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Internal Spatiotemporal Population Dynamics of Infection with Three *Wolbachia* Strains in the Adzuki Bean Beetle, *Callosobruchus chinensis* (Coleoptera: Bruchidae)

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The adzuki bean beetle, *Callosobruchus chinensis*, is infected with three distinct lineages of endosymbiotic bacteria belonging to the genus *Wolbachia,* which were designated wBruCon, wBruOri, and wBruAus. In an attempt to understand the mechanisms underlying the infection with these three organisms, the spatiotemporal infection dynamics of the three *Wolbachia* strains was investigated in detail by using a quantitative PCR technique. During the development of *C. chinensis*, the wBruCon, wBruOri, and wBruAus infection levels consistently increased but the growth patterns were different. The levels of infection plateaued at the pupal stage at approximately 3×10^8, 2×10^8, and 5×10^7 *wsp* copy equivalents per insect for wBruCon, wBruOri, and wBruAus, respectively. At the whole-insect level, the population densities of the three *Wolbachia* types did not show remarkable differences between adult males and females. At the tissue level, however, the total densities and relative levels of the three *Wolbachia* types varied significantly when different tissues and organs were compared and when the same tissues derived from males and females were compared. The histological data obtained by in situ hybridization and electron microscopy were concordant with the results of quantitative PCR analyses. Based on the histological data and the peculiar *Wolbachia* composition commonly found in nurse tissues and oocytes, we suggest that the *Wolbachia* strains are vertically transmitted to oocytes not directly, but by way of nurse tissue. On the basis of our results, we discuss interactions among the three coinfecting *Wolbachia* types, reproductive strategies of *Wolbachia*, and factors involved in the different cytoplasmic incompatibility phenotypes.

Members of the genus *Wolbachia* constitute a group of rickettsial endosymbiotic bacteria in the α-subdivision of the *Proteobacteria*. Infection with *Wolbachia* is found frequently in insects (39, 40, 41) and less frequently in mites (8), spiders (28), crustaceans (6), and nematodes (2). It has been shown that *Wolbachia* infection causes reproductive alterations in the arthropod hosts, such as cytoplasmic incompatibility (CI), parthenogenesis, feminization, and male killing (for a review see reference 29). Because *Wolbachia* is inherited solely through the maternal lineage of the host by transovarial transmission, these reproductive changes can increase the frequency of infected females in host populations, often at the expense of host fitness (for a review see reference 38).

Since *Wolbachia* strains are transovarially transmitted through the hosts and manipulate host reproduction, they are expected to be associated with germ line tissues of the host organisms. Hence, much of the research on *Wolbachia* infections has been directed toward host reproductive tissues. In contrast, descriptions of *Wolbachia* interactions with nonreproductive tissues are primarily fragmentary (3, 25, 32, 42). Recently, however, it has been recognized that *Wolbachia* infections are also found in a variety of somatic tissues in different insects (13, 14, 23, 26). To understand the relationships between *Wolbachia* infection and the phenotypic effects of *Wolbachia* on a host, it is necessary to understand *Wolbachia* tissue tropism and infection densities in different host tissues. Several studies have suggested that the density of *Wolbachia* in a host is positively correlated with the intensity of CI expression (7, 9, 10, 27). In the "popcorn" *Wolbachia* infection in *Drosophila melanogaster*, extensive infection of somatic tissues of adult insects resulted in a significant reduction in the life span of the insects (26). In these studies, however, the density of *Wolbachia* was estimated only for the whole body (7, 27), for eggs (9), or for sperm cysts (10) or was assessed qualitatively (26). No study has quantitatively estimated the levels of *Wolbachia* infection in different tissues and organs of the same host. Throughout growth and development of a host insect, the density and localization of *Wolbachia* in various tissues and organs may exhibit dynamic and highly controlled changes. However, spatial and temporal infection dynamics of *Wolbachia* have not been described in any host system.

The adzuki bean beetle, *Callosobruchus chinensis* (Coleoptera: Bruchidae), is a pest of stored adzuki bean, *Vigna angularis*. In previous studies (23, 24), it was demonstrated that infection with three phylogenetically distinct strains of *Wolbachia*, designated wBruCon, wBruOri, and wBruAus, is widespread in Japanese populations of *C. chinensis*. Interestingly, the three *Wolbachia* strains caused different levels of CI; wBruCon caused complete CI, wBruOri caused partial CI, and

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wBruAus caused no or very weak CI. In addition, notably, the densities of the three *Wolbachia* strains in adult insects were consistently different; the density of wBruOri was 10^7 to 10^8 *wsp* copy equivalents per insect, the density of wBruCon was 10^7 to 10^8 *wsp* copy equivalents per insect, and the density of wBruAus was 10^6 to 10^7 *wsp* copy equivalents per insect. These results suggest that there are potential interactions among the three *Wolbachia* strains, including competition for space and resources, differential tissue tropism, and various infection dynamics during host development.

To further understand these aspects of *Wolbachia* superinfection in *C. chinensis*, we investigated the spatiotemporal infection dynamics of the three *Wolbachia* types during host development by using a quantitative PCR technique and other molecular and histological methods.

MATERIALS AND METHODS

Materials. A long-established laboratory strain of *C. chinensis*, strain jC, was used in this study. The insects were reared in petri dishes filled with adzuki beans at 30°C and a relative humidity of 70% by using a long-day regimen (16 h of light and 8 h of darkness). The materials used in this study are available upon request from the corresponding author.

Definition of developmental stages of *C. chinensis*. To collect strictly staged eggs of *C. chinensis*, mated females were allowed to oviposit on adzuki beans in petri dishes for 1 h. The eggs were harvested 1, 6, 12, 18, 24, 48, 72, and 96 h after the start of oviposition. To collect first-instar larvae, we gently detached and collected eggs from the substratum and allowed them to hatch in a petri dish. To collect older larvae and pupae, infested beans were broken with forceps under a dissecting microscope. Based on size, second-, third-, and fourth-instar larvae were separated. Pupae that were white were considered early pupae, whereas pupae that displayed the coloration of the adult cuticle were considered late pupae. Adult insects were collected within 1 day after emergence.

Preparation of samples for DNA analysis. To monitor the infection dynamics of the three *Wolbachia* types during *C. chinensis* development, strictly staged eggs, larvae, pupae, and adults were collected and immediately preserved in acetone (17). Samples were fixed and dehydrated in acetone, dried in air, weighed individually, and subjected to DNA extraction. To investigate the titers of the three *Wolbachia* strains in tissues and organs of *C. chinensis*, fat bodies, Malpighian tubules, guts, nurse tissues, oocytes, and testes were isolated from adult insects. Individual insects were carefully dissected with fine forceps under a dissecting microscope in a petri dish covered with silicon rubber and filled with phosphate-buffered saline containing Tween 20 (PBT) (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 175 mM NaCl, 0.1% Tween 20 [pH 7.4]). Isolated tissues and organs were individually washed several times with fresh PBT to minimize possible microbial contamination and immediately subjected to DNA extraction.

DNA extraction. A sample was placed in a 1.5-ml plastic tube, combined with 200 μl of lysis buffer containing sodium dodecyl sulfate and proteinase K, homogenized with a plastic pestle, incubated at 55°C for 3 h or longer, and subjected to DNA extraction with a QIAamp tissue kit (QIAGEN). Total DNA of a sample was eluted with 200 μl of TE buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA).

PCR detection. Collective PCR detection of *Wolbachia* was conducted by using two sets of universal primers for the *ftsZ* and *wsp* genes as previously described (24). A 0.75-kb *ftsZ* segment was amplified by using primers ftsF and ftsR, and a 0.6-kb *wsp* fragment was amplified by using primers wspF and wspR. Specific PCR detection of wBruCon, wBruOri, and wBruAus was performed by using specific reverse PCR primers wspConR, wspOriR, and wspAusR in combination with universal forward primer wspF, which produced an approximately 0.4-kb *wsp* gene segment (24). Three plasmids, one containing the *wsp* gene of wBruCon, one containing the *wsp* gene of wBruOri, and one containing the *wsp* gene of wBruAus, were used as negative and positive control samples for specific detection. The PCR products were electrophoresed in agarose gels, stained with ethidium bromide, and observed on a UV transilluminator.

Quantitative PCR. Real-time fluorescence detection quantitative PCR was performed by using the TaqMan PCR and ABI Prism 7700 sequence detection system (PE Applied Biosystems) as previously described (24). To estimate the titers of the three *Wolbachia* strains, the copy number of the *wsp* gene was quantified. A double-fluorescence-labeled probe for detection, TQwspPRB, was targeted to a conserved region of the three types of *wsp* sequences. The following highly specific amplifying primers were designed for variable flanking regions between the three sequences: TQwspCF and TQwspCR, TQwspOF and TQwspOR, and TQwspAF and TQwspAR (24). The probe and these primers were designed by using the Primer Express 1.0 program package (PE Applied Biosystems). Standard curves were drawn by using standard plasmid samples that contained the different *wsp* genes at concentrations of 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 copies/μl. To estimate the amount of a host gene, the mitochondrial cytochrome oxidase II (COII) gene was quantified. Quantification was conducted by using a probe, TQAzkCIIPrb (5′-FAM-ATGCAACCCCGGGTCGTCTTAA-TAMRA-3′), and primers TQAzkCIIF (5′-CTTGAACTATCCCATCTCTAGG AGT-3′) and TQAzkCIIR (5′-AACCAGCTCGATTAATAATAAATCTG-3′).

Whole-mount in situ hybridization. All incubations were carried out in 1.5-ml plastic tubes on a shaker. Dissected tissues and organs from adult insects were fixed with 4% paraformaldehyde in PBT for 2 h. After samples were washed twice with PBT for 5 min, they were dehydrated with a water-ethanol series (25, 50, 75, and 100% ethanol in PBT, 5 min for each step). Then the samples were rehydrated with an ethanol-water series (100, 75, 50, and 25% ethanol in PBT, 5 min for each step), washed twice with PBT for 5 min, and treated with 6 μg of proteinase K per ml in PBT for 3 min at 37°C to improve the infiltration of probe and reagents. After incubation with 2 mg of glycine per ml in PBT for 3 min to inhibit proteinase activity, the samples were washed with PBT for 5 min, fixed with 0.2% glutaraldehyde–4% paraformaldehyde in PBT for 20 min, and washed twice with PBT for 3 min. Then PBT was replaced with hybridization buffer (HB) (20 mM Tris-HCl [pH 8.0], 0.9% NaCl, 0.01% sodium dodecyl sulfate, 30% formamide) by incubation three times (10 min each). A digoxigenin-labeled oligonucleotide probe, DIG-Wol16S49 (5′-digoxigenin-ACCATAGCAAGCTA-CAAT-3′), was designed in this study for specific detection of *Wolbachia*. Note that this probe recognizes wBruCon, wBruOri, and wBruAus. A universal probe for eubacteria, DIG-EUB338 (5′-digoxigenin-GCTGCCTCCCGTAGGAGT-3′) (1), was also used as a positive control probe. Probe DIG-Wol16S49cmp (5′-digoxigenin-ATTGTAGCTTGCTATGGT-3′), which is complementary to the specific probe DIG-Wol16S49, was used for negative control experiments. A reaction with no probe was also conducted as a negative control. The samples were hybridized with 66 pmol of probe per ml in HB overnight. Detection of the bound probe was performed with a DIG nucleic acid detection kit (Boehringer Mannheim).

Electron microscopy. Nurse tissues and Malpighian tubules were dissected from adult females under a dissecting microscope in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) (19 mM NaH_2PO_4, 81 mM Na_2HPO_4; pH 7.4), prefixed with the fixative at 4°C overnight, postfixed with 2% osmium tetroxide in 0.1 M PB at 4°C for 60 min, and subjected to block staining with 0.5% uranyl acetate for 1 h. The tissues were dehydrated with an ethanol series and embedded in Epon 812. Ultrathin sections were cut with a ultramicrotome (Leichert-Nissei Urtracut-N), mounted on collodion-coated copper mesh, stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (Hitachi H-7000) at 75 kV.

RESULTS

Infection dynamics of the three *Wolbachia* strains during development. The populations of wBruCon, wBruOri, and wBruAus were monitored throughout development of *C. chinensis* by using a quantitative PCR technique. Figure 1 shows the infection dynamics in terms of *wsp* copies per insect. The populations of wBruCon, wBruOri, and wBruAus consistently increased as development of the host proceeded. Notably, however, the three *Wolbachia* strains exhibited different population growth patterns. In early eggs, wBruOri was the most abundant (around 4×10^5 *wsp* copy equivalents per insect), the amount of wBruCon was intermediate (around 4×10^4 *wsp* copy equivalents per insect), and wBruAus was the least abundant (around 1×10^2 *wsp* copy equivalents per insect). In late eggs and first-instar larvae, the sizes of the populations of wBruCon, wBruOri, and wBruAus were almost the same, around 6×10^5 *wsp* copy equivalents per insect. At the second-instar and later stages, the sizes of the populations of wBruCon and wBruOri were comparable whereas the populations of wBruAus were consistently and significantly smaller. In prepu-

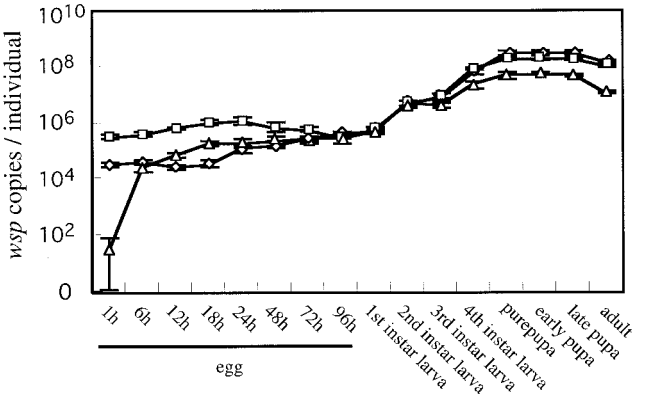

FIG. 1. Dynamics of wBruCon, wBruOri, and wBruAus infection during the development of *C. chinensis*, expressed in terms of number of *wsp* copies per insect. Symbols: ◇, wBruCon; □, wBruOri; △, wBruAus. The error bars indicate standard deviations ($n = 10$). The pupae and adults were females. Sex could not be distinguished at the other stages.

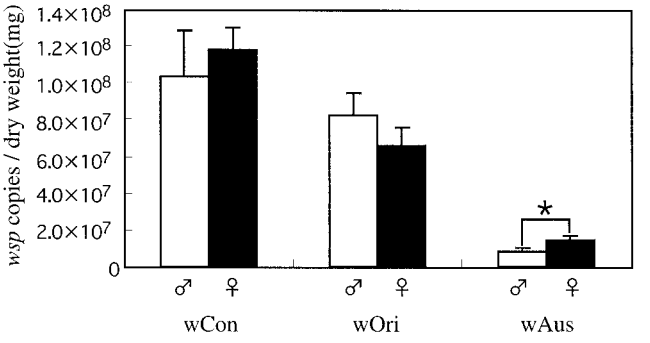

FIG. 2. Comparison of the population densities of wBruCon (wCon), wBruOri (wOri), and wBruAus (wAus) in females and males of newly emerged adults of *C. chinensis*, expressed in terms of number of *wsp* copies per milligram (dry weight) of insect. The error bars indicate standard deviations ($n = 10$). The asterisk indicates a statistically significant difference (Mann-Whitney U test, $P < 0.01$).

pae and pupae, the populations of the *Wolbachia* strains reached plateaus; the concentrations of wBruCon, wBruOri, and wBruAus were around 3×10^8, 2×10^8, and 5×10^7 *wsp* copy equivalents per insect, respectively.

Infection densities of the three *Wolbachia* strains in adult males and females. Figure 2 shows a comparison of the infection densities of wBruCon, wBruOri, and wBruAus in adult males and females of *C. chinensis*. In general, the difference in the densities between the sexes was not remarkable for each of the *Wolbachia* strains. However, the difference was statistically significant for wBruAus.

Densities of the three *Wolbachia* strains in tissues and organs. To quantitatively investigate the localization of wBruCon, wBruOri, and wBruAus in *C. chinensis* tissues, dissected tissues and organs from adult insects were subjected to quantitative PCR analysis. In order to compare different tissues and organs, the *wsp* gene copy number of the *Wolbachia* strains was standardized by using the copy number of a host gene, mitochondrial COII. It was found that both the density and the composition of the three *Wolbachia* strains were specific for tissues and organs of *C. chinensis*.

The total densities of the three *Wolbachia* strains differed significantly in different tissues and organs (Fig. 3). For the tissues and organs examined, for example, higher levels of *Wolbachia* infection were detected in the nurse tissue and fat body than in the gut and testis. The total densities of the *Wolbachia* strains also showed sex-related differences. For example, in the fat bodies the *Wolbachia* density was higher in females than in males, while in Malpighian tubules the *Wolbachia* density was lower in females than in males.

The compositions of the three *Wolbachia* strains were also significantly different in different tissues and organs (Fig. 4). In fat bodies, wBruCon accounted for around 80% of the total *Wolbachia* population, wBruOri accounted for about 15 to 18%, and wBruAus accounted for a small fraction. In guts and testes, wBruCon comprised about 50 to 70% of the total *Wolbachia* population, wBruOri comprised around 30 to 40%, and wBruAus comprised around 10% or less. In Malpighian tubules, a sex-related difference was detected; in males wBruOri was the predominant strain, whereas in females wBruCon was the major component. In nurse tissues and oocytes, strikingly, wBruOri accounted for more than 80% of the total *Wolbachia* population, whereas wBruCon and wBruAus accounted for only small fractions.

In situ hybridization and electron microscopy of *Wolbachia*. To directly observe *Wolbachia* in tissues and organs of *C. chinensis*, histological analyses were performed by using in situ hybridization and electron microscopy. In these experiments, the three *Wolbachia* strains were visualized collectively, not differentially.

Figure 5 shows the results of whole-mount in situ hybridization of dissected internal organs when an oligonucleotide probe targeted to *Wolbachia* 16S rRNA was used. *Wolbachia* signals were detected in all the tissues and organs examined, although the intensity and mode of staining were not uniform. In female ovarioles, nurse tissues and maturing oocytes exhibited particularly dense signals, while very young oocytes were stained only faintly (Fig. 5A). At the junction of nurse tissues and young oocytes, *Wolbachia* signals were observed frequently, as if the *Wolbachia* cells were transported between these tissues (Fig. 5A). Malpighian tubules were not uniformly stained but exhibited a dotted staining pattern (Fig. 5B). Although the posterior midgut exhibited particularly deep staining, the signal was due to endogenous alkaline phosphatase activity (Fig. 5B). In negative control experiments (i.e., complementary probe control and control containing no probe), most of these signals were not detected; the only exception was the signal in the posterior midgut (data not shown). In positive control experiments performed with a universal eubacterial probe, similar staining patterns were observed (data not shown).

Figure 6 is an electron microscopic image of a nurse tissue cell. A number of bacterial cells whose morphology was typical of *Wolbachia* cells were observed intracellularly. The same bacteria were also found in the cells of Malpighian tubules (data not shown).

DISCUSSION

This study was the first detailed quantitative analysis of the spatiotemporal infection dynamics of *Wolbachia* endosymbi-

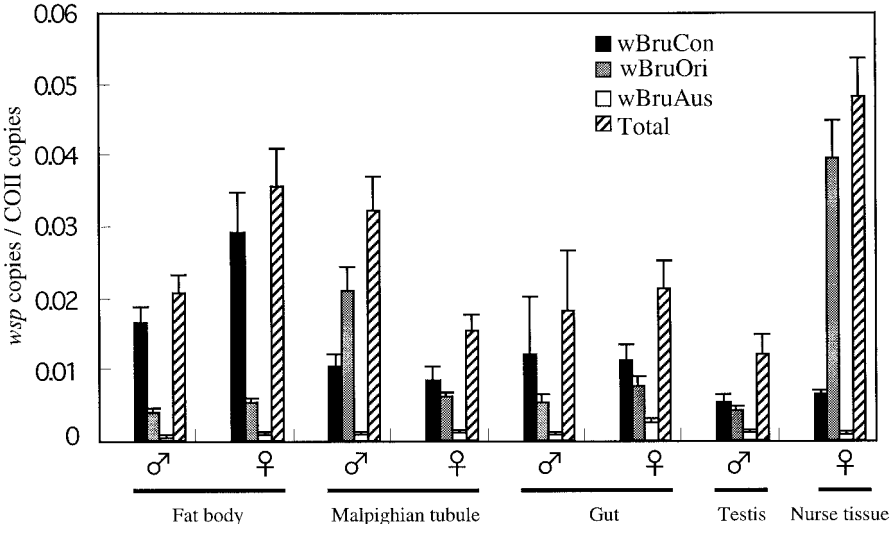

FIG. 3. Infection densities of wBruCon, wBruOri, and wBruAus in different tissues and organs of *C. chinensis* expressed in terms of number of *wsp* copies per COII copy. The mean and standard deviation for five samples are shown for each tissue or organ. Newly emerged adults were subjected to the analysis.

onts during development of an insect host. The real-time fluorescence detection quantitative PCR technique allowed us to perform reliable, robust, and efficient quantification of the *Wolbachia* strains in DNA samples extracted from individual insects or tissues in terms of *wsp* gene copy number. The insect which we examined, *C. chinensis*, was shown to be stably infected with three strains of *Wolbachia*, wBruCon, wBruOri, and wBruAus, which are phylogenetically distinct from each other and exhibit different CI phenotypes (24). From this study, therefore, it was expected that we would be able to gain insight into interactions among the three *Wolbachia* strains in the same host body, the reproductive strategies which the three *Wolbachia* strains adopt for survival and transmission, and factors that are involved in the different CI phenotypes.

Monitoring the populations of wBruCon, wBruOri, and wBruAus throughout the development of *C. chinensis* showed that the three *Wolbachia* strains exhibited different population growth patterns (Fig. 1). As a consequence, the relative amounts of the three *Wolbachia* strains changed as development of the host proceeded. In eggs wBruOri was the predominant strain, whereas in old larvae, pupae, and adults wBruCon constituted the majority. At all stages, the amount of wBruAus was the smallest. These results suggested that the three *Wolbachia* strains might be subject to differential control of proliferation during development of the host.

By using tissue-specific quantitative PCR analyses, we demonstrated that there are interesting patterns of differential tissue tropism of wBruCon, wBruOri, and wBruAus in the same host (Fig. 3 and 4). Although the mechanism is unknown, there are two possible components that may contribute to the tissue-dependent differences in density and composition of these three *Wolbachia* strains. One component is tissue tropism inherent in the *Wolbachia* strains. The other component is interactions between coinfecting *Wolbachia* strains in the same tissue. These two components could be sorted out by examining the density and localization of the *Wolbachia* strains in singly infected and doubly infected lines of *C. chinensis*.

The results of the whole-mount in situ hybridization analysis performed with a probe targeted to 16S rRNA of *Wolbachia* (Fig. 5) were concordant with the results of the tissue-specific quantitative PCR analyses (Fig. 3 and 4). All the tissues and organs examined were *Wolbachia* positive, although the intensities of the staining revealed differences among them. Nurse tissue, whose *Wolbachia* content was estimated to be high, was stained deeply. Malpighian tubules were not uniformly stained but exhibited a dotted staining pattern, which suggests that the *Wolbachia* strains do not infect all cells uniformly but preferentially infect specific cells that are distributed rather sparsely in this tissue. The cytological identity of the infected cells is unknown. In the in situ hybridization analysis, wBruCon, wBruOri, and wBruAus were collectively detected by using a *Wolbachia*-specific probe. To investigate the localization of the three *Wolbachia* strains in greater histological detail, it is necessary to develop highly specific probes for each of them.

At the level of the whole body, there were no remarkable differences in the densities and compositions of the populations of the three *Wolbachia* strains between males and females (Fig. 2). At the level of tissues and organs, in contrast, significant sex-related differences were identified (Fig. 3 and 4). The

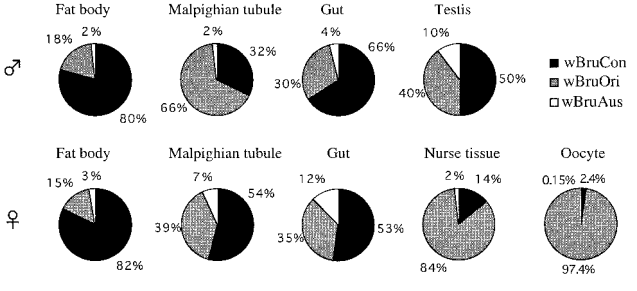

FIG. 4. Relative amounts of wBruCon, wBruOri, and wBruAus in different tissues and organs of *C. chinensis*. The mean values in Fig. 3 were used to construct the pie graphs.

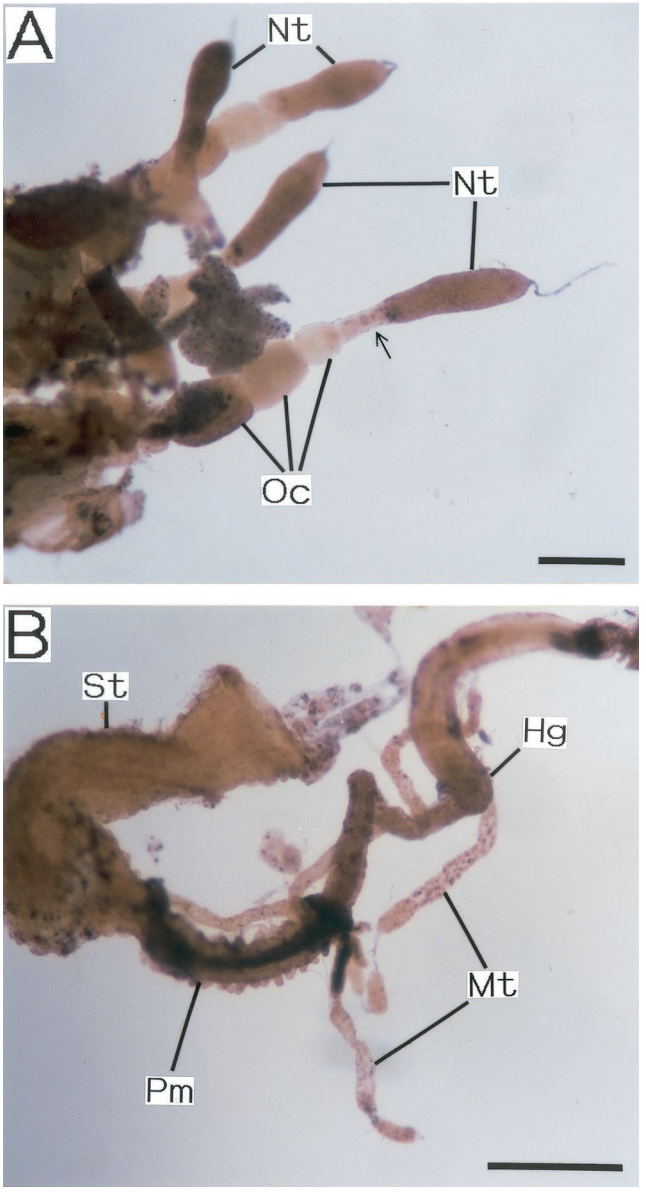

FIG. 5. Whole-mount in situ hybridization of dissected tissues and organs of *C. chinensis* performed with oligonucleotide probe DIG-Wol16S49 targeted to 16S rRNA of *Wolbachia*. (A) Ovarioles from an adult female. (B) Gut and Malpighian tubules from an adult male. Bars = 0.5 mm. Abbreviations: Hg, hindgut; Mt, Malpighian tubule; Nt, nurse tissue; Oc, oocyte; Pm, posterior midgut; St, stomach. The arrow indicates the signal of *Wolbachia* presumably in the process of vertical transmission from nurse tissue to oocyte.

densities and compositions of the three *Wolbachia* strains were markedly different in testes and oocytes (Fig. 4), which might be relevant to the different CI and vertical transmission phenotypes. In several somatic tissues, such as Malpighian tubules and fat bodies, the densities and compositions of the populations of the three *Wolbachia* strains also were different in the two sexes (Fig. 3 and 4), although the biological significance of the differences is not obvious.

In *C. chinensis*, the three *Wolbachia* strains caused different levels of CI; wBruCon caused complete CI, wBruOri caused partial CI, and wBruAus caused no or very weak CI (24). The expression and intensity of *Wolbachia*-induced CI are thought to be affected by the following factors: (i) the CI-inducing mechanism inherent in the *Wolbachia* strain (7, 18, 30, 33, 34); (ii) the density of the *Wolbachia* strain in the host (7, 9, 10, 27, 36); and (iii) the genetic background of the host (5, 7, 31). One of these factors, the effect of the host genetic background, can be disregarded in this study because of the superinfection of the same host used. The effect of *Wolbachia* density in the host may explain the absence of detectable CI with wBruAus, because the density of wBruAus was consistently and significantly less than that of wBruCon and that of wBruOri. On the other hand, the infection densities of wBruCon and wBruOri were largely comparable throughout the development of the host. Therefore, the differences in CI phenotype between wBruCon and wBruOri cannot simply be attributed to the density effect.

Although molecular mechanisms of CI are not understood, the modification-rescue model has been proposed to explain the phenomena observed in *Wolbachia*-induced CI (19, 20, 37). According to this model, it is expected that *Wolbachia* cells infecting testes are involved in the modification process and *Wolbachia* cells that accumulate in oocytes are responsible for the rescue process. In this study, we quantified the bacterial titer and composition of the CI-inducing *Wolbachia* strains, wBruCon and wBruOri, in testes and oocytes (Fig. 3 and 4). In testes, the proportions of wBruCon and wBruOri were relatively high, 50 and 40%, suggesting that these two *Wolbachia* types have equal potential access to modify the sperm during spermatogenesis. In oocytes, interestingly, wBruCon accounted for only 2.4% of the *Wolbachia* population, whereas wBruOri accounted for 97.4%. The complete CI and rescue caused by wBruCon (24) suggest that the small amount of wBruCon in oocytes can efficiently rescue the modified sperm. In contrast, the complete rescue of wBruOri-modified sperm (24) may be explained by the high infection titer of wBruOri in oocytes. The partial CI caused by wBruOri may be due to the weak modification ability of this strain.

In general, intracellular symbiotic bacteria of arthropods, including *Wolbachia*, are inherited by transovarial transmission to developing oocytes in the ovarioles in the maternal body. Histologically, it has been observed that such symbionts either actively find their way to eggs or are transferred there by specialized cells or via other structures (11, 21). However, the detailed processes of vertical transmission for *Wolbachia* have been poorly described. In this study, of the tissues examined, only nurse tissue and oocytes exhibited a peculiar *Wolbachia* composition in which wBruOri was highly predominant (Fig. 4). In the process of insect egg formation, proteins, lipids, RNAs, and other cytoplasmic components are actively synthesized by nurse cells and are transported to developing oocytes through nutritive cord with trophic flow (4, 16). One plausible explanation for the similar *Wolbachia* compositions of nurse tissue and oocytes is that the *Wolbachia* first infect and proliferate in the nurse tissue and are subsequently transported to oocytes by the nutritive cord. This idea was supported by the in situ hybridization results, which showed that *Wolbachia* cells were present in the nutritive cord, presumably being transported (Fig. 5A). The peculiar *Wolbachia* composition in nurse tissue and oocytes also suggests that wBruOri may have some

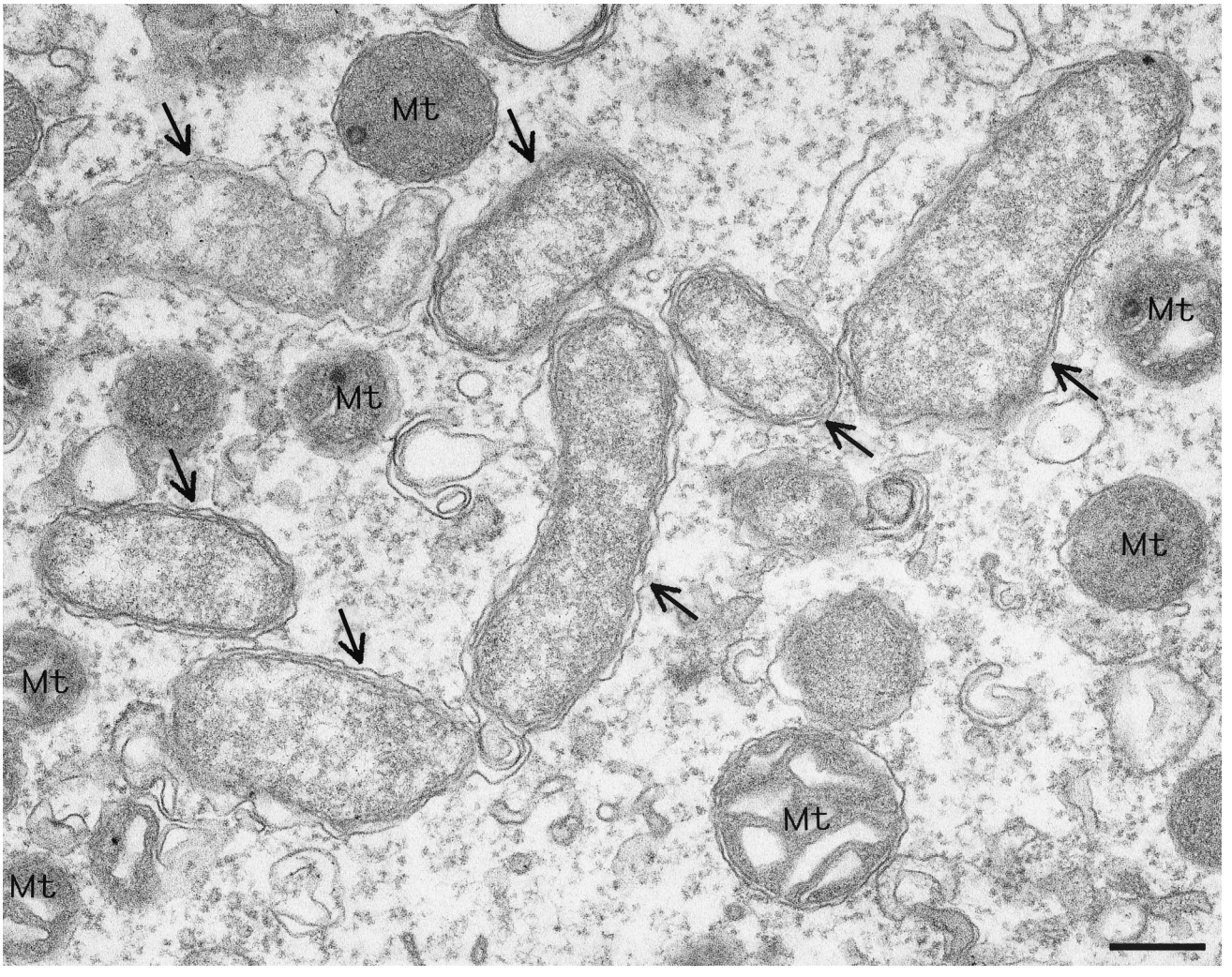

FIG. 6. Electron micrograph of *Wolbachia* in nurse tissue of *C. chinensis*. The arrows indicate *Wolbachia* cells. Bar = 0.2 μm. Mt, mitochondrion.

adaptation for transmission to and proliferation in the female germ tissues.

In natural populations of *C. chinensis*, wBruCon, wBruOri, and wBruAus exhibited very high infection frequencies (24). How the three different *Wolbachia* strains are stably maintained in the host populations is an interesting problem. Considering that they showed remarkable differences in CI expression, tissue tropism, and infection dynamics during host development, the three *Wolbachia* strains may have adopted different strategies for survival, reproduction, and transmission. Theoretically, the following strategies can favor maintenance of maternally inherited endosymbionts in host populations: (i) efficient vertical transmission, (ii) positive fitness effect on the host, (iii) reproductive manipulation, such as CI, and (iv) mechanism for horizontal transmission (12, 15, 35, 36). Since wBruCon caused complete CI and its bacterial titer in eggs was small, maintenance of wBruCon may principally be realized through an efficient mechanism for CI. In contrast, wBruOri caused partial CI, and its bacterial titer in eggs was very high, suggesting that maintenance of wBruOri may be mainly attributed to a sophisticated mechanism for vertical transmission. How wBruAus, which caused no CI and accounted for a very small fraction of the bacteria in eggs, can be stably maintained in host populations is puzzling. At present, we have no data for fitness effects and horizontal transmission of the three *Wolbachia* strains. Therefore, future examination of these aspects of the *Wolbachia* infection in *C. chinensis* is needed.

Using the quantitative PCR technique, we successfully quantified the three *Wolbachia* strains in the same DNA samples from individual insects or tissues in terms of *wsp* gene copy number. Since *Wolbachia* strains are difficult to culture, gene copy number is a reliable and useful index of bacterial amount. It should be noted, however, that gene copy number is not always in agreement with microbial cell number obtained by standard quantification methods, such as CFU determination or direct cell counting. There may be multiple copies of some target genes in the genome (for example, 16S ribosomal DNA in many bacteria). In *Buchnera*, an essential endosymbiont of aphids, the number of copies of whole genomic DNA per

bacterial cell is amplified more than 100 times (22). When quantitative PCR is used, DNA copies derived from dead *Wolbachia* cells and cell-free exogenous DNA present in the host are also counted. Considering these factors, we emphasize that the *wsp* copy number obtained in this study may not strictly reflect the real number of *Wolbachia* cells. To assess the *Wolbachia* densities in tissues and organs, the copy number of a mitochondrial gene, the COII gene, was quantified to normalize *wsp* copy number. However, we recognized the possibility that mitochondrial densities might be different in different tissues and organs and might be affected by the physiological condition of the host.

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