AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

MINIREVIEW

The Jekyll and Hyde Symbiont: Could *Wolbachia* Be a Nutritional Mutualist?

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ABSTRACT The most common intracellular symbiont on the planet—*Wolbachia pipientis*—is infamous largely for the reproductive manipulations induced in its host. However, more recent evidence suggests that this bacterium may also serve as a nutritional mutualist in certain host backgrounds and for certain metabolites. We performed a large-scale analysis of conserved gene content across all sequenced *Wolbachia* genomes to infer potential nutrients made by these symbionts. We review and critically evaluate the prior research supporting a beneficial role for *Wolbachia* and suggest future experiments to test hypotheses of metabolic provisioning.

KEYWORDS Insects, metabolites, mutualism, symbiosis

Wolbachia is a ubiquitous bacterial symbiont of nematodes, insects, and other arthropods. It is infamous for its prevalence across insects, where 40 to 60% of species harbor the infection (1, 2). One way that *Wolbachia* can spread through insect populations is by reproductive manipulations, which include sperm-egg incompatibility (so-called cytoplasmic incompatibility [CI]), parthenogenesis induction, male killing, and feminization (3). However, reproductive manipulation alone cannot explain *Wolbachia's* prevalence and frequency within insects (4). For example, *Wolbachia* bacteria that do not cause any reproductive manipulations, such as strain *w*Au, can spread quite efficiently (5), suggesting that *Wolbachia* provides some other benefit to its hosts that allows them to increase in frequency. Additionally, *Wolbachia* infections entering new populations will find it quite difficult to establish themselves using reproductive manipulations alone; assuming a net fitness cost to the host, the *Wolbachia* infection frequency in a population must be above some threshold for reproductive manipulations to be effective at maintaining the infection (6–8). This begs the question: how is *Wolbachia* maintained without reproductive manipulation?

One way that *Wolbachia* may be maintained in insect populations is by providing mutualistic benefits. Although *Wolbachia* is occasionally horizontally transmitted (9) or introgressed into new host backgrounds (10), it is primarily maternally transmitted (3, 11). Therefore, *Wolbachia*'s fitness is dependent on that of the host. Any mutualistic benefit *Wolbachia* can provide to the host, assuming that the immediate cost to itself is not too great, would increase its own fitness (12). Indeed, many maternally transmitted bacterial symbionts provide nutrients to their insect hosts, and by examining the genomic content of a symbiont, one can hypothesize as to potential nutrients supplied (13). But could *Wolbachia* be both Jekyll and Hyde (14)? Here, we review what is known and unknown about *Wolbachia* with regard to nutritional mutualism. Based on a large-scale genomic comparison (natural experiments of gene gain and loss), we suggest methods to directly test hypotheses of nutrient supplementation by *Wolbachia*.

WOLBACHIA IS NOT MONOLITHIC: STRAIN AND HOST DIVERSITY

Wolbachia strains infect a diversity of hosts, from spiders to isopods and from nematodes to insects (1, 2). We have the most genomic sampling from filarial nematode

Citation Newton ILG, Rice DW. 2020. The Jekyll and Hyde symbiont: could *Wolbachia* be a nutritional mutualist? J Bacteriol 202:e00589-19. https://doi.org/10.1128/JB.00589-19.

Editor William Margolin, McGovern Medical School

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Accepted manuscript posted online 28 October 2019
Published 29 January 2020

and insect-infecting strains (clades A, B, C, and D). In addition, *Wolbachia*-induced effects have been best explored in these two broad host types. The symbionts, however, will differ in regard to the phenotype induced in the host and also their tissue localization. For example, in leaf-cutting ants, *Wolbachia* is found in the foregut, both intra- and extracellularly, and therefore seems well poised to provide nutritional supplementation (15). Importantly, to understand the data on *Wolbachia* nutritional mutualisms, we must highlight that *Wolbachia* strains infecting the filarial nematodes are quite different from the insect-associated strains. Filarial nematode-infecting *Wolbachia* strains (clades C and D) have smaller genomes than their arthropod-associated counterparts (clades A and B) (16–18), and their genomes harbor fewer predicted type IV secretion system effectors (16, 19). These facts, coupled to prior observations that nematode-associated *Wolbachia* strains exhibit a pattern of cocladogenesis with their hosts (20, 21) and that clearing of the symbiont is detrimental to the nematode (22), have led researchers to suggest that the C and D clade *Wolbachia* may be mutualistic (18). *Wolbachia* is believed to supplement a large array of nutrients in the nematodes, ranging from heme to riboflavin/flavin adenine dinucleotide (FAD) (16, 23), although few have been experimentally tested. Therefore, because their hosts and symbiotic contexts are so different, and because the *Wolbachia* clades have been diverging for millions of years (20, 24), there are likely significant differences in the mechanisms by which the different clades of symbionts interact with their hosts. Evidence taken from experiments based on one specific host and symbiont may not apply to other *Wolbachia* strains.

MAKING A CASE FOR NUTRITIONAL MUTUALISM

When the genome of the *Wolbachia pipientis* strain *w*Mel was first sequenced, it was hypothesized that *Wolbachia* strains are auxotrophs, consuming host amino acids, as they cannot synthesize their own (25). More recently, comparative genomics and metabolic modeling support the idea of *Wolbachia* strains as metabolic parasites (26). However, although the microbes may acquire amino acids from the host, many new studies have suggested that *Wolbachia* strains are nutritional mutualists (a comprehensive review of older literature can be found in reference 12). The use of comparative genomics to infer *Wolbachia's* nutritional contributions to the host are fraught with caveats (Fig. 1). If a *Wolbachia* genome is truly missing the genes which encode enzymes in a specific pathway, then that *Wolbachia* strain probably does not make that product. However, the absence of a gene is often not confirmed, as *Wolbachia* assemblies are often incomplete and highly fragmented due to repetitive regions in the genome. On top of that, if a *Wolbachia* genome includes the genes for enzymes to synthesize a specific product, this does not necessarily imply that it shares that product with the host, and even if *Wolbachia* could share the product, the host may not need it. Indeed, all *Wolbachia* genomes encode the ability to convert fructose to phosphoenolpyruvate via the glycolytic pathway, but this pathway is largely redundant with the host metabolic capabilities and perhaps is not useful under most ecological contexts. So, in order for *Wolbachia*'s nutritional supplementation to be selected for, (i) *Wolbachia* has to be capable of making the nutrient, (ii) must supply it to the host somehow (either *Wolbachia* is consumed intracellularly, the nutrient leaks through the membrane, or there is a specialized transporter), and (iii) the nutrient should be relevant to host fitness (Fig. 1). Most of the evidence in support of *Wolbachia* as a nutritional mutualist has come from genomic studies, and in few cases have authors cleared hosts of *Wolbachia* and supplemented them with nutrients assumed to be provisioned.

In the literature, several metabolic pathways in the host have been speculated to be supplemented or modulated by *Wolbachia* (15, 23, 27–32), from B vitamins to nucleotides. Comparative genomics is a first step to identifying metabolic capabilities across *Wolbachia* strains that could be involved in nutritional mutualism. Additionally, identifying *Wolbachia* strains that differ in their abilities to make specific nutrients leads to straightforward laboratory assays to confirm the nutritional supplementation suggested by genomic content. Below, we analyze the 36 publicly available *Wolbachia*

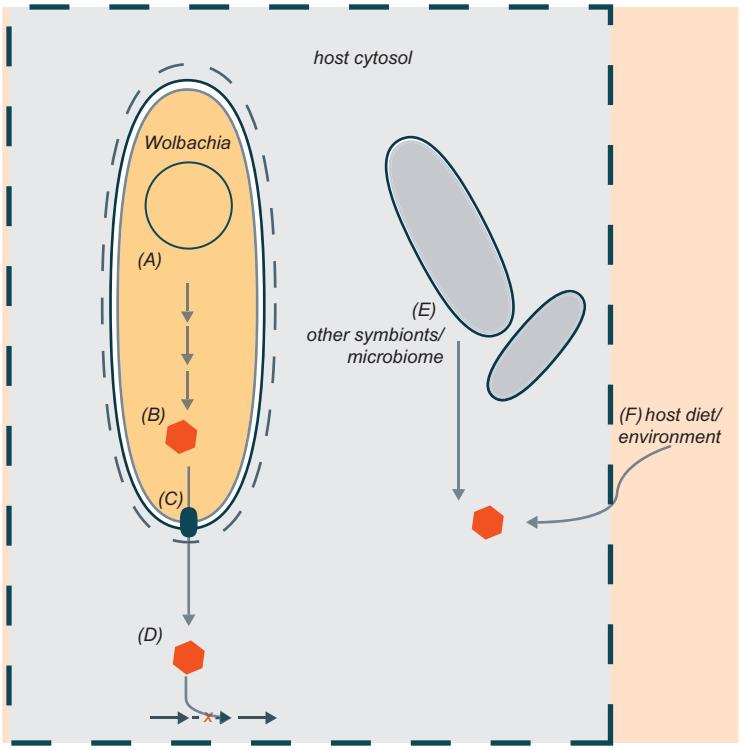

FIG 1 Schematic representation of *Wolbachia* supplementation of host nutrients. *Wolbachia* must (A) encode the enzymes for synthesis of said nutrient in its genome, (B) express the enzymes and generate the nutrient, and (C) export the nutrient somehow to the host, where it (D) complements a missing pathway important for host physiology. Ecological context impinges upon any nutrient supplementation if (E) other symbionts and the microbiome can supply the nutrient or (F) the host can acquire it from the surrounding environment or its diet.

genomes spanning clades A, B, C, D, E, F, and L for the presence/absence of genes encoding enzymes involved in the production of these nutrients (Fig. 2). We discuss previous experiments performed to support the provisioning of specific nutrients and suggest future areas of research to test *Wolbachia's* role as a "Jekyll and Hyde" symbiont.

EVIDENCE FOR WOLBACHIA AS A NUTRITIONAL MUTUALIST

B vitamins. The most obvious case where we find clear evidence for *Wolbachia*'s role as a nutritional mutualist in insects is in the bedbug, where *Wolbachia pipientis* strain *w*Cle, naturally infecting the bedbug *Cimex lectularius*, provisions B vitamins to its host. In this system, we see clear cocladogenesis between the bedbugs and the *Wolbachia* infecting them (33). If cleared of the infection with antibiotics, bedbugs survive only if supplemented with B vitamins (34). It was subsequently suggested that biotin (B_7) might be the vitamin in question, as genomic analyses found a biotin operon in the genome of strain *w*Cle (35). Importantly, *Wolbachia* in the bedbug is found in bacteriocytes (34), specialized cells that house nutritional symbionts in various insects. *Wolbachia* is also bacteriocyte associated in the termite, infected by the related strain *w*Ctub; this localization suggests that these specific symbionts may be nutritional mutualists. Interestingly, the *bioA* gene was amplified from the *w*Ctub symbionts, suggesting that they may also harbor the biotin biosynthesis operon (30). This operon was unique to the F clade symbionts until the *Wolbachia* strains that infect *Nomada* bees (*w*Nfla/*w*Nleu) were sequenced (28). The biotin operon in the *Nomada* bee *Wolbachia* is flanked by insertion sequence (IS) elements, suggesting that it may have been horizontally acquired. So far, the biotin biosynthesis loci (*bioABCDFH*) have been identified only in these two disparate clades of *Wolbachia*, the F and A clades (Fig. 2).

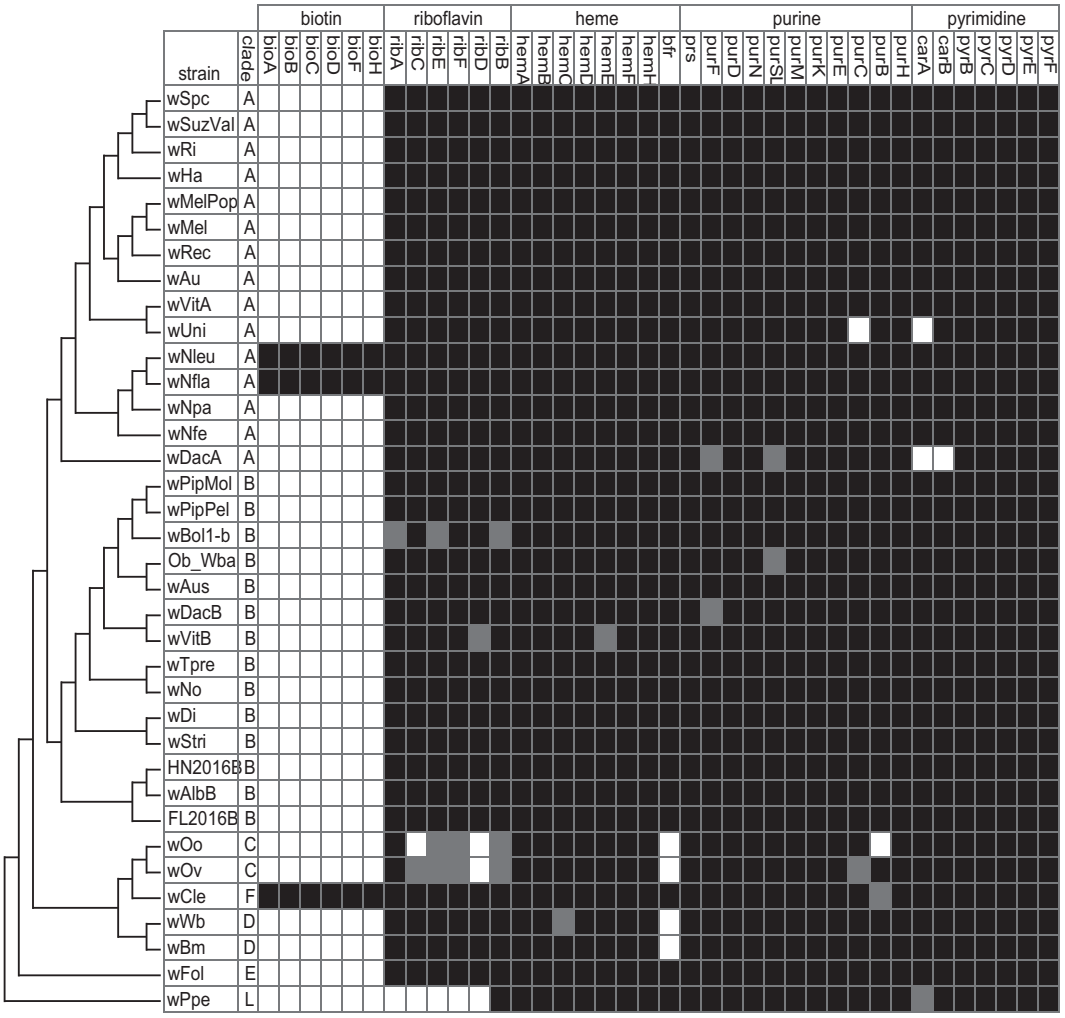

FIG 2 The presence of enzyme-encoding loci involved in biotin, riboflavin, heme, purine, and pyrimidine biosynthesis is shown across 36 publicly available *Wolbachia* genomes. Existing functional annotation data (NCBI's Prokaryotic Genome Annotation Pipeline [PGAP]) were used to identify enzymes across draft genomes and orthologs defined by complete linkage clustering of reciprocal blast hits. Black, present in the assembly; gray, present but pseudogenized (as defined by NCBI); white, absent from the assembly.

Based on the genomics of conserved B-vitamin pathways in *Wolbachia* genomes, riboflavin (vitamin B_2) has also been suggested as something the symbiont could contribute. The enzymes encoding the synthesis of riboflavin are conserved across many *Wolbachia* genomes (32) (although, interestingly, not in the clade C or L symbionts) (Fig. 2) and experiments in the bedbug system suggest that this nutrient also benefits the host (32). One might assume, then, that if the host were unable to take up riboflavin from its environment, it could rely on *Wolbachia* to generate that vitamin. In contrast, when mosquito cells harboring *Wolbachia* strain *w*Stri (from the leafhopper *Laodelphax striatellus*) are inhibited from taking up riboflavin in their media, *Wolbachia* loads are reduced intracellularly (27); this is perhaps not what you would expect if the symbiont is specifically provisioning riboflavin. It could be that the host consumes *Wolbachia* intracellularly and therefore avails itself of all *Wolbachia*-derived nutrients, leading to the loss of the infection. However, the experiments are confounded by the fact that *w*Stri is not the native *Wolbachia* strain for *Aedes albopictus*. Further research is needed to understand the mechanism by which host and *Wolbachia* B vitamin metabolism are related, the conditions under which this vitamin is provisioned, and how the vitamin is provided to the host.

Heme and iron homeostasis. Does *Wolbachia* provide the host with heme or modulate host iron metabolism (29)? Cells use iron for a variety of purposes, including energy conservation, oxygen transport, and production of heme. Heme itself is an important cofactor in many metalloproteins, the most famous of which is hemoglobin, which coordinates the transfer of oxygen throughout the bloodstream (36, 37). Heme is also found in a variety of other metalloproteins, where it is involved in electron transfer and catalysis. The loci encoding the enzymatic components of this pathway are *hemABCDEFH* and are found complete across all sequenced *Wolbachia* genomes; therefore, all sequenced *Wolbachia* strains seem metabolically capable of synthesizing heme (Fig. 2). Importantly, since many sequenced nematodes do not encode enzymes to synthesize heme and other vitamins (16, 38–40), the suggestion that *Wolbachia* might provide them may be true. In contrast, *Caenorhabditis elegans* does not encode heme biosynthesis and is thought to acquire it from its environment; therefore, just because the animal cannot make a nutrient does not necessarily mean that it requires a symbiont to provide it (Fig. 1).

Evidence for *Wolbachia*'s supplementation of host heme in filarial nematodes comes by way of both comparative genomics and chemical inhibition (23, 41). Because the filarial nematode genome of *Brugia malayi* does not encode enzymes for heme biosynthesis and the associated *Wolbachia* strain *w*Bm does, it is hypothesized that *Brugia* might get heme from *Wolbachia* (23). In a subsequent study, the authors identified a chemical inhibitor of the *w*Bm δ-aminolevulinic acid dehydratase (encoded by the *hemB* gene in the *Wolbachia* species strain *w*Bm genome) (41). Treatment of a different *Wolbachia*-infected filarial nematode (*Litomosoides sigmodontis*) with this inhibitor resulted in death of the nematode (41), suggesting that *Wolbachia*'s supplementation of heme to the host is a critical component of the mutualism. However, we cannot rule out that *hemB* is critical to *Wolbachia*'s own ability to function and that disruption of *Wolbachia* cell biology results in filaricide. Nonetheless, targeting heme biosynthesis is a promising avenue for pursuit of antifilarial therapies (23).

How would *Wolbachia*-synthesized heme be transported to the host cell? Many other pathogenic, invading microbes perform the opposite task, scavenging iron from the host for survival, and many do this via heme transporters (42). For example, the outer membrane heme transporter HutA in *Vibrio cholerae* binds to host heme and is upregulated under low iron conditions (43, 44). *Wolbachia*, if providing heme to the host, would have to have a transporter function in the opposite direction (Fig. 1). All sequenced *Wolbachia* genomes encode an inner membrane heme exporter (*ccmA* and *ccmC*, based on existing NCBI annotations), and in *Escherichia coli*, these proteins are predicted to be involved in the microbe's cytochrome maturation (45). However, although uncharacterized outer membrane proteins are encoded by *Wolbachia* genomes (46, 47), no evidence exists that these proteins participate in heme transport.

Regardless of whether *Wolbachia* supplements host heme, as an intracellular bacterial symbiont, *Wolbachia* likely acquires iron from the host. Therefore, *Wolbachia* infection may alter host iron metabolism in ways that are phenotypically perceptible. Indeed, *Wolbachia pipientis* strain *w*Mel seems to increase fecundity in fruit flies (*Drosophila melanogaster*) under high-iron diets; rearing flies on diets rich in $FeCl_3$ reduces fecundity, but a *Wolbachia* infection seems to modulate that reduction (48). Under a low-iron diet, the results were more mixed, with a modest and less consistent effect of *Wolbachia* infection (48). In a related study, *Wolbachia* bacterioferritin gene expression increased in the presence of high iron in three different host backgrounds, while host ferritin was downregulated (31), suggesting that *Wolbachia* may be modulating iron homeostasis; *Wolbachia* bacterioferritin may be binding available iron, leaving the host with a reduced need for cytosolic ferritin (31). Interestingly, the *Wolbachia* infecting filarial nematodes (clades C and D) lack bacterioferritin in their genomes (Fig. 2). As *Wolbachia* completely depends on the host for iron, it and the host must compete or share the iron in the host's diet. As yet, it is unclear what the ecological relevance of *Wolbachia*'s modulation of high iron toxicity would be for infected insects; certainly, the importance of the phenotype depends on the host's diet.

If hosts encounter high-iron environments with some frequency, then *Wolbachia* could provide an iron homeostasis benefit.

Nucleotides. Many rickettsia species are well-known nucleotide parasites, encoding their own ATP/ADP translocase to siphon off this important currency from host cells (49). Metabolic modeling suggests that many rickettsia species may also consume host GMP and UMP (50). *Wolbachia* genomes, however, do not encode a translocase. Instead, it was recently suggested that *Wolbachia* might actually provision ATP (17). In a nematode symbiont (*w*Oo), researchers found that *Wolbachia* highly expresses nucleotide and nucleoside metabolism proteins (17). In contrast, the authors suggested that vitamins and cofactors are not exchanged because enzymes for these pathways were poorly represented in their proteomics analysis (17). Based on genomic comparisons across *Wolbachia*, a connection between iron metabolism and nucleotide provisioning was suggested; perhaps *Wolbachia* take up host iron to synthesize components of their electron transport chain, by which they can synthesize ATP for the host (29). The nematode-associated strain *w*Bm, for example, retains the *de novo* nucleotide biosynthesis pathways, and perhaps, when demand is high, *w*Bm could supply these to the host (16). Indeed, *de novo* nucleotide biosynthesis genes of both purines and pyrimidines are conserved across most *Wolbachia* strains sequenced (Fig. 2). Therefore, many *Wolbachia* strains could potentially supplement host nucleotide metabolism.

Future directions. *Wolbachia* is a primarily maternally transmitted symbiont that is most well known for its ability to manipulate host reproduction (3). Because *Wolbachia* is primarily maternally transmitted, we expect that benefits provided to the host would be selected for, as they would increase host fitness and, by proxy, *Wolbachia* fitness (48). Additionally, because many *Wolbachia* strains do not manipulate host reproduction (4), the maintenance of *Wolbachia* within host populations must rely on other phenotypes, possibly nutritional mutualism. From B vitamins to iron to nucleotides, all of the hypotheses outlined above for *Wolbachia*'s nutritional mutualism require testing. The only strong physiological evidence for direct metabolic provisioning comes from the bedbug-*w*Cle model, where B-vitamin supplementation rescues *Wolbachia* clearing. However, the comparative genomics analyses performed here suggest natural comparisons that can be performed across host-symbiont combinations (Fig. 2). For example, nutritional provisioning in the case of the *Nomada* bees and their biotin operon-containing *Wolbachia* strains is questionable, given the vitamin content of pollen (28). However, this hypothesis can be tested, as two *Wolbachia* strains infecting the *Nomada* bees (*w*Nfla and *w*Nleu) would be expected to express the biotin biosynthesis operon, and this expression should be absent from the related strains *w*Nfe and *w*Npa (Fig. 2), which lack the operon. One might also expect that bees with strain *w*Nfla or *w*Nfleu would be able to subsist on diets low in biotin, while the opposite would be true of bees with strains *w*Nfe or *w*Npa.

We propose the *Drosophila* system as a powerful and straightforward genetic model system in which to identify metabolites supplemented by *Wolbachia*. *Drosophila melanogaster* is an excellent model host that has been used successfully to mechanistically identify *Wolbachia*-host interactions (51–57). Additionally, the flies can be transinfected with other *Wolbachia* strains, both in cell lines and in whole animals, and the microbiome easily controlled. Based on the genomic analyses, for example, strain *w*Mel could supplement host heme, purines, pyrimidines, and riboflavin but not biotin (Fig. 2). Hypotheses based on genomic content would be straightforward to test using defined diets coupled to fly genetics and labeled precursors. For example, one could test whether *Wolbachia* infection increases fecundity using a holidic diet without riboflavin. A negative control could be a diet excluding biotin, which *Wolbachia* species strain *w*Mel is not expected to synthesize and therefore would not rescue. Similarly, one could track the production of nucleotides by *Wolbachia* by providing labeled amino acid precursors to a *Drosophila* mutant unable to generate its own nucleotides (mutations in enzymes such as rudimentary or rudimentary-like [58]). We would then expect that any mRNAs produced by the host would include the label from *Wolbachia*-derived nucleotides.

In summary, comparative genomics is a powerful starting point in understanding host-microbe symbioses, and increasing evidence suggests that *Wolbachia* can supplement host nutrition in certain contexts. This nutritional mutualism could explain some of *Wolbachia*'s prevalence across insect populations and fitness effects for which we do not understand the underlying mechanism (8). For the filarial-nematode infecting strains, nutritional mutualisms could be targets for drug development, as knocking out *Wolbachia* results in death of the nematode or reduced fecundity. In the future, we expect that specific metabolic contributions from *Wolbachia* strains to host metabolism and fitness will be identified. The evolution of these biosynthetic pathways across both the *Wolbachia* and host phylogeny could unveil selective pressures on the symbiosis.

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