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Genetic and Functional Characterization of the Type IV Secretion System in *Wolbachia*▽†

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A type IV secretion system (T4SS) is used by many symbiotic and pathogenic intracellular bacteria for the successful infection of and survival, proliferation, and persistence within hosts. In this study, the presence and function of the T4SS in *Wolbachia* strains were investigated by a combination of genetic screening and immunofluorescence microscopy. Two operons of *virB-virD4* loci were found in the genome of *Wolbachia pipientis* strain *w*Atab3, from the Hymenoptera *Asobara tabida*, and strain *w*Ri, infecting *Drosophila simulans*. One operon consisted of five *vir* genes (*virB8*, *virB9*, *virB10*, *virB11*, and *virD4*) and the downstream *wspB* locus. The other operon was composed of three genes (*virB3*, *virB4*, and *virB6*) and included four additional open reading frames (*orf1* to *orf4*) orientated in the same direction. In cell culture and insect hosts infected with different *Wolbachia* strains, the bona fide *vir* genes were polycistronically transcribed, together with the downstream adjacent loci, notably, as *virB8* to *virD4* and *wspB* and as *virB3*, *virB4*, *virB6*, and *orf1* to *orf4*. Two peptides encompassing conserved C and N termini of the *Wolbachia* VirB6 protein were used for the production of polyclonal antibodies. Anti-VirB6 antibodies could detect the corresponding recombinant protein by chemifluorescence on Western blots of total proteins from *Escherichia coli* transformants and *Wolbachia* strains cultured in cell lines. Using immunofluorescence microscopy, we further demonstrated that the VirB6 protein was produced by *Wolbachia* strains in ovaries of insects harboring *w*Atab3 or *w*Ri and cell lines infected with *w*AlbB or *w*MelPop. As VirB6 is known to associate with other VirB proteins to form a membrane-spanning structure, this finding suggests that a T4SS may function in *Wolbachia*.

Wolbachia species are obligate intracellular alphaproteobacteria belonging to the order *Rickettsiales* (1, 21, 44). They are found in associations with numerous invertebrates, including arthropods and nematodes (53, 64). The latest investigations have reported that *Wolbachia* strains infect from 66 to 76% of species belonging to different orders of arthropods, thus identifying these bacteria as the most widespread intracellular symbionts yet described (26, 31). The interactions between *Wolbachia* strains and their hosts are very complex and range from parasitism to mutualism. In filarial nematodes, *Wolbachia* is required for host biology (5). In contrast, *Wolbachia* strains are mostly parasites which affect reproduction of arthropods; they alter host reproduction in a number of ways to enhance their own transmission through maternal inheritance from infected females to progeny (63). Some of these reproductive alterations include parthenogenesis induction in parasitoid wasps (54, 56), the feminization of genetic male isopods (8), and the induction of male killing (29) and cytoplasmic incompatibility (37, 55).

Despite the increasing number of investigations on *Wolbachia*-host interactions, little is known about the molecular determinants and mechanisms involved in the observed pleiotropic phenotypes. Research in this field is hampered by the uncultivable status of *Wolbachia*. However, the development of alternative cellular and molecular technologies has led to the suggestion that *Wolbachia* may excrete effectors that interfere with host development and reproduction programs (46, 58, 59). Analyzing the potential excretory machinery of *Wolbachia* may help in the identification of those effectors and orientate them to the corresponding potential targets in the host.

Bacteria have adapted series of machineries to mediate the transport of macromolecules across their envelopes. These transport apparatus play essential roles in bacterial life and adaptation in different environments, including the acquisition of nutrients, the expression of pathogenicity, or the establishment of symbiosis (9, 13, 22, 28, 67). Type IV secretion systems (T4SSs) are an example of these transport apparatus. T4SSs are broadly distributed among gram-negative and gram-positive bacteria (10, 24). T4SS family members have been grouped into three major subfamilies: (i) conjugation systems mediating DNA transfer from donor to recipient cells, (ii) effector translocator systems that transport molecules directly into the cytoplasm of eukaryotic cells, and (iii) DNA uptake or release systems mediating the exchange of DNA with the milieu by contact-independent mechanisms (10, 14, 20). By their versatility, T4SSs are involved in a wide range of biological processes. Conjugation through T4SSs is implicated in the transfer of genes involved in resistance to antibiotics or heavy metals, as well as in the acquisition of symbiotic or pathogenic islands, thus contributing to bacterial genome plasticity and evolution (10, 19, 22). Several pathogens, such as *Helicobacter pylori*, *Legionella pneumophila*, *Anaplasma phagocytophilum*, and

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Rickettsia spp., are known or suspected to secrete toxins via T4SSs of the effector translocator subfamily (1, 12, 38, 39, 60).

The *Agrobacterium tumefaciens* T4SS provides the hallmark of the T4SS-mediated transfer of bacterial effectors directly into eukaryotic cells, and it serves as a model system for basic research on the determinants and comprehensive mechanisms involved (27, 42). The bacterium *A. tumefaciens* uses the T4SS to transfer oncogenic DNA into susceptible plant cells, disturbing the plant's hormonal balance and causing the crown gall disease (69). This T4SS consists of a single operon encoding the mating pair formation (Mpf) VirB proteins and the coupling protein VirD4. The *virB* gene products elaborate a cell envelope-spanning structure required for substrate transfer, and the VirD4 coupling protein recruits the bacterial substrate to be delivered into the host cell (11).

In the past decade, *virB* and *virD4* homologues clustered into a single operon have been found in many other bacteria by genetic screening and/or whole-genome sequencing (10, 13, 14). Split *virB-virD* operons in obligate intracellular bacteria of the order *Rickettsiales*, including *Rickettsia* spp., *A. phagocytophilum*, and *Ehrlichia chaffeensis*, have been described previously (2, 43). In *Wolbachia pipientis*, the presence of a T4SS was first evidenced by hybridization and PCR screening (40). Thereafter, the complete genome sequences of *W. pipientis* strains *w*Mel from *Drosophila melanogaster* and *w*Bm from the human filarial parasitic nematode *Brugia malayi* confirmed the existence of split *virB* and *virD4* homologues (23, 66).

In the context of studying the potential role of the T4SS in *Wolbachia*, here we describe structural and functional analyses of *virB-virD4* gene clusters in two strains that differ drastically in the phenotype induced in the host. The strain *w*Ri induces strong cytoplasmic incompatibility, resulting in 100% offspring mortality in incompatible crosses between *Drosophila simulans* flies (45), whereas the strain *w*Atab3 is required for oogenesis in the wasp *Asobara tabida*, a rare case among arthropods of obligatory interdependence, as aposymbiotic females fail to produce eggs (18). *Wolbachia virB* and *virD* genes were cloned and sequenced, and their transcriptional activities were assayed. We developed antibodies against VirB6, an integral membrane protein constituting one of the T4SS structural core components (32, 33), and monitored the target protein in insect cell lines and insect tissues hosting different *Wolbachia* strains.

MATERIALS AND METHODS

Biological material. *A. tabida* (Hymenoptera: Braconidae) is an endoparasitoid wasp associated with several *Drosophila* species. As females lay eggs in fly larvae, within which parasitic larvae feed and develop, parasitoids were bred on a *Wolbachia*-free line of *Drosophila melanogaster* originating from Ste. Foy-les-Lyon (France). *A. tabida* is naturally infected with three *Wolbachia* strains: *w*Atab1 and *w*Atab2 induce cytoplasmic incompatibility, and *w*Atab3 is required for host oogenesis (16, 18). The tri-infected line named Pi(123) originated from Pierrefeu, France, and is hereinafter referred to as *A. tabida* Pierrefeu. The line Pi(3), singly infected with *w*Atab3, was obtained using moderate rifampin antibiotic treatment (18). An *A. tabida* North American line infected with the three *Wolbachia* strains (*w*Atab1, *w*Atab2, and *w*Atab3) was also used. Aposymbiotic females were obtained from the North American line by antibiotic treatment (17). *D. simulans* flies naturally infected with the *Wolbachia* strain *w*Ri, uninfected *D. melanogaster* flies, and *A. tabida* insects were reared on a standard-medium diet (15) at 20°C in a cycle of 12 h of light and 12 h of darkness and 70% relative humidity.

Mosquito cell lines infected with *Wolbachia*, RML12 from *Aedes aegypti* infected with strain *w*MelPop (C. J. McMeniman, I. Iturbe-Ormaetxe, A. M. Lane, D. T. Voronin, R. Yamada, E. McGraw, and S. L. O'Neill, submitted for publication) and Aa23 infected with strain *w*AlbB, as well as uninfected cells, were grown in 25-cm^2 culture flasks (Greiner Bio-One, Frickenhausen, Germany) at 26°C in equal volumes of Mitsuhashi-Maramorosch medium (Bioconcept, Switzerland) and Schneider's insect medium (Sigma, France), supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum and penicillin-streptomycin (50 U and 50 μg per ml, respectively [Gibco; Invitrogen]). The infection status was verified by PCR amplification of the *wsp* gene as described previously (41).

DNA purification. Genomic DNA was isolated from adult *A. tabida* insects singly infected with *Wolbachia* strain *w*Atab3, adult *D. simulans* insects singly infected with *Wolbachia* *w*Ri, and adult *D. melanogaster* insects singly infected with *w*Mel by using the DNeasy tissue kit according to the recommendations of the manufacturer (Qiagen) with some modifications. Briefly, insects (30 mg) were crushed in 360 μl of buffer ATL (Qiagen). The mixture was treated with 2 mg of lysozyme (Euromedex) ml^{-1} for 3 h at 37°C and then with proteinase K from the DNeasy tissue kit for 12 h at 56°C as recommended by the manufacturer (Qiagen). The mixture was centrifuged twice at 16,000 × *g* for 1 min each time. The supernatant was transferred into a new tube and treated with 1 mg of RNase A ml^{-1} for 2 min at room temperature. Then 330 μl of buffer AL (Qiagen) was added, and the sample was incubated for 10 min at 70°C. After the addition of 330 μl of 96 to 100% ethanol, the sample was mixed thoroughly by being subjected to a vortex and was pipetted into the DNeasy mini spin column from the DNeasy tissue kit as recommended by the manufacturer (Qiagen). DNA was eluted with 100 μl of TE buffer (10 mM Tris-Cl, pH 7.5, and 1 mM EDTA, pH 8) and stored at −20°C until being used. Recombinant plasmid DNA was extracted from *Escherichia coli* transformants by using a QIAminiprep kit (Qiagen).

RNA extraction and reverse transcription. Total RNA was isolated from 500 ovaries of *A. tabida* females singly infected with *Wolbachia* strain *w*Atab3, from 15 whole adult flies, and from 10^8 mosquito cells. Ovaries were dissected in a phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). Ovaries, insects, or cells were crushed in Trizol reagent (Invitrogen) with glass beads 212 to 300 μm in diameter (Sigma). After separation with chloroform, the aqueous phase was transferred into a new tube and the RNA was precipitated overnight with 2 M LiCl. Then samples were centrifuged at 16,000 × *g* and 4°C for 15 min. After the supernatant was discarded, pellets containing total RNA were washed twice with 70% ethanol by centrifugation at 16,000 × *g* and 4°C for 15 min each time and air dried for 30 min. The RNA obtained was dissolved in RNase-free water by incubation for 15 min at 37°C and then treated with DNase using a TURBO DNA-free kit as recommended by the manufacturer (Ambion). After the verification of the absence of contaminating DNA by PCR, aliquots of RNA were stored at −80°C until being used.

RNAs (1 to 3 μg) were reverse transcribed using SuperScript III reverse transcriptase (RT; Invitrogen) with random primers (Invitrogen). The procedure was done according to the manufacturer's protocols, but the final heat denaturation of the RT was omitted. Synthesized cDNA molecules were resuspended in 100 μl of H_2O and treated with 3 U of RNase H (Promega) for 20 min at 37°C. cDNA molecules were purified using a QIAquick PCR purification kit (Qiagen) and eluted with 15 μl of nuclease-free water (Ambion).

PCR, cloning, and sequencing. Oligonucleotide primers targeting *vir* genes and adjacent regions (Table 1) were designed according to the complete nucleotide sequence of the *W. pipientis* strain *w*Mel genome (GenBank accession number AE017196) by using the software Oligo 5.1. To produce recombinant VirB6 protein, PCR primers were designed according to the nucleotide sequence of the *virB6* gene of *Wolbachia* strain *w*Atab3, with the addition of restriction sites (Table 1). The oligonucleotides were synthesized by Invitrogen (France). PCR amplifications were done in a 25-μl reaction mixture containing the DNA template (30 to 90 ng) in 1× polymerase reaction buffer (Invitrogen), 1.5 mM $MgCl_2$, a 200 μM concentration of each deoxynucleoside triphosphate, a 200 nM concentration of each primer, and 0.5 U of *Taq* polymerase (Invitrogen). PCRs were performed in a T1 thermocycler (Biometra) under the following conditions: initial denaturation at 95°C for 1 min; 35 cycles of denaturation (94°C for 1 min), annealing (1 min at a melting temperature depending on the primers), and extension (72°C for 40 s to 2 min, depending on the fragment length); and a final extension at 72°C for 10 min. For nested PCRs, only 25 cycles were done. The PCR products were purified with a QIAquick PCR purification kit (Qiagen) and ligated into the PCR2.1-TOPO vector by using a TOPO-TA cloning kit (Invitrogen). After the transformation of competent *E. coli* DH5α cells, recombinant plasmids were extracted as described above and cloned inserts were subjected to sequencing at GenoScreen (Lille, France). All sequences were analyzed by the

TABLE 1. Primers, genes, and regions

Purpose	Gene(s)	Primer name[a]	Primer sequence (5′–3′)	Amplicon size (bp)/melting temp (°C)	GenBank accession no.[b] for *w*Ri/*w*Atab3 or reference
PCR	*ribA*, *virB8*, *virB9*	Vir2F	CGTCTGTTGGTGAATAATTTT	1,721/52	EF427901, EF423637/DQ887624,
		Vir2Fb	TGGAAGAGGYATTGGTTTAAC	1,191/53	DQ887625
		Vir2R	TTTTTAACTTTTTCTCCTTCC		
	virB9, *virB10*	Vir3F	CGGTGGTTTTTAGTCAGGGTT	1,979/56	EF423637, EF427902/DQ887625,
		Vir3Fb	TGCSTTAKGAAGATGGTGAAG	1,361/53	EF427903
		Vir3R	TCATCATAAACAGACTTGCTT		
	virB10	FB10m	CCAAAAGAAACTCCTGTGTCA	408/51	EF427902/EF427903
		RB10m	AAATGAATAACTGCCTATCAA		
	virB10, *virB11*	FB10e	TGATGCTGTTCTTGAAACTGC	879/50	EF427902, EF423636/EF427903,
		RB10e	CTTGAAATATGCCTTGTAATG		DQ887626
	virB10, *virB11*, *virD4*	Vir4F	GGTAGAGCAGGAATAGCAGGA	1,835/56	EF427902, EF423636,
		Vir4Fb	TCCACAGGCAATATTAAATCA	1,278/53	AY833076/EF427903,
		Vir4R	TATATAACTCTGGATGATGCC		DQ887626, DQ887627
	virD4	VirD4F	TTGTTGGCATCATCCAGAGTT	1,530/51	AY833076/DQ887627
		VirD4Fb	TGGTTGACGATGTGCAGAAAA	1,024/53	
		VirD4R	GCAGAAAGAACTTTATTTGGG		
	virD4, *wspB*	U21	ACCCAATTAAATCAAAGAAAA	622/49	AY833076, EU095939/
		RRT5	TAGACAAATTTTTGATACCTG		DQ887627, EU095938
	virD4, *wspB*	FRT5	GATAAGTTTGAAGATGATGAC	1,029/50	AY833076, EU095939/
		RwspB	TAAGCTTTGCTGGCAAAATGG		DQ887627, EU095938
	wspB, WD0010	F2wspB	AGGTAGCAAAGTAACTGAGGA	470/50	EU095939/EU095938
		R2wspB	ATGGTGGTATTGATGGTTGA		
	WD0856, *virB6*	VirB6F	ACGACCAGAAGAAAATTTACA	1,246/50	EF423639/DQ887628
		VirB6R	GTTTTTGTCCTTTATGTTCAT		
	virB6	VirB6Fb	CTTGTCCGGTTGTTCATTTTT	1,316/50	EF423639/DQ887628
		VirB6Rb	AATGTAGAGTTCTCAAAGCAG		
		FvirB6NdeI	ACGGAATTCATATGATAGCTGCAATTGTT	1,014/52	
		RvirB6BamHI	GGCGGATCCTTTCAGCCTTTTCTG		
	virB6, *virB4*	VirB4F	TGCCCGTTTTTCTCACTGCTT	1,285/50	EF423639, EF423640/DQ887628,
		VirB4R	TAGATAATCCAGTTTTTGCAC		DQ887629
	virB4	VirB4Fb	AAAAACATCCCGATAGACACT	1,338/50	EF423640/DQ887629
		VirB4Rb	TTTTTACAACTTCCTTACGAG		
	virB4, *virB3*	VirB3F	CGACAGAGCGTTATCCAATGA	1,576/49	EF423640, EF423641/DQ887629,
		VirB3R	GTCCATAACCAGTTTCAAATA		DQ887630
	virB3, WD0860	VirB3Fb	GAGCATTGGAGGTCTTGTAAG	1,334/50	EF423641/DQ887630
		VirB3Rb	TAAGAGGATATAAAACCCCAT		
RT-PCR	*virB8*, *virB9*	FRT1	TGGGTAACGATGCTCTTCAGG	377/50	
		RRT1	TTTTTAACTTTTTCTCCTTCC		
	virB9, *virB10*	FRT2	TTGTAAAAGGCTGTTATTTGG	339/50	
		RRT2	GATCAGTTATTATTCTTTCAG		
	virB10, *virB11*	FRT3	AAGCAATCGGGAAATCAATCG	383/50	
		RRT3	GCTTCTTCGCTAAGTTTTTGT		
	virB11, *virD4*	FRT4	GATCTGCGAGTGAAAATGCTT	282/50	
		RRT4	TATATAACTCTGGATGATGCC		
	virD4, *wspB*	FRT5	GATAAGTTTGAAGATGATGAC	350/50	
		RRT5	TAGACAAATTTTTGATACCTG		
	WD0853, WD0854	F853	CAATCTCTTTGTCTTCCTTAT	263/48	
		R854	TTCATCTTCAATCCTCTTCTC		
	WD0854, WD0855	F854	TCCAAGATGTCCATGCACCATTAACT	603/53	66
		R855	CTTTGGCGTGTTAGGTAATTTAGCCT		66
	WD0855, WD0856	F855	ATTTGATTTCCTCACCCTTAC	282/51	
		R856	TGACGTACCTGACTGTTGAGA		
	WD0856, *virB6*	FRT6	CCCATTTCTGCTCGTTGACA	225/50	
		RRT6	CTAAGAAGTTGAGTGGTGGAG		
	virB6, *virB4*	FRT7	TCTGCAAGCACTGGATATGAG	245/48	
		RRT7	TCAATGCTGTAGTAGCTAGAA		
	virB4, *virB3*	FRT8	CTTTTGTCATCAAGGTTGTGC	360/48	
		RRT8	TTGGTGTGAGTTATATATTTG		
	virB3, WD0860	FRT9	GAGCATTGGAGGTCTTGTAAG	379/48	
		RRT9	ATATCATCTGAAACTGCAATG		
Nested RT-PCR	*virB8*, *virB9*	FB8/B9n	ATTTACACGGAAAGATGGAAG	272/47	
		RB8/B9n	TACTTCATTAGGGCTATACAC		
	virB9, *virB10*	FB9/B10n	GGCGTAACAATTCAGAAGATG	101/50	
		RB9B10n	AAGCACTAAAATAATGACCAT		
	virB10, *virB11*	FB10/B11n	CAATGAAATGAACTATGCTGC	169/47	
		RB10/B11n	CAAATGGTTAAGATCGAATAC		
	virB11, *virD4*	FB11/D4n	GCGTACTTGAGTTTTGCTTCT	110/50	
		RB11/D4n	AGGGAAAGGCGTCAAACTAGG		
	virB6, *virB4*	FB6/B4n	CTGGATATGAGAGTAAAAAGT	222/47	
		RB6B4n	AGCTAGAATAGACTTAAAAGG		

[a] F, forward; R, reverse. Restriction site designations incorporated into primer names are underlined, as are the corresponding sequences.
[b] GenBank accession numbers correspond to sequences obtained in this study.

BLASTn program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and deposited in GenBank (Table 1).

For the cloning of *virB6* into an expression vector, PCR amplification was done with the high-fidelity Vent DNA polymerase (Ozyme) by employing an initial denaturation at 95°C for 3 min and two cycles of denaturation at 94°C, annealing at 40°C, and extension at 72°C for 1 min each, followed by a second round of denaturation at 95°C for 1 min 30 s; 35 cycles of denaturation (94°C for 1 min), annealing (52°C for 1 min), and extension (72°C for 1 min); and a final extension at 72°C for 10 min. The PCR amplicon was purified as described above, and a single adenosine was added to the 3′ ends of the double-stranded DNA molecules. Products were purified and cloned into the PCR2.1-TOPO vector as described above. The recombinant plasmid was extracted from *E. coli*, double-restricted with NdeI and BamHI, and subjected to gel electrophoresis. The expected *virB6* fragment was excised from the gel, purified using a QIAquick gel extraction kit (Qiagen), ligated into the restricted pIVEX2.4d vector (Roche Applied Science, Germany), and introduced into *E. coli* DH5α by transformation. Five transformant colonies were selected, and the corresponding pIVEX2.4d-*virB6* recombinant plasmids were purified and submitted to sequencing. One recombinant plasmid containing the expected *virB6* sequence was used to chemically transform *E. coli* BL21-AI (Invitrogen).

Production of anti-VirB6 peptides and recombinant VirB6 protein and immunoblotting. Two peptides encompassing residues 43 to 57 (FNSTGSFSRAS NPDC) and residues 687 to 701 (LDESYKNEQPDKSYE) from the VirB6 sequence of *w*Atab3 were synthesized by Covalab, France. The antisera were obtained by the immunization of rabbits with both oligopeptides coupled with keyhole limpet hemocyanin, and specific antibodies were purified from the antisera by affinity column chromatography using a mixture of the corresponding peptides fixed on the column (Covalab, France).

To produce VirB6 protein, the transformant *E. coli* BL21-AI/pIVEX2.4d-*virB6* was grown overnight at 37°C in modified Luria-Bertani medium (10 g of Bacto tryptone [Difco] liter^{-1}, 5 g of yeast extract [Difco] liter^{-1}, 5 g of NaCl liter^{-1}) containing 50 μg of carbenicillin ml^{-1}. Cultures (250 ml) were induced by L-arabinose at a 0.02% final concentration. Cell cultures were harvested by centrifugation at 6,500 × *g* for 20 min at 4°C. The pellet was resuspended in sample buffer (62.5 mM Tris-HCl [pH 6.8], 1% [wt/vol] sodium dodecyl sulfate [SDS], 100 mM dithioerythritol), the suspension was boiled at 95°C for 5 min, and a 25-μl sample was used to determine the protein concentration by the Bradford method with an assay kit from Bio-Rad. Then 10% glycerol and 0.001% bromophenol blue were added to the protein extract, and the mixture was heated for 5 min at 95°C. A 10-μg sample of proteins was separated by electrophoresis on a 1-mm-thick slab gel containing 12% polyacrylamide and 0.32% SDS in the upper buffer only (34). After electrophoresis, proteins were stained with Coomassie blue and the overexpressed protein band was excised and used for sequencing by liquid chromatography-tandem mass spectrometry at the Institut de Biologie et Chimie des Proteines, Université Lyon 1, Lyon, France.

To extract proteins from *Wolbachia*, the mosquito cell line RML12 infected with strain *w*MelPop and uninfected cells were used. Five 175-cm^2 flasks containing 35 ml of medium were used to grow cells using the medium and temperature described above. Cells were recovered when they reached 80% confluence (10^7 cells/ml). To isolate *Wolbachia*, cell cultures were centrifuged, the pellet was resuspended in 6 ml of fresh medium, and the suspension was divided into two 3-ml fractions, which were then homogenized thoroughly by being subjected to a vortex for 20 min in the presence of 3-mm borosilicate glass beads. Lysates were combined and centrifuged at 300 × *g* for 5 min, and the supernatant enriched with *Wolbachia* bacteria was centrifuged at 8,150 × *g* and 4°C for 15 min. Pellets containing bacteria were resuspended in 200 μl of 1× PBS, and the suspension was layered onto 800 μl of 250 mM sucrose in a 1.5-ml Eppendorf tube and centrifuged at 21,600 × *g* for 5 min at 4°C. Supernatant was removed, the pellet of *Wolbachia* cells was suspended in 50 μl of Laemmli buffer (35) containing 4% SDS, and the suspension was boiled for 5 min at 95°C and then used in SDS-polyacrylamide gel electrophoresis (PAGE). When flies were used as starting materials, five *D. simulans* adults infected with strain *w*Ri and five uninfected individuals were directly homogenized in 50 μl of Laemmli buffer, and the homogenates were boiled and centrifuged briefly to pellet debris before being used.

For Western blotting, separated proteins were transferred onto a nitrocellulose membrane (PerkinElmer) according to the method of Kyhse-Andersen (34). The transfer was done at 150 mA for 2 h. For dot blotting, crude boiled insect extracts were transferred onto a nitrocellulose membrane by using a Bio-Dot apparatus (Bio-Rad) with standard protocols. Membranes were soaked in 4% dried milk in 1× TBST buffer (20 mM Tris [pH 7.5], 130 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. Membranes were washed three times in 1× TBST buffer and incubated overnight at 4°C with anti-VirB6 at a 1:200 dilution in 1× TBST. Incubation was followed by three washes with 1× TBST, and then the membranes were incubated for 45 min at room temperature with the conjugate goat anti-rabbit immunoglobulin G–peroxidase at a 1:15,000 dilution in 1× TBST. Membranes were washed three times as described above. Hybridizing bands were revealed using an ECL kit as recommended by the manufacturer (Amersham).

Immunofluorescence microscopy. To obtain samples of ovaries, *D. simulans* females infected with *w*Ri, *A. tabida* Pierrefeu females singly infected with *w*Atab3, *A. tabida* North American females tri-infected with *Wolbachia w*Atab1, *w*Atab2, and *w*Atab3, and uninfected *A. tabida* North American females were dissected in 1× PBS. Recovered ovaries were rinsed once with 1× PBS before the fixation procedure. Cells of mosquito lines RML12 and Aa23 that were uninfected or infected with the *Wolbachia* strains *w*MelPop and *w*AlbB were also used for bacterial detection by immunofluorescence. A total of 10^4 to 10^5 cells of each cell line were separately transferred into Trac bottles containing a glass slide (Fisher Scientific) and grown in 1 ml of medium until approximately 80% confluence was achieved. The medium was removed, and cells attached to the glass were washed once with 1× PBST (PBS containing 1% Triton X-100) before the application of a fixative. Samples of cells and ovaries were fixed with 4% formaldehyde in 1× PBST for 20 min at room temperature. After being washed three times in 1× PBST (5 min each time), samples were incubated with anti-VirB6 (diluted 1:200 in 1× PBST and 1% bovine serum albumin Σ) for 1 h at room temperature. Samples were washed in 1× PBST three times for 10 min each time and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (diluted 1:500 in 1× PBST and 1% bovine serum albumin). At the end of the 1-h incubation time, samples were washed as described above and then mounted in 80% glycerol with 1× PBS. The samples were observed by using a fluorescence microscope (Axio ImagerZ1; Zeiss).

Nucleotide sequence accession numbers. The gene sequences obtained in this study have been deposited in GenBank under accession numbers AY833076, DQ887624, DQ887625, DQ887626, DQ887627, DQ887628, DQ887629, DQ887630, EF427901, EF427902, EF427903, EF423636, EF423637, EF423639, EF423640, EF423641, EU095938, and EU095939.

RESULTS

Identification of the *virB* and *virD4* loci in *Wolbachia* strains *w*Atab3 and *w*Ri. Based on the genome sequence of *Wolbachia* strain *w*Mel (66), oligonucleotide primers targeting *virB* and *virD4* genes were designed and used in PCR amplifications. Eight orthologs of *virB3*, *virB4*, *virB6*, *virB8*, *virB9*, *virB10*, *virB11*, and *virD4* were successfully amplified using genomic DNA extracted from *A. tabida* and *D. simulans* singly infected with *Wolbachia* strains *w*Atab3 and *w*Ri, respectively. Sequences of genes and intergenic regions indicated that the genomic organizations of these T4SS loci in the two strains are similar (Fig. 1 and Table 1). The *vir* genes are arranged into two separate clusters; one cluster (herein designated cluster I) contains the genes *virB8*, *virB9*, *virB10*, *virB11*, and *virD4*, and the other cluster (herein designated cluster II) is composed of *virB3*, *virB4*, and *virB6* (Fig. 1). This gene composition and clustering are similar to those of the T4SS determinants present in other *Wolbachia* strains, including *w*Mel (66), *w*Bm (23), *w*Ana (http://www.ncbi.nlm.nih.gov), and *w*Vul (GenBank accession numbers AY967767 and AY967766), as well as in the closest relatives, *Anaplasma* spp., *Ehrlichia* spp., and *Rickettsia* spp. (2, 43). Genes *virB1*, *virB2*, *virB5*, and *virB7* of *A. tumefaciens* seem to be absent in most *Rickettsiales* investigated so far.

All the Vir protein sequences of *w*Ri and *w*Atab3 showed higher levels of similarity (90 to 100%) to their homologues in *Wolbachia* strains *w*Mel and *w*Ana from insects than to Vir protein sequences of *Wolbachia* strains *w*Vul from the isopod *Armadillidium vulgare* and *w*Bm from the nematode *B. malayi* (81 to 90%) (see Table ST1 in the supplemental material).

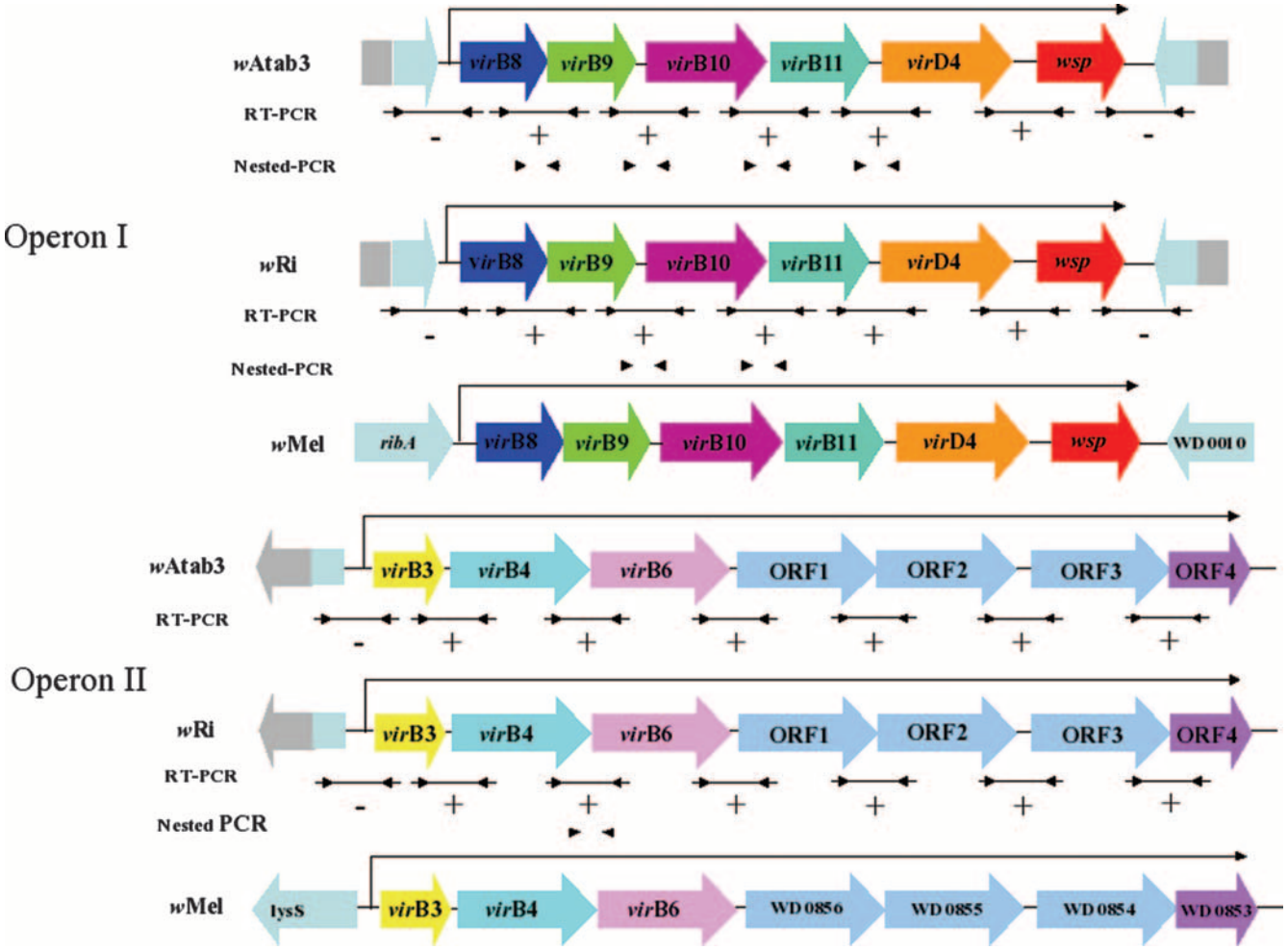

FIG. 1. Genomic organization and expression of the T4SS in *Wolbachia* strains *w*Atab3 and *w*Ri. ORFs are represented as open boxes, with arrowheads indicating their orientations. Lines with facing arrows at the ends indicate the regions subjected to RT-PCR transcriptional analysis. Arrows indicate positions of primers. Facing arrowheads indicate nested PCR. + or − indicates positive or negative transcription. The *w*Mel transcription pattern is depicted based on data from Wu et al. (66).

Alignments with available VirB and VirD4 protein sequences from other alphaproteobacteria showed the highest levels of amino acid similarity to Vir proteins from members of the closest genera, *Anaplasma* spp. and *Ehrlichia* spp. (33 to 78%), and the more distantly related *Rickettsia* spp. (11 to 28%), as expected (data not shown). These results are in accordance with the fact that all these four genera belong to the same order, *Rickettsiales* (21).

To further analyze the relationships among VirB and VirD orthologs of *w*Atab3 and *w*Ri and those of other alphaproteobacteria, we constructed a phylogenetic tree based on concatenated amino acid sequences. Concatenated VirB and VirD amino acid sequences of *Wolbachia* strains clustered into the same group, as did those of *Anaplasma* spp., *Ehrlichia* spp., and *Rickettsia* spp. (Fig. 2). The four groups were promiscuously located. The VirB-VirD phylogeny presented in Fig. 2 and the species phylogeny (65) are congruent, suggesting that no recent lateral acquisition of *vir* genes from distant bacteria by *Rickettsiales* has occurred.

As in the *w*Mel genome, in both *w*Ri and *w*Atab3, cluster I is positioned downstream of the *ribA* gene, encoding the GTP cyclohydrolase II, and upstream of the *wspB* gene, encoding an outer membrane protein, whereas cluster II is located downstream of the *lysS* gene, encoding lysyl-tRNA synthetase, and upstream of a gene encoding a hypothetical membrane protein (the WD0856 gene). The intergenic regions between *virD4* and *wspB* and between *virB6* and WD0856 are short to harbor potential promoters for these genes, and in addition, the *wspB* and WD0856 genes in the two strains are in the same orientation as the respective neighboring *vir* loci, which may suggest coregulation.

Transcriptional analysis of *virB* and *virD* genes in *Wolbachia* strains. To analyze the cotranscription of *vir* genes of the two clusters in strains *w*Atab3 and *w*Ri, RT-PCR was used with oligonucleotide primers that overlapped contiguous genes (Fig. 1). Results showed that the cDNA amplicons obtained had the expected sizes, as did the control DNA amplicons (Fig. 3). No amplification was achieved in the absence of RT, whatever the quantity of RNA used, up to 9 ng, thus excluding genomic DNA contamination. The sequencing of RT-PCR

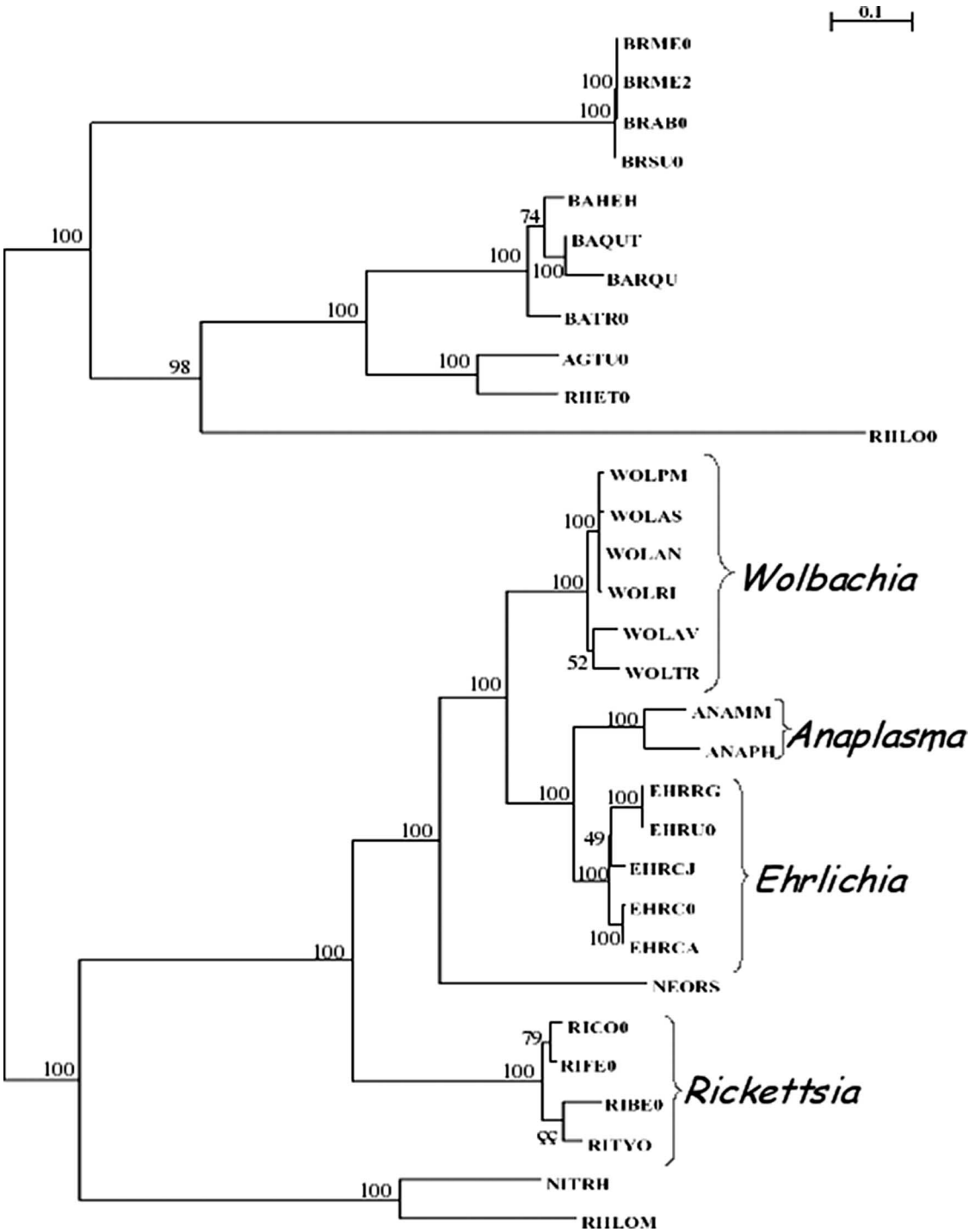

FIG. 2. T4SS tree topology of strains belonging to the *Rickettsiales* and other alphaproteobacteria. This tree was inferred from concatenated alignments of amino acid sequences corresponding to VirB3, VirB4, VirB8, VirB9, and VirB11. The topology and branch lengths are according to the results of a maximum-likelihood analysis of the five protein sequences with 100 bootstraps replicates. BRME0, *Brucella melitensis* biovar *suis*; BRME2, *Brucella melitensis* biovar *abortus*; BRAB0, *Brucella abortus* biovar 1 strain 9-941; BRSU0, *Brucella suis* 1330; BAHEH, *Bartonella henselae* strain Houston-1; BAQUT, *Bartonella quintana* strain Toulouse; BARQU, *Bartonella quintana*; BATR0, *Bartonella tribocorum*; AGTU0, *Agrobacterium tumefaciens* strain C58; RHET0, *Rhizobium etli*; WOLPM, *Wolbachia* strain *w*Mel; WOLAS, *Wolbachia* strain *w*Atab3; WOLAN, *Wolbachia* strain *w*Ana; WOLRI, *Wolbachia* strain *w*Ri; WOLAV, *Wolbachia* strain *w*Vul; WOLTR, *Wolbachia* strain *w*Bm; ANAMM, *Anaplasma marginale* strain St. Marie; ANAPH, *Anaplasma phagocytophilum* HZ; EHRRG, *Ehrlichia ruminantium* strain Gardel; EHRU0, *Ehrlichia ruminantium* strain Welgevonden; EHRCJ, *Ehrlichia canis* strain Jake; EHRC0, *Ehrlichia chaffeensis*; EHRCA, *Ehrlichia chaffeensis* strain Arkansas; NEORS, *Neorickettsia sennetsu* strain Miyayama; RICO0, *Rickettsia conorii* strain Malish; RIFE0, *Rickettsia felis* URRWXCAL2; RIBE0, *Rickettsia bellii* RML369-C; RITYO, *Rickettsia typhi* strain Wilmington; NITRH, *Nitrobacter hamburgensis* X14; RHLOM, *Mesorhizobium loti*.

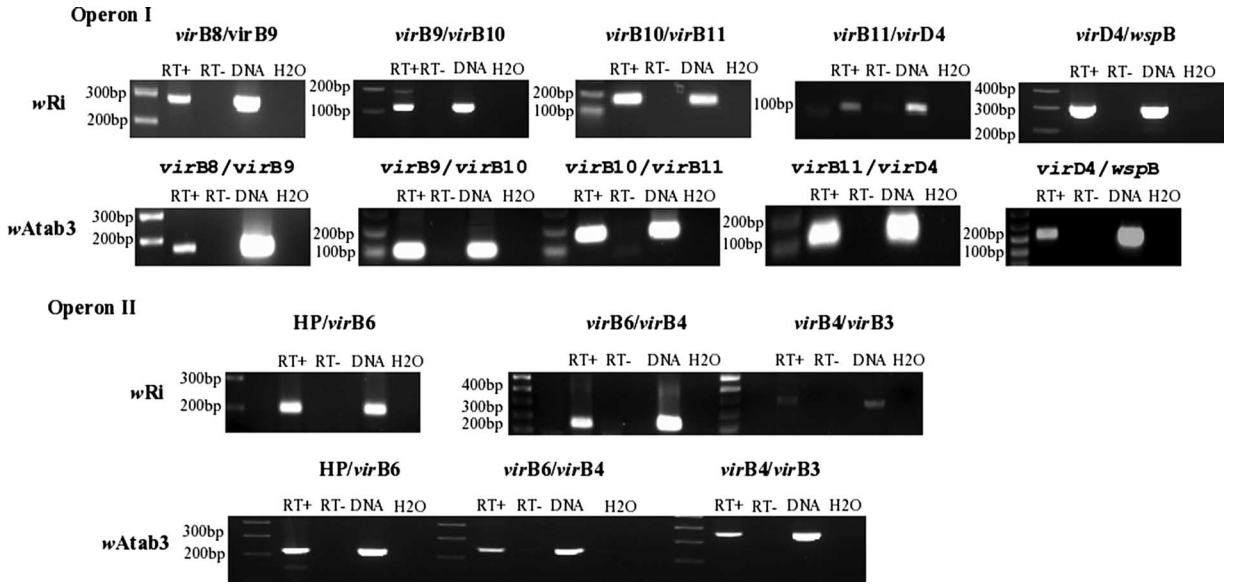

FIG. 3. Transcriptional analysis of *virB-virD4* operons in *Wolbachia* strains *w*Ri and *w*Atab3. RT-PCR was used to analyze cotranscription. The primers used and their positions are shown in Table 1 and Fig. 1. RT+ and RT− indicate the presence and absence of RT in the reaction mixtures. The DNA control lanes (containing 30 ng of genomic DNA from insects singly infected with *w*Ri or with *w*Atab3) show the specificity of amplification with each pair of primers. HP, ORF1.

products ascertained the presence of the expected overlapping *vir* genomic regions (data not shown). These results indicate that *virB8* to *virB11* and *virD4* of cluster I are cotranscribed, that *virB3*, *virB4*, and *virB6* of cluster II are cotranscribed, and that these clusters constitute two operons.

As some genes adjacent to *vir* loci were reported previously to be cotranscribed in *Wolbachia w*Mel from *D. melanogaster* (66), further transcriptional examination of contiguous genes in each cluster was performed using RNA extracted from insect tissues and oligonucleotide primers as depicted in Fig. 1. We found that *wspB* of cluster I and the four open reading frames (ORFs) of cluster II were cotranscribed with the genes of their respective operons (Fig. 1 and 3). Successful transcription of bona fide *virB* and *virD* genes and adjacent loci was also achieved using RNA extracted from mosquito RML12 and Aa23 cells infected with *Wolbachia w*MelPop and *w*AlbB (data not shown).

All together, these results clearly showed that T4SS genes of several *Wolbachia* strains from arthropods, notably, *w*Atab3 and *w*Ri, as well as *w*AlbB and *w*MelPop, are conserved and are transcriptionally expressed both in cellulo and in vivo.

Immunodetection of *Wolbachia* VirB6 protein in infected tissues. A comparative analysis of amino acid sequences of VirB6 showed high levels of similarity (up to 99% identity) among the proteins of the three *Wolbachia* strains from arthropods (*w*Mel, *w*Atab3, and *w*Ri). Polyclonal anti-VirB6 antibodies were produced by the immunization of rabbits with two peptides corresponding to conserved and antigenic C- and N-terminal *Wolbachia* VirB6 sequences (see Materials and Methods). Therefore, the anti-VirB6 peptides produced may detect these strains in the immunochemistry experiments.

To examine the quality of anti-VirB6 to be used for detecting the corresponding *Wolbachia* protein, an analysis was done by Western blotting of crude protein extracts from *E. coli* harboring a recombinant form of VirB6 from the *Wolbachia w*Atab3 strain. The results revealed a chemiluminescent specific band of the expected size in the induced *E. coli* culture overproducing recombinant VirB6 protein, and no signal was detected in the uninduced culture (Fig. 4A). The identity of the VirB6 protein was also confirmed by the sequencing of the corresponding band extracted from the polyacrylamide gel (data not shown). In addition, the production of VirB6 protein was investigated using the *Wolbachia* protein extract purified from infected mosquito cells. The anti-VirB6 antibodies detected a unique and specific band of approximately 200 kDa exclusively in the fraction of *Wolbachia*-infected cells (Fig. 4B). The detected 200-kDa protein was more than twice the expected size of *Wolbachia* VirB6 protein (90 kDa). SDS-PAGE

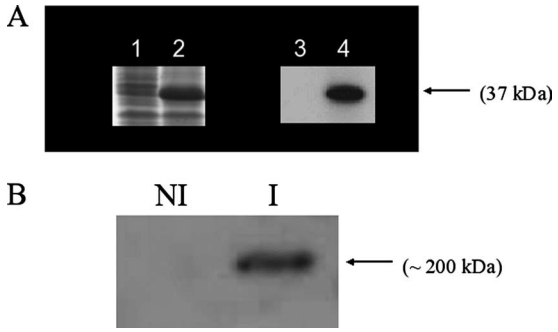

FIG. 4. Western blots of VirB6 proteins. (A) SDS-PAGE profile of *E. coli* BL21-AI harboring pIVEX2.4d-*vir*B6 (lanes 1 and 2) and the corresponding immunoblot with anti-VirB6 (lanes 3 and 4). Lanes 1 and 3, uninduced culture; lanes 2 and 4, culture induced by L-arabinose. (B) Western blot of *Wolbachia* protein revealed by anti-VirB6. NI, extract from *Aedes aegypti* RML12 cells not infected with *Wolbachia*; I, extract from *Wolbachia*-infected *Aedes aegypti* RML12 cells.

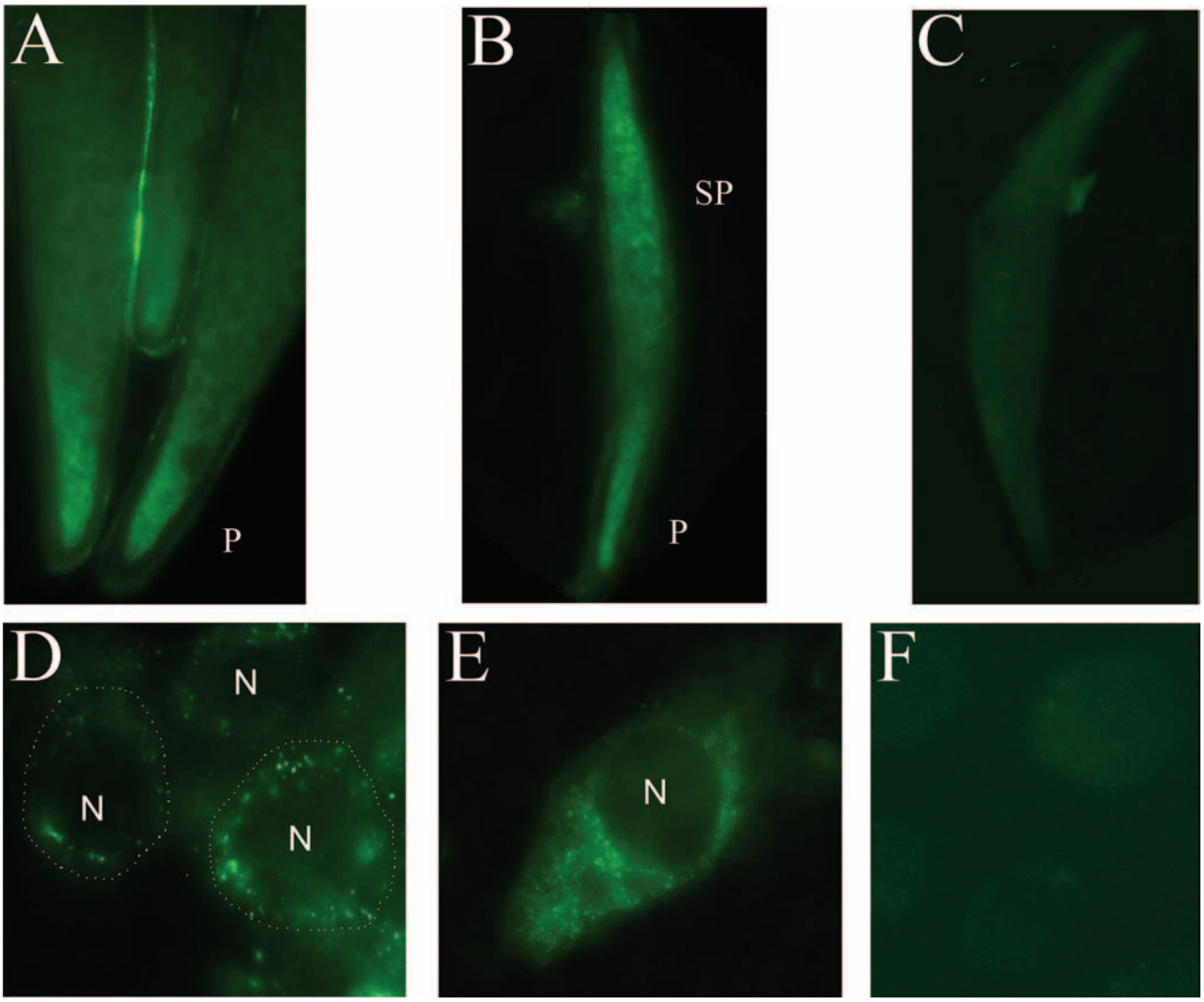

FIG. 5. Immunodetection of *Wolbachia* by anti-VirB6 peptides in cellulo and in insecta. Tissues were labeled with anti-VirB6 and fluorescent conjugates. (A to C) Oocytes of *A. tabida* singly infected with strain *w*Atab3 (A) and infected with the three strains *w*Atab1, *w*Atab2, and *w*Atab3 (B) and of an uninfected *A. tabida* line generated by antibiotic treatment (C). P and SP indicate polar and subpolar regions, respectively. (D) Low-magnification image showing *Wolbachia w*MelPop in a group of *Aedes aegypti* RML12 cells with boundaries manually delineated by circles. (E) High-magnification image of an enlarged *Wolbachia*-infected RML12 cell demonstrating the cell shape. (F) Uninfected RML12 cell. N designates the nucleus.

and Western blotting have previously revealed VirB6 and VirD4 proteins with unusually high molecular masses in *Agrobacterium* (25). The authors of the previous study suggested that this finding resulted from aggregates formed during boiling, as seen for some membrane proteins with high molecular masses. Other authors have reported difficulties and failures in detecting *Agrobacterium* VirB6 protein in Western blot assays due to the fact that VirB6 protein may form an SDS-resistant oligomer that does not enter the gel (32, 33). We took advantage of the aggregation behavior of VirB6 protein to perform a dot blot assay using adult flies. Results showed strong signals in the extracts from flies harboring *Wolbachia* strain *w*Ri, whereas weak signals corresponding to nonspecific bindings were seen in the samples from uninfected flies (see Fig. S1 in the supplemental material).

To detect VirB6 expression in vivo, we first used ovaries of *A. tabida* Pierrefeu females monoinfected with *Wolbachia* strain *w*Atba3. The analysis showed specific signals mainly in the cytoplasm of posterior oocytes hosting *w*Atab3 (Fig. 5A), which is in accordance with the usual location of this strain as shown previously (18). To ascertain that the signals corresponded to *Wolbachia* VirB6, we performed the same experiment with ovaries of the *A. tabida* North American line triply infected with *Wolbachia* and with ovaries of uninfected *A. tabida* North American females. In contrast to *A. tabida* Pierrefeu females, females of the North American line treated with

antibiotic to remove *Wolbachia* still have the ability to produce a certain number of eggs (17) and thus provided us with a negative control. The results confirmed strong specific signals in the poles of infected oocytes, albeit more extended toward the subpole areas of the oocytes than in the singly infected lines, indicating high *Wolbachia* cell density due to tri-infection (Fig. 5B). No signal was detected in uninfected ovaries, as expected (Fig. 5C). Similar specific immunofluorescent signals were also obtained using ovaries from *D. simulans* flies singly infected with strain *w*Ri (data not shown). Moreover, we applied immunofluorescence microscopy approaches to detect *Wolbachia* in the mosquito cell cultures by using anti-VirB6. Results showed fluorescent signals in the cytoplasm of cells infected with *Wolbachia* *w*MelPop (Fig. 5D and E) and *w*AlbB (data not shown), whereas no signal was seen in uninfected cells (Fig. 5F) used as a negative control.

These results indicate that the synthetic anti-VirB6 peptides produced in this study can specifically detect the VirB6 protein expressed by different *Wolbachia* strains (notably, *w*Atab3 and *w*Ri, as well as *w*AlbB and *w*MelPop) in host tissues.

DISCUSSION

The *vir* genes of the T4SS were investigated for their presence and expression in *Wolbachia* strains by PCR and RT-PCR amplifications, as well as by Western blotting and immunofluorescence microscopy using polyclonal antibodies to VirB6, a transmembranal core protein of the VirB-VirD4 apparatus. We have identified and sequenced two *vir* gene clusters from *Wolbachia* strains *w*Atab3 and *w*Ri, which had infected *A. tabida* and *D. simulans*, respectively. One cluster comprises *virB8*, *virB9*, *virB10*, *virB11*, and *virD4* located upstream from the *wspB* gene, and the other contains *virB3*, *virB4*, and *virB6* found upstream from the WD0856 homologue. The deduced amino acid sequences corresponding to these *vir* genes showed high levels of similarity (81 to 100% identity) to previously described *vir* loci in other *Wolbachia* strains (23, 40, 66).

The *virB8* to *virB11* and *virD4* genes of *w*Atab3 and *w*Ri were polycistronically transcribed, probably through a promoter located between the upstream gene *ribA* and *virB8*, as in *Wolbachia* strain *w*Tai (40). Here, this operon was found to extend to the downtream *wspB* gene, as in *w*Mel (66). Two other *wsp* genes are present in the *Wolbachia* genome, of which one (*wspA*) is usually used in strain identification and phylogeny analyses (68), although currently these analyses are completed by multilocus sequence typing (3, 4, 47). The *wsp* locus encodes an outer surface protein (Wsp) whose homologue in *Wolbachia* strain *w*Dm from the nematode *Dirofilaria immitis* is able to inhibit the apoptosis of human granulocytes (6). A gene for an antigenic outer membrane protein, a member of the p44 multigene family, is also cotranscribed with the *virB-virD4* cluster in *A. phagocytophilum* (43). The fact that *wspB* is cotranscribed with the *virB8* to *virD4* genes suggests its possible role in *Wolbachia* T4SS functioning. This role remains to be established, as *wspB* is interrupted by insertion sequence elements in some *Wolbachia* strains (49).

In *w*Atab3 and *w*Ri, we showed that the clustered *virB3*, *virB4*, and *virB6* genes were also cotranscribed as an operon, together with four downstream ORFs. This polycistronic transcription pattern is similar to that demonstrated previously for *Wolbachia* *w*Mel (66). Among the four adjacent cotranscribed ORFs, three are annotated as genes for putative membrane-spanning proteins (WD0854 to WD0856) in the *w*Mel genome whereas the products of the corresponding coding sequences in the *w*Bm genome are classified as VirB6 paralogs (Wbm0793 to Wbm0795); thus, the proteins corresponding to these ORFs are possibly some of the T4SS components. The fourth ORF encodes a typical hypothetical protein.

The architectural organization into two separate operons of tandemly arranged *virB* and *virD* genes in *Wolbachia* is similar to that found in members of closely related bacterial genera of the order *Rickettsiales*, including *Anaplasma* spp., *Ehrlichia* spp., and *Rickettsia* spp. (2, 43). This pattern is in contrast to the single *virB-virD* locus found in other free-living or facultative intracellular bacteria, like *A. tumefaciens*, *Mesorhizobium loti*, *Bordetella pertussis*, and *Bartonella* spp. (51, 57, 61, 62). Surprisingly, orthologs of *virB1*, *virB2*, *virB5*, and *virB7* seem to be absent in most members of the order *Rickettsiales* investigated so far. VirB2 and VirB5 are pilus components (36, 50), and VirB7 is a pilus-associated protein (48), whereas VirB1 is not an essential component of transport systems (7). Because *Wolbachia* and closely related genera are strict intracellular bacteria, they may be able to excrete molecules across their cell walls through the core T4SS membrane-spanning structure directly into the cytoplasm of the host cell, or the related pilus-encoding genes may be present in the genomes but have extremely low levels of similarity to the corresponding *virB* genes that prevent them from being detected by a simple sequence comparison.

An increasing amount of literature has shown the importance of T4SSs in intracellular bacteria for the successful infection of and survival, proliferation, and persistence within their hosts (10, 13, 52). For instance, the secretion of AnkA protein by *A. phagocytophilum* through a T4SS was found to facilitate infection (38). Therefore, the T4SS is expected to play a significant role in the wide range of phenotypes induced by *Wolbachia* strains in their invertebrate hosts. Of primary significance, in this study, genes encoding T4SS machinery were transcribed in insect tissues infected with *Wolbachia* strains *w*Atab3 and *w*Ri, as well as in cultured cell lines harboring *w*AlbB and *w*MelPop. To further investigate the functionality of the *Wolbachia* T4SS, polyclonal antibodies raised against VirB6 were produced and tested in different tissues infected with *Wolbachia*. The *A. tumefaciens* VirB6 protein (343 amino acids [aa], corresponding to GenBank accession number AAK90538) is considerably smaller than the *Wolbachia* protein (854 aa). The level of similarity between the two proteins is relatively low (11%), and identical amino acids are found mainly in stretches of 1 to 2 aa scattered along the central part of the *Wolbachia* VirB6 protein. VirB6 is a polytopic integral membrane protein that forms complexes with the core structure of T4SS. Several studies have shown the crucial role of VirB6 in both the assembly and functioning of T4SS apparatus (30, 32, 33). An examination of ovaries recovered from *A. tabida* and *D. simulans* females hosting *Wolbachia* strains *w*Atab3 and *w*Ri, respectively, showed the presence of VirB6 protein, as identified by immunofluorescence methods. Moreover, specific fluorescent VirB6 signals were observed in the cytoplasm of mosquito cell lines infected with *Wolbachia* strain *w*MelPop from the *D. melanogaster* w1118 line and

*w*AlbB from *Aedes albopictus*. To our knowledge, this was the first study to combine both transcriptional and immunolocalization analyses to monitor the expression of *vir* loci of *Wolbachia* in cellulo and in vivo.

In this study, we detected the presence of the *virB* and *virD* genes in the genomes of two strains of *Wolbachia*, *w*Ri and *w*Atab3, and determined their expression in host tissues. These two strains are involved in two different phenotypes; *w*Ri induces cytoplasmic incompatibility in *D. simulans*, whereas *w*Atab3 is directly involved in the production of oocytes in *A. tabida*. Could the T4SS help to elucidate the answers to some fundamental questions about the interactions between *Wolbachia* strains and their hosts? What kinds of molecules are secreted across the bacterial membrane via the T4SS? Candidates are proteins with ankyrin domains that are encoded in *Wolbachia* genomes (23, 66). Are the excreted molecules involved in the interactions between *Wolbachia* strains and their hosts? Are they essential for the persistence of the bacteria in eukaryotic cells? Our investigations open new avenues for exploring T4SS function at the protein level in *Wolbachia*.

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REFERENCES

1. **Anderson, C. L., and T. L. Karr.** 2001. *Wolbachia*: evolutionary novelty in rickettsial bacteria. BMC Evol. Biol. **1:**10.
2. **Andersson, S. G., A. Zomorodipour, J. O. Andersson, T. Sicheritz-Pontén, U. C. Alsmark, R. M. Podowski, A. K. Näslund, A. S. Eriksson, H. H. Winkler, and C. G. Kurland.** 1998. The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. Nature **396:**133–140.
3. **Baldo, L., J. C. Dunning Hotopp, K. A. Jolley, S. R. Bordenstein, S. A. Biber, R. R. Choudhury, C. Hayashi, M. C. Maiden, H. Tettelin, and J. H. Werren.** 2006. Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. Appl. Environ. Microbiol. **72:**7098–7110.
4. **Baldo, L., and J. H. Werren.** 2007. Revisiting *Wolbachia* supergroup typing based on WSP: spurious lineages and discordance with MLST. Curr. Microbiol. **55:**81–87.
5. **Bandi, C., J. W. McCall, C. Genchi, S. Corona, L. Venco, and L. Sacchi.** 1999. Effects of tetracycline on the filarial worms *Brugia pahangi* and *Dirofilaria immitis* and their bacterial endosymbionts *Wolbachia*. Int. J. Parasitol. **29:**357–364.
6. **Bazzocchi, C., S. Comazzi, R. Santoni, C. Bandi, C. Genchi, and M. Mortarino.** 2007. *Wolbachia* surface protein (WSP) inhibits apoptosis in human neutrophils. Parasite Immunol. **29:**73–79.
7. **Berger, B. R., and P. J. Christie.** 1994. Genetic complementation analysis of the *Agrobacterium tumefaciens virB* operon: *virB2* through *virB11* are essential virulence genes. J. Bacteriol. **176:**3646–3660.
8. **Bouchon, D., T. Rigaud, and P. Juchault.** 1998. Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. Proc. Biol. Sci. **265:**1081–1090.
9. **Brüggemann, H., C. Cazalet, and C. Buchrieser.** 2005. Adaptation of *Legionella pneumophila* to the host environment: role of protein secretion, effectors and eukaryotic-like proteins. Cur. Opin. Microbiol. **9:**1–9.
10. **Cascales, E., and P. J. Christie.** 2003. The versatile bacterial type IV secretion systems. Nat. Rev. Microbiol. **1:**137–149.
11. **Cascales, E., and P. J. Christie.** 2004. Definition of a bacterial type IV secretion pathway for DNA substrate. Science **304:**1170–1173.
12. **Censini, S., C. Lange, Z. Xiang, J. E. Crabtree, P. Ghiara, M. Borodovsky, R. Rappuoli, and A. Covacci.** 1996. cag, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. Proc. Natl. Acad. Sci. USA **93:**14648–14653.
13. **Christie, P. J., and J. P. Vogel.** 2000. Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. Trends Microbiol. **8:**354–360.
14. **Christie, P. J., K. Atmakuri, V. Krishnamoorthy, S. Jakubowski, and E. Cascales.** 2005. Biogenesis, architecture, and function of bacterial type IV secretion systems. Annu. Rev. Microbiol. **59:**451–485.
15. **David, J., and M. F. Clavel.** 1967. Influence of temperature during the course of development on various biometric characteristics of adult *Drosophila melanogaster* Meigen. J. Insect. Physiol. **13:**717–729.
16. **Dedeine, F., F. Vavre, D. D. Shoemaker, and M. Boulétreau.** 2004. Intra-individual coexistence of a *Wolbachia* strain required for host oogenesis with two strains inducing cytoplasmic incompatibility in the wasp *Asobara tabida*. Evolution Int. J. Org. Evolution **58:**2167–2174.
17. **Dedeine, F., M. Boulétreau, and F. Vavre.** 2005. *Wolbachia* requirement for oogenesis: occurrence within the genus *Asobara* (*Hymenoptera*, *Braconidae*) and evidence for intraspecific variation in *A. tabida*. Heredity **95:**394–400.
18. **Dedeine, F., F. Vavre, F. Fleury, B. Loppin, M. E. Hochberg, and M. Boulétreau.** 2001. Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. Proc. Natl. Acad. Sci. USA **98:**6247–6252.
19. **de la Cruz, F., and J. Davies.** 2000. Horizontal gene transfer and the origin of species: lessons from bacteria. Trends Microbiol. **8:**128–133.
20. **Ding, Z., and P. J. Christie.** 2003. *Agrobacterium tumefaciens* twin-arginine-dependent translocation is important for virulence, flagellation, and chemotaxis but not type IV secretion. J. Bacteriol. **185:**760–771.
21. **Dumler, J. S., A. F. Barbet, C. P. J. Bekker, G. A. Dasch, G. H. Palmer, S. C. Ray, Y. Rikihisa, and F. Rurangirwa.** 2001. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order *Rickettsiales*: unification of some species of *Ehrlichia* with *Anaplasma* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int. J. Syst. Evol. Microbiol. **51:**2145–2165.
22. **Duttan, C., and A. Pan.** 2002. Horizontal gene transfer and bacterial diversity. J. Biosci. **27:**27–33.
23. **Foster, J., M. Ganatra, I. Kamal, J. Ware, K. Makarova, N. Ivanova, A. Bhattacharyya, V. Kapatral, S. Kumar, J. Posfai, T. Vincze, J. Ingram, L. Moran, A. Lapidus, M. Omelchenko, N. Kyrpides, E. Ghedin, S. Wang, E. Goltsman, V. Joukov, O. Ostrovskaya, K. Tsukerman, M. Mazur, D. Comb, E. Koonin, and B. Slatko.** 2005. The *Wolbachia* genome of *Brugia malayi*: endosymbiont evolution within a human pathogenic nematode. PLoS Biol. **3:**0599–0614.
24. **Grohmann, E., G. Muth, and M. Espinosa.** 2003. Conjugative plasmid transfer in gram-positive bacteria. Microb. Mol. Biol. Rev. **67:**277–301.
25. **Hapfelmeier, S., N. Domke, P. C. Zambryski, and C. Baron.** 2000. VirB6 is required for stabilization of VirB5 and VirB3 and formation of VirB7 homodimers in *Agrobacterium tumefaciens*. J. Bacteriol. **182:**4505–4511.
26. **Hilgenboecker, K., P. Hammerstein, P. Schlattmann, A. Telschow, and J. H. Werren.** 2008. How many species are infected with *Wolbachia*? A statistical analysis of current data. FEMS Microbiol. Lett. **281:**215–220.
27. **Hooykaas, P. J. J., and A. G. M. Beijersbergen.** 1994. The virulence system of *Agrobacterium tumefaciens*. Annu. Rev. Phytopathol. **32:**157–179.
28. **Hubber, A., A. C. Vergunst, J. T. Sullivan, P. J. Hooykaas, and C. W. Ronson.** 2003. Symbiotic phenotypes and translocated effector proteins of the *Mesorhizobium loti* strain R7A VirB/D4 type IV secretion system. Mol. Microbiol. **54:**561–574.
29. **Hurst, G. D., J. H. Graf von der Schulenburg, T. M. Majerus, D. Bertrand, I. A. Zakharov, J. Baungaard, W. Volkl, R. Stouthamer, and M. E. Majerus.** 1999. Invasion of one insect species, *Adalia bipunctata*, by two different male-killing bacteria. Insect Mol. Biol. **8:**133–139.
30. **Jakubowski, S. J., V. Krishnamoorthy, and P. J. Christie.** 2003. *Agrobacterium tumefaciens* VirB6 protein participates in formation of VirB7 and VirB9 complexes required for type IV secretion. J. Bacteriol. **185:**2867–2878.
31. **Jeyaprakash, A., and M. A. Hoy.** 2000. Long PCR improves *Wolbachia* DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. Insect Mol. Biol. **9:**393–405.
32. **Judd, P. K., D. Mahli, and A. Das.** 2005. Molecular characterization of the *Agrobacterium tumefaciens* DNA transfer protein VirB6. Microbiology **51:**3483–3492.
33. **Judd, P. K., R. B. Kumar, and A. Das.** 2005. The type IV secretion apparatus protein VirB6 of *Agrobacterium tumefaciens* localizes to a cell pole. Mol. Microbiol. **55:**115–124.
34. **Kyhse-Andersen, J.** 1984. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Methods **10:**203–209.
35. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **277:**680–685.
36. **Lai, E. M., and C. I. Kado.** 1998. Processed VirB2 is the major subunit of the promiscuous pilus of *Agrobacterium tumefaciens*. J. Bacteriol. **180:**2711–2717.
37. **Laven, H.** 1967. Eradication of *Culex pipiens fatigans* through cytoplasmic incompatibility. Nature **216:**383–384.
38. **Lin, M., A. den Dulk-Ras, P. J. Hooykaas, and Y. Rikihisa.** 2007. *Anaplasma phagocytophilum* AnkA secreted by the type IV secretion system is tyrosine phosphorylated by Abl-1 to facilitate infection. Cell. Microbiol. **9:**2644–2657.
39. **Malek, J. A., J. M. Wierzbowski, W. Tao, S. A. Bosak, D. J. Saranga, L. Doucette-Stamm, D. R. Smith, P. J. McEwan, and K. J. McKernan.** 2004.

Protein interaction mapping on a functional shotgun sequence of *Rickettsia sibirica*. Nucleic Acids Res. **32:**1059–1064.
40. **Masui, S., T. Sasaki, and Y. Ishikawa.** 2000. Genes for the type IV secretion system in an intracellular symbiont, *Wolbachia*, a causative agent of various sexual alterations in arthropods. J. Bacteriol. **182:**6529–6531.
41. **Mavingui, P., V. Tran Van, E. Labeyrie, E. Rancès, F. Vavre, and P. Simonet.** 2005. Efficient procedure of purification of obligate intracellular *Wolbachia pipientis* and representative amplification of its genome by multiple-displacement amplification. Appl. Environ. Microbiol. **71:**6910–6917.
42. **McCullen, C. A., and A. N. Binns.** 2006. *Agrobacterium tumefaciens* and plant cell interactions and activities required for interkingdom macromolecular transfer. Annu. Rev. Cell Dev. Biol. **22:**101–127.
43. **Ohashi, N., N. Nzi, Q. Lin, and Y. Rikihisa.** 2002. Characterization and transcriptional analysis of gene clusters for type IV secretion machinery in human granulocytic and monocytic ehrlichiosis agents. Infect. Immun. **70:** 2128–2138.
44. **O'Neill, S. L., R. Giordano, A. M. E. Colbert, T. L. Karr, and H. M. Robertson.** 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc. Natl. Acad. Sci. USA **89:**2699–2702.
45. **O'Neill, S. L., and T. L. Karr.** 1990. Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. Nature **348:**178–180.
46. **Pannebakker, B. A., B. Loppin, C. P. Elemans, L. Humblot, and F. Vavre.** 2007. Parasitic inhibition of cell death facilitates symbiosis. Proc. Natl. Acad. Sci. USA **104:**213–215.
47. **Paraskevopoulos, C., S. R. Bordenstein, J. Wernegreen, J. H. Werren, and K. Bourtzis.** 2006. Toward a *Wolbachia* multilocus sequence typing system: discrimination of *Wolbachia* strains present in *Drosophila* species. Curr. Microbiol. **53:**388–395.
48. **Sagulenko, E., V. Sagulenko, J. Chen, and P. J. Christie.** 2001. Role of *Agrobacterium* VirB11 ATPase in T-pilus assembly and substrate selection. J. Bacteriol. **183:**5813–5825.
49. **Sanogo, Y. O., S. L. Dobson, S. R. Bordenstein, and R. J. Novak.** 2007. Disruption of the *Wolbachia* surface protein gene *wspB* by a transposable element in mosquitoes of the *Culex pipiens* complex (Diptera, Culicidae). Insect Mol. Biol. **16:**143–154.
50. **Schmidt-Eisenlohr, H., N. Domke, C. Angerer, G. Wanner, P. C. Zambryski, and C. Baron.** 1999. Vir proteins stabilize VirB5 and mediate its association with the T pilus of *Agrobacterium tumefaciens*. J. Bacteriol. **181:**7485–7492.
51. **Schulein, R., and C. Dehlio.** 2002. The VirB/VirD4 type IV secretion system of *Bartonella* is essential for establishing intraerythrocytic infection. Mol. Microbiol. **46:**1053–1067.
52. **Sexton, J. A., and J. P. Vogel.** 2002. Type IVB secretion by intracellular pathogens. Traffic **3:**178–185.
53. **Stevens, L., R. Giordano, and R. F. Fialho.** 2001. Male killing, nematode infections, bacteriophage infection, and virulence of cytoplasmic bacteria in the genus *Wolbachia*. Annu. Rev. Ecol. Syst. **32:**519–545.
54. **Stouthamer, R., J. A. Breeuwer, R. F. Luck, and J. H. Werren.** 1993. Molecular identification of microorganisms associated with parthenogenesis. Nature **361:**66–68.
55. **Stouthamer, R., J. A. J. Breeuwer, and G. D. D. Hurst.** 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Annu. Rev. Microbiol. **53:**71–102.
56. **Stouthamer, R., R. F. Luck, and W. D. Hamilton.** 1990. Antibiotics cause parthenogenetic *Trichogramma* (*Hymenoptera*/*Trichogrammatidae*) to revert to sex. Proc. Natl. Acad. Sci. USA **87:**2424–2427.
57. **Sullivan, J. T., J. R. Trzebiatowski, R. W. Cruickshank, J. Gouzy, S. D. Brown, R. M. Elliot, D. J. Fleetwood, N. G. McCallum, U. Rossbach, G. S. Stuart, J. E. Weaver, R. J. Webby, F. J. De Bruijn, and C. W. Ronson.** 2002. Comparative sequence analysis of the symbiosis island of *Mesorhizobium loti* strain R7A. J. Bacteriol. **184:**3086–3095.
58. **Taylor, M. J., C. Bandi, and A. Hoerauf.** 2005. *Wolbachia* bacterial endosymbionts of filarial nematodes. Adv. Parasitol. **60:**245–284.
59. **Tram, U., and W. Sullivan.** 2002. Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility. Science **29:**1124–1126.
60. **Vogel, J. P., and R. R. Isberg.** 1999. Cell biology of *Legionella pneumophila*. Curr. Opin. Microbiol. **2:**30–34.
61. **Ward, J. E., D. E. Akiyoshi, D. Regier, A. Datta, M. P. Gordon, and E. W. Nester.** 1988. Characterization of the *virB* operon from an *Agrobacterium tumefaciens* Ti plasmid. J. Biol. Chem. **263:**5804–5814.
62. **Weiss, A. A., F. D. Johnson, and D. L. Burns.** 1993. Molecular characterization of an operon required for pertussis toxin secretion. Proc. Natl. Acad. Sci. USA **90:**2970–2974.
63. **Werren, J. H.** 1997. Biology of *Wolbachia*. Annu. Rev. Entomol. **42:**587–609.
64. **Werren, J. H., and D. M. Windsor.** 2000. *Wolbachia* infection frequencies in insects: evidence of a global equilibrium? Proc. Biol. Sci. **267:**1277–1285.
65. **Williams, K. P., B. W. Sobral, and A. W. Dickerman.** 2007. A robust species tree for the *Alphaproteobacteria*. J. Bacteriol. **189:**4578–4586.
66. **Wu, M., L. V. Sun, J. Vamathevan, M. Riegler, R. Deboy, J. C. Brownlie, E. A. McGraw, W. Martin, C. Esser, N. Ahmadinejad, C. Wiegand, R. Madupu, M. J. Beanan, L. M. Brinkac, S. C. Daugherty, A. S. Durkin, J. F. Kolonay, W. C. Nelson, Y. Mohamoud, P. Lee, K. Berry, M. B. Young, T. Utterback, J. Weidman, W. C. Nierman, I. T. Paulsen, K. E. Nelson, H. Tettelin, S. L. O'Neill, and J. A. Eisen.** 2004. Phylogenomics of the reproductive parasite *Wolbachia pipientis w*Mel: a streamlined genome overrun by mobile genetic elements. PLoS Biol. **2:**0327–0341.
67. **Zhong, Q., S. H. Shao, L. L. Cui, R. H. Mu, X. L. Ju, and S. R. Dong.** 2007. Type IV secretion system in *Helicobacter pylori*: a new insight into pathogenicity. Chin. Med. J. (England) **120:**2138–2142.
68. **Zhou, W., F. Rousset, and S. O'Neill.** 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc. R. Soc. Lond. B **265:**509–515.
69. **Zupan, J. R., and P. Zambryski.** 1995. Transfer of T-DNA from *Agrobacterium* to the plant cell. Plant Physiol. **107:**1041–1047.