *B. suis* VirB5 and VirB8 production in MM. *B. suis* grown in TS broth to the stationary phase was washed, resuspended in MM at pH 4.5, and incubated at 37°C. After 3 h bacteria were divided into aliquots and centrifuged. The pellets were resuspended in MM at pH 4.5. After various incubation times at 37°C (3 to 24 h, as indicated), VirB protein production was evaluated by using purified VirB5 antiserum (lower panel) or VirB8 antiserum (upper panel). For direct comparison, lane 24* was loaded with bacteria incubated continuously for 24 h at pH 7.0. The data are data from a single representative experiment that was repeated three times.

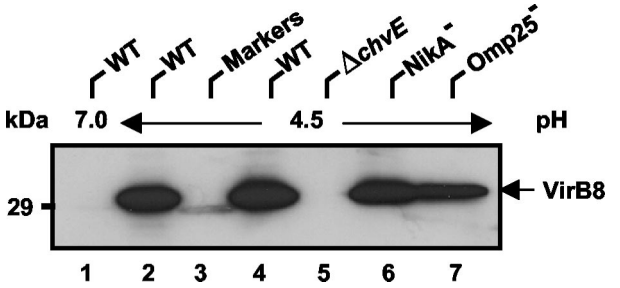

FIG. 4. Analysis of VirB8 induction in various *B. suis* mutants. Wild-type *B. suis* (WT) grown in TS medium to the stationary phase was washed and resuspended in MM at either pH 7.0 (lane 1) or pH 4.5 (lanes 2 and 4). *B. suis* mutants Δ*chv*E (lane 5), *nikA*::*kan* (NikA$^-$) (lane 6), and *omp25*::*kan* (Omp25$^-$) (lane 7) were grown in TS medium with kanamycin (50 μg/ml) and similarly resuspended in MM at pH 4.5. Cell cultivation was carried out for 6 h at 37°C. Lane 3 contained molecular weight standards (Markers).

acidic MM. Whereas the experiments described above provided information about the VirB5 and VirB8 levels after 5 to 6 h of incubation in the *virB*-inducing medium, we sought to determine the in vitro onset of VirB protein synthesis. *B. suis* was incubated in MM at pH 4.5 for periods ranging from 1 to 24 h. Synthesis of the VirB5 protein started after 2 h of cultivation in acidic MM (data not shown) and was clearly apparent after 3 h (Fig. 3, lower panel). In contrast, the VirB8 protein was first detected after 4 h, and the amount increased steadily after that (Fig. 3, upper panel). Thus, we concluded that under inducing conditions *B. suis* required about 3 h to produce detectable amounts of VirB5 and VirB8. Since the VirB8- and VirB5-specific antisera gave similar results and the former exhibited lower background reactivity, it was used in most subsequent experiments, and a standard incubation time of 6 h was considered suitable for obtaining *virB*-induced cells. Next, we evaluated whether *B. suis* VirB8 production was affected either by ChvE, as it is in *A. tumefaciens*, or by an *omp25* gene defect, since the absence of Omp25 resulted in attenuated strains.

***B. suis chvE* operon is not required for induction of the *virB* operon.** Recent animal trials indicated that Δ*omp25 Brucella* mutants were attenuated in cattle (19), goats (20), and mice (21). Furthermore, these *omp25* mutant strains raised a protective immune response against a later challenge with wild-type strains, and use of these strains as vaccines is under investigation (20). Moreover, attenuated *B. abortus bvrR-bvrS* mutants were recently reported to be unable to produce the outer membrane protein Omp25 (25). To establish whether the attenuation of *omp25* mutants is caused by their inability to synthesize VirB proteins, we included the *B. suis omp25* kanamycin insertion mutant in our analysis. As a control, another *B. suis* mutant was included (*nikA*::*kan*), which was generated by the same method. The *B. suis* mutants impaired in Omp25 production and nickel uptake (*nikA*) produced VirB8 like the wild type (Fig. 4). In contrast, VirB8 was not detected in lysate from the *chvE* mutant (Fig. 4, lane 5). This finding prompted us to evaluate whether alterations of the *chvE* operon could lead to an attenuated behavior in mice. A comparison of the infection kinetics of the *B. suis* wild-type strain with the infection kinetics of the Δ*chvE* variant indicated that the bacterial charges of both strains decreased similarly 1 week after infection (Fig. 5A). After this, the mutated strain persisted in mouse spleens even longer (8 weeks) than the parental strain (3 weeks). Slower elimination of a mutant than of wild-type *B. suis* was reported previously in the case of the Δ*clpA* mutant (22). Although the reason for the difference is not clear, our results suggested that the Δ*chvE* strain was still able to produce VirB proteins required for *Brucella* virulence. In an attempt to reconcile these conflicting in vitro and in vivo data, the effects of various carbon sources on VirB8 production were investigated. It was previously shown that galactose uptake, glucose uptake, and erythritol uptake were totally, partially, and not dependent on expression of the *chvE* operon, respectively (1). Growth in the presence of each of these sugars allowed production of VirB8 by the wild type (Fig. 5B). In contrast, the VirB8 protein was produced by the Δ*chvE* mutant in the presence of erythritol (lane 8) and to some extent also in the presence of glucose, but no VirB protein was ever detected in the presence of galactose. These experiments demonstrated that the ChvE protein was not required for in vivo or in vitro VirB8 production, as long as carbon sources other than galactose were available to the bacteria. The obvious specificity of the ChvE protein for uptake of glucose and galactose suggests that this protein likely does not play a general role in the regulation of VirB protein production in the natural infection process.

***B. melitensis*, *B. abortus*, and *Brucella ovis* produce VirB5 and VirB8 in rich TS medium.** Due to the high levels of similarity among orthologous genes in brucellae, it was anticipated that our antisera would detect VirB5 and VirB8 in all *Brucella* strains. Since the *B. abortus virB* promoter was activated in rich culture medium during the stationary phase (47), we analyzed the presence of the VirB components in various *Brucella* strains grown in rich TS medium (Fig. 6). *B. suis* did not produce detectable amounts of either VirB protein, and only small amounts were detected in *Brucella canis* (Fig. 6, lanes 1 and 2). In contrast, *B. melitensis*, *B. abortus* 544, and *B. ovis* produced easily detectable quantities of VirB5 and VirB8 (Fig. 6, lanes 3 to 5). Interestingly, the VirB5 form detected under these growth conditions corresponded almost exclusively to the

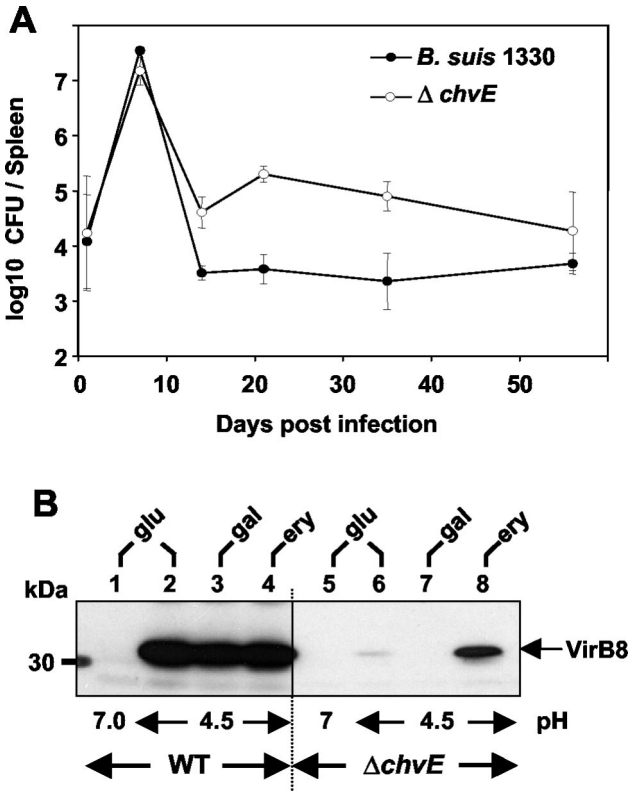

FIG. 5. Comparison of mouse infection and VirB8 production for wild-type *B. suis* and the Δ*chvE* mutant. (A) Mice were infected with *B. suis* wild-type strain 1330 (●) and the Δ*chvE* mutant (○). The infections were assessed by determining the number of bacteria present in the spleen. The data are means ± standard deviations for five animals per condition. The results obtained with the transporter Δ*gguA* mutant were not significantly different from the results obtained with the Δ*chvE* mutant. (B) Wild-type *B. suis* (WT) (lanes 1 to 4) and the isogenic Δ*chvE* mutant (lanes 5 to 8) were cultivated for 6 h in MM containing 10 mM D-(+)-glucose (glu) at pH 7.0 (lanes 1 and 5) or at pH 4.5 (lanes 2 and 6) or in pH 4.5 medium in which glucose was replaced by either D-(+)-galactose (gal) (lanes 3 and 7) or *meso*-erythritol (ery) (lanes 4 and 8). The data are representative of the results of the four experiments performed. The VirB8 levels obtained under acidic conditions in the presence of glucose and galactose were sometimes higher than those shown in lanes 6 and 8, while VirB8 was never detected in the presence of galactose.

higher-molecular-mass form detected in *B. suis* in acidic MM conditions (Fig. 2 and 3).

Acidic MM enhances VirB8 production in different *Brucella* species. Because some *Brucella* species produced VirB proteins in rich TS medium, we determined whether the *B. suis* *virB*-inducing conditions were also relevant for the other strains. To do this, *Brucella* strains grown to the stationary phase were incubated for 6 h in MM at pH 7.0 or 4.5 (Fig. 7). There were no significant changes in the VirB8 levels at pH 7.0 in the various strains compared to the levels reached in TS medium (Fig. 7, lanes 1, 3, 5, 7, 9, 11, 13, and 15), but in all strains the VirB8 content increased under the *B. suis virB*-inducing conditions (lanes 2, 4, 6, 8, 10, 12, 14, and 16). In the case of *B. ovis*, however, a decreased VirB8 content was observed, which probably resulted from poor adaptation of this

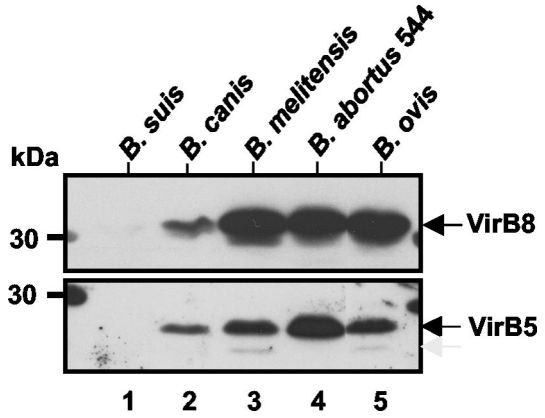

FIG. 6. Comparison of the VirB5 and VirB8 contents in various *Brucella* wild-type strains growth in rich TS medium. The wild-type bacteria *B. suis* (lane 1), *B. canis* (lane 2), *B. melitensis* (lane 3), *B. abortus* 544 (lane 4), and *B. ovis* (lane 5) were grown to the stationary phase in TS medium, washed once with PBS, and centrifuged. VirB8 (upper panel) and VirB5 (lower panel) protein contents were evaluated after SDS-PAGE and electrotransfer by Western blotting by using VirB8 antiserum or affinity-purified VirB5 antiserum. The data are the data from one experiment that was replicated once with similar results.

strain to the acidic conditions, as indicated by the dramatic protein degradation seen after SDS-PAGE (data not shown).

Because of the importance of the *virB* operon for the pathogenicity of *Brucella*, we considered the possibility that attenu-

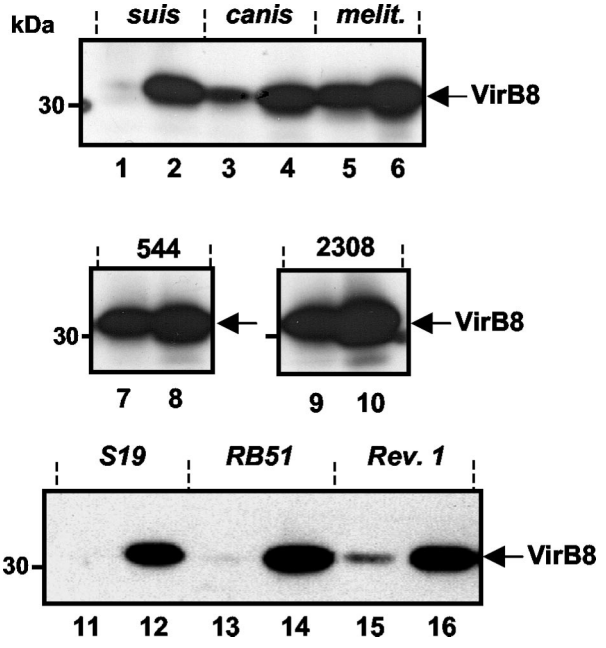

FIG. 7. Constitutive and inducible VirB8 production in the various *Brucella* strains. The wild-type bacteria *B. suis*, *B. canis*, *B. melitensis*, *B. abortus* 544, and *B. abortus* A2308 and the vaccine strains S19, RB51, and Rev.1 were grown to the stationary phase in TS medium and washed in PBS. After 6 h of incubation in MM at either pH 7.0 (lanes 1, 3, 5, 7, 9, 11, 13, and 15) or pH 4.5 (lanes 2, 4, 6, 8, 10, 12, 14, and 16), VirB8 protein production was evaluated by Western blotting by using the VirB8 antiserum. The data are the data from one experiment that was representative of the three experiments performed.

ated *Brucella* strains used as vaccine strains were affected in the ability to produce T4SS. To examine this, we determined the VirB8 contents in *B. abortus* S19 (also referred to as B19) and in the rough *B. abortus* RB51 strain, as well as in *B. melitensis* Rev.1 (23). Compared to the levels in the wild-type strains, only low levels of VirB8 were detected at neutral pH (Fig. 7, lanes 11, 13, and 15), but all vaccine strains produced substantial amounts of VirB8 protein in MM at pH 4.5 (Fig. 7, lanes 12, 14, and 16). This observation demonstrated that the vaccine strains were not impaired in terms of the ability to express the *virB* genes in an acidic environment, which is believed to be required for intracellular multiplication in the natural infection process. Interestingly, the in vitro regulation of VirB8 production in these vaccine strains was similar to that in *B. suis* and *B. canis* and notably different from that in the parental *B. abortus* and *B. melitensis* strains.

DISCUSSION

Different reports have highlighted the importance of *virB* operon integrity for the virulence of *Brucella* species. In addition, the operon-encoded VirB proteins exhibit significant sequence similarity to the proteins encoded by the well-studied T4SS of *A. tumefaciens*. However, so far there is no evidence concerning the proper assembly of the VirB proteins into a secretory apparatus, and substrates of the *Brucella* T4SS were not identified. Nevertheless, recent studies have indicated that the absence of some VirB proteins leads to alterations in the early steps of infection, cell entry (52), or intracellular trafficking (13), thus providing clues about the possible role(s) of VirB proteins. Similarly, analysis of the environmental conditions in which *Brucella* expresses its *virB* operon certainly should contribute to a better understanding of the functions of these gene products in the various steps of *Brucella* infection. With this in mind, induction and regulation of the *virB* operon, previously studied by using gene reporters (8, 47, 50), were investigated further. VirB5- and VirB8-specific antisera were raised to determine the presence of these proteins in various *Brucella* wild-type strains and mutants.

The first part of this study, which was devoted to analysis of induction of VirB proteins specifically in *B. suis*, demonstrated that VirB5 and VirB8 were either absent or only weakly produced at neutral pH either in MM or in rich TS medium. In accord with the analysis of mRNA levels (8), we showed for the first time that growth in acidic MM triggers the synthesis of both VirB5 and VirB8 proteins, although VirB5 was detected before VirB8. A comparison of the VirB8 and VirB5 contents after induction of the *B. suis* wild type and of *virB5* and *virB12* mutants further confirmed that the *virB5* and *virB12* genes belong to the same operon. Knockout of the 12th open reading frame of the *virB* operon does not prevent production of VirB8, indicating that VirB12 is not essential for expression of the *virB* operon. The absence of Omp25 in putative vaccine strains of *B. suis* did not affect their ability to produce VirB8 in MM at pH 4.5. Similarly, *B. suis chvE* deletion strains produced VirB proteins provided that suitable sugar sources were available. Whereas the conditions for induction of the *virB* operon of this organism share suggestive features with the conditions for induction of the *virB* operon of *A. tumefaciens*, the *B. suis chvE* strain survived well in macrophages (1), as well as in infected mice. Therefore, exogenous sugars may not play a role in the intracellular induction of *virB* expression. In addition to the requirement for acidic conditions, growth in MM was apparently another prerequisite for *virB* expression in *B. suis*. The intracellular vacuole is believed to be a nutrient-poor environment, and this is consistent with the recent finding that many attenuated transposon insertion mutants were affected in their basic metabolic functions (29). Thus, intracellularly, *B. suis* is in a nutrient-poor environment, and the bacteria may link the starvation response to induction of their virulence functions.

Incubation of *B. suis* in MM at pH 4.5 for 3 to 4 h was required for accumulation of detectable amounts of VirB5 and VirB8. This relatively long lag time might prevent the bacteria from inadequate induction of the *virB* operon under transiently changing environmental conditions. It is also possible that *virB* operon expression requires the synthesis of another sensing system, and this may account for the delay, similar to the delay observed for the *pmrA* and *pmrB* genes induced by the PhoP/PhoQ sensor in *Salmonella* (54). If present, such regulatory *Brucella* gene products would then induce production of VirB proteins leading to T4SS assembly. The observed in vitro lag time is in accord with the finding that intracellular survival was inhibited after neutralization of the acidic *Brucella*-containing phagosome with NH_4Cl 1 h postinfection (39). However, when the phagosome was neutralized 7 h postinfection, bacterial multiplication was not affected. This suggests that all *Brucella* signals were transmitted to the eukaryotic cells within the first 7 h and ensured inhibition of the phagolysosome fusion, allowing its survival and its replication.

In the second part of this study, we demonstrated the different abilities of various *Brucella* strains to produce both VirB5 and VirB8 at neutral pH in nutrient-rich TS medium. First, *B. canis* and *B. suis* biovar 1, whose genomic physical maps are identical (33), produced only marginal amounts of VirB8 at neutral pH. After cultivation in acidic MM, both organisms produced easily detectable amounts of VirB8. Thus, these two organisms can be considered *virB* operon-inducible bacteria. Second, the other *Brucella* wild-type strains produced substantial amounts VirB8 in nutrient-rich TS medium or in MM at neutral pH and can be considered *virB* constitutive. In acidic induction medium, however, *B. abortus* biovar 1 (strains 544 and 2308) and *B. melitensis* increased their VirB8 contents further.

With regard to these two VirB expression patterns, the results obtained after analysis of the *B. abortus* and *B. melitensis* vaccine strains more closely resembled the results obtained with the inducible organism *B. suis* than the results obtained with their parental strains. The vaccine strains exhibited reduced T4SS production at neutral pH in a medium presumably encountered in the body fluids. The similarity between the vaccine strains and *B. suis*, however, is not limited to the low VirB content at neutral pH. Similarly, the kinetics of mouse infection by *B. suis*, which are characterized by a rapid decrease in the bacterial content after only 1 week (Fig. 5A), are very similar to the kinetics of the attenuated vaccine strains *B. abortus* S19 (43) and *B. melitensis* Rev.1 (27; data not shown). This finding differs from the results of previous studies of the mouse colonization patterns of virulent *B. abortus* 2308 (7, 43) and *B. melitensis* 16M (27). In both cases, a plateau phase with

very high numbers of brucellae per spleen was observed, which lasted 8 weeks before there was a gradual reduction in bacterial content. The constitutive production of VirB proteins may therefore be a marker of wild-type virulence of *B. abortus* and *B. melitensis*. In the future, it will be very interesting to assess whether the rapid elimination of *B. suis* and the vaccine strains in the mouse infection model is solely due to the low production of VirB proteins under the neutral and nutrient-rich conditions of body fluids.

The concomitant presence of VirB5 and VirB8 in strains which constitutively expressed *virB* may be taken as an indication of proper assembly of all the VirB proteins into the T4SS. If this occurs, the T4SS apparatus should protrude outside the bacterial cell surface, and, besides its putative intracellular function, it may play a role in the early events of infection. Indeed, it was recently shown that a functional *virB* operon determined the mode of entry of the wild-type *B. abortus* strain in mouse bone marrow-derived macrophages (53). This mode of internalization required the integrity of cell surface lipid rafts and the presence of an intact *virB* operon in *B. abortus*. The presence of intact lipid rafts was also required for entry and short-term survival of *B. suis* (34), but the role of the *virB* operon in the initial infection steps of this species remains to be elucidated.

In summary, development of specific VirB5 and VirB8 antisera permitted for the first time monitoring of the production of the corresponding VirB proteins in the wild type, in vaccine strains, and in mutants of different *Brucella* strains. Analysis of the presence of VirB5 and VirB8 indicated that these two *virB* products are produced simultaneously and revealed that there is differential in vitro regulation of the *virB* operon in the *Brucella* strains. This may reflect in vivo differences in the requirement for the T4SS function(s) during the infection process. The distinction between *virB*-constitutive and *virB*-inducible strains underlines the fact that the limited differences between *Brucella* genomes (51) can influence bacterial virulence by modulation of the expression of essential genes. In the future, these antisera could be used to assess whether other interesting attenuated mutants have altered VirB protein levels. Among the candidates for such an analysis are strains that are not able to replicate in macrophages or epithelial cells, such as the *B. abortus* mutants with mutations in the *hfq* gene (40) and in the BvrR/BvrS system (48). Interestingly, the *A. tumefaciens* BvrR/BvrS orthologs are involved in acid sensing (32), suggesting that this two-component system may confer the acid inducibility of the *virB* operon.

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