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Sequence and Expression Analysis of *virB9* of the Type IV Secretion System of *Ehrlichia canis* Strains in Ticks, Dogs, and Cultured Cells

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***Ehrlichia canis virB9* was cloned and expressed. The sequences of *virB9* from six geographic locations were identical. *virB9* was transcribed by *E. canis* in dogs, ticks, and cell culture. Infected dogs had antibodies to recombinant VirB9, indicating that VirB9 was produced by *E. canis* in dogs and was antigenic.**

Ehrlichia canis, the etiologic agent of canine monocytic ehrlichiosis (CME), is a gram-negative obligate intracellular bacterium that replicates in monocytes and macrophages (21, 29). CME has been recognized as a significant canine disease that causes considerable levels of morbidity and mortality worldwide (13, 21). *E. canis* is primarily transmitted by the brown dog tick, *Rhipicephalus sanguineus* (16, 18, 29). Without antibiotic treatment, dogs infected with *E. canis* remain infected; in some cases, the infection can persist even after antibiotic treatment (15, 29). There is currently no vaccine available for CME. The type IV secretion system (TFSS) machinery is a multicomponent pore that allows the delivery of virulence factors from bacteria across the bacterial and host membranes into the cytoplasm of the host cell (3). The TFSS has been shown to be essential for the intracellular survival of several facultative intracellular bacteria, including *Brucella*, *Bartonella*, and *Legionella* (1, 19, 24, 26). In a previous study, *virB*/*D* operons encoding the TFSS machinery in the human monocytic ehrlichiosis agent *Ehrlichia chaffeensis* and in the human granulocytic ehrlichiosis agent *Anaplasma phagocytophilum* were identified (20). VirB9 is one of a few outer membrane components of the TFSS machinery. It has been shown that the *virB9* gene of *A. phagocytophilum* was transcribed in blood specimens from human patients and in an experimentally infected mouse and horse (20). However, it is not known whether VirB9 is expressed and immunogenic in infected mammalian hosts, or whether *virB9* is transcribed in ticks. In the present study, we cloned and expressed *virB9* from *E. canis* and investigated these questions.

Cloning and sequencing of *virB9*. Primers EcavB9f1 and EcavB9r2 (Table 1) were designed to amplify the entire *virB9* sequence from *E. canis* Oklahoma^T DNA extracted from purified bacteria as previously described (7, 23). PCR products were cloned into the pCRII TA cloning vector and sequenced (12). Analysis of the nucleic acid sequence of the 1,047-bp PCR product showed that it contained an open reading frame of 825 bp encoding a 275-amino-acid protein with a molecular mass of 31,948 Da. The deduced amino acid sequence of the *E. canis* Oklahoma^T VirB9 protein included a preferred cleavage site for signal peptidases (Ser-X-Ser) at amino acid positions 25 to 27, leading to a predicted N-terminal cleavable signal peptide of 27 amino acids. The CLUSTAL V method was used to align the nucleic acid sequence of the *E. canis* Oklahoma^T *virB9* gene, including the signal sequence, with those of *E. chaffeensis* Arkansas^T and *A. phagocytophilum* HZ. The results showed that the *E. canis* Oklahoma^T *virB9* base sequence shared 78.0 and 46.5% identity with those of *E. chaffeensis* and *A. phagocytophilum*, respectively. The alignment of the predicted amino acid sequence of *E. canis* Oklahoma^T *virB9* with those of *Wolbachia* sp. strain *w*Tai, *Wolbachia* sp. strain *w*KueYo, and *Neorickettsia sennetsu* Miyayama^T showed identity levels ranging from 25.0 to 77.3%. A total of 47 amino acids (17% of total amino acids) were consistently conserved across all VirB9 proteins belong to the family *Anaplasmataceae* (Fig. 1A). A phylogenetic tree consisting of the amino acid sequences of VirB9 from 14 different gram-negative bacteria, including *E. canis*, was constructed based on the estimated evolutionary distances. VirB9 sequences from *E. canis*, *E. chaffeensis*, *A. phagocytophilum*, a *Wolbachia* sp., *N. sennetsu*, and two *Rickettsia* spp. formed a cluster, as shown in Fig. 1B. By using the Protean program (DNASTAR Inc., Madison, Wis.), nine surface-exposed regions containing 5 to 18 amino acids were identified by the Emini method on *E. canis* VirB9 that corresponded to the surface-exposed regions of *E. chaffeensis* VirB9 (data not shown). The surface-exposed regions of *E. canis* VirB9 had a high antigenic index by the method of Jamison-Wolf, similar to those of *E. chaffeensis* VirB9. *E. canis* VirB9 was predicted to contain seven T-cell motifs by the AMPHI method.

Sequence analysis of the *virB9* gene from different geographic locations. Full-length *virB9* genes (959 bp) of *E. canis* were amplified by using the primer pair EcavB9of and EcavB9or (Table 1), from four dog blood 16S rRNA gene-based PCR-positive DNA samples derived from Arizona (strain Arizona-1), California, New Mexico (strain New Mexico-1), and Hawaii and from a DNA sample from the *E. canis* VDE strain culture isolated from a dog in Venezuela (26). The resulting PCR products were sequenced. These five DNA sequences were aligned with the Oklahoma sequence by the CLUSTAL V method, and all six were found to be 100% identical.

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TABLE 1. *virB9* primer pairs used in this study

Primer	Sequence	Purpose
EcavB9f1	5′-GAATTTACCCATCCAAGTGGC-3′	Cloning and sequencing
EcavB9r2	5′-CCAACAACGTTTACATTCTCTTC-3′	
EcavB9f3	5′-TCGGGATCCGAGCATAGCTGATAA CCCTGTTAC-3′	Cloning and expression
EcavB9r3	5′-AGTGCGGCCGCTTATAGTTTTTTGT TAGTTATTTTCACAG-3′	
EcavB9of	5′-CATTATCATTTCAATACGTAACTC-3′	Cloning and sequencing
EcavB9or	5′-TTTTGATTTTCTTCTGACATAGTG-3′	
EcavB9f	5′-GCACACTCCATAAGCATAGCTG-3′	Expression analysis
EcavB9r	5′-CTCTTAGGTCAGAGTACTCGTC-3′	

Analysis of *virB9* expression. Two 1- to 2-year-old specific-pathogen-free female beagles were infected by intravenous inoculation with naturally infected samples of dog blood from Arizona (strain Arizona-2) and New Mexico (strain New Mexico-2), respectively. Two 1- to 2-year-old specific-pathogen-free female mixed-breed dogs, designated dogs B and C, were intravenously inoculated with *E. canis* Oklahoma^T cultivated in DH82 cells (27). Peripheral blood mononuclear cells (PBMCs) were isolated from dog blood samples, as described elsewhere (11). Oklahoma^T and VDE were cultured in DH82 cells. A pool of *E. canis* 16S rRNA-positive ticks was removed from each of five dogs in Arizona (a total of 11 ticks) (12). RNA was extracted from dog PBMCs, cell culture, and tick specimens and treated with DNase and reverse transcribed as previously described (10). The resulting cDNA template was amplified with the primers EcavB9f and EcavB9r (Table 1). As a negative control, an equivalent sample of RNA was subjected to the same procedure in the absence of reverse transcriptase, to exclude the possibility that the RNA preparation was contaminated with genomic DNA. *virB9* expression was detected in two dogs experimentally infected with *E. canis* Arizona-2 and New Mexico-2 throughout the 6-week period after intravenous inoculation of infected dog blood (Fig. 2). *virB9* expression was

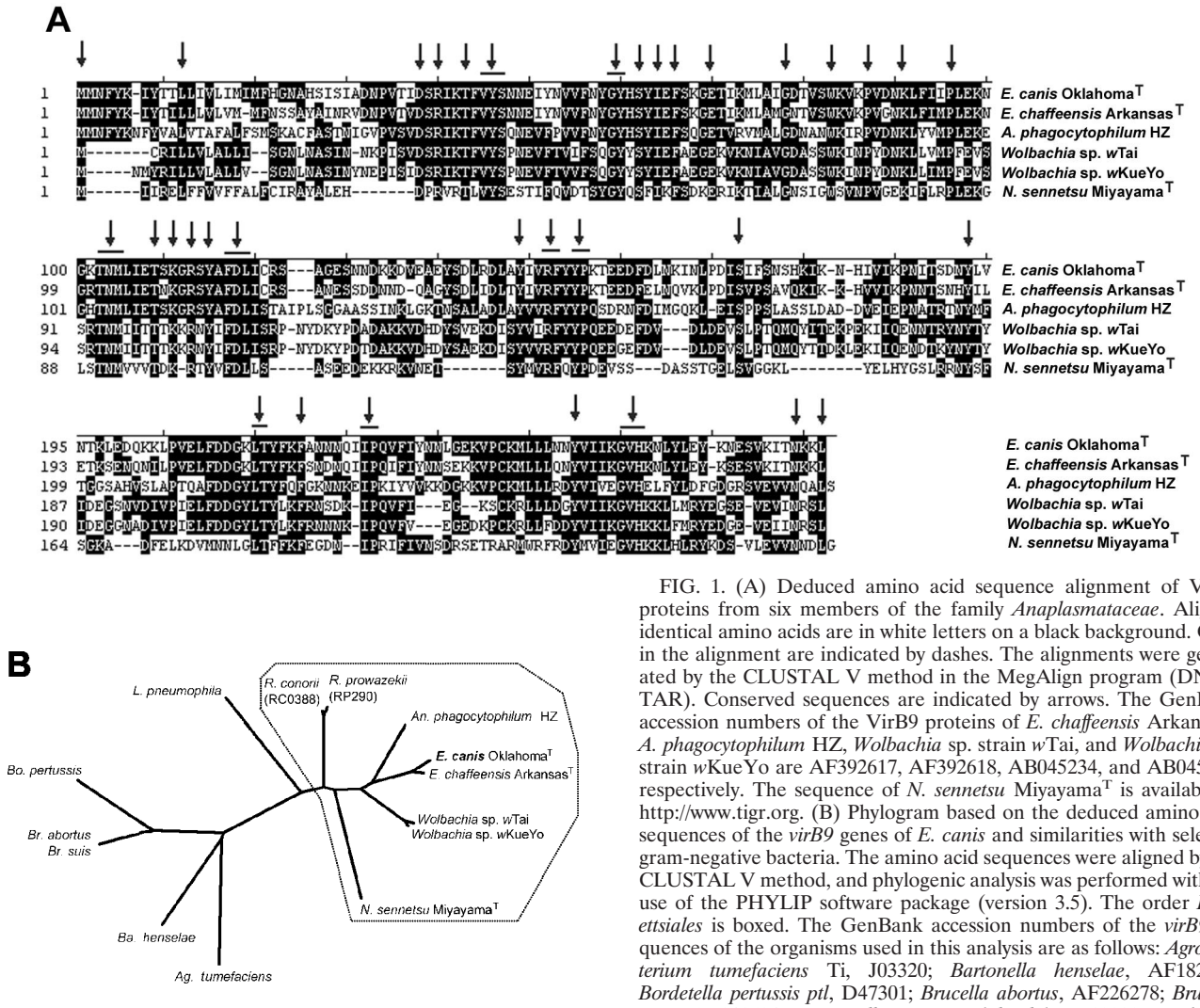

FIG. 1. (A) Deduced amino acid sequence alignment of VirB9 proteins from six members of the family *Anaplasmataceae*. Aligned identical amino acids are in white letters on a black background. Gaps in the alignment are indicated by dashes. The alignments were generated by the CLUSTAL V method in the MegAlign program (DNASTAR). Conserved sequences are indicated by arrows. The GenBank accession numbers of the VirB9 proteins of *E. chaffeensis* Arkansas^T, *A. phagocytophilum* HZ, *Wolbachia* sp. strain *w*Tai, and *Wolbachia* sp. strain *w*KueYo are AF392617, AF392618, AB045234, and AB045235, respectively. The sequence of *N. sennetsu* Miyayama^T is available at http://www.tigr.org. (B) Phylogram based on the deduced amino acid sequences of the *virB9* genes of *E. canis* and similarities with selected gram-negative bacteria. The amino acid sequences were aligned by the CLUSTAL V method, and phylogenic analysis was performed with the use of the PHYLIP software package (version 3.5). The order *Rickettsiales* is boxed. The GenBank accession numbers of the *virB9* sequences of the organisms used in this analysis are as follows: *Agrobacterium tumefaciens* Ti, J03320; *Bartonella henselae*, AF182718; *Bordetella pertussis ptl*, D47301; *Brucella abortus*, AF226278; *Brucella suis*, AF141604; *Legionella pneumophila lvh*, Y19029; *Rickettsia prowazekii*, AJ235271; *Rickettsia conorii*, NC_003103.

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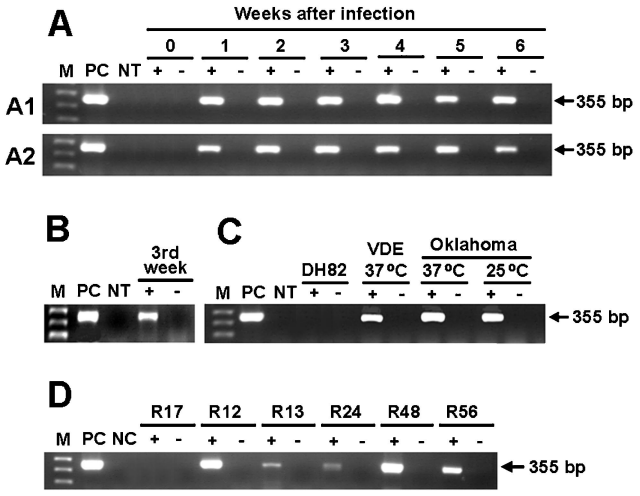

FIG. 2. *virB9* expression by *E. canis* in naturally infected *R. sanguineus* tick groups from Arizona and in samples of dog blood. Total RNA was extracted and subjected to reverse transcription-PCR, as described in Materials and Methods. The amplified products were separated on agarose gels and visualized with ethidium bromide. Lane M, molecular size marker (1 Kb Plus DNA ladder; Invitrogen); lane PC, product amplified by PCR with DNA from *E. canis* culture as a template and respective primer pairs, used as a positive control for PCR and as a determinant of amplicon size on the gel; lane NT, no template (negative control). Samples were run in the presence (+) and absence (−) of reverse transcriptase, as a control for DNA contamination. DH82, uninfected DH82 cells. (A) Weekly expression levels of *virB9* between day 0 and 6 weeks after infection of dog PBMCs (A1, Arizona-2; A2, New Mexico-2); (B) *virB9* expression in dog C (Oklahoma^T, *virB9* expression was analyzed only at the third week after infection in dog C); (C) *virB9* expression in culture of *E. canis* VDE and Oklahoma^T in DH82 cells; (D) *virB9* expression in naturally infected tick groups (R12, one engorged female; R13, two engorged females and one unengorged male; R24, one male and one semiengorged female; R48, two males and one semiengorged female; R56, two unengorged females; and R17 [*E. canis* 16S rRNA-negative ticks as a control], two engorged females and one male).

also detected 3 weeks after a dog was inoculated with the Oklahoma^T strain, although this was the only time point examined for that animal. *virB9* was transcribed in the VDE and Oklahoma strains of *E. canis* in culture at 37°C. *virB9* was also transcribed in the Oklahoma strain when it was incubated at 25°C, which approximates the natural tick temperature. *virB9* expression was detected in all five groups of naturally infected ticks (Fig. 2).

Expression of recombinant VirB9 (rVirB9) in *Escherichia coli* and Western blot analysis. A pair of primers (EcavB9f3 and EcavB9r3) (Table 1) was designed to clone and express the VirB9 protein. The resulting PCR products were digested with *Bam*HI and *Not*I, ligated into the *Bam*HI and *Not*I sites of the pET33b vector (Novagen Inc., Madison, Wis.), and amplified in *E. coli* Novablue cells (Novagen). *E. coli* BL21(DE3)pLys was transformed with the recombinant plasmid for protein expression. rVirB9 was harvested after 4 h of induction at 27°C with isopropylthio-β-D-galactoside and affinity purified by His-Bind resin (Novagen) column chromatography. *E. coli* transformed with the *E. canis virB9* expression plasmid (designated pET33beca*virB9*) produced a 287-amino-acid fusion protein (32,932 Da). The protein included 39 amino acids at the N terminus derived from pET33b. The expressed rVirB9 fusion protein was purified by affinity chromatography and detected as a single band of approximately 33 kDa on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Fig. 3A). Western blot analysis was performed using the samples from four experimentally infected dogs as described above, along with three other naturally infected dog plasma samples (Ohio, Arizona-3, and New Mexico-3), to detect antibodies to rVirB9 as previously described (22). All plasma samples from *E. canis*-infected dogs reacted with rVirB9, as determined by Western blot analysis. None of the samples tested reacted with the *E. coli* lysate, included as a negative control (Fig. 3B). Three *E. canis* indirect-fluorescent-antibody-negative plasma samples (from noninfected dogs) did not react with either rVirB9 or *E. canis* lysate (data not shown). These results showed that VirB9 is expressed by *E. canis* in infected dogs and that it is highly immunogenic.

VirB9 is a channel protein and forms heterodimers with VirB7 that localize at the outer membrane and play a critical role in stabilizing other VirB proteins during assembly of the TFSS machinery (5). Previously, eight *virB* and *virD* genes were

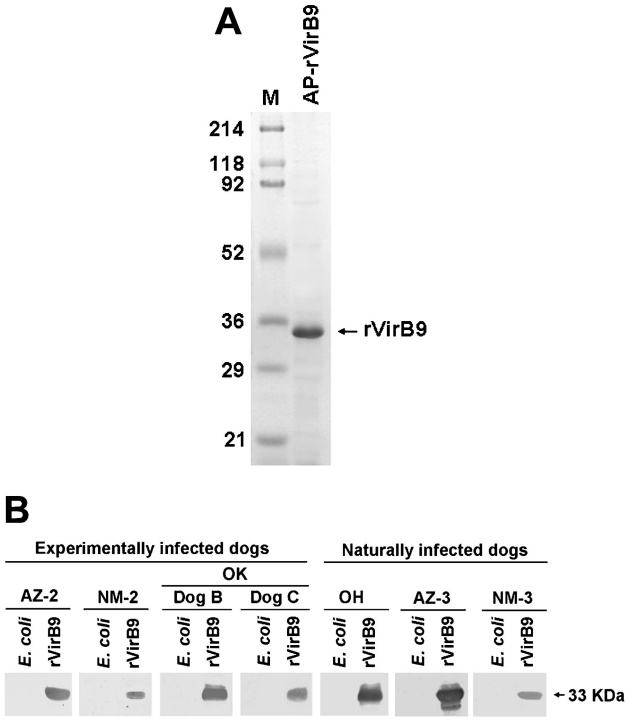

FIG. 3. (A) SDS-polyacrylamide gel electrophoresis analysis of purified *E. canis* Oklahoma^T rVirB9. Affinity-purified (AP) rVirB9 (2 μg) was subjected to SDS-polyacrylamide gel electrophoresis followed by Coomassie blue staining. M, molecular size marker. The numbers on the left are molecular masses, in kilodaltons. (B) Western immunoblot analysis to detect *E. canis* VirB9-specific antibody in plasma samples from infected dogs. pET33b-transformed *E. coli* was used as a negative control. rVirB9, affinity-purified recombinant fusion protein of *E. canis*. Antigens (15 μg of *E. coli* and 2 μg of rVirB9) were subjected to Western blot analysis with serum derived from the blood of various infected dogs (OK, Oklahoma; OH, Ohio; AZ, Arizona; NM, New Mexico). The arrow on the right indicates the apparent molecular mass of rVirB9, based on broad-range prestained standards (Bio-Rad Laboratories, Richmond, Calif.).

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detected in the human ehrlichiosis agents *E. chaffeensis* and *A. phagocytophilum*. All eight *virB* and *virD* genes were found to be expressed by *E. chaffeensis* and *A. phagocytophilum* in culture (20). Five of these genes (*virB8*, *virB9*, *virB10*, *virB11*, and *virD4*) were polycistronically transcribed and controlled through at least two tandem promoters located upstream of *virB8*. Thus, it is likely that *virB9* is transcribed by *E. canis* as a part of the *virB*/*D* operon in cell culture. Transcription of the *virB* operon in *Agrobacterium tumefaciens* has been reported to be regulated in vitro by various environmental factors, including temperature (2, 4). Expression of VirB proteins in *Brucella suis* was greater in cultures grown at 37 than at 20°C (4). We found that both the Oklahoma and VDE strains of *E. canis* expressed *virB9* in DH82 canine macrophage cell cultures at 37°C and that the Oklahoma strain also expressed *virB9* at 25°C. Since the cultures were not synchronized and reverse transcription-PCR was not quantitative, we did not compare the relative *virB9* expression levels in this study. It has been clearly established that the TFSS is essential for the survival and replication of intracellular pathogens, such as *Brucella*, *Bartonella*, and *Legionella pneumophila*, in that the TFSS prevents bacterial inclusions from trafficking to lysosomes (1, 5, 6, 9, 14, 19). *E. canis* is an intracellular pathogen that resides in the membrane-bound inclusion that does not fuse with lysosomes in monocytes and macrophages. The function of *E. canis virB9* may be related to facilitating intracellular survival and replication, similar to its function in other intracellular pathogens. Previous studies investigating the role of the TFSS in *L. pneumophila* showed that the organism has two types of TFSS. The Lvh system functions as a DNA conjugation system but does not contribute to pathogenesis, whereas the Dot/Icm system acts in protein delivery and is required for virulence (5, 17). Curiously, *virB9* sequences of *E. canis* as well as other members of the order *Rickettsiales* are more closely related to the ortholog sequence in the Lvh system than to that in the Dot/Icm system. In the present study, we found that the *virB9* sequences of *E. canis* strains derived from six geographic regions were identical, showing that *virB9* is more highly conserved than either *p30-10*, which encodes one of the major outer membrane proteins (12), or the 16S rRNA genes of *E. canis* (27, 28). For vaccine development, the ideal antigenic epitopes should be conserved to protect against all strains (8, 25, 29). *E. canis* VirB9 seems to be both highly antigenic and expressed in both mammalian and tick hosts. Based on these facts, we speculate that inhibition of VirB9 function may inhibit the intracellular survival of *E. canis* and thus may serve as a useful vaccine candidate for canine ehrlichiosis.

Nucleotide sequence accession numbers. The GenBank accession numbers for the *E. canis virB9* genes are as follows: Oklahoma^T, AY205339; Arizona-1, AF546158; California, AY205340; Hawaii, AY205341; New Mexico-1, AY205342; and VDE, AY205343.

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