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Characterization and Transcriptional Analysis of Gene Clusters for a Type IV Secretion Machinery in Human Granulocytic and Monocytic Ehrlichiosis Agents

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***Anaplasma* (*Ehrlichia*) *phagocytophila* and *Ehrlichia chaffeensis*, the etiologic agents of granulocytic and monocytic ehrlichioses, respectively, are obligatory intracellular bacteria that cause febrile systemic illness in humans. We identified and characterized clusters of genes for a type IV secretion machinery in these two bacteria, and analyzed their gene expression in cell culture and mammalian hosts. Eight *virB* and *virD* genes were found in each bacterial genome, and all of the genes were transcribed in cell culture. Although the gene order and orientation were similar to those found in other bacteria, the eight *virB* and *virD* genes were clustered at two separate loci in each genome. Five of the genes (*virB8*, *virB9*, *virB10*, *virB11*, and *virD4*) were located downstream from a *ribA* gene. These five genes in both *A. phagocytophila* and *E. chaffeensis* were polycistronically transcribed and controlled through at least two tandem promoters located upstream of the *virB8* gene in human leukemia cell lines. The *virB9* gene of *A. phagocytophila* was transcriptionally active in peripheral blood leukocytes from human ehrlichiosis patients and experimentally infected animals. Three of the remaining genes (*virB3*, *virB4*, and *virB6*) of both *A. phagocytophila* and *E. chaffeensis* were arranged downstream from a *sodB* gene and cotranscribed with the *sodB* gene through one or more *sodB* promoters in human leukocytes. This suggests that transcription of the three *virB* genes in these two *Anaplasma* and *Ehrlichia* spp. is regulated by factors that influence the *sodB* gene expression. This unique regulation of gene expression for the type IV secretion system may be associated with intracellular survival and replication of *Anaplasma* and *Ehrlichia* spp. in granulocytes or monocytes.**

Human granulocytic ehrlichiosis (HGE) and human monocytic ehrlichioses (HME) are newly discovered tick-borne febrile illnesses of increasing importance in the United States (1, 7) and have been recognized in several countries in Europe and Africa and in Mexico. The clinical signs of HGE and HME are similar. The patients frequently require hospitalization, and they may die when the treatment is delayed or they are immunocompromised (15, 30). HGE and HME are caused by obligatory intracellular bacteria, *Anaplasma* (*Ehrlichia*) *phagocytophila* (7, 13) and *Ehrlichia chaffeensis* (1), which have tropism for granulocytes and monocytes, respectively. These agents reside and replicate within membrane-bound inclusions (parasitophorous vacuoles) in the cytoplasm of phagocytes. The 16S rRNA gene sequences of *A. phagocytophila* and *E. chaffeensis* are 7.5% divergent (7), and their ultrastructures, antigenic compositions, inclusion compartments, sensitivities to intracellular iron depletion, and properties of the major outer membrane protein gene family are distinct (5, 6, 20, 24, 25, 28, 29, 32, 42–44).

Type IV secretion systems are ancestrally related to the bacterial conjugal system and are thought to function to deliver effector macromolecules produced by parasitic or symbiotic bacteria into eukaryotic target cells. A set of genes orthologous to *virB* and *virD* genes for a type IV secretion machinery of *Agrobacterium tumefaciens*, a phytopathogen, has been found in chromosomes or plasmids of more than a dozen different gram-negative bacterial species to date (8–10, 14). The *vir* transport system of *Agrobacterium tumefaciens* mediates the transfer of T-DNA on the Ti plasmid into the nuclei of infected plant cells, and the integration of this DNA into the plant genome leads to a plant tumor (16). The *virB* operon (containing 11 *virB* genes) and the *virD4* gene encode the components of the type IV secretion machinery, and the assembled gene products serve as a transporter for delivering the substrate complex (T-DNA, VirE2, VirD2, and VirF) from *Agrobacterium tumefaciens* into recipient plant cells. The T-DNA transport system is related to conjugal systems (Tra, Trw, and Trb) of the pKM101 (IncN), R388 (IncW), RP4 (IncP), and F (IncF) plasmids in *Escherichia coli* in sequence similarity and structure-function studies (9, 10). For obligate intracellular bacteria, the genes for a type IV secretion machinery have been described only for *Rickettsia prowazekii*, the agent of epidemic typhus (2); *Rickettsia conorii*, the agent of Mediterranean spotted fever (23); and *Wolbachia* spp., symbionts of aphids (19). In *R. prowazekii* and *R. conorii*, 16 *vir* genes (1 *virB3*, 2 *virB4*, 5 *virB6*, 1 *virB7*, 2 *virB8*, 2 *virB9*, 1 *virB10*, 1 *virB11*, and 1 *virD4*) were identified by genome sequencing and further analysis (2, 23, 37), but the transcriptional activities of these genes have not been examined. In *Wolbachia* spp., five *vir* genes (*virB8*, *-B9*, *-B10*, *-B11*, and *-D4*) were identified, and these genes were polycistronically transcribed (19); however, other *vir* genes have not been reported, and the promoter

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region for the wolbachial *virB8-D4* transcript has not been determined. In the present study, we identified and characterized (i) eight *vir* genes (*virB3*, *-B4*, *-B6*, *-B8*, *-B9*, *-B10*, *-B11*, and *-D4*) in two distinct and major human ehrlichiosis agents, *A. phagocytophila* (causing HGE) and *E. chaffeensis* (causing HME); (ii) the transcriptional mode of the *vir* genes of these two bacteria in cell culture, human patients, and experimentally infected animals; and (iii) the promoter regions for the transcription of the *vir* gene clusters. A unique feature found in both *A. phagocytophila* and *E. chaffeensis* is the cotranscription of a superoxide dismutase gene (*sodB*) and three *virB* genes (*virB3, -B4*, and *-B6*) through *sodB* promoters. This is the first description and expression analysis of the type IV secretion system in the genera *Anaplasma* and *Ehrlichia*.

MATERIALS AND METHODS

Organisms and culture. *A. phagocytophila* (strains HZ and LL and isolates I-NY31, I-NY36, and I-NY37) was cultivated in HL-60 cells as described elsewhere (32, 44; Q. Lin, N. Zhi, N. Ohashi, H. W. Horowitz, M. E. Aguero-Rosenfeld, G. P. Wormser, John Rattalli, and Y. Rikihisa, submitted for publication). Strain HZ was used in all experiments, and four other strains and isolates were used for an experiment for Fig. 3. *E. chaffeensis* strain Arkansas was grown in DH82 cells (canine monocytic cell line) or THP-1 cells as described previously (6, 31). These organisms were purified from the infected cells by Sephacryl S-1000 chromatography (31) or Percoll density gradient centrifugation (25) for preparation of genomic DNA.

Cloning of the genes for a type IV secretion machinery in *A. phagocytophila* and *E. chaffeensis*. We designed several degenerate primer pairs based on the conserved sequences of *virB4* and *virB11* between *Agrobacterium tumefaciens* (33) and *R. prowazekii* (2) for PCR. By using these degenerate primers, DNA fragments containing partial sequences of these two genes were amplified from each genomic DNA of *A. phagocytophila* and *E. chaffeensis*, cloned into a pCRII vector (Invitrogen-Life Technology, San Diego, Calif.), and sequenced using the dideoxy chain termination method. For assembly of the entire *vir* region, the overlapping DNA fragments with unknown flanking sequences, which were amplified by the adapter PCR method using the Universal Genome Walker kit (Clontech Laboratories, Palo Alto, Calif.) or by multiplex restriction site PCR (4, 17, 39), were cloned into a pCRII vector and sequenced.

Genomic Southern blot analysis. PCR products amplified with the primer pairs shown in Table 1 were labeled with [α-^{32} P]dATP by using the random primer method (Amersham Pharmacia Biotech, Piscataway, N.J.) and used as gene-specific DNA probes. Southern blot analyses with genomic DNAs from purified organisms were performed by a procedure described previously (25). Hybridization was carried out under high-stringency conditions (65°C), and after being washed, the membrane was exposed to a Hyperfilm (Amersham Pharmacia Biotech).

Specimens from infected animals and humans. Peripheral blood leukocytes (PBLs) from a mouse and a horse experimentally infected with strain HZ of *A. phagocytophila* were obtained on days 4 and 8 postinoculation, respectively, as previously described (43). PBLs were prepared from three HGE patients as described elsewhere (Lin et al., submitted).

Transcriptional analysis. Reverse transcription-PCR (RT-PCR) was performed by a procedure described previously (24, 42). Total RNA was prepared from 5×10^6 *A. phagocytophila*-infected HL-60 cells (70% infectivity), 5×10^6 *E. chaffeensis*-infected THP-1 cells (70% infected cells), or the PBL specimens by using the TRIzol reagent (Invitrogen-Life Technologies) or the RNeasy Mini Kit (Qiagen, Valencia, Calif.). After DNase I treatment, the RNA (0.5 to 2.5 μg) was reverse transcribed using Superscript II (Invitrogen-Life Technologies) with random hexamer primers at 42°C for 50 min. The PCR conditions were 30 to 35 cycles consisting of 1 min of denaturation at 94°C, 1 min of annealing at 54°C, and 1 min of extension at 72°C. For cDNA synthesis of long transcripts (approximately 2 to 6 kb), the DNase I-treated total RNA (5 μg) prepared from the tissue culture was reverse transcribed by using a Thermotranscript kit (Invitrogen-Life Technologies) according to the manufacturer's instructions with a gene-specific primer at 54°C for 50 min. The PCR conditions were 35 cycles consisting of 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 3 to 7 min of extension at 72°C. The PCR products were electrophoresed and visualized by ethidium bromide staining. The primers used for all RT-PCRs are shown in Table 1.

TABLE 1. Oligonucleotide primers used in RT-PCR, 5′RACE, and Southern blotting

Target	Amplicon size (bp)[a]	5′ primer (5′RACE primer 2)	3′ primer (5′RACE primer 1)	RT primer	Purpose
A. phagocytophila					
virB4	268	5′-TCTCTTATGTTGTACGTAAAG-3′	5′-AACACCCGCGGAGAAAACTT-3′		Southern blotting (probe B)
virB9	358	5′-TGGACAACAAGTTATACGTC-3′	5′-AGAGAAACGTGAGCACTTC-3′	Random hexamer	Southern blotting (probe E), RT-PCR
virB11	190	5′-CTCGAGGGAAATTGTCCTAT-3′	5′-CCCAGTATTAATAGCCCTCA-3′		Southern blotting (probe A)
virB8-virD4	5,979	5′-AAGATCGGAGATTGGTATCATGACAGATAC-3′	5′-ACAACCTCATCATCTAAAAGCTCATCCTCA-3′	5′-TCTTCTCCAAATTCATCC-3′	LA-RT-PCR
virD4-p44-22 omp	557	5′-GATAGCAGTAACGGAAGAC-3′	5′-CCTAGATAACAGGTCTCCA-3′	Random hexamer	RT-PCR
sodB-virB4	3,240	5′-AGCCGTATATTTCTTCCCATCTCTTAGATC-3′	5′-TACCTGACAAAACGTTTATCGTGTCATCGA-3′	5′-AACCACACATTTGGATCA-3′	LA-RT-PCR
virB4-virB6	1,995	5′-GCGAGTCGCATGATGATGTGGCA-3′	5′-GCAGCCATCACTGTCTTCTTAAGC-3′	5′-TCCCCTGCACTTTGTACC-3′	LA-RT-PCR
Upstream of *virB8*	370 (407), 383 (420), 417 (454)	5′-TACCTCGTCGGCAGAATACTGT-3′	ACGAAAGAAGACGTACTCTCGT	5′-GTTACTAGGACGTATCCA-3′	5′RACE
Upstream of *sodB*	289 (326), 296 (333)	5′-TCGGCTTCATGGATTGCCAATA-3′	5′-TACAAGCCATACCCATCCACTA-3′	5′-TTCGCTGTGCTAACAACT-3′	5′RACE
E. chaffeensis					
virB4	273	5′-TTTTGAATATATAGTTCGTAAAG-3′	5′-AAGAAAATCCTTGGTGAAAAC-3′		Southern blotting (probe D)
virB11	508	5′-TATTTCCTCCAGCCTGTGAA-3′	5′-GCCTGTATTAATTGCTCTTAA-3′		Southern blotting (probe C)
virB8-virD4	3,734	5′-GTGAGTTCCGTAACTGGATAAGGC-3′	5′-GGATTATGCCACCAGTGTTCTACG-3′	5′-CTTAGGATACCTATTGGT-3′	LA-RT-PCR
sodB-virB4	3,370	5′-CATCTGGAGATTTAGGATCTAGAGC-3′	5′-TACTGCACCTACTCCCTGTTTCAC-3′	5′-ATTTGGATCATCTCCTACTTCT-3′	LA-RT-PCR
virB4-virB6	3,258	5′-AGAACCAAGACATCCTACAGGATTC-3′	5′-GCCTGTTCTCCTACACTACCCTG-3′	5′-TGCAGCATCACCAGCTCCT-3′	LA-RT-PCR
virB6-ORFa	1,939	5′-AGGAATTCCTTTGTGGCCTCAAGTT-3′	5′-GTCTTCCAGCTAGTACCAAATCCG-3′	5′-ACCACATCTTTTGTATTATTAC-3′	LA-RT-PCR
ribA	294	5′-TGGTGACCTACTTAACAGC-3′	5′-ATGCCGTGATTCTGAATTTC-3′	Random hexamer	RT-PCR
ribA-virB8	354	5′-GAAATTCAGAATCACGGCAT-3′	5′-ACGACTGACGTGCCAACT-3′	Random hexamer	RT-PCR
pstA	347	5′-ACATGGATCCTATTAGTGAG-3′	5′-TTGTTGATATGGAAGTTCCG-3′	Random hexamer	RT-PCR
pstA-sodB	351	5′-TGATAAGATACGATCCTTCC-3′	5′-TTGTTGATATGGAAGTTCCG-3′	Random hexamer	RT-PCR
sodB-virB3	453	5′-TTGGGATTTTGCAGCTCAG-3′	5′-GCTTTAACAGTACCTGCCA-3′	Random hexamer	RT-PCR
Upstream of *virB8*	356 (391), 462 (497)	5′-TGAGTATTGTTTGACTGATACAGG-3′	5′-AGGGTCAAAAAGCTCTCTAGATC-3′	5′-ACTCACTGTATGTTGTTTG-3′	5′RACE
Upstream of *sodB*	363 (397)	5′-CCTGTAGGAGATCCTCCACCA-3′	5′-CATCCATGCTCCCAAAATCCTC-3′	5′-AAACCAACCATACCCATC-3′	5′RACE

[a] Sizes of 5′RACE amplicons are shown as base pairs from the transcriptional initiation site to 5′ end of 5′RACE primer 2. Numbers in parentheses include 35 to 37 bp of a 5′RACE abridged anchor primer.

RACE. The 5′ rapid amplification of cDNA ends (5′RACE) experiment was performed using the protocol provided by the manufacturer (Invitrogen-Life Technologies). DNase I-treated total RNA (5 μg) was reverse transcribed by using Superscript II with a gene-specific primer. The cDNA was tailed by adding cytosine or adenosine residues at the 3′ end by using terminal transferase and amplified by PCR with a primer set consisting of a second gene-specific primer and an oligo(dG)- or oligo(dT)-linked amplification primer. The PCR conditions were 35 cycles consisting of 1 min of denaturation at 94°C, 1 min of annealing at 54°C, and 1 min of extension at 72°C. The primary PCR products were further amplified by a nested gene-specific primer and the amplification primer without the oligo(dG) or oligo(dT) anchor. The secondary PCR products were purified and cloned. The inserts of 25 to 30 clones in each sample were sequenced. The primers used for all 5′RACE procedures are shown in Table 1.

Sequence analysis. A database search was carried out with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Protein localization site and signal sequence were analyzed with the PSORT program (http://psort.nibb.ac.jp/). Multiple alignments were done using the CLUSTAL V method in the DNASTAR program. Phylogenetic analysis was performed with the PHYLIP (Phylogeny Inference Package) version 3.5p. GenBank or EMBL accession numbers of published Vir protein sequences used for the phylogenetic analysis are as follows: *Agrobacterium tumefaciens* Ti, J03320; *Bartonella henselae*, U23447 and AF182718; *Bordetella pertussis ptl*, A47301, B47301, C47301, D47301, E47301, F47301, and G47301; *Brucella abortus*, AF226278; *Brucella suis*, AF141604; *Legionella pneumophila lvh*, Y19029; pKM101, U09868 and AF109305; *R. prowazekii*, AJ235270, AJ235271, and AJ235273; *R. conorii*, NC_003103; *Wolbachia* sp. strain *w*Tai, AB045234; and *Wolbachia* sp. strain *w*KueYo, AB045235.

Nucleotide sequence accession numbers. Sequence data for the *virB-D* regions of *A. phagocytophila* and *E. chaffeensis* determined in the present study have been assigned GenBank accession numbers AF392618 (*virB8-p44-22 omp*) and AF392616 (*pstA-virB6*) for *A. phagocytophila* and AF392617 (*ribA-p120*) and AF392615 (*pstA*-ORFa) for *E. chaffeensis*.

RESULTS

Gene organization and genetic loci of *virB* and *virD* regions in genomes of *A. phagocytophila* (causing HGE) and *E. chaffeensis* (causing HME). With the degenerate primer pairs, we successfully amplified DNA fragments including partial sequences of *virB4* and *virB11* genes from each of genomic DNAs of *A. phagocytophila* and *E. chaffeensis*. Several other *virB* and *virD* genes that were located upstream and downstream from the *virB4* and *virB11* genes were assembled by cloning and sequencing, using two kinds of genome walking procedures as described in Materials and Methods. By this approach, a total of eight genes orthologous to *virB3*, *virB4*, *virB6*, *virB8*, *virB9*, *virB10*, *virB11*, and *virD4* for a type IV secretion machinery were found in each genome of *A. phagocytophila* strain HZ and *E. chaffeensis* strain Arkansas (Fig. 1). The gene organizations of the *virB* and *virD* regions in both bacteria were similar to each other, and they were also similar to the corresponding regions of the type IV secretion systems in other gram-negative bacteria (9). However, unlike the single locus of the clustered *virB* and *virD* genes in most of other bacteria (9), the *vir* genes of *A. phagocytophila* and *E. chaffeensis* were clustered in two separate loci of each genome, one consisting of five tandem genes (*virB8*, *virB9*, *virB10*, *virB11*, and *virD4*) and another consisting of three tandem genes (*virB3*, *virB4*, and *virB6*) (Fig. 1). Four genes orthologous to *virB1*, *virB2*, *virB5*, and *virB7* of *Agrobacterium tumefaciens* were not found in these two loci of either *A. phagocytophila* or *E. chaffeensis*. Southern blot analyses supported that these two loci (each) in *A. phagocytophila* and *E. chaffeensis* are separated in the respective genomes (Fig. 2). Moreover, the blot result showed that *A. phagocytophila* strain HZ may have an additional *virB4* paralog (asterisks in Fig. 2B) like *R. prowazekii* (2) and *R. conorii* (23), because the *virB4* probes

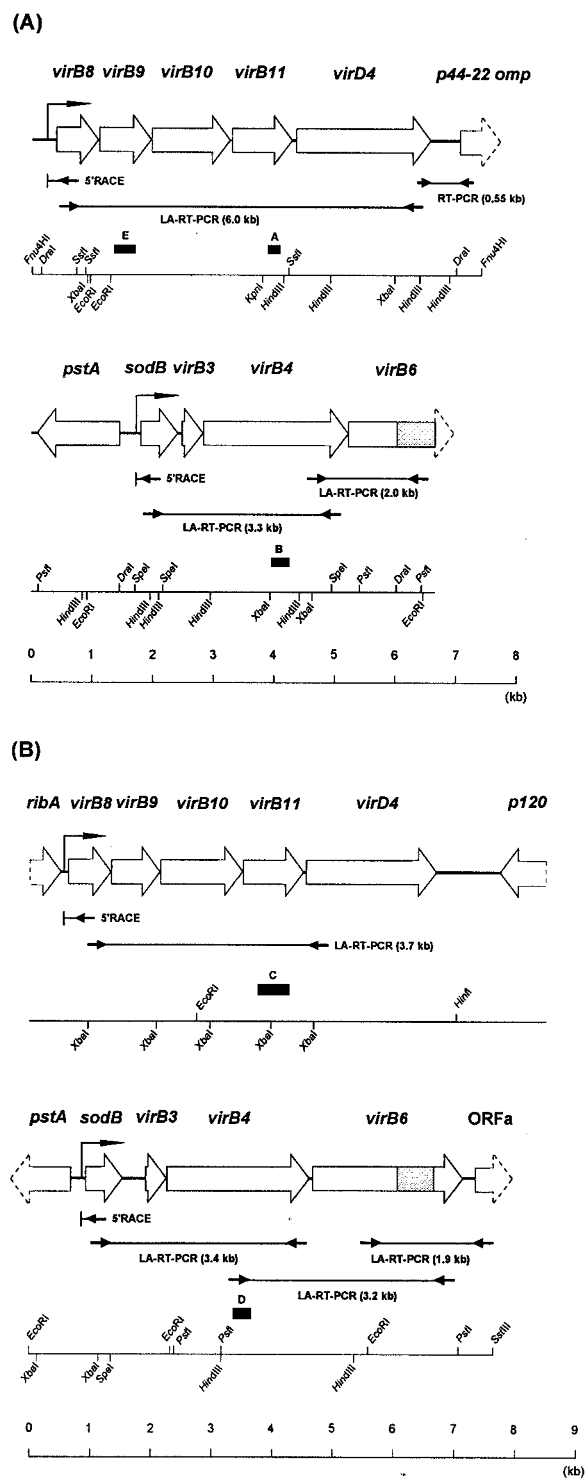


FIG. 1. Schematic representation of the gene organization of the *virB* and *virD* regions in *A. phagocytophila* HZ (A) and *E. chaffeensis* Arkansas (B). ORFs are represented as open boxes, with arrowheads indicating their orientation. The region homologous to *R. prowazekii* RP104 (one of *virB6* paralogs) is shaded within the *virB6* ortholog from each species. Lines with facing arrows at both ends indicate the LA-RT-PCR or RT-PCR regions in the transcriptional analysis. Bent arrows located upstream of *virB8* or *sodB* represent the transcriptional promoter regions for the respective clustered *virB* and *virD* genes which were deduced by 5′RACE analysis (arrows with "5′RACE"). Closed boxes with letters indicate the DNA probes used for genomic Southern blot analysis.

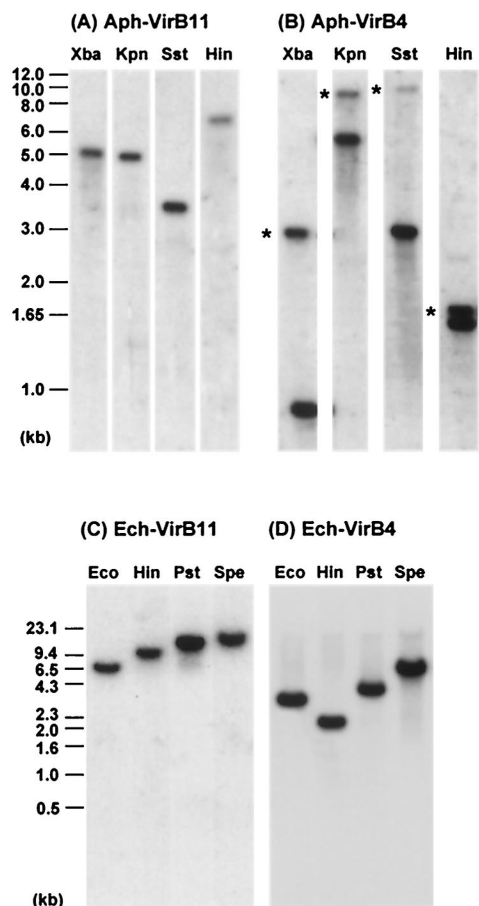


FIG. 2. Genomic Southern blot analyses of the clustered *virB* and *virD* genes in *A. phagocytophila* HZ (A and B) and *E. chaffeensis* Arkansas (C and D). Panels A to D show the hybridization patterns with probes A to D, respectively, shown in Fig. 1. Asterisks indicate second, more weakly hybridized fragments. Hin, *Hin*dIII; Kpn, *Kpn*I; Pst, *Pst*I; Spe, *Spe*I; Sst, *Sst*I; Xba, *Xba*I.

used did not have *Xba*I, *Kpn*I, *Sst*I, and *Hin*dIII restriction sites, whereas *E. chaffeensis* strain Arkansas has a single *virB4* gene (Fig. 2D). Among strains HZ and LL and three human isolates of *A. phagocytophila* (Fig. 3), the hybridization patterns with *virB9* or *virB4* probes were almost identical, suggesting the conservation of genetic loci of the *virB* and *virD* genes in strain LL and three isolates of *A. phagocytophila*. The blot results also revealed the presence of two *virB4* paralogs in all of five *A. phagocytophila* strains and isolates (Fig. 3B).

Five genes (*virB8* to *virD4*) within one of the two loci each in *A. phagocytophila* and *E. chaffeensis* were clustered upstream from an antigenic protein gene (Fig. 1): in *A. phagocytophila* a *p44* major outer membrane protein gene (designated *p44-22 omp*) which belongs to a *p44* polymorphic multigene family (42, 43) and in *E. chaffeensis* a 120-kDa antigenic protein gene (*p120*) in the opposite orientation (41). The intergenic space between *virD4* and *p44-22 omp* (486 bp) in *A. phagocytophila* was shorter than that between *virD4* and *p120* (998 bp) in *E. chaffeensis* (Fig. 1). A *ribA* gene that encodes a bifunctional enzyme with the activities of both 3,4-dihydroxy-2-butanone 4-phosphate synthase and GTP cyclohydrolase II, which catalyze two essential steps in riboflavin biosynthesis, was located at 134 bp upstream from *E. chaffeensis virB8*. No open reading frame (ORF) was found within 385 bp upstream from the start codon of the *A. phagocytophila virB8* gene. Three genes (*virB3*, *-B4*, and *-B6*) and a *sodB* gene encoding a superoxide dismutase (SOD) were located downstream from a *pstA* gene encoding a phosphate ABC transporter permease in the opposite orientation. The *sodB* gene probably encodes an iron-containing SOD, because the deduced amino acid sequence was significantly more homologous to known iron-containing SODs than to manganese-containing SODs. No gene homologous to a partial ORF located downstream from *E. chaffeensis virB6* (designated ORFa) was found in a database search. An additional difference between *A. phagocytophila* and *E. chaffeensis* is the length of the intergenic space between the *sodB* and *virB3* genes. The space in *A. phagocytophila* is shorter (92 bp) than that in *E. chaffeensis* (396 bp).

Proteins encoded by *virB* and *virD* genes of *A. phagocytophila*

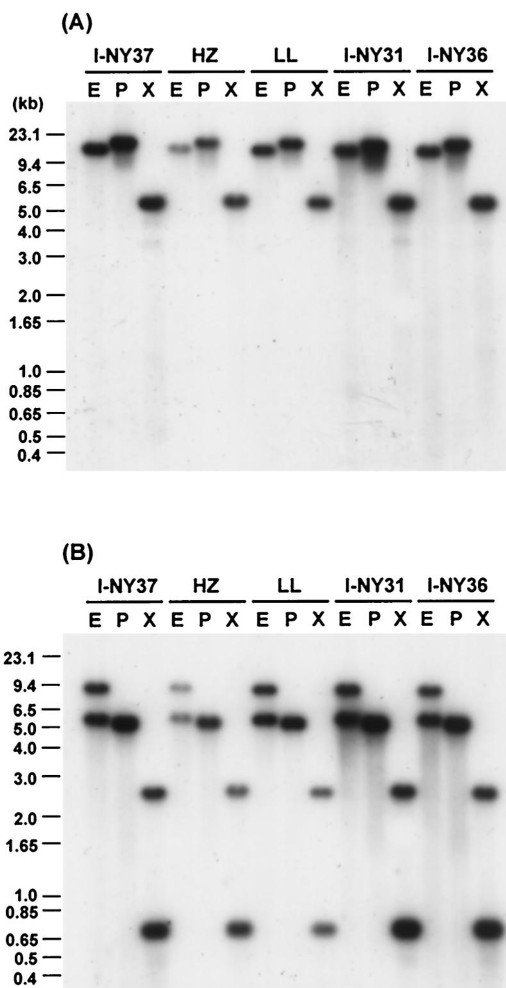


FIG. 3. Comparative genomic Southern blot analyses of *virB* and *virD* loci among strains HZ and LL and three human isolates of *A. phagocytophila*. (A) *virB9* (probe E); (B) *virB4* (probe B). The positions of the probes on the maps are shown in Fig. 1. E, *Eco*RI; P, *Pst*I; X, *Xba*I. The labels above each panel indicate *A. phagocytophila* strains (HZ and LL) or isolates (I-NY31, I-NY36, and I-NY37) from which genomic DNAs were prepared.

TABLE 2. Predicted localization of ehrlichiosis agent Vir proteins, amino acid (aa) identities, and amino acid numbers of Vir orthologs of selected gram-negative bacteria compared with those of *E. chaffeensis*

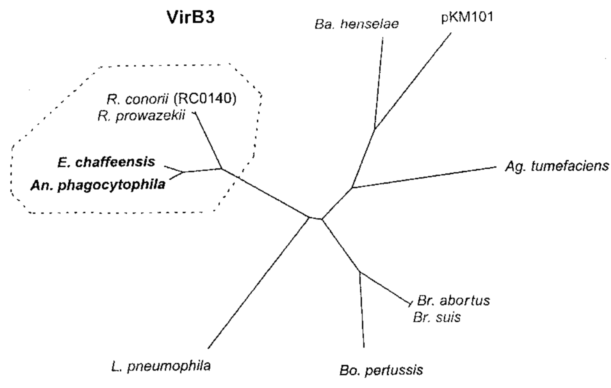
Vir protein	PSORT analysis[a]	Localization[b]	% aa identity						
			E. chaffeensis (aa no.)	*A. phagocytophila* (aa no.)	*R. prowazekii* (locus, aa no.)	*R. conorii* (locus, aa no.)	*Wolbachia* sp. strain *w* Tai (aa no.)	*Wolbachia* sp. strain *w* TueYo (aa no.)	*Agrobacterium tumefaciences* (aa no.)
VirB3	IM	OM	100 (97)	76.3 (97)	44.2 (ND[c], 95)	42.1 (RC0140, 95)			16.5 (108)
VirB4	IM	Integral IM	100 (800)	78.1 (801)	57.0 (RP103, 805)	57 (RC00141, 805)			27.4 (788)
					21.1 (RP784, 810)	21.0 (RC1217, 805)			
VirB6	IM	Polytopic IM	100 (826)	37.2 (486, partial)	12.8 (RP104, 1124)	11.7 (RC0142, 993)			15.6 (294)
					16.8 (RP105, 672)	17.1 (RC0143, 661)			
					12.5 (RP106, 971)	12.7 (RC0144, 966)			
					11.3 (RP107, 888)	12.1 (RC0145, 891)			
					10.5 (RP108, 1155)	10.4 (RC0146, 1153)			
VirB8	IM	IM	100 (237)	62.0 (234)	13.9 (RP287, 247)	13.4 (RC0385, 232)	50.0 (226)	45.6 (226)	16.0 (237)
					26.6 (RP289, 202)	24.9 (RC0387, 243)			
VirB9	OM	OM	100 (273)	46.2 (281)	31.2 (RP290, 157)	30.6 (RC0388, 157)	39.8 (261)	40.2 (266)	16.1 (293)
					20.8 (R286-TrbG, 250)	20.4 (RC0384-TrbG, 250)			
VirB10	IM	Bltopic IM	100 (447)	43.1 (434)	19.2 (RP291, 483)	20.1 (RC0389, 482)	33.1 (503)	35.1 (486)	16.7 (377)
VirB11	IM	Peripheral IM	100 (332)	72.6 (332)	66.6 (RP292, 334)	67.5 (RC0390, 334)	78.6 (330)	78.5 (330)	25.6 (344)
VirD4	IM	Integral IM	100 (714)	73.9 (740)	62.9 (RP293, 591)	63.3 (RC0391, 591)	74.9 (666)	74.5 (667)	22.0 (665)

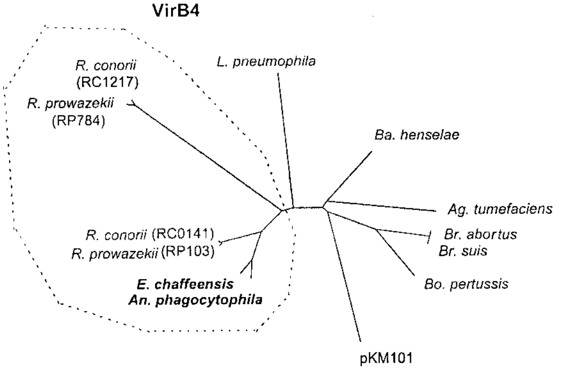
[a] Localization of ehrlichiosis agent Vir orthologs predicted by the PSORT program. IM, inner membrane; OM, outer membrane.
[b] Localization of T-DNA transfer system proteins and their orthologs shown by Christie (9).
[c] ND, not determined in previous studies (2, 37). The *virB3* locus of *R. prowazekii* was found between RP102 and RP103 from the genome sequence (2) in this study.

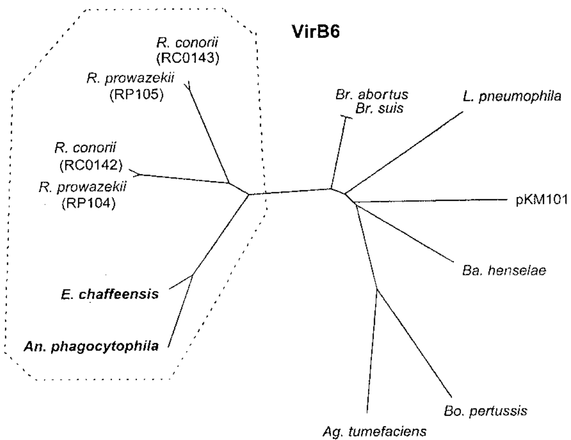
and *E. chaffeensis*. The predicted localization of Vir orthologs in *A. phagocytophila* and *E. chaffeensis* deduced by the PSORT program was identical to that of T-DNA transfer system proteins and their orthologs shown by Christie (9), except for that of VirB3 (Table 2). In addition, the PSORT analysis identified putative N-terminal signal sequences of three proteins of eight Vir orthologs in *A. phagocytophila* and *E. chaffeensis* (data not shown). The VirB9 proteins of *A. phagocytophila* and *E. chaffeensis* as well as those of other bacteria have cleavable signal sequences to translocate into the outer membranes (9). The VirB6 and VirD4 proteins of *A. phagocytophila* and *E. chaffeensis* have uncleavable or cleavable signal sequences, probably to anchor and locate in the inner membranes. Comparison with the database available for more than a dozen gram-negative bacteria showed that the VirB and VirD orthologous proteins of these two *Anaplasma* and *Ehrlichia* spp. had highest amino acid identities with those of *Wolbachia* spp. or *Rickettsia* spp. (Table 2 and Fig. 4). *Anaplasma* spp., *Ehrlichia* spp., *Wolbachia* spp., and *Rickettsia* spp. are obligatory intracellular bacteria that belong to the α1-proteobacteria. Among the more than one dozen bacteria for which the type IV secretion system has been described, *Wolbachia* spp., followed by *Rickettsia* spp., are closest to *Anaplasma* and *Ehrlichia* spp. on the basis of 16S rRNA gene sequence comparison (34). Of these obligate intracellular bacterial species, *E. chaffeensis* had higher identities with *A. phagocytophila* in three VirB orthologs (VirB8, -B9, and -B10), whereas VirB11 and VirD4 of *E. chaffeensis* showed the highest identities with those of *Wolbachia* spp. rather than with those of *A. phagocytophila* (Table 2). Between *A. phagocytophila* and *E. chaffeensis*, the four orthologs of VirB3, -B4, -B11, and -D4 are more conserved (72.6 to 78.1%) than the remaining orthologs of VirB6, -B8, -B9, and -B10 (37.3 to 62.0%). Among the VirB and VirD orthologs, the VirB6 proteins of *E. chaffeensis*, *R. prowazekii*, and *R. conorii* (661 to 1,155 amino acids) were significantly larger than those of *Agrobacterium tumefaciens*, *Bartonella henselae*, *Bordetella pertussis*, *L. pneumophila*, and *Brucella* spp. (294 to 436 amino acids) and retained the VirB6-homologous region in a central part of the molecules (Fig. 1) (2, 37). Because the VirB6 protein is involved in assembling the conjugal pore at the inner membrane and may interact with the effector molecules to be delivered (8, 22), the diversity of ehrlichial and rickettsial VirB6 proteins suggests a type of effector molecules different from those of other bacteria.

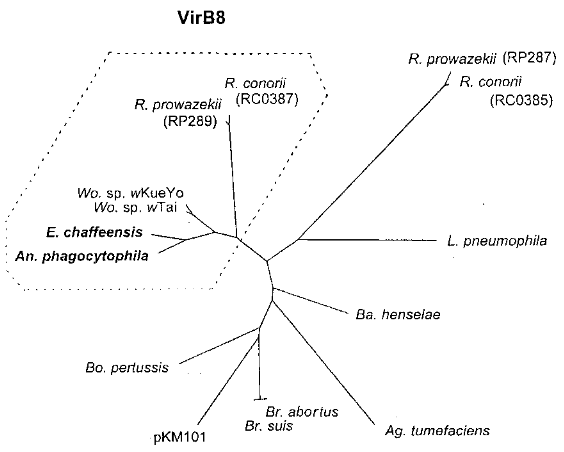
To analyze the relationship among VirB and VirD orthologs from *Anaplasma* and *Ehrlichia* spp. and several other bacteria, we constructed phylogenetic trees based on the deduced amino acid sequences (Fig. 4). Sixteen Vir proteins from *A. phagocytophila* and *E. chaffeensis*, 10 Vir proteins from *Wolbachia* spp., and 22 Vir proteins from *R. prowazekii* and *R. conorii* made a tight clade segregated from the remaining bacterial species in each of eight phylogenic trees (Fig. 4), except for VirB8 paralogs of *R. prowazekii* (RP287) and *R. conorii* (RC0385). However, in the eight trees most of the orthologs from facultative intracellular bacteria and the pKM101 plasmid were promiscuously located. The conservation of Vir proteins among obligate intracellular bacteria and the diversity of Vir proteins among facultative intracellular bacteria and the plasmids suggest that type IV secretion systems in obligate intracellular

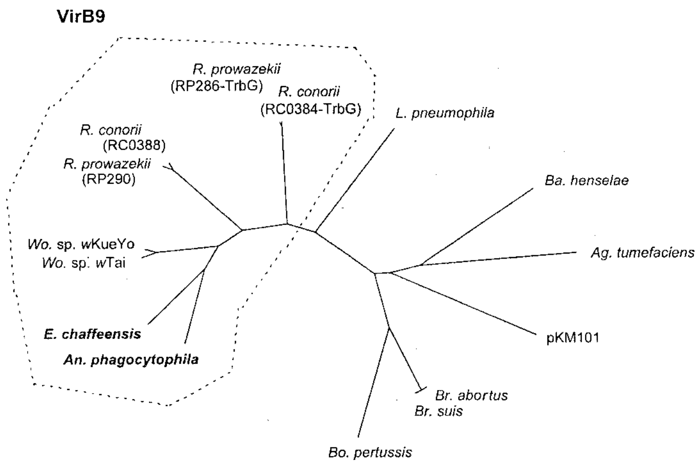
FIG. 4. Phylograms of VirB and VirD proteins in *A. phagocytophila*, *E. chaffeensis*, and selected gram-negative bacteria. The amino acid sequences were aligned with the CLUSTAL V method, and trees were constructed by using the neighbor-joining method. Two VirB6 proteins of *R. prowazekii* (RP104 and PR105) and two VirB6 proteins of *R. conorii* (RC0142 and RC0143) were used as representatives of six paralogs of each rickettsia (Table 2). Vir proteins of the obligate intracellular bacteria are surrounded by dotted lines, except for RP287 of *R. prowazekii* and RC0385 of *R. conorii* (*virB8* paralogs).

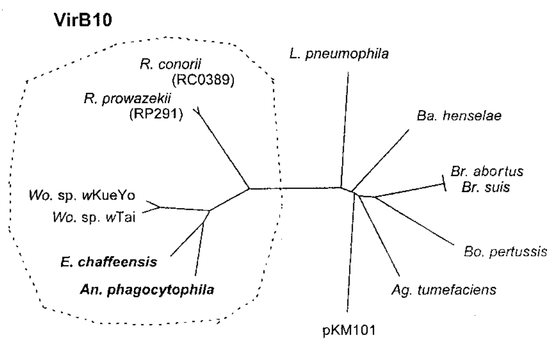

VirB3
Ba. henselae
pKM101
R. conorii (RC0140)
R. prowazekii
E. chaffeensis
An. phagocytophila
Ag. tumefaciens
Br. abortus
Br. suis
L. pneumophila
Bo. pertussis

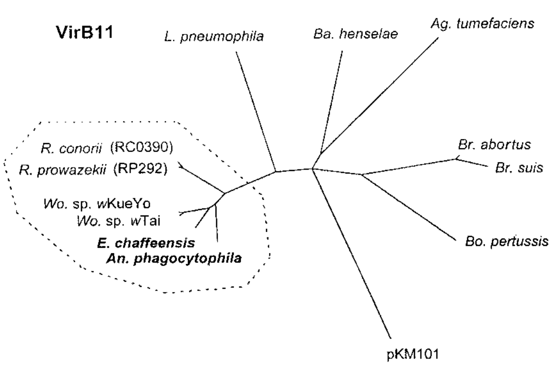

VirB4
R. conorii
(RC1217)
R. prowazekii
(RP784)
L. pneumophila
Ba. henselae
Ag. tumefaciens
R. conorii (RC0141)
R. prowazekii (RP103)
Br. abortus
Br. suis
E. chaffeensis
An. phagocytophila
Bo. pertussis
pKM101

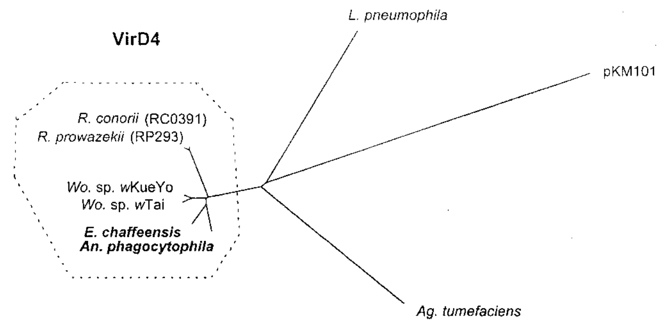

VirB6
R. conorii
(RC0143)
R. prowazekii
(RP105)
Br. abortus
Br. suis
L. pneumophila
R. conorii
(RC0142)
R. prowazekii
(RP104)
pKM101
Ba. henselae
E. chaffeensis
An. phagocytophila
Bo. pertussis
Ag. tumefaciens


VirB8
R. prowazekii (RP287)
R. conorii
(RC0385)
R. conorii
(RC0387)
R. prowazekii
(RP289)
Wo. sp. wKueYo
Wo. sp. wTai
E. chaffeensis
An. phagocytophila
L. pneumophila
Ba. henselae
Bo. pertussis
Br. abortus
Br. suis
pKM101
Ag. tumefaciens


VirB9
R. prowazekii
(RP286-TrbG)
R. conorii
(RC0384-TrbG)
L. pneumophila
R. conorii
(RC0388)
R. prowazekii
(RP290)
Ba. henselae
Wo. sp. wKueYo
Wo. sp. wTai
Ag. tumefaciens
E. chaffeensis
An. phagocytophila
pKM101
Br. abortus
Br. suis
Bo. pertussis


VirB10
R. conorii
(RC0389)
R. prowazekii
(RP291)
L. pneumophila
Ba. henselae
Wo. sp. wKueYo
Wo. sp. wTai
Br. abortus
Br. suis
E. chaffeensis
An. phagocytophila
Bo. pertussis
Ag. tumefaciens
pKM101


VirB11
L. pneumophila
Ba. henselae
Ag. tumefaciens
R. conorii (RC0390)
R. prowazekii (RP292)
Br. abortus
Br. suis
Wo. sp. wKueYo
Wo. sp. wTai
E. chaffeensis
An. phagocytophila
Bo. pertussis
pKM101


VirD4
L. pneumophila
pKM101
R. conorii (RC0391)
R. prowazekii (RP293)
Wo. sp. wKueYo
Wo. sp. wTai
E. chaffeensis
An. phagocytophila
Ag. tumefaciens

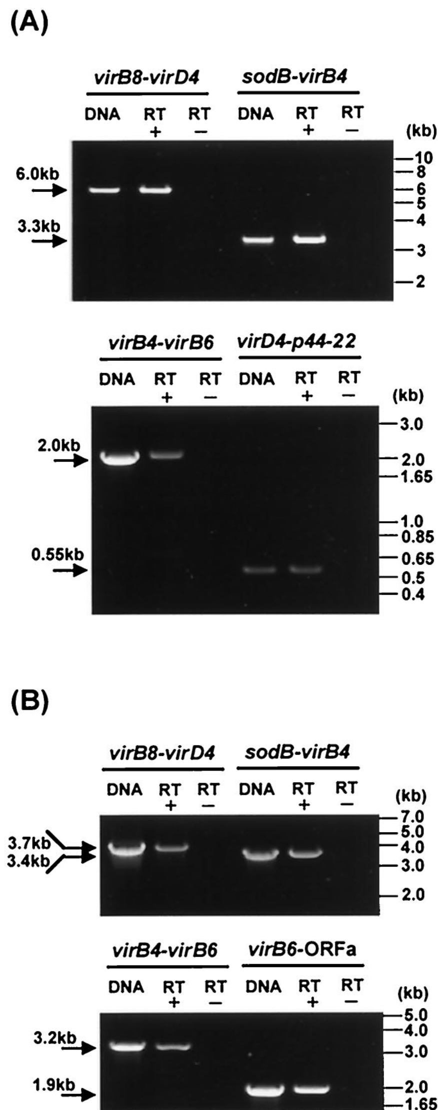


FIG. 5. Transcriptional analysis of the clustered *virB* and *virD* genes in *A. phagocytophila* (A) and *E. chaffeensis* (B). LA-RT-PCR or RT-PCR was used for transcriptional analysis of the genes in the *virB* and *virD* regions. Primers used and their positions are shown in Table 1 and Fig. 1. The DNA template control (0.1 ng of genomic DNA from purified organisms) shows the intensity and specificity of the band detected with each pair of primers. RT+ and RT− indicate the presence and absence of reverse transcriptase in the reaction, respectively. The numbers on the left indicate the respective amplicon sizes.

bacteria play roles different from those in facultative intracellular parasitism.

Transcription of the clustered genes in the *virB* and *virD* regions of *A. phagocytophila* and *E. chaffeensis*. Because of a short intergenic space between the genes within the *virB* and *virD* regions of *A. phagocytophila* and *E. chaffeensis*, as shown in Fig. 1, it is presumed that these clustered genes are polycistronically transcribed. Therefore, we primarily utilized a long and accurate RT-PCR (LA-RT-PCR) method to analyze the expression of the clustered *virB* and *virD* genes by these two bacteria cultivated in a human leukemia cell line. The sequences and positions of the primers and amplicon sizes are shown in Table 1 and Fig. 1. DNA template controls are shown for each primer pair to demonstrate the specificity of the primer to amplify the target sequence (Fig. 5). Without reverse transcriptase, no amplicon was detected in RT-PCR analyses using any of the primer pairs, indicating the absence of contamination of genomic DNA in the RNA preparation. As expected, a 6.0-kb LA-RT-PCR product including *virB8*, *-B9*, *-B10*, *-B11*, and *virD4* within one of the *virB-D* regions was detected in *A. phagocytophila* cultured in HL-60 cells, indicating the polycistronic transcription of these genes (Fig. 5A). Furthermore, a 3.3-kb LA-RT-PCR product including *sodB*, *virB3*, and *virB4* and a 2.0-kb LA-RT-PCR product including *virB4* and *-B6* within another *virB-D* region were detected, showing that not only *virB* orthologs but also the *sodB* gene were cotranscribed. The transcript of two adjacent genes (*virD4* and *p44-22 omp*) connected by a 487-bp intergenic space at the 3′ end of the *virB-D* region was detectable, indicating that one of the *p44* multigene family members was cotranscribed with the *virD* genes. Similar results regarding the expression of *virB* and *virD* genes were obtained for *E. chaffeensis* cultivated in THP-1 cells (Fig. 5B). ORFa of *E. chaffeensis* was cotranscribed with *virB6*. The results shown in Fig. 5 are diagrammed in Fig. 1. These findings suggest that the transcriptional promoters for the clustered *virB* and *virD* genes are present upstream of the *virB8* and *sodB* genes.

We further examined the transcription of *ribA*, *pstA*, and three sets of two adjacent genes (*ribA-virB8*, *pstA-sodB*, and *sodB-virB3*), including their intergenic spaces, in *E. chaffeensis* cultivated in THP-1 cells by RT-PCR. As shown in Fig. 6, the transcripts of *ribA*, *pstA*, and a set of two adjacent genes (*sodB-virB3*) with a long intergenic space (396 bp) were detected, but two other sets of two adjacent genes (*ribA-virB8* and *pstA-sodB*), including their intergenic spaces, were RT-PCR negative, showing that the *sodB* and *virB3* genes were cotranscribed but that the *ribA* and *virB8* genes or the *pstA* and *sodB* genes were not. In addition to the LA-RT-PCR results described above, these results support that the promoters for the clustered *virB* and *virD* genes exist within the intergenic spaces between the *ribA* and *virB8* genes and between the *pstA* and *sodB* genes but probably do not exist between the *sodB* and *virB3* genes.

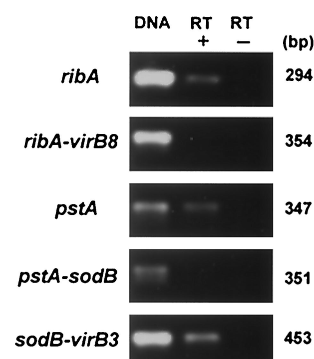


FIG. 6. RT-PCR analysis of *ribA*, *pstA*, and *sodB* and their intergenic spaces in *E. chaffeensis*. DNA, 0.1 ng of genomic DNA from purified *E. chaffeensis*; RT+, with reverse transcriptase; RT−, without reverse transcriptase. The primers used are shown in Table 1. The numbers on the right show the respective amplicon sizes.

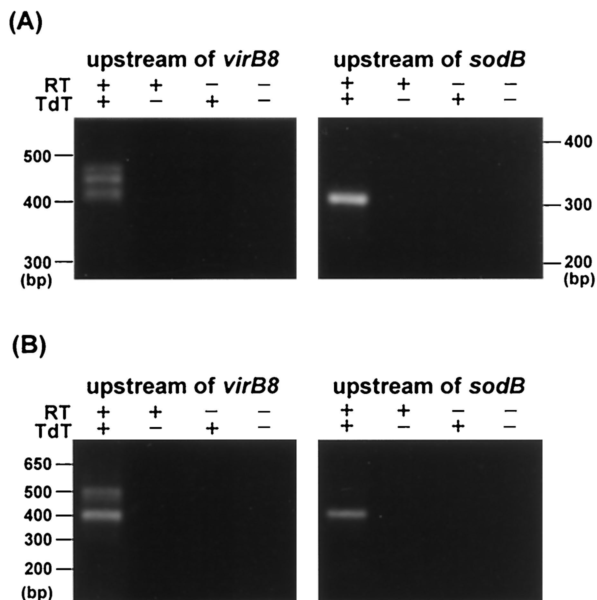


FIG. 7. 5′RACE analysis of *virB8* and *sodB* in *A. phagocytophila* (A) and *E. chaffeensis* (B). RT+, with reverse transcriptase; RT−, without reverse transcriptase; TdT+, with terminal deoxynucleotidyl transferase; TdT−, without terminal deoxynucleotidyl transferase.

Characterization of promoter regions for the clustered *virB* and *virD* genes in *A. phagocytophila* and *E. chaffeensis*. To characterize *vir* promoter regions, we analyzed a transcriptional initiation site upstream from *virB8* and *sodB* by 5′RACE procedures using the addition of a polymeric dC tail at the 3′ end of the cDNA. Three bands and a single band of the major 5′RACE products for *virB8* and *sodB*, respectively, were detected in *A. phagocytophila* cultivated in HL-60 cells (Fig. 7A). The same results were obtained when we performed 5′RACE with the addition of a poly(dA) tail at the 3′ end of the cDNA (data not shown). In *E. chaffeensis*-infected THP-1 cells, two bands and a single band of the major products for *virB8* and *sodB*, respectively, were detected (Fig. 7B). These 5′RACE products were cloned and sequenced to determine the transcriptional initiation site. One of the results is shown in Fig. 8. By sequencing *A. phagocytophila* 5′RACE clones with both poly(dC) and poly(dA) tails, we determined the initiation site of the AP3 transcript to be a guanine at −120 bp from the start codon of *virB8* but probably not to be an adenine at −119 bp (Fig. 8 and 9). In total, we identified three initiation sites at −73 bp, −86 bp, and −120 bp from the *virB8* start codon and two initiation sites at −21 bp and −30 bp from the *sodB* start codon in *A. phagocytophila* transcripts (Fig. 9). In *E. chaffeensis*, two initiation sites at −58 or −59 bp and at −165 bp from the *virB8* start codon and one initiation site at −69 bp from the *sodB* start codon were identified by sequencing the 5′RACE clones with poly(dC) tails. Putative sequences of −10 and −35 promoter regions were found upstream of the respective transcriptional initiation sites, which were similar to σ^{70}-type consensus promoter sequences of *E. coli* (26). One of the promoter regions for *virB8* in *E. chaffeensis* (EP2) is likely to be located within the 3′ coding region of the *ribA* gene, suggesting that the expression of *ribA* may influence the transcription of clustered *virB8-virD4* genes.

Transcription of the *virB* gene in HGE patients and in experimentally infected animals. We examined by RT-PCR whether the *virB* gene is expressed by *A. phagocytophila* in acute-stage HGE patients or in experimentally infected animals. The clinical signs and laboratory parameters for three patients were described in detail elsewhere (Lin et al., submitted). A transcript of *A. phagocytophila virB9* was detected in PBLs from each patient's blood collected at 20 days (patient NY31) and 3 days (patients NY36 and NY37) after recognition of initial clinical signs such as fever (Fig. 10A). The transcript was also detected in PBLs from blood collected on days 4 and 8 after inoculation of *A. phagocytophila* in an experimentally infected mouse and horse, respectively (Fig. 10B). These findings show that the *virB-D* region of *A. phagocytophila* is transcriptionally active in infected humans and animals at the acute stage as well as in cell cultures and suggest that the type IV secretion system is involved in HGE pathogenesis.

DISCUSSION

The present study characterized the genes for the type IV secretion machinery in *A. phagocytophila* (agent of HGE) and *E. chaffeensis* (agent of HME) and their transcription. Of primary significance is that these genes were transcribed not only in cell cultures but also in the blood of acute-stage patients and experimentally infected animals. Therefore, the type IV secretion system is expected to have a significant role in both HGE and HME. The type IV systems have been recently classified into two groups (9). One group consists of genes orthologous to the *virB* and *virD* genes of *Agrobacterium tumefaciens*. The type IV systems in most bacteria, including *L. pneumophila lvh*, belong to this group. Another group contains the *dot-icm* genes of *L. pneumophila* (36, 38) and *colIb* genes (IncI) of *Shigella flexneri* (9). The Lvh system of *L. pneumophila* functions as a DNA conjugation system, while the *dot-icm* system is required for the bacterial virulence (36–38). The type IV secretion systems of *A. phagocytophila* and *E. chaffeensis* which were identified in the present study belong to the former group. A number of unique features were found in the type IV secretion systems from these two *Anaplasma* and *Ehrlichia* spp. Unlike

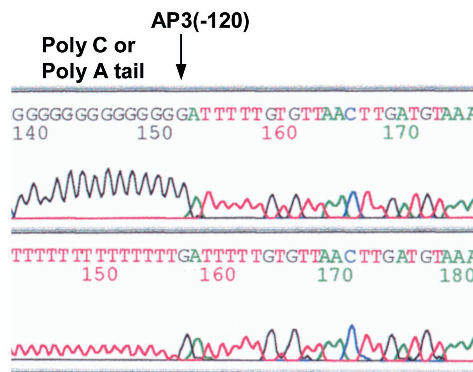
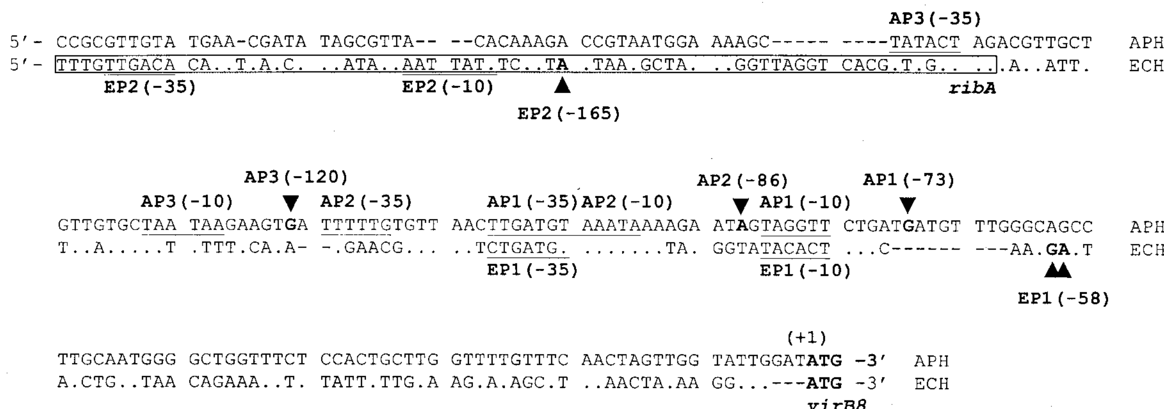


FIG. 8. Transcriptional initiation site of a *virB8* transcript (AP3) in *A. phagocytophila*. An arrow indicates the AP3 initiation site that was determined by sequencing the 5′RACE clones with poly(dC) and poly(dA) tails. The location of the AP3 site on the upstream sequence of *virB8* is shown in Fig. 9.



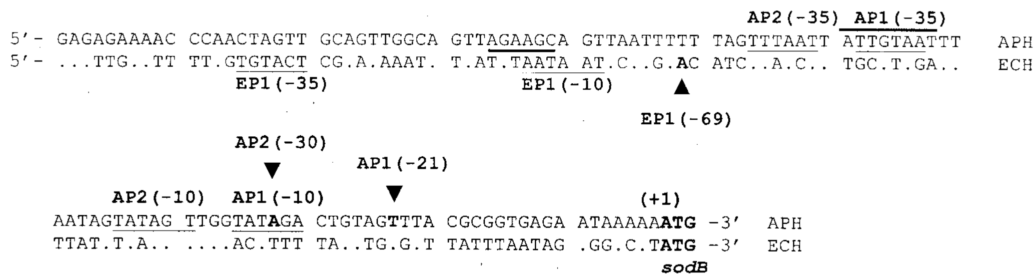
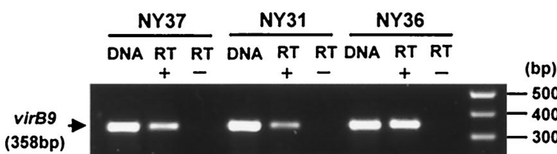


FIG. 9. Alignment of DNA sequences in the promoter regions of *virB8* (A) and *sodB* (B) of *A. phagocytophila* and *E. chaffeensis*. The putative −10 and −35 regions of each promoter are underlined. The initiation site (base) of each transcript of *A. phagocytophila* (AP1 to AP3) and *E. chaffeensis* (EP1 and EP2) is in boldface and indicated by arrowheads. Sequences with boldface bars in panel B, 5′dATTGTAAT3′ (*A. phagocytophila*) and 5′dTGTAAT3′ (*E. chaffeensis*), show putative *vir* boxes. The numbers in parentheses indicate the position upstream from the start codon of *virB8* or *sodB* (+1). The sequence enclosed by a box shows a 3′-end coding region of *ribA*. Gaps in the alignment are indicated by dashes.

the single locus of *virB* and *virD* genes in extracellular or facultative intracellular bacteria, the *virB* and *virD* genes of *Anaplasma* and *Ehrlichia* spp. were found in two separate loci like in *Rickettsia* spp. (three loci). This may allow for an independent transcriptional control of separate loci in *Anaplasma* and *Ehrlichia* spp., which ensures that only when two conditions are met is the complete type IV system assembled.

The type IV secretion systems of *Anaplasma* and *Ehrlichia* spp. are phylogenetically close to that of *Rickettsia* spp. but significantly different in *virB* gene numbers or their genetic loci. Most notably, *virB3*, *-B4*, and *-B6* in *Anaplasma* and *Ehrlichia* spp. were tandemly arranged downstream from the *sodB* gene and polycistronically transcribed through the *sodB* promoters. In *Rickettsia* spp., the *virB3* genes are not located downstream from a *sodB* gene, and between the *sodB* and *virB3* genes in the genome more than 240 other genes are present (2, 23). Therefore, the rickettsial *virB3* genes probably could not be cotranscribed with their *sodB* genes, although transcriptional analysis of these genes has not been done. In *Wolbachia* spp., *sodB*, *virB3*, *virB4*, and *virB6* so far have not been described, but a *virB-D* gene cluster (*ribA*, *virB8*, *virB9*, *virB10*, *virB11*, *virD4*, and *wsp*) with a genetic locus and polycistronic transcription similar to those of *Anaplasma* and *Ehrlichia* spp. has been identified (19). As far as we know, there



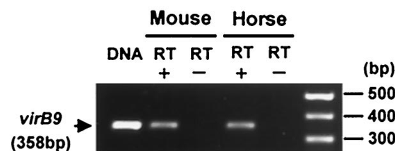


FIG. 10. RT-PCR analysis of a *virB9* transcript in PBLs from three human patients (A) and animals (B) infected with *A. phagocytophila*. The blood samples from three HGE patients are indicated as NY31, NY36, and NY37. DNA, 0.1 ng of genomic DNA from the respective organisms of purified *A. phagocytophila* isolates (I-NY31, I-NY36, or I-NY37) (A) or strain HZ (B). RT+, with reverse transcriptase; RT−, without reverse transcriptase. Primers are shown in Table 1. Numbers on the left of each panel show the respective amplicon sizes.

has been no report that the *sodB* and *virB* genes are polycistronically transcribed in the type IV secretion systems of other bacteria or plasmids so far identified. Therefore, the cotranscription of *sodB* and *virB* genes found in the present study is truly unique and conserved for *Anaplasma* and *Ehrlichia* spp. This result suggests that the activation of the *sodB* gene is required for construction of the type IV secretion machinery of *Anaplasma* and *Ehrlichia* spp.

The *sodB* gene of *Anaplasma* and *Ehrlichia* spp. probably encodes an iron-containing SOD (FeSOD), based on a database search. The SODs are metalloenzymes that function by catalyzing the dismutation of O_2^- to H_2O_2 and O_2. There are two additional types of SODs, which can be distinguished by the prosthetic metals present at the active site: manganese (MnSOD [*sodA*]) or copper or zinc (Cu/ZnSOD [*sodC*]). Cytoplasmic MnSODs and FeSODs are known to defend bacterial cells against reactive oxygen species generated from bacterial metabolism and protect DNA from oxidative damage, whereas Cu/ZnSOD localized in the periplasm is proposed to play a role in the defense of bacteria against external reactive oxygen. *sodB* gene expression is known to be controlled by iron uptake systems (12, 18). Therefore, the *virB3*, *-B4*, and *-B6* genes of *Anaplasma* and *Ehrlichia* spp. are expected to be induced under iron-rich conditions such as inside blood-feeding ticks and in eukaryotic cytoplasm.

Analysis of upstream regions of the *virB8* and *sodB* genes of both *A. phagocytophila* and *E. chaffeensis* showed that the polycistronic transcription of the *vir* genes seems to be regulated by multiple σ^{70}-type promoters. However, the transcriptional regulation mechanism of the *vir* genes still remains to be elucidated. In the Ti plasmids of *Agrobacterium tumefaciens*, VirA and VirG are members of the family of two-component regulatory systems and are required for induction of all other *vir* genes. VirA, a signal sensor/histidine kinase transmembrane protein, is autophosphorylated in response to phenolic compounds released by wounded plant cells and then phosphorylates VirG, a transcriptional activator. The phosphorylated VirG activates other *vir* operons through binding to the consensus sequence (*vir* box) of the respective promoter regions. The consensus sequence (14 bp) is 5′dPu(T/A)TDCAATTG HAAPy3′ (H = A, C, or T; D = A, G, or T) (11). The *vir* box of the closely related Ri plasmid *vir* genes has the 6-bp sequence dTG(A/T)AA(C/T) (3), which is equivalent to the 3′ half of the Ti plasmid *vir* box. In the present study, the sequence 5′dATTGTAAT3′, equivalent to the 3′ region (8 bp) of the Ti plasmid *vir* box, was found to overlap with the −35 region of AP1 in the *sodB* promoter of *A. phagocytophila*. In addition, the sequence dTGTAAT, equivalent to the Ri plasmid *vir* box (6 bp), overlapped with the −10 region of EP1 in the *sodB* promoters of *E. chaffeensis*. This suggests that a two-component regulatory system similar to that of *Agrobacterium tumefaciens* Ti plasmids (11) may control the polycistronic transcription of the *sodB-virB6* genes in *Anaplasma* and *Ehrlichia* spp.

Another unique feature is that antigenic outer membrane protein genes were colocalized with the *virB* and *virD* genes in *Anaplasma*, *Ehrlichia*, *Wolbachia*, and *Bartonella* spp. A *p44-22 omp* gene of *A. phagocytophila*, a member of the *p44* multigene family (42, 43), was located downstream of *virD4* and seems to be cotranscribed with the clustered *virB8-virD4* genes. This locus of *A. phagocytophila* probably is one of the expression sites for the multigene family. A *p120* gene of *E. chaffeensis* was also located downstream of *virD4* but with the opposite orientation, suggesting a lack of cotranscription of the *p120* and *virD4* genes. A *wsp* gene of *Wolbachia* spp. was positioned downstream of *virD4* like in *A. phagocytophila* (19). Although transcriptional analysis has not been performed, the *wsp* and *virD4* genes may also be cotranscribed. A 17-kDa antigen gene of *B. henselae* was located between *virB4* and *virB6* orthologs, missing *virB5*, within a single *virB* locus (27, 35), suggesting the cotranscription of the antigen gene and *virB* genes. Thus, except in *E. chaffeensis*, these bacterium-specific outer membrane antigens may be associated with the respective type IV secretion systems.

Eight *vir* orthologs (*virB3*, *-B4*, *-B6*, *-B8*, *-B9*, *-B10*, *-B11*, and *virD4*) present in most bacteria or plasmids that have evolved the type IV secretion systems (9) were found in *A. phagocytophila* and *E. chaffeensis*. The *virB1*, *-B2*, *-B5*, and *-B7* genes seem to be missing in *Anaplasma* and *Ehrlichia* spp.; however, these genes may also be required for the architecture of their type IV secretion machinery (e.g., the VirB2 protein is an F-pilin ortholog). Therefore, the related genes may be present in the genomes of these two bacteria but probably have extremely low homology with *virB* orthologs of other bacteria.

The etiologic agents of HGE and HME, *A. phagocytophila* and *E. chaffeensis*, infect mainly granulocytes and monocytes, respectively, primary host defensive cells with powerful bactericidal activities. Since these agents cannot survive outside host cells, they must enter granuclocytes or monocytes. For intracellular survival, *A. phagocytophila* and *E. chaffeensis* form a unique membrane-bound niche that does not fuse with lysosomes (5, 6, 20), and *A. phagocytophila* (agent of HGE) delays apoptosis of the infected neutrophils (40) and inhibits generation of superoxide anion by neutrophils (21). The type IV secretion system is considered to have evolved in intracellular bacteria to modulate eukaryotic cells for their intracellular survival (9). Further study is required for clarification of the roles of the type IV secretion system for survival of *Anaplasma* and *Ehrlichia* spp. in granulocytes or monocytes. The present data on the transcriptionally active type IV secretion systems in two major agents of HGE and HME are expected to facilitate analysis of the type IV secretion systems in the obligate intracellular pathogens.

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