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Determination of *Wolbachia* Genome Size by Pulsed-Field Gel Electrophoresis

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Genome sizes of six different *Wolbachia* strains from insect and nematode hosts have been determined by pulsed-field gel electrophoresis of purified DNA both before and after digestion with rare-cutting restriction endonucleases. Enzymes *Sma*I, *Apa*I, *Asc*I, and *Fse*I cleaved the studied *Wolbachia* strains at a small number of sites and were used for the determination of the genome sizes of *w*MelPop, *w*Mel, and *w*MelCS (each 1.36 Mb), *w*Ri (1.66 Mb), *w*Bma (1.1 Mb), and *w*Dim (0.95 Mb). The *Wolbachia* genomes studied were all much smaller than the genomes of free-living bacteria such as *Escherichia coli* (4.7 Mb), as is typical for obligate intracellular bacteria. There was considerable genome size variability among *Wolbachia* strains, especially between the more parasitic A group *Wolbachia* infections of insects and the mutualistic C and D group infections of nematodes. The studies described here found no evidence for extrachromosomal plasmid DNA in any of the strains examined. They also indicated that the *Wolbachia* genome is circular.

Wolbachia spp. are maternally inherited obligate intracellular bacteria belonging to the α-*Proteobacteria*. They infect a broad range of insect species, a number of noninsect arthropods such as isopods and mites, and most species of filarial nematodes (3, 34, 37, 38). In arthropods they have been implicated in several host reproductive modifications, including cytoplasmic incompatibility in various insect species (16), parthenogenesis in wasps (33), feminization in isopods (29), and virulence in *Drosophila melanogaster* (25). Within the Nematoda, it appears that *Wolbachia* spp. are required for fertility and normal development of the filarial worms they infect (22, 34).

Most research attention to date has been focused on the phenomenology of *Wolbachia*-host interactions. Little is known about the molecular genetics of *Wolbachia*. For example, there has been little characterization of *Wolbachia* genes other than a few loci that have been cloned and used mainly for phylogenetic purposes. This is largely due to the fastidious nature of *Wolbachia* and the difficulty in obtaining large amounts of pure material for laboratory studies.

In preparation for complete genome sequencing, we have determined the genome sizes of a number of *Wolbachia* strains using pulsed-field gel electrophoresis (PFGE) and have developed a method to rapidly purify *Wolbachia* chromosomal DNA in quantities sufficient for library construction.

MATERIALS AND METHODS

***Wolbachia* strains.** The seven *Wolbachia* strains used in this study are listed in Table 1. *Drosophila simulans* Riverside previously treated with tetracycline (DSRT) was used as a *Wolbachia*-free control insect strain.

***Wolbachia* purification from *Drosophila*.** *Drosophila* organisms were reared on standard corn flour-sugar-yeast medium at 25°C. Young adults were harvested for extraction of *Wolbachia*, except for *Drosophila melanogaster* w^{1118}, which harbors *w*MelPop. The infection density in this strain rises dramatically with age (25). Newly emerged adults of this strain were transferred to standard egg-laying bottles for aging. Twenty-day-old flies were harvested for *w*MelPop purification.

All purification methods published to date have been unable to separate *Wolbachia* from *Drosophila* mitochondria. In this report, the methods used to purify mitochondria from *Drosophila* (28, 35) were modified to prepare DNA from *Wolbachia* in quantities that could be visualized by ethidium bromide staining of agarose gels. Around 5 ml of adult flies (about 1,000) was collected and then homogenized in buffer as previously described (9), except without Lubrol (90 mM KCl, 55 mM $CaCl_2$, 15 mM $MgSO_4$, 30 mM NaCl, 250 mM sucrose) using a Dounce tissue grinder (Wheaton, Millville, N.J.). The homogenate was filtered through a 95-μm-pore-size nylon mesh. The filtrate was centrifuged at 200 × g_{max} for 25 min at 4°C to pellet *Drosophila* nuclei. The supernatant was then centrifuged at 4,100 × g_{max} for 5 min at 4°C to pellet *Wolbachia*. The pellet was resuspended at 56°C in a mixture consisting of 1 volume of Tris-EDTA (TE) plus 1 volume of 2% molten GPG low-melting-point agarose (American Bioanalytical, Natick, Mass.), and the resuspension was loaded into a plug module (Bio-Rad, Hercules, Calif.). Plugs were treated with 40 μg of DNase I (Roche, Basel, Switzerland)/ml in DNase I reaction buffer (10 mM Tris-HCl [pH 8.0], 1 mM $MgCl_2$) for 40 min at room temperature (RT) (25°C). After DNase I treatment, the plugs were incubated overnight at 56°C in the lysis buffer (2) (100 mM EDTA [pH 8.0], 10 mM Tris-HCl [pH 8.0], 1% [wt/vol] *N*-lauroylsarcosine sodium salt [Sigma, St. Louis, Mo.], 200 μg of proteinase K [Roche]/ml). The plugs were stored in this lysis buffer at 4°C before restriction digestion or electrophoresis.

***Wolbachia* purification from nematodes.** Adult female nematodes were selected for purification of *Wolbachia* not only because they are larger than certain other life cycle stages (e.g., microfilariae) that also have high densities of endosymbionts but also because they were more readily and uniformly homogenized using the Dounce tissue grinder. Typically, 5 mature adult females of *Dirofilaria immitis* or approximately 275 mature females of the smaller *Brugia malayi* were used for extractions. The purification procedure for *Wolbachia* from nematodes was essentially the same as the one for *Wolbachia* from insects, but with the modifications discussed below.

Live worms, supplied by TRS Laboratories (Athens, Ga.), were placed in a petri dish on ice and chopped into small pieces using a sterile razor blade. The homogenization buffer used was physiological saline (0.85% NaCl) supplemented with 0.001% Nonidet P-40 detergent (Sigma). Inclusion of this very low concentration of detergent in the homogenization buffer was found to decrease

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TABLE 1. *Wolbachia* strains studied

Strain	Host	Phenotype
*w*Ri	*D. simulans* Riverside	mod^+ $resc^+$
*w*MelCS	*D. melanogaster* Canton-S	mod^- $resc^+$
*w*Mel	*D. melanogaster* yw^{67c23}	mod^+ $resc^+$
*w*MelPop	*D. melanogaster* w^{1118}	Virulence
*w*Dim	*D. immitis*	Mutualism
*w*Bma	*B. malayi*	Mutualism
*w*AlbB	*A. albopictus*	mod^+ $resc^+$

the amount of *Wolbachia* pelleting with the worm tissue fragments without causing any noticeable increase in degradation of DNA. The filtrate was passed through two layers of cheesecloth (Veratec, Walpole, Mass.). The first centrifugation was carried out at 350 × g_{max} for 25 min at 4°C to pellet nematode nuclei. The supernatant was then centrifuged at 4,100 × g_{max} for 5 min at 4°C to pellet *Wolbachia*. This final pellet was resuspended in an equal volume of saline without detergent and 2 volumes of molten 2% SeaPlaque (low-melting-point) agarose (FMC, Rockland, Maine) in 0.5× Tris-borate-EDTA (TBE) to give a final concentration of 1% agarose. The sample was allowed to set in 75-μl plug molds (Bio-Rad). Following DNase I treatment, the plugs were transferred to proteinase K lysis buffer (6) (0.5 M EDTA [pH 8.0], 1% lauroylsarcosine, sodium salt supplemented with 2 mg of proteinase K [Gibco BRL, Gaithersburg, Md.]/ml) and incubated at 55°C for 48 h. The proteinase K was diffused out of the plugs by performing a minimum of six washes, each of 30 min, in TE at RT. The plugs were stored short term in TE at 4°C.

Optimization of DNase I treatment. After formation of plugs, DNase I was used to digest any fragmented DNA produced during homogenization. Several concentrations of DNase I (10, 20, 30, 40, 50, and 60 μg/ml) in combination with a time course (0, 5, 10, 15, 22, 28, 35, 40, or 50 min) were used at RT to determine the best conditions for digestion. The limited amounts of nematode sample precluded optimization of DNase I treatment as was carried out for the *Wolbachia* from *Drosophila*. The conditions determined optimal for *Drosophila* (40 μg of DNase I/ml for 40 min at RT) were applied.

Plug preparation from cell line culture. *Aedes albopictus* cell line Aa23 containing *Wolbachia* strain *w*AlbB was used (27). Cells were maintained in a 25-mm^2 flask at 25°C in 5 ml of medium (45% Mitsuhashi and Maramorosch insect medium [Sigma], 45% Schneider medium [Sigma], and 10% heat-inactivated fetal bovine serum). The cells were harvested and washed in phosphate-buffered saline twice. The end pellet was then used to make agarose plugs as described above and directly placed into the lysis buffer and treated at 56°C overnight.

Restriction digestion of *Wolbachia* genome DNA. The *Drosophila Wolbachia* plugs were washed twice with TE buffer, treated twice with TE buffer supplemented with 40 μg of phenylmethylsulfonyl fluoride (Life Technologies, Rockville, Md.)/ml to inactivate the proteinase K, and then washed twice with TE buffer again. All washes were done for 30 min each at RT. For the nematode *Wolbachia* plugs, the proteinase K was diffused out of the plugs before they were stored in TE buffer.

The *Drosophila Wolbachia* plugs were equilibrated with restriction endonuclease buffer for 1 h at RT and transferred to fresh buffer for endonuclease digestion overnight. Four restriction enzymes were used: *Asc*I (GG^CGCGCC), *Apa*I (GGGCC^C), *Fse*I (GGCCGG^CC), and *Sma*I (CCC^GGG) (New England Biolabs, Beverly, Mass.). The nematode *Wolbachia* plugs were equilibrated on ice for 2 h in restriction enzyme buffer containing 50% of the final number of enzyme units. The remaining 50% of the enzyme was then added, and the endonuclease digestion continued for 3 h at the appropriate reaction temperature.

PFGE. Contour-clamped homogeneous electric field (CHEF) (10) gels were run to separate DNA fragments that included at least one fragment with a size greater than 50 kb, using either a CHEF Mapper XA (Bio-Rad) or a CHEF-DR II (Bio-Rad). For resolution of DNA fragments of less than 50 kb, field inversion gel electrophoresis (7) gels were used with only the CHEF Mapper XA. All of the electrophoresis was carried out at 14°C using 0.5× TBE as the running buffer. Plugs prepared from filarial nematode samples were equilibrated in 0.5× TBE prior to electrophoresis, while *Drosophila Wolbachia* plugs underwent no treatment before electrophoresis. The migration profiles were determined using CHEF Mapper XA interactive software, version 1.2 (Bio-Rad). Fragment lengths and the presence of multiple fragments were determined using Gel-doc and Quantity One one-dimensional analysis software (Bio-Rad).

Southern hybridization. The *Wolbachia* surface protein (*wsp*) gene fragment from *w*Ri was amplified by PCR using total DNA from DSRT flies as the template and *wsp*-specific primers 81F (5′-TGGTCCAATAAGTGATGAAGAAAC-3′) and 691R (5′-AAAAATTAAACGCTACTCCA-3′) as a probe (9). The nematode *ftsZ* gene fragment was amplified with total DNA from *B. malayi* as the template and with *ftsZ*-specific primers *fts*Z1F (5′-GTTGTCGCAAATACCGATGC-3′) and *fts*Z1R (5′-CTTAAGTAAGCTGGTATATC-3′) as a probe (39). The mitochondrial 12S rRNA gene fragment was amplified with primer pair 12SAI (5′-AAACTAGGATTAGATACCCTATTAT-3′) and 12SBI (5′-AAGAGCGACGGGCGATGTGT-3′) (32). The amplification conditions were the same as those previously described (9). Total DNA of *Drosophila* was extracted using the Holmes-Bonner method (18). PCR products were gel purified with either β-agarase (New England Biolabs) or Qiagen (Valencia, Calif.) gel extraction kits. These probes were radioactively labeled using either the Random Primed DNA labeling kit (Roche) or the NEBlot kit (New England Biolabs) according to the manufacturer's instructions.

A cocktail of seven probes derived from *w*Bma and known from preliminary mapping to be well dispersed around the *Wolbachia* genome was also prepared. These were a 16S rRNA fragment, a 23S rRNA fragment, HSP-60 (GroEL homologue), a DNA mismatch repair protein homologue, the DNA polymerase III γ subunit, the RNA polymerase β subunit, and serine hydroxymethyltransferase. These *Wolbachia* sequences had been identified among the expressed sequence tags reported from *B. malayi* as part of the Filarial Genome Project (see http://neb.com/fgn/filgen1.html). The sequences were amplified from the appropriate phage stocks representing these cDNA clones using T3 and T7 primers (New England Biolabs). The PCR products were precipitated to remove excess primers, and nucleotides and then labeled by hot PCR (15) with the same primers but using a nucleotide mixture that contained dATP at 1/10 the concentration of the other deoxynucleoside triphosphates but that included [^{32}P]dATP. The labeled PCR products were purified using QIAquick PCR purification columns (Qiagen) according to the manufacturer's instructions and pooled to make a *Wolbachia* probe cocktail.

After gel electrophoresis, Southern transfer was done with a VacuGene XL vacuum blotting system (Amersham Pharmacia Biotech, Uppsala, Sweden), and the filters were hybridized at either 60 or 65°C and washed under high-stringency conditions.

RESULTS

***Wolbachia* purification.** The methods used led to the successful enrichment of *Drosophila Wolbachia*. When run on a PFGE gel, *Wolbachia* genomic DNA could be resolved with ethidium bromide staining (Fig. 1a, lane 2, b, lanes 2 and 3, and c, lane 2). This band was not visible in preparations from uninfected *Drosophila*. Moreover, Southern hybridization using a *wsp* gene fragment as the probe confirmed that the visible band represented *Wolbachia* genomic DNA (Fig. 1a, lane 3, b, lanes 4 and 5, and c, lane 3). This procedure also showed that much of the uncut *Wolbachia* DNA still remained in the loading wells (Fig. 1). Southern hybridization using labeled total DNA from *Wolbachia*-free strain DSRT as a probe produced a faint background smear, which indicated the presence of trace amounts of degraded *Drosophila* DNA but which did not hybridize to any distinct fragments. No extrachromosomal DNA was detected on the gel, indicating an absence of plasmids.

Optimal DNase I reaction conditions were found to be 40 μg of DNase I/ml for 40 min of treatment at RT. A time course study with DNase I (40 μg/ml) showed that the background DNA smear on gels gradually decreased and finally disappeared with the increasing reaction time (data not shown). Southern hybridization with a mitochondrial DNA 12S rRNA gene fragment as the probe showed that the mitochondrial genome migrated at approximately 500 kb (owing to its circular conformation) and was cleared with increasing DNase I treatment (Fig. 2). Excessive DNase I treatment was found to digest *Wolbachia* DNA completely as well. The final conditions used were found to remove mitochondrial DNA while still maintaining a good yield of *Wolbachia* DNA.

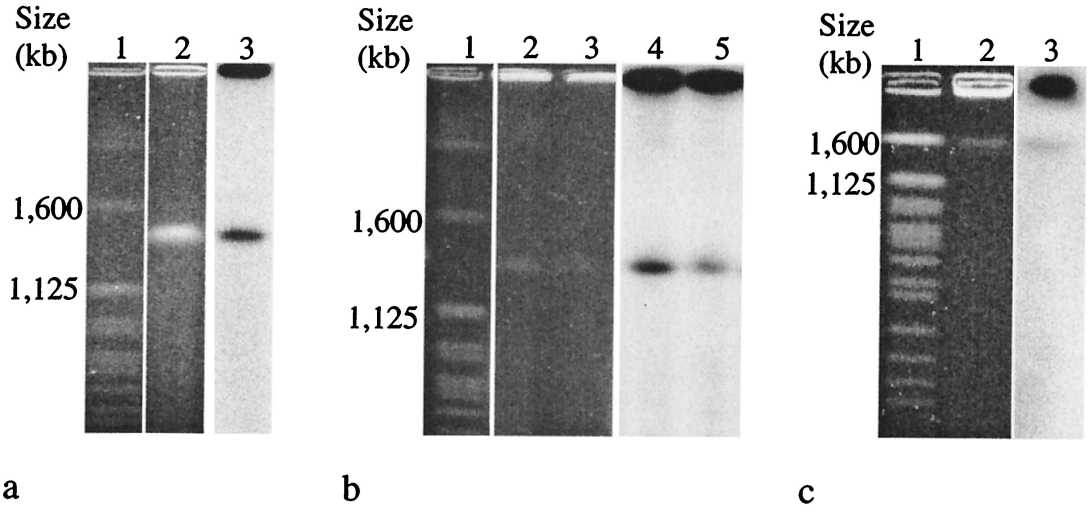

FIG. 1. Composite ethidium bromide-stained gel and corresponding autoradiograph of Southern blot probed with a *w*Ri *wsp* gene fragment. (a) Lane 1, yeast chromosomal size marker; lane 2, undigested *w*MelPop genome fragment; lane 3, Southern blot. (b) Lane 1, yeast chromosomal size marker; lane 2, undigested *w*MelCS genome fragment; lane 3, undigested *w*Mel genome fragment; lane 4, Southern blot (undigested *w*MelCS genome fragment); lane 5, Southern blot (undigested *w*Mel genome fragment). (c) Lane 1, yeast chromosomal size marker; lane 2, undigested *w*Ri genome fragment; lane 3, Southern blot.

The methods described above also led to the successful enrichment of *Wolbachia* from the filarial nematodes. Staining pulsed-field gels with ethidium bromide showed bands of around 1,100 and 950 kb for extracts made from *B. malayi and D. immitis*, respectively (Fig. 3). There was a sufficient amount of *Wolbachia* DNA in the extracts made from *D. immitis* worms for hybridization with the *ftsZ* gene fragment probe, confirming that the 950-kb band represented *Wolbachia* genomic DNA. For the extracts made from the smaller *B. malayi* worms it was usually necessary to use the *Wolbachia* probe cocktail to demonstrate that the 1,100-kb band was the *Wolbachia* genome (Fig. 3). The majority of uncut *Wolbachia* was found to remain in the well of the gel, paralleling the situation observed for extracts from *Drosophila*.

Restriction digestion of *Wolbachia* genomic DNA. The sequences of several genes previously cloned from *Wolbachia* strains suggest that the genomes of these bacteria are A+T rich (8, 9, 17, 23). Therefore, to find rare-cutting restriction enzymes, we utilized those with six- or eight-base GC recognition sites initially using *w*MelPop and *w*Ri genomic DNA.

Out of a total of 15 restriction enzymes screened, *Apa*I, *Sma*I, *Asc*I, and *Fse*I produced small numbers of fragments that facilitated the calculation of the genome size for *w*MelPop. Among them, *Apa*I and *Sma*I cut *w*MelPop into multiple fragments, *Asc*I cut this chromosome into two fragments, and *Fse*I cut only once (Fig. 4a). The size of the genome of *w*MelPop was calculated by digestion with multiple enzymes to be 1.36 Mb (Table 2). Digestions of the closely related *w*Mel and *w*MelCS strains with *Asc*I indicated genome sizes equal to that for *w*MelPop (Fig. 4b; Table 2).

The size of the genome of *w*Ri was obtained via restriction enzyme digestion with *Apa*I, *Sma*I, and *Asc*I (Fig. 4c and d). The size estimated from *Apa*I digestion and *Apa*I and *Asc*I double digestion was 1.66 Mb (Table 2), while that from *Sma*I digestion and *Sma*I and *Asc*I double digestion was 1.65 Mb (Table 2). Thus the genome size of *w*Ri was designated 1.66 Mb, the mean of 1.65 and 1.66 Mb.

A panel of restriction enzymes having GC-rich recognition sequences were also tested for their ability to cut the nematode *Wolbachia* DNA into only one or a few linear fragments. Several enzymes were found not to cut the genomes of *w*Bma or *w*Dim, while others cut the genome into multiple fragments. The optimal enzyme for *w*Bma was found to be *Apa*I, whose cut produced only four large fragments (Fig. 3), the sum of which was approximately 1,100 kb (Table 2), confirming the genome size determined from analysis of uncut DNA. Similarly, the *Wolbachia* genome from *D. immitis* extracts was cut into four bands totaling 950 kb by enzyme *Apa*I, again con-

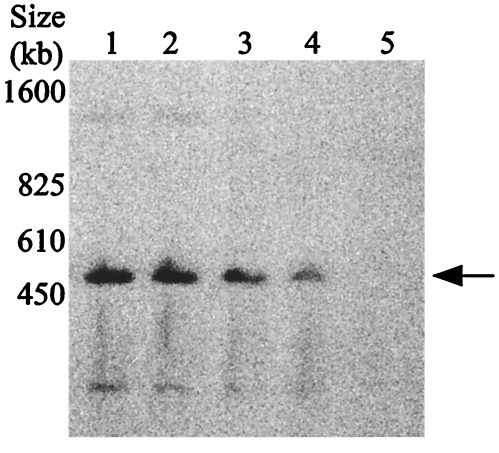

FIG. 2. Autoradiograph of Southern blot probed with mitochondrial 12S rRNA gene fragment. Shown is a DNase I (40 μg/ml) reaction time course at RT (25°C). Lane 1, 15 min; lane 2, 22 min; lane 3, 28 min; lane 4, 35 min; lane 5, 40 min. Arrow, *Drosophila* mitochondrial genome fragment.

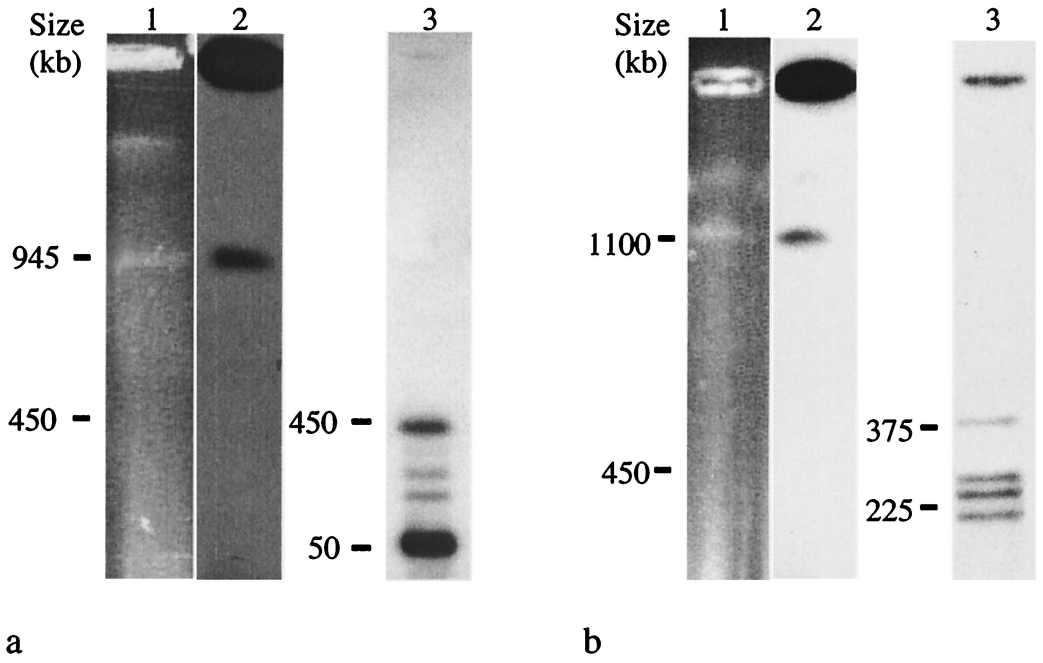

FIG. 3. Pulsed-field gel sizing of *Wolbachia* genomes from *D. immitis* (a) and *B. malayi* (b). Lanes 1, ethidium bromide staining of the uncut genomes; lanes 2, corresponding autoradiographs, which were probed with the *ftsZ* gene fragment (a) and with the *Wolbachia* probe cocktail (b); lanes 3, autoradiographs of the genomes after digestion with *Apa*I and probing with the *Wolbachia* probe cocktail. Sizes of selected DNA standards (yeast chromosome and MidRange II pulsed-field gel markers; New England Biolabs) are indicated.

firming the genome size by an independent method (Fig. 3; Table 2). For the *Wolbachia* genomes enriched from nematode tissues it was necessary to hybridize Southern blots of digested DNA with the probe cocktail in order to identify the individual fragments.

Conformation of the *Wolbachia* chromosome. To determine if the *Wolbachia* chromosome was linear or circular, plugs containing intact mosquito cells which harbored *Wolbachia* *w*AlbB were prepared (27). The plugs of intact Aa23 (*w*AlbB) were digested with *Asc*I and *Fse*I, respectively. Then, PFGE

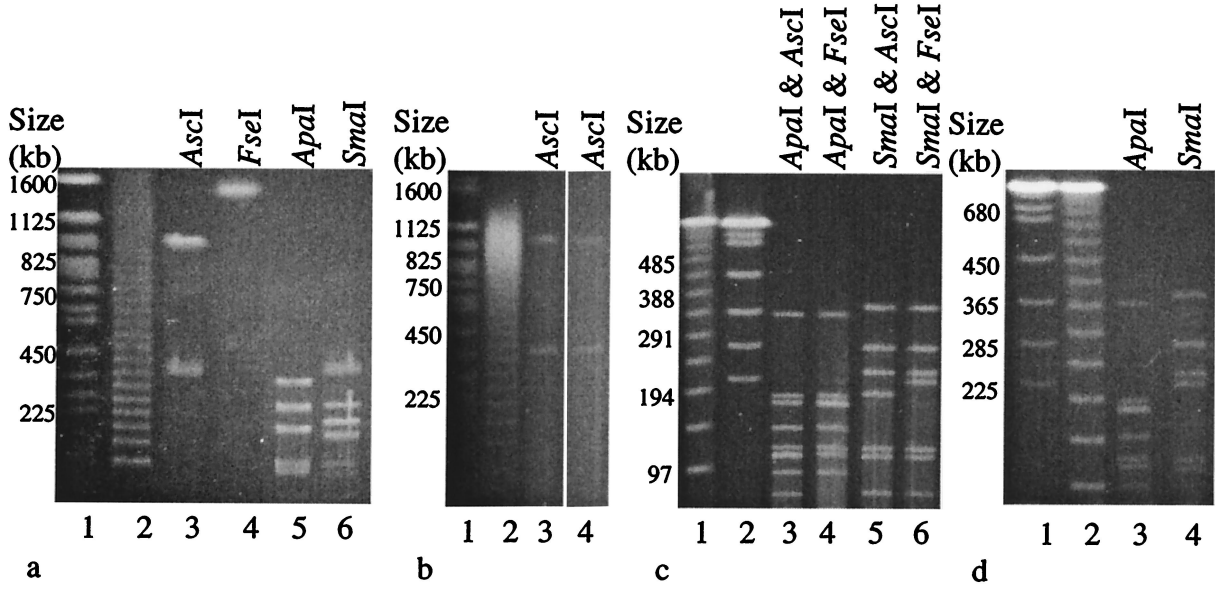

FIG. 4. CHEF gels of digested genomes of arthropod *Wolbachia* strains *w*MelPop (a), *w*MelCS and *w*Mel (b), and *w*Ri (c and d). (a) Lane 1, *Saccharomyces cerevisiae* chromosomal size marker; lane 2, lambda ladder; lanes 3 to 6, digested *w*MelPop genome. (b) Lane 1, *S. cerevisiae* chromosomal size marker; lane 2, lambda ladder; lane 3, digested *w*MelCS genome; lane 4, digested *w*Mel genome. (c) Lane 1, lambda ladder; lane 2, *S. cerevisiae* chromosomal size marker; lanes 3 to 6, digested *w*Ri genome. (d) Lane 1, *S. cerevisiae* chromosomal size marker; lane 2, lambda ladder; lanes 3 and 4, digested *w*Ri. Each lane is labeled with the enzyme(s) used.

TABLE 2. Sizes of DNA fragments produced by digestions of *Wolbachia* genomes with selected restriction enzymes

Strain	Sizes (kb) of DNA fragments[a] produced by digestions with restriction enzyme(s)					
	*Fse*I	*Asc*I	*Apa*I	*Apa*I + *Asc*I	*Sma*I	*Sma*I + *Asc*I
*w*MelPop	1,380	986, 376 (1,362)	343, 230,[b] 154, 146, 47, 45, 42, 36, 33, 33, 18 (1,357)		389, 245, 190, 173, 129, 125, 48, 29, 18, 6, 6 (1,358)	
*w*MelCS			986, 376 (1,362)			
*w*Mel			986, 376 (1,362)			
*w*Ri			363, 200, 191,[b] 155, 128, 119, 100, 58, 47, 36, 33, 20, 18 (1,659)	363, 200, 191, 155, 128, 119,[b] 100, 72, 58, 47, 36, 33, 20, 18 (1,659)	381, 287, 243, 226, 130, 121, 79, 57, 45, 33, 28, 20 (1,653)	381, 287, 243, 206, 130, 121, 79, 57, 45, 33, 28, 23, 20 (1,650)
*w*Dim			420, 290, 225, 60 (995)			
*w*Bma			380, 272, 250, 215 (1,117)			

[a] Total numbers of kilobases are in parentheses.
[b] Two fragments comigrated.

was performed for both digested and undigested plugs. After PFGE, the gel was Southern blotted. Hybridization with the *w*Ri *wsp* gene fragment clearly showed that uncut and *Fse*I-digested *Wolbachia* DNAs were retained in the loading well, while *Asc*I-digested *Wolbachia* DNA migrated into the gel (Fig. 5). These results suggest that the *Wolbachia* chromosome is circular.

DISCUSSION

Previous studies of *Wolbachia* have focused heavily on ultrastructure, reproductive phenotypes, and phylogeny. In the past, the difficulty in culturing and purifying the bacteria has hindered the progress of genetic and biochemical studies. A new protocol based on the purification of *Drosophila* mitochondria proved to be suitable for purification of *Drosophila Wolbachia*. Three modifications were key: (i) a change to the composition of the homogenization buffer, (ii) incorporation of a DNase I digestion step to obtain purer *Wolbachia* DNA, and (iii) the use of *Drosophila* adults as starting material. The homogenization buffer had previously been shown to be effective in separating *Wolbachia* from host materials (9). The detergent Lubrol was removed from the original recipe, however, since its presence increased degradation of DNA (data not shown). A digestion step with DNase I was added to remove sheared DNA, generated during homogenization. Addition of the DNase I step also appeared to remove contaminating host mitochondrial DNA from the preparation. Levels of purification of *Drosophila Wolbachia* from both adults and embryos were compared, and the latter generated very poor results (data not shown).

Bacterial chromosomes demonstrate different forms by PFGE studies; not all bacteria have circular genomes (5, 13). In circular forms, DNA with a large size is not expected to migrate into pulsed-field gels (31). As such, the fragments resolved on PFGE gels without restriction digestion (Fig. 1a and 3) are likely to be the result of nicking during homogenization. This is also consistent with the observation that most of the *Wolbachia* DNA was retained in the loading wells (Fig. 1a and 3). Furthermore, *Fse*I was determined to be a single cutter for the *w*MelPop strain. If the genome of *w*MelPop were linear, then *Fse*I digestion should have resulted in two fragments (complete digestion) or three fragments (partial digestion). However, digestion resulted in a single fragment. Similarly, digestion with *Asc*I produced two fragments. Comparing restriction patterns of *w*MelPop from single digestion with *Apa*I or *Sma*I to those from double digestions with *Apa*I and *Asc*I or *Sma*I and *Asc*I clearly showed that *Asc*I cut the chromosome in two places. These data strongly suggest that the *Wolbachia* chromosome is circular.

Studies using either PFGE or whole-genome sequencing have revealed a diversity of bacterial genome sizes, ranging from as low as 0.58 Mb to as high as 9.5 Mb. For all characterized bacterial genomes, the sizes of free-living species are generally larger than the sizes of intracellular species. Within the α-*Proteobacteria*, reported genome sizes of the free-living

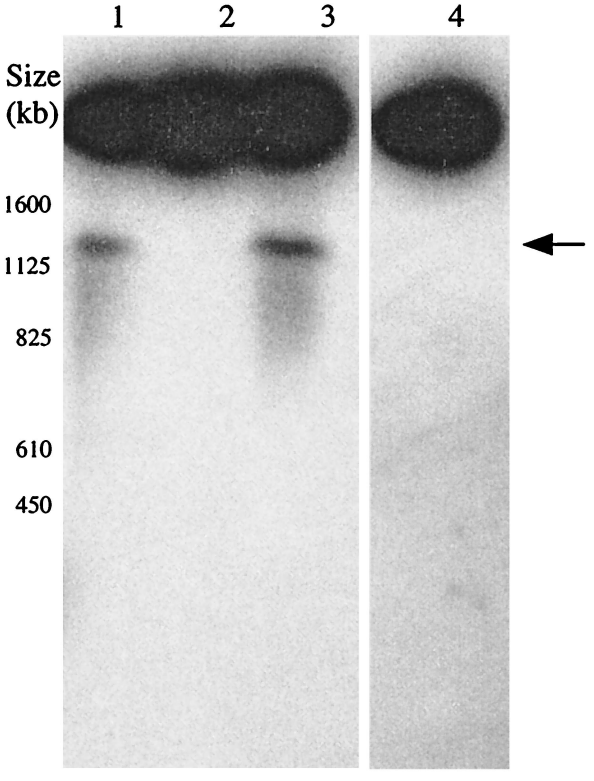

FIG. 5. Autoradiographs of Southern blot of *w*AlbB probed with a *w*Ri *wsp* gene fragment. Lanes 1 to 3, plugs of Aa23 cells (*w*AlbB) digested with restriction enzymes *Asc*I (lane 1), *Fse*I (lane 2), and *Asc*I and *Fse*I (lane 3); lane 4, uncut DNA. Arrow, *Wolbachia* genome fragment that migrated into the gel after *Asc*I digestion.

species are typically above 3.0 Mb: 3.8 Mb for *Rhodobacter capsulatus* (14), 3.8 to 4.0 Mb for *Caulobacter crescentus* (11, 12), 3.4 Mb for *Rhizobium meliloti* (19), and 8.7 Mb for *Bradyrhizobium japonicum* (21). The strictly obligate species, on the other hand, typically have genome sizes below 2.0 Mb: 1.6 Mb for *Bartonella bacilliformis* (20), 1.1 Mb for *Rickettsia prowazekii*, and 0.9 to 1.5 Mb for *Ehrlichia* spp. (30). Consistent with these previous results we have also demonstrated reduced genome sizes for *Wolbachia*: 0.95 and 1.1 Mb for the *Wolbachia* infecting nematodes and 1.4 to 1.6 Mb for the different A group *Wolbachia* strains infecting *Drosophila*.

At the present time four major monophyletic clades of *Wolbachia* are recognized and referred to as *Wolbachia* groups A, B, C, and D. The A and B groups are found in a range of arthropods and crustaceans. The C and D groups are restricted to filarial nematodes. Infections with A and B group *Wolbachia* strains are associated with various parasitic traits that indicate a conflict between their own vertical transmission and the normal reproduction of their host. In these cases *Wolbachia* has evolved various mechanisms to increase its vertical transmission including cytoplasmic incompatibility, parthenogenesis, and feminization phenotypes in the hosts they infect (24). In addition phylogenetic and experimental studies indicate that these infections are capable of moving horizontally among hosts, albeit as presumably rare events (26). In contrast C and D group *Wolbachia* strains infecting nematodes appear to be more like classical mutualists, being required for normal reproduction and development of their hosts, presumably through the supply of metabolic products required by the worm (4, 22). In addition the phylogeny of these *Wolbachia* strains mirrors that of the host worms, indicating a long period of concordant evolution between host and symbiont (3). At the present time, the lack of a suitable outgroup has prevented resolution of the evolutionary relationships among the four *Wolbachia* clades.

The large difference between the genome sizes of representatives from these different groups is intriguing. Nematode *Wolbachia* strains have a genome 30% smaller than those of the A group counterparts. The reduction in the genome sizes of these strains is consistent with the reduced genome sizes reported for other mutualistic symbionts (1, 36).

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