Evolutionary origin of insect–*Wolbachia* nutritional mutualism

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Edited by Nancy A. Moran, University of Texas at Austin, Austin, TX, and approved June 3, 2014 (received for review May 20, 2014)

Obligate insect–bacterium nutritional mutualism is among the most sophisticated forms of symbiosis, wherein the host and the symbiont are integrated into a coherent biological entity and unable to survive without the partnership. Originally, however, such obligate symbiotic bacteria must have been derived from free-living bacteria. How highly specialized obligate mutualisms have arisen from less specialized associations is of interest. Here we address this evolutionary issue by focusing on an exceptional insect–*Wolbachia* nutritional mutualism. Although *Wolbachia* endosymbionts are ubiquitously found in diverse insects and generally regarded as facultative/parasitic associates for their insect hosts, a *Wolbachia* strain associated with the bedbug *Cimex lectularius*, designated as *w*Cle, was shown to be essential for host's growth and reproduction via provisioning of B vitamins. We determined the 1,250,060-bp genome of *w*Cle, which was generally similar to the genomes of insect-associated facultative *Wolbachia* strains, except for the presence of an operon encoding the complete biotin synthetic pathway that was acquired via lateral gene transfer presumably from a coinfecting endosymbiont *Cardinium* or *Rickettsia*. Nutritional and physiological experiments, in which *w*Cle-infected and *w*Cle-cured bedbugs of the same genetic background were fed on B-vitamin–manipulated blood meals via an artificial feeding system, demonstrated that *w*Cle certainly synthesizes biotin, and the *w*Cle-provisioned biotin significantly contributes to the host fitness. These findings strongly suggest that acquisition of a single gene cluster consisting of biotin synthesis genes underlies the bedbug–*Wolbachia* nutritional mutualism, uncovering an evolutionary transition from facultative symbiosis to obligate mutualism facilitated by lateral gene transfer in an endosymbiont lineage.

Symbiotic associations are ubiquitous in the biological world, in which obligate insect–bacterium endosymbiotic associations are among the most sophisticated forms wherein the host and the symbiont are integrated into a coherent biological entity and cannot survive without the partnership (1, 2). For example, in the aphid–*Buchnera* nutritional mutualism, the host depends on the symbiont for supply of essential amino acids that are needed for host's protein synthesis but are scarce in the host's plant sap diet (3). In the tsetse–*Wigglesworthia* nutritional mutualism, the symbiont provides B vitamins that are deficient in vertebrate blood the host exclusively feeds on (4). Through the intimate relationship over evolutionary time, these and other endosymbiont genomes have been reduced drastically, losing many genes needed for independent life and streamlined for specific biological roles to support their hosts (5, 6). Novel biological properties acquired through endosymbiosis have played substantial roles in adaptation, evolution, and diversification of insects and other organisms (1, 2). Although currently comprising elaborate symbiotic systems, such endosymbionts must have originally been derived from free-living ancestors. How highly specialized obligate endosymbionts have arisen from less specialized bacterial associates is of evolutionary interest.

Members of the genus *Wolbachia* are well known as facultative bacterial endosymbionts ubiquitously associated with diverse insects, generally conferring negative fitness consequences to their hosts and often causing hosts' reproductive aberrations to enhance their own transmission in a selfish manner (7, 8). Recently, however, a *Wolbachia* strain associated with the bedbug *Cimex lectularius*, designated as *w*Cle, was shown to be essential for normal growth and reproduction of the blood-sucking insect host via provisioning of B vitamins (9). Hence, it is expected that a transition from facultative association to obligate mutualism may have occurred in an ancestor of *w*Cle. What evolutionary processes and mechanisms are involved in the emergence of the insect–*Wolbachia* nutritional mutualism?

In this study, we determined the complete genome of *w*Cle, which was similar in size and composition to the genomes of facultative *Wolbachia* endosymbionts associated with other insects, except for the presence of an operon encoding biotin synthesis pathway that was presumably acquired via lateral gene transfer from an unrelated bacterium. Using *w*Cle-infected and *w*Cle-cured bedbug strains under the same genetic background, we experimentally demonstrated that *w*Cle is capable of synthesizing biotin and *w*Cle-provisioned biotin significantly contributes to the host fitness, thereby uncovering a genomic basis of the insect–*Wolbachia* nutritional mutualism. Through comprehensive

Significance

How sophisticated mutualism has arisen from less-intimate associations is of general interest. Here we address this evolutionary issue by looking into the bedbug. *Wolbachia* endosymbionts are generally regarded as facultative/parasitic bacterial associates for their insect hosts, but in the bedbug, exceptionally, *Wolbachia* supports the host's growth and survival via provisioning of vitamins. In the bedbug's *Wolbachia* genome, we identified a gene cluster encoding the complete synthetic pathway for biotin (vitamin B7), which is not present in other *Wolbachia* genomes and is presumably acquired via lateral transfer from a coinfecting endosymbiont. The *Wolbachia*-provisioned biotin contributes to the bedbug's fitness significantly, uncovering an evolutionary transition from facultative symbiosis to obligate mutualism facilitated by lateral gene transfer in the endosymbiont lineage.

Author contributions: N.N., T.H., M.M., and T.F. designed research; N.N., T.H., M.M., and K.O. performed research; M.H. and T.F. contributed new reagents/analytic tools; N.N., T.H., M.M., K.O., and M.H. analyzed data; and T.F. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The sequences reported in this paper have been deposited in the DNA Data Bank of Japan database, www.ddbj.nig.ac.jp/index-e.html (accession nos. AP013028 and AB934986–AB934989).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1409284111/-/DCSupplemental.

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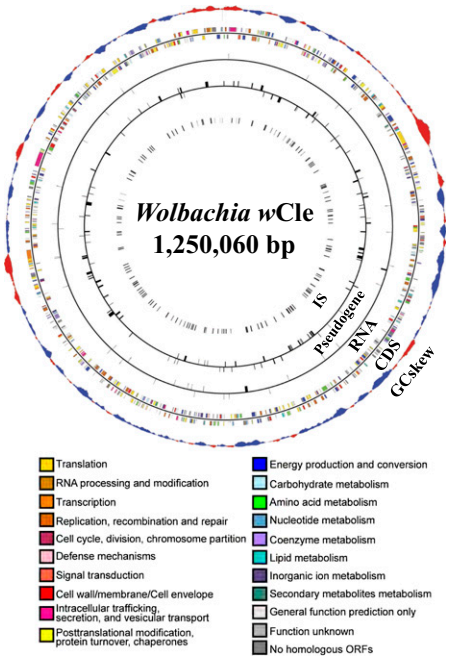

Fig. 1. Circular view of the *w*Cle genome. On the GC skew circle, red and blue indicate GC rich and poor, respectively. On the CDS circle, colors indicate functional categories as shown at the bottom.

survey of *Wolbachia* genomic data, we discuss evolutionary hypotheses as to how and when the biotin operon was acquired by *w*Cle in the course of insect–*Wolbachia* coevolution.

Results and Discussion

Determination of *w*Cle Genome. We carefully dissected 26 adult bedbugs of the monosymbiotic strain JESC infected with *w*Cle only without secondary symbionts (9), collected their bacteriomes, and extracted total DNA from the symbiotic organs. The DNA sample was subjected to shotgun library construction and Sanger sequencing. Of 18,432 reads obtained, 12,491 reads were assembled into 30 major contigs with sequence similarity to known *Wolbachia* genomes, and gap filling yielded a 1,250,060-bp circular bacterial genome (Fig. 1). The genome of *w*Cle encoded 1,216 putative protein-coding ORFs with an average size of 771.6 bp, which covered 75% of the whole genome (Table S1). The genome size, GC content, coding capacity and density, and abundance of pseudogenes and insertion sequences of *w*Cle were similar to those of facultative insect-associated *Wolbachia* strains such as *w*Mel, *w*Ri, and *w*Pip (Table S1). The absence of remarkable genome degeneration in *w*Cle suggests that, unlike the ancient aphid–*Buchnera* and tsetse–*Wigglesworthia* nutritional mutualisms (3, 4), the bedbug–*Wolbachia* mutualism is of relatively recent evolutionary origin. Of the 1,216 protein-coding genes, 816 were assigned to putative biological functions, 321 matched hypothetical proteins of unknown function, and 79 were not assigned to any genes in the databases. Overall, cluster of orthologous groups category composition of the *w*Cle genome was similar to those of other insect-associated *Wolbachia* genomes (Table S2), indicating basically similar metabolic capacities among the different *Wolbachia* strains.

Identification of *w*Cle-Specific Synthetic Pathways for B Vitamins. However, inspection of synthetic pathways for B vitamins revealed a notable peculiarity of the *w*Cle genome. Although all known insect-associated *Wolbachia* genomes commonly possess a complete pathway for riboflavin (vitamin B2) and partial pathways for pyridoxine (vitamin B6) and folate (vitamin B9), the *w*Cle genome additionally contained a complete pathway for biotin (vitamin B7) and a partial pathway for thiamine (vitamin B1) (Fig. 2 *A* and *B*). The biotin synthesis genes *bioC*, *bioH*, *bioF*, *bioA*, *bioD*, and *bioB* formed a compact operon on the *w*Cle genome. Almost the same operon structure was identified on the genome of a facultative endosymbiont *Cardinium hertigii* (Bacteroidetes) causing cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella* (10), on the genome of a swine pathogen *Lawsonia intracellularis* (Deltaproteobacteria) (11), and also on the plasmid of a *Rickettsia* strain (Alphaproteobacteria) isolated from the tick *Ixodes scapularis* (12), but not on the genomes of *Wolbachia* and allied alphaproteobacteria (Fig. 3*A*). Molecular phylogenetic analyses of these biotin synthesis genes consistently exhibited similar evolutionary patterns: closely allied to the corresponding genes of *Cardinium*, *L. intracellularis*, and the *Rickettsia* plasmid and also related to the corresponding genes from diverse bacterial lineages representing Alphaproteobacteria, Deltaproteobacteria, Gammaproteobacteria, Cyanobacteria, Chlamydiae, and others (Fig. S1 *A–F*). These pat terns suggest that the biotin synthesis genes were acquired as a whole operon by an ancestor of *w*Cle from an unrelated bacterium, which was presumably a facultative endosymbiont (likely either *Cardinium* or *Rickettsia*) coinfecting the same insect host.

In addition, three genes involved in a thiamine salvage pathway, *tenA1*, *thiD*, and ψ*thiM* (wherein the last one is a pseudogene), formed a compact operon on the *w*Cle genome. Similar operon configuration was identified on the genome a fish francisellosis pathogen *Francisella noatunensis* (Gammaproteobacteria) (13), but not on the genomes of *Wolbachia* and allied alphaproteobacteria (Fig. 3*B*). Molecular phylogenetic analyses of these genes consistently exhibited similar evolutionary patterns: closely allied to the corresponding genes of *F. noatunensis*, *Brachyspira hyodysenteriae*, and *Legionella drancourtii* and also related to the corresponding genes from diverse bacterial lineages representing Gammaproteobacteria, Bacteroidetes, Spirochaetes, and others (Fig. S2 *A–C*). These patterns suggest that the thiamine synthesis genes *tenA1*, *thiD*, and ψ*thiM* were acquired as a partial operon by an ancestor of *w*Cle from an unrelated bacterium. Here it should be noted that, although *tenA1*

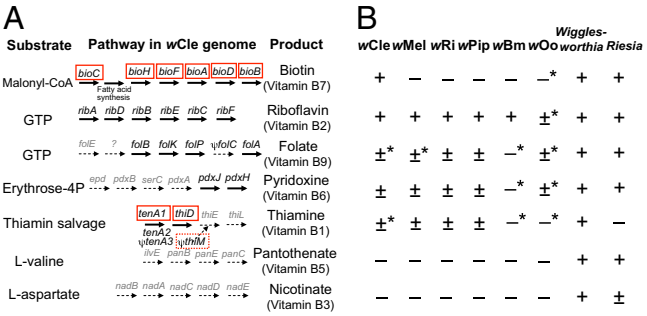

Fig. 2. Biosynthetic pathways for B vitamins in the *Wolbachia* genomes. (*A*) Biosynthetic pathways for B vitamins in the *w*Cle genome. Solid arrows indicate genes present in the *w*Cle genome, whereas dotted arrows show genes absent in the *w*Cle genome. Genes in gray color and smaller font are missing genes, whereas genes with prefix ψ are pseudogenes. Red rectangles highlight genes presumably acquired via lateral gene transfer from unrelated bacteria. (*B*) Presence/absence of biosynthetic pathways for B vitamins in *Wolbachia* strains *w*Cle of bedbug *C. lectularius*, *w*Mel of fruit fly *D. melanogaster*, *w*Ri of fruit fly *D. simulans*, *w*Pip of mosquito *C. quinquefasciatus*, *w*Bm of filarial nematode *B. malayi*, and *w*Oo of filarial nematode *O. ochengi*, and also obligate endosymbionts of blood-feeding insects *Wigglesworthia glossinidia* of tsetse fly *G. brevipalpis* and *Riesia pediculicola* of human louse *Pediculus humanus*. +, pathway is complete; ±, pathway is incomplete; –, pathway is absent; *, pathway contains pseudogene(s). For more details, see Table S3.

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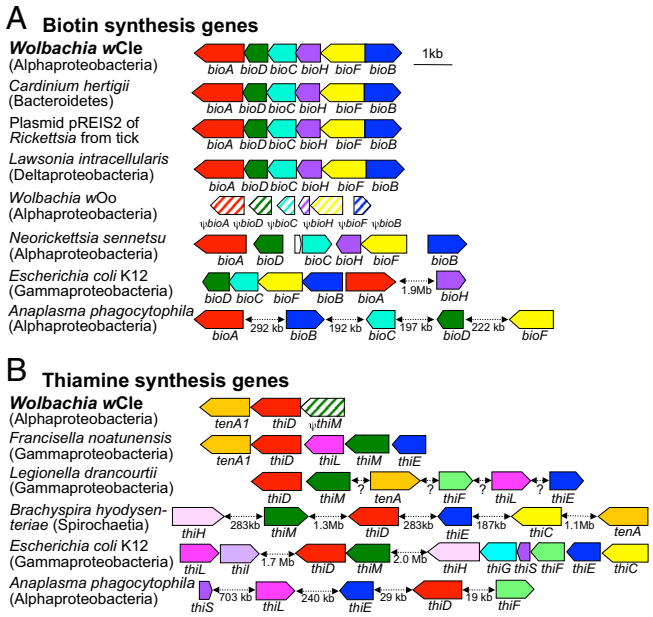

Fig. 3. Operon structure of vitamin B synthesis genes on the genomes of *w*Cle, allied alphaproteobacteria, and other bacteria. (*A*) Biotin synthesis genes. (*B*) Thiamine synthesis genes. Filled arrows and hatched arrows indicate intact genes and pseudogenes, respectively.

is specific to *w*Cle, its paralogs *tenA2* and *tenA3* are present in all of the insect-associated *Wolbachia* strains and likely represent *tenA* gene copies authentic to the *Wolbachia* lineage (Fig. S2*A*). It is also notable that ψ*tenA3* has been pseudogenized specifically in *w*Cle (Fig. 2*A*, Fig. S2*A*, and Table S3), highlighting complicated evolutionary trajectories of the *tenA* gene families among the *Wolbachia* strains.

From all these results taken together, it is conceivable, although speculative, that the synthetic pathways for biotin, thiamine, and other B vitamins have been moving across diverse bacterial lineages in a dynamic manner (14), presumably as evolutionarily cohesive functional modules or selfish operons, like antibiotic resistance genes and restriction modification genes, that can confer immediate functional advantage to the recipient organisms (15, 16).

***w*Cle Provisions Biotin and Riboflavin to Host Bedbug.** By rearing newborn nymphs of the *w*Cle-infected bedbug strain JESC on normal rabbit blood and rifampicin-supplemented rabbit blood, we generated symbiotic insects and symbiont-deficient insects of the same genetic background. By this antibiotic treatment, *w*Cle in these insects was killed, although remnant *Wolbachia* DNA tended to be detectable by PCR. These insects were able to grow to adults but suffered significantly impaired growth rate, body size, and fertility (9). When these insects were subjected to extraction and quantification of B vitamins at the fourth instar, the symbiont-deficient insects exhibited significantly lower titers of some B vitamins, including biotin and riboflavin, but not thiamine and pyridoxine, in comparison with the symbiotic insects (Fig. 4 *A*–*D*). These results indicate that *w*Cle is capable of provisioning biotin and riboflavin, but not thiamine and pyridoxine, to the host bedbug, which is concordant with the *w*Cle genome data (Fig. 2).

***w*Cle-Provisioned Biotin Significantly Contributes to Fitness of Host Bedbug.** Symbiont-mediated provisioning of riboflavin has been experimentally demonstrated or suggested in aphid–*Buchnera*, tsetse–*Wigglesworthia*, louse–*Riesia*, and other insect–bacterium symbiotic associations (17–19), and the complete synthetic pathway for riboflavin is consistently retained in these endosymbiont genomes (3, 4, 20) and also in most of the *Wolbachia* genomes determined to date (Fig. 2*B*). Hence, it is suggested that riboflavin provisioning by these symbionts, including *Wolbachia*, generally has biological roles in the symbiotic associations. Here, our focal interest is whether the *w*Cle-specific *Wolbachia*'s capability of biotin provisioning is biologically meaningful for the host bedbug. By rifampicin treatment, we established a *w*Cle-cured bedbug strain JESC^{-wCle} from the *w*Cle-infected bedbug strain JESC (9). By feeding with rabbit blood supplemented with all B vitamins, the *w*Cle-cured insects restored normal growth and reproduction (9), whereby we were able to maintain the *w*Cle-cured bedbug strain continuously. When biotin was selectively omitted from the B vitamin-supplemented blood meal, the *w*Cle-cured insects exhibited significantly reduced adult emergence rates in comparison with the *w*Cle-cured insects reared on the blood meal supplemented with all B vitamins (Fig. 4*E*), confirming that the biotin synthetic pathway of *w*Cle plays an important role for the host bedbug. By contrast, when thiamine was selectively omitted from the B vitamin-supplemented blood meal, no significant fitness decline was observed in the *w*Cle-cured insects (Fig. 4*F*), which probably reflects the incomplete synthetic pathway for thiamine in the *w*Cle genome (Fig. 2*A*) and consequent absence of the symbiont-derived thiamine supply (Fig. 4*D*).

Survey of Biotin Synthesis Genes of Diverse *Wolbachia* Strains. These results strongly suggest that the *w*Cle-specific biotin operon, which was presumably acquired via lateral gene transfer from a coinfecting endosymbiont, pivotally underpins the *Wolbachia*–bedbug nutritional mutualism. Here, our focal interest is the origin of the biotin synthesis genes in the *Wolbachia* evolution. We surveyed all complete and draft genomes of insect-associated

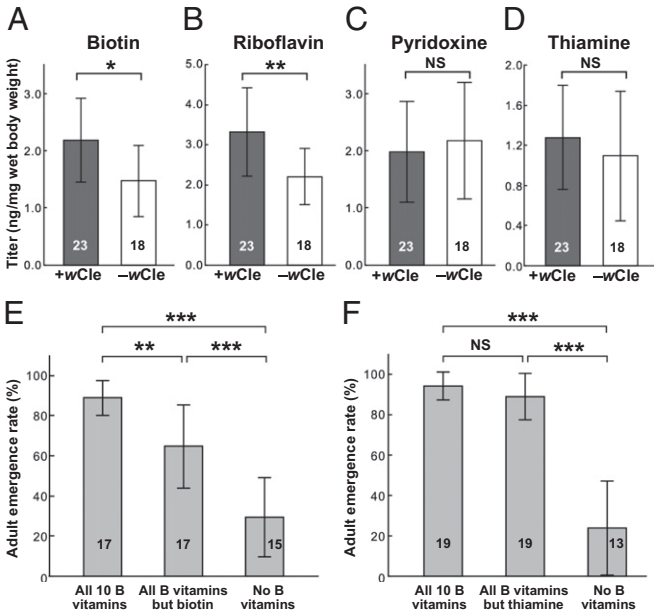

Fig. 4. *w*Cle-mediated provisioning of B vitamins to the host bedbug. Quantification of biotin (*A*), riboflavin (*B*), pyridoxine (*C*), and thiamine (*D*) in whole-body extract of *w*Cle-infected (+*w*Cle) and *w*Cle-cured (−*w*Cle) fourth instar nymphs. Effects of omission of biotin (*E*) and thiamine (*F*) from the B vitamin-supplemented rabbit blood meal on adult emergence rates of *w*Cle-cured insects. Means and SDs are shown with sample sizes. Asterisks indicate statistically significant differences (likelihood ratio test: $*P < 0.01$; $**P < 0.001$; $***P < 0.0001$; NS, no significant difference).

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Wolbachia strains available in the DNA databases (*SI Materials and Methods*), but no biotin synthesis genes were detected. Meanwhile, when we surveyed all genomic data of nematode-associated *Wolbachia* strains (*SI Materials and Methods*), a degenerate biotin operon, in which all biotin synthesis genes had been pseudogenized, was identified in the complete genome of *w*Oo from *Onchocerca ochengi* (HE660029) (21) and the draft *Wolbachia* genome from *O. volvulus* (ASM33837v1) (22). Although the biotin operon in the *w*Cle genome was 5.4 kb in size and encoding six intact genes, the biotin operon in the *w*Oo genome was 4.1 kb in size, wherein all six genes were disrupted by a number of stop codons, frame shifts, and deletions (Fig. 3*A* and Table S3).

Hypotheses on the Evolutionary Origin of Biotin Synthesis Genes in *Wolbachia*. Notably, the biotin synthesis pseudogenes ψ*bioC*, ψ*bioH*, ψ*bioF*, ψ*bioA*, ψ*bioD*, and ψ*bioB* on the *w*Oo genome (Fig. 3*A*) were closely allied to the corresponding genes on the *w*Cle genome (Fig. S3 *A–F*). Phylogenetic analysis based on 52 ribosomal protein sequences showed that *w*Cle and *w*Oo form a well-supported clade in the *Wolbachia* phylogeny (Fig. 5), although *w*Cle belonging to the *Wolbachia* F supergroup and *w*Oo representing the *Wolbachia* C supergroup diverged early in the *Wolbachia* diversification (23). These patterns are in favor of the hypothesis that the biotin operon was acquired by the common ancestor of *w*Cle and *w*Oo via lateral gene transfer, which has subsequently been retained in the *w*Cle lineage but disrupted in the *w*Oo lineage (Fig. S4*A*). On the other hand, the flanking regions of the biotin operon were not conserved between the *w*Cle genome and the *w*Oo genome (Fig. 6*A*), and the syntenic patterns between the *w*Cle genome and the *w*Oo genome did not favor the notion that the biotin operon of *w*Cle is located at the homologous genomic region of *w*Oo (Fig. 6*B*). Considering the wandering nature of the biotin operon in the bacterial evolution (Figs. S1 and S3), these patterns may favor the hypothesis that the lineage of *w*Cle and the lineage of *w*Oo acquired the biotin operon from the common bacterial source independently (Fig. S4*B*). The common bacterial source seems likely a coinfecting endosymbiont lineage on account of the frequent coinfection of *Wolbachia* with other endosymbionts including *Cardinium* and *Rickettsia* (24) and the presence of a closely related biotin operon in the *Cardinium* genome and the *Rickettsia* plasmid (10, 11).

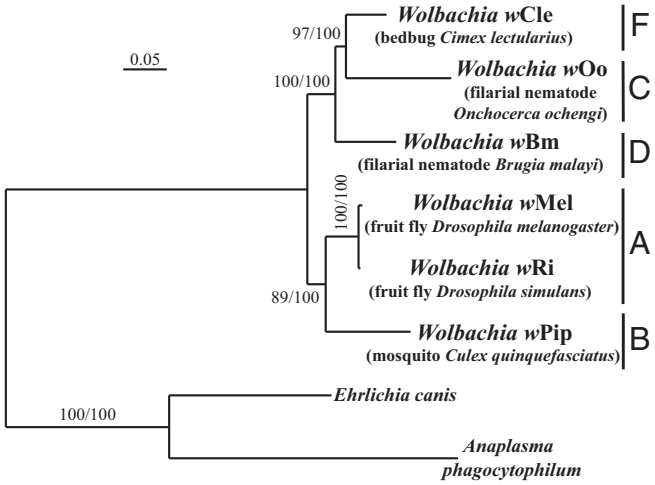

Fig. 5. Phylogenetic relationship of *Wolbachia* strains on the basis of 52 ribosomal protein sequences. Unambiguously aligned 7,007 amino acid sites are concatenated and subjected to the analysis. Bootstrap probabilities of maximum likelihood analysis and posterior probabilities of Bayesian analysis are indicated at the nodes.

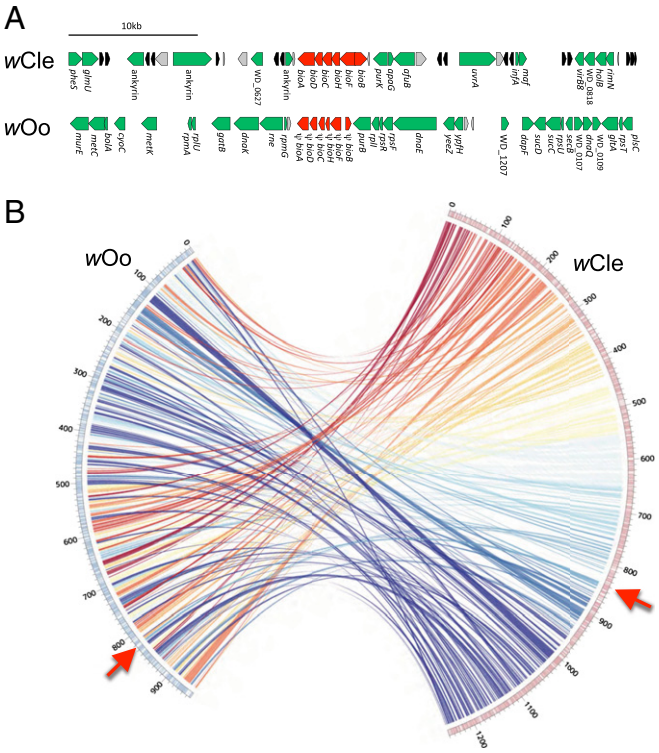

Fig. 6. Location of biotin operon on the genomes of *w*Cle and *w*Oo. (*A*) Structure of biotin operon and flanking regions on the genomes of *w*Cle and *w*Oo. Protein-coding sequences are shown in green, transposases are in black, and hypothetical genes are in gray. (*B*) Structural comparison between the genomes of *w*Cle and *w*Oo. Locations of biotin operon are highlighted by red arrows. Corresponding genes are connected between the *w*Cle genome and the *w*Oo genomes by lines, whose colors are arranged in gradient on the *w*Cle genome.

Meanwhile, it should be noted that the rampant intra- and intergenomic recombinations commonly observed in *Wolbachia* genomes (25) may account for the apparent divergence of the flanking regions. Fig. S4 *C* and *D* depicts, although seemingly less likely, representative alternative hypotheses on the evolutionary origin of the biotin operon in *Wolbachia*. To address these evolutionary issues conclusively, it is necessary to examine more *Wolbachia* genomes, in particular those representing the C, D, and F supergroups.

Contrasting Mutualistic *Wolbachia* Genomes Associated with Bedbug and Filarial Nematodes. Previous antibiotic curing experiments showed that the *Wolbachia* strains *w*Bm, *w*Oo, and *w*Cle are essential for their hosts *B. malayi*, *O. ochengi*, and *C. lectularius*, respectively (9, 26, 27). Although *w*Cle was shown to provide B vitamins to its bedbug host (Fig. 4) (9), it has been elusive what mechanisms underlie the essentiality of *w*Bm and *w*Oo for their nematode hosts (21, 28). Most of the synthetic pathways for B vitamins are either absent or eroded in the *w*Bm and *w*Oo genomes (Fig. 2*B* and Table S3), indicating that the major biological role of *w*Bm and *w*Oo cannot be provisioning of B vitamins. Although genome sizes of *w*Bm (1.08 Mb) and *w*Oo (0.96 Mb) are remarkably smaller than those of insect-associated facultative *Wolbachia* strains (1.27–1.48 Mb), such a genome reduction is not observed with the *w*Cle genome (1.25 Mb; Table S1). Taken together, the bedbug–*w*Cle mutualism seems to have evolved independently of the *B. malayi*–*w*Bm and *O. ochengi*–*w*Oo mutualisms.

Conserved Biotin Operon Inserted in the *Wolbachia* Genome Among Bedbug Populations and Allied Cimicid Species. In addition to the bedbug strain JESC mainly used in this study, three

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additional bedbug strains TUA (from Tokyo, Japan), TIH (from Toyama, Japan), and SYDL (from Sydney, Australia), and also an allied cimicid species, the Japanese bat bug *Cimex japonicus* (from Hokkaido, Japan), were subjected to cloning and sequencing of the insertion site of the biotin operon on the *Wolbachia* genome. The 7,724-bp region on the *w*Cle genome, which encodes six biotin synthesis genes franked by an ankyrin repeat gene and a *purK* gene, exhibited 100% (7,724/7,724) nucleotide sequence identity among the Japanese and Australian populations of *C. lectularius* (Fig. S5*A*). The biotin operon of the same structure was identified at the same location of the *Wolbachia* genome associated with *C. japonicus*, which was 7,721 bp in size and exhibited 98.1% (7,576/7,724 including indels) nucleotide sequence identity to that of *w*Cle (Fig. S5*B*). These results indicate that the biotin operon laterally transferred to the *Wolbachia* genome was already present in the common ancestor of *C. lectularius* and *C. japonicus* and has stably been maintained in natural populations of *C. lectularius*. In *C. japonicus*, notably, frame shift mutations were identified in *bioC* and *bioH* genes on the inserted biotin operon (Fig. S5*B*). The partial erosion of the biotin synthesis genes may be, although speculative, relevant to biological, ecological, and evolutionary differences between the bedbug and the bat bug, such as different nutritional compositions of human blood and bat blood and different facultative/gut microbiotae associated with the bedbug and the bat bug.

Placement of *w*Cle in the *Wolbachia* F Supergroup. To gain further insights into the evolution of the bedbug-*Wolbachia* mutualism, we analyzed the phylogenetic placement of *w*Cle within the *Wolbachia* F supergroup on the basis of 16S rRNA gene sequences available in the DNA databases. In the *Wolbachia* F clade, *w*Cle was the most closely related to the *Wolbachia* strain associated with the Japanese bat bug *C. japonicus* and also clustered with *Wolbachia* strains associated with mite, bat fly, louse fly, grasshopper, ant, and termites (Fig. S6). Some F *Wolbachia* strains are obligatorily associated with *Mansonella* spp. and other filarial nematodes (29–31). However, they were placed in distinct lineages from the arthropod-associated F *Wolbachia* strains including *w*Cle, although statistical supports for the groupings were not necessarily significant (Fig. S6). In the bat fly, the louse fly, the grasshopper, and the termites, the F *Wolbachia* infections are not fixed in the host populations, suggesting facultative nature of these host–symbiont associations (32–35). These observations suggest that (*i*) the bedbug–*w*Cle nutritional mutualism evolved independently of the nematode–*Wolbachia* mutualism within the *Wolbachia* F supergroup, (*ii*) the origin of the bedbug–*w*Cle mutualistic association is more recent than the origin of the nematode–*Wolbachia* mutualistic association, (*iii*) plausibly, an ancestor of the cimicid bugs acquired *w*Cle from an unrelated arthropod host, (*iv*) in the donor arthropod, *w*Cle was likely a facultative endosymbiotic associate, and (*v*) the establishment of the bedbug–*w*Cle association presumably entailed an evolutionary transition from facultative symbiosis to obligate nutritional mutualism for the symbiont side. For the host side, it is elusive whether the acquisition of *w*Cle entailed establishment of a novel nutritional mutualism or replacement of a preexisting nutritional symbiont. In other words, *w*Cle may have been acquired by the common ancestor of cimicid bugs or acquired later in a cimicid lineage via symbiont replacement. To address this evolutionary issue, a comprehensive endosymbiont survey is needed for the family Cimicidae, which embraces more than 22 genera and 74 species of blood-sucking bugs in the world (36).

Conclusion and Perspective. With all these results taken together, we strongly suggest that acquisition of a single gene cluster consisting of biotin synthesis genes underlies the evolution of the bedbug–*Wolbachia* nutritional mutualism. This finding provides an impressive case of evolutionary transition from facultative symbiosis to obligate mutualism that was facilitated by lateral gene transfer in an endosymbiont lineage. Here it should be noted that two lateral transfer events at different levels are involved in the evolution of the bedbug–*Wolbachia* nutritional mutualism: (*i*) acquisition of the biotin operon by an ancestor of *w*Cle via lateral gene transfer and (*ii*) acquisition of the biotin operon-bearing *Wolbachia* strain by an ancestor of the bedbug. Plausibly, the biotin synthetic capability of *w*Cle played no major role in the original nonbedbug host, but, once acquired by the bedbug, it conferred significant fitness advantage to the host that feeds solely on vertebrate blood deficient in biotin and other B vitamins. In this context, it is conceivable, although speculative, that the *w*Cle-allied facultative *Wolbachia* strains associated with the bat fly and the louse fly (Fig. S6) may have some auxiliary nutritional roles in the blood-sucking insect hosts.

In this study, we elucidated the genomic basis of the exceptional *Wolbachia*-mediated biotin provisioning in the bedbug, wherein lateral gene transfer underpins the peculiar metabolic capacity of *w*Cle. Notably, another peculiarity of *w*Cle resides in its cellular tropism. Although most of insect-associated facultative *Wolbachia* strains are sparsely distributed in various host cells and tissues, *w*Cle densely and specifically populates bacteriocytes of the host bedbug (9). Here, the evolutionary transition from facultative symbiosis to obligate mutualism might have occurred in parallel with the transition from systemic infection to bacteriocyte localization, which are consistently directing toward a higher level of host–symbiont integrity. Molecular, cellular, and genetic mechanisms underlying the *w*Cle-specific bacteriocyte localization are, although currently unknown, of evolutionary interest and deserving future studies.

Recent studies have revealed that lateral gene transfers sometimes entail evolutionary consequences to adaptive ecological and physiological traits in insects and other organisms (37–40). *Wolbachia* endosymbionts occur ubiquitously (7) and rampantly exchange their genetic materials with their cosymbionts and hosts (25, 41). Our results suggest that the biotin synthetic operon of *w*Cle was acquired from a facultative endosymbiont (likely *Cardinium* or *Rickettsia*) coinfecting the same insect host. It was recently reported that in the mealybug *Planococcus citri*, strikingly, the prokaryotic biotin synthesis genes *bioA*, *bioD*, and *bioB*, which are phylogenetically close to those found in *w*Cle, *Cardinium,* and *Rickettsia* (Figs. S1 *D–F* and S3 *D–F*), are encoded in the host nuclear genome, significantly expressed in the bacteriome, and presumably functioning in the endosymbiotic system (42). These findings highlight the evolutionary importance of endosymbiotic associations, wherein a host genome and multiple symbiont genomes are continuously integrated into a coherent system, as arenas for symbiont–symbiont and symbiont–host exchanges of genetic materials, which potentially lead to biological novelties and innovations including capability of synthesizing essential nutrients. In this context, the origin of *Wolbachia*–bedbug nutritional mutualism compiles an additional dimension to the dynamic evolutionary perspective of symbiosis.

Materials and Methods

Insect Rearing. The *w*Cle-infected bedbug strain JESC was maintained in plastic Petri dishes with a piece of filter paper at 25 °C under constant darkness. The insects were fed with purchased rabbit blood (Kohjin Bio) warmed at 37 °C once per week using an artificial membrane feeding system as previously described (9). For curing of *w*Cle, rifampicin was added to the blood meal at a concentration of 10 μg/mL. For rearing the *w*Cle-cured insects, B vitamins were supplemented to the blood meal as previously described (9).

Genome Analysis. For *w*Cle genome sequencing, we dissected 26 healthy young adult bedbugs, collected 51 bacteriomes, and extracted DNA from the symbiotic organs. The DNA sample (2 μg) was subjected to whole-genome

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shotgun sequencing and assembly as previously described (4). Gene prediction and annotation were performed as previously described (43).

Molecular Phylogenetic Analysis. Molecular phylogenetic analyses were conducted essentially as previously described (43). Multiple alignment was performed using MAFFT 5.6 (44). Maximum likelihood and Bayesian phylogenies were constructed using RAxML Version 7.7.6 (45) and MrBayes 3.1.2 (46).

Vitamin Analysis. Newborn nymphs of the *w*Cle-infected bedbug strain JESC were allocated to two experimental groups: one reared on normal rabbit blood and the other kept on the rabbit blood supplemented with 10 μg/mL rifampicin. Fourth-instar nymphs, which were collected 10 d after the last feeding at the third instar and weighed individually, were homogenized and hydrolyzed in 0.1 N HCl at 100 °C for 30 min. After adjusting to pH 4.5 with 2.5 M sodium acetate, Takadiastase (Sigma-Aldrich) was added to each sample and incubated at 37 °C for 16 h. After adding methanol for protein precipitation, the supernatant was lyophilized, suspended in 0.05% formic acid, 1.25 mM ammonium formate, and 50% methanol, and purified using a polymer-based column (GL-Tip SDB; GL Sciences). The purified sample was lyophilized and quantitatively analyzed using a high-performance LC system (Prominence; Shimadzu) coupled with a mass spectrometer (LCQ Duo; Thermo Fisher Scientific).

Fitness Measurement. First-instar nymphs of the *w*Cle-cured bedbug strain, whose parents had been maintained on rabbit blood supplemented with all B vitamins, were reared to adulthood on nonsupplemented rabbit blood to eliminate transgenerational carryover of B vitamins. These adult insects were allowed to mate and lay eggs, and nymphs from these eggs were randomly allocated to the following experimental groups: (*i*) all 10 B vitamins were added to the blood meal, (*ii*) all B vitamins except biotin or thiamine were added to the blood meal, and (*iii*) no B vitamins were added to the blood meal. These insects were fed once a week and monitored until all of the insects either became adult or died.

See *SI Materials and Methods* for complete details on the materials and methods.

ACKNOWLEDGMENTS. This study was supported by the Program for Promotion of Basic and Applied Researches for Innovations in Bio-Oriented Industry and by Grants-in-Aid for Scientific Research on Innovative Areas (Grants 22128001 and 22128007) from Japan Society of the Promotion of Science.

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