Wolbachia as a bacteriocyte-associated nutritional mutualist

Takahiro Hosokawa, Ryuichi Koga, Yoshitomo Kikuchi, Xian-Ying Meng, and Takema Fukatsu[1]

National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8566, Japan

Many insects are dependent on bacterial symbionts that provide essential nutrients (ex. aphid–*Buchnera* and tsetse–*Wiglesworthia* associations), wherein the symbionts are harbored in specific cells called bacteriocytes that constitute a symbiotic organ bacteriome. Facultative and parasitic bacterial symbionts like *Wolbachia* have been regarded as evolutionarily distinct from such obligate nutritional mutualists. However, we discovered that, in the bedbug *Cimex lectularius*, *Wolbachia* resides in a bacteriome and appears to be an obligate nutritional mutualist. Two bacterial symbionts, a *Wolbachia* strain and an unnamed γ-proteobacterium, were identified from different strains of the bedbug. The *Wolbachia* symbiont was detected from all of the insects examined whereas the γ-proteobacterium was found in a part of them. The *Wolbachia* symbiont was specifically localized in the bacteriomes and vertically transmitted via the somatic stem cell niche of germalia to oocytes, infecting the incipient symbiotic organ at an early stage of the embryogenesis. Elimination of the *Wolbachia* symbiont resulted in retarded growth and sterility of the host insect. These deficiencies were rescued by oral supplementation of B vitamins, confirming the essential nutritional role of the symbiont for the host. The estimated genome size of the *Wolbachia* symbiont was around 1.3 Mb, which was almost equivalent to the genome sizes of parasitic *Wolbachia* strains of other insects. These results indicate that bacteriocyte-associated nutritional mutualism can evolve from facultative and prevalent microbial associates like *Wolbachia*, highlighting a previously unknown aspect of the parasitism-mutualism evolutionary continuum.

B vitamins | bacteriome | *Cimex lectularius* | nutritional mutualism

The rise of sophisticated mutualism from less-intimate biological associations is of evolutionary interest. Obligate insect-bacterium endosymbioses are among the most impressive cases of such mutualism, wherein the partners are integrated into a coherent symbiotic entity and can no longer survive independently. For example, aphids harbor *Buchnera aphidicola*, which provide essential amino acids that are deficient in their plant sap diet, in specialized cells called bacteriocytes (1). Tsetse flies possess *Wigglesworthia glossinidia*, which synthesize B vitamins that are devoid in their blood meal, in bacteriocytes (2). Acquisition of novel biological properties via endosymbiosis seems to have played important roles in adaptation, evolution, and diversification of insects (3, 4). However, it is poorly understood how sophisticated endocellular symbionts such as *Buchnera* and *Wigglesworthia* have evolved from less-specialized bacterial ancestors.

Wolbachia represents the most prevalent endosymbiotic bacterial group, associated with over 60% of insect species (5). In infected insects, *Wolbachia* symbionts are localized in diverse cells and tissues (6) and usually affect host fitness negatively, often manipulating host reproduction to enhance their own transmission (7). Unlike *Buchnera* in aphids and *Wigglesworthia* in tsetse flies, *Wolbachia* is generally not regarded as obligate bacteriocyte-associated nutritional symbiont.

The common bedbug *Cimex lectularius* (Fig. 1*A*) is notorious as a pest that feeds on human blood, whose recent resurgence is causing hygienic, economical, and social problems (8). Since the early 1920s, the presence of bacteriocyte-associated symbionts has been recognized in *C. lectularius*. A pair of symbiotic organs consisting of many bacteriocytes, called bacteriomes, are located adjacent to the gonads (Fig. 1 *B* and *C*), wherein numerous bacteria were observed endocellularly (9–12). Early experimental works suggested a role for the symbiont in the reproduction of the bedbug (13, 14), but its microbiological nature has been elusive to date. Previous works detected 16S rRNA gene sequences of a *Wolbachia* strain and an unnamed γ-proteobacterium from the bedbug (15, 16). A recent review of the issue suggested that either the γ-proteobacterium or other undescribed symbionts may be required for reproduction of the bedbug (17).

Here we report the discovery that *Wolbachia* is the bacteriocyte-associated nutritional mutualist essential for survival and reproduction of the bedbug *C. lectularius*, which unveils an evolutionary pathway toward obligate nutritional mutualist and highlights the parasitism-mutualism evolutionary continuum.

Results and Discussion

Characterization of Bacterial Symbionts from Bacteriome of Bedbugs.
We carefully dissected bacteriomes from individual insects and conducted PCR, cloning, and sequencing of a bacterial 16S rRNA gene fragment. Two types of sequences were reproducibly obtained from two Japanese strains (TUA and TIH) and two Australian strains (SYDW and SYDL). These sequences were completely or nearly identical to the *Wolbachia* sequence and the γ-proteobacterial sequence that had been identified previously from US strains (15, 16). Meanwhile, in JESC, a Japanese strain, only the *Wolbachia* sequence was identified (Tables S1 and S2). Molecular phylogenetic analyses of the 16S rRNA gene sequences indicated that the *Wolbachia* sequences derived from different bedbug populations were phylogenetically coherent, as were the γ-proteobacterial sequences (Fig. 2). Molecular phylogenetic analysis of *Wolbachia* ftsZ gene sequences confirmed phylogenetic coherence of the *Wolbachia* sequences (Fig. S1). The ftsZ gene sequences obtained in this study were identical to the ftsZ gene sequence of a *Wolbachia* strain from *C. lectularius* that was placed in the F supergroup and genotyped by the multilocus strain typing system in a previous study (18). Sequences from no other bacterial types were detected from the bacteriome. Of 105 bedbugs examined, the *Wolbachia* sequence was identified from every insect, whereas the γ-proteobacterial sequence was detected from only 53% of them (Table S1). These results indicated that two bacterial symbionts, the *Wolbachia* and the γ-proteobacterium, are present in the bacteriome of *C. lectularius*. It seemed unlikely that the γ-proteobacterium is an essential symbiont for the bedbug because it was not present in all samples.

EVOLUTION

Fig. 1. The common bedbug *Cimex lectularius* and localization of its symbiotic bacteria. (*A*) A mating pair of adult bedbugs. (*B* and *C*) Gonad-associated bacteriomes in adult male (*B*) and female (*C*) highlighted by orange arrows, from Davis (10). (*D* and *E*) FISH detection of *Wolbachia* in adult male (*D*) and female (*E*); orange arrows and arrowheads indicate bacteriome infections and ovarial infections, respectively. (*F* and *G*) FISH detection of *Wolbachia* and γ-proteobacteria in bacteriomes of monosymbiotic bedbug strain JESC (*F*) and disymbiotic bedbug strain TUA (*G*). (*H* and *I*) Transmission electron microscopy of rod-shaped *Wolbachia* cells in a bacteriocyte of JESC insect (*H*) and a tubular γ-proteobacterial cell in addition to *Wolbachia* cells in a bacteriocyte of TUA insect (*I*). (*J* and *K*) Infection patterns of *Wolbachia* and γ-proteobacteria in germalia of JESC female (*J*) and TUA female (*K*); white arrowheads and arrows indicate *Wolbachia* infections in somatic stem cell niche and nutritive cord, respectively. (*L* and *M*) Localization of *Wolbachia* and γ-proteobacteria at posterior pole of developing oocytes in JESC female (*L*) and TUA female (*M*). (*N*) Localization of *Wolbachia* in primordial bacteriome (orange arrow) in developing embryo. Abbreviations: Mn, mandible; Mx, maxilla; Lb, labium; T1–3, first-third thoracic appendages. In *D–G* and *J–N*, red, green, and blue signals indicate *Wolbachia*, γ-proteobacteria and insect nuclei, respectively.

Localization of Bacterial Symbionts in Bedbugs. Quantitative PCR assays unveiled a striking tissue tropism of the *Wolbachia* symbiont in *C. lectularius*. The *Wolbachia* densities in the bacteriome were approximately 30 times higher than those in the ovary, and 2,000–900,000 times higher than in other organs (Fig. 3). These results indicated that, except for the bacterial fraction passed to the ovary, the *Wolbachia* infection is substantially restricted to the bacteriome. This is atypical of *Wolbachia* symbionts, whose infection is generally observed in multiple types of cells and tissues of their host insects (6). Fluorescent in situ hybridization and electron microscopy
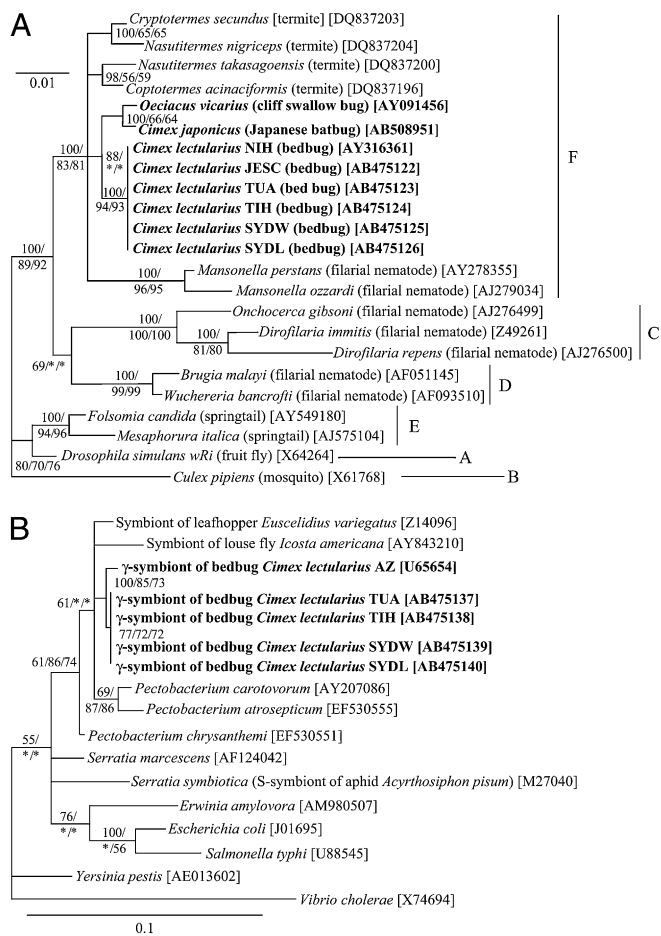
Fig. 2. Phylogenetic placement of symbiotic bacteria from *C. lectularius* and allied bugs. (*A*) A 16S rRNA gene phylogeny of *Wolbachia* from the bedbugs (1,441 aligned nucleotide sites). (*B*) A 16S rRNA gene phylogeny of γ-proteobacteria from the bedbugs (1,089 sites). Bayesian (BA) phylogenies are shown. Host insect names are in italic, accession numbers in brackets, and *Wolbachia* supergroups A–F on the right side are shown in *A*. Posterior probabilities for BA analyses and bootstrap probabilities for maximum parsimony (MP) and maximum likelihood (ML) analyses greater than 50% are indicated at the nodes in the order of BA/MP/ML.

unequivocally demonstrated the bacteriome-specific localization of the *Wolbachia* symbiont: in males, the *Wolbachia* signals were restricted to the testis-associated bacteriomes (Fig. 1*D*), whereas in females, the signals were found in the bacteriomes and the ovaries (Fig. 1*E*). In the monosymbiotic JESC strain, the bacteriocytes were occupied solely by the rod-shaped *Wolbachia* cells (Fig. 1 *F* and *H*), whereas in the disymbiotic TUA strain, tubular γ-proteobacterial cells were found at low densities in addition to the *Wolbachia* cells (Fig. 1 *G* and *I*). The γ-proteobacterial symbiont was sporadically observed in diverse cells and tissues, particularly in Malpighian tubules and ovariole pedicels (Fig. S2).

Ovarial Transmission of Bacterial Symbionts in Bedbugs. Fluorescent in situ hybridization using *Wolbachia*-specific probes revealed characteristic localization patterns of *Wolbachia* symbiont in the ovary, which were suggestive of the route and mechanism of its vertical transmission. At the tip of the ovarioles, the *Wolbachia* signals were found inside specific cells in a middle region of the germalia (Fig. 1 *J* and *K*, arrowheads), where the somatic stem cell niche is presumably located. In *Drosophila melanogaster*, the somatic stem cell niche was demonstrated to be the entry point of *Wolbachia* infection to germline (19). The *Wolbachia* signals were
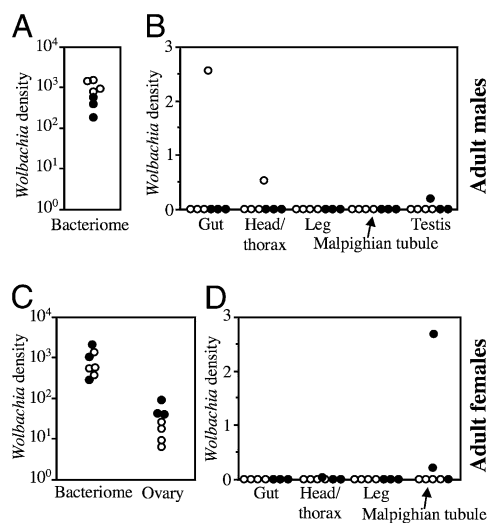

Fig. 3. Quantification of *Wolbachia* density in different organs of *C. lectularius*. (*A* and *B*) Infection densities in male insects; and (*C* and *D*) infection densities in female insects in terms of *Wolbachia* wsp gene copies per insect elongation factor 1α gene copy. Filled and open dots indicate insects of the monosymbiotic strain JESC and the disymbiotic strain TUA, respectively.

also found in the nutritive cord connecting nurse cells and developing oocytes (Fig. 1 *J* and *K*, arrows), which probably indicate migrating symbiont cells with trophic flow from the germalia to the oocytes. In the oocytes, the *Wolbachia* signals were concentrated at a posterior pole region (Fig. 1 *L* and *M*), the location of germ plasm destined to form germline cells. Such colocalization with the germ plasm has been known from a wide variety of *Wolbachia* and other insect endosymbionts (11, 20), which may facilitate stable vertical transmission of the maternally inherited microbes. During embryonic development, the *Wolbachia* signals migrated to a specific region associated with the germ band, participating in the formation of the bacteriome (Fig. 1*N*). From these results, we concluded that *Wolbachia*, rather than the γ-proteobacterium, is the major bacteriocyte-associated symbiont in the bedbug, whose localization and infection dynamics are comparable to those of obligate mutualistic bacteriocyte symbionts like *Buchnera* in aphids and *Wigglesworthia* in tsetse flies.

Wolbachia as an Essential Symbiont for Bedbugs. On account of the elaborate symbiotic configuration, we predicted that the *Wolbachia* symbiont would play a biological role for the bedbug host. By making use of an artificial feeding system (Fig. 4 *A* and *B*), we performed antibiotic treatments of the monosymbiotic JESC strain. Continuous rearing of newly-emerged adult insects on a rifampicin-supplemented blood meal resulted in complete elimination or drastic reduction (1/100~1/10,000) of the *Wolbachia* infection (Fig. 4*C*; Fig. S3). The aposymbiotic insects normally survived and laid eggs, as did the control insects (Fig. 4*D*). However, the rate of normally developing eggs was much reduced compared to the controls (Fig. 4*E*). Usually red eyespots appeared on the control eggs within five days after oviposition, whereas the antibiotic-affected eggs were darkened and deformed (Fig. 4*F*). When newly-hatched nymphs were continuously reared on the antibiotic-supplemented diet, the insects suffered a significantly lower adult emergence rate (Fig. 4*G*) and a prolonged nymphal period (Fig. 4*H*). These results clearly indicated that *Wolbachia* is essential for normal growth and reproduction of the bedbug.

Wolbachia as Supplier of B Vitamins for Bedbugs. What is the basis of the essentiality of *Wolbachia* for the bedbug? In other blood-sucking insects like tsetse flies, their bacterial symbionts synthe-
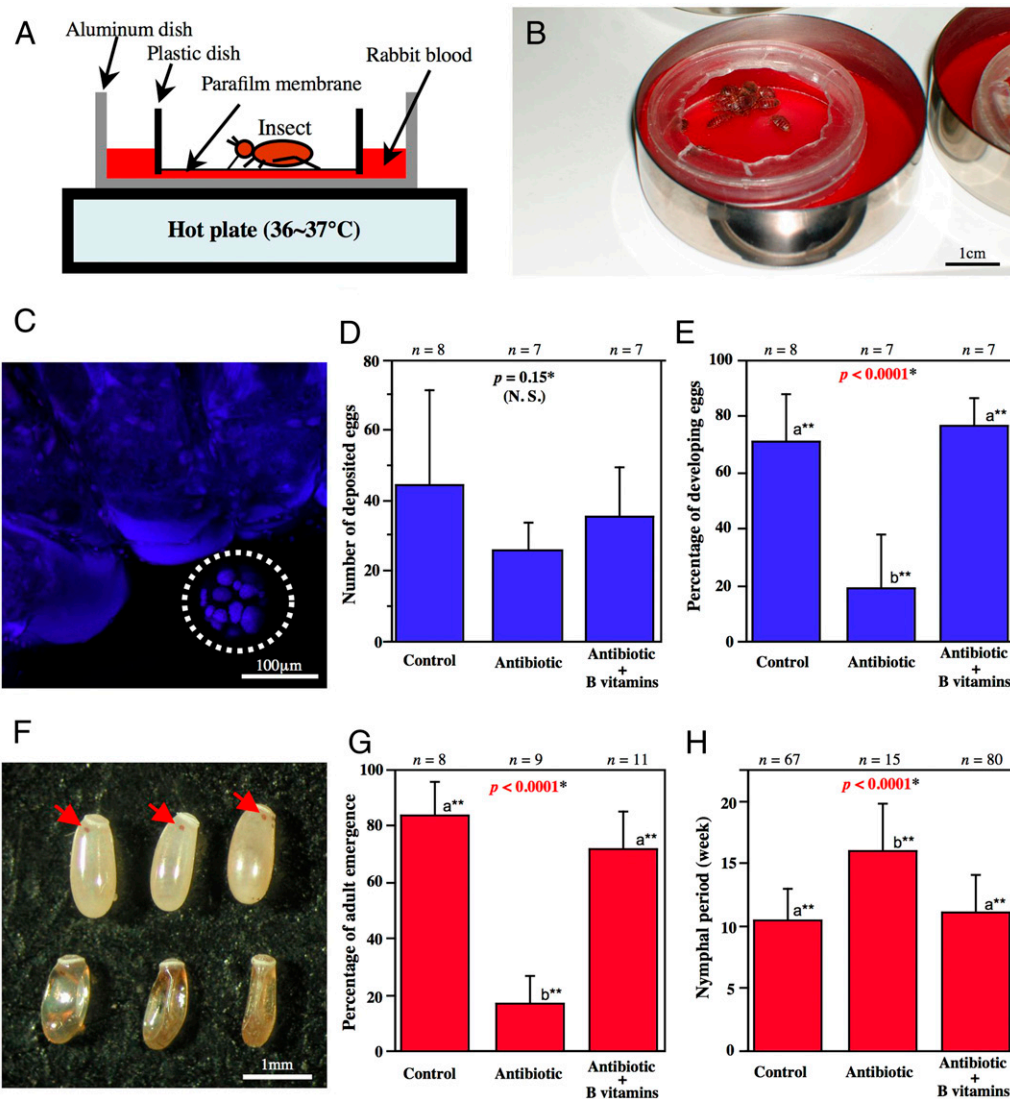
Fig. 4. Biological role of *Wolbachia* for *C. lectularius* demonstrated by antibiotic treatment and nutritional supplementation using an artificial feeding system. (*A* and *B*) An illustration (*A*) and a photo (*B*) of the artificial feeding system for bedbug. (*C*) FISH of an antibiotic-treated adult insect, wherein *Wolbachia* signal in the bacteriome disappeared (dotted circle). (*D* and *E*) Effects of antibiotic treatment and vitamin B supplementation on adult insects: (*D*) number of deposited eggs; and (*E*) percentage of developing eggs. (*F*) Eggs 5 days after oviposition: (*Upper*) eggs laid by control insects, wherein arrows indicate red eyespots; (*Lower*) eggs laid by antibiotic-treated insects, which are darkened, deformed, and dead. (*G* and *H*) Effects of antibiotic treatment and vitamin B supplementation on nymphal growth and development: (*G*) percentage of adult emergence; and (*H*) nymphal period.

size B vitamins that are deficient in their blood meal (2). When we supplemented B vitamins to every blood meal for the experimental bedbugs, strikingly, the aposymbiotic insects were almost completely restored to normal egg development, adult emergence rate and nymphal period (Fig. 4 *E, G,* and *H*). These results strongly suggested that the bacteriocyte-associated *Wolbachia* symbiont is the obligate nutritional mutualist for the bedbug, whose major biological role is provisioning of B vitamins.

Wolbachia as Bacteriocyte-Associated Nutritional Mutualist for Bedbug. In conclusion, *Wolbachia* has evolved bacteriocyte-specific localization and nutritional mutualism in the bedbug, similar to the ancient bacteriocyte-associated nutritional mutualists *Buchnera* in aphids and *Wigglesworthia* in tsetse flies. *Wolbachia* symbionts are generally regarded as parasitic, affecting the host fitness negatively and often causing cytoplasmic incompatibility or other reproductive aberrations (7). It should be noted, however, that several intermediate forms of *Wolbachia* that tend

toward mutualism have been recognized. Some *Wolbachia* strains (those in which pathogen resistance, nutritional supplementation, or other mechanisms might be involved) were shown to improve the host fitness conditionally (21–26). In a parasitoid wasp, *Wolbachia* elimination inhibits the development of ovaries, wherein the symbiont does not necessarily benefit the host but somehow modifies the host to be dependent on the symbiont infection (27, 28). In filarial nematodes, *Wolbachia* symbionts are regarded as mutualistic, although the exact benefits conferred by the bacteria have been elusive (29). The discovery of *Wolbachia* as bacteriocyte-associated nutrient supplier in the bedbug provides an unprecedented case of *Wolbachia*-insect mutualism. Considering that over 60% of insect species are estimated to carry *Wolbachia* infections (5), we expect that the bedbug case cannot be an exception but many more *Buchnera*- or *Wigglesworthia*-like *Wolbachia* symbionts are to be discovered. In a book louse, a bacteriome-associated *Rickettsia* symbiont was identified (30).

Evolutionary Origin of Bedbug–Wolbachia Mutualism. The aphid–*Buchnera* and tsetse–*Wigglesworthia* associations are of ancient evolutionary origins dating back to over 100 million years ago, leading to drastically reduced symbiont genomes (1, 2). How old is the bedbug–*Wolbachia* bacteriocyte-associated nutritional mutualism? Among diverse bedbugs, *Wolbachia* strains allied to that of *C. lectularius* have been characterized only from the closely-related genera *Cimex* and *Oeciacus* in the subfamily Cimicinae (Fig. 2*A* and Fig. S1) (16, 17). Pulsed-field gel electrophoresis of the bacteriome of *C. lectularius* estimated the *Wolbachia* genome size as 1.3 Mb (Fig. 5), which is closer to the genome sizes of parasitic *Wolbachia* strains of insects (1.3–1.6 Mb) rather than to those of mutualistic *Wolbachia* strains of filarial nematodes (1.0–1.1 Mb) (7). Hence, the *Wolbachia* symbiont of the bedbug may be at an early evolutionary stage of bacteriocyte-associated obligate mutualism. Genome sequencing of the *Wolbachia* symbiont would lead to insights into how elaborate endocellular mutualists like *Buchnera* in aphids and *Wigglesworthia* in tsetse flies have evolved from much less specialized but more prevalent microbial associates like *Wolbachia*. The mutualistic *Wolbachia* of *C. lectularius* belongs to the F supergroup, which also contains *Wolbachia* strains from termites and filarial nematodes (Fig. 2 and Fig. S1). For the termite strains, their phenotypic aspects are unknown (31), whereas the nematode strains are suspected to be mutualistic (32). No reproductive parasite strains have been known from the F supergroup. Hence it is currently elusive whether the ancestor of the bedbug strains was a reproductive parasite, a commensal, or already a mutualist. Finally, we point out the possibility that the mutualistic *Wolbachia* symbiont could be a target for controlling this cosmopolitan insect pest.

Materials and Methods

Insects. The samples of *C. lectularius* used in this study are listed in Table S1. The JESC and TIH strains were maintainable using an artificial feeding system (Fig. 4 *A* and *B*). A Parafilm membrane was stretched on a Petri dish, through which the insects were allowed to feed every two weeks on rabbit blood (Kohjin Bio) warmed at 37 °C. Otherwise the insects were kept in Petri dishes with several pieces of filter paper at 25 °C under constant darkness. The TUA insects could not be maintained with the system, plausibly because of adaptation to the avian host, and thus were collected at the quail coop upon necessity. The SYDW and SYDL insects were provided as acetone-preserved specimens. The samples of *Cimex japonicus* were collected from a bat colony and preserved in acetone.

PCR, Cloning, and Sequencing. Dissected tissues or whole insects were individually subjected to DNA extraction using NucleoSpin Tissue Kit (Machery-

Nagel). A bacterial 16S rRNA gene (1.5 kb) and two *Wolbachia* genes, wsp (0.56 kb) and ftsZ (0.75 kb), were amplified by PCR as described elsewhere (33, 34). An insect gene, elongation factor 1α ($ef1\alpha$; 0.54 kb), was amplified with primers efF2 (5′-GCT GGT ACT GGW GAA TTY GAA-3′) and efR1 (5′-CAC DGA CTT DAC TTC AGT GGT-3′). The PCR products were subjected to cloning, restriction fragment length polymorphism genotyping, and sequencing as described (34).

Molecular Phylogenetic Analysis. Multiple alignments of the nucleotide sequences were generated using Clustal W (35). Phylogenetic analyses were conducted by three methods: maximum parsimony, maximum likelihood and Bayesian using PAUP 4.0b10 (36) and MrBayes v3.0b4 (37).

Diagnostic PCR. A wsp gene fragment (0.56 kb) of the *Wolbachia* symbiont was amplified by PCR as described (33). A 16S rRNA gene fragment (1.0 kb) was amplified with primers CLsecF (5′-CGG TAA GGT TAA TAA CCT TGC-3′) and 16SB1 (5′-TAC GGY TAC CTT GTT ACG ACT T-3′).

Quantitative PCR. *Wolbachia* wsp gene copies and insect $ef1\alpha$ gene copies were quantified using SYBR green and Mx3000P QPCR system (Stratagene) as described elsewhere (38) with primers CLwspF (5′-CGG CTC TTA TGG CGC TAG C-3′) and CLwspR (5′-CTT TTT GGT TGT ATC GCC AGG A-3′) for wsp and with primers CLefF (5′- GCA AAT GCC TTA TTG AAG CTC TC-3′) and CLefR (5′-GGA AGC CTA AGA GGC TTG TCA G-3′) for $ef1\alpha$.

Histological Procedures. For in situ hybridization targeting bacterial 16S rRNA, adult insects were removed of head, thorax, and dorsal or ventral plates, and the dissected abdomens were fixed with Carnoy's solution (ethanol: chloroform: acetic acid = 6:3:1 [vol/vol]) for several days and were treated with 6% H_2O_2 in 80% ethanol for a week to eliminate autofluorescence of the tissues (39). The *Wolbachia* symbiont was detected with two oligonucleotide probes whose 5′ end was labeled with AlexaFluor555, TsWol944R (5′-AAC CGA CCC TAT CCC TTC G-3′) and TsWol1187R (5′-CTC GCG ACT TTG CAG CCC A-3′). The γ-proteobacterial symbiont was visualized with a probe whose 5′ end was labeled with Alexafluor647, CimexSec1229R (5′-TTG CTC TCG CGA GGT CGC TT-3′). The stained samples were observed under an epifluorescent microscope or a laser confocal microscope. For transmission electron microscopy, dissected bacteriomes were fixed with glutaraldehyde and osmium tetroxide, embedded in Spurr resin, processed into ultrathin sections, stained with uranyl acetate and lead citrate, and observed under an electron microscope as described (34).

Feeding Treatments. For antibiotic treatment, rifampicin solution in methanol was added to the blood meal at a final concentration of 10 μg/mL For nutritional supplementation, aqueous solution of B vitamins was supplied to the blood meal in addition to the antibiotic at final concentrations described in Table S3.

Symbiont Effects on Adult Fecundity and Fertility. Adult insects of the JESC strain were divided into groups consisting of three males and three females within 2 weeks of emergence. Each of the groups was randomly assigned to one of the following experimental treatments: (*i*) control treatment, wherein only methanol was added to every blood meal; (*ii*) antibiotic treatment, wherein rifampicin was added to every blood meal; and (*iii*) antibiotic + B vitamins treatment, wherein both rifampicin and B vitamins were added to every blood meal. In all groups, the insects were fed for 20 min every week. They were allowed to reproduce for 8 weeks, and the number of deposited eggs and hatching rate of the eggs were recorded for each of the groups.

Symbiont Effects on Nymphal Growth and Survival. The first instar nymphs of the JESC strain were divided into groups of 10 insects within a week of hatching. Each of the groups was randomly assigned to one of the above experimental treatments. Their growth and survival were monitored until all of the insects either became adult or died.

Pulsed Field Gel Electrophoresis. Bacteriomes dissected from 25 adult insects were homogenized in 50 μL of a PBS and embedded in 1% low melting point agarose. The agarose plug was incubated in a lysis buffer containing proteinase K at 50°C overnight. After thorough washing, the plug was treated with a restriction enzyme, cut and set on an agarose gel, and subjected to pulsed field gel electrophoresis using CHEF Mapper (BioRad).
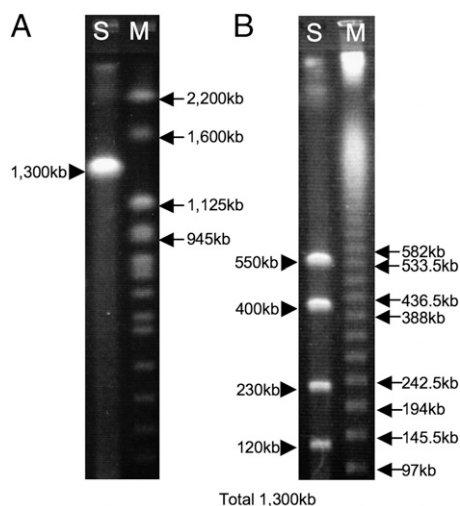
Fig. 5. Genome size of *Wolbachia* from the bacteriome of *C. lectularius* estimated by pulsed field gel electrophoresis. (*A*) *I-CeuI* digestion yielded a single band of 1.3 Mb. (*B*) *AscI* digestion produced four bands, which were summed up to 1.3 Mb. Lanes S and M indicate samples and DNA size markers, respectively.

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