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The impact of host plant on the abundance and function of symbiotic bacteria in an aphid

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Summary

The black-bean aphid *Aphis fabae* bears populations of coccoid symbiotic bacteria *Buchnera* spp. at 2.0–3.2×10^{7} cells mg^{-1} aphid mass and rod-shaped secondary symbionts of uncertain taxonomic affiliation at 0.1–0.6×10^{7} cells mg^{-1} aphid mass. *Buchnera* provides essential amino acids, supplementing the poor supply in the aphid diet of plant phloem sap. Comparison of the performance of *A. fabae* containing and experimentally deprived of their bacteria showed that the bacteria caused increased larval mass of aphids reared on *Chenopodium album* and *Papaver dubium* plants, but not when reared on *Lamium purpureum*. In the aphids reared on *L. purpureum*, the density of the bacteria, especially the secondary symbionts, was significantly elevated, and bacterial-mediated production of the essential amino acid threonine was reduced, even though the essential amino acid content of phloem exudates from *L. purpureum* had a low threonine content. It is proposed that the shortfall in threonine, possibly compounded by the high density of secondary symbionts, may contribute to the poor performance of the aphids on *L. purpureum*. This study offers the first evidence to suggest plant-mediated interference with the nutritional function of symbiotic bacteria in any phytophagous insect.

Key words: aphid, *Aphis fabae*, symbiosis, *Buchnera* spp., threonine, essential amino acid, plant resistance.

Introduction

The utilization of plants by many taxa of phytophagous insects is promoted by symbiotic micro-organisms that provide the insect with nutrients or detoxify plant allelochemicals (Buchner, 1966; Douglas, 1989; Dowd, 1991). It has been proposed that these micro-organisms may also influence the plant range of phytophagous insects. They may expand the plant range of insects by improving insect utilization of otherwise marginal plants (Jones, 1984), or restrict it, either because they are susceptible to anti-microbial compounds in certain plant taxa (Jones, 1984; Dowd, 1991) or because their nutritional function is of advantage only to insects on plants of particular nutrient profiles (Douglas, 1998; Douglas, 2000). However, it is generally difficult to explore the interaction between plant utilization by insects and the microbiota of insects because the function of the micro-organisms in most phytophagous insects is little understood and techniques to study and manipulate the symbioses are poorly developed.

One group of phytophagous insects, the aphids, are exceptional in that the basic biology of their bacterial symbiosis is now established (Baumann et al., 1995; Douglas, 1998). The microbiology of virtually all aphids is dominated by one bacterium, a γ-Proteobacterium of the genus *Buchnera* (Munson et al., 1991), which is obligately vertically transmitted *via* the ovaries of the female aphid and is located in specialised cells, bacteriocytes, in the insect haemocoel (body cavity) (Buchner, 1966). *Buchnera* has a nutritional function, specifically providing the insect with essential amino acids (Douglas, 1998) that the insect cannot synthesize and are in short supply in the aphid diet of plant phloem sap (Douglas, 1993; Sandström and Pettersson, 1994; Sandström and Moran, 1999). This function is reflected in the gene complement of *Buchnera* which, despite large-scale genomic reduction compared to related free-living bacteria (Charles and Ishikawa, 1999; Wernergreen et al., 2000), bears most genes for essential amino acid synthesis (Shigenobu et al., 2000). Although the aphid–*Buchnera* symbiosis remains technically difficult in some respects (e.g. *Buchnera* is unculturable, and the symbiosis cannot be re-synthesized from the separate partners), an array of methods is now in place to assess nutritional function (e.g. Febvay et al., 1995; Febvay et al., 1999), quantify the population of *Buchnera* in an aphid (e.g. Baumann and Baumann, 1994; Humphreys and Douglas, 1997) and to eliminate the *Buchnera* from the symbiosis using antibiotics at doses that have minimal nonspecific effects on aphid feeding or metabolism (Wilkinson, 1998). In addition, the recently completed genome sequence of *Buchnera* (Shigenobu et al.,

2000) provides the basis for genomic approaches to explore *Buchnera* function.

Many aphids bear one to several bacterial taxa, phylogenetically distinct from *Buchnera* and informally known as ‘secondary symbionts’, associated with the bacteriocytes or free in the insect haemocoel (Buchner, 1966; Douglas, 1989). No specific function has been attributed to the secondary symbionts, and they are not universal in some aphid species reported to bear them (Chen and Purcell, 1997; Fukatsu et al., 2000; Sandstrom et al., 2001; Darby et al., 2001). The secondary symbionts are eliminated by the antibiotic treatments used to generate *Buchnera*-free aphids, and aphids deprived of their symbiotic bacteria are termed aposymbiotic aphids, as distinct from the symbiotic aphids containing their ‘normal’ complement of bacteria.

We have selected the black bean aphid *Aphis fabae* Scop. as an experimental system to investigate the impact of plant species on the aphid–bacterial symbiosis. The parthenogenetic morphs of this species are polyphagous, with 80–100 species recorded as host plants in the field (Stroyan, 1984; Thieme, 1987); the host plants include several crops, e.g. sugar beet, broad bean (Blackman and Eastop, 1984). In our first analysis (Adams and Douglas, 1997), the capacity of symbiotic and aposymbiotic *A. fabae* to utilize a panel of 16 plants, including species recorded as hosts and non-hosts in the field, was compared. The aposymbiotic aphids performed poorly on all plant species, and symbiotic aphids performed significantly better than the aposymbiotic aphids on many, but not all, of the plants. These data suggest that the symbiotic bacteria in *A. fabae* do not generally enhance utilization of marginal hosts by this aphid, but the data are compatible with a role for the bacteria in defining the limits to its plant range.

The purpose of this study was to identify the basis for the restriction of bacterial-mediated promotion of aphid performance to certain plant species. We focused particularly on one plant, the labiate *Lamium purpureum* (‘red dead nettle’), which is occasionally colonised by *A. fabae* in the field (Blackman, 1974; A. E. Douglas, unpublished observations) and supports poor performance of symbiotic aphids in the laboratory (Adams and Douglas, 1997) and field (Raymond et al., 2000). The principal objective was to explore whether the population or nutritional function of the symbiotic bacteria are suppressed in *A. fabae* on *L. purpureum*, by comparison with two alternative host plants, *Chenopodium album* (‘fat hen’) and *Papaver dubium* (‘long-headed poppy’), which support vigorous colonies of *A. fabae* in the field. Although the focus of this study is the nature of the interactions between insect–bacterial symbioses and plants, this area of research may also offer insight into factors promoting crop resistance to the many insect pests with symbiotic bacteria.

Materials and methods

The plants and aphids

The plants, *Chenopodium album* (family Chenopodiaceae), *Lamium purpureum* (Lamiaceae), *Papaver dubium* (Papaveraceae) and *Trifolium repens* (Leguminosae) were grown from seed (Herbiseed, UK) in medium nutrient compost (John Innes F2) under glass at 20±3 °C and used as pre-flowering plants at 3–4 weeks post-sowing. The experimental aphids were *Aphis fabae* Scop. subspecies *fabae* clone HR91/3, originally collected from a pre-flowering *Vicia faba* crop in 1991 and maintained on *V. faba* cv. The Sutton at 20 °C with an 18 h:6 h light:dark regime. Clone HR91/3 bears both *Buchnera* and secondary symbionts.

Each experiment was initiated by allowing adult apterae from the culture on *V. faba* to larviposit over 24 h (day 0 to day 1) on a chemically defined diet of formulation A ($0.15\,mol\,l^{-1}$ amino acids and $0.5\,mol\,l^{-1}$ sucrose; Prosser and Douglas, 1992) with or without the antibiotic rifampicin at $50\,\mu g\,ml^{-1}$ diet (Rahbé et al., 1994). 1 day later, replicate groups of larvae (described as 2 days old) were transferred to the test plants. Unless stated otherwise, they were harvested 7 days later (when 9 days old). Light microscopical analysis of serially sectioned 9-day-old aphids confirmed that the symbiotic bacteria *Buchnera* in the rifampicin-treated aphids were disrupted (see Results), i.e. these insects were aposymbiotic.

Aphids were individually weighed to the nearest µg on a Mettler MT5 microbalance. For analysis of the intrinsic rate of population increase (r_m), the aphids were caged individually in clip-cages (3 cm diameter), and the time from birth to reproduction (the prereproductive period, T) and number of offspring deposited in the time equivalent to T (M_d) were quantifed. r_m was calculated as $0.738(\ln M_d)/T$ (Wyatt and White, 1977).

Quantification of Buchnera *in aphids*

The symbiosis was assessed in nine serially sectioned symbiotic and aposymbiotic aphids from each plant species, for which none of the sections had been damaged or lost during preparation. Each aphid was fixed in modified Bouin-Dubosq solution (Ponsen, 1976), embedded in Histoplast paraffin wax, sectioned (8 µm) and stained with Gills Haemotoxylin and alcoholic Eosin (Kiernan, 1981).

Embryos were dissected from aphids in ice-cold $50\,mmol\,l^{-1}$ Tris-HCl, pH 7.5, using fine pins at ×100 magnification, and carefully separated from surrounding maternal tissues with multiple washes in Tris-buffer. To quantify the density of bacteria, each aphid or embryo preparation was homogenized in a known volume of ice-cold $50\,mmol\,l^{-1}$ Tris-HCl, pH 7.5, with $0.25\,mol\,l^{-1}$ sucrose and 3.9×10^7 polystyrene ‘ultraspheres’ (2.1 µm diameter) ml^{-1} (final density). Eight replicate samples of each homogenate were examined by phase-contrast microscopy at ×1000 magnification, and the numbers of bacterial cells and ultraspheres in a single field of view per sample were scored. The volume of homogenate per field of view was calculated from the number of ultraspheres scored, and this was used to transform the number of bacteria scored to density per unit homogenate volume. The mass of the embryos could not be quantified accurately (Douglas, 1996) and therefore, for comparisons of the bacterial

populations in the total aphid tissues and embryos, bacterial density was expressed per unit of aphid protein. The protein content of each homogenate was quantified using a protein assay kit from BioRad Chemical Co., following the manufacturer's instructions for the microassay, with bovine serum albumin as standard.

Amino acid analysis

The amino acids in plant phloem exudates, aphid honeydew and methanol extracts of aphids were quantified. The phloem sap of plants was sampled by the EDTA exudation technique (King and Zeevart, 1974) as modified by Douglas (Douglas, 1993). Briefly, the top fully expanded leaf from 10 plants of each species was excised with sharp scissors and its cut surface was immediately immersed in 0.2 ml 5 mmol l^{-1} Na_2EDTA solution and incubated at 25 °C for 90 min in the dark with a saturated solution of KH_2PO_4 to maintain high humidity. Honeydew deposited over 2 days onto circles of tinfoil placed below groups of ten 7- to 9-day-old aphids was collected into known volumes of filter-sterilized distilled water; the tinfoil was then air-dried, and the weight of honeydew collected determined from the difference in mass of the tinfoil before and after honeydew collection. Individual pre-weighed 9-day-old aphids were homogenized in 0.1 ml ice-cold 80 % methanol in a glass hand-held tissue grinder; the homogenate was centrifuged at 20,000 ***g*** for 15 min and the supernatant was retained. All samples were frozen at –20 °C prior to analysis.

Amino acids in the samples were derivatized with *o*-phthaldialdehyde (Jones et al., 1981) and separated by reverse-phase high-pressure liquid chroamtography (HPLC) on a Beckman HPLC System Gold delivery system using a C_{18}-ultrasphere column and Shimadzu RF-551 fluorescence detector. The reference amino acids were AA-S-18 (Sigma Chemical Co.) supplemented with asparagine, glutamine and tryptophan. All protein amino acids except proline and cysteine can be detected by this methodology. In some analyses, certain amino acids with similar elution times could not be discriminated with complete confidence and they are displayed as amino acid pairs: histidine + serine, alanine + tyrosine and tryptophan + methionine.

Radiochemical analysis

More than 90 % of the amino acids in *A. fabae* are in protein, the amino acid composition of which does not vary with diet or aposymbiosis (Douglas et al., 2001). To quantify the incorporation of essential amino acids synthesized by the symbiotic bacteria into protein, the non-essential amino acid [U-^{14}C]glutamic acid (1 mCi ml^{-1} in 2 % ethanol, ICN Radiochemicals Ltd; purity confirmed by thin-layer chromatography) was injected into 8-day-old aphids reared on each plant species. Each aphid was immobilised on its back by suction from a vacuum and 50 nl of radioactivity was administered *via* the lateral abdomen with a fine glass needle attached to a fixed-dose Nanoliter Injector (World Precision Instruments). The aphids were returned to the plant and harvested 24 h later, when the amount of radioactivity incorporated into the protein fraction was maximal (A. E. Douglas and L. B. Minto, unpublished results).

To analyse the distribution of radioactivity among protein-bound amino acids in the aphids, each replicate group of 5–10 aphids was homogenized in ice-cold 50 mmol l^{-1} Tris-HCl, pH 7.5, incubated with 10 % trichloroacetic acid (TCA) for 30 min on ice and centrifuged at 14,000 ***g*** for 15 min at 4 °C. The pellet was washed twice with 10 % TCA and once with ether, and then dried and hydrolysed in a vacuum-sealed ampoule with 6 mol l^{-1} HCl at 110 °C for 24 h, to hydrolyse the protein. The hydrolysate was neutralised with NaOH, dried, dissolved in 80 % methanol and analysed by HPLC (as above). The eluate from the HPLC delivery system was collected with a fraction collector (30 s per sample) and each sample was mixed with 4 ml scintillation fluid (Ultima Gold XR, Packman). Radioactivity was quantified in a scintillation counter (Tri-Carb Packard) with preset ^{14}C windows and quench curve. The counts obtained for control aphids injected with 50 nl 2 % ethanol were subtracted from the experimental data. The amino acid identifications were confirmed by thin layer chromatography (TLC) with autoradiography, following the procedure of Wang and Douglas (Wang and Douglas, 1999).

Statistical methods

The data sets were analysed by parametric statistical tests after confirmation of normal distributions with homogenous variances by the Kolmogorov–Smirnoff one-sample test and Bartlett's test, respectively. Logarithmic and arcsine-square root transformations were required where shown. One-way analysis of variance (ANOVA) was used to investigate the impact of plant species on numbers of bacteriocytes in the maternal tissues of symbiotic aphids, the threonine content of phloem exudates and aphid honeydew, and the incorporation of radioactivity from ^{14}C-glutamate into amino acids. The effects of rearing plant and antibiotic treatment on aphid amino acid content was explored by two-way ANOVA, and the effects of rearing plant, type of bacteria (*Buchnera* or secondary symbionts) and aphid tissue (total or embryos) on bacterial density were tested by three-way ANOVA. A Bonferroni correction for multiple tests (α'=0.05) were included where appropriate and Tukey's honestly significant difference method was adopted for pairwise comparisons that contributed to significant ANOVA differences.

The amino acid composition of phloem exudates from the three test plant species was compared by multivariate analysis of variance (MANOVA) of balanced, single-factor design with log-transformed data (nmol amino acid exuded per sample) as the dependent variable. Principal components analysis (PCA) was performed with the relative amount of each amino acid (mol %) and correlation matrix to standardize variables.

Results

Aphid performance

The three test plant species, *Chenopodium album*, *Lamium purpureum* and *Papaver dubium* were colonised by the 2-day-

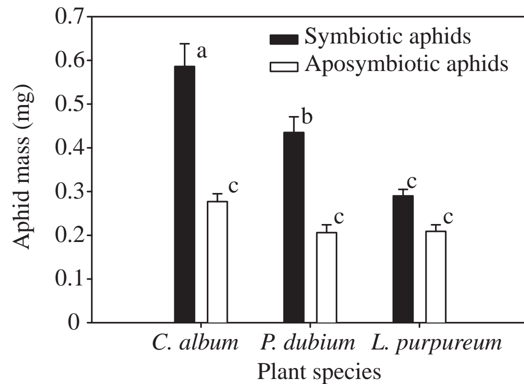

Fig. 1. The mass of 9-day-old larvae of *A. fabae* reared for 7 days on the three test plant species. Values are means ± S.E.M. (N=10). Values with different letters are significantly different (P>0.05) by Tukey's test. ANOVA: for plant species: $F_{2,54}$=17.37, P<0.001; for symbiosis: $F_{1,54}$=77.03, P<0.001; for interaction: $F_{2,54}$=4.11, 0.05>P>0.01.

old symbiotic and aposymbiotic aphids, which settled readily and fed, as indicated by honeydew production. Over the following week (i.e. to day 9), the aphids on all plant species developed to the fourth (final) larval stadium. The mass of aposymbiotic aphids did not vary significantly with the species of rearing plant, but the symbiotic aphids on *P. dubium* and *C. album* were more than double the mass of the symbiotic aphids on *L. purpureum*, which were not significantly heavier than the aposymbiotic aphids on any of the plants (Fig. 1). These data suggest that the bacterial symbiosis may be depressed in symbiotic aphids on *L. purpureum*.

On all plant species, the larval development time of the symbiotic aphids was 9–12 days and the adults produced offspring, generating long-term cultures on all three plant species, although the cultures were conspicuously more vigorous on *C. album* and *P. dubium* than on *L. purpureum*. The intrinsic rate of population increase, r_m, of aphids on *C. album* and *P. dubium* was 0.27±0.011 (N=14) and 0.32±0.007 (N=9) aphids aphid^{-1} day^{-1}, respectively. Aphid fecundity on *L. purpureum* was low, and consistent values of r_m could not be obtained because the mortality of these aphids in early adulthood was relatively high and varied between experiments.

The aposymbiotic aphids persisted for 2–7 weeks on all plant species. They remained as fourth-instar larvae for 12–40 days before dying or moulting to adulthood. A few adults produced one to several offspring, but these were dead at birth or died without developing beyond the first larval stadium.

Subsequent experiments investigated the density and nutritional function of the bacteria in symbiotic aphids, using 9-day-old larvae to ensure that the insects under all treatments were of comparable developmental age. Bacterial density and aphid amino acid metabolism are known to vary with developmental age (e.g. Whitehead and Douglas, 1993; Baumann and Baumann, 1994; Wilkinson and Douglas, 1995; Douglas, 1996).

Light microscopy of sectioned aphids

Every serially sectioned 9-day-old symbiotic aphid on all three test plant species contained bacteriocytes, with cytoplasm packed with *Buchnera*, in both the maternal haemocoel and embryos. The number of bacteriocytes in the maternal haemocoel could be scored readily and did not vary among the plant species: 64.6±6.29 in aphids on *C. album*, 66.0±5.66 in aphids on *L. purpureum* and 66.6±8.40 in aphids on *P. dubium* (means ± S.E.M., 10 replicates; $F_{2,24}$=0.20, P>0.05). The aposymbiotic aphids bore no identifiable bacteriocytes or bacterial cells in either the maternal haemocoel or in the embryos, confirming that the antibiotic treatment had disrupted the symbiosis.

In the sectioned material, the embryos in all symbiotic aphids were structurally intact and at a range of developmental stages, including some with appendages, but the aposymbiotic aphids on all plants bore few embryos, most of which appeared structurally disorganised. Abnormalities in other organ systems were not generally evident in any aphids. However, the stomach (anterior midgut) in both symbiotic and aposymbiotic aphids on *C. album* was distended, and its lumen in the stained sections of aphids contained dense aggregations of basophilic droplets. Parallel dissections of fresh aphids confirmed that the stomach was enlarged in aphids reared on *C. album*, up to twice the length of the stomach in aphids of the same age and size on *P. dubium*, and opaque due to the accumulation of white material in the lumen.

Bacterial density in aphid homogenates

The homogenates of the symbiotic aphids bore two morphological forms of bacteria: cocci of diameter 2–4 μm, identified as *Buchnera*, and rods of length 1–5 μm, referred to as 'secondary symbionts'. The density of *Buchnera* in the aphids, in the range 2.0–3.2×10^{7} cells mg^{-1} fresh mass, was consistently greater than that of secondary symbionts, at 0.1–0.6×10^{7} cells mg^{-1}. In Fig. 2, the bacterial populations are expressed on the basis of protein content to permit direct comparison between the bacterial density in the total aphid tissues and their embryos. All the main factors in the ANOVA (summarised in the legend to Fig. 2) are statistically significant: the bacterial density varied with plant species and was higher in the total aphid tissues than embryos, and the density of *Buchnera* was higher than that of the secondary symbionts. The significant interaction terms of the ANOVA indicate that the difference between the density of *Buchnera* and secondary symbionts was greater in the embryos than in total tissues and varied with the test plant species. Application of Tukey's test to the data revealed that the populations of secondary symbionts were significantly depressed in aphids on *C. album* and *P. dubium*, relative to aphids on *L. purpureum*, and in embryos relative to total aphid tissues.

Amino acid synthesis in the aphids

To explore whether bacteria in aphids on *L. purpureum* are functional, essential amino acid synthesis by aphids was investigated. Radiolabelled glutamic acid was used as the

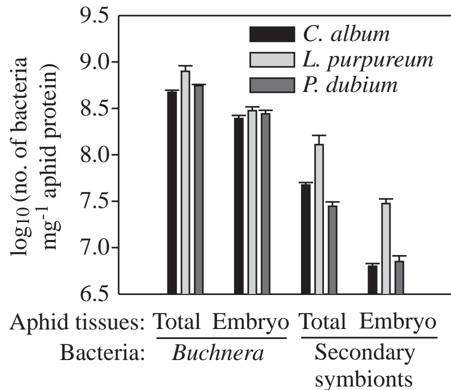

Fig. 2. The density of *Buchnera* and secondary symbionts in symbiotic aphids. Data were tested for significance by ANOVA (on logarithmic-transformed data): plant species, $F_{2,78}$=51.52, P<0.001; tissue (total tissues *versus* embryos), $F_{1,78}$=238.67, P<0.001; bacterial type (*Buchnera versus* secondary symbionts) $F_{1,78}$=1298.5, P<0.001; plant species × tissue, $F_{2,78}$=1.29, P>0.05; plant species × bacterial type, $F_{2,78}$=23.62, P<0.001; bacterial type × tissue, $F_{1,78}$=29.40, P<0.001; plant species × bacterial type × tissue, $F_{2,78}$=2.95, P>0.05. Density is expressed per unit protein; the total aphid tissues comprised 43 μg protein mg^{-1} fresh mass (without significant variation among rearing plant species, P>0.05). Values are means ± S.E.M.

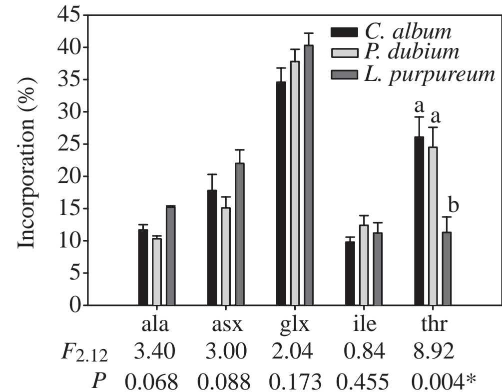

Fig. 3. Incorporation of [U-^{14}C]glutamic acid into amino acids of the protein fraction of symbiotic aphids, expressed as a percentage of total incorporation. ala, alanine; asx, aspartic acid + asparagine; glx, glutamic acid + glutamine; ile, isoleucine; thr, threonine. The F values for ANOVA tests for each amino acid after arcsine-square root transformation are shown. The critical value for significance of the ANOVA tests after Bonferroni correction for 5 tests is P=0.01; *significantly different value. Mean values with different letters are significantly different (P>0.01) by Tukey's test.

precursor because bacterial-mediated synthesis of the essential amino acids isoleucine, lysine and threonine has previously been demonstrated for *A. fabae* reared on chemically defined diets (Douglas et al., 2001). The radiolabelled amino acids identified in protein hydrolysates by HPLC were the non-essential glutamic acid, aspartic acid and alanine in both symbiotic and aposymbiotic aphids, and isoleucine, threonine and a trace of lysine in symbiotic aphids. Parallel TLC analysis confirmed these identifications and additionally established that the non-essential proline (which cannot be detected by HPLC) was radiolabelled in both symbiotic and aposymbiotic aphids. The distribution of radioactivity among the amino acids readily detectable by HPLC in protein hydrolysates of symbiotic aphids on the three test plant species is shown in Fig. 3 (lysine was excluded from the analysis because its ^{14}C content was too low to be quantified reliably). In aphids on all plants, the amino acid with the highest radioactivity content was glutamic acid/glutamine, and 10–26 % of the radioactivity was recovered from each of the other amino acids. For threonine, but none of the other amino acids, the percentage incorporation varied significantly among the aphids on the different plant species, and was significantly depressed in aphids on *L. purpureum*.

We reasoned that bacterial-mediated threonine production could be reduced in response to either (1) high threonine levels in the phloem sap of *L. purpureum* or (2) a plant factor other than phloem threonine content, resulting in threonine deficiency in the aphids reared on *L. purpureum*. To address these possibilities, the amino acid content of plant phloem sap and aphids was assessed.

Amino acids in plant phloem sap

Initial studies revealed that phloem sap collection from severed aphid stylets was not feasible because the stylets from aphids feeding on both *C. album* and *L. purpureum* consistently failed to exude. It was, therefore, not possible to quantify the absolute concentrations of amino acids in plant phloem sap. As an alternative way of establishing whether *L. purpureum* phloem sap has high threonine levels, two complementary approaches were adopted: quantification of the amino acid content of phloem exudates collected in EDTA solution, which prevents sieve element sealing and is known to provide reliable measurements of amino acid composition but not the absolute concentration in sieve elements (e.g. Weibull et al., 1990); and amino acid concentration of aphid honeydew, which is positively correlated with the absolute concentration of amino acids ingested by aphids (Prosser et al., 1992).

Every sample of phloem exudate from *C. album*, *L. purpureum* and *P. dubium* contained detectable levels of all 18 protein amino acids, quantifiable by HPLC. For all the plant species, the dominant amino acid was the non-essential glutamine, which, on average, accounted for 58 % of the amino acids in *L. purpureum*, and 32 % in *P. dubium* and 24 % in *C. album*; and the concentrations of glutamic acid in exudates from *C. album* and aspartic acid in exudates of *P. dubium* also exceeded 20 % of the total (Fig. 4A). The composition of amino acids varied significantly between test plant species when tested by MANOVA (Table 1) and, in the complementary PCA, 73 % of the variance in the data could be explained by the first two principal components, which effectively separated the three plant species (Fig. 4B). The first principal component axis divided the data largely by a group

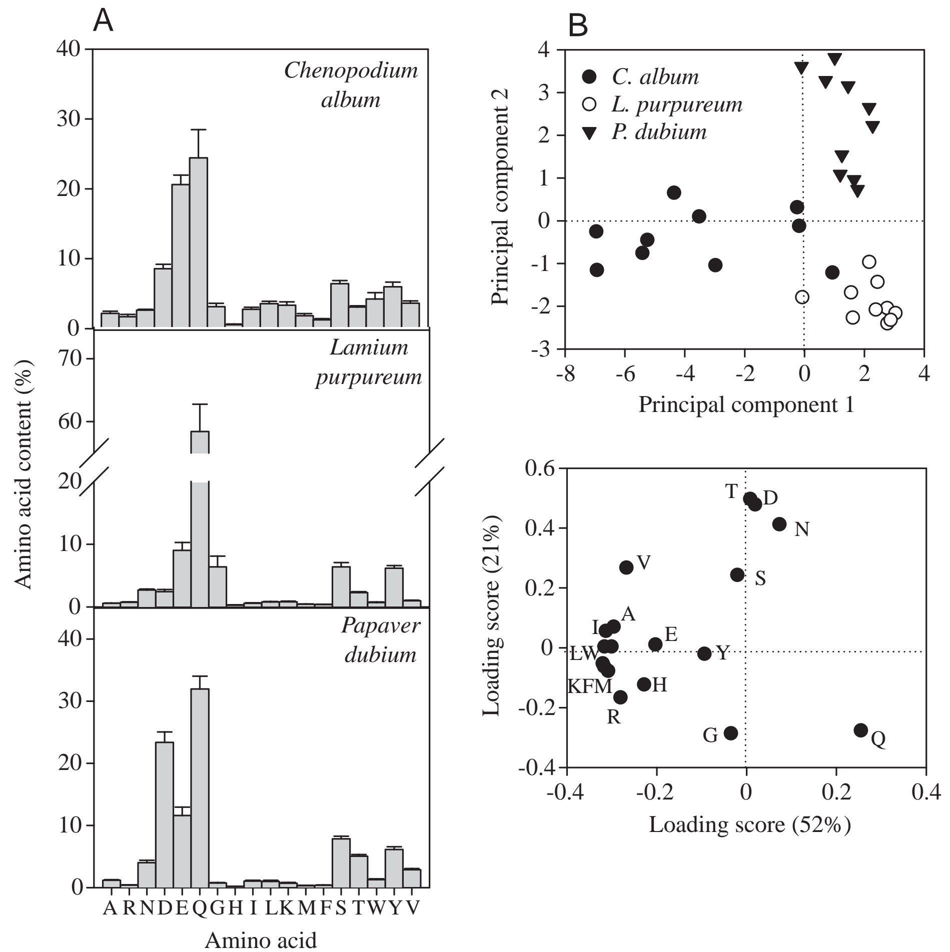

Fig. 4. Amino acid composition of phloem exudates of test plants. (A) percentage composition; values are means ± S.E.M. (N=10). (B) Principal components analysis of the percentage composition data. A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

of mostly essential amino acids, including isoleucine, leucine, lysine, methionine, phenylalanine and tryptophan (high in most samples of *C. album*); and the second principal component axis was strongly correlated with the amount of threonine and aspartic acid in the exudates (high in *P. dubium*, low in *L. purpureum*). Consistent with the data in Fig. 4B, the %-threonine content varied significantly between plant species (ANOVA after arcsine-square root transformation: $F_{2,29}$=54.26, P<0.001) and Tukey's test revealed that the mean value for *L. purpureum* (2.29 %) was significantly lower than that of *C. album* (3.10 %) and *P. dubium* (5.05 %) (P<0.05).

Table 1. *Multivariate analysis of variance (MANOVA) of amino acid concentrations (pmol exuded) in phloem exudates from the test plant species*

Test	Statistic	F-ratio	Degrees of freedom	Probability P
Wilk's	0.00006	68.60	20,36	<0.001
Lawley–Hotelling	352.06	88.0	18,36	<0.001
Pillai's	1.98	52.89	22,36	<0.001
Roy's	301.89			

s=2, m=7.5, n=4.0.

The concentration of amino acids in honeydew from symbiotic aphids feeding from *P. dubium* was almost two orders of magnitude greater than for aphids on *C. album* and *L. purpureum*. After logarithmic transformation of the data to obtain normal distributions, the total concentration of amino acids and concentration of threonine was significantly higher in honeydew from aphids on *P. dubium* than the other plants, but as a percentage of the total amino acids, the threonine content did not vary significantly with plant species (Table 2).

In summary, the level of threonine is not elevated either as a percentage of the total amino acids in phloem-exudates of the phloem sap of *L. purpureum* or as the absolute concentration in the honeydew of aphids feeding on *L. purpureum*. These data do not support the hypothesis posed above that bacterial-mediated threonine synthesis is reduced in response to high threonine levels in *L. purpureum*.

The free amino acid pools of the aphids

The approach to exploring the impact of depressed threonine production in symbiotic aphids on *L. purpureum* derives from current understanding of the metabolic consequences of eliminating the bacterial symbiosis from aphids (Wilkinson, 1998). Aposymbiotic aphids have elevated free amino acid

Table 2. *Amino acids in the honeydew of aphids*

Plant species	Log_{10}(nmol amino acids μg^{-1} honeydew) Total	Threonine	Threonine content (%)
Chenopodium album	3.49±0.22^{b}	1.60±0.13^{b}	2.0±0.61
Lamium purpureum	3.57±0.12^{b}	1.17±0.39^{b}	1.4±0.46
Papaver dubium	5.39±0.13^{a}	3.73±0.14^{a}	2.4±0.38
ANOVA $F_{2,24}$	43.14, P<0.001	29.81, P<0.001	1.97, P>0.05*

*After arcsine-square root transformation of the data.
Values are means ± S.E.M. (N=9).
Mean values with different letters are significantly different (P<0.05) by Tukey's test.

concentrations relative to symbiotic aphids but the concentration of one-to-several essential amino acids is significantly lower in aposymbiotic aphids than symbiotic aphids, e.g. phenylalanine, isoleucine and threonine in diet-reared *Acyrthosiphon pisum* (Prosser and Douglas, 1991) and leucine in diet-reared *Aphis fabae* (Douglas et al., 2001). This distinctive pattern has been interpreted as a limitation of the synthesis of protein (which has a relatively invariant amino acid composition) in aposymbiotic aphids by the essential amino acid(s) with depressed free concentrations, and accumulation of other non-limiting amino acids in the free amino acid pool (e.g. Prosser and Douglas, 1991; Liadouze et al., 1995; Wilkinson, 1998). By extension of this reasoning, if depressed threonine synthesis limits protein synthesis and growth of symbiotic aphids on *L. purpureum*, then the threonine concentration in the free amino acid pools of these aphids is predicted to be significantly lower than in aphids on other plants.

The total free amino acid content of the aphids on all test plants was significantly elevated by aposymbiosis (Table 3), and most amino acids contributed to the difference between symbiotic and aposymbiotic aphids (Fig. 5). The only amino acids with lower mean concentration in aposymbiotic aphids than symbiotic aphids were the essential amino acids threonine, for aphids on all plants, and isoleucine, for aphids on *P. dubium*, and the non-essential amino acids alanine/tyrosine (these amino acids could not be separated with confidence in all samples).

The pattern of variation in threonine and isoleucine concentrations was examined further by ANOVA (Table 3). For threonine, the impact of aposymbiosis varied with plant species (the interaction term is significant) and the threonine concentration in symbiotic aphids on *L. purpureum* was significantly lower than in symbiotic aphids on *C. album* and *P. dubium*, but did not differ significantly from aposymbiotic aphids on any plant species. The concentration of isoleucine varied significantly with plant species (it was significantly elevated in aphids on *C. album* relative to aphids on *L. purpureum* and *P. dubium*), but was not significantly influenced by aposymbiosis. The low concentration of alanine/tyrosine (both non-essential amino acids) in the aposymbiotic plant-reared aphids probably parallels the significantly depressed alanine (but not tyrosine) content in aposymbiotic diet-reared *A. fabae* (Douglas et al., 2001) and,

Table 3. *Amino acids in the free amino acid fraction of aphids*

	Total amino acids (nmol mg^{-1} aphid mass)		Threonine (nmol mg^{-1} aphid mass)		Isoleucine (nmol mg^{-1} aphid mass)	
Plant species	Symbiotic aphids	Aposymbiotic aphids	Symbiotic aphids	Aposymbiotic aphids	Symbiotic aphids	Aposymbiotic aphids
Chenopodium album	44.8±2.40^{b}	104.6±3.35^{a}	1.81±0.165^{a}	1.04±0.130^{b,c}	1.19±0.107^{a}	1.28±0.162^{a}
Lamium purpureum	35.6±1.31^{b}	87.8±5.51^{a}	0.71±0.134^{c}	0.62±0.030^{c}	0.51±0.079^{b,c}	0.68±0.118^{b}
Papaver dubium	39.72±2.79^{b}	97.0±5.40^{a}	1.41±0.109^{a,b}	1.05±0.049^{b,c}	0.37±0.047^{b,c}	0.15±0.089^{c}
ANOVA[1]						
Plant species						
$F_{2,42}$	7.21, 0.01>P>0.001		24.54, P<0.001		43.00, P<0.001	
Symbiosis						
$F_{1,42}$	398.21, P<0.001		19.04, P<0.001		0.03, P>0.05	
Interaction						
$F_{2,42}$	0.09, P>0.05		4.45, 0.01<P<0.05		1.84, P>0.05	

[1]Data for total amino acid content were logarithmically transformed to obtain homogeneous variance.
Values are means ± S.E.M. (N=8).
Mean values with different letters are significantly different (P<0.05) by Tukey's test.

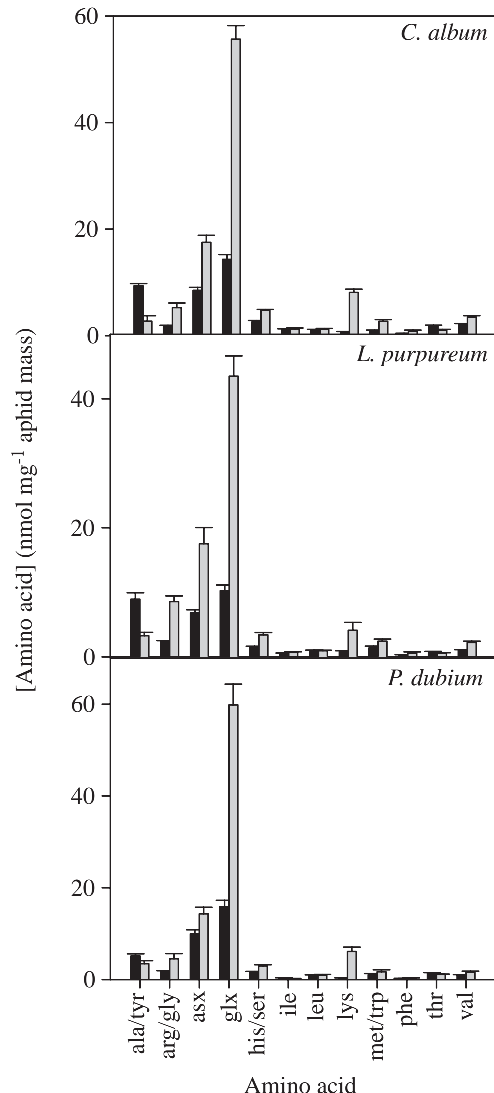

Fig. 5. Amino acid composition of the free amino acid fraction in symbiotic aphids (closed bars) and aposymbiotic aphids (open bars). ala/tyr, alanine + tyrosine; arg/gly, arginine + glycine; asx, aspartic acid + asparagine; glx, glutamic acid + glutamine; his/ser, histidine + serine; ile, isoleucine; leu, leucine; lys, lysine; met/trp, methionine + tryptophan; phe, phenylalanine; thr, threonine; val, valine. Values are means ± S.E.M. (*N*=8).

although the metabolic basis of this effect is unknown, it was not investigated further here.

Supplementary experiments were conducted to address whether the low free threonine concentration in symbiotic aphids on *L. purpureum* (Table 3) is a general chacteristic of small or slowly growing aphids. On the plant *Trifolium repens*, *A. fabae* grows and develops slowly, linked to poor settling and low feeding rates (Adams and Douglas, 1997). The pattern of amino acids in aphids reared to 9 days on this plant broadly resembled that of aphids on *C. album* and *P. dubium*. The threonine concentration was significantly lower in aposymbiotic aphids (0.83±0.158 nmol mg^{-1} aphid mass) than in symbiotic aphids (1.55±0.126 nmol mg^{-1}) (mean ± S.E.M., 8 replicates, t_{14}=3.54, 0.01>*P*>0.001), even though the total free amino acid concentration was significantly higher in aposymbiotic aphids (98.5±7.13 nmol mg^{-1}) than in symbiotic aphids (44.3±5.06 nmol mg^{-1}) (t_{14}=6.19, *P*<0.001).

Discussion

Aphis fabae has an absolute requirement for its complement of symbiotic bacteria. When the bacteria are eliminated by antibiotic treatment, larval growth is impaired and reproductive output is essentially abolished (the few live offspring that are deposited die without developing or growing) in *A. fabae* reared both on plants (this study) and on chemically defined diets (Douglas et al., 2001). Other aphid species display broadly comparable dependence on the bacterial symbiosis (e.g. Douglas, 1988; Douglas, 1992; Sasaki et al., 1991). The clone of *A. fabae* used in this study bore both *Buchnera*, the ‘primary symbiont’ of most aphids, and ‘secondary symbionts’ of unknown taxonomic affiliation, and both forms were eliminated by the antibiotic treatment. It is very likely that the insect requires the *Buchnera* but not the secondary symbionts because in the pea aphid *Acyrthosiphon pisum*, whose microbiology is better studied than that of *A. fabae*, secondary symbionts are not universal and can impair aphid performance (Chen and Purcell, 1997; Chen et al., 2000; Fukatsu et al., 2000; Sandstrom et al., 2001; Darby et al., 2001). However, definitive evidence on the significance of *Buchnera* and secondary symbionts to *A. fabae* must await further study of the microbiology of *A. fabae*.

This first analysis of the impact of the species of rearing plant on the bacterial symbiosis in any phytophagous insect has revealed plant-linked variation in both the density and function of the symbiotic bacteria. The principal topics addressed here are the nature of the plant factors causing the differences in the symbiosis and the relationship between the various impacts of the plant species on bacterial symbiosis. To tease apart the various issues, bacterial symbiosis in aphids reared on *Chenopodium album* and *Papaver dubium*, plant species on which the symbiosis promotes aphid growth, is considered first, and followed by an analysis of the symbiosis in aphids reared on *Lamium purpureum*, where the performance of symbiotic aphids is selectively depressed (see Fig. 1).

The aphid-bacterial symbiosis on Chenopodium album *and* Papaver dubium

Two lines of evidence indicate that *C. album* and *P. dubium* are not nutritionally equivalent for *A. fabae*. First, their phloem exudates differ in amino acid composition (Fig. 4), with *P. dubium* relatively enriched in the essential amino acid threonine and many samples of *C. album* enriched in several essentials, including isoleucine. Second, the dominant compounds contributing to the mass of aphid honeydew are sugars derived from ingested sucrose (Klingauf, 1987), and the high concentration of amino acids per unit mass of honeydew produced by aphids on *P. dubium* (Table 2) is indicative of a low sugar:amino acid ratio in the phloem sap of this plant

species. The excellent performance of *A. fabae* on these plant species is consistent with data on diet-reared aphids, indicating that this species is tolerant of a range of dietary amino acid inputs (e.g. Leckstein and Llewellyn, 1973; Douglas et al., 2001).

In *A. fabae* on chemically defined diets, the density and function of the symbiotic bacteria vary with dietary amino acid content. Specifically, the density of symbiotic bacteria, especially secondary symbionts, is increased in aphids on diets of high sucrose:amino acid content (T. L. Wilkinson and A. E. Douglas, manuscipt in preparation) and bacterial-mediated synthesis of isoleucine and threonine from ^{14}C-glutamic acid is elevated on diets from which the same amino acid is omitted (this effect is significant for isoleucine) (Douglas et al., 2001). The striking feature of the response of *A. fabae* to *C. album* and *P. dubium* is that it does not involve detectable differences in the bacterial populations (Fig. 2) or function (Fig. 3). These results raise the possibility that the aphid–bacterial symbiosis does not necessarily respond to variation in amino acid composition of plants and diets in the same way, either because the nutritional differences between plants are less extreme than in those used in diet studies or because of fundamental differences between plants and diets as a food source (e.g. plant phloem sap is under pressure and is nutritionally far more complex than diets, and plants provide multiple olfactory and mechanosensory cues that are absent from diets). A further difference between the plant- and diet-reared *A. fabae* is that the essential amino acid specifically depressed in the free amino acid pool of aposymbiotic aphids is threonine (Fig. 5) for aphids reared on plants and leucine (Douglas et al., 2001) for diet-reared insects, suggesting that protein synthesis and growth of aposymbiotic aphids may be limited by the loss of bacterial-derived threonine on plants and leucine on diets.

In other respects, the bacterial symbiosis in *A. fabae* reared on *C. album* and *P. dubium* is comparable to other systems. The density of *Buchnera* in these plant-reared aphids, at $2.0–3.2\times10^7$ cells mg^{-1} aphid fresh mass, is broadly comparable to published values for *A. fabae* on chemically defined diets, at 2.5×10^7 cells mg^{-1} (Douglas et al., 2001), and slightly higher than for plant-reared *Schizaphis graminum*, at 1.2×10^7 cells mg^{-1} (Baumann and Baumann, 1994), and *Acyrthosiphon pisum*, at 1.5×10^7 cells mg^{-1} (Humphreys and Douglas, 1997). The plant-reared *A. fabae* synthesize the same array of amino acids from ^{14}C-glutamic acid (Fig. 3) as previously reported for diet-reared *A. fabae* (Douglas et al., 2001) and *Ac. pisum* (Febvay et al., 1995). However, detailed between-study comparisons of the quantitative pattern of ^{14}C incorporation into amino acids would be inappropriate because the mode of delivery of radioisotope and timescale of experiments differed between studies.

The aphids on *C. album* had a distended stomach bearing white precipitate. This feature is chacteristic of aphids feeding on plants of the family Chenopodiaceae (Edwards, 1964) and, although the specific cause of this response is not fully understood, it is commonly associated with poor aphid performance and premature mortality (Kift et al., 1996; Williams et al., 1997). For *A. fabae* on *C. album*, however, no such negative effects were evident in either laboratory or field experiments (this study; Raymond et al., 2000), although the enlarged stomach may have contributed to the significantly greater mass of the 9-day-old larval *A. fabae* on *C. album* than those on *P. dubium* (Fig. 1).

The aphid-bacterial symbiosis on Lamium purpureum

Symbiotic aphids reared on *L. purpureum* performed poorly, had low rates of bacterial-mediated threonine synthesis and bore a high density of symbiotic bacteria, especially the secondary symbionts, compared to aphids on *C. album* and *P. dubium*. On the basis of our current understanding of dietary impacts on the symbiosis, these characteristics cannot readily be attributed to amino acid composition of the phloem sap in *L. purpureum*. There are two issues. First, the composition of amino acids is more unbalanced in the phloem exudates of *L. purpureum* than in those of *C. album* and *P. dubium*, with just one non-essential amino acid, glutamine, accounting for more than 50 %, and essential amino acids contributing less than 10 %, of the total amino acid content (Fig. 4A). Other host plants of *A. fabae,* however, have phloem amino acids as unbalanced as in *L. purpureum*. For example, *Vicia faba*, which supports high growth and reproductive rates of *A. fabae* subsp. *fabae* (Douglas, 1997) has phloem sap with the non-essential asparagine at greater than 50 % and essential amino acids at less than 10 % (Douglas, 1993). The implication is that essential amino acid synthesis by symbiotic bacteria may be of particular nutritional value to *A. fabae* on *L. purpureum*, but that the amino acid composition of *L. purpureum* phloem sap is anticipated to be compatible with high performance of *A. fabae*. The second issue is that *L. purpureum* phloem sap has a low content of the essential amino acid threonine compared to that in *C. album* and *P. dubium* (Fig. 4). The depressed threonine production in symbiotic aphids on *L. purpureum* (Fig. 3) is the reverse of the prediction from studies of diet-reared *A. fabae*, which revealed that bacterial-mediated threonine synthesis is elevated (although not significantly) in *A. fabae* reared on threonine-free diets (Douglas et al., 2001).

The negative effects of *L. purpureum* on the performance of *A. fabae* cannot readily be ascribed to a generalized malaise of the aphids linked to plant toxicity or impaired aphid feeding because the two consequences of such effects, namely reduced larval development rates and depressed performance of aposymbiotic aphids relative to aposymbiotic aphids on susceptible plant species (D. Adams and A. E. Douglas, 1997; unpublished results), were not observed. The key performance characteristics of the aphids on *L. purpureum* are depressed larval mass gain of symbiotic, but not aposymbiotic, aphids and low but sustained reproduction sufficient to sustain the aphid population over multiple generations. These are the characteristics anticipated of aphids whose bacterial symbiosis has been reduced, but not abolished.

The sole symbiotic property of aphids on *L. purpureum* known to be depressed is bacterial-mediated synthesis of the essential amino acid threonine. Although further work is

required to establish whether the synthesis of other essential amino acids (not derived from glutamic acid) may also be reduced, the significantly lower mean threonine content of the free amino acid fraction of symbiotic aphids on *L. purpureum* than on the other test plants (Table 3) and low threonine content of both phloem exudates of *L. purpureum* (Fig. 4) and honeydew of aphids feeding from this plant species (Table 2) together raise the possibility that threonine may be a limiting amino acid for these aphids. The key outstanding issue, however, is the extent to which the shortfall in bacterial-derived threonine contributes to the poor performance of symbiotic aphids on *L. purpureum*. Threonine injections into the insect haemolymph ('blood') did not significantly improve aphid performance on *L. purpureum* (T. L. Wilkinson, L. B. Minto and A. E. Douglas, unpublished results), but this and other 'negative' results with injected amino acid supplements (e.g. Wilkinson and Ishikawa, 2000) cannot be interpreted readily in terms of aphid nutritional requirements because much of the threonine or other amino acids injected into *A. fabae* is rapidly used as a respiratory substrate and so is unavailable for biosynthetic metabolism (Wilkinson et al., 2001).

The metabolic basis of reduced net threonine synthesis in symbiotic aphids on *L. purpureum* may be linked to the role of threonine as an intermediate in isoleucine synthesis. Increased carbon flux from threonine to isoleucine, resulting in reduced net threonine synthesis relative to isoleucine, would arise from increased threonine deamination to α-ketobutyrate (the first step in isoleucine synthesis from threonine), as occurs, for example, by derepression of threonine deaminase in isoleucine-overproducing strains of the bacterium *Corynebacterium glutamicum* (Sahm et al., 1999). Diversion of threonine to non-protein fates (e.g. respiratory loss) may also contribute to depressed threonine production in the symbiotic aphids.

The density of symbiotic bacteria was elevated in symbiotic aphids on *L. purpureum*, but it is difficult to interpret this result in relation to aphid performance because, although the broad patterns of regulation of the aphid-bacterial symbiosis are fairly well established, the underlying mechanisms are obscure. Briefly, the complement of bacteria acquired by each aphid embryo from its mother is partitioned among the bacteriocytes as these cells differentiate and the bacteriocytes undergo several rounds of division in the embryos. After birth of the aphid, the bacteriocytes display little or no further division and they accommodate the proliferating *Buchnera* population in their cytoplasm by growth (i.e. cell expansion). In late larval development and during adult life, individual bacteriocytes lyse and their *Buchnera* population is destroyed, such that post-reproductive aphids have few or no bacteriocytes (Lamb and Hinde, 1967; Douglas and Dixon, 1987; Wilkinson and Douglas, 1998). The association between the aphid and its secondary symbionts is cytologically less intimate: the secondary symbionts are located both in sheath cells surrounding the bacteriocytes and, especially in older aphids, free in the haemolymph (Douglas and Dixon, 1987; Chen and Purcell, 1997; Fukatsu et al., 2000). The numbers of bacteriocytes in the maternal haemocoel of the 9-day-old, final-instar larvae of *A. fabae* reared on *L. purpureum* were comparable to those in aphids reared on the other test plant species, indicating that regulatory processes at the level of the bacteriocyte are intact in these insects. The elevated bacterial density in the aphids reared on *L. purpureum* (Fig. 2) could be interpreted as a host-mediated upregulation of the bacteria, perhaps in response to a shortfall of key essential amino acids (especially threonine), or as a consequence of impaired insect-mediated suppression of bacterial proliferation rates arising from the poor performance of the aphids on this plant. The outcomes of these contrary interpretations would potentially be to alleviate and exacerbate, respectively, the negative effects of *L. purpureum* on the aphid–bacterial symbiosis. Although there are as yet insufficient data to distinguish between these two interpretations, the increased density of secondary symbionts, which in *A. pisum* can be deleterious to the insect (Chen et al., 2000; Fukatsu et al., 2000), gives some credence to the view that the elevated bacterial density may contribute to the poor performance of symbiotic aphids on *L. purpureum*. However, plant-mediated promotion of secondary symbiont abundance is unlikely to be the sole cause of poor aphid performance on *L. purpureum* because this plant is generally a poor host for *A. fabae* (Blackman, 1974), some clones of which apparently lack secondary symbionts (A. E. Douglas, unpublished results).

In summary, the balance of evidence suggests that low threonine production by the bacterial symbiosis in aphids contributes to the poor performance of symbiotic aphids on *L. purpureum*, a plant with phloem sap of relatively low threonine content. However, definitive evidence will only come from elucidation of the metabolic basis of the depressed threonine production and identification of the plant factors responsible. *L. purpureum*, like other plant species of the family Lamiaceae, has a rich array of bioactive secondary metabolites (e.g. Damtoft, 1992; Pederson, 2000) which, in principle, could include compounds that specifically target the aphid-bacterial symbiosis.

Concluding comments

Plant secondary compounds with bacteriostatic and bacteriocidal properties have been described (Harborne, 1993) but, to date, no plant is known specifically to suppress the population of symbiotic bacteria in aphids or any other phloem-feeding insect. Possibly, plant-mediated disruption of these symbiotic bacteria is not a widespread plant resistance mechanism because phloem-mobile antibacterials would be incompatible with sustained sieve element function. This study provides the first empirical evidence to indicate plant-mediated interference with the nutritional function of symbiotic bacteria in aphids although, as indicated above, definitive evidence must await biochemical characterisation of the active compounds and their mode of action. Plant-mediated disruption of the aphid-bacterial nutritional interactions may be a widespread but unrecognised cause of poor insect

performance on certain plants. Alternatively, this phenonemon may be rare, and the *A. fabae–L. purpureum* interaction exceptional, because of strong selection pressure on the insect not to utilize plants detrimental to the symbiosis. There is field evidence that *L. purpureum* may be included as an occasional host of *A. fabae*, despite poor aphid performance, because of the very low incidence of natural enemies of *A. fabae* on this plant, i.e. *L. purpureum* provides enemy-free space (Raymond et al., 2000).

Although much uncertainty remains as to the incidence and nature of plant resistance factors that target the bacterial symbiosis in aphids, they should not be neglected as a factor shaping the range of plant species utilized by aphids and other insects dependent on symbiotic micro-organisms. Exploitation of the bacterial symbiosis may additionally offer novel routes for improved crop protection against the many symbiotic insect pest species.

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