Riboflavin Provisioning Underlies *Wolbachia*'s Fitness Contribution to Its Insect Host

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ABSTRACT Endosymbiotic bacteria of the genus *Wolbachia* represent the most successful symbiotic bacteria in the terrestrial ecosystem. The success of *Wolbachia* has been ascribed to its remarkable phenotypic effects on host reproduction, such as cytoplasmic incompatibility, whereby maternally inherited bacteria can spread in their host populations at the expense of their host's fitness. Meanwhile, recent theoretical as well as empirical studies have unveiled that weak and/or conditional positive fitness effects may significantly facilitate invasion and spread of *Wolbachia* infections in host populations. Here, we report a previously unrecognized nutritional aspect, the provision of riboflavin (vitamin B_2), that potentially underpins the *Wolbachia*-mediated fitness benefit to insect hosts. A comparative genomic survey for synthetic capability of B vitamins revealed that only the synthesis pathway for riboflavin is highly conserved among diverse insect-associated *Wolbachia* strains, while the synthesis pathways for other B vitamins were either incomplete or absent. Molecular phylogenetic and genomic analyses of riboflavin synthesis genes from diverse *Wolbachia* strains revealed that, in general, their phylogenetic relationships are concordant with *Wolbachia*'s genomic phylogeny, suggesting that the riboflavin synthesis genes have been stably maintained in the course of *Wolbachia* evolution. In rearing experiments with bedbugs (*Cimex lectularius*) on blood meals in which B vitamin contents were manipulated, we demonstrated that *Wolbachia*'s riboflavin provisioning significantly contributes to growth, survival, and reproduction of the insect host. These results provide a physiological basis upon which *Wolbachia*-mediated positive fitness consequences are manifested and shed new light on the ecological and evolutionary relevance of *Wolbachia* infections.

IMPORTANCE Conventionally, *Wolbachia* has been regarded as a parasitic bacterial endosymbiont that manipulates the host insect's reproduction in a selfish manner, which tends to affect a host's fitness negatively. Meanwhile, some theories predict that, at the same time, *Wolbachia* can directly affect the host's fitness positively, which may potentially reconcile the negative effect and facilitate spread and stability of the symbiotic association. Here we demonstrate, by using comparative genomic and experimental approaches, that among synthetic pathways for B vitamins, the synthetic pathway for riboflavin (vitamin B_2) is exceptionally conserved among diverse insect-associated *Wolbachia* strains, and *Wolbachia*'s riboflavin provisioning certainly contributes to growth, survival, and reproduction in an insect. These findings uncover a nutritional mechanism of a *Wolbachia*-mediated fitness benefit, which provides empirical evidence highlighting a "Jekyll and Hyde" aspect of *Wolbachia* infection.

Endosymbiotic bacteria of the genus *Wolbachia* are widely found in diverse insects and other arthropods, and also in some filarial nematodes (1, 2). Over one-half of the world's insect species have been estimated to be associated with *Wolbachia* endosymbionts, which represent the most successful symbiotic bacteria in terrestrial ecosystems (3, 4). The success of *Wolbachia* has been understood in relation to its remarkable phenotypic effects on host reproduction, such as cytoplasmic incompatibility, parthenogenesis induction, feminization, and male killing. Since *Wolbachia* endosymbionts are vertically transmitted via ovarial passage in the host matriline, the reproductive manipulations are regarded as the symbiont's selfish strategies to increase its infection frequencies in its host populations, often at the expense of its host's fitness (1, 2).

In general, *Wolbachia* endosymbionts are not essential for their insect hosts (bar a few exceptions of parasitoid wasps and bedbugs [5, 6]), being commensal or parasitic with neutral or negative fitness consequences. Here, it should be noted that the fitness effects are partitioned into two major components: negative/neutral fitness effects due to the presence/absence of selfish reproductive phenotypes, and negative/neutral/positive fitness effects directly affecting the host's growth, survival, and fecundity. Hence,

TABLE 1 Presence of synthesis pathways for B vitamins encoded in 7 complete and 14 draft insect-associated *Wolbachia* genomes[a]

Status of synthesis pathway	No. of genome(s) (%) with pathway for:						
	Riboflavin (B$_2$)	Biotin (B$_7$)	Folate (B$_9$)	Pyridoxine (B$_6$)	Thiamine (B$_1$)	Pantothenate (B$_5$)	Nicotinate (B$_3$)
Complete	16 (76)	1 (5)	0	0	0	0	0
Incomplete	5 (24)	0 (0)	21 (100)	21 (100)	20 (95)	0	0
Absent	0 (0)	20 (95)	0 (0)	0 (0)	1 (5)	21 (100)	21 (100)

[a] For further details, see Table S1 in the supplemental material.

interestingly, *Wolbachia* infections may sometimes entail negative effects in the former component and positive effects in the latter component simultaneously; thus, these infections have been termed "Jekyll and Hyde" infections (7, 8). Theories predict that even weak and/or conditional positive fitness effects may facilitate invasion and spread of cytoplasmic incompatibility-inducing *Wolbachia* endosymbionts in their host populations (9–11). In the absence of such reproductive phenotypes, the weak and/or conditional positive fitness effects will constitute "stand-alone benefits" of the *Wolbachia* infections, which are likely to exhibit larger net benefits to the host fitness than the "Jekyll and Hyde" infections (8). In this context, although conventionally researchers have mainly paid attention to *Wolbachia*'s reproductive phenotypes, recent studies have highlighted the importance of slight but significant beneficial effects of *Wolbachia* infections found among diverse insects (8, 10, 12–16).

How *Wolbachia* infections affect host fitness positively has been poorly understood, although several mechanisms have been proposed with experimental supporting evidence. For example, in a variety of mosquitoes and fruit flies, *Wolbachia* infections suppress further infections of pathogenic viruses, bacteria, protists, and/or nematodes, thereby attenuating pathology and hindering pathogen transmission (17–20). In *Drosophila melanogaster*, *Wolbachia* infection confers a positive fecundity benefit under iron-restricted or iron-overloaded diet conditions, suggesting *Wolbachia*'s involvement in iron metabolism (21). In *Drosophila innubila*, *Wolbachia* infection boosts the fecundity of nutrient-deprived host insects, suggesting *Wolbachia*'s provisioning of an unknown nutritional factor(s) (16). In the parasitoid wasp *Asobara tabida*, *Wolbachia*-cured insects fail to develop normal ovaries and become sterile, suggesting *Wolbachia*'s involvement in oogenesis (5, 22). In the bedbug *Cimex lectularius*, *Wolbachia*-cured insects exhibit retarded growth and reduced fecundity on normal blood meal, but supplementation of biotin (vitamin B$_7$) to the blood meal restores these fitness defects, indicating that *Wolbachia*'s capability of biotin provisioning, which was acquired via lateral gene transfer from an unrelated bacterium, plays an essential role (6, 23). It should be noted, however, that whether these mechanisms actually operate in natural insect populations is still under debate (8).

Phylogenetically, diverse *Wolbachia* strains are classified into evolutionarily coherent lineages called "supergroups," which are markedly different in their host distribution and biology (24, 25). The above-mentioned *Wolbachia* strains associated with diverse insects mostly belong to either supergroup A or supergroup B (26, 27). In contrast, some *Wolbachia* strains associated with filarial nematodes are phylogenetically distinct from the insect-associated *Wolbachia* strains, and these constitute supergroup C (with *Onchocerca*, *Dirofilaria*, etc.) or su-

pergroup D (with *Brugia*, *Wuchereria*, *Litomosoides*, etc.) (28). These nematode-associated *Wolbachia* strains are not only phylogenetically but also biologically distinct from the insect-associated *Wolbachia* strains in the following respects: (i) smaller genomes versus larger genomes; (ii) host-symbiont cocladogenesis versus host-symbiont phylogenetic promiscuity; (iii) no reproductive manipulation versus a variety of reproductive manipulations; (iv) generally obligatory host-symbiont associations versus generally facultative host-symbiont associations (2, 29, 30). These patterns suggest that the insect-associated *Wolbachia* lineages and the nematode-associated *Wolbachia* lineages have experienced distinct evolutionary trajectories. Filarial nematodes cured of *Wolbachia* by antibiotic treatments tend to exhibit reduced motility, viability, and reproduction, indicating nematodes' dependence on *Wolbachia* infections, but mechanisms underlying the dependence are elusive (29–31). The supergroup F contains *Wolbachia* strains associated with both insects (termites, bedbugs, grasshoppers, etc.) and filarial nematodes (*Mansonella*, etc.) (23, 32). The supergroups E, G, and H are relatively small *Wolbachia* assemblages associated with springtails, spiders, and termites, respectively (33–35).

In this study, mainly focused on the insect-associated *Wolbachia* lineages, we highlight a nutritional mechanism of the *Wolbachia*-mediated fitness contribution, namely, provision of riboflavin (vitamin B$_2$), based on results of comparative genomic and experimental approaches.

RESULTS AND DISCUSSION

Comparison of B vitamin synthesis pathways among *Wolbachia* strains. Since the first genome sequencing of the *Wolbachia* strain *w*Mel of the fruit fly *D. melanogaster* (36), dozens of *Wolbachia* genomes from diverse insects and filarial nematodes have been determined. When we compared synthesis pathway genes for B vitamins encoded in 7 complete and 14 draft insect-associated *Wolbachia* genomes available in the databases, a striking pattern emerged. Only the synthesis pathway for riboflavin was conserved among the insect-associated *Wolbachia* genomes: there were 16 genomes with the complete pathway, 5 genomes with incomplete pathways, and no genome without the pathway (Table 1; see also Table S1 in the supplemental material). Here it should be noted that the apparently incomplete pathways in some of the draft genomes might be due to incompleteness of the genomes. On the other hand, the synthesis pathways for folate, pyridoxine, and thiamine were mostly incomplete, while the synthesis pathways for pantothenate, nicotinate, and biotin were mostly absent (Table 1; see also Table S1). For biotin, only 1 insect-associated *Wolbachia* genome retained the complete synthesis pathway: the *Wolbachia* strain *w*Cle, associated with the bedbug *C. lectularius*, wherein the biotin synthesis operon was acquired via lateral gene transfer for
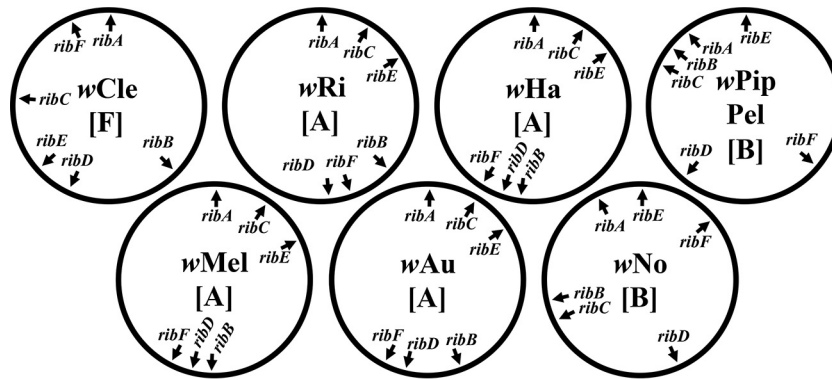
FIG 1 Distribution of riboflavin synthesis genes on the complete genomes of insect-associated *Wolbachia* strains *w*Cle, *w*Mel, *w*Ri, *w*Au, *w*Ha, *w*No and *w*Pip Pel, with the corresponding *Wolbachia* supergroup shown in brackets.

nutritional supplementation of the biotin-deficient blood meal for the host insect (23). In the complete genomes of nematode-associated *Wolbachia* strains, the synthesis pathway for riboflavin was complete in *w*Bm. and incomplete in *w*Oo (*ribD* lacking; *ribB*, *ribC*, and *ribF* degenerate), whereas synthesis pathways for folate, pyridoxine, and thiamine were either absent or degenerate (see Table S1). These patterns suggest that (i) riboflavin synthesis may play some biological roles in the diverse insect-associated *Wolbachia* strains in general, (ii) the partial synthesis pathways for folate, pyridoxine, and thiamine may also play some roles in the insect-associated *Wolbachia* strains, and (iii) pantothenate, nicotinate, and biotin are generally not provided by the *Wolbachia* strains.

Evolutionary conservation of riboflavin synthesis genes across *Wolbachia* strains. Hence, we focused on the riboflavin synthesis genes of the insect-associated *Wolbachia* strains. The riboflavin synthesis genes *ribA*, *ribB*, *ribC* *ribD*, *ribE*, and *ribF* were not found as a coherent operon but scattered on the *Wolbachia* genomes (Fig. 1). The distribution patterns were not conserved among the *Wolbachia* strains, which were plausibly driven by intra- and intergenomic recombinations in *Wolbachia* evolution (37–39), but in agreement with the phylogenetic relationship to some extent; for example, the arrangement of *ribA*, *ribC*, and *ribE* is shared between the *Wolbachia* genomes of supergroup A (Fig. 1). On the other hand, flanking regions of each riboflavin synthesis gene were highly conserved across the diverse *Wolbachia* strains: *ribA* flanked by *dnaA* on the 5′ side and by *virB8-11* and *virD4* on the 3′ side; *ribB* flanked by *greA*, *atpA*, and *atpH* on the 5′ side and by *fabG* and *aprE* on the 3′ side; *ribC* flanked by *cvpA* on the 3′ side; *ribD* flanked by *dgt*, *erpA*, and *lolD* on the 5′ side; *ribE* flanked by *yidC* and *secF* on the 5′ side and by *nusB* on the 3′ side; *ribF* flanked by *grxC* on the 5′ side and by *lspA* on the 3′ side (Fig. 2). These flanking genes were conserved not only among the insect-associated *Wolbachia* strains representing the supergroups A, B, and F but also among the nematode-associated *Wolbachia* strains representing the supergroups C and D; furthermore, we also found in the alphaproteobacterial outgroup taxa such as *Anaplasma* and *Ehrlichia* (Fig. 2). These observations suggest that the riboflavin synthesis genes were present in the common ancestor of the extant *Wolbachia* supergroups and have been stably maintained in the evolutionary course of the diverse insect-associated *Wolbachia* strains.

General congruence between phylogenies of riboflavin synthesis genes and *Wolbachia* genomic phylogeny. Given that the

riboflavin synthesis genes have been stably maintained during *Wolbachia* evolution, it is expected that phylogenies of the riboflavin synthesis genes should mirror the *Wolbachia* genomic phylogeny. Figure S1 in the supplemental material shows the phylogeny of the *Wolbachia* strains inferred from 52 ribosomal protein sequences, wherein the *Wolbachia* supergroups A, B, C, D, and F were identified as distinct clades or lineages with strong statistical support, but relationships within the supergroups A and B were resolved poorly. The phylogenies based on RibA, RibC, RibD, RibE, and RibF protein sequences exhibited essentially the same patterns: the supergroups A, B, C, D, and F were identified as distinct clades or lineages (see Fig. S2A and C to F in the supplemental material).

In contrast, the phylogeny based on RibB protein sequences exhibited a strange pattern: two large sister clades, one consisting of sequences from the supergroups A, B, C, D, and F (tentatively designated "presumable vertical transmission type") and the other containing sequences from supergroups A and B only (tentatively called "presumable lateral gene transfer type"), were identified. Within each of the sister clades, the supergroups A and B formed distinct clades. In the latter sister clade, notably, the supergroup A sequences exhibited few sequence variations, and this was also true for the supergroup B sequences (see Fig. S2B in the supplemental material). These patterns may reflect complex evolutionary trajectories of *ribB* during *Wolbachia* diversification. A possible hypothesis to account for these patterns is, although speculative and complicated, that the *ribB* gene of the "presumable vertical transmission type" was replaced by the *ribB* gene of the "presumable lateral gene transfer type" at least twice, once in supergroup A and once in supergroup B, via lateral gene transfer events. However, the origin of the transferred *ribB* genes is totally unknown. Phylogenetic analyses of *greA*, *atpA*, and *fabG*, which flank *ribB* in the *Wolbachia* genomes, exhibited the typical phylogenetic patterns reflecting the *Wolbachia* genomic phylogeny (see Fig. S3A to C in the supplemental material), making the situation even more puzzling.

In conclusion, overall, the conserved riboflavin synthesis pathway across the diverse *Wolbachia* strains (Fig. 2; see also Table S1 in the supplemental material) and the congruence between the phylogenies of the riboflavin synthesis genes (except for *ribB*) and the *Wolbachia* genomic phylogeny (see Fig. S1 and S2 in the supplemental material) suggest that the riboflavin synthesis pathway
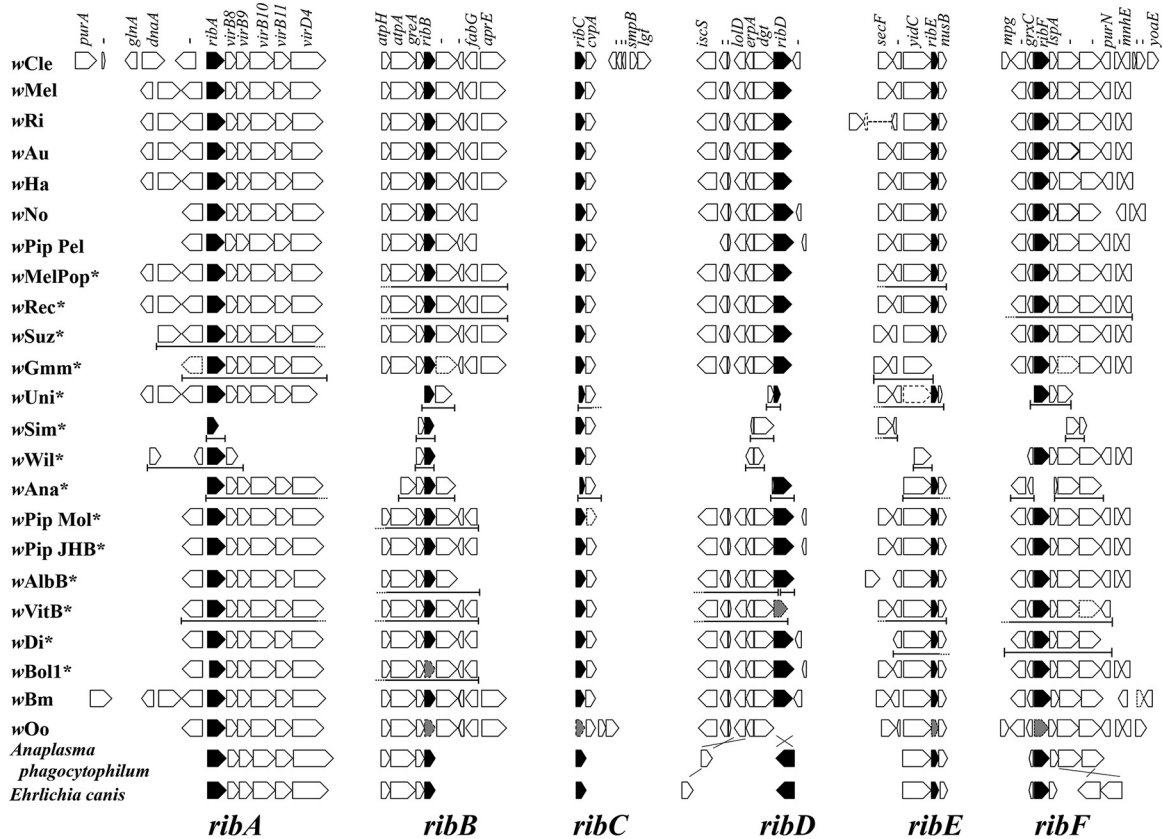
FIG 2 Genomic regions flanking riboflavin synthesis genes *ribA*, *ribB*, *ribC*, *ribD*, *ribE*, and *ribF* in the genomes of 21 insect-associated *Wolbachia* strains, two nematode-associated *Wolbachia* strains, and *Anaplasma phagocytophilum* and *Ehrlichia canis*. Filled and open pentagons depict riboflavin synthesis genes and their flanking genes, respectively, with a lateral pentagonal apex indicating the coding direction. Intact and pseudogenized riboflavin synthesis genes are shown in black and gray, respectively. Dashed pentagons are pseudogenes. Contigs mapped to partially sequenced genomic regions are indicated by horizontal lines under the regions. On the top, dashes indicate unannotated genes. In *A. phagocytophilum* and *E. canis*, crossed lines show inversions of the genes. Asterisks indicate draft *Wolbachia* genomes.

has been stably maintained in the evolutionary course of the diverse insect-associated *Wolbachia* strains.

Hypothesis testing using an artificial blood-feeding system for *Wolbachia*-cured bedbugs. The evolutionary conservation of the riboflavin synthesis pathway suggests the possibility that the riboflavin synthetic capability of the *Wolbachia* endosymbionts may be important for their own survival directly or indirectly via positively affecting their host's fitness. Here we hypothesized that these *Wolbachia* strains may confer positive fitness effects on their host insects, particularly under riboflavin-deficient diet conditions. Previous studies on the *Wolbachia* strain *w*Cle of the bedbug *C. lecturalius* demonstrated that, by using an artificial blood-feeding system and an antibiotic-mediated symbiont-curing technique, (i) *Wolbachia*-cured bedbugs fed with normal rabbit blood suffered drastically reduced fitness, (ii) the fitness defects were restored by supplementation of B vitamins to the blood meal, and therefore, (iii) *Wolbachia*'s provisioning of B vitamins is essential for normal growth, survival, and reproduction of the blood-feeding host insect (6, 23). As for the contribution of each B vitamin, the importance of biotin and the lack of involvement of thiamine were shown experimentally (23), whereas involvement of the other B vitamins has been elusive. In this study, by making use of *w*Cle-cured bedbugs reared on rabbit blood supplemented with various B vitamins, we tested the hypothesis that *Wolbachia*'s

riboflavin provisioning may affect the host fitness under riboflavin-deficient diet conditions.

Dietary omission of riboflavin significantly reduces fitness of *Wolbachia*-cured bedbugs. *w*Cle-cured bedbugs reared on rabbit blood supplemented with all B vitamins except for riboflavin exhibited a significantly lower survival rate, lower adult emergence rate, and lower fecundity than those reared on rabbit blood supplemented with all B vitamins (Fig. 3A to C), indicating that *w*Cle's riboflavin provisioning significantly contributes to growth, survival, and reproduction of the host bedbug. On the other hand, *w*Cle-cured bedbugs reared on rabbit blood supplemented with all B vitamins except for riboflavin exhibited a significantly higher survival rate and higher adult emergence rate than those reared on rabbit blood without supplementation of B vitamins (Fig. 3A and B), suggesting that not only riboflavin but also other B vitamins must be provisioned by *w*Cle to the host bedbug.

Dietary supplementation of riboflavin and biotin almost fulfills the B vitamin requirements of *Wolbachia*-cured bedbugs. In our previous study, it was demonstrated that *w*Cle provides the host bedbug with biotin but not thiamine (23), which prompted us to investigate the relative contributions of riboflavin, biotin, and thiamine to the fitness of the host bedbug. *w*Cle-cured bedbugs reared on rabbit blood supplemented with riboflavin and biotin exhibited similar levels of survival, adult emergence rate,
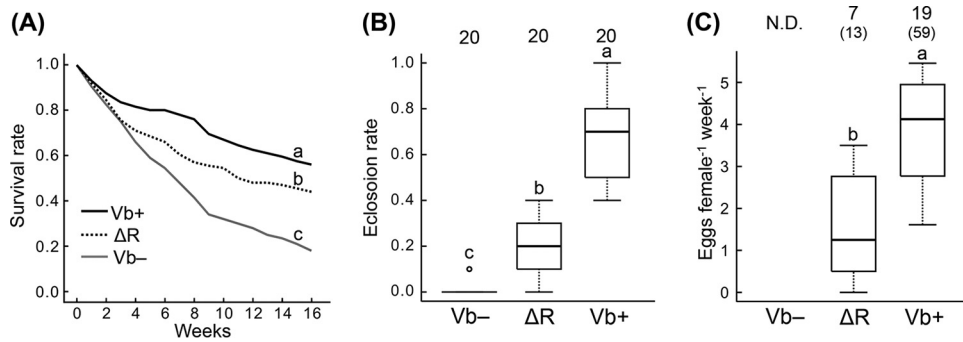
FIG 3 Fitness effects of riboflavin depletion on *Wolbachia*-cured bedbugs. (A) Effect on survival rate. *w*Cle-cured bedbugs were reared on rabbit blood supplemented with different B vitamin cocktails for 16 weeks. Each survival curve represents average results for 20 replicates, each consisting of 10 insects. (B) Effect on adult emergence rate. Numbers above the boxes indicate numbers of replicates. (C) Effect on egg production rate. Numbers above the bars indicate numbers of replicates (upper) and total numbers of females (lower, in parentheses). ND, no data for the Vb- group due to no emergence of ovipositing females; Vb+, reared on blood meal supplemented with all B vitamins; ΔR, reared on blood meal supplemented with all B vitamins except for riboflavin; Vb-, reared on blood meal without nutritional supplements (see Table S3 in the supplemental material for the composition of the supplements). Different lowercase alphabetical letters indicate statistically significant differences ($P < 0.05$; GLMM followed by *post hoc* multiple comparisons). The box-and-whisker plots indicate the median (bold line), the 25th and 75th percentiles (box edges), the range (whiskers), and outliers (open circles) that are 1.5 times higher or lower than the interquartile range from the box edge.

and fecundity as those reared on rabbit blood supplemented with all B vitamins (Fig. 4A to C), indicating that the two B vitamins, riboflavin and biotin, can largely account for the B vitamin requirement of nonsymbiotic bedbugs. *w*Cle-cured bedbugs reared on rabbit blood supplemented with riboflavin, biotin, and thiamine exhibited similar patterns, confirming the importance of riboflavin and biotin for the host bedbug, although fecundity was slightly but significantly reduced in comparison with that in those reared on rabbit blood supplemented with all B vitamins (Fig. 4A to C). These results are also in agreement with the fact that, among the synthesis pathways for B vitamins encoded in the *w*Cle genome, only the synthesis pathways for riboflavin and biotin are complete, while those for thiamine and other B vitamins are either incomplete or absent (23).

Evolutionarily conserved riboflavin provisioning and laterally acquired biotin provisioning by bedbug-associated *Wolbachia* strains. From our results, taken together, we conclude that in the bedbug *C. lectularius*, *Wolbachia* contributes to the host fitness, mainly via provisioning of riboflavin and biotin. Notably,

the riboflavin synthesis pathway and the biotin synthesis pathway in the *Wolbachia* genome have experienced distinct evolutionary trajectories: the riboflavin synthesis genes are of ancient origin and have been stably maintained across diverse *Wolbachia* strains, whereas the biotin synthesis genes were recently acquired via lateral gene transfer from an unrelated bacterium to the *Wolbachia* genome.

***Wolbachia*'s conserved capability of riboflavin provisioning and potential relevance to *Wolbachia*'s positive fitness effects.** The conservation of the riboflavin synthesis pathway among the diverse insect-associated *Wolbachia* strains may reflect the necessity of riboflavin for their own metabolism. However, the experimental validation of *Wolbachia*'s riboflavin provisioning suggests an alternative, but not mutually exclusive, possibility that riboflavin provisioning has been maintained in *Wolbachia* evolution based on its contribution to host fitness. The majority of insect-associated *Wolbachia* strains are facultative bacterial symbionts that are commensal or parasitic in nature, often causing cytoplasmic incompatibility or other selfish reproductive phenotypes at
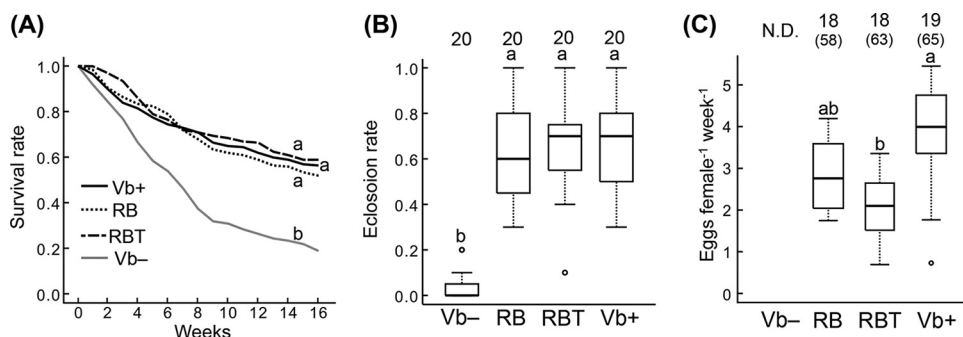

FIG 4 Fitness effects of riboflavin, biotin, and thiamine supplementation on *Wolbachia*-cured bedbugs. (A) Effect on survival rate. *w*Cle-cured bedbugs were reared on rabbit blood supplemented with different B vitamin cocktails for 16 weeks. Each survival curve represents average results for 20 replicates, each consisting of 10 insects. (B) Effect on adult emergence rate. Numbers above the bars indicate numbers of replicates. (C) Effect on egg production rate. Numbers above the bars indicate numbers of replicates (upper) and total number of females (lower, in parentheses). ND, no data for the Vb- group due to no emergence of ovipositing females; Vb+, reared on blood meal supplemented with all B vitamins; RB, reared on blood meal supplemented with riboflavin and biotin; RBT, reared on blood meal supplemented with riboflavin, biotin, and thiamine; Vb-, reared on blood meal without nutritional supplements (see Table S3 in the supplemental material for details on the compositions of the supplements). Different lowercase alphabetical letters indicate statistically significant differences ($P < 0.05$; GLMM followed with *post hoc* multiple comparisons). The box-and-whisker plots are shown as described for Fig. 3.

the expense of their host's fitness (1, 2). Relatively slight but significantly positive effects of *Wolbachia* infections on their host's growth, survival, and reproduction have been reported in a variety of insects, including mosquitoes (10, 13), fruit flies (14–16), parasitoid wasps (12), and many others (8). Here, we propose a hypothesis that some, if not all, of the beneficial *Wolbachia* infections have a nutritional basis wherein riboflavin provisioning may play a role. Nutritionally, some insect diets, such as plant sap, vertebrate blood, and seeds, are deficient in riboflavin and other B vitamins (40). In the following insects that live on such diets, obligate symbiotic bacteria or fungi are known to supply the deficient nutrients: blood-sucking tsetse flies (41, 42), lice (43, 44), and bedbugs (6, 23); plant sap-sucking aphids (45, 46); seed-sucking bugs (47); grain/flour-feeding anobiid beetles (48); and weevils (49). In other insects that live on such diets without obligate symbionts, like mosquitoes, flour beetles, etc., *Wolbachia*-derived riboflavin would confer substantial positive effects on host fitness. Even in other insects whose diets are not deficient in B vitamins, although this is speculative, the *Wolbachia*-derived nutrients might provide beneficial fitness consequences under starvation, hibernation, or other stressful conditions. Using insects other than bedbugs, the hypothesis of *Wolbachia*-mediated riboflavin provisioning should be verified experimentally. Note that the bedbug-associated *w*Cle is an atypical *Wolbachia* strain that is localized to the host bacteriomes at high densities as an obligate nutritional mutualist (6, 23), whereas the majority of *Wolbachia* strains are facultative bacterial associates that exhibit systemic infection throughout the host insect body at relatively low densities (50).

Conclusion and perspective. In conclusion, comparative genomics revealed that the riboflavin synthesis pathway is highly conserved among diverse insect-associated *Wolbachia* strains, and nutritional experiments using a specific insect system confirmed that *Wolbachia*-provisioned riboflavin certainly contributes to host fitness. Our finding provides a physiological basis upon which *Wolbachia*-mediated positive fitness consequences are manifested and sheds new light on the ecological and evolutionary relevance of context-dependent fitness effects of microbial associates. Future studies should focus on, in particular, experimental verification of *Wolbachia*-mediated riboflavin provisioning in other insect-*Wolbachia* symbiotic systems. We point out that, to this end, mosquitoes and flour beetles would provide promising model systems. The Asian tiger mosquito *Aedes albopictus*, which is associated with cytoplasmic incompatibility-inducing *Wolbachia* strains, can be reared on B vitamin-deficient blood meal, and their survival and fecundity were reported to be positively affected by *Wolbachia* infections (10, 13). Therefore, fitness effects of B vitamin-supplemented blood meals can be readily evaluated using *Wolbachia*-infected and uninfected mosquitoes. A recent study reported that depletion of host cell riboflavin reduces *Wolbachia* infection levels in cultured mosquito cells (51), and this may provide an alternative experimental system. The flour beetle *Tribolium confusum* infected with a cytoplasmic incompatibility-inducing *Wolbachia* strain is easily maintainable on B vitamin-deficient flour meal (52, 53). Traditionally, the flour beetle was a model system for insect nutritional physiology, and its requirement for B vitamins has been investigated in detail (54, 55). On the basis of this background, fitness evaluation of *Wolbachia*-infected and uninfected beetles can be conveniently performed using B vitamin-supplemented flour meals. Why not synthesis pathways

for other B vitamins but only the synthesis pathway of riboflavin is conserved among the diverse insect-associated *Wolbachia* strains is an intriguing issue whose physiological and evolutionary implications deserve future studies. Considering that synthesis pathways for riboflavin and other B vitamins are often retained in many genomes of facultative bacterial symbionts and also in some genomes of obligate bacterial symbionts (see Table S2 in the supplemental material), the possibility should be taken into account that symbiont-mediated provisioning of B vitamins may play important roles not only in blood-sucking, seed-sucking, and grain-eating insects for nutritional supplementation, but also in diverse insects associated with facultative bacterial symbionts for maintaining their infections in natural host populations (2, 8, 56–58).

MATERIALS AND METHODS

Insect materials. We used a laboratory strain of the bedbug *C. lectularius* that had been maintained at the Japan Environmental Sanitation Center, Kanagawa, Japan. This bedbug strain is associated with the *Wolbachia* strain *w*Cle but is free of the facultative gammaproteobacterial symbiont found in some bedbug populations (6). The insects were reared in plastic petri dishes (90 mm in diameter, 15 mm high) containing several pieces of filter paper (ca. 15 mm by 30 mm) at 25°C under constant darkness. Commercially purchased rabbit blood (Kohjin Bio, Saitama, Japan) was fed to the insects once a week using an artificial feeding system consisting of a petri dish and parafilm membranes placed on a heater at 37°C as described previously (6).

Genomic and molecular phylogenetic analyses. To compare synthesis pathway genes for B vitamins, tBLASTn searches (59) against a custom database consisting of genome sequences of symbiotic bacteria from the GenBank database (accession numbers are listed in Tables S1 and S2 in the supplemental material) were conducted using the following proteins of *Escherichia coli* or the *Wolbachia* strain *w*Cle of the bedbug *C. lectularius* as query sequences: RibA, RibB, RibC, RibD, RibE, and RibF for riboflavin; BioA, BioB, BioC, BioD, BioF, and BioH for biotin; FolA, FolB, FolC, FolE, FolK, and FolP for folate; SerC, PdxA, PdxB, PdxH, and PdxJ for pyridoxine; ThiC, ThiD, ThiE, ThiF, ThiG, ThiH, ThiI, ThiL, ThiM, and TenA for thiamine; PanB, PanC, and PanE for pantothenate; NadA, NadB, NadC, NadD, and NadE for nicotinate. For molecular phylogenetic analyses, protein-coding gene sequences were analyzed after conversion to amino acid sequences. Pseudogenes were translated into conceptual amino acid sequences by comparison with closely related gene sequences and applying frameshifts, by which sites containing either a stop codon or frameshift were removed. Multiple alignments were generated by using MAFFT 5.6 (60); gap-containing sites and unambiguously aligned sites were excluded, whereas sites with missing data due to insufficient sequencing coverage were included and treated as missing. The substitution models for protein sequences were selected using ProtTest 3 (61). Maximum likelihood phylogenies and Bayesian phylogenies were constructed using RAxML version 8.2.0 (62) and MrBayes 3.1.2 (63), respectively. Bootstrap probabilities were generated by 1,000 resamplings for maximum likelihood phylogenies, whereas posterior probabilities were calculated for Bayesian phylogenies. The following 52 ribosomal proteins were subjected to phylogenetic analysis of the *Wolbachia* genomes: RplA, RplB, RplC, RplD, RplE, RplF, RplI, RplJ, RplK, RplL, RplM, RplN, RplO, RplP, RplQ, RplR, RplS, RplT, RplU, RplV, RplW, RplX, RplY, RpmA, RpmB, RpmC, RpmE, RpmF, RpmG, RpmH, RpmI, RpmJ, RpsB, RpsC, RpsD, RpsE, RpsF, RpsG, RpsH, RpsI, RpsJ, RpsK, RpsL, RpsM, RpsN, RpsO, RpsP, RpsQ, RpsR, RpsS, RpsT, and RpsU.

Nutritional experiments. Experimental procedures for evaluating fitness effects of vitamin supplementation were essentially as described elsewhere (23). A *Wolbachia*-cured bedbug strain was established by rifampin administration in blood meal at a final concentration of 10 μg/ml and was subsequently maintained on blood meal supplemented with all B vitamins and vitamin-like nutrients (see Table S3, Vb+ data, in the supplemental

material) (64). Prior to nutritional experiments, offspring of the *Wolbachia*-cured bedbugs were transferred to and reared on nonsupplemented blood meal (see Vb- data in Table S3) for a generation, by which effects of maternal carryover of B vitamins were eliminated. After the first feeding of nonsupplemented blood meal, newborn nymphs were randomly allocated to either of the five treatment groups: Vb-, fed nonsupplemented blood meal; Vb+, fed blood meal supplemented with all B vitamins and vitamin-like nutrients; ΔR, fed blood meal supplemented with all B vitamins and vitamin-like nutrients except for riboflavin; RB, fed blood meal supplemented with riboflavin and biotin; RBT, fed blood meal supplemented with riboflavin, biotin, and thiamine (see Table S3). During the experiments, 10 nymphs were kept in each plastic petri dish (53 mm in diameter, 12 mm high) with a piece of filter paper and fed once a week. Each petri dish population was monitored for survival rate, adult emergence rate, and number of eggs per female for 16 weeks.

Statistics. Fitness parameters were statistically compared among treatment groups under a generalized linear mixed model (GLMM) framework, in which individual random effects were taken into account to avoid overdispersion. Survival rates and adult emergence rates were fitted to a model that assumed a binomial error structure with a logit link function. For survival rates, pseudoreplication from the longitudinal data was removed by incorporating time effects as a random intercept (65). To evaluate oviposition frequency, the total number of eggs was offset with the accumulative female number in a model with a Poisson error structure and a log link function. We set the preoviposition period to 2 weeks, which was excluded from the calculation. When a chi-square test of deviance reduction exhibited a significant treatment effect, a Tukey-type multiple comparison was performed between each treatment group. All statistic analyses were performed using R version 3.1.2 (66).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01732-15/-/DCSupplemental.

Figure S1, PDF file, 0.05 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.1 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.
Table S3, PDF file, 0.05 MB.

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