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Culture and Phenotypic Characterization of a *Wolbachia pipientis* Isolate

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The recent isolation of *Wolbachia pipientis* in the continuous cell line Aa23, established from eggs of a strain of the Asian tiger mosquito *Aedes albopictus*, allowed us to perform extensive characterization of the isolate. Bacterial growth could be obtained in C6/36, another *A. albopictus* cell line, at 28°C and in a human embryonic lung fibroblast monolayer at 28 and 37°C, confirming that its host cell range is broader than was initially thought. The bacteria were best visualized by Diff-Quik and May-Grünwald-Giemsa staining. Proteins from 213 to 18 kDa with two major protein bands of 65 and 25 kDa were observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. By Western blotting with specific polyclonal mouse and rabbit antisera, dominant immunoreactive antigens were found at approximately 100, 80, and 30 kDa. The genome size was calculated to be 1,790 ± 17 kb by pulsed-field gel electrophoresis. The sequence of the citrate synthase gene (*gltA*) of *W. pipientis* was determined by gene walking. Its position in the phylogenetic tree constructed with *gltA* confirmed that found in a phylogenetic tree constructed with 16S rRNA genes and that it belongs in the α subgroup of the class *Proteobacteria* and that it is closely related to but independent from the genera *Ehrlichia*, *Anaplasma*, and *Neorickettsia*.

Bacteria of the genus *Wolbachia* are maternally inherited and are known to infect a wide range of arthropods and filarial nematodes (31, 37). The first description of *Wolbachia* was made in 1924, when it was detected in the ovaries of the mosquito *Culex pipiens* and classified as an unnamed *Rickettsia* (11). The bacterium was subsequently named *Wolbachia pipientis* (10), to honor Wolbach, who first made these observations. On the basis of its association with arthropods and its intracellular location, it was classified in the family *Rickettsiaceae*, tribe *Wolbachieae* (36). The advent of molecular tools for phylogenetic and taxonomic studies, especially 16S rRNA gene sequencing, has dramatically modified classification of the bacteria belonging to the family *Rickettsiaceae* (35, 36). *W. pipientis* was found to be in the α subgroup of the class *Proteobacteria* and closely related to the genus *Rickettsia* (7). On the basis of analysis of 16S rRNA genes, *groESL*, and surface protein genes, the members of the order *Rickettsiales* have recently been reorganized in the families *Rickettsiaceae* and *Anaplasmataceae* (7). *W. pipientis* was then included in the family *Anaplasmataceae* with the genera *Ehrlichia*, *Neorickettsia*, and *Anaplasma*. The increasing use of gene sequencing for the characterization of insect endosymbionts has demonstrated the extreme diversity of the *Wolbachia* genus (21). Phylogeny based on genes such as *ftsZ* and *wsp* has revealed the existence of four major clades within the genus: clades A and B in insects, mites, and crustaceans and clades C and D in filarial nematodes (1, 2, 38). However, no consensus opinion exists at present for description of members of these clades as species or subspecies (16). Since the original description of the genus *Wolbachia*, the inability to culture these endosymbionts outside of the invertebrate host has limited research on these bacteria. Until now, as no isolate of *Wolbachia* was maintained in vitro, extensive study of bacteria of the *Wolbachia* genus has not been performed. However, the recent establishment of a strain of *W. pipientis* in a cell line originating from *Aedes albopictus* (i.e., the Aa23 cell line) allows the production and study of significant numbers of bacteria (22). Here, we report on the culture, purification, and molecular characterization of this bacterium, which has also been named strain *wAlbB* on the basis of *wsp* sequencing (40).

MATERIALS AND METHODS

Strain cultivation. Details of the isolation and routine culture of the *W. pipientis* strain in the Aa23 mosquito cell line are provided elsewhere (22). Briefly, *W. pipientis* was cultured in Aa23 cell monolayers grown in 150-cm^2 cell culture flasks with 30 ml of a mixture (1:1; vol/vol) of Mitsuhashi-Maramorosh insect medium (Sigma, St. Louis, Mo.) and Schneider's insect medium (Sigma). The mixture was supplemented with 10% fetal bovine serum (Gibco, Cergy Pontoise, France). The culture flasks were incubated at 28°C. Medium was replaced every week. For the production of large amounts of bacteria, infected cells from one flask were harvested every 5 days and inoculated into three cell culture flasks with fresh medium (1:3 split). Aa23 cells were cured of infection with *W. pipientis* by adding 10 μg of doxycycline per ml in culture medium for three passages. For uninfected cells, all cell culture flasks were incubated at 28°C. Medium was replaced every week.

Subculture assays. Propagation of *W. pipientis* was attempted on human embryonic lung (HEL) fibroblast monolayers (CCL-137; American Type Culture Collection, Manassas, Va.) at 28 and 37°C under previously described conditions (24), and in C6/36 cells (CRL-1660; American Type Culture Collection), another *A. albopictus* cell line grown in Leibowitz-15 medium with L-glutamine and

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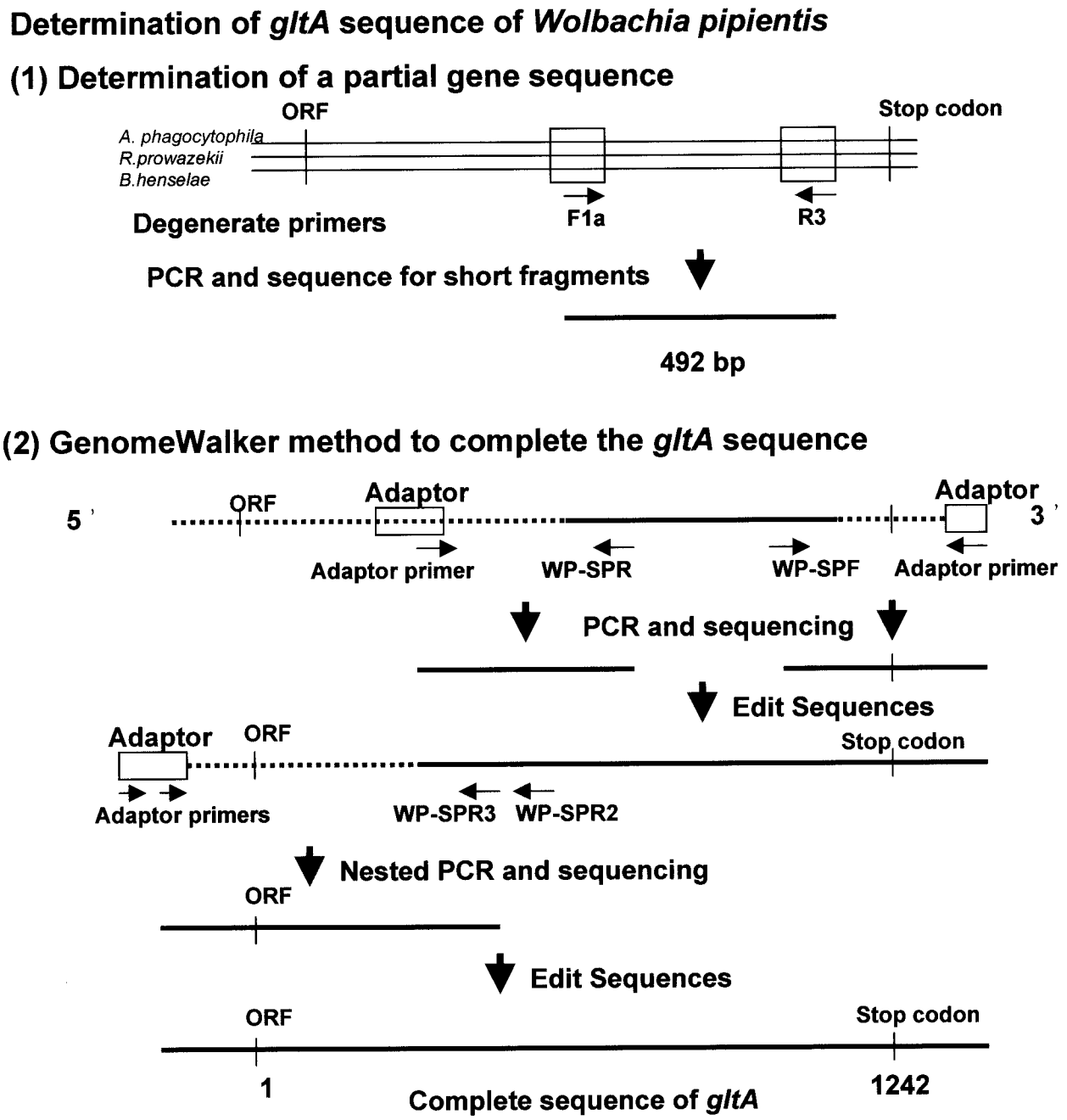

FIG. 1. Strategy for determination of citrate synthase gene (*gltA*) of the strain of *W. pipientis.* Primers F1a and R3 were created after alignment of *gltA* of *A. phagocytophilum*, *R. prowazekii*, and *B. henselae.* After determination of the partial sequence, the unknown sequences of both the 3′ and the 5′ ends of the gene were amplified by PCR with an adaptor primer provided in the Universal GenomeWalker kit and the strain-specific primers based on the partial sequence. Alignment and assembly of these sequences allowed the determination of the complete *gltA* sequence of *W. pipientis*. ORF, open reading frame.

L-amino acids (Gibco), 5% (vol/vol) fetal bovine serum, and 2% (vol/vol) tryptose phosphate (Gibco) at 28°C. For subculture in these cell lines, the bacteria were first grown in Aa23 cells for 10 days without medium replacement. The supernatant was then harvested, filtered through 0.80-μm-pore-size filters in order to remove intact cells, and then centrifuged at 9,000 × *g* for 30 min. The resulting pellet was resuspended in fresh culture medium (which varied according to the cell line used for subculture) and inoculated into a 25-cm^2 cell culture flask. In order to objectively measure bacterial growth in HEL cells at 28°C, we also performed quantitative detection of the DNA of *Wolbachia* growing in cells. Inoculation of five 25-cm^2 cell culture flasks containing HEL cells was performed as described above. From the day of inoculation and every 3 days, the flasks were tested for *Wolbachia* DNA quantification by a real-time PCR targeting the *wsp* gene under previously described conditions (8).

Purification of *W. pipientis* from Aa23 cells. Aa23 cells infected with the bacterium were harvested from 20 150-cm^2 cell culture flasks. The suspension was subjected to sonication, after which unlysed cells were removed by centrifugation at 100 × *g* for 15 min. The supernatant was layered onto a 25% (wt/vol) sucrose solution in phosphate-buffered saline (PBS). After centrifugation at

TABLE 1. Oligonucleotide primers and restriction genome libraries used for genome walking of the *W. pipientis* citrate synthase gene

Primer	Sequence	Restriction genomic library
R1b	CGA-TGA-CCA-AAA-CCC-AT	*Eco*RV and *Dra*I
WP-SPR1	CTA-CCA-ATC-TGA-CAG-CTG-C	*Eco*RV and *Dra*I
WP-SPR2	GCT-GAC-AAA-TTT-GCA-AAA-CAT-G	*Eco*RV and *Dra*I
WP-SPR3	TGG-AAA-TGC-TTT-GAT-TAC-ATT-TG	*Eco*RV and *Dra*I
F1b	GAT-CAT-GAR-CAR-AAT-GCT-TC	*Stu*I
WP-SPF	GCA-AAA-AAA-CTT-GAA-GAA-ATA-GC	*Stu*I

9,000 × *g* for 30 min at 4°C, the bacterium-containing pellet was resuspended in 2 ml of PBS and carefully layered onto a 25 to 45% (wt/vol in PBS) step density gradient (Gastrografine; Shering, Lys Lez Lannoy, France). This gradient was subjected to centrifugation at 130,000 × *g* for 1 h at 4°C; and the bacteria were harvested and washed twice in PBS, resuspended in sterile distilled water in the smallest possible volume, and then frozen at −80°C. To assess the purity of the bacteria, a PCR targeting the sequence of the 18S rRNA gene was performed before and after the purification of *W. pipientis* from Aa23 cells, as described previously (13).

Animal immunization. Production of mouse and rabbit polyclonal antibodies was performed as follows. Six- to 8-week-old immunocompetent BALB/c mice were inoculated subcutaneously with 0.5 ml of a suspension of 1 mg of the bacterium obtained from purified bacteria and Freund's complete adjuvant. Inoculations were repeated on days 10, 20, and 30. On day 40, the mice were anesthetized and killed. Blood was sampled by intracardiac puncture, and serum was frozen at −80°C for further studies. Rabbits were immunized by intradermal inoculation of a total of 1 mg of purified bacteria and Freund's complete adjuvant. The rabbits were given a booster immunization on day 20, and serum sampled 10 days later was frozen at −80°C for further studies. Production of rabbit polyclonal antibodies was performed as described below.

Morphological studies. (i) Staining for light microscopy. Infected cells and the supernatant were separately cytocentrifuged for staining with Gram, Gimenez, Diff-Quik (Dade Behring, Marburg, Germany), and May-Grünwald-Giemsa stains (4, 9, 24).

(ii) Electron microscopy. Infected cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 0.1 M sucrose for 1 h at 4°C. Fixed cells were washed overnight with the same buffer and then postfixed for 1 h at room temperature with 1% osmium tetroxide in 0.1 M cacodylate buffer. Dehydration was performed through washes in increasing concentrations (25 to 100%) of acetone. The cells were then embedded in Araldite (Fluka, St. Quentin Fallavier, France). Thin sections were cut from blocks with embedded cells with an Ultracut microtome (Reichert-Leica, Marseille, France) and were poststained with a saturated solution of methanol-uranyl acetate and lead nitrate with sodium citrate in water before examination with a Jeol 1220 electron microscope (Jeol, Croissy sur Seine, France).

SDS-PAGE and Western blot immunoassay. To characterize the total protein profile of *W. pipientis*, Renografin-purified preparations of bacterial suspensions of the *Wolbachia* strain cultured in Aa23 cells were spectrophotometrically adjusted to 1 $mg \cdot ml^{-1}$. *Neorickettsia sennetsu* (Miyayama strain), *Ehrlichia chaffeensis* (Arkansas strain), and *Anaplasma phagocytophilum* (Webster strain) were cultured under previously described conditions (26) and were resuspended in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (0.625 M Tris [pH 8.0], 2% [wt/vol] SDS, 5% [vol/vol] 2-mercaptoethanol, 10% [vol/vol] glycerol, 0.002% [wt/vol] bromophenol blue) for protein immunoblotting. An aliquot of each was then heated for 10 min at 100°C, and then heated and unheated aliquots were loaded onto 10% polyacrylamide gels (18 cm by 20 cm by 1.5 mm). Proteins were electrophoretically resolved at a constant current of 60 mA/gel at 10°C until the dye front reached the bottom of the gel (15). Proteins were visualized with silver stain by a modified Blum procedure (18). For Western immunoblotting procedures, proteins were extracted and heated as described above for SDS-PAGE. A total of 10 μg of total protein was loaded per lane. Following electrophoresis, the proteins were electroblotted onto nitrocellulose membranes at 100 V for 1 h at 10°C. The membranes were incubated with 5% (wt/vol) nonfat dry milk in Tris buffer (10 mM Tris-HCl [pH 8.8], 150 mM NaCl, 0.1% Tween 20) overnight and were then incubated with either mouse or rabbit antiserum and mouse or rabbit preimmune serum diluted 1:100 in 0.5% (wt/vol) nonfat dry milk in PBS for 1 h at room temperature. After three 10-min washing steps in Tris buffer, the membranes were incubated with goat anti-mouse immunoglobulin G (IgG) plus IgM (Jackson ImmunoResearch Laboratories, Inc.) and with donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) diluted 1:200 in PBS with 0.5% (wt/vol) nonfat dry milk. After three washes in PBS, bound peroxidase was revealed by using 4-chloro-1-naphthol as the substrate.

Genomic studies. (i) PFGE. Purified bacteria were suspended in PBS to an optical density of 1.1 to 1.2 at a λ value of 260 nm and were immediately used for preparation of pulsed-field gel electrophoresis (PFGE) plugs. Plug preparation, enzymatic digestion, and PFGE migration were performed as described previously (25). A 1% agarose gel was used for determination of the molecular weight of the restricted fragment. In order to resolve the low- and medium-molecular-weight DNA fragments, electrophoresis conditions of 6 V/cm with a ramped pulse time from 1 to 3 s for 8 h, 6 V/cm with a ramped pulse time from 1 to 5 s for 9 h, and then 6 V/cm with a ramped pulse time from 1 to 10 s for 10 h were used. Determination of medium-molecular-weight DNA fragments was performed at 5.7 V/cm with a ramped pulse time from 20 to 40 s for 10 h. A 0.6% agarose gel and a ramped pulse time of 5 to 120 s for 33 h at 4.5 V/cm were used for entire chromosomal DNA migration as well as for determination of the sizes of fragments with high molecular weights. Several restriction endonucleases (*Bss*HII, *Sac*II, *Sma*I, *Bstz*I, *Not*I, *Xba*I, *Sfi*I, *Sal*I) were tested for their abilities to determine the genome sizes of *Ehrlichia*, *Anaplasma*, and *Neorickettsia* (26). Finally, it was found that *Bss*HII, *Sac*II, and *Sal*I were suitable for the study.

(ii) Study of the citrate synthase gene (*gltA*). Genomic DNA was extracted from purified bacteria by using the QIAamp tissue kit (Qiagen GmbH, Hilden, Germany) according to the recommendations of the manufacturer. The strategy used to determine the *gltA* sequences of *W. pipientis* used in the present study is summarized in Fig. 1. First, a partial sequence of *gltA* was determined by PCR with degenerate primers F1 (5′-CAT-GAR-CAR-AAT-GCT-TC-3′) and R3 (5′-CNG-CCC-ANC-CAG-ACG-T-3′) and sequencing. Both primers were designed after alignment of the conserved region of *gltA* among *A. phagocytophilum*, *Rickettsia prowazekii*, and *Bartonella henselae.* For the amplification, the reaction mixture contained 50 pmol of each primer, 1.5 U of *Taq* DNA polymerase (Gibco BRL, Gaithersburg, Md.), 20 mM each deoxynucleoside triphosphate, 10 mM Tris-HCl, 50 mM KCl, 1.6 mM $MgCl_2$, and 5 μl of template DNA with a final volume of 50 μl. The amplification was performed in a Peltier model PTC-200 thermal cycler (MJ Research, Inc., Watertown, Mass.) with the following program: an initial 5 min of denaturation at 95°C; 34 cycles of denaturation (95°C for 30 s), annealing (51°C for 30 s), and extension (72°C for 90 s); and 5 min of extension at 72°C. Distilled water and *B. henselae* DNA were included in the PCR as negative and positive controls, respectively. The amplification products were visualized on a 1% ethidium bromide-stained agarose gel after electrophoretic migration of the amplified material. A positive amplification product of the expected size (492 bp) was purified for sequence analysis. After determination of the partial sequence, the unknown sequences of both the 3′ and 5′ ends of the gene were amplified by PCR by using the Universal GenomeWalker kit (Clontech Laboratories, Palo Alto, Calif.). Briefly, genomic DNA was digested with *Eco*RV, *Dra*I, *Pvu*II, *Stu*I, and *Sca*I. The DNA fragments were ligated with a GenomeWalker adaptor, which had one blunt end and one end with a 5′ overhang. The ligation mixture of the adaptor and the genomic DNA fragments was used as the template for PCR. This PCR was performed with an adaptor primer supplied by the manufacturer and *W. pipientis gltA*-specific primers to walk downstream on the DNA sequence (Table 1). For the amplification, 1.5 U of Elongase (Boehringer Manheim) was used with 10 pmol of each primer, 20 mM each deoxynucleoside triphosphate, 10 mM Tris-HCl, 50 mM KCl, 1.6 mM $MgCl_2$, and 5 μl of template with a final volume of 50 μl. Distilled water was included in each PCR as a negative control. The following program was used for the amplification: an initial 2 min of denaturation at 94°C; 44 cycles of denaturation (94°C for 30 s), annealing (53°C for 60 s), and extension (68°C for 60 s); and 3 min of extension at 68°C. The PCR products for DNA sequencing were purified with QIAquick PCR purification kits (Qiagen) and were sequenced directly by using PCR primers when a single clear band was observed on the ethidium bromide-stained agarose gel. When multiple bands including bands of the expected sizes were obtained in a PCR, a Gel Extraction kit (Qiagen) was used to purify the expected bands from the gel. The fluorescent dideoxynucleotide technology (Perkin-Elmer, Applied Biosystems Division, Foster City, Calif.) was used for the DNA sequencing reactions. The sequenced fragments were separated, and data were collected on an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer). The sequences collected were assembled and edited with the AutoAssembler program (version 1.4; Perkin-Elmer). To avoid editorial errors by use of the GenomeWalking method, the sequences of the citrate synthase-coding region, including open reading frames at the 5′ end and stop codons at the 3′ end, were confirmed by PCR with primers WP-M91F (5′-TGA-CCG-CTT-AAT-AC G-GTT-AAT-G-3′) and WP1274R (5′-TTG-AGG-ACG-AAT-TGT-GAA-TG A-3′) and sequenced. The *gltA* sequences of the following species were analyzed

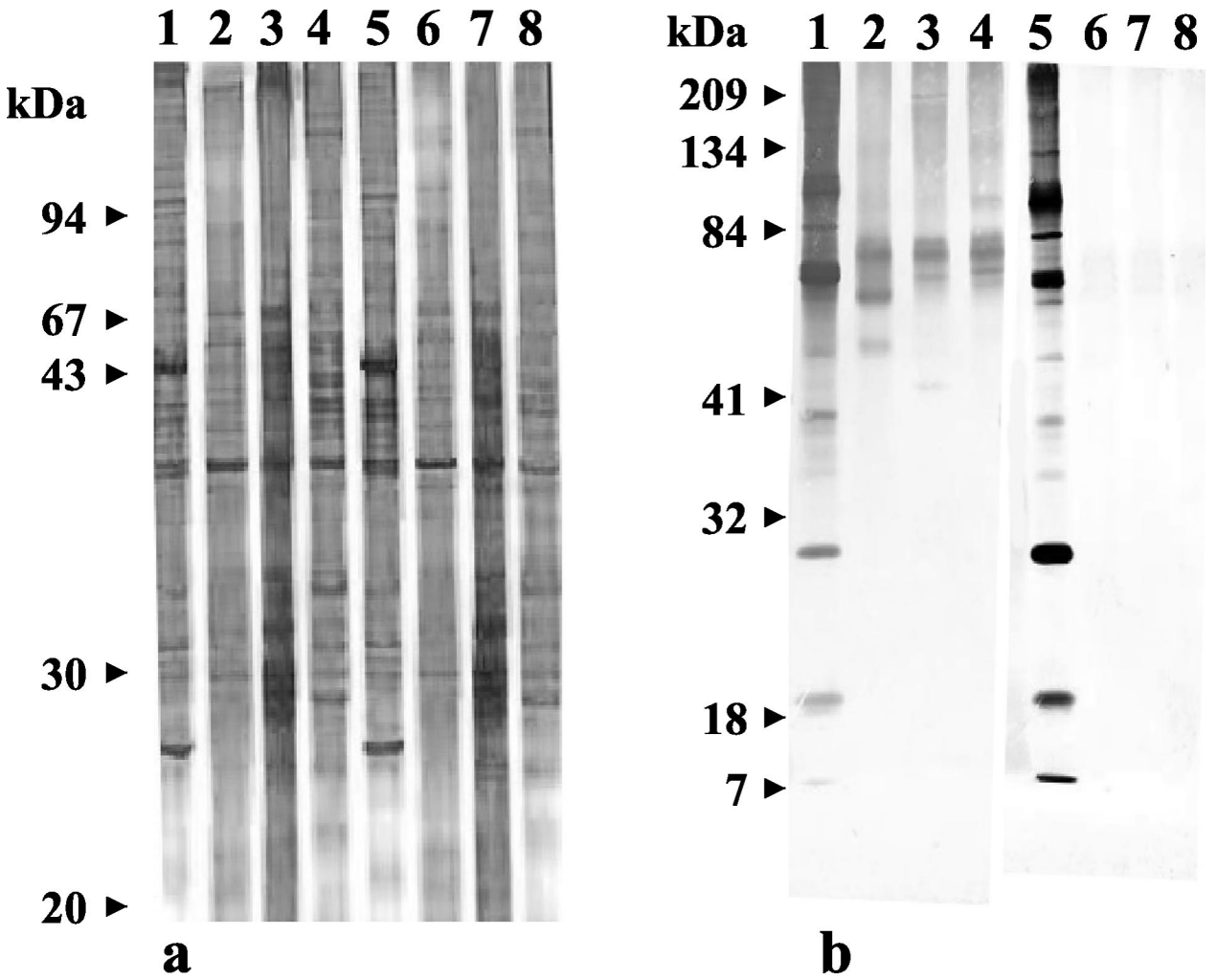

FIG. 2. (a) Silver-stained SDS-polyacrylamide gel of whole-cell protein preparations of *W. pipientis*. Lanes 1 and 5, *W. pipientis*; lanes 2 and 6, *A. phagocytophilum*; lanes 3 and 7, *E. chaffeensis*; lanes 4 and 8, *N. sennetsu*; lanes 1 to 4, unheated samples; lanes 5 to 8, heated samples. (b) Western blot of the strain reacted with mouse and rabbit polyclonal antisera. Lanes 1 and 5, *W. pipientis*; lanes 2 and 6, *A. phagocytophilum*; lanes 3 and 7, *E. chaffeensis*; lanes 4 and 8, *N. sennetsu*; lanes 1 to 4, mouse polyclonal serum; lanes 5 to 8, rabbit polyclonal serum.

for levels of similarity and phylogenetic relationships: *E. chaffeensis*, *Ehrlichia muris*, an *Ehrlichia* sp. detected in *Ixodes ovatus* ticks, *Ehrlichia canis*, *Ehrlichia ruminantium*, *A. phagocytophilum* (composed of the former species *Ehrlichia phagocytophila*, *Ehrlichia equi*, and the human granulocytic ehrlichia agent), *Anaplasma marginale*, *Anaplasma centrale*, *W. pipientis*, *N. sennetsu*, *Neorickettsia risticii*, *Neorickettsia helminthoeca*, *R. prowazekii*, and *B. henselae*. Pairwise percent identities of the sequences with all gaps omitted were calculated by use of a program designed by H. Ogata (Ogata@igs.cnrs-mrs.fr), Institut de Génomique Structurale, Centre National de la Recherche Scientifique-Unité Mixte de Recherche, Marseille, France. Multiple-sequence alignment analysis, distance matrix calculation, and construction of phylogenetic trees were performed with the ClustalW program (version 1.8) (33) at the DNA Data Bank of Japan (Mishima, Japan [http://www.ddbj.nig.ac.jp/htmls/E-mail/clustalw-e.html]). The distance matrices for the aligned sequences with all gaps ignored were calculated by the Kimura two-parameter method (14), and the neighbor-joining method was used to construct phylogenetic trees (28). The stability of the tree obtained was estimated by bootstrap analysis with 1,000 replications by using the ClustalW program. Tree figures were generated with the Tree View program (version 1.61) (23). The same analyses of similarity and phylogenetic relationships were also performed for the deduced amino acid sequences of *gltA* and the 16S rRNA gene. The GenBank accession numbers of the *gltA* sequences used in this study are as follows: *E. chaffeensis*, AF304142; *E. muris*, AF304144; *Ehrlichia* sp. detected in *I. ovatus* ticks, AF304145; *E. canis*, AF304143; *E. ruminantium*, AF304146; *A. phagocytophilum*, AF304138; *A. phagocytophilum* (formerly *E. equi*), AF304137; *A. phagocytophilum* (formerly the human granulocytic ehrlichia agent), AF304136; *A. marginale*, AF304139; *A. centrale*, AF304141; *N. sennetsu*, AF304148; *N. risticii*, AF304147; *N. helminthoeca*, AF304149; *R. prowazekii*, M17149; *B. henselae* L38987; and *Escherichia coli*, J01619. The GenBank accession numbers of the 16S rRNA gene sequences used to calculate percent identities and construct phylogenetic trees are as follows: *E. chaffeensis*, M73222; *Ehrlichia* sp. detected in *I. ovatus* ticks, AF260591; *E. muris*, U15527; *E. canis*, M73221; *E. ruminantium*, AF069758; *A. phagocytophilum*, M73224; *A. phagocytophilum* (MRK strain), M73223; *A. phagocytophilum* (a Webster strain), U02521; *A. marginale*, M60313; *A. centrale*, AF283007; *W. pipientis*, AF179630; *N. sennetsu*, M73225; *N. risticii*, M21290; *N. helminthoeca*, U12457; *R. prowazekii*, M21789; and *B. henselae*, AJ223779.

Nucleotide sequence accession number. The *gltA* sequence of *W. pipientis* has been deposited in the GenBank database under accession number AF332584.

RESULTS

Morphological studies. Bacteria were best visualized by the Diff-Quik and May-Grünwald-Giemsa staining methods. On Gimenez staining, the bacteria stained poorly and appeared as pale pink structures. On Gram staining, the bacteria also stained poorly and always appeared to be gram negative. The

TABLE 2. Sizes of linearized DNA fragments after restriction endonuclease digestion for *W. pipientis*, as determined by PFGE[a]

Size (kb) of linearized fragment(s)		
*Bss*HII	*Sac*II	*Sal*I
291[b]	542	398
210[b]	339	251[b]
169	242	140
160	194	120[b]
130	170	110[b]
121	140	90
110	80	56
97	50	50
	40	46
	10	31

[a] The total sizes after digestion with *Bss*HI, *Sac*II, and *Sal*I were 1,789, 1,807, and 1,773 kb, respectively (mean ± standard deviation, 1,790 ± 17).
[b] Two fragments.

bacteria appeared as small cocci. No obvious cytopathic effect was observed in Aa23 cells that remained persistently infected. The level of infection in chronically infected cells was variable within a single flask, with some cells containing high numbers of bacteria and others containing few detectable bacteria. Due to the presence of some heavily infected cells that coexisted with lightly infected or uninfected cells, it is likely that some cell lysis was compensated for by the concomitant multiplication of uninfected cells. The infection level within a single flask was also variable over time. When HEL and C6/36 cells were inoculated with *W. pipientis*, the cells became progressively filled with bacteria. After 2 weeks, the appearance on Giemsa staining was the same as that observed with Aa23 cells, and the cells remained constantly infected without an evident cytopathic effect. The same variations in levels of infection observed in Aa23 cells were observed in HEL and C6/36 cells. These infected HEL and C6/36 cells remained chronically infected for at least 3 months. Bacteria were seen in vacuoles within HEL cells by electron microscopy. For determination of the growth of *W. pipientis* in HEL cells at 28°C by PCR assay, a primary inoculum with approximately 10^3 DNA copies/ml was evaluated. The growth of *W. pipientis* was exponential, with a nearly 2-log increase in the bacterial load after a 9-day incubation of the cultures.

Purification of *W. pipientis*, SDS-PAGE analysis, and Western blot immunoassay (Fig. 2). The PCR targeting the 18S rRNA sequence was positive before purification and negative after purification. SDS-PAGE showed the presence of proteins from 18 to 213 kDa. The main difference between *W. pipientis*, *A. phagocytophilum*, *N. sennetsu*, and *E. chaffeensis* was the presence of two major bands of approximately 65 and 25 kDa for *W. pipientis* that were lacking for the other bacteria. By the immunofluorescence assay, the titers of antibodies to *W. pipientis* in rabbit and mouse sera were 3,200 and 1,600, respectively. Polyclonal rabbit serum recognized several *W. pipientis* antigens, with dominant bands of approximately 100, 80, and 30 kDa and several less intense bands of 96 and 18 kDa. There was no cross-reactivity with *A. phagocytophilum*, *N. sennetsu*, or *E. chaffeensis*. Polyclonal mouse serum recognized several *W. pipientis* antigens with dominant bands of approximately 100, 80, and 30 kDa and several less intense bands of 96 and 18 kDa. It also recognized a band of approximately 82 kDa, in common with *A. phagocytophilum*, *N. sennetsu*, and *E. chaffeensis*. No cross-reactivity was observed with mouse or rabbit preimmune serum (data not shown).

Genomic studies. By PFGE, *W. pipientis* was digested only with the enzymes listed in Table 2, and the genome size was calculated to be 1,790 ± 17 kb. A 1,242-bp open reading frame extending from the ATG start codon down to the TAA stop codon of the *gltA* sequence was determined by the GenomeWalker PCR method. The G+C content of the gene was calculated to be 34 mol%. A phylogenetic tree constructed on the basis of the multiple-sequence alignments of the *gltA* nucleotide sequence was compared with the tree constructed on the basis of the multiple-sequence alignments of the 16S rRNA gene sequences (Fig. 3). A phylogenetic tree based on the deduced amino acid sequences was also constructed (Fig. 4). The positions of *W. pipientis* in the phylogenetic trees constructed by use of the *gltA* nucleotide sequences and amino acid sequences were almost the same as that in the tree derived from the 16S rRNA gene sequences.

DISCUSSION

Bacteria of the genus *Wolbachia* are associated with arthropods and nematode worms. These bacteria are responsible for sex manipulation in insects that causes a number of reproductive alterations in their arthropod hosts, including cytoplasmic incompatibility between strains and related species, male killing, parthenogenesis, and feminization (37). A variant strain detected in *Drosophila melanogaster* was demonstrated to proliferate massively in adults, leading to widespread degeneration of tissues followed by early death of the insect (17). The bacteria may be associated with nematodes and thus may be indirectly responsible for pathogenesis in human filariasis (27, 32).

Wolbachia is extremely widespread and has been found in up to 16% of insect species, including each of the major insect orders. A recent study that used a more sensitive "long" PCR detection method suggested that this value might be considerably higher (12). All nematode worms responsible for human diseases studied so far by PCR are infected with *Wolbachia*, with the possible exception of *Loa loa* worms (3, 31). Nematodes infected with *Wolbachia* bacteria are confined to the superfamily *Filaroidea*, raising the possibility that these parasites were infected via their arthropod hosts (31). No evidence for the recent transfer of bacteria between arthropods and nematodes has been found.

For a long time, *Wolbachia* research has been limited to laboratories with insect-rearing facilities due to the inability to cultivate these bacteria outside of invertebrate hosts. It was not until 1997 that a strain of *W. pipientis* was established in vitro. A new study has demonstrated that *Wolbachia* infections can be simply established and stably maintained in vitro by using standard tissue cell cultures in shell vials (6). Insect cell lines have been stably infected with *Wolbachia* isolates from *Drosophila simulans*, *Cadra cautella*, and *Culex pipiens*. These infections represent a phylogenetically diverse range of *Wolbachia* types from a broad range of invertebrate hosts. *Wolbachia* infections were realized in cell lines derived from *A. albopictus* (Aa23, C6/36), *Spodoptera frugiperda* (SF9), and *D. melanogaster* (S2). Another recent study has demonstrated that *Wol-*

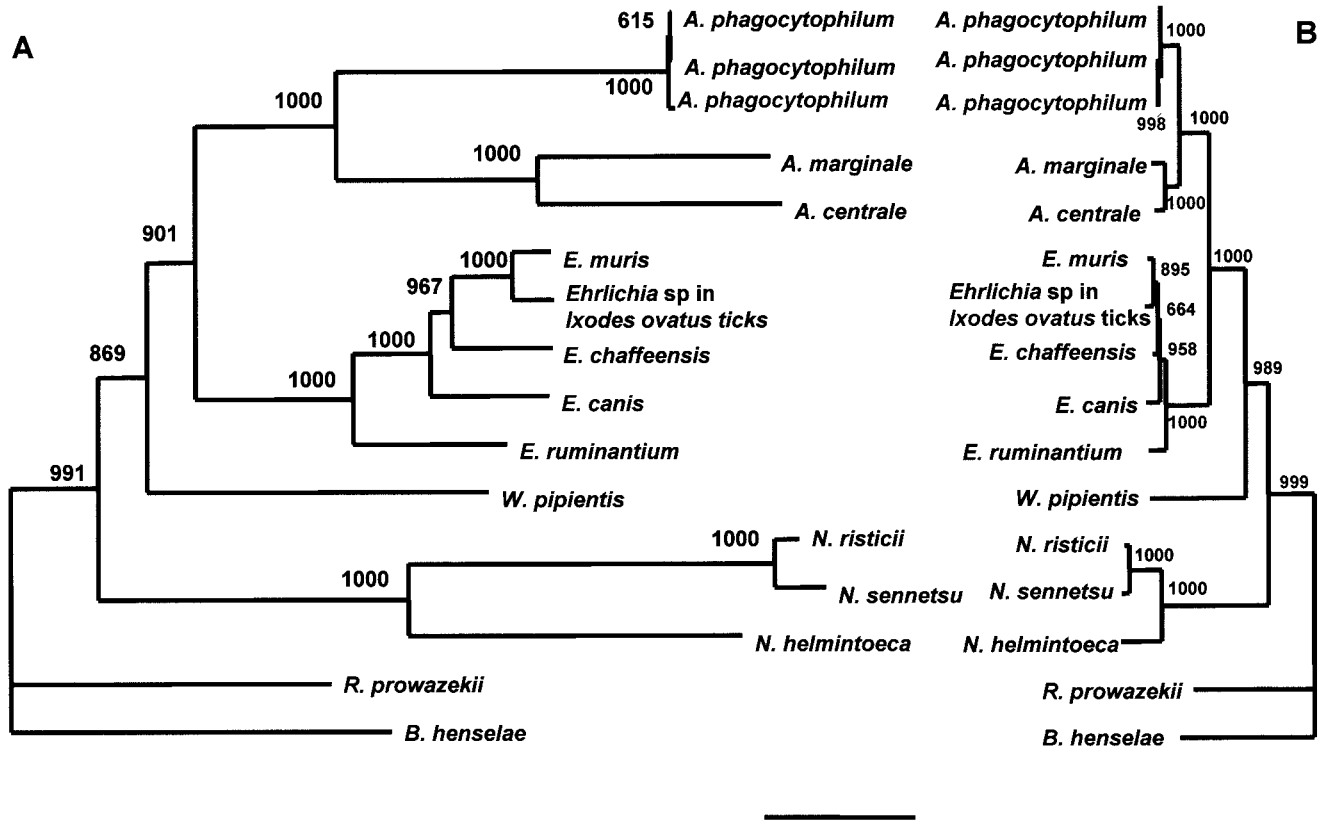

FIG. 3. Phylogenetic relationship of *W. pipientis* and closely related species based on the nucleotide sequences of the citrate synthase gene (A) and the 16S rRNA gene (B). The neighbor-joining method was used to construct the phylogenetic tree by using the ClustalW program. The scale bar represents 1% divergence. The numbers at the nodes are the proportions of 1,000 bootstrap resamplings that support the topology shown.

bachia proliferates not only in a lepidopteran (*Heliothis zea*) cell line but also in a mammalian cell line obtained from a mouse (L929) (19). These successes in establishing infections have suggested that the *Wolbachia* host cell range is broader than was previously thought. These results were confirmed with the C6/36 and mammalian cell lines and were extended to the culture of *Wolbachia* in human cells.

The purification of bacteria was obtained by using a Renografin density gradient and allowed the characterization of whole-cell proteins by SDS-PAGE, the preparation of polyclonal antisera, the characterization of the proteins by Western blot immunoassay, and the determination of the *W. pipientis* genome size. By SDS-PAGE, the main differences between *W. pipientis* and the other genera of the family *Anaplasmataceae* was the presence of two major bands of approximately 65 and 25 kDa. By Western blotting, polyclonal mouse and rabbit antisera have low degrees of cross-reactivity with the other genera of the family *Anaplasmataceae*. By PFGE, the molecular size of *W. pipientis* chromosomal DNA is slightly larger than those of *E. ruminantium* (1,576 kb) (5), *E. chaffeensis* (1,226 kb), *A. phagocytophilum* (1,494 kb), and *A. marginale* and *Rickettsia* spp. (1,138 to 1,660 kb) (26) and differs considerably from those of *N. sennetsu* and *N. risticii* (about 880 kb) (26). This determination confirms the heterogeneity in the genome size for representative genomes of the family *Anaplasmataceae*. The molecular size of *W. pipientis* chromosomal DNA is nearly the same as those previously described for *Wolbachia* strains infecting *Drosophila* (1,400 to 1,600 kb), but is larger than those of *Wolbachia* strains infecting nematodes (950 and 1,100 kb) (30), confirming the large difference in genome sizes between these different groups.

With the availability of molecular biology-based techniques, it has become evident that bacteria of the *Wolbachia* group are more diverse than was previously believed. The use of *gltA* in this work helped to confirm the position of *W. pipientis* among the closely related α subgroup of the class *Proteobacteria*, but only determination of the sequence of this gene in other *W. pipientis* isolates will allow verification that it is a good tool for the distinction of several groups, as has previously been described with genes such as *ftsZ* and *wsp* (29). Nevertheless, if the data obtained with the *ftsZ* and *wsp* genes suggest the presence of different species in the genus *Wolbachia*, only the establishment of new strains followed by DNA-DNA hybridization and comparison with the *W. pipientis wAlbB* strain, the sole available strain of the species, will allow definition of novel species (34).

In the phylogenetic trees constructed by use of the 16S rRNA and *gltA* gene sequences, *W. pipientis* and the *Wolbachia* spp. belong to the α subgroup of *Proteobacteria* and are close to but independent from the genera *Ehrlichia*, *Cowdria*, and *Anaplasma*. *W. pipientis* and the *Wolbachia* spp. occupy a position intermediate between the two tick-transmitted genera

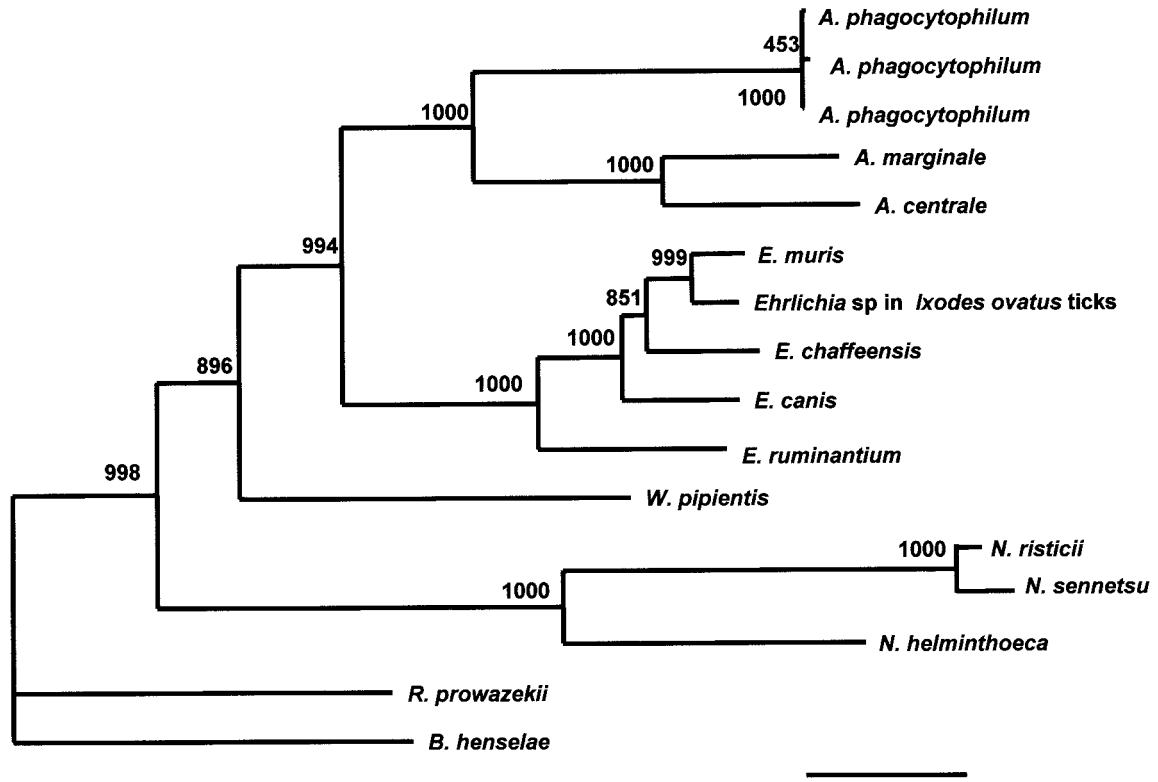

FIG. 4. Phylogenetic relationship of *W. pipientis* and closely related species based on the deduced amino acid sequences of the citrate synthase gene. The neighbor-joining method was used to construct the phylogenetic tree by using the ClustalW program. The scale bar represents 1% divergence. The numbers at the nodes are the proportions of 1,000 bootstrap resamplings that support the topology shown.

(*Ehrlichia* and *Anaplasma*) and the helminth-borne genus (*Neorickettsia*). The deduced amino acid sequences of *Wolbachia* sp. outer membrane proteins exhibit similarity to those of the major outer membrane proteins of *A. marginale*, *A. phagocytophilum*, *E. chaffeensis*, *E. canis*, and *E. ruminantium*, corroborating the phylogenetic position of *W. pipientis* (20, 39). However, *W. pipientis* is not recognized as a vertebrate pathogen, since mammalian infections have never been documented.

In conclusion, our results show that the *W. pipientis* host range is broader than was initially thought. The ability to establish in vitro infections should now permit new approaches to the investigation of *Wolbachia*. Thus, an important step in the future will be the establishment of a nematode strain of *W. pipientis* in vitro, provided that the host range in nematodes is as large as that in arthropods.

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