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Integrated Metabonomic−Proteomic Analysis of an Insect−Bacterial Symbiotic System

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The health of animals, including humans, is dependent on their resident microbiota, but the complexity of the microbial communities makes these associations difficult to study in most animals. Exceptionally, the microbiology of the pea aphid *Acyrthosiphon pisum* is dominated by a single bacterium *Buchnera aphidicola* (*B. aphidicola*). A ^{1}H NMR-based metabonomic strategy was applied to investigate metabolic profiles of aphids fed on a low essential amino acid diet and treated by antibiotic to eliminate *B. aphidicola*. In addition, differential gel electrophoresis (DIGE) with mass spectrometry was utilized to determine the alterations of proteins induced by these treatments. We found that these perturbations resulted in significant changes to the abundance of 15 metabolites and 238 proteins. Ten (67%) of the metabolites with altered abundance were amino acids, with nonessential amino acids increased and essential amino acids decreased by both perturbations. Over-represented proteins in the perturbed treatments included catabolic enzymes with roles in amino acid degradation and glycolysis, various cuticular proteins, and a C-type lectin and regucalcin with candidate defensive roles. This analysis demonstrates the central role of essential amino acid production in the relationship and identifies candidate proteins and processes underpinning the function and persistence of the association.

Keywords: *Acyrthosiphon pisum* • amino acid metabolism • aphid • differential protein expression • metabonome • proteome;

Introduction

All multicellular animals have indigenous symbiotic microbes that interact with the hosts biochemically and physiologically. The importance of the resident microbiota is recognized most widely in relation to human health, particularly the development of the immune system, resistance to pathogens, and the efficiency of nutrient utilization, including the propensity to obesity and even affecting drug metabolism.[1−4] There is also accumulating evidence for the central role of microorganisms in the physiology of insects[5−7] and increasing interest in exploiting the resident insect microbiota for pest management.[8,9] Certain insect pests can, in principle, be eliminated by disrupting required microorganisms, and the traits of some insects (e.g., capacity to utilize crop plants or to vector disease) can be modified by manipulating associated microorganisms.[10,11]

This study focuses on the association between the pea aphid *Acyrthosiphon pisum* and its bacterial partner, *Buchnera aphidicola*. This symbiosis is uniquely amenable to global analyses of interactions between animals and their resident microbiota for two reasons. First, it is the only animal symbiosis for which the genome of both partners has been sequenced (http://www.iagc.org).[12] Second, *B. aphidicola* is the dominant, and often sole, microbial partner of aphids,[13] enabling the animal−microbial interaction to be anchored to a single microorganism of known gene content. By contrast, the microbiota of many animals, including humans, is complex, comprising hundreds-to-thousands of interacting microbial taxa, and function cannot be coupled with confidence to any single species.[14−16] In this way, the aphid-*B. aphidicola* symbiosis has great potential as a model system for animal interactions with their resident microbiota. A further reason to study this symbiosis is that aphids are major crop pests that depress crop yield by their feeding activity and transmission of plant pathogens.[17] Aphids are currently controlled principally by chemical insecticides,

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and there is a dearth of viable, alternative control strategies. An understanding of the molecular basis of the aphid−*B. aphidicola* relationship should yield specific targets for novel aphid control strategies.

The aphid−*B. aphidicola* association has three key features.[18,19] First, it has a nutritional basis; *B. aphidicola* provides the insect with essential amino acids, nutrients that animals generally cannot synthesize and are in short supply in the aphid diet of plant phloem sap. Second, the association is obligate for both partners; *B. aphidicola* has a small genome (0.64 Mb for the strain in pea aphids) and is unculturable, while the aphid grows poorly and is reproductively sterile when its complement of *B. aphidicola* is eliminated by antibiotic treatment. Finally, *B. aphidicola* is intracellular, restricted to large cells known as bacteriocytes in the aphid body cavity and is vertically transmitted via the egg or embryo. Bacteriocytes containing symbiotic microorganisms have been described in various insects, including other pests exploiting plant sap (whitefly, planthoppers) and vertebrate blood (e.g., anopluran lice, tsetse fly, cimicid bugs). An understanding of the symbiosis in aphids could promote the development of novel control strategies for these important pests.

The specific goal of this study was to understand the interaction between aphids and *B. aphidicola* in molecular terms through parallel global analyses of the proteome and metabonome of the pea aphid, *Acyrthosiphon pisum*. The metabonomic approach employed ^{1}H nuclear magnetic resonance (NMR) spectroscopy with interpretation by multivariate statistical analysis, methods that have been successfully applied to study the mechanism of the interaction between the host and microbes.[20,21] The proteome analysis used two-dimensional difference gel electrophoresis (DIGE) coupled with mass spectrometry. Similar approaches have been used previously to investigate interactions in other animal−microbe associations.[22,23]

Our experimental rationale builds on the demonstration that the core function of *B. aphidicola* is to provide essential nutrients to its insect host. We, therefore, analyzed the response of *A. pisum* to perturbations in the supply of essential amino acids, reducing either the exogenous supply (by rearing aphids on a diet deficient in essential amino acids) or the endogenous supply (by eliminating *B. aphidicola* from the aphids). Our experimental approach used aphids reared on chemically defined diets, so that the dietary inputs were known and could be manipulated precisely, so avoiding the considerable nutritional variability of the natural diet of plant phloem sap.[6] Our comparison between the aphid response to perturbations of the endogenous and exogenous nutrient supply offers new insight into the mechanisms by which the dietary and symbiotic inputs to the amino acid nutrition of the insect are regulated.

Materials and Methods

Insects. *Acyrthosiphon pisum* clone LL01 was maintained on preflowering *Vicia faba* (cv. The Sutton) at 20 °C with the 18 L:6D regime. The aphids were confirmed to lack gut microorganisms by microscopical examination and polymerase chain recation (PCR) assays using the general 16S rRNA gene primers 16SA1 and 16SB1 and DNA from isolated aphid guts as a template; and no secondary symbionts were detected by diagnostic PCR assays[13] with total aphid DNA as a template (data not shown). Additionally, surface-sterilized aphids yielded no microorganisms that grew on standard microbiological media.

The experimental aphids were generated by allowing plant-reared adults to larviposit over 24 h onto the chemically defined diet of Douglas et al.[24] with 0.5 M sucrose and 0.15 M amino acids (essential/nonessential amino acids in a ratio of 1:1). This diet is designated diet-H (high essential amino acids). For the dietary perturbation, aphids were reared on diets with essential/nonessential amino acids in the ratio 1:3 diet-L (low essential amino acids). The antibiotic-treated aphids were generated by rearing newborn larvae on diet-H supplemented with 50 μg of rifampicin per mL for 2 days before transfer to antibiotic-free diet-H; the control aphids containing the unmanipulated complement of *B. aphidicola* were reared on diet-H without rifampicin throughout. This treatment eliminates the *B. aphidicola* while having minimal nonspecific deleterious effects on the aphid.[25] All aphids were assayed at day 7, when they were in the final (fourth) larval stadium. The relative growth rates of 2−7-day-old control aphids did not differ significantly ($p > 0.05$) between the two diet treatments, at 0.317 ± 0.016 g g^{-1} day^{-1} (mean ± standard error (se), n = 20) on diet-H and 0.308 ± 0.021 g g^{-1} day^{-1} (n = 20) on diet-L, but the relative growth rate of the antibiotic-treated aphids on diet-H, at 0.110 ± 0.114 g g^{-1} day^{-1} (n = 11), was significantly depressed relative to the control aphids ($p < 0.05$). Mortality of all aphids over this time period was less than 10%.

^{1}H NMR Spectroscopy. The metabolite analysis was conducted on 0.05−0.15 mg of aphids per treatment, obtained by pooling four replicate samples collected over a period of 4 weeks. The 7-day-old aphids were snap-frozen in liquid nitrogen and stored at −80 °C prior to analysis. A total of eight samples per treatment were used in metabonomic analysis. Each aphid sample was homogenized in 1.5 mL of 50% methanol using TissueLyser (Qiagen, Germany) and mixed with 0.7 mL of chloroform with vortexing for 1 min followed by centrifugation at ~3500g_{av} for 25 min. The aqueous phase was decanted, and the methanol was removed under N_2 gas before freeze-drying the samples. The lipid phase was pipetted out, and chloroform was removed under N_2 gas.

The aqueous extracts were reconstituted in 700 μL of water containing 10% D_2O (which acts as a field frequency lock for the spectrometer) and 0.01% sodium 3-(trimethylsilyl) propionate-2,2,3,3-d_4 (TSP), which was used as a chemical shift reference. The lipid extracts were reconstituted in 0.6 mL of deuterated chloroform. The resulting samples were centrifuged at 14 000g_{av} for 10 min to remove particulates, and a portion of the supernatant (0.60 and 0.55 mL for aqueous and lipid extracts, respectively) was pipetted to a 5 mm NMR tube for spectroscopic analysis. ^{1}H NMR spectra were acquired using a Bruker DRX 600 NMR spectrometer operating at 600.13 MHz for ^{1}H equipped with a 5 mm triple resonance probe with an inverse detection (Bruker, Germany). A standard one-dimensional NMR spectrum was acquired for aqueous extracts using the first increment of the pulse sequence [RD-90°-t_1-90°-t_m-90°-ACQ]. Water suppression was achieved by irradiation of the water peak during the recycle delay (RD = 2 s) and mixing time, t_m (100 ms). t_1 was set to 3 μs. A single pulse excitation pulse sequence was used for lipid extracts. The 90° pulse length was adjusted to approximately 10 μs. The spectral width was set to 20 ppm, and 64 transients were collected into 32 k data points for each spectrum. For assignment purposes, several two-dimensional (2D) NMR spectra including correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear multiple bond correlation (HMBC), and heteronuclear single quantum coherence (HSQC) were also acquired in

a 800 MHz NMR spectrometer for a selected sample following the method described by Bax et al.[26] and Hurd.[27]

All free induction decays were multiplied by an exponential function equivalent to a 0.3 Hz line-broadening factor prior to Fourier transformation. The spectra were corrected for phase and baseline distortions, referenced using XwinNMR 3.5 (Bruker Analytik, Rheinstetten, Germany). The spectra of aqueous extracts were referenced using the TSP peak at δ 0.00 whereas the lipid extract spectra were referenced using the chloroform peak at δ 7.26. The aqueous extracts spectra over the range δ 0.7−10.0 were digitized using a MATLAB script developed in-house (Dr. O. Cloarec, Imperial College London). The region δ 4.68−5.02 was removed from the analysis to avoid the effect of imperfect water suppression. The lipid extracts spectra were digitized in the same way as aqueous extract spectra over the range δ 0.57−7.00. Normalization to the total sum of the spectrum was carried out on the data prior to pattern recognition analyses. Projection to latent structure with an inbuilt orthogonal signal correction filter (orthogonal projection to latent structures discriminant analysis, O-PLS-DA),[28] removing the variation in the NMR spectra unrelated to the variation between the different treatment groups was used to analyze the NMR spectra using an unit variance scaling method in a MATLAB 7.0 environment with a MATLAB script developed in-house.[29] This procedure involved the combination of orthogonal signal correction and PLS discriminant analysis. In addition, back transformation of the loadings plot was utilized to improve the interpretation together with a coefficient incorporating the weight of the variable contributing the discrimination in the models for displaying the loadings.[29] The models were constructed using the NMR data as the *X* matrix and the class information as the *Y* variables, with unit variance scaling of the NMR data. The models were validated using a 7-fold cross validation method, i.e., constructing models repeatedly by leaving out one-seventh of the data and predicting them back into the model. The quality of the model was described by the cross-validation parameter Q^2, indicating the predictability of the model, and R^2, representing the total explained variation for the *X* matrix.

Sample Preparation for Proteomics. For each treatment, three replicate protein samples were prepared, each comprising five aphids macerated in 150 μL of DIGE lysis buffer (9.5 M urea; 2% CHAPS; 0.8% 30 mM Tris pH 9.5), sonicated (Vibra Cell Ultra Sonic Processor, Sonics, U.K) at 4 °C by 5 × 10 s bursts and then centrifuged at 2 500g_{av} for 2 min to pellet the debris. The supernatant was purified using the 2D-clean up kit (GE Healthcare, U.K.), and the final pellet was solublized in 30 μL of DIGE lysis buffer. The protein content of each sample was quantified using a modified Bradford assay.[30]

For the antibiotic-treated aphids, sample preparation was modified to include *B. aphidicola* cells, so as to minimize the contribution of *B. aphidicola* proteins differentially represented in the DIGE comparison of antibiotic-treated and untreated aphids. Each antibiotic-treated aphid sample was supplemented with *B. aphidicola* cells isolated from fourth-instar larvae prior to homogenization. To obtain isolated *B. aphidicola*, 20 aphids were homogenized in 50 mM Tris pH 7.5 + 0.25 M sucrose (buffer A), and the homogenate was passed through muslin and centrifuged at 1 000g_{av} for 30 s. The supernatant was removed, and the pellet was mixed with 0.3 mL of buffer A + 0.01% Nonidet P40 and centrifuged at 4 000g_{av} for 30 s. The pellet was washed three times in 1.4 mL of buffer A, and resuspended in 100 μL of DIGE lysis buffer. A total of 25 μL of the suspension (equivalent to the *Buchnera* from five aphids) was added to each homogenate of antibiotic-treated aphids in 75 μL of DIGE lysis buffer.

DIGE Labeling, 2D PAGE, Gel Imaging, and Image Analysis. The DIGE comparisons were between untreated and antibiotic-treated aphids (comparison A) and between aphids reared on diet-H/diet-L (comparison D). Two internal standards (one for each analysis) were prepared with equal fractions (40 μg protein) from each sample. Proteins were labeled according to the Ettan DIGE minimal labeling protocol (GE Healthcare, U.K.). For each sample, 40 μg of protein was labeled with 400 pmol of CyDye. Each internal standard was bulk labeled with 400 pmol of Cy5, and individual samples were labeled with 400 pmol of Cy3. Two samples of the *B. aphidicola* preparation (50 μg) were also labeled with 400 pmol of Cy3. Equal volumes of a 2× buffer (7 M urea, 2 M thiourea, 2% CHAPS, 2% DTT, 2% Pharmalyte pH 3−10) were added to each of the labeled samples.

Equal amounts of internal standard were added to each aphid sample and to the *B. aphidicola* preparations and prepared for isoelectric focusing (IEF) by diluting with rehydration buffer (8 M urea, 0.5% w/v CHAPS, 0.2% w/v DTT, 1.6% Pharmalyte, pH 3−10). Samples were added to 24 cm nonlinear pH gradient immobilized pH gradient (IPG) strips of pH 3−10 (GE, Healthcare, U.K.) and rehydrated overnight in an Immobiline DryStrip Reswelling Tray (GE Healthcare, U.K.). Strips were focused using an Ettan IPGphor II IEF unit (GE Healthcare, U.K.) following the method of Rabiloud et al.[31] IEF was performed using an IPGphor apparatus (GE, Healthcare, U.K.) for 75 000 V h at 20 °C and a maximum current setting of 50 A per strip. The strips were then equilibrated by reduction and alkylation as in Gorg et al.,[32] loaded onto 12% polyacrylamide gels, and sealed with 1% agarose for a second dimension separation using the Ettan DALT12 system (GE, Healthcare, U.K.) overnight at 1 W/gel at 15 °C.

A set of six gels were used for analysis of each comparison, i.e., three diet-L versus three diet-H and three antibiotic-treated versus three control aphids. Gels were scanned using a Typhoon 9410 imager (GE, Healthcare, U.K.), and images were imported into Progenesis SameSpots v.2 (Non Linear Dynamics, U.K.) for gel alignment, spot detection, and spot analysis. Protein spots were normalized to the internal standards, and those with expression levels greater than 1.3-fold difference between the control and treated samples and at least $p < 0.05$ (*t*-test) were defined as being differentially expressed.

Spot Processing and Sample Preparation for Mass Spectrometry. Preparative 2-D and reference *B. aphidicola* gels, comprising 600 μg of whole aphid preparation and 50 μg of *B. aphidicola*, respectively, were run as above and stained with Plus One Silver Staining Kit (GE, Healthcare, U.K.). Gels were digitalized using a GS-800 calibrated densitometer coupled with Discovery Series QuantOne software (v 4.4; BioRad, Sweden), which were opened in Progenesis software (Non Linear Dynamics, U.K.). Differentially expressed spots were checked against the *B. aphidicola* reference gels to ensure that only aphid-specific proteins were chosen for identification. Spots were then located on the preparatory gels and excised manually. Spot plugs were destained and subjected to purification and in-gel trypsin digestion as in Shevchenko et al.[33]

A sample (0.5 μL) of each digest was applied directly to the matrix-assisted laser desorption ionization (MALDI) target plate, followed immediately by an equal volume of α-cyano-cinnamic acid (5 mg/mL, Sigma, Ireland) per milliliter of 50%

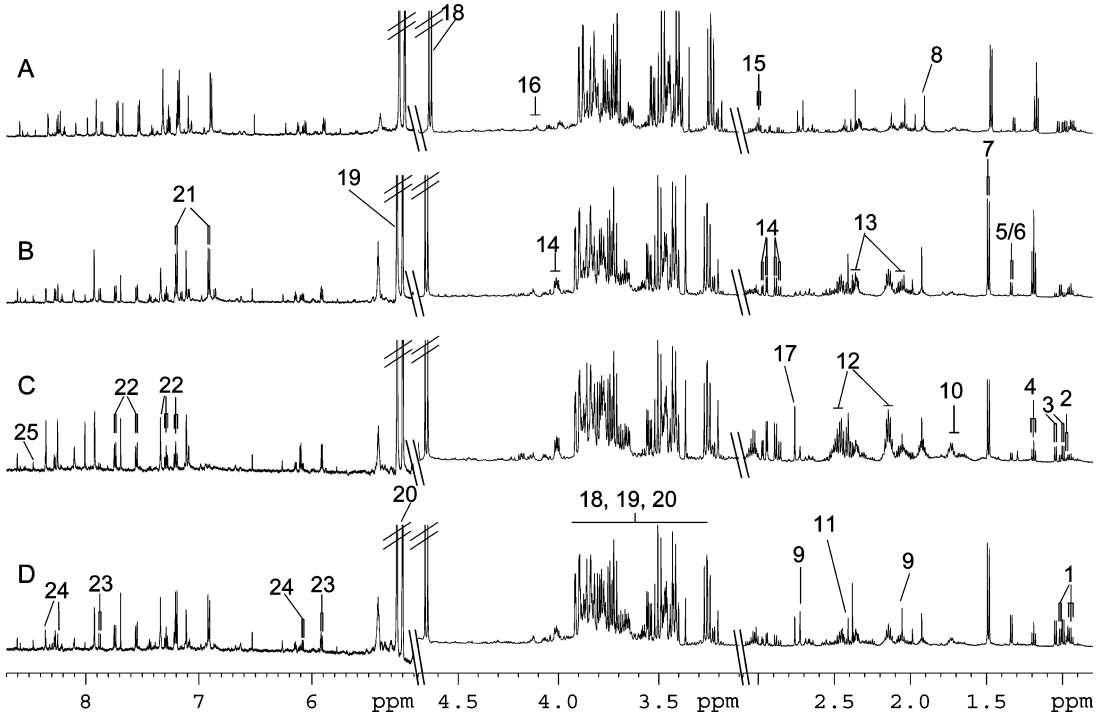

Figure 1. Representative one-dimensional 600 MHz ^{1}H NMR spectra of aqueous extracts of aphids fed on diet-L (A) and diet-H (B) and antibiotic-treated aphids (C) and untreated aphids (D). Key: 1, isoleucine; 2, leucine; 3, valine; 4, ethanol; 5, lactate; 6, threoine; 7, alanine; 8, acetate; 9, *N*-methylacetamide; 10, arginine; 11, succinate; 12, glutamine; 13, glutamate; 14, asparagine; 15, lysine; 16, proline; 17, unknown; 18, β-glucose; 19, α-glucose; 20, trehalose; 21, tyrosine; 22, tryptophan; 23, uridine; 24, inosine; 25, formate.

aqueous (v/v) acetonitrile containing 0.1% TFA (v/v). Positive-ion MALDI mass spectra were obtained using an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA). Settings for the mass spectrometer and collisionally induced dissociation−tandem mass spectrometry (CID−MS/MS) followed those outlined in Hewitson et al.[34]

Mass Spectrometry and Database Searches. Mass spectral data obtained in batch mode were submitted to database searching using a locally running copy of the Mascot program (Matrix Science Ltd., version 2.1). Batch-acquired MS and MS/MS spectral data were submitted to a combined peptide mass fingerprint and MS/MS ion search through the Applied Biosystems GPS Explorer software interface (version 3.6) to Mascot against the National Center for Biotechnology Information (NCBI) nonredundant database (April 2009), including sequences and gene predictions resulting from the *A. pisum* genome assembly (available at http://www.hgsc.bcm.tmc.edu/projects/aphid) and an *A. pisum* expressed sequence tags (EST) database (http://www.aphidests.org/ comprising 120 257 ESTs assembled into clusters). The genomic sequence and databases used for peptide/protein searches were produced and made available by the Human Genome Sequencing Centre at Baylor College of Medicine (www.hgsc.bcm.tmc.edu/) and the International Aphid Genomics Consortium (IAGC). Search criteria included maximum missed cleavages, 1; variable modifications, oxidation (M), carbamidomethyl; peptide tolerance, 100 ppm; MS/MS tolerance, 0.1 Da. Where identification was based on matches to EST clusters, BLASTN searches[35] were conducted of the EST sequence specifically against reference sequence (Ref Seqs) mRNA transcripts for *A. pisum* using the Basic Local Alignment Search Tool (BLAST) search facility on AphidBase (The Aphid Genome Database; http://www.aphidbase.com/aphidbase). Only Ref Seqs with >98% sequence similarity to the EST query were assumed to be an orthologous match. mRNA Ref Seqs and their corresponding genomic sequences were inspected via GBrowse for transcript support, annotation, completeness of sequence and to obtain the unique ACYPI identifier for each matched protein. Gene ontology (GO) terms were assigned using BLAST2GO (http://www.blast2go.org/),[36] and for each predicted sequence, the domain/family/motif was mapped using InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/). Additional GO terms were assigned to proteins involved in metabolism using AcypiCyc, a dedicated BioCyc[37] database for *A. pisum* metabolic pathways (http://pbil.univlyon1.fr/software/cycads/ acypicyc/home) using the ACYPI identifier for each matched protein.

Results

Whole Organism Metabonome Analysis. Representative ^{1}H NMR spectra of aqueous extracts of the aphids are shown in Figure 1. The compounds identified with the aid of the two-dimensional ^{1}H NMR methods COSY, TOCSY, HMBC, and HSQC included the amino acids leucine, isoleucine, valine, alanine, lysine, threonine, asparagine, tyrosine, tryptophan, proline, glutamine, and glutamate; the sugars α-glucose, β-glucose, and trehalose; the nucleosides uridine and inosine; and the acetate *N*-methylacetamide. The NMR resonance of *N*-methylacetamide has previously been assigned only tentatively,[38] and confirmation of this metabolite is provided here by utilizing HMBC, HSQC, and statistical total correlation spectroscopy[39] in Supplementary Figure S1 (in Supplemental File 1) in the Supporting Information. The complete NMR resonance assignment and their multiplicity are listed in Supplementary Table S1 (in Supplemental File 1) in the Supporting Information. The metabolic profiles of the lipid phase extracts were dominated by triglycerides and were not significantly affected by diet or antibiotic treatments; they were not considered further.

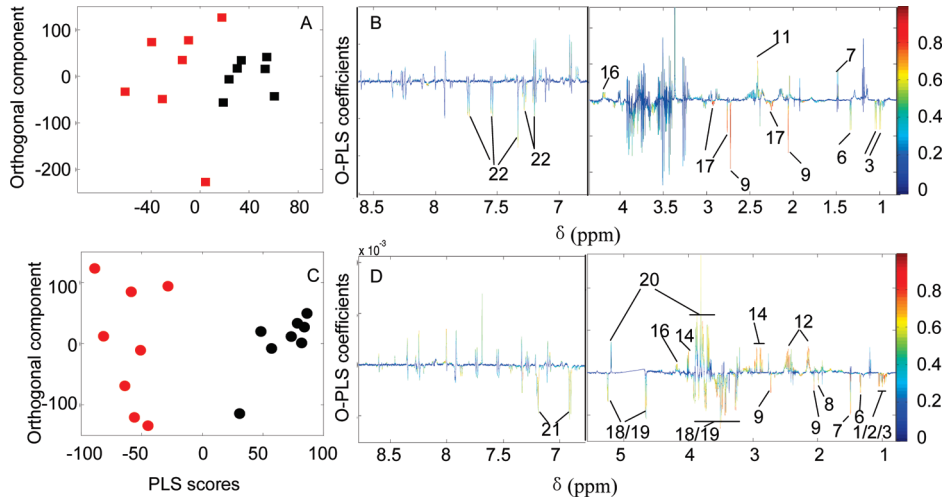

Figure 2. The cross-validated O-PLS-DA scores plots for (A) comparison D between aphids on diet-H (black) and diet-L (red) and (C) comparison A between untreated (black) and antibiotic-treated (red) aphids. The corresponding coefficient plots, as calculated from the covariance matrix, for (B) comparison D and (D) comparison A. For the coefficient plots, the direction of the signals indicates the changes of relative concentration of the metabolites in the class of interest with respect to the other classes in the model, and the color of signals represents the significance of metabolites contributing to the class separation, following the color scaling-map.

For the aqueous extracts, the quality of the O-PLS-DA model used for between-treatment comparisons was described by the cross-validation parameter Q^2 (index of the predictability of the model) and R^2 (representing the total explained variation for the X matrix, i.e., the NMR data). The values of Q^2 were 0.57 for the diet comparison (comparison D) and 0.59 for the antibiotic comparison (comparison A), and the equivalent values for R^2 were 0.35 and 0.43, respectively. The cross-validated scores plots show unambiguous separation between the ^{1}H NMR spectra of aphids in both comparisons (parts A and C of Figure 2). The metabolites contributing significantly ($p < 0.05$) to the separation between treatment groups are displayed in the corresponding O-PLS-DA coefficient plot, as calculated from the covariance matrix (parts B and D of Figure 2, respectively). The peaks directed upward indicate elevated metabolites in aphids fed on diet-L and antibiotic-treated aphids, and the color of signals in the plot represents the significance of metabolites contributing to the separation between treatment groups. On the basis of the number of samples in each group, a coefficient ≥ 0.71 was regarded as being significant in the separation of compared groups ($p < 0.05$) and these metabolites are shown in parts B and D of Figure 2 and also listed in Supplementary Table S2 (in Supplementary File 1) in the Supporting Information.

Of the 15 identified metabolites with significantly different abundance, 10 (67%) were amino acids, including 5 essential amino acids that were depressed in aphids fed on diet-L or treated with antibiotic (Figure 3). Among the five nonessential amino acids, three were elevated in antibiotic-treated aphids or aphids on diet-L, one (tyrosine) was depressed in antibiotic-treated aphids, and one (alanine) was depressed in antibiotic-treated aphids and elevated in aphids on diet-L. Other differences included depressed glucose and acetate and increased trehalose in antibiotic-treated aphids and increased succinate in aphids on diet-L.

Proteome Analysis. A total of 1138 protein spots were detected reproducibly on 2D gels from the comparisons between untreated and antibiotic-treated aphids (comparison A); and 1008 protein spots were detected for the comparison between aphids on diet-H and diet-L (comparison D). No

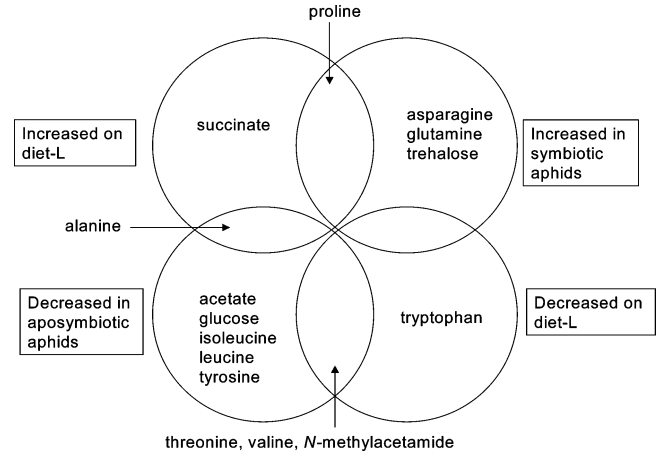

Figure 3. Metabolites with abundance that differs significantly across the treatments

difference in the qualitative representation of proteins between the control aphid and antibiotic-treated aphid plus *B. aphidicola* preparations was detected, indicating that normalizing the antibiotic-treated sample to include *B. aphidicola* proteins was successful. For comparison A, 222 statistically significant differentially expressed (SSDE) proteins were calculated, and 16 SSDE proteins were obtained for comparison D. The *B. aphidicola* gel (Supplementary Figure S2 (in Supplementary File 1) in the Supporting Information) was used to determine which differentially expressed proteins were bacterial in origin, ensuring that only aphid proteins were subjected to mass spectrometry. To validate this strategy, a number of spots matching those found on the *B. aphidicola* gel were excised from the preparative whole aphid gels and identified using MALDI-time-of-flight (TOF)-MS (results not shown) and were identified as *B. aphidicola* proteins.

Of the spots suitable for MS analysis, identities could be assigned to 35 protein spots (Table 1) comprising 29 unique proteins in comparison A (15 upregulated, 12 downregulated in antibiotic-treated aphids, and 1 protein identified as both up and down regulated from different spots). The equivalent analysis of comparison D yielded 9 proteins (Table 2), all but

Table 1. Statistically Supported Differentially Expressed Proteins Identified by MS for Antibiotic-Treated (Comparison A) Aphids

spot no.[a]	name	NCBI ref Seq or EST cluster	ACYPI identifier	fold change[b]	*p*-value	MASCOT score	no. of peptides
A5	cuticular protein CPG12	gi|193713854	ACYPI003765	3.4	0.002	259	7
A12	dicarbonyl/L-xylulose reductase	gi|89473706	ACYPI000088	−2.4	3.67×10^{-5}	108	2
A16	cuticular protein CPG12	gi|193657470	ACYPI002477	−2.2	6.04×10^{-4}	216	5
A18	cuticular protein CPG12	gi|193716064	ACYPI007782	2.2	0.017	73	3
A19	cuticular protein CPG12	gi|193716064	ACYPI007782	2.2	0.003	220	8
A50	C-type lectin (CTL)	gi|193632049	ACYPI001013	−1.9	1.14×10^{-4}	56	2
A53	phosphatidylethanolamine-binding protein	gi|240848795	ACYPI002878	1.9	8.00×10^{-3}	224	5
A60	RR2 cuticle protein 3	gi|193647888	ACYPI000291	1.8	1.80×10^{-2}	49	2
A61	ribosomal protein S7E	APD17476[c]	ACYPI010042	−1.8	3.00×10^{-3}	31	1[d]
A63	conserved hypothetical protein	gi|193580244	ACYPI004357	1.8	6.35×10^{-4}	106	2
A72	cuticular protein CPG12	gi|193706873	ACYPI005547	1.7	6.00×10^{-3}	106	4
A73	conserved hypothetical protein	gi|193678837	ACYPI002332	1.7	0.004	82	2
A86	cuticular protein 15	gi|193620175	ACYPI008503	1.6	1.00×10^{-3}	233	8
A95	aldehyde dehydrogenase	gi|193617714	ACYPI005866	−1.6	0.012	523	8
A96	conserved hypothetical protein	gi|240848531	ACYPI005249	−1.6	3.04×10^{-5}	539	8
A104	cuticular protein 111	gi|193683772	ACYPI001278	−1.6	1.00×10^{-3}	139	3
A114	cuticular protein 15	gi|193620175	ACYPI008503	1.5	0.003	87	4
A118	proteasome subunit α type 2	gi|237874223	ACYPI001003	1.5	1.00×10^{-2}	293	4
A119	conserved hypothetical protein	gi|240848531	ACYPI005249	−1.5	8.32×10^{-5}	223	3
A123	fructose 1,6-bisphosphate aldolase	gi|193591901	ACYPI007027	−1.5	3.34×10^{-6}	180	4
A135	senescence marker protein-30	gi|240849259	ACYPI003308	−1.5	3.00×10^{-3}	122	2
A140	tropomyosin	gi|239789378	NA	−1.5	1.00×10^{-3}	432	6
A145	tropomyosin 1	gi|193704626	ACYPI000969	−1.5	2.00×10^{-3}	596	9
A154	glutamine synthetase	APD4276[c]	ACYPI001461	−1.5	4.76×10^{-4}	86	2
A158	isovaleryl-CoA dehydrogenase	gi|193645728	ACYPI009436	−1.4	8.21×10^{-4}	228	6
A167	fructose 1,6-bisphosphate aldolase	gi|193591901	ACYPI007027	−1.4	6.28×10^{-4}	676	6
A174	translationally controlled tumor protein	gi|193713599	ACYPI008080	1.4	1.70×10^{-2}	126	2
A183	cuticular protein CPG12	gi|193716064	ACYPI007782	1.4	1.50×10^{-2}	271	6
A190	Eb1 microtubule-associated protein	gi|239788557	ACYPI005363	−1.4	7.00×10^{-3}	210	3
A201	replication protein A 70 kDa DNA-binding subunit	gi|193667016	ACYPI006150	1.4	3.00×10^{-3}	76	2
A205	cuticular protein 15	gi|193620175	ACYPI008503	−1.4	1.00×10^{-2}	68	2
A219	heat shock protein hsp21.4	gi|193618009	ACYPI003907	−1.4	5.00×10^{-3}	162	2
A222	β-1 tubulin	gi|193690653	ACYPI008874	−1.4	0.024	696	14
A266	chaperonin subunit 6a ζ	gi|193687012	ACYPI000575	1.3	2.00×10^{-3}	300	6
A285	(bicaudal) β-NAC-like protein	gi|187121188	ACYPI000087	1.3	7.00×10^{-3}	222	3

[a] Spot no. refers to the master number generated by Progenesis Same Spots Software, and the spot location is shown in Figure 4. [b] A positive fold change value indicates that the protein was more abundant in untreated aphids than antibiotic-treated aphids. [c] Spots were identified or obtained increased confidence scores after searches against the EST data set. [d] MS/MS spectra for single peptide-based identifications are presented in Supplemental File 2 in the Supporting Information.

Table 2. Statistically Supported Differentially Expressed Proteins Identified by MS for the Dietary (Comparison D) Analysis

spot no.[a]	name	NCBI ref Seq or EST cluster	ACYPI identifier	fold change[b]	*p*-value	MASCOT score	no. of peptides
D3	cuticle protein	APD00460[c]	NA	−1.9	0.022	145	3
D9	β-1-tubulin	gi|193690653	ACYPI008874	−1.6	0.026	696	14
D11	conserved hypothetical protein	APD00108[c]	ACYPI000294	−1.5	0.009	48	2
D13	conserved hypothetical protein	gi|193678837	ACYPI002332	−1.5	0.004	82	2
D17	E1 subunit of pyruvate dehydrogenase	gi|193669457	ACYPI008371	1.4	0.032	64	2
D23	ATP synthase-β subunit	gi|89574469	ACYPI000061	−1.4	0.001	1240	15
D24	*Buchnera aphidicola* flagellar hook protein flgE	APD10095[c]	NA	−1.4	0.028	74	1[d]
D25	enolase	gi|193669445	ACYPI005806	−1.4	0.041	494	8
D30	aldehyde dehydrogenase	gi|193617714	ACYPI005866	−1.3	0.045	523	8

[a] Spot no. refers to the master number generated by the Progenesis Same Spots Software, and the spot location is shown in Figure 4. [b] A negative fold change value indicates that the protein was more abundant in aphids reared on diet-L than diet-H. [c] Spots were identified or obtained increased confidence scores after searches against EST data sets. [d] MS/MS spectra for single peptide-based identifications are presented in Supplemental File 2 in the Supporting Information.

one of which was upregulated in aphids on diet-L. The differentially expressed proteins identified by MALDI-TOF/TOF are listed in Tables 1 and 2, and the position of each protein spot on the preparative 2D gels is given in Figure 4. The MASCOT results file for all peptides identified by MS for both treatments is provided in Supplementary File 2 in the Supporting Information.

GO and KO annotation of the differentially expressed proteins were obtained with BLAST2GO and AcypiCyc yielding 8 metabolism proteins, 7 cuticle proteins, 4 proteins with no obvious orthologues, 2 heat shock/chaperonin proteins, and single representatives of various other functional classes. A summary of the gene ontology and InterProScan results obtained from BLAST2GO for all identified proteins is

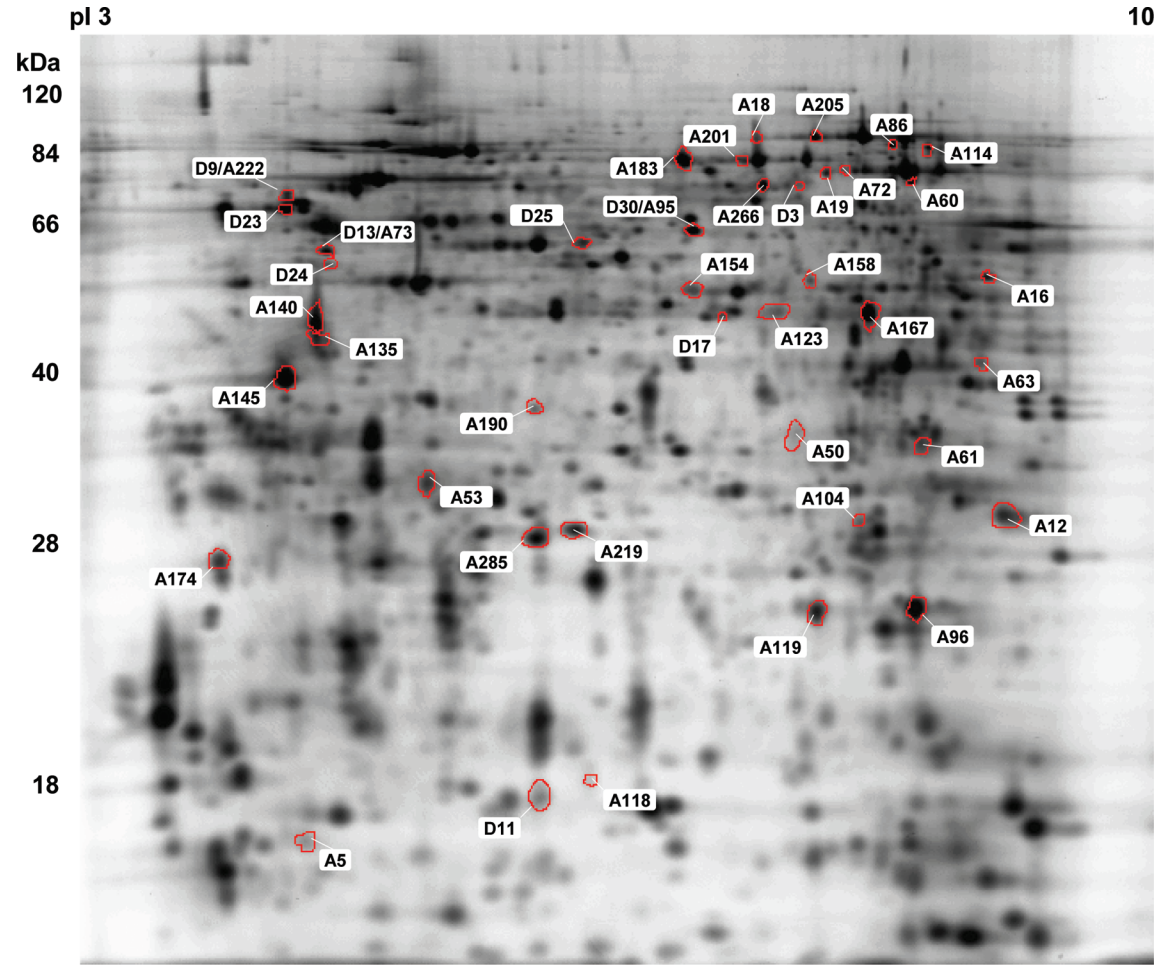

Figure 4. 2-DE image of whole aphid extract indicating the identified differentially expressed proteins from the diet (comparison D) and antibiotic (comparison A) perturbations. Spots were located on a 600 μg silver stained preparative gel and excised for analysis with MS.

provided as Supplementary File 3 in the Supporting Information.

Proteome Comparison between Untreated and Antibiotic-Treated Aphids (Comparison A). Five SSDE metabolic proteins, all of which were more abundant in antibiotic-treated aphids (Supplementary Figure S3 (in Supplementary File 1) in the Supporting Information), were identified as aldehyde dehydrogenase (Aldh, ACYPI005866), dicarbonyl/L-xylulose reductase (Dcxr; ACYPI000088), fructose 1,6 bisphosphate-aldolase (Aldo, ACYPI007027), isovaleryl-CoA dehydrogenase (Ivd, ACYPI009436), and glutamine synthetase (GlnA, ACYPI001461). Two isoforms of fructose 1,6 bisphosphate-aldolase were detected at spots A123 and A167 both with comparable levels of differential expression (upregulated 1.5- and 1.4-fold in antibiotic-treated aphids).

Four SSDE proteins in comparison A could be assigned to the functional class of genetic information processing. One protein, ribosomal protein S7E (RP-S7e; ACYPI010042) was more abundant in antibiotic-treated aphids. The three proteins more abundant in untreated aphids were proteasome subunit α type-2 (25 kDa subunit, ACYPI001003), replication protein A 70 kDa DNA-binding subunit (RPA1, ACYPI006150), and β-NAC-like protein (BTF3, ACYPI000087, orthologue of the *Drosophila* gene *bicaudal*, which plays a role in processing proteins as they emerge from the ribosome).[40]

Seven proteins were categorized as constituents of the cuticle after GO annotation. Of these, three were more abundant in antibiotic-treated aphids: one RR3 type cuticle protein (ACYPI001278) and two glycine-rich cuticular proteins [ACYPI002477 (CPG12 type) and ACYPI008503 (cuticle protein 15 type)]. Of the five cuticular proteins with elevated abundance in untreated aphids, one was RR2 type (ACYPI000291) and the remaining four were glycine-rich (ACYPI007782, ACYPI003765, ACYPI005547, and ACYPI008503). Most of the cuticular proteins were identified from single spots, but two of the glycine-rich proteins over-represented in untreated aphids were identified from three spots (A18, A19, and A183 for ACYPI007782 and A86, A114, and A205 for ACYPI008503). Thus, ACYPI08503 was identified from spots both over- and under-represented in the Comparison A.

Three cytoskeletal proteins were more abundant in antibiotic-treated aphids: tropomyosin 1 (ACYPI000969), a tropomyosin homologue matching to a full length cDNA sequence deposited to NCBI (gi|239789378|dbj|BAH71317.1) and β-1 tubulin (Tubb, ACYPI008874). The fact that six unique peptides were identified from ESTs rather than genome-derived sequences may reflect limitations in the annotation or incomplete genome sequence coverage. Two heat shock proteins/chaperonins were also differentially represented: chaperonin subunit 6a ζ (CCT6, ACYPI000575) was more abundant in untreated aphids, and

HSP21.4 (ACYPI003907) was more abundant in antibiotic-treated aphids.

Of the additional SSDE proteins identified, the orthologue of a translationally controlled tumor protein (Tctp) (ACYPI008080), phosphatidylethanolamine-binding protein (ACYPI002878), and two conserved hypothetical proteins (ACYPI002332, ACYPI004357) were more abundant in untreated aphids. The over-represented proteins in antibiotic-treated aphids were the Eb1 microtubule-associated protein (MAPRE, ACYPI005363), a calcium-binding regucalcin protein [also known as senescence marker protein-30, (SMP-30 ACYPI003308)], a protein with a C-type lectin-like domain (CTLD, ACYPI001013), and a hypothetical protein, ACYPI005249 (identified from spots A96 and A119).

Proteome Comparison between Diets (Comparison D). Of the 9 pea aphid proteins identified by MS, 8 and 1 were upregulated in aphids feeding from diet-L and diet-H, respectively. These include four proteins with a function in metabolism (Supplementary Figure S3 (in Supplementary File 1) in the Supporting Information): enolase (Eno, ACYPI005806), aldehyde dehydrogenase (Aldh, ACYPI005866), and ATP synthase β subunit (ATP5B, ACYPI000061), which were both more abundant in aphids on diet-L, and pyruvate dehydrogenase (Pdah, ACYPI008371), which was more abundant in the diet-H group. Other proteins that were present in higher abundance in aphids on diet-L were a cuticle protein (matching to EST cluster APD00460), β-1-tubulin (ACYPI008874), and two conserved hypothetical proteins, ACYPI000294 and ACYPI002332. A single *B. aphidicola* protein was also upregulated in the aphids on diet-L: the flagellar hook protein FlgE.

Discussion

Patterns in the Aphid Response to Perturbations. The parallel analysis of the proteome and metabonome of the pea aphid indicated very limited overlap in the aphid response to low exogenous supply (diet-L) and low endogenous supply (antibiotic treatment) of essential amino acids. Among the 15 differentially represented metabolites identified, just five (33%) were represented in both perturbations (Figure 3); and for the proteome analysis, just two proteins were significantly differentially expressed in both perturbations: an aldehyde dehydrogenase and β-tubulin. These data suggest that different processes at the level of both protein abundance and metabolism dominate the aphid responses to a deficit of exogenous and endogenous essential amino acids.

The proteome and metabonome were affected more substantially by antibiotic treatment than by dietary manipulation, as indicated by the higher number of significantly differentially expressed proteins and metabolites in the antibiotic-treated aphids than in aphids reared on diet-L (222 of 1138 vs 16 of 1008 proteins and 13 vs 7 metabolites, respectively). One factor contributing to this difference might be the changes in the physiology of antibiotic-treated aphids linked to their slow growth rates (such differences would not arise for the dietary comparison because the aphids on diet-L and diet-H grew at comparable rates; see Materials and Methods and ref 41). Additionally, a wider range of aphid physiological systems is expected to be affected by elimination of *B. aphidicola* than by dietary manipulation. Consistent with the latter, identified aphid proteins with a metabolic role have a greater proportional representation in the dietary comparison (4/8, 50%) than antibiotic treatment (5/29, 17%).

The primary focus of this study is the metabolic consequences of perturbed amino acid supply to aphids, as discussed below. Nevertheless, several differentially represented proteins, especially in the antibiotic treatment, may be important for the function and persistence of the symbiosis. These include various cuticular proteins, the best known role of which is in determining the mechanical properties of insect cuticle. However, it would be premature, on these data, to conclude that the aphid exoskeleton is modified by the elimination of *B. aphidicola*. This is because the genes for some cuticular proteins are expressed in internal organs in both the pea aphid and other insects, where they may play a role in tracheal structure, be synthesized internally for export to the epidermis, or possibly have functions unrelated to the cuticle. Differences in expression of genes for cuticular proteins have also been obtained across aphids with different reproductive modes[42] and in the beetle *Leptinotarsa decemlineata* in response to an organophosphorous insecticide and to water stress.[43] Three additional proteins with function not directly related to metabolism are also of particular note. First, the translationally controlled tumor protein is an abundantly expressed protein in many animals, including insects,[44] and plays a role in the regulation of insect growth.[45] The significance of its reduced abundance in antibiotic-treated aphids remains to be established definitively, but it is probably linked to the slow growth and small size of these insects. Second, proteins with a C-type lectin-like domain (CTLD proteins) are broadly implicated in immune function, including protection from pathogens,[46,47] and the over-representation of the CTLD protein in antibiotic-treated aphids raises the possibility that suppression of this defensive function may contribute to the persistence of the symbiosis. Finally, the over-representation of regucalcin in antibiotic-treated aphids is intriguing because this protein has recently been reported in the saliva of untreated aphids and may play a role in the suppression of Ca^{2+}-mediated plant defenses against aphid feeding.[48] Although no substantive difference between the feeding patterns of untreated and antibiotic-treated aphids has been identified, salivation is significantly extended in antibiotic-treated aphids.[49] This raises the possibility of symbiosis-dependent effects on the regucalcin content of aphid saliva with potentially important implications for plant utilization. Additionally or alternatively, regucalcin may have symbiosis-related functions unlinked to its role in saliva. Implicated by these data in both the obligate symbiosis and plant relations, regucalcin is a candidate target for novel agents in aphid pest control.

Metabolic Consequences of Perturbations. Aphids are rich in lipids and sugars, especially the blood sugar trehalose. The metabonomic analysis identified no evidence that the perturbations affected the lipid composition, a result which confirms and extends the demonstration that eliminating *B. aphidicola* has no detectable impact on the triglyceride composition of the pea aphid.[50] The trehalose pool, however, is significantly elevated, apparently at the expense of glucose (which is reduced) in antibiotic-treated aphids (Figure 3). There is no evidence for a direct involvement of *B. aphidicola* in the sugar metabolism of aphids,[24,51] and the change in the sugar pools is, therefore, interpreted as an indirect consequence of eliminating the symbiosis. The proteome analysis provides evidence that this effect might be underpinned by increased glycolysis (which consumes glucose) and that it may be linked to the shortfall of essential amino acids. Specifically, the abundance of glycolytic enzymes, fructose 1,6-bisphosphate aldolase and

enolase, is increased by the antibiotic treatment and diet-L, respectively. Interestingly, the E1 α subunit of the pyruvate dehydrogenase is downregulated in aphids on diet-L, potentially providing pyruvate as a substrate for the synthesis of alanine, which is over-represented in these aphids. Furthermore, the effect of low essential amino acid supply on carbohydrate metabolism may extend beyond glycolysis, as is suggested by the upregulation of dicarbonyl/L-xylulose reductase (DCXR), an enzyme that mediates the interconversion between xylitol and xylulose in the uronate cycle of glucose metabolism, in antibiotic-treated aphids and of the ATP synthase β-subunit in aphids on diet-L.

The metabonomic analysis offers spectacular confirmation of the central role of amino acid relations in the aphid symbiosis with *B. aphidicola*. Of the 15 metabolites identified with differential abundance across treatments, 10 (67%) are amino acids in the free amino acid pool (the protein-bound amino acids were not quantified). Furthermore, the impact of the shortfall of exogenous and endogenous essential amino acids (diet-L and antibiotic treatment, respectively) on the free amino acid pools of the aphids was similar, involving a decrease in essential amino acids and increase in nonessential amino acids. The two exceptions to this pattern are the nonessentials alanine and tyrosine, both of which are decreased in antibiotic-treated aphids (Figure 3), confirming previous results.[52] It remains to be established why alanine is depressed in antibiotic-treated aphids, but the low tyrosine content can be explained by the aphid-mediated synthesis of this amino acid from phenylalanine, which is produced by *B. aphidicola*.[53]

The impact of antibiotic treatment on the amino acid profile of aphids has been interpreted previously as limitation of aphid protein synthesis by the shortfall of essential amino acids derived from *B. aphidicola*, with the accumulation of nonessential amino acids, which are not limiting.[18] It has also been argued that nonessential amino acids are selectively degraded in antibiotic-treated aphids, generating ammonia; and the increased ammonia load is alleviated by its condensation with glutamate to form glutamine, via the enzyme glutamine synthetase. Previous supportive evidence comes from the elevated glutamine concentration in the honeydew and glutamine synthetase activity in tissues of antibiotic-treated aphids.[54] The over-representation of glutamine in the NMR detected metabonome (Figure 3) and of glutamine synthetase in the proteome of antibiotic-treated aphids (Table 1) offers crucial support for the significance of this interaction. The elevated titer of asparagine in the antibiotic-treated aphids suggests that this nitrogen-rich compound may also be a repository for excess ammonia in these insects.

Nevertheless, other data obtained here suggest that this interpretation of the amino acid profile of antibiotic-treated aphids may be incomplete in two complementary ways. First, the aphids have comparable protein growth rates on diet-L and diet-H, but those on diet-L have modified amino acid profiles, similar to antibiotic-treated aphids (see above). A likely explanation is that aphids with a shortfall of dietary essential amino acids may maintain smaller pool sizes to conserve essential amino acids. Second, the enzyme isovaleryl-CoA dehydrogenase, which mediates a reaction in the degradation of valine, is significantly upregulated in antibiotic-treated aphids, raising the possibility that the elevated amino acid degradation may include this essential amino acid. The upregulation of one of 13 annotated aldehyde dehydrogenases of the pea aphid, specifically ACYPI005866, in both antibiotic-treated aphids and aphids on diet-L may also be relevant because these enzymes transform aldehydes to carboxylic acids, including in the degradation of several essential amino acids (valine, leucine, isoleucine, tryptophan, lysine, and histidine). Further research is required to determine the specificity of the enzyme coded by ACYPI005866, but a role in the oxidation of acetaldehyde to acetic acid is unlikely because acetate and its aminated product, *N*-methylacetamide, are decreased in the aphids with elevated aldehyde dehydrogenase levels. These indications that degradation of essential amino acids is elevated under essential amino acid deficiency are counterintuitive and require direct investigation by metabolic analysis.

The production of essential amino acids by *B. aphidicola* is likely to contribute to two further metabolites identified by the metabonomic analysis: succinate and acetate. The metabolic network of *B. aphidicola* has been reconstructed and the metabolic flux through the network, as investigated by flux balance analysis, results in the net production of both succinate and acetate, i.e., these compounds are "overflow metabolites".[55] Succinate is a product of succinyl-diaminopimelate desuccinylase (DapE), a reaction in the lysine biosynthetic pathway; and *B. aphidicola* lacks the genetic capacity to utilize succinate. The increased succinate content of aphids on diet-L may, therefore, relate to an increased production of the essential amino acid lysine by *B. aphidicola* in these aphids. *B. aphidicola* can mediate three reactions involving acetate: as a substrate of acetate kinase (AckA) and as a product of both acetylornithine deacetylase (ArgE) in arginine synthesis and cysteine synthase (CysK) in cysteine synthesis. In the flux balance models, flux through acetate kinase is small compared especially to ArgE, and the net production of acetate is, thus, largely linked to arginine synthesis. We hypothesize that the low acetate levels in antibiotic-treated aphids is a marker of the loss of arginine production by *B. aphidicola*. These specific hypotheses on the molecular basis of the nutritional interaction between the insect and its symbiotic bacteria have been generated by combining global metabonomic analysis with systems level metabolic network analysis, an approach that is feasible because the relationship involves a single microbial partner with a sequenced genome.

In conclusion, this parallel metabonomic and proteomic analysis has provided an excellent demonstration of the central role of amino acid metabolism in the aphid symbiosis with *B. aphidicola* and identified several key proteins and processes (e.g., CTLD protein, regucalcin) with candidate role in the function and persistence of the symbiosis. These have potential as targets for novel insect pest control strategies. More generally, this investigation of an insect association with a single microbial taxon demonstrates the value of combined proteomic and metabonomic analysis for the study of interactions between animals and their resident microbiota.

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Supporting Information Available: Supplemental File 1 contains Supplementary Figure S1, NMR assignment for *N*-methylacetamide; Supplementary Figure S2, representative 2D DIGE images of *Buchnera aphidicola* isolation (A) and pooled standard (B) used in the Comparison A experiments; Supplementary Figure S3, sections of 2-DE gels and log normalized volume comparisons for identified statistically significant differentially expressed (SSDE) proteins involved in metabolic processes; Supplementary Table S1, NMR resonance assignment and multiplicity; Supplementary Table S2, the coefficients of metabolites contributing to the separation between aphids subjected to dietary perturbation and antibiotic treatment. Supplemental File 2 containes peptides and proteins identified using MALDI-TOF-MS and searches against NCBI nonredundant and aphid EST databases, and MS/MS spectra annotated with observed masses have been provided for single peptide-based identifications. Supplemental File 3 contains BLAST2Go derived GO terms and InterProScan results for the differentially expressed proteins identified from the D and A comparisons. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Kelly, D.; Campbell, J. I.; King, T. P.; Grant, G.; Jansson, E. A.; Coutts, A. G. P.; Pettersson, S.; Conway, S. Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. *Nat. Immunol.* **2004**, *5* (1), 104–112.

(2) Backhed, F.; Ding, H.; Wang, T.; Hooper, L. V.; Koh, G. Y.; Nagy, A.; Semenkovich, C. F.; Gordon, J. I. The gut microbiota as an environmental factor that regulates fat storage. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (44), 15718–15723.

(3) Clayton, T. A.; Lindon, J. C.; Cloarec, O.; Antti, H.; Charuel, C.; Hanton, G.; Provost, J. P.; Le Net, J. L.; Baker, D.; Walley, R. J.; Everett, J. R.; Nicholson, J. K. Pharmaco-metabonomic phenotyping and personalized drug treatment. *Nature* **2006**, *440* (7087), 1073–1077.

(4) Nicholson, J. K.; Holmes, E.; Wilson, I. D. Gut microorganisms, mammalian metabolism and personalized health care. *Nat. Rev. Microbiol.* **2005**, *3* (5), 431–438.

(5) Moran, N. A. Symbiosis as an adaptive process and source of phenotypic complexity. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104* (Suppl.), 8627–8633.

(6) Douglas, A. E. The microbial dimension in insect nutritional ecology. *Funct. Ecol.* **2009**, *23* (1), 38–47.

(7) Bourtzis K., Miller T. A. *Insect Symbiosis*; CRC Press: Boca Raton, FL, 2008.

(8) Douglas, A. E. Symbiotic microorganisms: untapped resources for insect pest control. *Trend Biotechnol.* **2007**, *25* (8), 338–342.

(9) Miller, T. A. Pest and disease challenges and insect biotechnology solutions. *Entomol. Res.* **2008**, *38* (1), 34–40.

(10) Durvasula, R. V.; Sundaram, R. K.; Kirsch, P.; Hurwitz, I.; Crawford, C. V.; Dotson, E.; Beard, C. B. Genetic transformation of a corynebacterial symbiont from the chagas disease vector triatoma infestans. *Exp. Parasitol.* **2008**, *119* (1), 94–98.

(11) Durvasula, R. V.; Gumbs, A.; Panackal, A.; Kruglov, O.; Aksoy, S.; Merrifield, R. B.; Richards, F. F.; Beard, C. B. Prevention of insect-borne disease: An approach using transgenic symbiotic bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94* (7), 3274–3278.

(12) Shigenobu, S.; Watanabe, H.; Hattori, M.; Sakaki, Y.; Ishikawa, H. Genome sequence of the endocellular bacterial symbiont of aphids Buchnera sp APS. *Nature* **2000**, *407* (6800), 81–86.

(13) Haynes, S.; Darby, A. C.; Daniell, T. J.; Webster, G.; van Veen, F. J. F.; Godfray, H. C. J.; Prosser, J. I.; Douglas, A. E. Diversity of bacteria associated with natural aphid populations. *Appl. Environ. Microbiol.* **2003**, *69* (12), 7216–7223.

(14) Warnecke, F.; Luginbuhl, P.; Ivanova, N.; Ghassemian, M.; Richardson, T. H.; Stege, J. T. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature* **2007**, *450*, 560–565.

(15) Ley, R. E.; Lozupone, C. A.; Hamady, M.; Knight, R.; Gordon, J. I. Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat. Rev. Microbiol.* **2008**, *6* (10), 776–788.

(16) Dethlefsen, L.; McFall-Ngai, M.; Relman, D. A. An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature* **2007**, *449*, 811–818.

(17) van Emden H. *Aphids as Crop Pests*; CAB International: Wallingford, U.K., 2007.

(18) Douglas, A. E. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria buchnera. *Annu. Rev. Entomol.* **1998**, *43*, 17–37.

(19) Moran, N. A.; McCutcheon, J. P.; Nakabachi, A. Genomics and evolution of heritable bacterial symbionts. *Annu. Rev. Genet.* **2008**, *42*, 165–190.

(20) Li, M.; Wang, B. H.; Zhang, M. H.; Rantalainen, M.; Wang, S. Y.; Zhou, H. K.; Zhang, Y.; Shen, J.; Pang, X. Y.; Zhang, M. L.; Wei, H.; Chen, Y.; Lu, H. F.; Zuo, J.; Su, M. M.; Qiu, Y. P.; Jia, W.; Xiao, C. N.; Smith, L. M.; Yang, S. L.; Holmes, E.; Tang, H. R.; Zhao, G. P.; Nicholson, J. K.; Li, L. J.; Zhao, L. P. Symbiotic gut microbes modulate human metabolic phenotypes. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105* (6), 2117–2122.

(21) Martin, F. P. J.; Dumas, M. E.; Wang, Y. L.; Legido-Quigley, C.; Yap, I. K. S.; Tang, H. R.; Zirah, S.; Murphy, G. M.; Cloarec, O.; Lindon, J. C.; Sprenger, N.; Fay, L. B.; Kochhar, S.; van Bladeren, P.; Holmes, E.; Nicholson, J. K. A top-down systems biology view of microbiome-mammalian metabolic interactions in a mouse model. *Mol. Syst. Biol.* **2007**, *3*, 112.

(22) Engstrom, Y.; Loseva, O.; Theopold, U. Proteomics of the Drosophila immune response. *Trend Biotechnol.* **2004**, *22* (11), 600–605.

(23) Lefevre, T.; Thomas, F.; Schwartz, A.; Levashina, E.; Blandin, S.; Brizard, J. P.; Le Bourligu, L.; Demettre, E.; Renaud, F.; Biron, D. G. Malaria Plasmodium agent induces alteration in the head proteome of their Anopheles mosquito host. *Proteomics* **2007**, *7* (11), 1908–1915.

(24) Douglas, A. E.; Price, D. R. G.; Minto, L. B.; Jones, E.; Pescod, K. V.; Francois, C. L. M. J.; Pritchard, J.; Boonham, N. Sweet problems: insect traits defining the limits to dietary sugar utilisation by the pea aphid, *Acyrthosiphon pisum*. *J. Exp. Biol.* **2006**, *209* (8), 1395–1403.

(25) Wilkinson, T. L. The elimination of intracellular microorganisms from insects: an analysis of antibiotic-treatment in the pea aphid (*Acyrthosiphon pisum*). *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* **1998**, *119* (4), 871–881.

(26) Bax, A.; Davis, D. G. MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. *J. Magn. Reson.* **1985**, *65* (2), 355–360.

(27) Hurd, R. E. Gradient-Enhanced Spectroscopy. *J. Magn. Reson.* **1990**, *87* (2), 422–428.

(28) Trygg, J. O2-PLS for qualitative and quantitative analysis in multivariate calibration. *J. Chemom.* **2002**, *16* (6), 283–293.

(29) Cloarec, O.; Dumas, M. E.; Trygg, J.; Craig, A.; Barton, R. H.; Lindon, J. C.; Nicholson, J. K.; Holmes, E. Evaluation of the orthogonal projection on latent structure model limitations caused by chemical shift variability and improved visualization of biomarker changes in ^{1}H NMR spectroscopic metabonomic studies. *Anal. Chem.* **2005**, *77* (2), 517–526.

(30) Ramagli, L. S.; Rodriguez, L. V. Quantitation of microgram amounts of protein in two-dimensional polyacrylamide-gel electrophoresis sample buffer. *Electrophoresis* **1985**, *6* (11), 559–563.

(31) Rabilloud, T.; Valette, C.; Lawrence, J. J. Sample application by in-gel rehydration improves the resolution of 2-dimensional electrophoresis with immobilized pH-gradients in the first-dimension. *Electrophoresis* **1994**, *15* (12), 1552–1558.

(32) Gorg, A.; Postel, W.; Weser, J.; Gunther, S.; Strahler, J. R.; Hanash, S. M.; Somerlot, L. Elimination of point streaking on silver stained two-dimensional gels by addition of iodoacetamide to the equilibration buffer. *Electrophoresis* **1987**, *8* (2), 122–124.

(33) Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. *Anal. Chem.* **1996**, *68* (5), 850–858.

(34) Hewitson, J. R.; Harcus, Y. M.; Curwenb, R. S.; Dowle, A. A.; Atmadja, A. K.; Ashton, P. D.; Wilson, A.; Maizels, R. M. The secretome of the filarial parasite, *Brugia malayi*: proteomic profile of adult excretory-secretory products. *Mol. Biochem. Parasitol.* **2008**, *160* (1), 8–21.

(35) Altschul, S. F.; Madden, T. L.; Schaffer, A. A.; Zhang, J. H.; Zhang, Z.; Miller, W.; Lipman, D. J. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **1997**, *25* (17), 3389–3402.

(36) Conesa, A.; Gotz, S.; Garcia-Gomez, J. M.; Terol, J.; Talon, M.; Robles, M. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **2005**, *21* (18), 3674–3676.

(37) Karp, P. D.; Ouzounis, C. A.; Moore-Kochlacs, C.; Goldovsky, L.; Kaipa, P.; Ahren, D.; Tsoka, S.; Darzentas, N.; Kunin, V.; Lopez-Bigas, N. Expansion of the BioCyc collection of pathway/genome databases to 160 genomes. *Nucleic Acids Res.* **2005**, *33* (19), 6083–6089.

(38) Gard, J. K.; Feng, P. C. C.; Hutton, W. C. Nuclear magnetic resonance timecourse studies of glyphosate metabolism by microbial soil isolates. *Xenobiotica* **1997**, *27* (7), 633–644.

(39) Cloarec, O.; Dumas, M. E.; Craig, A.; Barton, R. H.; Trygg, J.; Hudson, J.; Blancher, C.; Gauguier, D.; Lindon, J. C.; Holmes, E.; Nicholson, J. Statistical total correlation spectroscopy: An exploratory approach for latent biomarker identification from metabolic 1H NMR data sets. *Anal. Chem.* **2005**, *77* (5), 1282–1289.

(40) Markesich, D. C.; Gajewski, K. M.; Nazimiec, M. E.; Beckingham, K. bicaudal encodes the *Drosophila* beta NAC homolog, a component of the ribosomal translational machinery. *Development* **2000**, *127* (3), 559–572.

(41) Reymond, N.; Calevro, F.; Vinuelas, J.; Morin, N.; Rahbe, Y.; Febvay, G.; Laugier, C.; Douglas, A.; Fayard, J. M.; Charles, H. Different levels of transcriptional regulation due to trophic constraints in the reduced genome of *Buchnera aphidicola* APS. *Appl. Environ. Microbiol.* **2006**, *72* (12), 7760–7766.

(42) Le Trionnaire, G.; Jaubert, S.; Sabater-Munoz, B.; Benedetto, A.; Bonhomme, J.; Prunier-Leterme, N.; Martinez-Torres, D.; Simon, J. C.; Tagu, D. Seasonal photoperiodism regulates the expression of cuticular and signalling protein genes in the pea aphid. *Insect Biochem. Mol. Biol.* **2007**, *37* (10), 1094–1102.

(43) Zhang, J.; Goyer, C.; Pelletier, Y. Environmental stresses induce the expression of putative glycine-rich insect cuticular protein genes in adult *Leptinotarsa decemlineata* (Say). *Insect Mol. Biol.* **2008**, *17* (3), 209–216.

(44) Lee, J. M.; Kusakabe, T.; Kawaguchi, Y.; Miyagawa, Y.; Takahashi, M.; Mon, H.; Nho, S. K.; Koga, K. Molecular cloning and characterization of the translationally controlled tumor protein gene in *Bombyx mori*. *Comp. Biochem. Physiol. B: Mol. Integr. Physiol.* **2004**, *139* (1), 35–43.

(45) Hsu, Y. C.; Chern, J. J.; Cai, Y.; Liu, M. Y.; Choi, K. W. *Drosophila* TCTP is essential for growth and proliferation through regulation of dRheb GTPase. *Nature* **2007**, *445* (7129), 785–788.

(46) Zelensky, A. N.; Gready, J. E. The C-type lectin-like domain superfamily. *FEBS J.* **2005**, *272* (24), 6179–6217.

(47) Marmaras, V. J.; Lampropoulou, M. Regulators and signalling in insect haemocyte immunity. *Cell Signal* **2009**, *21* (2), 186–195.

(48) Carolan, J. C.; Fitzroy, C. I. J.; Ashton, P. D.; Douglas, A. E.; Wilkinson, T. L. The secreted salivary proteome of the pea aphid *Acyrthosiphon pisum* characterised by mass spectrometry. *Proteomics* **2009**, *9* (9), 2457–2467.

(49) Wilkinson, T. L.; Douglas, A. E. Aphid feeding, as influenced by disruption of the symbiotic bacteria-an analysis of the pea aphid (*Acyrthosiphon-Pisum*). *J. Insect Physiol.* **1995**, *41* (8), 635–640.

(50) Rahbe, Y.; Delobel, B.; Febvay, G.; Chantegrel, B. Aphid-specific triglycerides in symbiotic and aposymbiotic *Acyrthosiphon-pisum*. *Insect Biochem. Mol. Biol.* **1994**, *24* (1), 95–101.

(51) Wilkinson, T. L.; Ashford, D. A.; Pritchard, J.; Douglas, A. E. Honeydew sugars and osmoregulation in the pea aphid *Acyrthosiphon pisum*. *J. Exp. Biol.* **1997**, *200* (15), 2137–2143.

(52) Prosser, W. A.; Douglas, A. E. The aposymbiotic aphid-an analysis of chlortetracycline-treated pea aphid, *Acyrthosiphon-Pisum*. *J. Insect Physiol.* **1991**, *37* (10), 713–719.

(53) Wilson, A.; Ashton, P. D.; Calevro, F.; Charles, H.; Colella, S.; Febvay, G. ; Jander D.; Kuahlan P. F.; Macdonald A. J.; Schwartz J. F.; Thomas, G. H.; Douglas, A. E. Genomic insight into the amino acid relations of the pea aphid Acyrthosiphon pisum with its symbiotic bacterium, *Buchnera aphidicola*. *Insect Mol. Biol.* In press.

(54) Wilkinson, T. L.; Douglas, A. E. Why pea aphids (*Acyrthosiphon-pisum*) lacking symbiotic bacteria have elevated levels of the amino-acid glutamine. *J. Insect Physiol.* **1995**, *41* (11), 921–927.

(55) Thomas, G. H.; Zucker, J.; Macdonald, S. J.; Sorokin, A.; Goryanin, I.; Douglas, A. E. A fragile metabolic network adapted for cooperation in the symbiotic bacterium *Buchnera aphidicola*. *BMC Syst. Biol.* **2009**, *3*, 24.

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