A Homologue of an Operon Required for DNA Transfer in Agrobacterium Is Required in Brucella abortus for Virulence and Intracellular Multiplication

RODRIGO SIEIRA,1 DIEGO J. COMERCI, 1,2 DANIEL O. SÁNCHEZ,1 AND RODOLFO A. UGALDE1*  
Instituto de Investigaciones Biotecnológicas, Universidad Nacional de General San Martín, CONICET, San Martín 1650,1 and Comisión Nacional de Energía Atómica, División Agropecuaria, Centro Atómico César François, Centenario, San Martín 1650, Buenos Aires, Argentina  
Received 2 March 2000/Accepted 16 June 2000

As part of a Brucella abortus 2308 genome project carried out in our laboratory, we identified, cloned, and sequenced a genomic DNA fragment containing a locus (virB) highly homologous to bacterial type IV secretion systems. The B. abortus virB locus is a collinear arrangement of 13 open reading frames (ORFs). Between virB1 and virB2 and downstream of ORF12, two degenerated, palindromic repeat sequences characteristic of Brucella intergenic regions were found. Gene reporter studies demonstrated that the B. abortus virB locus constitutes an operon transcribed from virB1 which is turned on during the stationary phase of growth. A B. abortus polar virB1 mutant failed to replicate in HeLa cells, indicating that the virB operon plays a critical role in intracellular multiplication. Mutants with polar and nonpolar mutations introduced in virB10 showed different behaviors in mice and in the HeLa cell infection assay, suggesting that virB10 per se is necessary for the correct function of this type IV secretion apparatus. Mouse infection assays demonstrated that the virB operon constitutes a major determinant of B. abortus virulence. It is suggested that putative effector molecules secreted by this type IV secretion system determine routing of B. abortus to an endoplasmic reticulum-related replication compartment.

Brucella spp. are facultative intracellular gram-negative bacteria that are pathogenic for many mammalian species including humans, causing a chronic infectious disease known as brucellosis, a major zoonosis in several countries (6). In humans, brucellosis is a serious debilitating disease characterized by diverse pathological manifestations like undulant fever, osteoarticular complications, endocarditis, and several neurological disorders. In domestic animals like cattle, goats, and sheep, the outstanding manifestation of the pathology is abortion in pregnant females and sterility of males due to colonization of placenta, fetal tissues, and sexual organs (15).

Brucella spp. belong, like Agrobacterium spp., Rhizobium spp., and Rickettsia spp., to the alpha-2 subgroup of the Proteobacteria (14). Most genera of this group are characterized by their ability to pericellularly or intracellularly with eukaryotic cells either as pathogens or as endosymbionts.

In Brucella spp., virulence is associated with their capacity to multiply inside the host cell. In view of recent data reported by Pizarro-Cerdá et al. (19), it is clear that intracellular survival and multiplication of Brucella depend on effectively avoiding the fusion of the phagosome-containing bacteria with the lysosome and replication in an endoplasmic reticulum-like vesicle. Genes that allow Brucella spp. to invade and reach the appropriate intracellular replication niche remain to be identified.

Recently, operons coding for export mechanisms specializing in transfer of a variety of multimolecular complexes across the bacterial membrane to the extracellular space or into other cells have been described. These complexes, named type IV secretion systems, are present in Bordetella pertussis (pil genes) (11, 29), Agrobacterium tumefaciens (virB genes) (13, 27), Escherichia coli (tra genes) (20, 30), Legionella pneumophila (dot/icm genes and hkh genes) (3, 23, 25), and Helicobacter pylori (cag genes) (7). The paradigmatic example of type IV secretion machinery is the virB operon of the phytopathogen A. tumefaciens, which comprises a collinear arrangement of 11 genes coding for a pilus-like structure necessary for transfer of T-DNA (transfer DNA) from the bacterium to the plant cell. The Agrobacterium virB operon shares high sequence similarities with E. coli tra genes, which code for the conjugative pilus and other components necessary for transfer of DNA from one bacterium to another (4). In B. pertussis, the pil operon codes for the apparatus that allows secretion of pertussis toxin. The H. pylori cag pathogenicity island is composed of 31 genes, six of them displaying homologies with others bacterial type IV secretion systems. The dot/icm virulence genes of the intracellular pathogen L. pneumophila encode a type IV secretion system that controls the intracellular trafficking of the bacteria. dot and icm mutants reside in phagosomes that rapidly fuse with lysosomes, resulting in a decrease in intracellular survival.

During the course of our work, it was reported that the virB region is essential for intracellular replication of Brucella suis 1330 in an in vitro infection model (17). Here, we describe the entire Brucella abortus virB region coding for a type IV secretion apparatus and demonstrate that it is a stationary-phase-induced operon that plays a critical role in virulence in vivo and intracellular multiplication within nonprofessional phagocytes.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. Bacterial strains and plasmids used in this work are listed in Table 1. Brucella strains were grown at 37°C on a rotary shaker (200 rpm) for 24 h in tryptic soy broth (TSB). E. coli strains were grown at 37°C on a rotary shaker (200 rpm) overnight in Luria-Bertani broth. When necessary, the following antibiotics were added to the indicated final concentrations: kanamycin (50 μg/mL), gentamicin (2.5 μg/mL), tetracycline (2 μg/mL), and ampicillin (50 μg/mL).

Cloning of virB region. In order to clone the virB region of B. abortus 2308, plasmid pBP2A3, containing a 1.5-kb DNA fragment with high homology to the virB9-virB10 genes of A. tumefaciens, was obtained from the B. abortus genome.
TABLE 1. Bacterial strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strains</th>
<th>Characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Brucella abortus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2308</td>
<td>Wild type, smooth, virulent, β-hemolysin, Nal&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>S19</td>
<td>Smooth, vaccine strain, Nal&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>virB1::Kan</td>
<td>2308 Nal&lt;sup&gt;+&lt;/sup&gt; Kan&lt;sup&gt;-&lt;/sup&gt;, polar mutant of virB1</td>
<td>This work</td>
</tr>
<tr>
<td>virB10::Gm</td>
<td>2308 Nal&lt;sup&gt;+&lt;/sup&gt; Gm&lt;sup&gt;-&lt;/sup&gt;, polar mutant of virB10</td>
<td>This work</td>
</tr>
<tr>
<td>virB10::lteC-Z-Gm</td>
<td>2308 Nal&lt;sup&gt;+&lt;/sup&gt; Gm&lt;sup&gt;-&lt;/sup&gt;, nonpolar mutant of virB10</td>
<td>This work</td>
</tr>
<tr>
<td>virB1::Kan virB10::lteC-Z-Gm</td>
<td>2308 Nal&lt;sup&gt;+&lt;/sup&gt; Kan&lt;sup&gt;-&lt;/sup&gt;, polar mutant of virB1, chromosomal transcriptional fusion virB10::zGm</td>
<td>This work</td>
</tr>
<tr>
<td>virB1::Kan(pVK8.3)</td>
<td><em>B. abortus</em> virB1::Kan harboring the plasmid pVK8.3</td>
<td>This work</td>
</tr>
<tr>
<td>virB10::Kan(pVK8.3)</td>
<td><em>B. abortus</em> virB10::Kan harboring the plasmid pVK8.3</td>
<td>This work</td>
</tr>
<tr>
<td>virB10::Gm(pBBR-virB10)</td>
<td><em>B. abortus</em> virB10::Gm harboring the plasmid pBBR-virB10</td>
<td>This work</td>
</tr>
<tr>
<td>virB1::Kan(pBBR4-virB1)</td>
<td><em>B. abortus</em> virB1::Kan harboring the plasmid pBBR4-virB1</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Brucella suis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1330</td>
<td>Wild type, smooth, virulent</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td><strong>Brucella ovis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>REO198</td>
<td>Wild type, rough, virulent</td>
<td>Laboratory stock</td>
</tr>
</tbody>
</table>

**Plasmids**

- pBBR1MCS-2
- pBBR1MCS-4
- pBB2A3
- pGEM-T-virB1
- pGEM-T-virB1::Kan
- pVK8.3
- pBB2A-virB1
- pBBR4-virB1

**Construction of pBBR-virB1 complementing plasmids.** PCR was carried out with primers 5'-GGGATCCGGCGGCCTGAGAGCAGAAT-3' and 5'-CCA CAGTGGGAAAGGATGAGGAA-3' to amplify a 1400-bp DNA fragment containing the complete (from the ATG to the stop codon) *B. abortus* 2308 virB10 gene. This PCR product was cloned into pGEM-T-Easy, generating plasmid pGEM-T-virB10. A 1.2-kb EcoRI fragment was excised from pGEM-T-virB10 and ligated to the EcoRI site of pBBR1MCS-2 or pBBR1MCS-4 (12) under the lacZ promoter. The resulting plasmid, named pBBR2-virB10 or pBBR4-virB10, respectively, was conjugated into the *B. abortus* virB10::Gm nonpolar mutant or the *B. abortus* virB10::Km polar mutant, respectively, by triparental mating.

**Construction of lacZ::Gm transcriptional fusion.** Plasmid pAB2001 (2) was digested with HindIII, producing a 4.5-kb fragment containing a lacZ::Gm<sup>+</sup> promoter-probe cassette. This DNA fragment was blunt ended with T4 DNA polymerase (New England Biolabs) and ligated into the NdeI site of pB2A3. The resulting plasmid, pB2A3::lacZ::Gm<sup>-</sup> (which does not replicate in *Brucella* spp.), was electroporated into *B. abortus* 2308 (5). Gm<sup>-</sup> Amp<sup>+</sup> colonies were selected as possible double-homologous recombinants by plating them on TSB agar containing gentamicin (2.5 µg/ml) or ampicillin (100 µg/ml). PCR and Southern blot analysis confirmed correct recombination events. This procedure generated strain *B. abortus* virB10::lacZ-Gm, which has a transcriptional fusion of a promoterless lacZ gene to virB10.

**Construction of the *B. abortus* virB1::Kan virB10::lacZ-Gm double mutant strain.** Plasmid pGEM-T-virB1::Kan was electroporated into the *B. abortus* virB10::lacZ-Gm strain. Bacteria were plated on TSB agar containing gentamicin (2.5 µg/ml) and kanamycin (50 µg/ml). Gm<sup>-</sup> Kan<sup>-</sup> Amp<sup>-</sup> colonies were selected as possible double-homologous recombinants, and gene replacement was confirmed by PCR analysis.
reaction mixture was centrifuged before the determination of $A_{420}\beta$-Galactosidase activity was expressed as $A_{420}/\text{volume} \times A_{420}$.

**Cell infection assays.** Log-phase growing cultures of *B. abortus* were prepared as described previously (19). HeLa cells seeded in 24-well plates ($10^5$ cells per well) were inoculated with 1 ml of minimal essential medium (Gibco, Paisley, Scotland) supplemented with 5% fetal calf serum and 2 mM glutamine (cell culture medium) containing $5 \times 10^7$ CFU of bacteria, without antibiotics. In order to ensure close contact between cells and bacteria, multiwell plates were centrifuged for 10 min at 141 $g$ at room temperature and placed in a 5% CO$_2$ atmosphere at 37$^\circ$C. After 1 h, wells were washed five times with phosphate-buffered saline (PBS) (pH 7.4) and further incubated with cell culture medium containing 100 $\mu$g of gentamicin per ml and 50 $\mu$g of streptomycin per ml to eliminate remaining extracellular brucellae. At different times, the number of intracellular viable *B. abortus* bacteria was determined as follows: cells were washed four times with PBS and treated for 10 min with 1 ml of 0.1% Triton X-100 in deionized sterile water, and lysates were serially diluted in PBS and plated on tryptic soy agar with the appropriate antibiotic to determine CFU.

**Experimental infection of mice.** The infection assay was carried out as described previously (5). Briefly, 60-day-old female BALB/c mice were injected intraperitoneally with $10^4$ CFU of *B. abortus* 2308 wild-type or mutant strains. At 15 days postinfection (p.i.), mice were sacrificed, and spleens were removed, weighed, and homogenized in 1 ml of 150 mM NaCl. Tissue homogenates were serially diluted and plated in duplicate on tryptic soy agar with the appropriate antibiotic. CFU were counted after 3 to 4 days of incubation at 37$^\circ$C.

**Nucleotide sequence accession number.** The DNA sequence of the complete *B. abortus* 2308 virB operon was deposited in GenBank under accession no. AF226278.

**RESULTS**

Identification of a homologue to the *A. tumefaciens virB* operon in *B. abortus* 2308. A partial genome project carried out in our laboratory led us to identify a clone, named pB2A3, consisting of a 1.5-kb DNA genomic fragment of *B. abortus* 2308 containing sequences highly similar to *A. tumefaciens* virB9 and virB10 genes and to ptIF and ptIG of *B. pertussis* (28). Plasmid pB2A3 was used as a probe to screen a pVK102 *B. abortus* 2308 genomic library. Cosmid pVK8.3 was selected, and the complete DNA sequence was obtained (see Materials and Methods).

Analysis of the sequence revealed the presence of a collinear array of 13 open reading frames (ORFs) with the same orientation, all of them having putative ribosome binding sites. These ORFs are organized in a manner very similar to that of the recently published *B. suis* 1330virB region (17) and of other operons coding for type IV secretion systems such as the *A. tumefaciens virB* and the *B. pertussis ptl* operons (Fig. 1).

Comparison of this operon with the recently described *B. suis* 1330 virB region revealed that the two sequences are 99.7% identical. However, a few differences were observed. *B. abortus* 2308 has a 9-bp in-frame deletion at position 8897 on the virB11 homologue gene and a 1-bp insertion at position 10739 on the virB11 homologue gene. This insertion resulted in a VirB11 predicted protein of 363 amino acids (aa) that differs from the previously reported *B. suis* 1330 VirB11, which is 397 aa long. The VirB11 homologues of *B. abortus* 2308 and *B. suis* 1330 are 99% identical in the first 309 aa, but the sequences diverge at the C-terminal portion of the predicted proteins due to the 1-bp insertion present in the *B. abortus* 2308 virB11 gene. In order to assess whether this difference is due to divergence between the two Brucella species or to a mistake in the sequencing process, we amplified, cloned, and subsequently sequenced the corresponding virB11 3′ region of *B. abortus* S-19, *B. suis* 1330, and the most divergent member of the group, *Brucella ovis* REO198. We found that all sequences analyzed code for predicted products of 363 aa identical to that of *B. abortus* 2308 (data not shown). Thus, the difference was due to a misreading in the recently published *B. suis* 1330 virB11 sequence.

Another important difference is the presence of an additional overlapping ORF between VirB11 and ORF12 of *B. abortus*. This ORF, named ORF13, encodes a hypothetical 83-aa-long protein and has a conserved ribosome binding site sequence, and, as in other ORFs of the *B. abortus virB* operon,
its ATG codon overlaps with the stop codon of the preceding ORF. ORF13 lacks similarity with any protein sequence deposited in GenBank databases.

It is interesting to note that *B. abortus* has two degenerated palindromic repeat sequences (RS in Fig. 1), one located between the VirB1 and VirB2 homologues and the other one downstream of ORF12. These RS are dispersed over the genome of all *Brucella* species and possibly behave as hot spots for insertion of IS711 (9). The presence of these flanking RS suggests that the *Brucella* VirB operon could have been acquired by horizontal transfer, although the region has a GC content similar to that of the rest of the genome. These RS have an organization similar to that found in the recently described *B. suis* virB region, except for an 18-bp inversion of the region flanked by the inverted repeats located at the end of the *B. abortus* operon (Fig. 1).

**virB** genes of *B. abortus* 2308 constitute an operon that is turned on in the stationary phase. To study how these genes are expressed in *B. abortus*, we constructed two kinds of transcriptional fusions. First, a promotorless lacZ::Gm cassette was ligated at the NruI site on the coding sequence of the virB10 gene. This procedure generated strain *B. abortus* 2308 virB10::lacZ::Gm. Second, to determine if *Brucella* virB genes function as an operon starting at virB1, a polar insertional virB1 mutant was constructed on a *B. abortus* virB10::lacZ::Gm strain to generate the double mutant strain *B. abortus* virB1::Km virB10::lacZ::Gm (Fig. 2).

Both strains were used to measure β-galactosidase activity during growth. Figure 3A shows that, after 20 h of growth, β-galactosidase activity starts to increase, reaching a 30-fold induction after 36 h of growth (optical density at 600 nm of 2.6 [Fig. 3B]), indicating that the *B. abortus* virB region is turned on at the beginning of the stationary phase. However, β-galactosidase was not induced when a polar mutation was introduced in the virB1 gene (Fig. 3A). This result indicates that the *B. abortus* virB region behaves as an operon transcribed from virB1.

The virB operon is essential for intracellular replication of *B. abortus*. The effect of the virB operon on intracellular replication in HeLa cells was studied with a virB1 polar mutant (*B. abortus* virB1::Kan) generated as described in Materials and Methods. Figure 4 shows that at 4 h p.i. the number of intracellular *B. abortus* virB1::Kan mutants was the same as that of the wild-type parental strain. However, at longer times p.i., the CFU of the mutant decreased and no bacteria were recovered after 48 h. It can be observed that the number of wild-type...
intracellular bacteria grew exponentially during this period, reaching $7.4 \times 10^7$ CFU/ml at 48 h p.i. These results indicate that the \textit{B. abortus} \textit{virB} operon is essential for intracellular survival and is not involved with the invasion process.

Plasmid pVK8.3, containing the entire \textit{virB} operon introduced into \textit{B. abortus} \textit{virB1::Kan}, restored intracellular multiplication of the mutant to wild-type levels. Plasmid pBBR4-\textit{virB1}, which contains only the complete \textit{virB} gene under the \textit{lacz} promoter, failed to complement the polar \textit{virB1} mutation. This result confirms the polar nature of the \textit{virB1::Kan} mutant.

**Role of \textit{virB10} in \textit{B. abortus} intracellular multiplication.** The \textit{A. tumefaciens} \textit{virB} operon is the best-studied type IV secretion system. Structure-function studies have been made with the T-complex transport machinery, assigning a possible role in transmembrane transport to most of its components. \textit{VirB10} is a member of this transport apparatus that has homologues in all type IV secretion systems described so far (4, 7). \textit{VirB10} is an inner membrane-bound protein that has a C-terminal periplasmic domain, as shown by PhoA fusion experiments (8).

To test the role of the \textit{B. abortus} \textit{virB10} gene in intracellular replication, two targeted insertion mutants were constructed. \textit{B. abortus} \textit{virB10::Kan} has an insertion of a polar kanamycin resistance cassette interrupting the \textit{virB10} sequence at an \textit{NruI} restriction site, and the \textit{B. abortus} \textit{virB10::Gm} mutant has an insertion of a nonpolar gentamicin resistance cassette interrupting the \textit{virB10} sequence at the same restriction site.

The intracellular behavior of both mutants was studied with \textit{HeLa} cells. As shown in Fig. 5A, during the first 8 h p.i. the numbers of intracellular bacteria for both mutants were similar to that of the parental wild-type strain. At 24 h p.i., both polar and nonpolar \textit{virB10} mutants were affected in their ability to survive inside \textit{HeLa} cells. However, the number of \textit{B. abortus} \textit{virB10::Gm} mutants remained significantly higher than the number of polar mutants. After 48 h p.i., no viable \textit{virB10} mutants were recovered, while the wild-type strain grew exponentially, reaching $1.38 \times 10^7$ CFU/ml. No host cell damage was observed with the wild type or the mutants during the course of the experiment. Thus, the lack of recovery of viable \textit{Brucella} bacteria was the result of effective control of intracellular survival rather than the result of cell damage.

Plasmid pBBR2-\textit{virB10} containing the complete \textit{virB10} gene under the \textit{lacz} promoter was used to complement the nonpolar \textit{B. abortus} \textit{virB10::Gm} mutant strain. The \textit{B. abortus} \textit{virB10::Kan} polar mutant was complemented either with cosmids pVK8.3 containing the entire \textit{virB} operon or with pBBR4-\textit{virB10}. In every case, the ability of mutants to replicate within \textit{HeLa} cells was restored, the bacteria reaching numbers similar to those of the wild-type strain (Fig. 5B). These results indicate that \textit{virB10} per se is essential for \textit{B. abortus} intracellular multiplication within nonprofessional phagocytes and that \textit{virB11-ORF12-ORF13} are not required for intracellular survival.

Since many \textit{virB} genes of \textit{A. tumefaciens} and \textit{B. abortus}, as well as those in other multicomponent secretion systems, overlap, it has been proposed that they are probably translationally coupled, suggesting that stoichiometry and order of expression may be important for the biogenesis of the secretion apparatus (4, 16). The fact that plasmid pBBR2-\textit{virB10} restored the intracellular replication of the \textit{B. abortus} \textit{virB10::Gm} mutant at
levels similar to those of the wild type suggests that this hypothesis may not be applicable to the B. abortus virB operon. **virB10 is essential for virulence in mice.** The effect of B. abortus virB10 on virulence was studied in a mouse infection assay. Equal numbers of bacteria of the viable B. abortus 2308 wild-type strain or the virB10 polar and nonpolar insertion mutants were injected intraperitoneally into 90-day-old female BALB/c mice. Mice were sacrificed 15 days p.i., spleens were removed, and the number of Brucella bacteria recovered from spleens was estimated as described in Materials and Methods. As shown in Fig. 6, 4 × 10^5 viable bacteria were recovered from spleens of mice receiving the wild-type B. abortus 2308 whereas no viable bacteria were obtained from spleens of mice inoculated with the B. abortus virB10::Kan polar mutant. The number of bacteria recovered from spleens of mice inoculated with the virB10 nonpolar mutant was significantly lower than the nonpolar recovered from viable bacteria inoculated with the B. abortus virB10::Kan polar mutant (3.5 × 10^2 CFU/spleen) but significantly higher than CFU recovered from mice inoculated with B. abortus virB10::Kan polar mutant (<10 CFU/spleen). B. abortus virB10::Kan harboring pVK8.3 and B. abortus virB10::Gm harboring pBBR2-plasmid were recovered virulence, reaching viable counts similar to those of the wild-type parental strain.

These results indicate that virB10 and downstream sequences (virB11-ORF12-ORF13) are essential for Brucella pathogenesis in mice and suggest that the integrity of the virB operon is required for wild-type virulence.

**DISCUSSION**

The mechanism of virulence of Brucella spp. is not yet fully understood. It has become evident that virulence is linked to intracellular survival in brucellae. The understanding of the molecular events that allow brucellae to reach an intracellular niche where the bacteria freely multiply without causing any cellular damage will shed light on the mechanism of pathogenesis. This intracellular compartment is out of the reach of antibiotics and bactericidal plasma factors such as complement and antibodies and may be determinant of the chronic nature of this worldwide zoonosis.

In some other bacteria, pathogenesis has been associated with the integrity of a type IV secretion system. In A. tumefaciens, a type IV secretion system participates in delivering oncogenic T-DNA from the bacterium to the plant cell, whereas in B. pertussis it participates in the secretion of pertussis toxin. It is interesting to note that these type IV secretion systems display high similarity to E. coli Tra proteins, a cluster of genes that code for the conjugal pilus and mating pore. The tra region is organized in a manner similar to that of the A. tumefaciens, B. abortus, B. suis, and B. pertussis virB regions. TraL has high similarity to VirB1, a gene product that is absent in B. pertussis, L. pneumophila, Ricetettia prowazekii, and H. pylori.

Recently, O’Callaghan et al. described the presence of a virB region of B. suis 1330 (17) containing 11 genes highly similar to the 11 virB genes of A. tumefaciens and an extra ORF12 that shares homology with an adhesin of Pseudomonas fluorescens. Between the virB1 and virB2 homologues and at the end of ORF12, palindromic RS characteristic of intergenic regions of the Brucella genome were noted. This finding raises the question whether the virB1 gene is part of the same transcript as the rest of the virB genes. The authors reported that mutants with mutations in virB5, virB9, or virB10 were highly attenuated in an in vitro infection model whereas an ORF12 mutant was not and stressed that they do not know whether the mutants analyzed were affected in only one protein or whether they were polar.

Here, we demonstrate that B. abortus 2308 virB is indeed an operon, transcribed from the virB1 promoter, and show that, besides being required for intracellular survival, the operon constitutes a major determinant of virulence in mice. Moreover, the β-galactosidase fusion experiments showed that the operon is turned on at the beginning of the stationary phase of vegetative growth. Recently, Robertson and Roop described a Brucella hfg gene required for stationary-phase stress resistance and intracellular multiplication (21). They pointed out that stationary-phase gene expression might be important for virulence. This hypothesis may be applicable to the virB operon, although the pattern of virB induction in an intracellular environment remains to be characterized.

The requirement of an active virB operon for intracellular survival of brucellae may have two possible explanations: (i) the virB operon is essential to reach a competent intracellular replication niche or (ii) the virB operon is needed for replication once the intracellular replication niche has been established. Future investigation will focus on the elucidation of these questions.

Sola-Landa et al. described a B. abortus mutant in a two-component regulatory system, bvrR-bvrS, that displayed a reduced ability to enter into HeLa cells (26). This is not the case for virB mutants, since they have the same penetration rate as the wild-type strain, thus indicating that the virB operon does not participate in the mechanism of entry into the eukaryotic cell.

Homologues of A. tumefaciens virB10 and virB11 are present in type IV transport mechanisms of E. coli, B. pertussis, H. pylori, R. prowazekii, and L. pneumophila, while homologues of other components are not (7), indicating that these two proteins may play a common role in all these systems. Recently, Segal et al. reported that a type IV secretion system is required for plasmid RSF1010 conjugation in L. pneumophila (hvg genes) (24) and suggested that it may have an origin different from that of the dot-icm system. Both systems have conserved VirB10 and VirB11 homologues. VirB11 also has homologues in some type II secretion systems and pilin biosynthesis components. It has been proposed previously that there is a minimum core structure necessary for an active type IV transport system.
system (7) that includes VirB4, VirB7, VirB9, VirB10, VirB11, and VirD4. The B. abortus virB operon contains homologues to all these proteins except for VirD4. In vitro cell infection assays showed that both polar and nonpolar B. abortus virB10 mutants failed to survive intracellularly, although some differences were observed. The results shown in Fig. 5 demonstrate that virB10 plays an essential role in intracellular survival in HeLa cells whereas virB11-ORF13-ORF12 do not. Moreover, both mutants showed different behaviors in mice depending on the nature of the mutation introduced in virB10. A polar mutation preventing the transcription of the virB10-virB11-ORF13-ORF12 renders Brucella unable to establish an infection process in mice, being completely cleared from the spleen. The virB10 nonpolar mutant showed a 1,000-fold reduction in spleen counts compared with those of the wild-type strain. This phenotype is probably a consequence of defective secretion of putative effector molecules, due to the presence of a type IV secretion system that works inefficiently in the absence of virB10.

The evolutionary linkage between DNA transfer and protein export systems has already been established; in some cases, they are functionally related and some components are capable of substituting for each other. One example is the A. tumefaciens T-DNA transport system, which is able to deliver IncQ plasmids to recipient cells (4). In view of the similarities that exist between the organization of the A. tumefaciens and B. abortus virB operons and considering that they are closely related species, the question whether the B. abortus type IV secretion system is competent to deliver non-self-transmissible DNA remains to be answered.

Although this report shows that the B. abortus virB operon is essential for intracellular multiplication and virulence, the effector molecule secreted by this system remains unknown. Future investigations directed toward the elucidation of the nature of the effector molecules will let us know the molecular mechanism underlying the infection process of this fascinating pathogen.

ACKNOWLEDGMENTS

We thank A. J. Parodi and I. J. Cazzulo for critical reading of the manuscript, J. E. Ugalde for helpful suggestions and for providing the gentamicin nonpolar resistance cassette, and F. Fraga for technical assistance. This work was supported by grant PICT97-01767 from the Agencia Nacional de Promoción Científica y Tecnológica, Argentina. D.S. and R.A.U. are members of the Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET, Argentina. R.S. and D.J.C. contributed equally to the work.

REFERENCES


Downloaded from http://jb.asm.org/ on November 9, 2017 by guest