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INVERTEBRATE MICROBIOLOGY

Infections with *Arsenophonus* Facultative Endosymbionts Alter Performance of Aphids (*Aphis gossypii*) on an Amino-Acid-Deficient Diet

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ABSTRACT Genetic polymorphism and endosymbiont infection are ubiquitous in aphid populations. It has been known that the obligate symbiont *Buchnera* provides aphids with essential amino acids which cannot be ingested from plant sap. *Buchnera* often coexists with facultative endosymbionts in aphids. However, it is unclear whether the facultative endosymbionts affect the aphid's amino acid requirements from diet. In this study, we found that the facultative endosymbiont status in populations of the cotton-melon aphid *Aphis gossypii* was associated with aphid genotype or host plant. The infection frequency of *Arsenophonus* in aphids living on cotton was significantly higher than that in aphids on cucumber, and cucumber leaves contained higher titers of free amino acids than cotton leaves, especially amino acids Leu, Arg, Ile, Val, and Phe. The net reproductive rates of five aphid genotypes infected with *Arsenophonus* were not different on the complete-amino-acid diet, but the values were significantly different among seven *Arsenophonus*-free aphid genotypes. Moreover, the net reproductive rates of aphids on the amino-acid-deficient diet were significantly affected by *Arsenophonus* infection and aphid genotype. *Arsenophonus* infection decreased aphid performance on the Phe-free diet but improved performance on the Leu-free diet and did not affect the performance on the Ile-free or Val-free diet. *Arsenophonus* infections altered aphid requirements for amino acids that were significantly different in cotton and cucumber leaves, suggesting this endosymbiont would modulate the host specialization of this aphid.

IMPORTANCE The facultative endosymbiont *Arsenophonus* plays an important role in regulating reproduction through son killing, enemy resistance, and the dietary breadth of its insect hosts. In this study, we found *Arsenophonus* could alter aphid performance on the amino-acid-deficient diets. *Arsenophonus* infection increased aphid requirements for the amino acid Phe, but decreased requirements for the Leu. Cotton and cucumber leaves contained drastically different titers of free amino acids Phe and Leu, and aphids living on these two plants were infected with different incidences of *Arsenophonus*. We hypothesize that host specialization or the host plant range of aphids may be mediated by *Arsenophonus*.

KEYWORDS *Aphis gossypii*, *Arsenophonus*, amino acid, genotype, host specialization, net reproductive rate

Aphids require amino acids to support survival and reproduction (1, 2). When *Myzus persicae* (Sulzer) aphids fed on histidine-, isoleucine-, or methionine-free diets, their body weights decreased (1). The pea aphid *Acyrthosiphon pisum* (Harris) needs 10 essential amino acids and one nonessential cysteine; individual omission of all these 11 amino acids leads aphids to decreased body weight and even no reproduction on the cysteine-, isoleucine-, methionine- or tryptophan-free diet (3). Amino acids cysteine, methionine, tryptophan, and phenylalanine are essential to the cotton-melon aphid

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Aphis gossypii Glover, whereas cystine and tyrosine are not required (4, 5). Some clones of *Aphis fabae* Scopoli displayed lower nymphal survival, nymphal growth rate, or intrinsic rate of population increase on diets omitting histidine, methionine, threonine, or valine (2).

Phloem sap of a plant contains insufficient and imbalanced nutrients for aphids, especially the essential amino acids (6, 7). It has been proved that endosymbionts are involved in the nutrient synthesis of aphids, which complements the insufficient nutrition in plant sap. It is well known that the obligate endosymbiont *Buchnera aphidicola* supplies aphids with essential amino acids lacking in their phloem sap diet (1, 8–11). Despite massive loss of genes in *Buchnera*, approximately 10% of the genome encoded biosynthesis of almost all essential amino acids (12–15). In addition, facultative endosymbionts can sometimes have nutritional roles that are not obligate for the host. *Serratia symbiotica* would compensate for the loss of *Buchnera* in *A. pisum* (16). The genome analysis suggested that *Hamiltonella defensa* might reciprocate with the production of heme, ubiquinone, and pyridoxal-5-phosphate vitamin B_6 (17). In whiteflies, it has been found that the facultative endosymbiont *Arsenophonus* was able to synthesize B vitamins (18). However, it is still unknown whether *Arsenophonus* is associated with the requirements of aphids for amino acids.

Aphid populations developed various genetic differentiations and formed obvious host specialization (19–21). Community structure of endosymbionts in aphids varies with the genetic backgrounds of hosts (22–25). Aphid populations were often infected with various facultative endosymbionts, such as *Serratia*, *Regiella*, *Rickettsia*, and *Spiroplasma* (26). In North America, eight species of facultative endosymbionts were detected in the pea aphid and displayed different frequencies between locations and host plants (27). Aphid populations feeding on different host plants were found to be infected with different endosymbionts and belong to different genotypes (27–29). In a previous study, we found that the population genetic structures were different between cotton-melon aphids on cotton and those on cucumber (29), and in this study we also found that the facultative endosymbiont *Arsenophonus* statuses were different in the aphid populations collected from cotton and cucumber. *Arsenophonus* has also been found in wild cotton-melon aphid populations (30, 31). Based on genetic differentiation, facultative endosymbionts, and host specialization of cotton-melon aphids, here, we hypothesized that the facultative endosymbiont *Arsenophonus* and genetic background of aphids were associated with the performance of cotton-melon aphids on different nutrient diets and consequently contribute to the host specialization. Therefore, in this study, we focused on the performance of cotton-melon aphids on diets lacking one of five amino acids, the titers of which in the cotton and cucumber leaves were significantly different, and five *Arsenophonus*-infected and seven *Arsenophonus*-free aphid genotypes originally collected from cotton and cucumber were used to address the role of *Arsenophonus* in mediating the amino acid requirements of aphids. The result will highlight the *Arsenophonus*-mediated host specialization in aphids.

RESULTS

Relationship between facultative endosymbiont status and genotype of aphids. Three facultative endosymbionts, *Arsenophonus*, *Spiroplasma*, and *Wolbachia*, were found in cotton-melon aphids collected from cotton, cucumber, zucchini, and cowpea in Nanjing, China, and their infection frequencies were approximately 44, 87, and 2%, respectively. Approximately 6% of the aphids were not infected with any known facultative endosymbionts (Table 1). The infection statuses of endosymbionts were significantly different among aphid genotypes ($\chi^2 = 668.3$, df $= 42$, $P < 0.001$). The infection frequencies of *Arsenophonus* were significantly higher in the aphid genotypes CA9 (81.7%), CA1 (66.7%), and AG5 (49.4%) and were lowest in genotypes CA8 and AG11 ($\chi^2 = 580.3$, df $= 6$, $P < 0.001$) (Table 1). Aphids collected from cotton and cucumber showed different infection patterns of facultative endosymbionts ($\chi^2 = 163.77$, df $= 2$, $P < 0.001$) (Fig. 1). The infection frequency of *Arsenophonus* in aphids on cotton was significantly higher than that of aphids on cucumber ($\chi^2 = 234.38$, df $= 1$, $P < 0.001$). On the

TABLE 1 Frequency of cotton-melon aphids belonging to 12 genotypes and infected with facultative endosymbionts

Genotype	Genotype in this study	Frequency of aphids with endosymbiont infection (%)[a]								
		Ars$^+$	Spi$^+$	Wol$^+$	Ars$^+$ + Spi$^+$	Ars$^+$ + Wol$^+$	Spi$^+$ + Wol$^+$	Ars$^+$ + Spi$^+$ + Wol$^+$	None	Total
AG1	CA2	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.08	0.17
AG2	CA3	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.08
AG3	CA8	0.17	7.75	0.00	0.42	0.00	0.17	0.00	0.33	8.83
AG4		0.00	2.92	0.00	1.08	0.00	0.00	0.00	0.08	4.08
AG5		0.67	2.25	0.00	2.58	0.00	0.08	0.00	1.00	6.58
AG6	CA4	0.25	14.67	0.00	2.17	0.00	0.42	0.08	1.25	18.83
AG7	CA9	5.08	5.25	0.00	27.75	0.17	0.00	0.83	2.33	41.42
AG8	CA1	0.33	0.92	0.08	2.08	0.00	0.00	0.08	0.25	3.75
AG9		0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.08	0.17
AG10		0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.08	0.33
AG11		0.00	14.17	0.00	0.25	0.00	0.17	0.08	0.83	15.50
AG12		0.00	0.17	0.00	0.08	0.00	0.00	0.00	0.00	0.25
Total		6.50	48.17	0.08	36.83	0.17	0.83	1.08	6.33	100

[a]Shown are results from a total of 1,200 aphid samples collected in 2016 in Nanjing, China. Ars$^+$, *Arsenophonus* infected; Spi$^+$, *Spiroplasma* infected; Wol$^+$, *Wolbachia* infected; None, not infected with any of nine endosymbionts.

contrary, the infection frequency of *Spiroplasma* in aphids on cucumber was higher than that of aphids on cotton ($\chi^2 = 50.74$, df = 1, $P < 0.001$). Aphids infected with *Wolbachia* were infrequent in populations on both cotton and cucumber (Fig. 1).

Compositions of free amino acids in cotton and cucumber leaves. The compositions of free amino acids in cotton and cucumber leaves were not significantly different based on multivariate analysis of variance (MANOVA) for titers of 15 free amino acids ($F_{1,\ 4} = 29.423$, $P = 0.137$). However, titers of seven free amino acids—cysteine (Cys), valine (Val), isoleucine (Ile), leucine (Leu), phenylalanine (Phe), histidine (His), and arginine (Arg)—were significantly higher in cucumber leaves than in cotton leaves (Fig. 2).

Performance of *Arsenophonus*-infected and *Arsenophonus*-free aphids on the complete-amino-acid diet. The net reproductive rates of five aphid genotypes infected with *Arsenophonus* were not significantly different on the complete amino acid diet ($F_{4,\ 10} = 1.215$, $P = 0.364$), but the values were significantly different among seven genotypes not infected with *Arsenophonus*, and these aphid genotypes from cucumber (MA10 to MA12) had significantly higher net reproductive rates than those (CA6 to CA9) from cotton ($F_{6,\ 14} = 14.54$, $P < 0.001$). When all five *Arsenophonus*-infected genotypes were treated using antibiotics to cure the symbiont, their net reproductive rates on the complete amino acid diet also became different among genotypes ($F_{4,\ 10} = 10.31$, $P = 0.001$). Elimination of *Arsenophonus* using antibiotics resulted in the decreased net reproductive rates of the three aphid genotypes CA2, CA3, and CA4, whereas there were no changes of the two genotypes CA1 and MA5 (Fig. 3). The

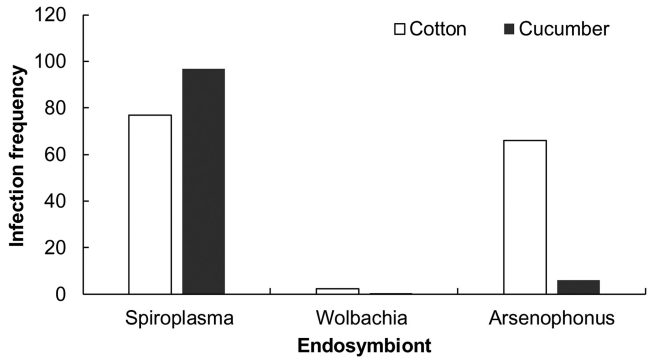

FIG 1 Infection frequency (%) of facultative endosymbionts in aphids collected from cotton and cucumber.

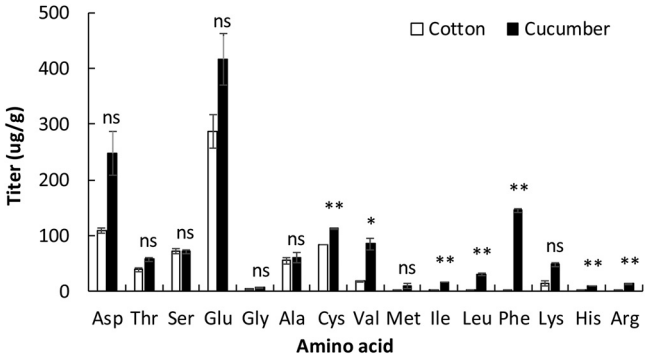

FIG 2 Titer of 15 free amino acids in cotton and cucumber leaves. * and ** indicate significant differences between cotton and cucumber using the *t* test followed by the Bonferroni correction at $P = 0.05/15$ and $P = 0.01/15$, respectively. ns, no significant differences at $P > 0.05/15$.

significant differences in the net reproductive rates were still maintained among seven *Arsenophonus*-free genotypes after antibiotic treatment ($F_{6, 14} = 25.45$, $P < 0.001$), but none of these seven genotypes except for genotype CA8 altered their net reproductive rates as the antibiotic treatment was performed (Fig. 3). *Arsenophonus* infection affected performance of some aphid genotypes on the complete-amino-acid diet.

Relative density of *Buchnera* in *Arsenophonus*-infected and *Arsenophonus*-cured aphids. The relative density of *Buchnera* in the MA5 aphids from cucumber hosting *Arsenophonus* was significantly higher than that in the CA1, CA2, CA3, and CA4 aphids from cotton ($F_{4, 10} = 39.14$, $P < 0.001$). When the facultative symbiont *Arsenophonus* was eliminated by antibiotics, the relative densities of obligate symbiont *Buchnera* in the CA1 and MA5 aphids increased, whereas the densities in CA2, CA3, and CA4 aphids remained constant (Fig. 4). The interactions between aphid genotype and *Arsenophonus* infection affected the *Buchnera* relative density in aphids ($F_{4, 20} = 4.32$, $P = 0.011$) (Fig. 4).

Performance of *Arsenophonus*-infected aphids on the amino-acid-deficient diet. Feeding on the complete-amino-acid diet, CA1 and MA5 aphids did not change their net reproductive rates when their *Arsenophonus* infections were cured by antibiotics (Fig. 5A). On diets lacking a specific amino acid, however, the cure of *Arsenophonus* resulted in significant changes in the net reproductive rates of CA1 and MA5 aphids (Fig. 5B to F). *Arsenophonus*-cured CA1 had a significantly lower net reproductive rate on the diets lacking one of the amino acids Leu and Val than the *Arsenophonus*-infected CA1 aphids (Fig. 5B and

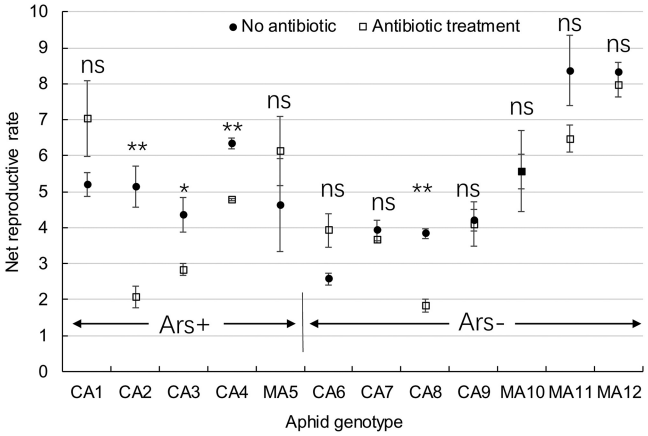

FIG 3 Net reproductive rates (R_0s) of 12 genotypes aphids (five *Arsenophonus* infected [Ars$^+$], and seven *Arsenophonus* free [Ars$^-$]) on the complete amino acid diet when they were treated or not treated with antibiotics. * and ** indicate significant differences in the net reproductive rates of a genotype between antibiotic treatment and no treatment at $P = 0.05$ and $P = 0.01$, respectively. ns, no significant differences.

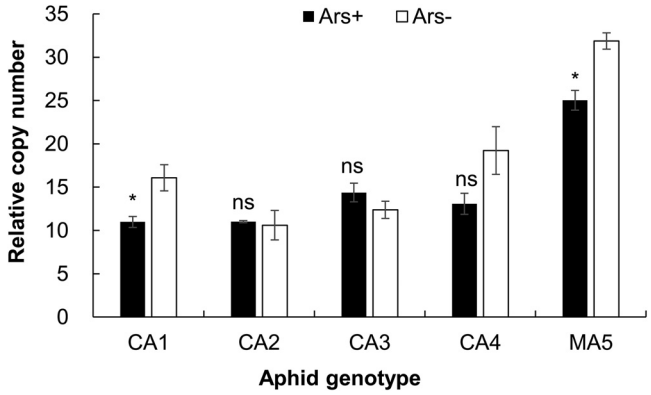

FIG 4 Relative density of *Buchnera* in *Arsenophonus*-infected (Ars^+) and *Arsenophonus*-cured (Ars^-) aphids. * indicates significant difference between infect and cured lineages at $P = 0.05$. ns, no significant differences.

E), whereas they had the same net reproductive rate as the *Arsenophonus*-infected CA1 on the diets lacking Arg, Ile, or Phe (Fig. 5C, D, and F). The *Arsenophonus*-cured MA5 aphids also showed a lower net reproductive rate on the diets lacking one of amino acids Leu, Arg, and Ile than the *Arsenophonus*-infected MA5 aphid (Fig. 5B to D), but they had a higher net reproductive rate on diets lacking Val or Phe than the *Arsenophonus*-infected one (Fig. 5E and F). Moreover, the net reproductive rates of CA2, CA3, and CA4 were significantly decreased on the complete-amino-acid diet when cured of *Arsenophonus* (Fig. 5A), but on the diets lacking one of amino acids Leu, Arg, Ile, Val, and Phe, their net reproductive rates would be lower than, equivalent to, or higher than the *Arsenophonus*-cured aphids, depending on the aphid genotype and amino acid (Fig. 5B to F).

Due to the increase of *Buchnera* density in CA1 and MA5 aphids after the elimination of *Arsenophonus* (Fig. 4), the aphid genotypes CA2, CA3, and CA4 with the same densities of *Buchnera* before and after antibiotic treatment were selected to reanalyze the role of *Arsenophonus* in the amino acid requirements of aphids by MANOVA. The result showed that both the aphid genotype and antibiotic treatment significantly affected

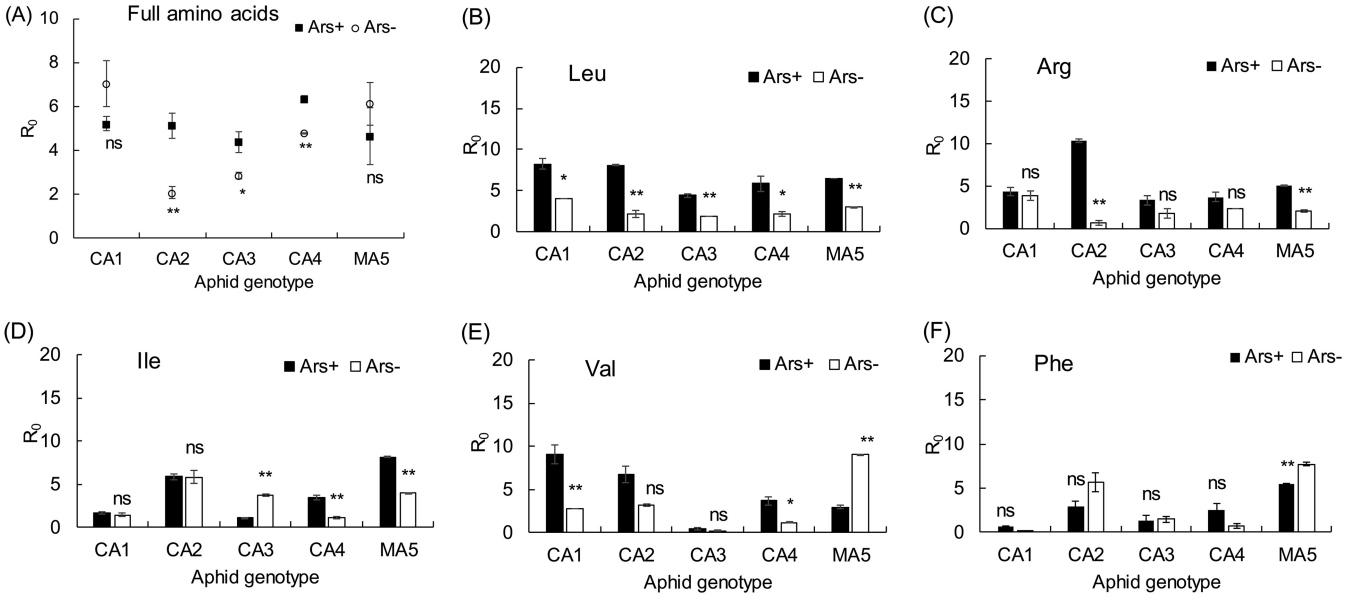

FIG 5 The net reproductive rates (R_0s) of five aphid genotypes on the complete-amino-acid diet (A) or Leu-free (B), Arg-free (C), Ile-free (D), Val-free (E), and Phe-free (F) diets before (Ars^+) and after (Ars^-) antibiotic treatment. * and ** indicate significant differences between antibiotic treatment and no treatment at $P = 0.05$ and $P = 0.01$, respectively. ns, no significant differences.

TABLE 2 Effects of aphid genotype and antibiotic treatment on the net reproductive rate of *Arsenophonus*-infected and -free aphids analyzed by MANOVA

Aphid type[a]	Effect	Roy's greatest root	*F*	df		*P* value
				Numerator	Denominator	
Ars$^+$	Genotype	36.192	57.907	5	8	<0.001
	Antibiotic	10.971	15.360	5	7	0.001
Ars$^-$	Genotype	32.260	145.169	6	27	<0.001
	Antibiotics	4.486	20.637	5	23	<0.001

[a]Ars$^+$, *Arsenophonus* infected; Ars$^-$, *Arsenophonus* free.

the net reproductive rates of both the *Arsenophonus*-infected and *Arsenophonus*-free aphids when data from all diets lacking Leu, Arg, Ile, Val, and Phe were pooled (Table 2), but on each of the amino-acid-deficient diets, the effects of antibiotic treatment on *Arsenophonus*-infected aphids were not quite the same as those on the *Arsenophonus*-free aphids (Table 3). Antibiotic treatment resulted in the significant change in net reproductive rates of both the *Arsenophonus*-infected and *Arsenophonus*-free aphids on the diet lacking Arg (Table 3 and Fig. 5C). On the contrary, antibiotic treatment did not lead to a significant change of the net reproductive rates in both the *Arsenophonus*-infected and *Arsenophonus*-free aphids on the diet lacking Ile or Val when data from all aphid genotypes were pooled (Table 3), because there were different effects among aphid genotypes (Fig. 5D and E). Overall, *Arsenophonus* infection did not affect the requirements of aphids for Ile and Val (Table 3). However, on the diets lacking Leu or Phe, antibiotic treatment produced a drastic change in the net reproductive rates of the *Arsenophonus*-infected aphids (Table 3 and Fig. 5B and F), but not the *Arsenophonus*-free aphids (Table 3). *Arsenophonus* infection altered the amino acid requirements of aphids for Leu and Phe. *Arsenophonus* infection improved the performance of aphids on the Leu-deficient diet (Fig. 5B), but it decreased the performance on the Phe-deficient diet (Fig. 5F).

DISCUSSION

Genetic background mainly affects nutrient requirements of insects. The amino acid requirements of two biotypes (C and J) in pea aphids were different; biotype C needed arginine, leucine, lysine, and tryptophan, but biotype J needed phenylalanine, threonine, and valine (32). Six clones in pea aphids displayed different adult masses on diets lacking individual essential amino acids (23). The result in this study showed that the net reproductive rate of the *Arsenophonus*-free *A. gossypii* on the complete-amino-acid diet varied with their genotypes or the host plants on which aphids were originally collected, and the net reproductive rates of aphid genotypes from cucumber were significantly higher

TABLE 3 Effects of antibiotic treatment on the net reproductive rate of *Arsenophonus*-infected and -free aphids on diet lacking one of the amino acids Leu, Arg, Ile, Val, and Phe as analyzed by MANOVA[a]

Aphid type[b]	Amino acid	Type III SS	df	*F*	*P* value
Ars$^+$	**Leu**	**13.508**	**1**	**20.889**	**0.0008**
	Arg	**6.581**	**1**	**16.344**	**0.0019**
	Ile	0.041	1	0.0894	0.7705
	Val	9.187E−05	1	0.0003	0.9871
	Phe	**9.213**	**1**	**14.012**	**0.0032**
Ars$^-$	Leu	0.287072	1	0.547157	0.4659
	Arg	**24.21308**	**1**	**85.63252**	**<0.0001**
	Ile	0.8	1	1.091195	0.3055
	Val	3.548563	1	6.432599	0.0173
	Phe	0.360469	1	0.590888	0.4487

[a]Bold text indicates the significant effect on this amino acid requirement of aphids at $P < 0.01$. SS, sum of squares.
[b]Ars$^+$, *Arsenophonus* infected; Ars$^-$, *Arsenophonus* free.

than that from cotton. The dominant aphid genotypes on different host plants were different (19, 29, 33), and contents of free amino acids in cotton and cucumber leaves were also different (34). In this study, we found at least seven amino acids (Leu, Arg, Ile, Val, Phe, His, and Cys) were different in titer between cotton and cucumber leaves, and the titers in cucumber leaves were generally higher than that in cotton leaves. *A. gossypii* aphids on cotton do not use cucumber and vice versa (21, 33–35). These results imply that the amino acid nutrition in a specific host plant might only meet the demand for some specific genotypes in aphid populations. Different aphid genotypes in *Sitobion avenae* (Fabricius) exhibited differential preference and performance for different barley genotypes (36). Those aphid genotypes depending closely on a specific nutrient or host plant genotype may find it easy to form host specialization. The nutrition requirements of aphids are associated with their genetic background.

However, in this study we also found that five aphid genotypes infected with *Arsenophonus* had the same net reproductive rates on the complete-amino-acid diet, but on the amino acid-deficient diet, the *Arsenophonus*-infected and *Arsenophonus*-cured aphids exhibited different levels of performance. This implied that besides genetic background, the nutrient requirements of aphids were also affected by facultative endosymbionts. There is a close nutritional relation between endosymbionts and their hosts. It has been well known that the obligate endosymbiont *Buchnera* supplies necessary nutrients for its insect hosts (1, 8, 12–14). On the other hand, a few studies also indicated or implied that some species of facultative endosymbionts, such as *S. symbiotica*, *H. defensa*, and *Regiella insecticola*, were involved in tryptophan biosynthesis or affected the aphids' performance on a low-amino-acid diet (23, 37, 38). In this study, we found that the facultative symbiont *Arsenophonus* improved aphid performance on the Leu-free diet, but decreased aphid performance on the Phe-free diet, and it did not affect aphid performance on the Ile- or Val-free diet. This result suggests that *Arsenophonus* infections can alter amino acid requirements of aphids. Our study provides evidence of a direct correlation between *Arsenophonus* and amino acid requirements in aphids.

Facultative endosymbionts may mediate dietary breadth in polyphagous aphids (28, 39, 40). *Arsenophonus* promoted the specialization of *Aphis craccivora* Koch on locust trees, and the *Arsenophonus*-free locust-tree-origin clones performed no better on locust trees than alfalfa-origin clones (39). *Aphis glycines* Matsumura soybean aphids infected with *Arsenophonus* performed better on a resistant soybean than their paired uninfected isolines (40). In this study, we further found that *Arsenophonus* affected the performance or fitness of cotton-melon aphids on the amino-acid-deficient diet. The absence of amino acid biosynthetic capabilities was found in the facultative endosymbionts. A previous study based on genome sequence showed that endosymbiont *Arsenophonus* presented no genes to metabolize histidine, arginine, and proline, but retained the conserved ABC transporters for proline, arginine, and methionine, suggesting that the endosymbiont might increase uptake of amino acids from its environment (41). Although *Arsenophonus* has no complete capacity to biosynthesize essential amino acids, some lineages or clades of this endosymbiont retain a part of enzymes in the biosynthetic pathways of isoleucine, valine (Val), phenylalanine (Phe), lysine, tryptophan, leucine (Leu), threonine, and arginine (Arg) (18). In this study, we found that *Arsenophonus* altered the performance of aphids on an amino-acid-deficient diet—especially on the Phe-free or Leu-free diet. Moreover, the effect of *Arsenophonus* on aphid performance on amino-acid-deficient diets was dependent on the genotype of aphids. These results suggest that the *Arsenophonus* may be associated with the nutrition metabolism of the cotton-melon aphids. Based on the result that *Arsenophonus* infection could alter *Buchnera* density in some aphid genotypes (for example, CA1 and MA5), we speculated that *Arsenophonus* might assist the obligate symbiont *Buchnera* to biosynthesize amino acids and then alter aphid performance on amino-acid-deficient diets. In addition, different strains of *Arsenophonus* might play different roles in determining host fitness; for example, the S-type *Arsenophonus* increased the susceptibility of its host brown planthopper *Nilaparvata lugens* to insecticides, but the N-type

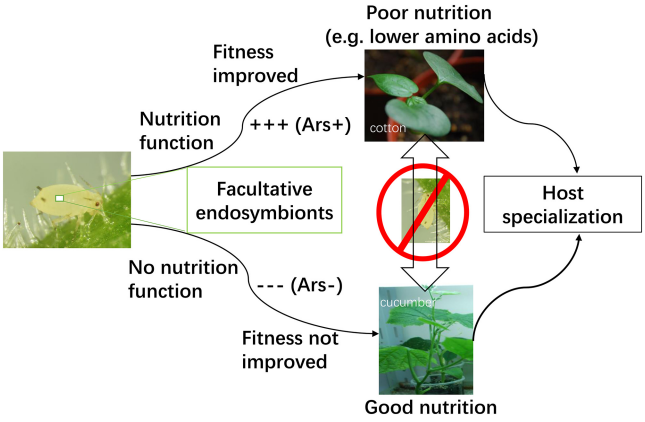

FIG 6 A mode of the facultative endosymbiont-mediated host specialization in aphids.

did not (42). The strains of *Arsenophonus* in aphids belonging to different genotypes might be different. Therefore, the effect of *Arsenophonus* on the performance of aphids varied with aphid genotype.

The *A. gossypii* aphids on cotton were infected with *Arsenophonus* more frequently than on cucumber, and the free-amino-acid titers in cotton leaves were generally lower than that in cucumber leaves (for example, Phe and Leu). *Arsenophonus* infection was also found in *A. gossypii* populations on cotton in Cameroon and northern China, but not found on melon in France (30, 43). The host-specialized biotypes in *A. gossypii* populations on cotton and cucurbits are ubiquitous in nature (19, 21, 33, 35). *Arsenophonus* infection in aphids feeding on cotton may compensate for the shortage of amino acids in this plant, due to the nutrition function of endosymbionts (Fig. 6). The aphids infected with a specific endosymbiont can use host plants with "poor" nutrition, but endosymbiont-free aphids cannot and only use host plants with "good" nutrition (abundant amino acids). Therefore, the endosymbiont-mediated host specialization will be promoted (Fig. 6). We speculated that the facultative endosymbiont *Arsenophonus* may play a role in promoting host specialization in *A. gossypii*.

As a facultative endosymbiont, the effects of *Arsenophonus* on host fitness were not consistently evident (40). *Arsenophonus*-reduced or -expanded dietary breadth in *A. craccivora* was dependent on host genotype (39). In this study, the effects of *Arsenophonus* on amino acid requirements of aphids were also inconsistent in different genotypes and for different amino acids. We considered that *Arsenophonus* was still a double-edged endosymbiont in aphids, with both positive and negative effects on its host. This phenomenon was also found in another facultative endosymbiont, *Regiella*, in the grain aphid *S. avenae* (44). The function in nutrient regulation of *Arsenophonus* will promote the coevolution of the facultative endosymbiont and aphid. Therefore, the mechanism of *Arsenophonus* to alter aphid performance on the amino-acid-deficient diet is worth studying.

MATERIALS AND METHODS

Cotton-melon aphids. The cotton-melon aphids, *Aphis gossypii* Glover, were collected from cotton and cucumber fields in Nanjing, China. Aphids from cotton were reared on cotton leaves, and those from cucumber were fed on cucumber leaves under conditions of 14 h of light and 10 h of darkness at 25°C. In order to explore the relationship between endosymbiont status and aphid genotype, 1,200 cotton-melon aphids from cotton, cucumber, zucchini, and cowpea (300 aphids per host plant) were collected from August to October of 2016 in Nanjing, China, and the infection frequencies of nine facultative endosymbionts and genotype of each of the aphids were examined by PCR.

PCR detection of aphid genotype and endosymbiont infection. All the aphids used in this study were genotyped by PCR using six microsatellite locus primers (45), and the analysis detected the presence of nine facultative endosymbionts, *Arsenophonus*, *Hamiltonella*, *Regiella*, *Rickettsia*, *Rickettsiella*, *Serratia*, *Spiroplasma*, *Wolbachia*, and X-type, using the diagnostic PCR method (28, 46–48). Only three facultative endosymbionts (*Arsenophonus*, *Spiroplasma*, and *Wolbachia*) were found in the cotton-melon aphids. Based on the endosymbiont status, a total of 12 genotype lineages of aphid were set up. Five

TABLE 4 Basic components of the complete-amino-acid diet for feeding aphids

Essential amino acid	Dose (mg/100 ml)	Nonessential amino acid	Dose (mg/100 ml)	Vitamin	Dose (mg/100 ml)	Mineral and sucrose	Dose (mg/100 ml)
Phenylalanine	40	Glutamate	140	Calcium pantothenate	5	Potassium dihydrogen phosphate	500
Methionine	80	Glutamine	150	Inositol	50	Magnesium chloride	200
Arginine	270	Aspartic acid	140	Ascorbic acid	100	Manganese chloride	0.26
Lysine	120	Asparagine	550	Choline chloride	50	Cupric chloride	0.14
Tryptophan	80	Cysteine	40	Biotin	0.1	Zinc chloride	0.28
Threonine	140	Alanine	200	Pyridoxine hydrochloride	2.5	Ferric citrate	0.67
Valine	80	Glycine	80	Folic acid	0.5	Sucrose	15,000
Isoleucine	80	Tyrosine	40	Thiamine hydrochloride	2.5	Deionized water	100,000
Leucine	80	Proline	80	Nicotinic acid	10		
Histidine	80	Serine	80				

genotype lineages (four from cotton [CA1 to -4] and one from cucumber [MA5]) were only infected with one species of facultative endosymbionts *Arsenophonus* (here called *Arsenophonus*-infected strains), and the other seven lineages (four from cotton [CA6 to -9] and three from cucumber [MA10 to -12]) were not infected with any known facultative endosymbionts (called *Arsenophonus*-free strains). All aphid lineages were infected with the obligate endosymbiont *Buchnera*. Aphid lineages were reared using leaves as their original host plant in petri dishes (diameter of 90 mm and height of 15 mm). The leaf in a dish was replaced with a fresh one every 3 to 4 days.

***Arsenophonus* elimination.** Selective elimination of *Arsenophonus* in aphids was conducted using antibiotics (400 μg/ml ampicillin, 200 μg/ml cephalosporin, and 200 μg/ml gentamicin) (49) added into the artificial diet (Table 4) containing 20 amino acids, vitamins, minerals, and sucrose (1, 50). Newborn nymphs were fed on the antibiotic diet for 6 days and then removed onto leaves of their original host plants. When antibiotic-treated aphids generated offspring, the mother and part of the offspring were chosen to examine the presence of *Arsenophonus* by PCR. These offspring from a mother whose *Arsenophonus* endosymbiont was cured were maintained on leaves in dishes as the *Arsenophonus*-cured lineage. All the 12 genotype aphid lineages infected or not infected with *Arsenophonus* were treated by antibiotics by the same method. These *Arsenophonus*-cured lineages were used for experiments after 20 generations had been maintained on leaves without antibiotics. Before experiments, these aphids were examined again in three successive generations in order to confirm the absence of *Arsenophonus*.

Examination of *Buchnera* density. The relative densities of *Buchnera* in aphids were measured by quantitative PCR (qPCR) based on the relative copy number of the *Buchnera groEL* gene to that of the *ef1*α gene of the aphid. The densities of *Buchnera* in the same genotype of aphids infected with *Arsenophonus* and eliminated from this symbiont by antibiotics were quantified before these genotypes were used in experiments. Total aphid genomic DNA was extracted from five 5-day-old aphids by the method of Zhang et al. (25). The qPCR was performed using SYBR *Premix Ex Taq* (TaKaRa) in an ABI 7500 real-time PCR system (Thermo Fisher Scientific). The forward primer *GroEL*-F (5′-GCCATCCAAAGCCGTATTAGTCA-3′) and the reverse primer *GroEL*-R (5′-AGTACCGCAACACCACCAGATA-3′) amplified a 116-bp fragment of the *groEL* gene with 107% efficiency. The forward primer *ef1*α-F (5′-TCACCATCATTGACGCACCTG-3′) and the reverse primer *ef1*α-R (5′-CCAGTACCAGCAGCAACGATAAG-3′) amplified a 103-bp fragment of the *ef1*α gene with 108% efficiency. The qPCR conditions were 95°C for 0.5 min, followed by 40 cycles of 95°C for 5 s, and 60°C for 34 s. All the melting curves had a unique peak. Standard curves (log concentration of DNA on the *x* axis and PCR cycle number on the *y* axis) for the *groEL* and *ef1*α genes were set up based on the method of Zhang et al. (25), and the gene copy number was calculated using the method described by Whelan et al. (51). The copy number of genes and the relative density of *Buchnera* in five *Arsenophonus*-infected lineages (CA1 to -4 and MA5) and their *Arsenophonus*-cured lineages reared for 20 generations were examined. The examination was performed for three biological replicates in each aphid lineage.

Detection of free amino acids in cotton and cucumber leaves. The free amino acids in seedling cotton and cucumber with five leaves were examined. Fresh leaves (0.2 g) were cut into pieces and ground well on ice with 1 ml 0.02 N hydrochloric acid solution. The grinding apparatus was washed using 0.02 N hydrochloric acid solution three times, and the solution mixture was filtered. The total extracted solution was adjusted to 10 ml using 0.02 N hydrochloric acid solution. The leaf extraction was maintained at 5°C for 24 h. Leaf extraction (0.8 ml) was transferred into a 2-ml microcentrifuge tube, and then 0.8 ml 4% aqueous sulfosalicylic acid was added, and the tube was shaken well. After 15 min, the mixture was centrifuged at 10,000 rpm at 4°C for 15 min, and the supernatant liquid was filtered with a 0.22-μm-pore aqueous-phase filter. One milliliter of filtrate was used to detect the concentrations of 16 free amino acids in a Hitachi Automatic Amino-Acid Analyzer L-8900 (Hitachi, Japan). Three replications were performed for each of the host plant leaves.

Aphid performance on the diet lacking an amino acid. We used the artificial diet described by Mittler (1) and Sun and Li (50) for rearing aphids (Table 4). The composition of amino acids in the diet was manipulated to assay the performance of aphids on a diet lacking a specific amino acid. The diet includes all 20 essential and nonessential amino acids and is considered the complete amino acid diet. One hundred fifty microliters of artificial diet was placed between two layers of thin Parafilm, which was fixed to one end of a glass tube (30 mm in diameter and 35 mm in height). Ten apterous adult aphids

were transferred onto the Parafilm in the tube, and then the other end of the tube was covered with one layer of Parafilm to prevent escape of aphids.

Ten newborn nymphs in a glass tube were left and considered the original cohort for the population life table. The survival and reproduction of the cohort were surveyed daily. When the original cohort reproduced, all offspring were recorded and then removed. The survey lasted until the death of the original cohort. During the survey, the artificial diet was replaced every 2 days. Life tables of 12 aphid genotypes were carried out on each artificial diet individually lacking phenylalanine (Phe), arginine (Arg), valine (Val), isoleucine (Ile), or leucine (Leu), and those amino acids were significantly different in titers between cotton and cucumber leaves as measured as described above. The aphids reared on the complete-amino-acid diet were the control. The experiments of the life table were conducted for all the *Arsenophonus*-infected, -free, and -cured aphid strains, and each strain experiment was replicated three times.

Data analyses. The relationship between endosymbiont status and genotype of aphids collected from cotton, cucumber, zucchini, and cowpea represented samples from more than 40 aphids, and the results were analyzed by Pearson's chi-square test. Effects of aphid genotype and *Arsenophonus* infection on the relative density of the obligate endosymbiont *Buchnera* in aphids were analyzed using the GLM model, and differences between *Arsenophonus*-infected and *Arsenophonus*-cured aphid lineages were analyzed using Student's *t* test. The titers of 15 free amino acids in cotton and cucumber leaves were analyzed by MANOVA in GLM to highlight the differences in host plants in amino acid contents, and the mean concentrations of each free amino acid between cotton and cucumber leaves were compared using the *t* test followed by the Bonferroni correction ($P < 0.05/15 = 0.003$). The life table parameters were calculated as net reproductive rate (R_0) = $\sum l_x m_x$, in which l_x is the proportion of individuals in the initial cohort alive at age x days, and m_x is the mean number of progeny produced per mother aphid alive on day x. The R_0s of seven aphid genotypes not infected with *Arsenophonus* (CA6 to -9 and MA10 to -12) and five genotypes infected with *Arsenophonus* (CA1 to -4 and MA5) on the complete diet were analyzed by ANOVA followed by the *post hoc* Tukey's test to distinguish the differences among different genotypes. The differences in R_0 between the *Arsenophonus*-infected and *Arsenophonus*-cured lineages were analyzed using Student's *t* test. The effects of aphid genotype and antibiotic treatment on the R_0 of *Arsenophonus*-infected and *Arsenophonus*-free aphids feeding on five amino-acid-deficient diets were analyzed using MANOVA in GLM, the R_0 on the completed diet was considered a covariant, and the R_0s on each amino-acid-deficient diet were considered multiple dependent variables. In order to eliminate the effect of *Buchnera* density on R_0, only data from three *Arsenophonus*-infected aphid genotypes CA2, CA3, and CA4 were used in this MANOVA, because these three genotypes had the same density of *Buchnera* before and after antibiotic treatment, and CA1 and MA5 were ruled out. All data analyses were performed using SPSS 25.0.

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We declare that we have no competing interests.

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