PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2011) **278**, 115–121
doi:10.1098/rspb.2010.1304
Published online 28 July 2010

Sources of variation in dietary requirements in an obligate nutritional symbiosis

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The nutritional symbiosis between aphids and their obligate symbiont, *Buchnera aphidicola*, is often characterized as a highly functional partnership in which the symbiont provides the host with essential nutrients. Despite this, some aphid lineages exhibit dietary requirements for nutrients typically synthesized by *Buchnera*, suggesting that some aspect of the symbiosis is disrupted. To examine this phenomenon in the pea aphid, *Acyrthosiphon pisum*, populations were assayed using defined artificial diet to determine dietary requirements for essential amino acids (EAAs). Six clones exhibiting dependence on EAAs in their diet were investigated further. In one aphid clone, a mutation in a *Buchnera* amino acid biosynthesis gene could account for the clone's requirement for dietary arginine. Analysis of aphid F_1 hybrids allowed separation of effects of the host and symbiont genomes, and revealed that both affect the requirement for dietary EAAs in the clones tested. Amino acid requirements were minimally affected by secondary symbiont infection. Our results indicate that variation among pea aphids in dependence on dietary amino acids can result from *Buchnera* mutation as well as variation in the host genotype.

Keywords: amino acid metabolism; nutritional symbiosis; *Acyrthosiphon pisum*; artificial diet; secondary symbiont; *Buchnera aphidicola*

1. INTRODUCTION

Nutritional symbiotic associations with bacteria are common in insects and have allowed many groups to exploit otherwise unsuitable food sources by synthesizing nutrients lacking in their host's diet [1]. Such relationships appear remarkably persistent over evolutionary time, with associations lasting hundreds of millions of years [2,3]. Over this duration, genetic drift and relaxed selection have led to extensive genome erosion, resulting in the loss of many genes in the genomes of bacterial symbionts [4]. Despite having some of the smallest known cellular genomes, these symbionts retain the biosynthetic capacity to produce the nutrients necessary to supplement the hosts' diet [5–8].

Buchnera aphidicola, the obligate bacterial symbiont of aphids, is one of the most extensively investigated nutritional symbionts. Experimental studies and genome sequences of *Buchnera* from several different aphid species have revealed that the symbiont synthesizes essential amino acids (EAAs) for the aphid that are limiting in the aphid's phloem sap diet [9,10]. *Buchnera* of distantly related aphid species retain most EAA biosynthesis genes [4,7,10,11]. Some losses may be associated with a shift in the aphid's diet, as is the case in *Buchnera* of *Schizaphis graminum*, which no longer reduces sulphur as part of cysteine biosynthesis, possibly owing to the availability of reduced sulphur in the phloem of the grasses the aphid feeds on [4]. Loss of functional amino acid biosynthesis genes in *Buchnera* might also be compensated for by the presence of secondary symbionts; in *Buchnera* of *Cinara cedri*, tryptophan appears to be synthesized cooperatively by *Buchnera* and the secondary symbiont *Serratia symbiotica* [12].

Intraspecific variation in amino acid requirements has been described in several species of aphid [13–15], suggesting that symbiont-produced nutrients are insufficient to sustain the host in some populations. One potential source of this variation is disruption of *Buchnera*'s ability to produce EAAs. This explanation has been proposed previously [14] but has not been tested. Differences among aphid genomes could also be the cause of this variation in dietary requirements, as host genes involved in amino acid synthesis, catabolism and transport, as well as genes that support the symbiont, may vary among aphid clones.

The source of variation in amino acid requirements has ramifications for the maintenance of this variation, aphid population dynamics, as well as its long-term effects on the evolution of this symbiosis. Loss of amino acid provisioning is irreversible in *Buchnera*, which does not recombine or acquire genes through lateral gene transfer [4]. Such loss may restrict the host range of the aphid, leading to population subdivision and divergence [16,17] or potentially to extinction of the lineage.

This study uses fitness assays to identify clones of the pea aphid, *Acyrthosiphon pisum*, which exhibit dietary requirements for EAAs. The contribution of *Buchnera* to this variation was determined by the examination of symbiont genes. The role of secondary symbionts was assessed both in a large panel of aphids with their naturally occurring symbionts as well as in aphid hosts with identical genetic backgrounds and different secondary symbionts. Finally, the fitness of F_1 hybrids was used to measure the contributions of host and *Buchnera* to the variation in aphid amino acid requirements.

2. MATERIAL AND METHODS

(a) *Aphid cultures*

Acyrthosiphon pisum clones were collected on various host plant species (referred to hereafter as 'host plant') from

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Electronic supplementary material is available at http://dx.doi.org/10.1098/rspb.2010.1304 or via http://rspb.royalsocietypublishing.org.

Received 17 June 2010
Accepted 6 July 2010

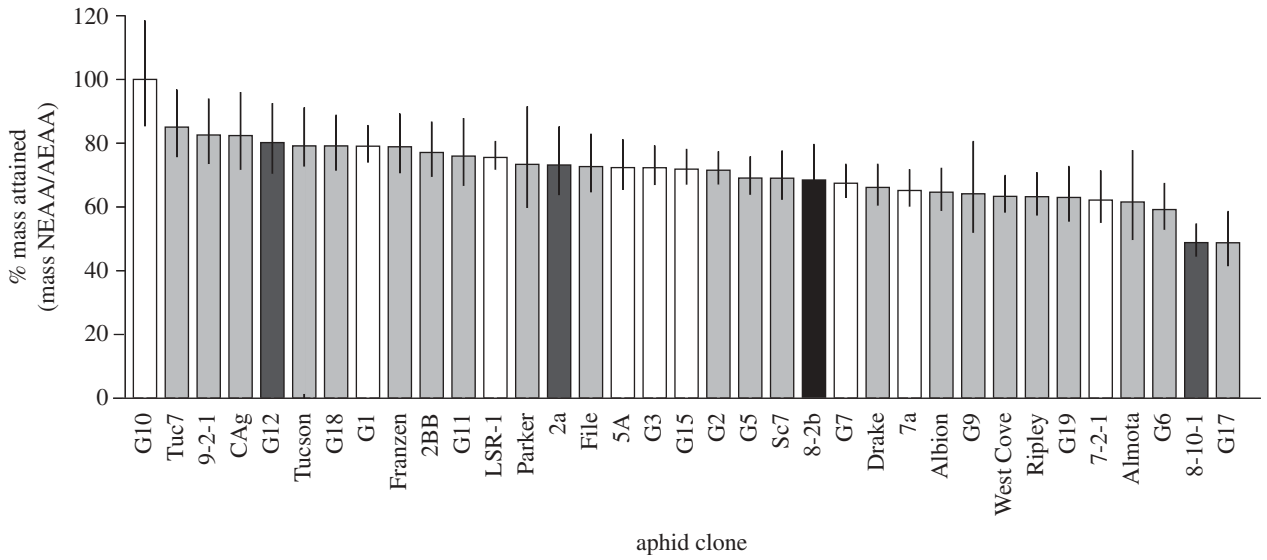

Figure 1. EAA-dependence of aphid clones presented as relative adult mass on diet without and with essential amino acids (AEAA versus NEAA). Aphid clones showed 0–52% average decrease in mass on the NEAA diet. Error bars indicate 95% CI of the mean. Shade of bars indicates secondary symbiont type: open bars, uninfected; black bars, infected with *Hamiltonella defensa*; light grey bars, infected with *Serratia symbiotica*; dark grey bars, infected with *Regiella insecticola*.

locations around the United States between 1999 and 2008 (electronic supplementary material, table S1). Each of the 35 clonal lineages was derived from single parthenogenetic females then divided into two to four sublines for at least three generations prior to experiments. These were reared on *Vicia faba* seedlings under 16 L : 8 D at 20°C in an environmental chamber.

(b) *Artificial diet and feeding assays*

Aphids were fed on an artificial diet to reveal dietary requirements for EAAs. The all-essential-amino acids (AEAA) diet was formulated after the AP3 diet of Febvay *et al.* [13], which contains sucrose, amino acids, vitamins and trace elements. The no-essential-amino acid (NEAA) diet was created by omitting all EAAs and cysteine from the AP3 diet and increasing the amounts of non-EAAs proportionally until the total nitrogen concentration was equivalent to that of the AEAA diet. Diets lacking individual EAAs were created by omitting a particular EAA and balancing total nitrogen by the addition of equal molar concentrations of glutamate. Adult *A. pisum* were placed on fresh *V. faba* seedlings 24 h prior to experimental set-up and allowed to deposit nymphs. Artificial diet feeding was a microtitre plate system with one to two nymphs and 300 µl of artificial diet per well [18]. Plates were kept at conditions described for the aphid cultures. After 7 days, individual aphids were weighed on a microbalance and their mass used as a proxy for fitness (electronic supplementary material, figure S1). Mass at birth was not measured, and variation in initial mass probably contributed to the variation in adult mass. All clones were tested on both the AEAA diet and the NEAA diet. A subset of clones was tested on diet lacking individual EAAs. For all dietary assays, clones were subject to feeding treatments twice independently, with a total of at least 30 aphids measured for each clone × treatment pair.

(c) *Sequencing of* Buchnera *genes*

Genomic DNA was isolated from clones exhibiting requirements for EAAs in the individual amino acid deletion assay; specifically these were clones 8-10-1, G17, G6 and G19. PCR was performed to amplify *Buchnera* genes underlying the biosynthesis of the required amino acid genes; PCR products were directly sequenced (electronic supplementary material, methods and table S2).

(d) *Secondary symbiont screening*

Presence of secondary symbiont was determined by PCR screen using symbiont-specific diagnostic primers that produce amplicons spanning the intergenic region between the 16S and 23S ribosomal RNA subunits. Forward primers (1279F_Ss, 1279F_Ri and 1279F_Hd) specific for each secondary symbiont were used along with a universal primer (35R) [19].

(e) *Establishment of F_1 clones*

To determine whether *Buchnera* mutations were responsible for fitness differences observed on the NEAA diet, clones 5A and 8-10-1 were sexually induced using the method of Moran & Dunbar [20] (electronic supplementary material, methods). Fundatrices were collected and initially reared on *V. faba* and *Medicago sativa* shoots. Single fundatrices were used to establish clones corresponding to independent genotypes for each direction of the sexual cross and were allowed to reproduce asexually for three generations prior to use in feeding experiments.

(f) *Statistical analyses*

For all experiments, aphid mass was normalized by natural log transformation and then subjected to a least-squares means analysis in JMP7 to determine significant effects within the model. For the AEAA versus NEAA comparisons and the individual amino acid deletion assays, significant differences between and within clones, respectively, were determined by a Tukey's HSD test. Significant differences within clones on the AEAA and NEAA diets were determined by a Student's *t*-test.

3. RESULTS

(a) *Aphid performance on AEAA and NEAA diets*

Aphid performance was substantially different between diets with and without EAAs (figure 1 and electronic supplementary material, table S3). Compared with their

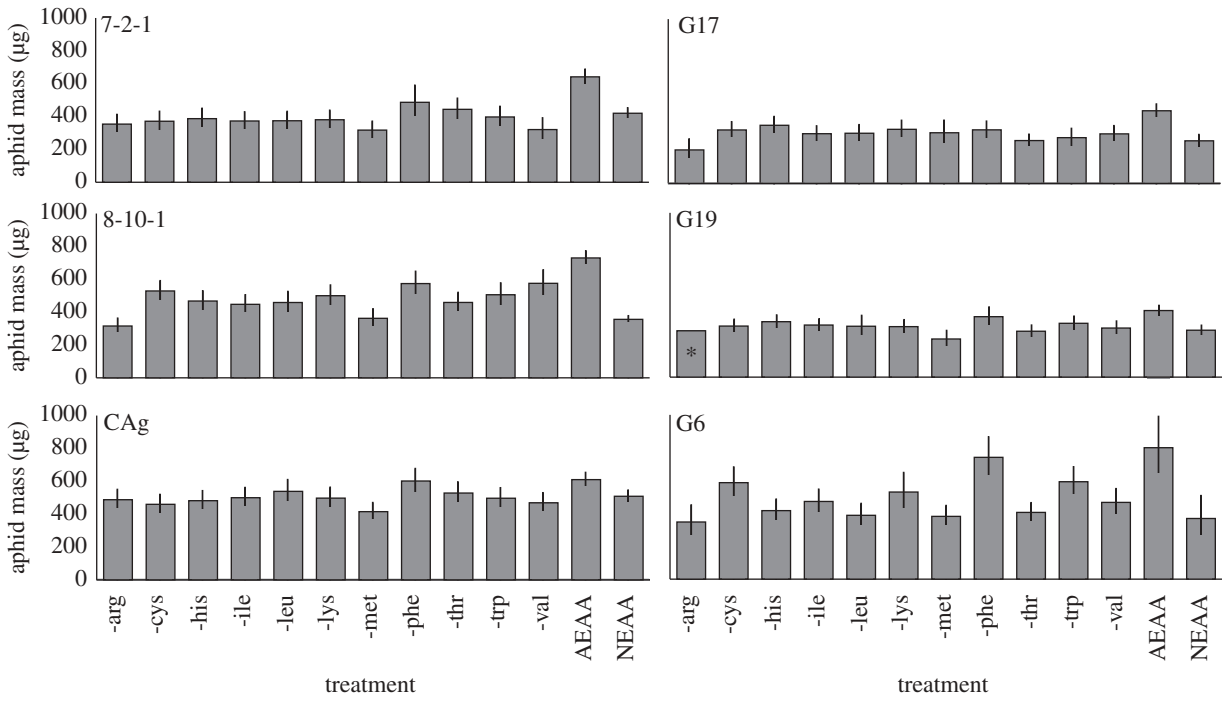

Figure 2. Adult mass for selected clones on diets lacking individual EAAs. Clone names appear in the upper left of each graph. X-axis shows the amino acid omitted from the diet. Error bars represent 95% CI of the mean. The asterisk denotes that only a single aphid from G19 survived the duration of the experiment.

mass on the AEAA diet, clones lost between 0 and 52 per cent of individual adult mass on the NEAA diet. Between-clone variation in individual adult mass attained on the NEAA diet relative to the AEAA diet was effectively continuous. Clones varied in dependence on EAAs in the diet, as indicated by a significant clone × diet interaction (electronic supplementary material, table S3; $F_{34,4064} = 14.1$, $p = 6.3\ \ 1.0 \times 10^{-16}$). The effect of the host plant by treatment was significant but minor, and probably reflects differences in the aphid genotype rather than an effect of the plant, as all clones were reared on *V. faba* prior to feeding assays (electronic supplementary material, methods and table S3).

Six clones exhibiting major loss of mass (greater than 37%) on the NEAA relative to the AEAA diet were G17, 8-10-1, G6, Almota, 7-2-1 and G19 (referred hereafter as EAA-dependent). These EAA-dependent clones were subjected to feeding assays using diets lacking individual EAAs to determine if specific amino acids were responsible for EAA-dependence (figure 2 and electronic supplementary material, figure S2). Clone CAg was also tested as controls for performance on these diets. When tested on a diet lacking individual EAAs, a significant effect of clone × treatment was observed (electronic supplementary material, table S3; $F_{60,1774} = 3.3$, $p = 3.5 \times 10^{-16}$) indicating variation among clones in the particular EAAs required in the diet. Elimination of individual amino acids from the diet affected all aphids tested, and most such eliminations resulted in significant decreases in aphid mass ($p < 0.01$, Tukey's HSD). We chose to focus on amino acids whose elimination had a major effect on aphid mass (see §4 for more information), particularly cases in which mass on individual amino acid deletion diets was no more than 102 per cent of mass on the NEAA diet. Using this criterion, several clones were dependent on arginine, while methionine, threonine, leucine, valine and histidine were each needed by at least one clone. Most clones required one to two EAAs. Clone G6 exhibited much greater variation in performance on individual amino acid deletion diets. Six of the diets lacking individual EAAs reduced G6's mass by more than 55 per cent.

(b) *Examining polymorphisms in* Buchnera *to identify causes of EAA-dependence*

To determine if biosynthetic capabilities of *Buchnera* caused the variation in dependence on particular EAAs, we sequenced the corresponding *Buchnera* biosynthetic genes and itemized all polymorphisms (table 1 and electronic supplementary material, table S4). Of 89 polymorphic sites, two were single base insertions/deletions, one in *argC* of 8-10-1 and another in the upstream region of *thrB* in both clones examined (G19 and G6).

Clone 8-10-1 had an insertion of a thymidine at the 610th base pair of *argC*, causing a frameshift and a premature stop codon at amino acid 206. *argC* encodes *N*-acetyl-gamma-glutamyl phosphate reductase, which catalyzes the third step in ornithine biosynthesis [21]. Inactivation of this gene would inhibit biosynthesis of ornithine, an essential precursor of arginine. All other gene sequences for all other clones lacked any obvious mutations (frameshift or nonsense), which would inhibit translation of the gene product.

Of the 87 single nucleotide changes, six were located in the region immediately upstream of a coding region, although none was found in a −10/−35 promoter region as identified by BPROM (http://www.softberry.com), suggesting that these mutations have no effect on gene expression. Of mutations in coding regions for which more than one clone was sequenced, 19 were

Table 1. Summary of mutations in selected regions of EAA-dependent *A. pisum* clones.

total mutations	89
SNPs[a]	87
indels	2
coding region	83
upstream	6
synonymous	62
non-synonymous	21
unique[b]	19
all lines[c]	16
segregating[d]	43

[a]All mutations are called relative to the genome of *Buchnera aphidicola* str. APS.
[b]Found only in a single clone examined, does not include mutations from *pLeu* of G6 as only this clone was sequenced for this region.
[c]Found in all four EAA-dependent clones.
[d]Found in more than one clone but not all clones examined.

unique to a single clone, and 16 were shared by all four EAA-dependent clones relative to the reference genome of *Buchnera* APS (NC_002528).

Of the 87 observed changes, 21 were non-synonymous mutations, but most resulted in a conservative amino acid change. Only a single mutation created a major change in the coded amino acid, defined as a penalty of −3 or more in the amino acid matrix of Muller *et al.* [22]. An A → T transversion in *argE* of the related matrilines 8-10-1 and 5A [23] changed an asparagine to isoleucine, but 5A is not an EAA-dependent clone, suggesting that this mutation has little effect. The other non-synonymous mutations involved less drastic changes in the amino acid; five had penalties of −1 to −2, and 15 had 0 or positive values in the matrix.

(c) *Effect of secondary symbiont infection on host EAA-dependence*

Among tested clones, one (8-2b) was infected with *Hamiltonella defensa*, four clones were infected with *Regiella insecticola* and 21 clones were infected with *S. symbiotica* (figure 1). *Serratia symbiotica* or *R. insecticola* did not differentially impact aphid mass on the NEAA and AEAA diet (electronic supplementary material, table S3; $F_{2,31} = 0.895$, $p = 0.42$). The impact of *H. defensa* could not be assessed as only one clone was infected.

To directly assess the effects of secondary symbionts on aphid EAA-dependence, sublines of a single clone (5A) artificially infected with each of the three secondary symbionts were tested on AEAA and NEAA diets (electronic supplementary material, figure S3). The three symbionts had different effects on aphid mass. Regardless of the treatment (AEAA or NEAA diet), sublines infected with *H. defensa* or *R. insecticola* were significantly smaller than *S. symbiotica*-infected or uninfected sublines ($p < 0.05$, Tukey's HSD). There was a significant interaction between diet and treatment (electronic supplementary material, table S3; $F_{3,1069} = 3.2$, $p = 0.022$), although the difference in per cent mass attained between clones was small (12%; electronic supplementary material, figure S2). Taken together, these data suggest that secondary symbionts exert a minimal effect on their host's EAA-dependence.

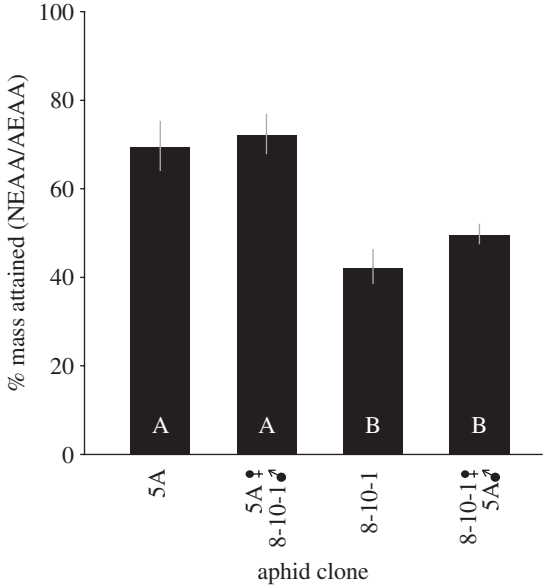

Figure 3. EAA-dependence of aphid clones representing full siblings from a sexual cross. Aphids from 8-10-1f × 5Am contain *Buchnera* with inactivated *argC*; aphids from 5Af × 8-10-1m contain *Buchnera* with intact *argC*. Error bars represent 95% CI. Different letters within bars indicate significant differences ($p < 0.05$, Tukey's HSD).

(d) *Effect of* Buchnera *genotype on EAA-dependence*

Since *Buchnera* is exclusively maternally transmitted, sexual crosses of *A. pisum* clones can be used to change the host genetic background independently of *Buchnera* genotype. In such crosses, F_1 progeny will be 50 per cent related on average at aphid loci and will contain the same *Buchnera* of the maternal clone. Clone 5A was reciprocally mated with clone 8-10-1 to assess whether the observed mutation in *Buchnera* of 8-10-1 was responsible for the clone's decrease in fitness on the NEAA diet.

Clones from both sexual crosses (8-10-1f × 5Am or 5Af × 8-10-1m) showed a significant decrease in mass on the NEAA diet when compared with the AEAA diet (8-10-1f × 5Am: $t\text{-ratio}_{1144} = -26.91$, $p = 1 \times 10^{-15}$; 5Af × 8-10-1m: $t\text{-ratio}_{718} = -10.48$, $p < 1 \times 10^{-15}$, Student's *t*-test), as did both parental clones (figure 1). The offspring of the 5Af × 8-10-1m cross had an average 28 per cent decrease in mass (figure 3), which is not significantly different from that observed for the parental 5A clone when tested simultaneously, but significantly different from parental 8-10-1, which lost an average of 56 per cent mass on the NEAA diet ($p < 0.05$, Tukey's HSD). The clones from the 8-10-1f × 5Am cross exhibited a 46 per cent decrease on average when reared on the NEAA diet, which was similar to the decrease observed for the 8-10-1 parental clone but significantly greater than the performance of the 5A parental clone ($p < 0.05$, Tukey's HSD). Thus, the *argC* mutation in *Buchnera* of 8-10-1 was a major factor in the EAA-dependence of the F_1 hybrids from the 8-10-1 matriline. The offspring of both crosses and 8-10-1 parental clones attained similar mass on the AEAA diet (data not shown).

(e) *Effect of host genotype on EAA-dependence*

Clones from the same cross showed highly significant differences in EAA-dependence (figure 4 and electronic

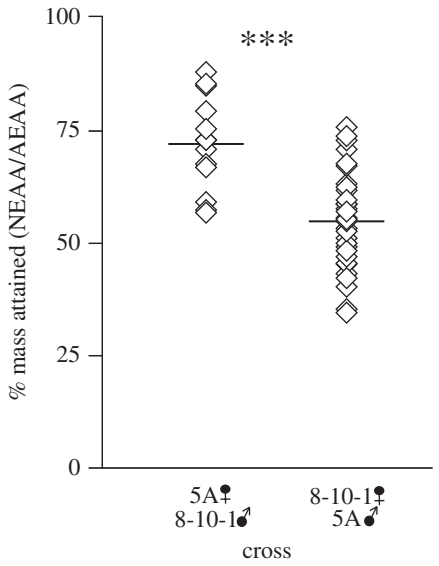

Figure 4. Effect of aphid genotype on EAA-dependence of F_1 hybrids. Diamonds indicate the mean value for each F_1 genotype within each direction of the cross. The horizontal lines denote the mean per cent mass attained for all genotypes from each direction of the cross. ***$p = 1.1 \times 10^{-15}$, standard least squares.

supplementary material, table S3; $F_{47,2153} = 12.5$, $p = 5.9 \times 10^{-79}$, standard least squares). Clones from the 8-10-1f × 5Am cross lost between 25 and 67 per cent of adult mass on the NEAA diet, while clones from 5Af × 8101m cross lost 12–43% of adult mass on the NEAA diet. These data imply that the *Buchnera argC* mutation does cause EAA-dependence, but that host-encoded factors also play a substantial role.

4. DISCUSSION

Aphid clones used in this study varied in requirements for dietary EAAs. In one instance, increased EAA-dependence reflected an inactivating mutation in an amino acid biosynthesis gene of *Buchnera*, but such inactivating mutations were absent in three of four clones exhibiting major EAA-dependence. The one inactivating mutation observed was confirmed as a factor in EAA-dependence through crossing experiments in which the *Buchnera* with the mutation were established in novel host-genetic backgrounds. However, different F_1 genotypes with identical *Buchnera* varied substantially in performance, implying that host genotype also contributes to EAA-dependence. Host genotypes are probably the source of variation among other aphid clones examined in these experiments, as no other mutations were found in *Buchnera* genes that could explain their requirement for dietary amino acids.

Elimination of individual EAAs from the diet significantly reduced aphid mass in almost all cases, suggesting that such elimination imposes an energetic or metabolic cost to the aphid–symbiont pair, even when *Buchnera* has the capacity to make the missing amino acid. Consistent with this hypothesis, the complete *Buchnera* genome of clone 8-10-1 exhibits no sequence differences in genes underlying any amino acid biosynthesis pathway except for the frameshift in *argC* found in this study [23], yet 8-10-1 showed small decreases in mass on diets lacking individual amino acids other than arginine.

Previous studies have failed to reveal an obvious connection between infection with secondary symbionts and aphid performance on artificial diets [24], though secondary symbiont infection appears to exacerbate deleterious effects of sub-optimal host plants on aphids [25]. Our experiments, carried out for three common secondary symbiont types infecting the same *A. pisum* clone, revealed no major impact of secondary symbionts on aphid amino acid requirements, though secondary symbionts did significantly reduce aphid mass on the AEAA diet. These results suggest that the impact of secondary symbionts on aphid fitness may not be related to amino acid nutrition. Population cage studies have shown that, in the absence of parasitoid pressure, the frequencies of *H. defensa* and *S. symbiotica* decrease, suggesting that infection with these symbionts imparts a cost to the aphid [26]. The recently completed genome sequences of *H. defensa* and *R. insecticola* revealed the absence of most EAA biosynthesis pathways, implying that these symbionts obtain these nutrients from the aphid's free amino acid pool [27,28].

In clone 8-10-1, the need for dietary arginine along with the frameshift in *argC* suggests that *Buchnera* cannot produce this EAA. While some eukaryotes have evolved the ability to synthesize arginine from aspartate and citrulline as part of the urea cycle, the *A. pisum* genome lacks genes encoding arginosuccinate synthase and arginosuccinate lyase, the two enzymes necessary to synthesize arginine in this manner [29]. Analyses of arginine levels in the phloem sap of several host plants of *A. pisum* reveal it to be one of the most abundant EAAs in the phloem sap of all host plants tested [30]. Phloem arginine levels in *V. faba* are unlikely to completely compensate for disrupted arginine biosynthesis by *Buchnera*, but the relatively higher level of this amino acid may reduce selection against the loss of arginine biosynthesis enough to allow fixation of this inactivating mutation in laboratory cultures. In wild populations, selection against such mutations is probably stronger and effective population sizes larger, resulting in more efficient purging of mutations in amino acid biosynthesis genes in *Buchnera*.

Recent re-sequencing of *Buchnera* genomes from *A. pisum* lineages has revealed that the symbiont's mutation rate is an order of magnitude higher than that of free-living relatives such as *Escherichia coli* [23]. This high mutation rate is counteracted by host-level selection that acts to maintain *Buchnera*'s amino acid production capabilities [31,32]. This study has shown that differences among *A. pisum* clones in dietary amino acid requirements can result from differences in the amino acid biosynthesis genes of *Buchnera*, but often reflect genetic variation in the aphid hosts. The underlying host genes could encode products that interact with the symbiont [33] or that affect chemosensory abilities, as certain amino acids, notably methionine, are known phagostimulants of *A. pisum* [34].

The variation in the other *A. pisum* clones' dietary requirements may affect their ability to use different plants, and thereby their population structure. Host plants of *A. pisum* vary in their phloem amino acid concentrations, and aphid performance is partly associated

with plant phloem amino acid levels [30]. Adaptation by either *A. pisum* or *Buchnera* to the phloem profile of specific host plants could help to affect the establishment of host races of *A. pisum* [35].

The authors would like to thank J. Stavrinides for assistance in developing the feeding assay, N. Gerardo and S. Eigenbrode for collection of aphid clones, K. Hammond, K. MacDow, K. Z. Sunitsch and E. Hartley for help in data collection and aphid maintenance, and three anonymous reviewers for their help in improving the manuscript. This work was funded by NSF grant 0723472 to N.A.M. K.J.V. was supported on an NSF IGERT fellowship and an NSF G-K12 fellowship during this research.

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