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ARTICLE

Biotin provisioning by horizontally transferred genes from bacteria confers animal fitness benefits

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Abstract

Insect symbionts are widespread in nature and lateral gene transfer is prevalent in insect symbiosis. However, the function of horizontally transferred genes (HTGs) in insect symbiosis remains speculative, including the mechanism that enables insects to feed on plant phloem deficient in B vitamins. Previously, we found there is redundancy in biotin synthesis pathways from both whitefly *Bemisia tabaci* and symbiotic *Hamiltonella* due to the presence of whitefly HTGs. Here, we demonstrate that elimination of *Hamiltonella* decreased biotin levels but elevated the expression of horizontally transferred biotin genes in whiteflies. HTGs proteins exhibit specific expression patterns in specialized insect cells called bacteriocytes housing symbionts. Complementation with whitefly HTGs rescued *E. coli* biotin gene knockout mutants. Furthermore, silencing whitefly HTGs in *Hamiltonella*-infected whiteflies reduced biotin levels and hindered adult survival and fecundity, which was partially rescued by biotin supplementation. Each of horizontally transferred biotin genes are conserved in various laboratory cultures and species of whiteflies with geographically diverse distributions, which shares an evolutionary origin. We provide the first experimental evidence that biotin synthesized through acquired HTGs is important in whiteflies and may be as well in other animals. Our findings suggest that B vitamin provisioning in animal-microbe symbiosis frequently evolved from bacterial symbionts to animal hosts through horizontal gene transfer events. This study will also shed light on how the animal genomes evolve through functional transfer of genes with bacterial origin in the wider contexts of microbial ecology.

Introduction

Insects are a highly successful group of animals, some of which are able to utilize a wide range of nutrient-depleted food resources from plant leaves to animal blood [1–3]. One of the driving forces shaping insect adaption to diverse feeding habits are microbial symbionts, which are widespread in nature [4, 5]. Insects bear primary symbionts, which can provide their hosts with essential nutrients deficient in their food, and secondary symbionts that may confer ecologically important traits depending on the environments and physiological states [6]. Many of these symbionts are localized in the gut and hemocoel or within specialized insect cells called bacteriocytes [6]. Generally speaking, symbionts housed in bacteriocytes are vertically transmitted and evolve to have highly reduced genomes compared with free-living relatives [7]. Thus, bacteriocyte adaptation to extreme genomic degeneracy of symbionts is critical for maintaining insect symbiosis [7]. Although considerable progress has been made, studies on the bacteriocyte-symbiont interface remain in the early stages [7].

Horizontal gene transfer events have tremendous impacts on the genomic evolution of both prokaryotes and eukaryotes, and it has been known to occur very frequently in prokaryotes [8, 9]. However, an increasing number of horizontal gene transfer from bacteria to eukaryotes,

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especially in insects and mites, has been reported [9–11]. For example, it was demonstrated that horizontally transferred carotenoid desaturase or phytoene desaturase is responsible for carotenoid production in aphids and spider mites and influences body color [12, 13]. In addition, horizontally transferred genes (HTGs) help insects to efficiently assimilate or detoxify plant produced metabolites [14, 15]. HTGs are widespread in insect symbiosis and they presumably function with other genes coded by the symbiont, making some metabolic pathways intact [1, 9, 16, 17]. Recent studies have shown that the horizontally transferred *RlpA4* with bacterial origin encodes a protein that is transported to an obligate endosymbiont in aphids [18], and silencing horizontally transferred *amiD* and *ldcA1* in aphids reduces *Buchnera* abundance [19]. It has been demonstrated that the mealybug protein encoded by the HTGs of bacterial origin is transported into the cytoplasm of symbiont *Moranella* for peptidoglycan synthesis [20]. However, functional validation for most HTGs is lacking, so that the metabolic, biological, and evolutionary role of HTGs in insect symbiosis remains largely unknown.

B vitamins perform various important functions in animals [21]. Animals cannot synthesize these compounds so that acquire them from their diet [21]. Vertebrate animal blood and plant sap are deficient in B vitamins. How groups of insects that feed on these diets deal with nutritional deficiencies is of great interest. It has been experimentally demonstrated that bedbugs, ticks and tsetse flies that feed on animal blood are able to derive B vitamins from their symbionts [2, 22–27]. Insects feeding on plant phloem and xylem could also obtain B vitamins from symbionts, but this has been mainly inferred from their genomic data [28–33]. Few studies have experimentally examined the role of bacterial symbionts that supply B vitamins for herbivorous insects [34–36]. Therefore, how herbivorous insects cope with B vitamin deficiency in plant sap needs to clarified.

The whitefly *Bemisia tabaci* species MEAM1 is a widespread invasive pest in agriculture damaging a wide range of crops [37–39]. The MEAM1 whitefly species bears two symbionts, *Portiera* and *Hamiltonella*, in the same bacteriocyte, which is maternally inherited [40, 41]. Previously, we found three horizontally transferred *bioA*, *bioD,* and *bioB* were encoded in the genome of the whitefly *B. tabaci* MEAM1 using genomic and transcriptomic analysis [17, 42]. Among them, *bioB* has acquired intron and all three genes are highly expressed in bacteriocytes as reported in the *Planococcus citri* mealybug [1, 17, 42]. These genes are presumably involved in biotin synthesis. However, the genome of the symbiont *Hamiltonella* in the whitefly also have biotin synthesis genes including *bioA*, *bioD,* and *bioB* [17, 42]. Thus, whether whitefly HTGs and *Hamiltonella* contribute to biotin synthesis independently or collectively is unknown. In this study, the function of horizontally transferred biotin synthesis genes in the whitefly *B. tabaci* MEAM1-*Hamiltonella* symbiosis system was investigated by combining immunohistochemistry, complementation assays, gene silencing, biotin assay, insect performance observation, and evolutionary analysis. We reveal that although *Hamiltonella* can synthesize biotin, biotin provisioned by whitefly HTGs affects the survival and fecundity of adult whiteflies.

Materials and methods

Insect rearing and plants

The whitefly *B. tabaci* MEAM1 was cultured on cotton plants (*Gossypium hirsutum*, cv. Shiyuan 321) grown in compost supplemented with Miracle-Gro Water Soluble All Purpose Plant Food. Cotton plants were cultivated to the 6–7 true-leaf stage for use in experiments described below. For more details, see the Supplementary Text.

PCR, quantitative PCR (qPCR) and qRT-PCR analysis

The presence of *Portiera*, *Hamiltonella* and *Rickettsia* in the *B. tabaci* colony was identified by diagnostic PCR in combination with Sanger sequencing. *Portiera*, *Hamiltonella,* and *Rickettsia* were quantified by qPCR using the copy number of *16 S rRNA*, *16 S rRNA*, and *gltA* genes, respectively, with the *B. tabaci β-actin* gene as internal standard for normalization. Relative gene expression was performed by qRT-PCR and calculated using the *β-actin* gene for transcript normalization in the symbiont elimination and gene silencing experiments. All primers used in this study are provided in Supplementary Table 1. Relative symbiont density and gene expression were calculated using the $2^{-\Delta Ct}$ method [43]. For more details, see the Supplementary Text.

Biotin measurement

A microbiological assay was used for biotin quantification in whiteflies using *Lactobacillus plantarum* ATCC 8014 (Beijing Landbridge technology Limited, Beijing, China) referring to the protocol as described previously [44]. Briefly, in an experiment, 100 male and 100 female adult whiteflies within 1 week after emergence were collected using a mouth aspirator and flash-frozen in liquid N_2. Insects from each treatment were pooled, weighed, and homogenized in citrate buffer using homogenizer MP FastPrep-24 (MP Biomedicals LLC, Santa Ana, USA) and incubated in sulphuric acid. Then samples were sterilized using a 0.2 μm pore size filter (PALL Life Science, New York, USA), mixed with vitamin B7-deficient Difco biotin

assay medium (thereafter called as B7DB; Becton, Dickinson and Company, USA) and inoculated with log-phase *L. plantarum* ATCC 8014. Cultures were incubated for 22–28 h at 37 °C. Standard concentrations of biotin (0–0.8 ng/mL; Sigma-Aldrich, St. Louis, MO USA) were mixed with the *L. plantarum* culture to create a standard curve. A negative control consisting of citrate buffer only was subjected to the complete procedure to ensure the retention of the initial biotin and a lack of additional biotin, respectively. Moreover, an additional negative control included only the assay medium lacking *L. plantarum* to exclude the contamination by other bacteria. The growth of *L. plantarum* was measured using a microplate reader (Versa Max Molecular Devices, Silicon Valley, USA) taking absorbance readings at 630 nm. Biotin in whiteflies was quantified using the standard curve and normalized to the weight of insects included in the homogenate. For more details, see the Supplementary Text.

Effects of *Hamiltonella* elimination by antibiotic treatment on whitefly biotin gene expression and biotin levels

Hamiltonella was eliminated by treating whitefly adults with a cocktail of antibiotics [45]. Hundreds of adult whiteflies within 0–7 days post emergence (F0) were fed a 25% (w/v) sucrose solution supplemented with 500 µg/mL each of 3 antibiotics (i.e., ampicillin, gentamycin, and cefotaxime) (BBI Life Sciences, Shanghai, China), for 4 days. The artificial diets with antibiotics were renewed every 2 days. Control insects were fed only a sucrose solution that was not supplemented with antibiotics. Following the diet treatments, whiteflies were transferred to cotton plants and allowed to lay eggs. Female and male adult whiteflies were collected within 0–7 days after emergence at the F1 stage. DNA was extracted from 8 female adult whiteflies and used for symbiont quantification by qPCR. RNA was extracted from 25 male and 25 female adult whiteflies from each of the 3 biological replicates and used to compare the expression levels of horizontally transferred *bioA*, *bioD,* and *bioB* in *Hamiltonella*-cured and *Hamiltonella*-infected whiteflies by qRT-PCR. Biotin was extracted from 100 male and 100 female adult whiteflies for each of the 3 biological replicates and quantified in *Hamiltonella*-cured and *Hamiltonella*-infected whiteflies by microbiological assay as described above.

To see a time series of biotin amounts over time after antibiotic treatment, *Hamiltonella* was eliminated as described above and biotin was quantified in 100 male and 100 female adults of *Hamiltonella*-cured and *Hamiltonella*-infected whiteflies at day 5, day 10, and day 15 after emergence at the F1 stage. Three biological replicates were conducted.

Fluorescence in situ hybridization (FISH)

Localization of *Portiera*, *Hamiltonella*, and *Rickettsia* in the whole body of *Hamiltonella*-cured and *Hamiltonella*-infected adult whiteflies and *Portiera* and *Hamiltonella* in the bacteriocytes of *Hamiltonella*-infected adult whiteflies was investigated by FISH following the protocol as described previously [46]. For more details, see the Supplementary Text.

Recombinant enzyme generation, antibody preparation, and western blot

The open reading frame of whitefly *bioA*, *bioD*, and *bioB* was cloned and recombinant enzymes were generated. Custom-made polyclonal antibodies against BioA (predicted size, 49 kDa), BioD (predicted size, 24 kDa), and BioB (predicted size, 38 kDa) proteins were produced by ProbeGene Life Sciences Co. Ltd. following previously described methods [18, 47]. The specificity of polyclonal antibodies was verified by western blot. For more details, see the Supplementary Text.

Immunofluorescence microscopy

Bacteriocytes and guts from adult female whiteflies infected with *Hamiltonella* and bacteriocytes from *Hamiltonella*-infected and *Hamiltonella*-cured adult female whiteflies at 7 days after emergence were dissected. Samples were fixed, permeabilized, and incubated with antibodies. Images were collected and analyzed on an FV3000 confocal microscope (Olympus, Japan). For more details, see the Supplementary Text.

Functional complementation of *E. coli* biotin auxotrophs with whitefly HTGs

To examine the metabolic function of horizontally transferred *bioA*, *bioD*, and *bioB*, the kanamycin resistance site was amplified from *E. coli* G11 using knockout primers (Supplementary Table 1). Then pKD46 plasmids expressing Lambda Red recombinase were transformed into the *E. coli* K-12 BW25113 by electroporation. Subsequently, the *E. coli* K-12 BW25113 *bioA*, *bioD,* and *bioB* knockout mutants (i.e., -Δ*bioA*, -Δ*bioD*, and -Δ*bioB*) were generated following the Lambda Red protocol as described previously [48–50]. *E. coli* mutants were screened using an LB media agar plate with 0.1 mg/mL kanamycin. PCR amplification was carried out using primers specific for deleted genes, the kanamycin resistance site and flanking locus to verify the loss of the parental fragment and gain of the kanamycin resistance site (Supplementary Table 1). The *E. coli* mutants were then made into competent cells by electroporation. For

the functional complement experiments, *E. coli* K-12 mutant cells were transformed with the plasmids pMD19-T-*bioA*, pMD19-T-*bioD*, pMD19-T-*bioB*, and pMD19-T empty vector (negative control), respectively, following the previous methods [34]. The *E. coli* wild-type K-12, mutant K-12, and mutant K-12 transformants were grown overnight in LB agar media with 0.1 mg/mL ampicilin at 37 °C. The mutant K-12 transformants were verified by PCR amplification using primers specific for inserted genes. Then *E. coli* wild-type K-12, mutant K-12, and mutant K-12 transformants were grown overnight in LB media and vitamin B7-deficient Difco biotin assay media at 37 °C. All *E. coli* cells were washed twice in sterile distilled water and resuspended to measure the cell density at OD_{600} using a microplate reader (Versa Max Molecular Devices, Silicon Valley, USA). Finally, aliquots (2 µL each) of cell suspensions were plated on LB media agar plates to assess cell growth after overnight incubation at 37 °C.

dsRNA preparation

dsRNAs specific to whitefly *bioA*, *bioD,* and *bioB* and *GFP* were synthesized using a T7 RiboMAX™ Express RNAi System kit (Promega, USA), following the manufacturer's instructions. For more details, see the Supplementary Text.

Effects of silencing horizontally transferred biotin genes on biotin levels and whitefly performance

To investigate whether silencing of horizontally transferred biotin genes influences biotin levels, ~400 male and 400 female adult whiteflies infected with *Hamiltonella* within 1 week after emergence were fed 30% (w/v) sucrose solution supplemented with ds*GFP*, ds*bioA*, ds*bioD*, or ds*bioB*, each at the concentration of 0.5 µg/µL for each biological replicate, for 3 days. ds*GFP* serves as a control. RNA was extracted from 25 male and 25 female adult whiteflies to examine the expression of *bioA* at day 1 and 3 using 6 biological replicates or *bioD* at day 2 and 3 and *bioB* at day 1 and 3 using 3 biological replicates after dsRNA treatment. To examine whether silencing of whitefly biotin genes affects protein expression levels in bacteriocytes, whitefly bacteriocytes were dissected, fixed, permeabilized, and incubated with antibodies as described above. Images were collected and analyzed on an FV3000 confocal microscope (Olympus, Japan). In parallel, 100 male and 100 female adult whiteflies at day 3 in each biological replicate after dsRNA treatment were collected for biotin analysis as described above.

To measure the effect of knockdown of horizontally transferred biotin genes on whitefly survivorship, 150 female adult whiteflies infected with *Hamiltonella* within 1 week after emergence were fed 30% (w/v) sucrose solution supplemented with ds*GFP*, ds*bioA*, ds*bioD*, or ds*bioB*, each at the concentration of 0.5 µg/µL, for 3 days. Mortalities of female whiteflies after 1, 2, and 3 days were recorded for each of the 3 biological replicates. To measure the effect of knockdown of horizontally transferred biotin genes on whitefly fecundity, ~50 male and 50 female adult whiteflies within 1 week after emergence were fed 30% (w/v) sucrose solution supplemented with ds*GFP*, ds*bioA*, ds*bioD*, or ds*bioB*, each at the concentration of 0.5 µg/µL, for 3 days. Subsequently, one female adult whitefly was released into a clip-cage attached to a cotton plant and allowed to lay eggs with 12, 12, 8, and 10 replicates for the treatment with ds*GFP*, ds*bioA*, ds*bioD*, and ds*bioB*, respectively. After 7 days, egg numbers were recorded. RNA was extracted from adult whiteflies to verify the expression of *bioA*, *bioD,* and *bioB*.

To detect whether silencing of whitefly biotin genes influences the abundance of *Hamiltonella*, 40 female adult whiteflies infected with *Hamiltonella* within 1 week after emergence were fed 30% (w/v) sucrose solution supplemented with ds*GFP*, ds*bioA*, ds*bioD* or ds*bioB*, each at the concentration of 0.5 µg/µL. ds*GFP* serves as a control. After feeding on dsRNAs for 3 days, whiteflies were collected. DNA was extracted from 8 dsRNA-fed female adult whiteflies and used for *Hamiltonella* quantification by qPCR as described above.

Effects of biotin supplementation on whitefly performance

To test whether biotin supplementation restores the survival of dsRNA-fed whiteflies, 150 female adult whiteflies within 1 week after emergence were fed 30% (w/v) sucrose solution supplemented with ds*GFP*, ds*bioA* or ds*bioA* plus biotin, each at a final concentration of 0.5 µg/µL for dsRNA and 167 ng/mL for biotin for 3 days. Mortalities of female whiteflies after 1, 2, and 3 days were recorded for each of the 3 biological replicates.

To investigate whether biotin supplementation restores the fecundity of dsRNA-fed whiteflies, ~50 male and 50 female adult whiteflies within 1 week after emergence were fed 30% (w/v) sucrose solution supplemented with ds*GFP*, ds*bioA* or ds*bioA* plus biotin, each at a final concentration of 0.5 µg/µL for dsRNA and 167 ng/mL for biotin for 3 days. Subsequently, one female adult whitefly was released into a clip-cage attached to a cotton plant and allowed to lay eggs with 10 replicates for each treatment. After 7 days, egg numbers were recorded.

Amino acid sequence alignment and phylogenetic tree analysis

Amino acid sequence alignments were conducted among *B. tabaci* MEAM1 [17], MED [51, 52], SSA-ECA, and Asia II

3 [53] and *Trialeurodes vaporariorum* and *Halmiltonella* using BioEdit v7.1.3.0. To construct the molecular phylogenetic tree for each of whitefly *bioA*, *bioD*, and *bioB*, a Bayesian inference analysis was conducted as described previously [17]. For more details, see the Supplementary Text.

Statistical analyses

For the egg numbers of ds*GFP*, ds*bioA*, ds*bioD*, and ds*bioB*-fed whiteflies, the egg numbers and mortality of ds*GFP*, ds*bioA* and ds*bioA* plus biotin-fed female whiteflies, and the OD values of the *E. coli* wild-type K-12, mutant K-12 and mutant K-12 transformants, statistical differences were evaluated using a one-way ANOVA at a significance threshold of 0.05 followed by LSD post-hoc tests. For the symbiont titer, biotin amount, gene expression level and mortality of ds*GFP* and ds*bioA*, ds*bioD* and ds*bioB*-fed female whiteflies, statistical differences were evaluated using a one-way ANOVA at a significance threshold of 0.05 level. Data in proportions were transformed by arcsine square root before analysis. All data analyses were conducted using the STATISTICA v6.1 software (StatSoft, Inc., Tulsa, OK, USA).

Results

Hamiltonella elimination decreases biotin levels and elevates expression of horizontally transferred biotin genes in whiteflies

The whitefly *B. tabaci* MEAM1 bears *Portiera* and *Hamiltonella* in the same bacteriocyte and *Rickettisa* in the whole body cavity, particularly in the abdomen (Fig. 1a, b). To investigate whether *Hamiltonella* contributes to the generation of biotin, *Hamiltonella* was eliminated by 86% through treating whitefly adults with a cocktail of antibiotics (Fig. 1a, c) ($P < 0.05$ for *Hamiltonella*). Both FISH and qPCR revealed that *Hamiltonella* elimination didn't influence the abundance of *Portiera* and *Rickettisa* (Fig. 1a–c) ($P > 0.05$ for *Portiera* and *Rickettisa*). *Hamiltonella* elimination decreased biotin level in whiteflies over time after antibiotic treatment

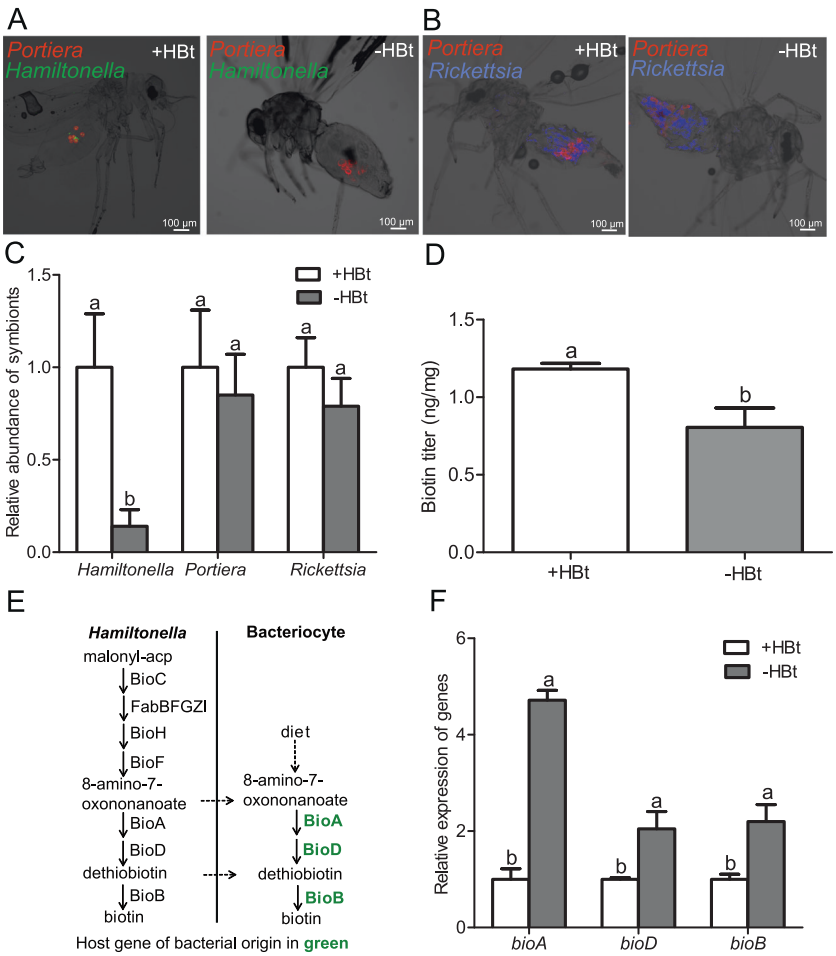

Fig. 1 Effects of *Hamiltonella* elimination on the biotin levels and HTG expression in whiteflies. a Localization of symbiotic bacteria *Portiera* (red) and *Hamiltonella* (green) in adult whiteflies. **b** Localization of symbiotic bacteria *Portiera* (red) and *Rickettsia* (blue) in adult whiteflies. **c** Effects of antibiotic treatments on the abundance of symbionts in whiteflies. **d** Effects of *Hamiltonella* elimination on biotin levels in whiteflies within 0–7 days after emergence at the F1 stage. **e** Genetic duplication of biotin synthesis for the whitefly *B. tabaci* HTGs and symbiont *Hamiltonella* (This figure is adapted from previous work [17, 42]). **f** Effect of *Hamiltonella* elimination on HTG expression in whiteflies. +HBt and -HBt represent *Hamiltonella*-infected and *Hamiltonella*-cured whiteflies, respectively. Data shown are mean ± SE. Different letters above the bars indicate significant differences between treatments at $P < 0.05$.

(Fig. 1d; $P < 0.01$ and Supplementary Fig. 1; $P < 0.05$). Our earlier work showed that there is redundancy in biotin biosynthesis from both whitefly and *Hamiltonella* because of the presence of whitefly HTGs [17, 42] (Fig. 1e). Interestingly, after *Hamiltonella* was eliminated, expression of horizontally transferred *bioA*, *bioD* or *bioB* was significantly elevated (Fig. 1f) ($P < 0.05$), indicating that horizontally transferred biotin genes are functional in the biotin synthesis of whiteflies. In addition, we found that *Rickettisa* genome has lost key genes (e.g., *bioA*, *bioD*, and *bioB*) involved in biotin synthesis (Supplementary Table 2) [42]. So *Rickettisa* is not able to synthesize biotin itself and the role of *Rickettsia* in biotin provisioning in this whitefly strain can be excluded.

Horizontally transferred biotin synthesis proteins have specific expression patterns in whitefly bacteriocytes

To examine the subcellular location of the proteins encoded by whitefly horizontally transferred *bioA*, *bioD*, or *bioB* in bacteriocytes, the recombinant proteins were successfully generated (Supplementary Fig. 2a–c). Then, polyclonal antibodies against BioA, BioD, and BioB proteins were produced using the purified recombinant protein. Polyclonal antibodies showed consistent specificity to those proteins, which was verified by western blot (Supplementary Fig. 2d–f). *Hamiltonella* was mainly distributed around bacteriocyte nuclei and *Portiera* occupied the cytoplasmic regions of bacteriocytes [40] (Fig. 2a). Immunofluorescence microscopy showed that BioA and BioD were mainly located in the peripheral regions of bacteriocytes in contact with external medium (Fig. 2b, c), while BioB was distributed both in the peripheral regions and around bacteriocyte nuclei in whiteflies infected with *Hamiltonella* (Fig. 2d). After *Hamiltonella* was cured, the protein expression levels and patterns were maintained in whiteflies (Supplementary Fig. 3), confirming that BioA, BioD, and BioB were not encoded by *Hamiltonella*. In contrast, no or a very weak signal was detected for these proteins in the guts as the control (Fig. 2e), where there were abundant *Rickettsia* as reported previously [54], indicating that BioA, BioD, and BioB were not encoded by *Rickettsia*, too. These data further suggest that BioA, BioD and BioB likely perform some functions in bacteriocytes.

Functional complementation of *E. coli* biotin auxotrophs with whitefly biotin genes

To test the hypothesis that *bioA*, *bioD* and *bioB* of the whitefly function in biotin synthesis, the *E. coli* K-12 *bioA*, *bioD* and *bioB* knockout mutant (-Δ*bioA*, -Δ*bioD*, or -Δ*bioB*) were generated using the Lambda Red protocol and functionally complemented *E. coli* K-12 mutant with whitefly *bioA*, *bioD*, and *bioB*, respectively. Compared with wild-type *E. coli*, *E. coli* K-12 knockout mutants (-Δ*bioA*, -Δ*bioD*, and -Δ*bioB*) grew poorly on LB media and did not grow on B7DB media lacking biotin (Fig. 3a–d). Significant differences in OD values among treatments were detected (Fig. 3a; $P < 0.001$ for *bioA*, *bioD*, and *bioB*). Although whitefly *bioA*, *bioD*, and *bioB* shared low amino acid sequence similarities with *E. coli* homolog genes (34.85%, 23.5%, and 50.87%, respectively) (Supplementary Fig. 4a–c), complementation with whitefly *bioA*, *bioD* and *bioB* rescued *E. coli* K-12 knockout mutants on LB and B7DB media (Fig. 3a–d). In contrast, cells transformed with

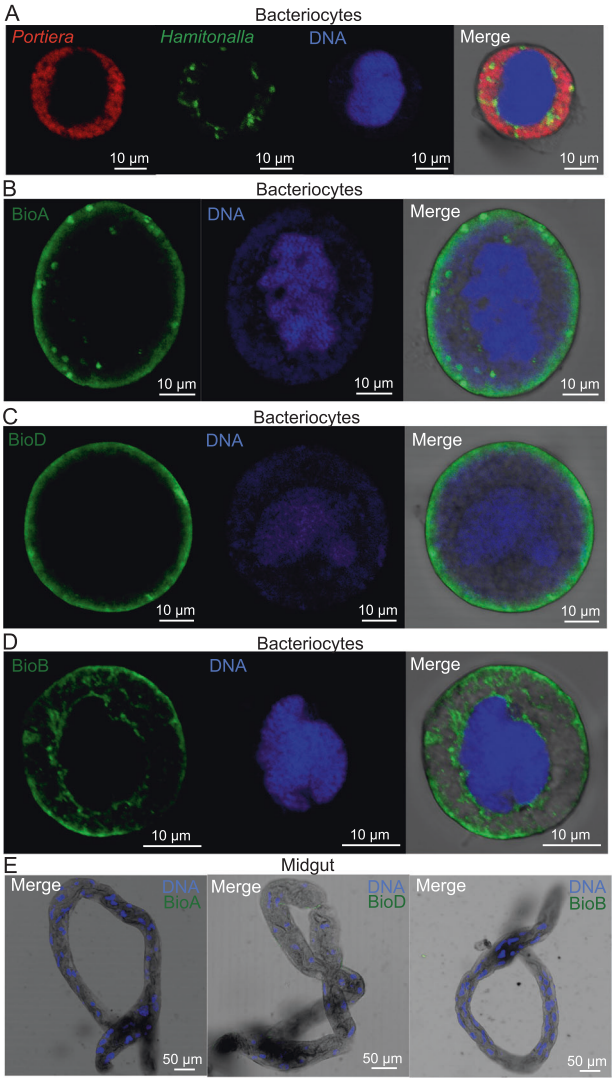

Fig. 2 Localization of horizontally transferred biotin synthesis proteins in whiteflies. a Localization of symbiotic bacteria *Portiera* (red) and *Hamiltonella* (green) in the bacteriocytes of adult whiteflies. Localization of BioA, BioD, and BioB proteins (green) in the bacteriocytes (**b–d**) and guts (**e**) of female adult whiteflies. DNA was stained with DAPI.

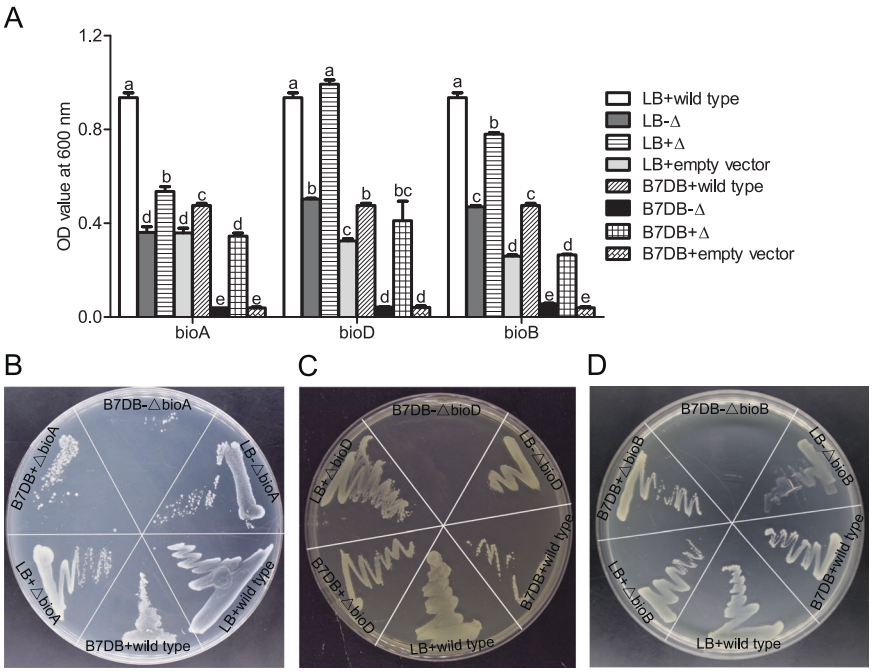

Fig. 3 Functional complementation of *E. coli* biotin auxotrophs. **a** *E. coli* K-12 knockout mutant cells were transformed with expression plasmids containing whitefly *bioA*, *bioD*, *bioB* or the negative control pMD19-T empty vector. The *E. coli* wild-type K-12, mutant K-12 (-Δ) and mutant K-12 transformants (+Δ) were grown overnight in LB and vitamin B7-deficient Difco biotin assay media (named as B7DB) at 37 °C. All *E. coli* cells were washed and resuspended to measure cell density at OD_{600}. *E. coli* wild-type K-12, mutant K-12 and mutant K-12 transformants were plated on LB and B7DB media agar plates for *bioA* (**b**), for *bioD* (**c**) and for *bioB* (**d**). Recovery of *E. coli* cell growth was assessed after overnight incubation at 37 °C. Data shown are mean ± SE. Different letters above the bars indicate significant differences between treatments at $P < 0.05$.

the pMD19-T empty vector grew poorly on LB media and did not grow on B7DB media without biotin supplementation (Fig. 3a–d).

Silencing horizontally transferred biotin genes reduces biotin levels and whitefly fitness while biotin supplementation restores whitefly performance

To confirm the metabolic function of horizontally transferred biotin genes, the gene silencing approach was applied in *Hamiltonella*-infected whiteflies. Expression of whitefly *bioA*, *bioD*, and *bioB* were reduced by 70–94% after feeding on dsRNAs for 3 days (Fig. 4a–c) ($P < 0.01$ for *bioA*; $P < 0.01$ for *bioD* and $P < 0.001$ for *bioB*). In addition, the protein expression levels of BioA, BioD, and BioB were significantly reduced in bacteriocytes after the RNAi treatment of these whiteflies (Fig. 4d–f). Silencing whitefly biotin genes significantly reduced biotin levels (Fig. 4g–i) ($P < 0.0001$ for *bioA*; $P < 0.05$ for *bioD* and $P < 0.001$ for *bioB*). As a result, gene silencing significantly increased the mortality of female adult whiteflies after 3 days, except for *bioD* silencing after day 2 and 3 (Fig. 4j–l) ($P < 0.05$ for *bioA* and *bioB* at day 1, 2, and 3; $P < 0.001$ for *bioD* at day 1 and $P > 0.05$ for *bioD* at day 2 and 3). After biotin supplementation in the artificial diet, the mortality of ds*bioA*-fed whiteflies was decreased over 3 days, which is close to that of ds*GFP*-fed whiteflies (Fig. 4m) ($P > 0.05$ for day 1 and 2; $P < 0.05$ for day 3). In addition, gene silencing significantly repressed the fecundity of female adult whiteflies (Fig. 4n) ($P < 0.0001$). Meanwhile, after biotin supplementation in the artificial diet, the fecundity of ds*bioA*-fed whiteflies was restored (Fig. 4o) ($P < 0.0001$). Furthermore, the abundance of *Hamiltonella* was not changed in RNAi treated whiteflies (Supplementary Fig. 5) ($P > 0.05$), suggesting that *Hamiltonella* could not complement the depleted roles of horizontally transferred biotin genes in whiteflies.

Evolutionary origin of horizontally transferred biotin genes in whiteflies

To investigate whether horizontally transferred biotin genes are ubiquitous in whitefly populations, the presence of *bioA*, *bioD*, and *bioB* was checked in multiple whitefly species and cultures. We found that all of nine whitefly cultures of five species, which are distributed in Asia, America, Europe and Africa, possess these horizontally transferred biotin genes (Supplementary Table 3). To examine the divergence of protein sequences, amino acid

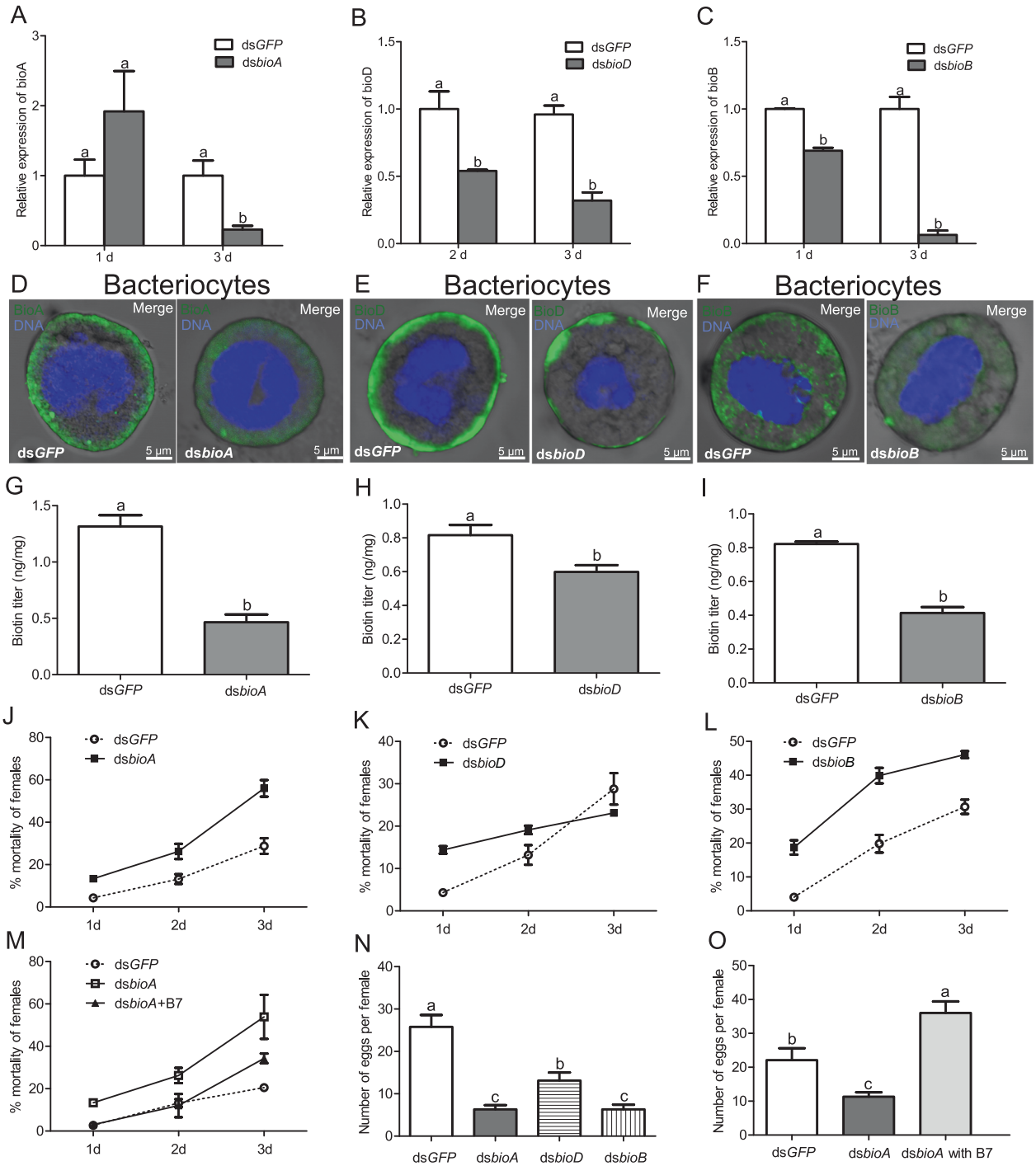

Fig. 4 Supplementation with biotin restores fitness of RNAi whiteflies. Expression of whitefly *bioA* (**a**), *bioD* (**b**), and *bioB* (**c**) after whiteflies fed on dsRNAs for 3 days. Localization of BioA (**d**), BioD (**e**), and BioB (**f**) proteins (green) in the bacteriocytes of female adult RNAi whiteflies. DNA was stained with DAPI. Biotin levels in whiteflies after feeding on ds*bioA* (**g**), ds*bioD* (**h**) and ds*bioB* (**i**) for 3 days. Mortality of female adult whiteflies after feeding on ds*bioA* (**j**), ds*bioD* (**k**), and ds*bioB* (**l**) for 3 days. **m** Mortality of female adult whiteflies after feeding on ds*bioA* and ds*bioA* plus B7 biotin for 3 days. **n** Fecundity of female adult whiteflies after feeding on dsRNAs for 3 days. **o** Fecundity of female adult whiteflies after feeding on ds*bioA* and ds*bioA* plus B7 biotin for 3 days. ds*GFP*-fed whiteflies were used as the control. Data shown are mean ± SE. Different letters above the bars indicate significant differences between treatments at $P < 0.05$.

sequences were aligned among 4 whitefly species and *Hamiltonella* for *bioA* and *bioD* and among 5 whitefly species and *Hamiltonella* for *bioB*. The amino acid sequence identity was high among all whitefly species

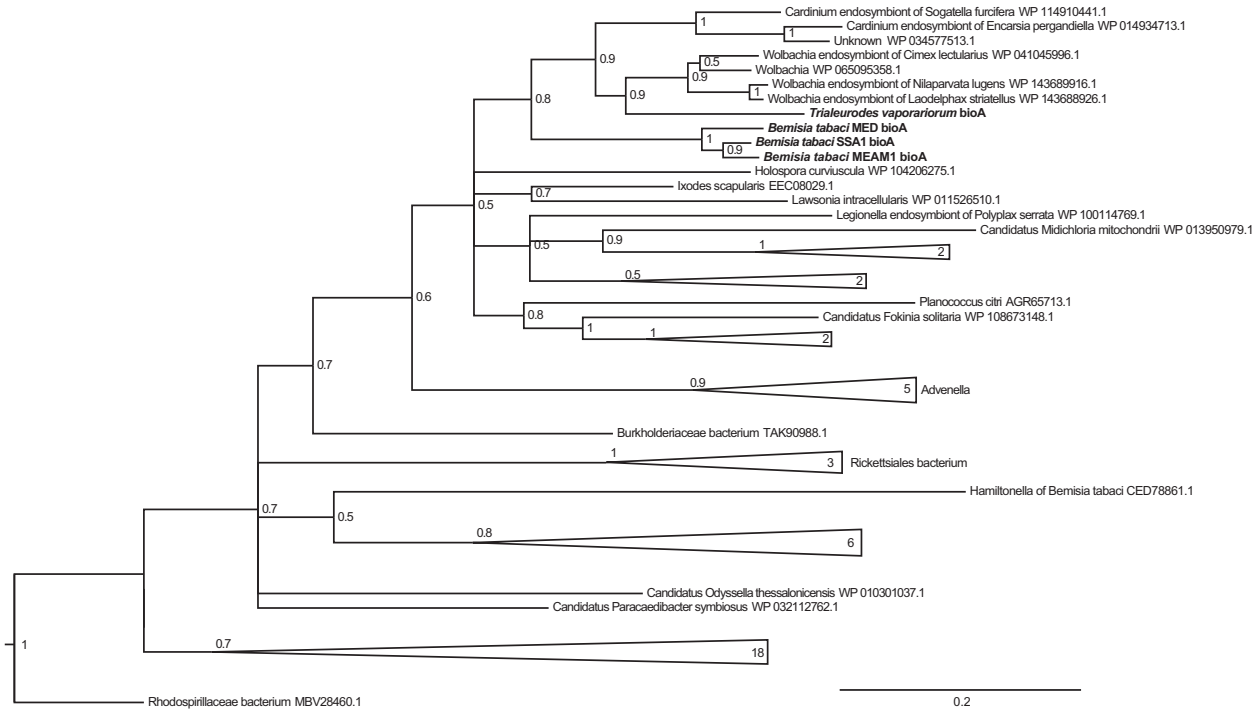

Fig. 5 Phylogenetic tree analysis of horizontally transferred BioA in the whitefly. Bayesian inference analysis was conducted for tree construction. A posterior probability of each node was shown.

(78–88%) but low between whiteflies and *Hamiltonella* (25–48%), indicating that *bioA*, *bioD*, and *bioB* were not horizontally transferred from *Hamiltonella* (Supplementary Fig. 4a–c). To gain insight into the evolution of these HTGs, a phylogenetic tree was constructed. Interestingly, BioA, BioD, and BioB of all whitefly species clustered within the same clade (Fig. 5; Supplementary Fig. 6a, b). Furthermore, whitefly BioA and BioD clustered with *Wolbachia* strains of the bedbug and planthoppers and *Cardinium* associated with the whitefly parasitioid and planthopper (Fig. 5; Supplementary Fig. 6a). BioB fell within the clade of *Rickettsia*, and clustered with *Wolbachia* strains of the bedbug and planthoppers and *Cardinium* associated with the whitefly parasitioid and planthopper (Supplementary Fig. 6b). Collectively, these data suggest that horizontally transferred BioA, BioD, and BioB share a common evolutionary origin in whiteflies.

Discussion

Using multi-disciplinary approaches, we have demonstrated that whiteflies are able to synthesize biotin via HTGs of bacterial origins. Because biotin is essential for whitefly fitness and horizontally transferred biotin synthesis genes are widespread in whiteflies, these results show that horizontal gene transfer contributes to the ability of whitefly to feed on B vitamin deficient phloem and so infest diverse crop plants. Our findings suggest that B vitamin provisioning in animal-microbe symbiosis frequently evolved from bacterial symbionts to animal hosts through horizontal gene transfer events.

Previously, the function of horizontally transferred biotin genes in whitefly and mealybug was speculated to be involved in biotin synthesis based on genomic and transcriptomic data [1, 17, 42]. Intron gain and duplication of the HTGs are thought to be the key steps when becoming functional in a eukaryotic genome [55]. In whiteflies, horizontally transferred *bioB* has acquired intron, and *bioA* (Bta01937 and Bta00841) and *bioD* (Bta00840 and Bta01938) were duplicated, of which, one of these duplicated genes (Bta00841 and Bta01938) was pseudogenized [42]. Our results support the hypothesis that intron gain and duplication of HTGs may help them become functional [55]. Interestingly, the elimination of *Hamiltonella* increased the expression of whitefly biotin genes. In addition, horizontally transferred BioA, BioD, and BioB proteins are enriched in bacteriocytes when compared with the guts. Localization in peripheral regions of bacteriocytes may help BioA and BioD access to precursors or intermediate metabolites from hemolymph for biotin synthesis. In contrast, distribution in the peripheral regions and around bacteriocyte nuclei could allow BioB to utilize dethiobiotin, the product of reaction mediated by BioD, from either HTGs or *Hamiltonella*, which mainly distributed around

bacteriocyte nuclei [40]. Cell-specific expression patterns of these proteins strongly suggest that they are functional. Furthermore, complementation with whitefly HTGs restored *E. coli* biotin gene knockout mutants, indicating that whitefly biotin genes serve the same function as *E. coli* homolog genes. Indeed, silencing whitefly HTGs reduced biotin levels. Biotin is the coenzyme of carboxylases and plays critical roles in central metabolic processes [21]. Not surprisingly, silencing whitefly HTGs hindered insect performance dramatically, which was restored by dietary biotin supplementation. To our knowledge, this study is the first to present empirical evidence that although *Hamiltonella* are able to produce biotin, whiteflies are able to synthesize biotin through acquired HTGs. A recent study shows that HTGs in a mealybug cooperate with genes of the bacterial symbiont *Moranella* to produce the peptidoglycan layer at the *Moranella* cell periphery [20]. These findings provide excellent genetic targets for insect control by selective interference.

There is redundancy in biotin synthesis from both whitefly and *Hamiltonella*. The biotin synthesized by *Hamiltonella* could be used for its growth and reproduction or provided to whiteflies. So, the importance of biotin and other B vitamins provisioned by *Hamiltonella* in whitefly fitness is worthy investigation in the future. This may explain why *Hamiltonella* appears to be a critical nutritional mutualist. In addition, the acquisition of biotin synthesis capability in whiteflies via HTGs may help whiteflies not to completely depend on the symbiont for biotin synthesis. Such metabolic redundancy reflects the coevolution of the host and symbiont.

Rickettsia can give fitness benefits in *B. tabaci* MEAM1 in USA [56], suggesting that this symbiont may synthesize biotin for the host. Because *Hamiltonella* elimination did not influence the abundance of *Rickettisa*, reduction of biotin level in *Hamiltonella*-cured whiteflies is not caused by changes of *Rickettisa* titer. Furthermore, *Rickettisa* genome has lost key biotin genes, revealing that *Rickettisa* is not able to synthesize biotin itself. Moreover, there is no possibility of non-target RNAi knockdowns for *Rickettisa* genes. Collectively, the role of *Rickettsia* in biotin provisioning in this whitefly strain can be excluded, and the results in this study are not due to the infection of these whiteflies with *Rickettsia*.

Notably, all nine whitefly cultures of the five species we tested harbored these horizontally transferred biotin genes. Among the five whitefly species, the whitefly *B. tabaci* MEAM1, MED, and *T. vaporariorum* are globally important pests in agriculture and the whitefly *B. tabaci* SSA-ECA and Asia II 3 are local pests found in Africa and Asia, respectively. It seems that the acquisition of horizontally transferred biotin genes is independent of whitefly species and their geographical region, suggesting a common evolutionary origin of these HTGs. The phylogenetic tree analysis revealed that BioA, BioD, and BioB cluster with *Wolbachia*, *Cardinium,* or *Rickettsia*. The whitefly *B. tabaci* MEAM1 harbors *Rickettsia* [57, 58], *B. tabaci* Asia II 3 bears *Rickettsia* and *Cardinium* [57], and *B. tabaci* MED and *T. vaporariorum* harbor *Rickettsia*, *Cardinium* and *Wolbachia* [59–61]. Therefore, HTGs were likely transferred to the common ancestor of whiteflies from *Rickettsia*, *Cardinium* or *Wolbachia* before the divergence of whiteflies infected with these symbionts. This hypothesis is supported by the fact that biotin synthesis genes are absent in the genomes of *Rickettsia*, *Cardinium*, and *Wolbachia* (accession No.: CP016430.1) associated with whiteflies [42, 61]. In addition, BioA, BioD, and BioB were very closely related to *Cardinium* associated with the whitefly parasitoid *Encarsia pergandiella* that is widely distributed in the world [62, 63]. Therefore, *Cardinium* likely switched hosts between the whitefly and parasitoid early in their evolution and then independently diverged in two hosts. Last, biotin genes were lost in *Cardinium* and transferred to the whitefly host. The ubiquity of horizontally transferred biotin genes in various whitefly populations indicates that they are essential for whitefly evolution, which may lead to the loss of biotin synthesis genes in whitefly symbionts, including *Hamiltonella*. This speculation is supported by the fact that biotin synthesis pathway of *Moranella* is degenerated in the mealybug, which bears horizontally transferred biotin genes of *Rickettsiales* origin [1] and also *bioH*, *bioA* and *bioD* of *Arsenophonus* [32] are pseudogenes in the whitefly *T. vaporariorum* which possesses horizontally transferred *bioA*, *bioD*, and *bioB*.

Our findings along with previous studies present three modes of B vitamin provisioning in insect symbiosis. The first is that symbionts such as *Francisella* and *Wigglesworthia* provide B vitamins including biotin for ticks and tsetse flies without being related to HTGs [23–27]. The second is that symbionts provide B vitamins for insects in bedbug/planthopper-*Wolbachia* symbiosis and potentially in aphid-*Erwinia* symbiosis through acquired HTGs for B vitamin synthesis [2, 22, 33, 36]. The biotin operon of *Wolbachia* is acquired via horizontal gene transfer, presumably from coinfecting *Cardinium* or *Rickettsia* in bedbugs [2] and from *Cardinium* in planthoppers [36], and *bioA*, *bioD* and *bioB* of *Erwinia* are horizontally transferred from *Sodalis* or *Sodalis*-like bacteria [33]. The results of this study uncovered a third mechanism in that both symbiont and insect HTGs synthesize biotin, but biotin synthesized by whitefly HTGs with bacteria origin determines insect performance. Interestingly, whitefly BioA, BioD and BioB clustered with *Wolbachia* strains of the bedbug and planthoppers, indicating that horizontal gene transfer events of bacteria-to-insect and bacteria-to-*Wolbachia* shares a common biological and evolutionary histories [2, 36]. In

addition, whitefly BioA, BioD, and BioB clustered with closely related mealybugs of the same Sternorrhyncha, suggesting that mealybugs also have biotin synthesis potential through horizontally transferred biotin genes [1]. Collectively, three mechanisms summarized here all point to the convergent role of biotin in insect symbiosis. Thus, this study suggests that the transition of B vitamins provisioned by symbionts to insect hosts is mediated through horizontal gene transfer. It will be worth investigating the role of HTGs in the synthesis of B vitamins in diverse insect symbiosis systems in the future.

Data availability

All relevant data supporting the findings of this study are included within the article and its Supplementary Information files.

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Author contributions JBL conceived the study. FRR conducted symbiont elimination, biotin assays, ecology experiments, gene expression analyses, and gene silencing. FRR, XS, TYW, and XZ performed the complementation experiments. XS carried out gene silencing, FISH and immunofluorescence experiments. YLY constructed the phylogenetic tree. YZH helped design the biotin assays. FRR, JBL, and YZH analyzed the data. JBL wrote the manuscript. All authors edited and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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