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ORIGINAL ARTICLE

Effects of facultative symbionts and heat stress on the metabolome of pea aphids

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We examined metabolite pools of pea aphids with different facultative symbiont infections, and characterized their effects on aphid metabolism in baseline and heat stress conditions. The bacterial symbiont *Serratia symbiotica* protects aphid hosts from the detrimental results of heat stress and shields the obligate symbiont *Buchnera* from effects of heat. We investigated whether broad effects on metabolism might correlate with this protection. Both facultative symbiont infection and heat treatment had large effects on the aphid metabolome. All three pea aphid facultative symbionts had similar effects on aphid metabolism despite their evolutionary diversity. Paradoxically, heat triggers lysis of many *S. symbiotica* cells and a correlated rapid reduction in *S. symbiotica* titres within aphid hosts. We conclude that facultative symbionts can have substantial effects on host metabolic pools, and we hypothesize that the protective effects of *S. symbiotica* may reflect the delivery of protective metabolites to aphid or *Buchnera* cells, after heat exposure.
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Introduction

Many animals have intimate symbioses with bacteria that live within cells or tissues and that are a normal part of the host. This fusion of two distinct metabolic systems has a variety of potential consequences for the host. In the case of nutritional symbionts, exemplified by *Buchnera aphidicola* the obligate symbiont of aphids, the primary metabolic impact is to provision hosts with essential cellular compounds that are not produced by the host metabolism and that are not provided in the diet (Shigenobu *et al.*, 2000; Akman and Douglas, 2009). By provisioning essential amino acids not present in phloem sap, *Buchnera* is an essential component of the fused host–symbiont metabolism.

Facultative symbionts are also expected to impact the metabolome of the host. Three additional symbiont lineages, *Serratia symbiotica*, *Hamiltonella defensa*, and *Regiella insecticola*, are commonly found infecting the pea aphid, *Acyrthosiphon pisum* (Moran *et al.*, 2005). Like *Buchnera*, they are maternally transmitted, but are facultative from the perspective of the aphid host. These symbionts appear to be maintained in pea aphid populations at least in part because of their ability to confer protection against various environmental stresses. The main benefit recorded for *S. symbiotica* is protection against heat stress (Chen *et al.*, 2000; Montllor *et al.*, 2002), whereas *H. defensa* and *R. insecticola* confer little or no heat protection (Russell and Moran, 2006) but are known to protect against natural enemies (Oliver *et al.*, 2003; Scarborough *et al.*, 2005).

At constant temperatures as low at 25 °C, pea aphids show decreased survival, mass, and fecundity and increases in development time compared with aphids kept at 20 °C (Chen *et al.*, 2000), and they cannot survive brief exposure to temperatures above 42 °C (Montllor *et al.*, 2002). Heat negatively affects numbers of *Buchnera* cells and bacteriocytes, which is likely reflected in the fitness of their aphid hosts. Experimental studies have shown that *S. symbiotica* protects bacteriocyte numbers as well as aphid fitness after heat exposure treatments of varying severity and duration. In this study, we examined symbiont density dynamics after heat-shock, and found evidence that, though *S. symbiotica* ameliorates the effects of heat on *Buchnera* cell density, as reported earlier (Montllor *et al.*, 2002; Russell and Moran, 2006), this protection occurs at the same time that many *S. symbiotica* cells lyse in response to heat exposure. One hypothesis for the mechanism underlying *S. symbiotica*-mediated protection is that lysis releases bacterial metabolites that are beneficial for maintenance of *Buchnera* cells and aphid fitness.

To date, no study has examined the effects of facultative symbionts on the overall metabolism

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of insect hosts. Such metabolic changes might be important as the mediators of phenotypic effects on hosts such as the enhanced heat tolerance conferred by *S. symbiotica*. We attempt to characterize the metabolites influenced by infection with *S. symbiotica* and two other common facultative symbionts in normal and heat stress conditions across several pea aphid genotypes.

Materials and methods

Aphid lineages

Aphids were taken from aphid colonies grown in a growth chamber at University of Arizona with a long day photoperiod (16L:8D) to ensure parthenogenetic reproduction. These aphid clones were established from collections within the United States and represent natural infections with facultative symbionts present at the time of collection, except for the 5AT, 5AU, 5AR, and Tucson-uninfected aphid sub-clones (Table 1). The Tucson-uninfected sub-clone was established from female adults treated with heat-shock consisting of 48 h at 30 °C. The mothers were allowed to reproduce for 7 days and the progeny were treated with heat-shock again, then placed on plants individually. The progeny of these aphids were tested for facultative symbiont infection using PCR with the primers 559F 5′-CGTGCCAGCAGCCGCGGTAATAC-3′ (Russell *et al*., 2003) and 35R 5′-CCTTCATCGCCTCTGA CTGC-3′ every few generations, and uninfected lines were kept. The 5AR, 5AT, and 5AU aphid sub-clones were established from the naturally uninfected 5A clone (collected in Madison, WI, USA, June 1999) by microinjection of body fluids containing symbionts from the Tucson, 8-2b, and 2a aphid clones, respectively, in the years indicated in Table 1. Aphid genotypes represented both active and inactive forms of the *Buchnera ibpA* promoter; hence, its effects were averaged across samples.

Quantitative PCR for symbiont density after heat-shock

Aphid heat-shock experiments were performed as described in Montllor *et al*. (2002) and Russell and Moran (2006). Briefly, mothers were placed on plants and allowed to reproduce for 24 h, and subsequently were removed. After 48 h (day 2) second instar nymphs were subjected to heat-shock in a growth chamber in which temperature gradually increased over 2 h from 20 to 39 °C, and was maintained at 39 °C (± 0.5 °C) for an additional 2 h. After 4 h the temperature was slowly decreased to 20 °C, and at 6 h the heat-shocked cultures were placed side-by-side with control treatments in the original growth chamber. In the control treatment, aphids were kept at 20 °C for the duration of the experiment. For symbiont density experiments, nymphs were sampled before heat-shock, then immediately after heat-shock treatment (after cooling) at various intervals by flash freezing.

Quantitative PCR was performed using a Lightcycler (Roche Molecular Biochemicals, Indianapolis, IN, USA) to estimate the number of *S. symbiotica* and *Buchnera* bacterial chromosomes present within an aphid, measured relative to the number of aphid chromosomes present. DNA was extracted from aphid samples using the Qiagen DNeasy kit (Valencia, CA, USA) eluted in 50 µl because of the small amount of starting material. We used primers to specifically amplify the *dnaK/hsp70* gene from *S. symbiotica, Buchnera,* and *A. pisum* using the primers ApRF1 5′-TGGCGGGTGATGTGAAG-3′ and 5′-ApRR1 5′-CGGGATAGTGGTGTTTTTGG-3′ for *S. symbiotica, Buchnera*HS70F2 5′-ATGGGTAAAAT TATTGGTAATTG-3′ and *Buchnera*HS70R2 5′-ATAG CTTGACGTTTAGCAGG-3′ for *Buchnera,* and A70F2 5′-TAAGAGGAAAACTAAAAAGGACG-3′ and A70R2 5′-GGAAQJ;ACTCGGGTGTAGAAATC-3′ for *A. pisum*. Quantitative PCR conditions were used as in Oliver *et al*. (2006), and DNA copies were quantified using an absolute standard curve specific for each gene. The regression lines for the standard curves had mean squared error <0.1, and fidelity of the amplication was checked using diagnostic melting curves for each amplicon.

Transmission electron microscopy

Aphids were heat treated as for quantitative PCR but without the 2 h cooling phase, and were immediately decapitated and fixed in 4% formaldehyde, 1% glutaraldehyde, and 0.1 M PO_4 and microwaved to facilitate infiltration. Samples were rinsed in 0.1 M PO_4 and 0.07% Ruthenium Red three times then post-fixed in 1% osmium in dH_2O. An ethanol series increasing in concentration from 30–100% was used for sample dehydration. The samples were embedded in Epon Araldite and cut into 60 nm sections before microscopy.

Metabolomics experimental design

Aphids were grown in large cages in a growth chamber at 20 °C, and adults (grandmothers) were placed onto plants in six individual cages per clone.

Table 1 Aphid clonal lineages used in this study

Aphid genotype	*Facultative symbiont*	*Year introduced to lab*
Tucson	*S. symbiotica*	1999
Tucson	Uninfected	2005
9-2-1	*S. symbiotica*	2001
2BB	*S. symbiotica*	1999
5AR	*S. symbiotica*	2000
5AT	*H. defensa*	2001
5AU	*R. insecticola*	2001
7a	Uninfected	2000
Sc7	*S. symbiotica*	2007
TucAUF	*S. symbiotica*	2008
8-2b	*H. defensa*	2000

Grandmothers were allowed to deposit nymphs for 2 days and were subsequently removed. After nymphs had matured (mothers) and reproduced, immature aphids (1st–4th instar) from each of the aphid clones were separated from adults and were divided and placed into two cages, for the control and heat-shock treatments. For each clone, six cages were heat shocked, and six cages were kept at 20 °C as controls. Heat-shock consisted of moving cages to be treated to a 20 °C incubator and slowly increasing its temperature to 39 ± 0.5 °C over 2 h, where it was maintained for an additional 2 h. At this point, aphids were collected, flash frozen, and weighed (average mass 25.6 mg).

Metabolite extraction, derivatization, and GC analysis
Samples were milled in frozen state for 2 min at a frequency of $25\,s^{-1}$ in a ball mill (MM301, with 3 mm metal balls, both Retsch Corp., Newton, PA, USA). Metabolites were extracted with 1 ml of a 3:3:2 (v/v/v) acetonitrile:isopropanol:water solvent mixture kept at −20 °C, vortexed for 10 s and shaken for 4–6 min at 4 °C. Centrifugation removed insoluble proteins and cell membrane components, and the supernatant was dried down using a speed vacuum (Labconco Centrivap cold trap, Kansas City, MO, USA). Pellets were washed in 500 ml of 50% acetonitrile:water to remove membrane and storage lipids and dried again. Thirty external standards were used to check for instrument performance in calibration series before and after the runs to ensure instrument drifts or other effects that would compromise quantification differences did not occur; 2 μl of internal retention index markers were added to the dried extracts (for automatic retention index calculation to allow for compound identifications) and derivatized as described by Fiehn *et al.* (2008). An agilent 6890 gas chromatograph (Santa Clara, CA, USA) and a Leco Pegasus IV time of flight spectrometer were used, controlled by the Leco ChromaTOF software vs 2.32 (St Joseph, MI, USA); 0.5 μl of sample was injected in split mode with a 1:5 ratio. Injector and oven temperatures, column specifications, ionization, and mass spectrometry were performed as described in Fiehn *et al.* (2008). ChromaTOF vs 2.32 was used for data preprocessing and resulting files were exported and further processed by a filtering algorithm implemented in the metabolomics BinBase database (Fiehn *et al.*, 2005). Metabolites were identified using retention index and mass spectrum as the two most important identification criteria and assigned a BinBase identifier number. All BinBase identifiers were matched against the Fiehn mass spectral library of 1200 authentic metabolite spectra and the NIST05 commercial library for chemical structure classification. All metabolites detected in more than 80% (for unidentified metabolites) or 50% (for structurally identified compounds) of samples in a study design class (as defined in the SetupX database (Scholz and Fiehn, 2007)) were quantified using peak height of the unique ion as default. All raw and processed data are downloadable at http://fiehnlab.ucdavis.edu:8080/m1/main_public.jsp.

Statistical analyses
Statistical analyses were performed using JMP7 (SAS Institute). Before normalization the concentrations of total metabolites observed among treatments were compared for global changes in metabolic activity. The metabolic profile for each sample was normalized by calculating the sum of the intensities of all metabolites in each sample and then dividing all data associated with that sample by its metabolite sum. The distribution of each metabolite was examined and transformed using either a log, square root, inverse, and square transformation (listed in Supplementary Table S1 for each metabolite) to improve the assumptions of normality required for multivariate analyses. Metabolite concentrations were inverse transformed with the following formula: $\text{inverse}(x) = -1/(x) + 2$.

Samples were divided into two groups; Tucson Genotype included all aphids derived from the Tucson aphid clone and allowed examination of symbiont and treatment effects for one aphid genotype, and All genotypes consisted of eight aphid clones (5AR, 5AT, 5AU, 2BB, 9-2-1, Tuc8, 7a, and Sc7), allowing averaging of symbiont and treatment effects across different aphid genotypes.

The percentage variance in overall aphid metabolism explained by experimental factors, such as treatments or symbiont infections could be investigated using principal components analysis and controlled partial least squares. To test for changes in metabolite concentrations in the Tucson clones, a model was fit to each metabolite with the following factors: symbiont, treatment, and symbiont × treatment. For the All genotypes experiments, an additional factor, genotype (symbiont) was added to control for the effects of aphid genotype. A Bonferroni correction was used to control the Type II error to 0.05 across all 485 comparisons, resulting in a critical *P*-value of $P < 0.0001$ for significant changes. Only statistically significant metabolites that changed >1.5-fold in concentration were considered. Metabolites that changed consistently because of symbiont infection in both the single aphid genotype and multiple aphid genotypes comparisons were identified using the criteria above for one of the comparisons, and more relaxed criteria for the other comparison ($P < 0.05$ for symbiont infected compared to uninfected concentrations, same direction of change).

For log transformations, fold change was determined using e^(difference between log transformed means). For other transformations, the fold change was approximated by back transforming means and calculating their ratio.

Results

Bacterial density dynamics in response to heat stress

Changes in densities for the facultative symbionts *S. symbiotica* and *H. defensa* during a constant 30 °C heat treatment are shown in Figure 1. Although *S. symbiotica* has a protective role in similar conditions (Chen *et al*., 2000), symbiont density decreases compared to controls from 24 h onwards, whereas *H. defensa* density remains unaffected by heat stress. This pattern is consistent for facultative symbionts infecting several genetically distinct aphid clones.

The ratios of symbiont densities in aphids subjected to control temperatures and to a 4 h, 39 °C heat-shock are shown in Figure 2, for both *Buchnera* and *S. symbiotica*. Aphids infected solely with *Buchnera* (no facultative symbionts) show dramatically decreased symbiont density starting at 24 h after the heat-shock treatment. In contrast, aphids infected with *Buchnera* and *S. symbiotica* do not show a decrease in *Buchnera* density after heat-shock, suggesting that *S. symbiotica* shields *Buchnera* cells from the effects of heat. *S. symbiotica* shows a reduction in density early after heat-shock to approximately 50% of the symbiont density in control aphids for both genetically distinct aphid clones measured, with recovery to normal levels somewhere between 24 and 72 h post treatment. Transmission electron microscopy shows that lysis of *S. symbiotica* cells correlates with the observed reduction in density of this symbiont immediately after heat treatment (Figure 3).

The effects of symbionts and heat on the aphid metabolome

Tucson aphids are naturally infected with *S. symbiotica*, but a sub-line cured of symbionts was used to evaluate the effects of adding a symbiont and its metabolism while controlling for the aphids' genotype. In addition, to look for symbiont effects that are consistent across several aphid genotypes, aphids of different genetic backgrounds and different facultative symbiont infections were examined. We reasoned that consistent effects of symbionts in both of these comparisons ('Tucson' and 'among genotypes') would be of interest as possible sources of protection against heat.

Totals of 134 identified metabolites and 351 unidentified metabolites were found among samples using the BinBase algorithm detailed in the 'Materials and methods' section. Before normalization, the sum of peak heights for all metabolites per unit wet weight increased 64% in heat shocked compared to control aphids (two sample t test on log transformed data, $t_{142} = 3.75$, two-tailed $P = 0.0003$), presumably because of dehydration resulting from heat exposure. The different facultative symbiont infections (*S. symbiotica*, *H. defensa*, *R. insecticola*, uninfected) did not cause significant differences in the levels of total metabolites (ANOVA, $F_{3,140} = 1.58$, $P = 0.197$).

Principal components analyses of all metabolites showed separation between infected and uninfected samples, and also between control and heat-shocked samples (Figure 4). In Tucson aphids, the first

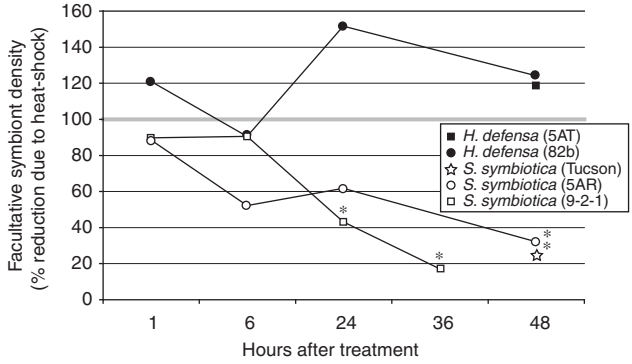

Figure 1 Facultative symbiont density changes after raising aphids at 30 °C (constant heat stress treatment) for several aphid genotypes. Per cent reduction because of heat-shock is the ratio of heat-shock-treated symbiont densities ($N = 4$) to control symbiont densities ($N = 4$, *represents a significant reduction in density $P < 0.05$). Symbiont density is determined as the ratio of the number of single-copy symbiont genes to single copy aphid genes (to correct for aphid body size). Although *S. symbiotica* density decreases over time because of heat stress, *H. defensa* density does not.

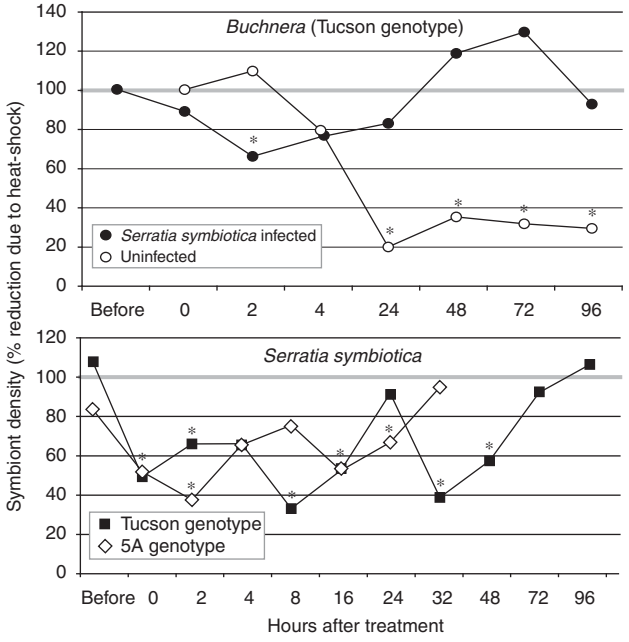

Figure 2 Symbiont density changes after a 4 h heat-shock treatment at 39 °C. Per cent reduction because of heat-shock is the ratio of heat-shock-treated symbiont densities ($N = 4$) to control symbiont densities ($N = 4$, *represents a significant reduction in density $P < 0.05$). Symbiont density is determined as the ratio of the number of single-copy symbiont genes to single copy aphid genes (to correct for aphid body size). *Buchnera* density decreases dramatically 24 h after heat-shock if no facultative symbiont is present, but if infected with *S. symbiotica*, its density is maintained at similar levels to controls. *S. symbiotica* density decreases early after heat-shock treatment compared to controls.

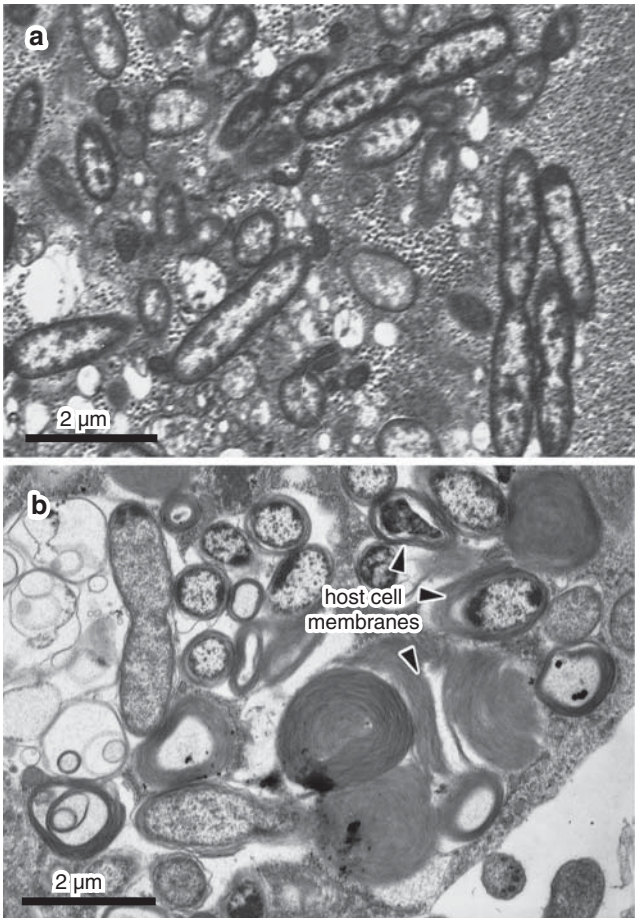

Figure 3 Transmission electron microscopy of aphids treated with control and heat-shock conditions. (**a**) *S. symbiotica* cells in an aphid bacteriocyte in control conditions. (**b**) Whorls of host membranes are engulfing lysed *S. symbiotica* cells after a heat-shock.

component separated *S. symbiotica*-infected aphid samples from uninfected aphid samples, and represented 20.1% of total metabolic variation, and the second component separated samples by treatment and explained 12.4% of total metabolic variation (Figure 4). All other aphid genotypes examined showed a similar pattern, with the first component separating infected and uninfected aphid samples and explaining 20.0% of total variance, and the second component differentiating between control and heat-shocked samples and representing 11.1% of total variance (Figure 4). The weight loadings for the top three metabolite increases and decreases are listed in Supplementary Table 5. Principal components analysis of all other aphid genotypes revealed that *S. symbiotica, H. defensa,* and *R. insecticola* have very similar effects on the metabolome.

Variation explained by facultative symbionts

Although many metabolites changed in concentration with facultative symbiont infection, fewer than 30% of the metabolites could be identified (Table 2; Supplementary Tables 1 and 2). The largest changes

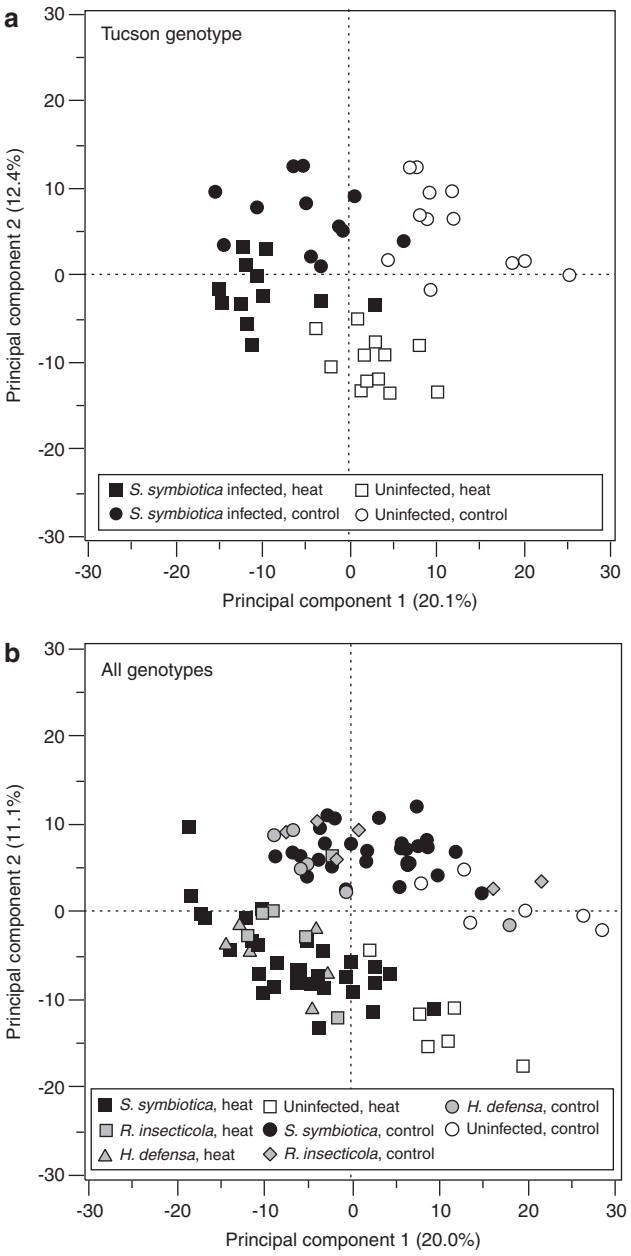

Figure 4 (**a**) Principal components analysis of Tucson genotype metabolites. (**b**) Principal components analysis of all aphid genotypes' metabolites.

in concentration occurred for unidentified metabolites, including two unknown metabolites (227585 and 227647) that changed 37.4- and 28.2–fold, respectively, because of *S. symbiotica* infection in Tucson aphids. Although these metabolites are not yet structurally identified, properties and spectra can be queried using the public BinBase website (http://eros.fiehnlab.ucdavis.edu:8080/binbase-compound/), featuring an automatic mass spectral similarity search for the selected unknown compound against all other BinBase metabolites and information about its presence in other species. These metabolites were also significantly increased for all three facultative symbionts in the 'all genotypes' comparison, but with

Table 2 Summary of changes that occur because of symbiont infection or heat treatment

	Tucson	*All genotypes*
Percentage of total variation attributed to treatment (PLS)		
Heat-shock	11.9%	11.7%
S. symbiotica	14.3%	3.6%
All symbionts	NA	8.6%
Number of metabolites that increase (I) or decrease (D) in concentration		
Heat-shock		
Identified	32 (15 I, 17 D)	31 (13 I, 18 D)
Unidentified	21 (8 I, 13 D)	42 (12 I, 30 D)
S. symbiotica		
Identified	19 (9 I, 8 D)	39 (19 I, 13 D)
Unidentified	56 (28 I, 30 D)	96 (29 I, 68 D)
All symbionts		
Identified	NA	34 (19 I, 15 D)
Unidentified	NA	107 (32 I, 75 D)
Range in fold-changes		
Heat-shock		
Identified	10.0 D–65.9 I	3.1 D–91.9 I
Unidentified	4.4 D–4.0 I	5.0 D–3.2 I
S. symbiotica		
Identified	3.1 D–4.0 I	4.3 D–4.6 I
Unidentified	6.3 D–37.4 I	4.8 D–7.9 I
All symbionts		
Identified	NA	9.3 D–4.8 I
Unidentified	NA	6.2 D–7.9 I

Abbreviation: PLS, partial least squares.
Significant changes identified using ANOVA, fold change >1.5 and $P<0.0001$, Tukey's HSD for comparisons between infected and uninfected samples in all genotypes, $P<0.05$. 'I' indicates an increase in concentration, and 'D' indicates a decrease.

fold changes less than 5 (227585: F ratio $=10.5$, $P=5.96e{-}06$, and 227647: F ratio $=16.5$, $P=1.58e{-}08$, *t*-tests between uninfected and infected aphids' means $P<0.05$).

The metabolic effects of infection with each of the three facultative symbionts, *S. symbiotica*, *H. defensa*, and *R. insecticola*, were highly correlated when compared with uninfected aphids. Metabolite concentrations in infected aphids always changed in the same direction or did not significantly change when compared with uninfected aphids' metabolite levels.

Twenty-two known metabolites changed consistently because of *S. symbiotica* infection in both the 'Tucson' and 'all genotypes' comparisons (Table 4). These included six amino acids, two nucleic acids, five sugars and sugar alcohols, and nine other metabolites. Only one of these metabolites, indole-3-lactate, showed a significant change in concentration because of both *S. symbiotica* (2.22-fold decrease for Tucson genotype, $t_{44}=4.54$, $P=4.36e{-}5$ and 1.30-fold decrease for all genotypes, $F_{11,84}=4.09$, $P=0.009$) and *H. defensa* infection (1.43-fold decrease), but was not influenced by *R. insecticola* infection. Forty-three unknown metabolites consistently changed in concentration in association with symbiont infection. Two of these changed because of *S. symbiotica* infection, but not because of *H. defensa* or *R. insecticola* infection.

In Tucson aphids, *S. symbiotica* infection is associated with increased concentrations of the amino acids arginine and serine, and for all aphid genotypes, increases occurred for lysine, histidine, arginine, phenylalanine, tyrosine, valine, and asparagine (ANOVA, fold change >1.5 and $P<0.0001$). Considering only the protein-coding amino acids, both essential and non-essential amino acids were positively associated with infection by all three facultative symbionts (Supplementary Figures 1 and 2, two sided *t*-test or Tukey's HSD among genotypes, $P<0.05$).

Variation explained by heat treatment
A higher percentage of the metabolites that changed in concentration because of heat-shock treatment were identifiable, compared with those associated with symbiont effects (Table 2; Supplementary Tables 3 and 4). Identified metabolites that changed in concentration included sugars and polyols, nucleic acids, amino acids, TCA cycle metabolites, a vitamin, a fatty acid, and several others (Table 3). The effects of heat treatment are very similar whether comparing within one aphid genotype or comparing across several aphid genotypes.

Interactions between facultative symbionts and heat-shock treatment
Four metabolites identified from Tucson aphids showed evidence for *S. symbiotica* effects that differed depending on the treatment the aphids received. These were fructose, sucrose, mannose-6-phosphate, and *N*-acetyl-D-mannosamine (Figure 5). Fructose, sucrose and *N*-acetyl-D-mannosamine decreased in concentration during control conditions because of *S. symbiotica* infection, whereas in heat-shock conditions, symbionts did not have an effect. Mannose-6-phosphate increased in concentration because of symbiont infection in heat-shock conditions, but not in control conditions.

In all aphid genotypes, three metabolites showed a significant interaction between symbiont infection and treatment: sucrose, isoleucine, and threonine (Figure 5). Sucrose concentration responded differently to symbionts and heat treatment, with facultative symbionts decreasing sucrose concentrations during heat, but not in control conditions. In uninfected aphids, sucrose concentration increased in hot conditions. Sucrose is likely from the aphids' diet, and may be increasing in concentration because of aphid dehydration. Isoleucine increased in infected heat-shocked aphids, but not in control aphids, and threonine decreased in infected control aphids, but not in heat-shocked aphids.

Discussion

Metabolic responses to symbiont infection
Metabolic responses because of symbiont infection include both metabolites produced by bacterial

Table 3 Known metabolites that change in concentration in response to heat-shock treatment in comparison to controls

Metabolite	*PubChem ID*	*Category*	*Fold change*	
			Tucson	*All genotypes*
Alanine	5950	Amino acids	NS	1.6
Arginine	232	Amino acids	0.4	0.2
Glutamine	5961	Amino acids	NS	0.6
Glycine	750	Amino acids	1.7	NS
Phenylalanine	6140	Amino acids	0.4	0.4
Proline	145742	Amino acids	NS	0.6
Lauric acid	5649	Fatty acids	0.6	NS
Adenine	190	Nucleic acids	2.0	2.0
Adenosine	60961	Nucleic acids	0.6	NS
2-aminoadipic acid	469	Other	0.6	NS
2-hydroxyglutaric acid	43	Other	NS	0.6
2-isopropylmalic acid	5280523	Other	0.3	0.4
3-hydroxypropionic acid	68152	Other	0.4	0.4
5′-deoxy-5′-methylthioadenosine	439176	Other	2.9	2.2
Aconitic acid	309	Other	1.8	NS
Aminomalonate	100714	Other	1.6	NS
β-Alanine	239	Other	NS	1.9
Erythronic acid lactone	6427168	Other	0.6	NS
Glutaric acid	743	Other	NS	0.6
Homoserine	12647	Other	0.5	0.5
Isothreonic acid	151152	Other	2.1	1.8
L-DOPA	6047	Other	NS	0.5
Malonic acid	867	Other	0.6	0.6
N-acetyl-D-mannosamine	65150	Other	2.6	2.1
Ornithine	6262	Other	0.4	0.3
Quinic acid	6508	Other	NS	0.7
Shikimic acid	8742	Other	1.8	NS
Threonic acid	5282933	Other	NS	0.5
Arabinose	229	Sugars and polyols	0.5	NS
Arabitol	94154	Sugars and polyols	0.5	0.4
Erythritol	222285	Sugars and polyols	10.3	5.4
Fructose	5984	Sugars and polyols	2.4	2.8
Galactitol	453	Sugars and polyols	26.2	50.4
Glucoheptose	76599	Sugars and polyols	2.2	2.4
Lyxitol	439255	Sugars and polyols	2.1	1.6
Mannitol	6251	Sugars and polyols	2.3	2.5
Mannose-6-phosphate	65127	Sugars and polyols	NS	2.2
Sorbitol	5780	Sugars and polyols	65.9	91.9
Sucrose	5988	Sugars and polyols	0.1	0.4
Fumaric acid	444972	TCA cycle	0.5	0.4
Malate	525	TCA cycle	0.6	0.4
Succinic acid	1110	TCA cycle	0.6	0.6
Pantothenic acid	6613	Vitamins	2.4	1.9

symbionts, and also metabolites produced by aphids and affected by symbiont presence. Many more metabolites that changed significantly because of symbiont infection remained unidentified compared to heat-shock influenced metabolites (25.3% compared to 60.4% in Tucson aphids, 26.7% compared to 42.5% in other genotypes). Our inability to identify many metabolites linked to symbiont infection reflects the greater diversity of bacterial metabolites and their lower representation in the library used for metabolite identification.

There were few consistent changes in metabolites attributed to symbiont infection between the Tucson genotype and other genotypes comparisons, suggesting that aphid or *S. symbiotica* genotype have a large influence on the metabolic effects of infection (Table 4 for identified metabolites). The *S. symbiotica* lineages in these aphids are closely related, with >99.7% similarity between 16S rDNA sequences, whereas the aphid clones used in this study represent several genotypes (Moran *et al*., 2005).

There are two conceivable ways in which *S. symbiotica* could protect against heat stress after cell lysis. One is production of protective metabolites not made by other facultative symbionts. Another possibility is that *S. symbiotica* lysis in heat stress is key for protection. Thus, lysis may provide a necessary vehicle for metabolite delivery even if the metabolites released are not unique to *S. symbiotica*. Only three metabolite changes were limited to aphids infected with *S. symbiotica* (and sometimes with *H. defensa,* which also provides some heat protection): indole-3-lactate and two unidentified metabolites (202885 and 227610).

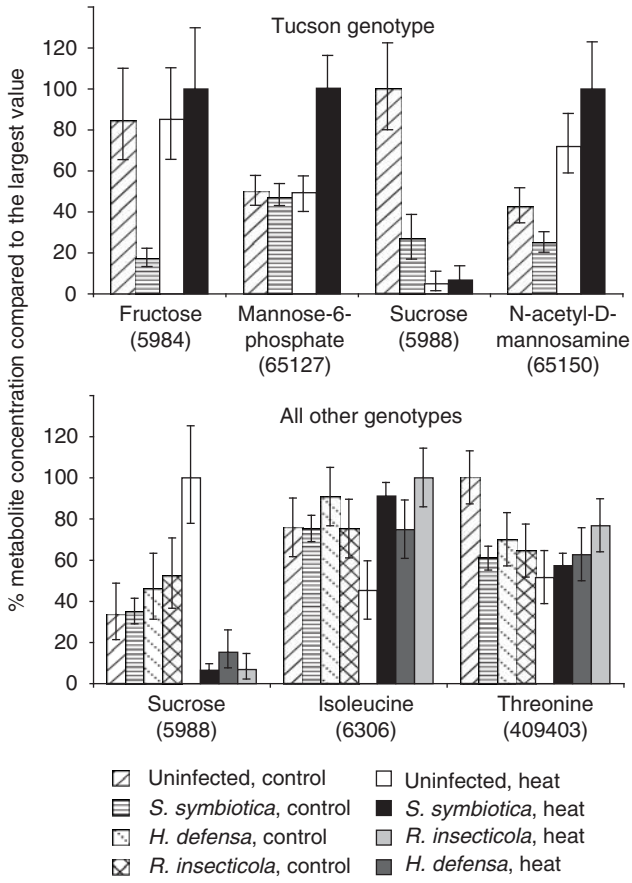

Figure 5 Metabolites whose concentration depends on both symbiont infection and treatment temperature. PubChem ID numbers are listed in parentheses below each metabolite.

Indole-3-lactate is an antioxidant (Poeggeler *et al.*, 1999), and although heat-shock can create oxidative stress conditions, it is not clear how this compound could be protective, as it decreased in concentration in association with *S. symbiotica* and *H. defensa* infection.

Many of the metabolites changing in association with *S. symbiotica* infection underwent similar changes for *H. defensa* and *R. insecticola* infection. These represent both metabolites produced in common by these symbionts as well as metabolites that reflect a general aphid response to facultative symbiont infection.

In general, aphids infected with facultative symbionts had higher concentrations of both essential and non-essential amino acids. On the basis of genome sequences, *H. defensa* and *R. insecticola* are only able to produce the essential amino acids lysine and threonine (Degnan *et al.*, 2009a, b), but other essential amino acids also increase because of infection with these symbionts. One possibility is that facultative symbiont infection somehow stimulates *Buchnera* to produce amino acids, as *Buchnera* is the only organism in the system capable of producing most essential amino acids (Shigenobu *et al.*, 2000). However, *Buchnera* cannot produce several of the non-essential amino acids that also increased in concentration, so this possibility does not fully explain the pattern. Another possibility is that proteases produced by facultative symbionts are degrading proteins, increasing free amino acid pools in pea aphids. The *H. defensa* and *R. insecticola* genomes possess several proteases that could be responsible for this effect (Degnan *et al.*, 2009a, b).

Symbiont responses to heat-shock

Of the many observed changes in metabolite concentrations because of symbiont infection, very few showed evidence for differing symbiont effects in baseline and hot temperature treatments (Figure 5). Similar levels of symbiont-associated metabolites are expected in control and heat-shocked aphids because aphids are sampled as symbiont lysis is occurring, and any metabolites released are contained within the aphid body. In Tucson aphids, sucrose, fructose, and *N*-acetyl-D-mannosamine decrease in concentration in control aphids with facultative symbiont infection, yet do not change significantly because of infection after heat treatment. *S. symbiotica* may deplete sucrose and fructose by using them as energy sources in control conditions, but may not be able to continue after stress, possibly because of metabolic shut-down or lysis. *N*-acetyl-D-mannosamine is a precursor for sialic acid, a protective metabolite during heat stress, and could also be produced by *S. symbiotica* when not under stress (sialic acid was not represented in both libraries used for metabolite identification). Mannose-6-phosphate targets newly synthesized hydrolytic enzymes to lysosomes, and its increased concentration in *S. symbiotica*-infected aphids in heat-shock conditions suggests its involvement in host degradation of lysed symbiont proteins (Sly and Fischer, 1982). In comparison, for all aphid genotypes, sucrose levels are not significantly altered by symbionts in control conditions. Although heat-shock is linked to an increase in sucrose in uninfected aphids, sucrose concentrations are significantly decreased compared to controls in association with symbiont infection after heat-shock.

Aphid and Buchnera responses to heat-shock

Several biochemical studies of insect responses to temperature shock have revealed that some insects accumulate sugar alcohols (polyols) to protect cell membranes and protein stability (Salvucci, 2000). Sorbitol, a metabolite known to protect *Bemisia tabaci* whiteflies during high temperatures, dramatically increased in aphids after heat stress (Wolfe *et al.*, 1998; Salvucci, 2000). In whiteflies, this sugar alcohol is made from fructose (derived from isomerization of sucrose) in conjunction with NADPH provided by the pentose phosphate pathway (Salvucci *et al.*, 1998, 1999). Our observation of

Table 4 Metabolites that changed in concentration consistently in Tucson aphids and in all genotypes because of facultative symbiont infection

Metabolite	*PubChem ID*	*Category*	*Fold change (Tucson)*	*t Ratio*[a]	P-*value*[b]	*Fold change (all genotypes)*			F *ratio*[c]	P-*value*[b]
			S. symbiotica			S. symbiotica	H. defensa	R. insecticola		
Arginine	232	Amino acids	1.8	4.6	3.2e−5	7.1[d]	5.8[d]	4.9[d]	33.6	2.3e−14
Asparagine	236	Amino acids	1.4	15.9	2.0e−4	1.7[d]	1.8[d]	1.8[d]	17.7	5.3e−9
Isoleucine	6306	Amino acids	1.5	31.8	1.2e−6	1.4[d]	1.4[d]	1.5[d]	6.4	6.0e−4
Lysine	5962	Amino acids	1.5	28.7	2.9e−6	4.1[d]	4.0[d]	4.8[d]	55.0	8.9e−20
Tyrosine	6057	Amino acids	1.3	35.6	3.8e−7	1.8[d]	1.8[d]	2.0[d]	24.0	2.7e−11
Valine	6287	Amino acids	1.3	36.1	3.3e−7	1.8[d]	1.8[d]	2.0[d]	24.9	1.3e−11
Guanosine	6802	Nucleic acids	1.4	22.5	2.2e−5	2.0[d]	2.3[d]	2.9[d]	13.9	1.9e−7
Inosine	6021	Nucleic acids	1.5	41.5	7.5e−8	2.0[d]	2.3[d]	3.0[d]	21.0	3.1e−10
Indole-3-lactate	92904	Other	0.5	20.6	4.4e−5	0.8[d]	0.7[d]	1.0	4.1	9.0e−3
Oxalic acid	971	Other	0.4	22.4	2.3e−5	0.4[d]	0.4[d]	0.5[d]	7.3	2.1e−4
Ornithine	6262	Other	1.2	5.0	0.03	2.6[d]	1.8[d]	1.9[d]	20.0	7.1e−10
Maleimide	10935	Other	1.7	17.2	1.5e−4	1.9[d]	1.8[d]	1.6[d]	9.8	1.3e−5
Citrulline	833	Other	1.3	4.8	0.034	1.6[d]	1.9[d]	2.0[d]	9.1	2.7e−5
Pelargonic acid	8158	Other	0.7	13.7	5.6e−4	0.5[d]	0.5[d]	0.7[d]	14.3	1.3e−7
Hydroxycarbamate	18931500	Other	0.7	18.2	1.0e−4	0.5[d]	0.4[d]	0.4[d]	13.4	3.1e−7
Malonic acid	867	Other	0.8	5.4	0.025	0.5[d]	0.5[d]	0.5[d]	15.8	3.2e−8
Hydroxylamine	787	Other	0.8	16.5	2.0e−4	0.6[d]	0.6[d]	0.6[d]	12.2	1.1e−6
Inulotriose	N/A	Sugars and polyols	0.3	26.8	5.3e−6	0.2[d]	0.3[d]	0.2[d]	10.9	4.1e−6
Mannitol	6251	Sugars and polyols	0.6	25.0	9.6e−6	0.5[d]	0.4[d]	0.3[d]	20.8	3.7e−10
Ribitol	827	Sugars and polyols	2.0	49.3	1.1e−8	1.5[d]	1.3	1.6[d]	4.3	7.4e−3
Trehalose	7427	Sugars and polyols	0.7	21.6	3.0e−5	0.6[d]	0.6[d]	0.7[d]	5.6	1.6e−3
Sucrose	5988	Sugars and polyols	0.4	18.1	1.1e−4	0.3[d]	0.5[d]	0.4[d]	21.6	1.9e−10

[a]This test was conducted with 47 degrees of freedom.
[b]In numbering notation, e represents the exponent base 10, for example 1.07e−04 is equivalent to 1.07×10^{4}.
[c]This test was conducted with 3, 84 degrees of freedom.
[d]Significantly different from uninfected aphids at level $P<0.05$.

a decrease in sucrose and increases in fructose and sorbitol after heat treatment is consistent with this mechanism for sorbitol metabolism. In all aphid genotypes, heat treatment resulted in increases of several sugar alcohols that are known to be protective under temperature stress, including erythritol and mannitol (Ruijter *et al.*, 2003; Michaud *et al.*, 2008; Rangel *et al.*, 2008) and galactitol and lyxitol. Mannitol production is considered to be the major response to heat stress in the cotton aphid *Aphis gossypii,* but in *A. pisum* we observed a much larger response for sorbitol than for mannitol, suggesting that pea aphids respond to heat in a manner more similar to whiteflies (Hendrix and Salvucci, 1998). Symbiont infection is negatively associated with fructose, mannitol, and sorbitol, so it is unlikely that these metabolites are of bacterial origin. They are likely produced by the aphids themselves as an intrinsic protective response.

The pentose phosphate pathway increases in activity relative to the TCA cycle in response to cold treatment for insects that accumulate polyols (Moreau *et al.*, 1977; Wood and Nordin, 1980). A similar decrease in TCA cycle and respiratory chain activity was seen in a proteomic study of the aphid *Macrosiphum euphorbiae* after heat and radiation stress (Nguyen *et al.*, 2009). In insects, cold and heat tolerance can involve the same metabolic responses, and the decrease in TCA cycle intermediates fumarate, malate, and succinate in *A. pisum* suggests a shift from glycolysis to the pentose phosphate pathway, providing NADPH for sugar alcohol production (Michaud *et al.*, 2008).

β-Alanine is a precursor for pantothenic acid, and both metabolites increased in pea aphids after heat stress. β-Alanine production is a conserved response to heat stress in organisms as distantly related as *Drosophila* and *Arabidopsis*, and β-alanine may be a chemical chaperone, preserving enzyme function during heat-shock (Kaplan *et al.*, 2004; Mehta and Seidler, 2005; Malmendal *et al.*, 2006). Polyamine production is another conserved response to heat in animals and plants, and, although increases were not observed in heat-shocked aphids, polyamine precursors arginine and ornithine decreased and by-product 5′-deoxy-5′-methylthioadenosine increased after heat-shock, suggesting that polyamine biosynthesis occurred (Michaud *et al.*, 2008). Increases in adenine could be a result of recycling of 5′-deoxy-5′-methylthioadenosine, or of recycling of plant cytokinins or nucleosides (Caspi *et al.*, 2008).

In *Drosophila*, several amino acids increase in concentration after severe heat-shock, possibly because of the breakdown of proteins (Malmendal *et al.*, 2006). In contrast, only glycine in Tucson aphids and alanine in other genotypes showed increases in concentration in response to heat, whereas several other amino acids decreased in concentration. Several metabolites involved in amino acid biosynthesis or degradation changed in concentration after heat-shock: 2-aminoadipic acid, involved in lysine biosynthesis and degradation; 2-isopropylmalic acid, involved in leucine biosynthesis; homoserine, involved in threonine, methionine, and homocysteine biosynthesis; shikimic and quinic acids, involved in phenylalanine, tyrosine, and tryptophan biosynthesis; and 2-hydroxyglutaric acid, which is made from α-ketoglutarate and is thought to be involved in NADH recycling and serine biosynthesis. Changes in these metabolites may be indicative of protein degradation and rapid recycling and biosynthesis because of heat stress.

Conclusions

Through examination of the effects of facultative symbionts on aphid metabolism, we have come to several new conclusions. Facultative symbionts have a large effect on aphid metabolism. Aphid genotype may influence *S. symbiotica*-associated metabolic changes. The density dynamics of a heat stress protective symbiont compared with other facultative symbionts during heat-shock is consistent with the hypothesis that *S. symbiotica* lysis is key for its protective role. The fact that very few *S. symbiotica* metabolites were influenced by temperature treatment is consistent with the observations that many *S. symbiotica* cells lyse in response to heat. Our samples included metabolites whether or not they were contained within symbiont cells, and metabolic activity of the symbionts is likely to be arrested by heat stress. The three facultative symbionts had very similar metabolic effects relative to uninfected aphids, but *S. symbiotica* appears to be the only one capable of rapid metabolite delivery through lysis in stressful conditions.

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References

Akman GE, Douglas AE. (2009). Symbiotic bacteria enable insect to use a nutritionally inadequate diet. *Proc Biol Sci* **276**: 987–991.

Caspi RFH, Fulcher CA, Kaipa P, Krummenacker M, Latendresse M, Paley S *et al.* (2008). The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Res* **36**: D623–D631.

Chen D-Q, Montllor CB, Purcell AH. (2000). Fitness effects of two facultative endosymbiotic bacteria on the pea aphid, *Acyrthosiphon pisum*, the blue alfalfa aphid, *A kondoi*. *Entomol Exp Appl* **95**: 315–323.

Degnan PH, Leonardo TE, Cass B, Hurwitz B, Stern D, Gibbs RA *et al.* (2009a). Dynamics of genome evolution in facultative symbionts of aphids. *Environ Microbiol* (e-pub ahead of print).

Degnan PH, Yu Y, Sisneros N, Wing RA, Moran NA. (2009b). *Hamiltonella defensa*, genome evolution of protective bacterial endosymbiont from pathogenic ancestors. *Proc Natl Acad Sci USA* **106**: 9063–9068.

Fiehn O, Wohlgemuth G, Scholz M. (2005). Setup and annotation of metabolomice experiments by integrating biological and mass spectrometric metadata. *Proc Lect Notes Bioinformatics* **3615**: 224–239.

Fiehn O, Wohlgemuth G, Scholz M, Kind T, Lee DY, Lu Y *et al.* (2008). Quality control for plant metabolomics: reporting MSI-compliant studies. *Plant J* **53**: 691–704.

Hendrix DL, Salvucci ME. (1998). Polyol metabolism in homopterans at high temperatures: accumulation of mannitol in aphids (Aphididae: Homoptera) and sorbitol in whiteflies (Aleyrodidae: Homoptera). *Comp Biochem Physiol A* **120**: 487–494.

Kaplan F, Kopka J, Haskell DW, Zhao W, Schiller KC, Gatzke N *et al.* (2004). Exploring the temperature-stress metabolome of Arabidopsis. *Plant Physiol* **136**: 4159–4168.

Malmendal A, Overgaard J, Bundy JG, Sorensen JG, Nielsen NC, Loeschcke V *et al.* (2006). Metabolomic profiling of heat stress: hardening and recovery of homeostasis in Drosophila. *Am J Physiol Regul Integr Comp Physiol* **291**: R205–R212.

Mehta AD, Seidler NW. (2005). Beta-alanine suppresses heat inactivation of lactate dehydrogenase. *J Enzyme Inhib Med Chem* **20**: 199–203.

Michaud MR, Benoit JB, Lopez-Maratinez G, Elnitsky MA, Lee Jr RE, Denlinger DL. (2008). Metabolomics reveals unique and shared metabolic changes in response to heat shock, freezing and desiccation in the Antarctic midge, *Belgica antarctica*. *J Insect Physiol* **54**: 645–655.

Montllor C, Maxmen A, Purcell AH. (2002). Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. *Ecol Entomol* **27**: 189–195.

Moran NA, Russell JA, Koga R, Fukatsu T. (2005). Evolutionary relationships of three new species of Enterobacteriaceae living as symbionts of aphids and other insects. *Appl Environ Microbiol* **71**: 3302–3310.

Moreau R, Gordoux L, Dutrieu J. (1977). Utilisation comparee du cycle des pentoses en fonction des variations thermiques chez deux lepidoteres *Bombyx mori* L and *Pieris bassicae* L. *Comp Biochem and Physiol B* **56**: 175–179.

Nguyen TT, Michaud D, Cloutier C. (2009). A proteomic analysis of the aphid *Macrosiphum euphorbiae* under heat and radiation stress. *Insect Biochem Mol Biol* **39**: 20–30.

Oliver KM, Moran NA, Hunter MS. (2006). Costs and benefits of a superinfection of facultative symbionts in aphids. *Proc Biol Sci* **273**: 1273–1280.

Oliver KM, Russell JA, Moran NA, Hunter MS. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc Natl Acad Sci USA* **100**: 1803–1807.

Poeggeler B, Pappolla MA, Hardeland R, Rassoulpour A, Hodgkins PS, Guidetti P *et al.* (1999). Indole-3-propionate: a potent hydroxyl radical scavenger in rat brain. *Brain Res* **815**: 382–388.

Rangel DE, Anderson AJ, Roberts DW. (2008). Evaluating physical and nutritional stress during mycelial growth as inducers of tolerance to heat and UV-B radiation in *Metarhizium anisopliae* conidia. *Mycol Res* **112**: 1362–1372.

Ruijter GJ, Bax M, Patel H, Flitter SJ, van de Vondervoort PJ, de Vries RP *et al.* (2003). Mannitol is required for stress tolerance in *Aspergillus niger* conidiospores. *Eukaryot Cell* **2**: 690–698.

Russell JA, Latorre AL, Sabater-Muñoz B, Moya A, Moran NA. (2003). Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. *Mol Ecol* **12**: 1061–1075.

Russell JA, Moran NA. (2006). Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. *Proc Biol Sci* **273**: 603–610.

Salvucci ME. (2000). Sorbitol accumulation in whiteflies: evidence for a role in protecting proteins during heat stress. *J Therm Biol* **25**: 353–361.

Salvucci ME, Hendrix DL, Wolfe GR. (1998). A thermoprotective role for sorbitol in the silverleaf whitefly, *Bemisia argentifolii*. *J Insect Physiol* **44**: 597–603.

Salvucci ME, Hendrix DL, Wolfe GR. (1999). Effect of high temperature on the metabolic processes affecting sorbitol synthesis in the silverleaf whitefly, *Bemisia argentifolii*. *J Insect Physiol* **45**: 21–27.

Scarborough CL, Ferrari J, Godfray HC. (2005). Aphid protected from pathogen by endosymbiont. *Science* **310**: 1781.

Scholz M, Fiehn O. (2007). SetupX—a public study design database for metabolomic projects. *Pac Symp Biocomput* **12**: 169–180.

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

Sly WS, Fischer HD. (1982). The phosphomannosyl recognition system for intracellular and intercellular transport of lysosomal enzymes. *J Cell Biochem* **18**: 67–85.

Wolfe GR, Hendrix DL, Salvucci ME. (1998). A thermoprotective role for sorbitol in the silverleaf whitefly. *J Insect Physiol* **44**: 597–660.

Wood Jr FE, Nordin JH. (1980). Activation of the hexose monophosphate shunt during cold-induced glycerol accumulation by *Protophormia terranovae*. *Insect Biochem* **10**: 87–93.

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