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# Role of Host Nutrition in Symbiont Regulation: Impact of Dietary Nitrogen on Proliferation of Obligate and Facultative Bacterial Endosymbionts of the Pea Aphid *Acyrthosiphon pisum*[▽]

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**The impact of host nutrition on symbiont regulation in the pea aphid *Acyrthosiphon pisum* was investigated. The population density of the obligate symbiont *Buchnera aphidicola* positively correlated with dietary nitrogen levels. In contrast, the population density of the facultative symbiont *Serratia symbiotica* increased in aphids reared on low-nitrogen diets, indicating distinct regulatory mechanisms in the same insect host.**

The endosymbiotic bacterial partners of aphids (Insecta: Sternorrhyncha) fall into two categories: the obligate "primary" symbiont *Buchnera* sp. found in almost all aphids and the facultative "secondary" bacteria whose presence is not universal (3, 6, 16). The association between aphids and *Buchnera* sp. is well documented: the bacteria are housed in specialized host cells, the bacteriocytes or mycetocytes, and they supplement the insects' diet through the provision of essential amino acids (see reference 6 for a full review). In contrast, the association between aphids and their secondary symbionts is less well understood, although the presence of secondary bacteria in symbiosis alongside *Buchnera* sp. has been known for many years (3, 13). The secondary bacteria are transmitted vertically between host generations (3, 4), but their distribution patterns within and between aphid populations suggest that occasional horizontal transmission must have occurred (5, 19).

The infection density of endosymbionts is one of the most important factors for understanding their biological effects. A reduction in infection density may result in imperfect vertical transmission and attenuated phenotypic effects, which could lead to loss of the infection in host populations. Excessive infection density may lead to enhanced negative or positive phenotypic effects on the host that could significantly influence host fitness and, at their extreme, could cause pathological damage. The proliferation of endosymbionts relies on the consumption of resources from the insect body; consequently, infection densities may be significantly influenced by the nutritional condition of the host. To date, however, no studies have investigated the impact of nutrition on the population density of insect endosymbionts.

In this study, the impact of nutrition on the population density of the obligate symbiont *Buchnera aphidicola* (17) and the facultative symbiont *Serratia symbiotica* (16) was investigated using the pea aphid *Acyrthosiphon pisum*. Two clonal lineages of pea aphid were used: (i) clone IS, a naturally *S. symbiotica*-infected line (9), and (ii) clone AIST$^{IS}$, in which the *S. symbiotica* infection was generated artificially by hemolymph injection (10). Both of these aphid clones also contain *B. aphidicola*. Continuous infection with *S. symbiotica* for all aphid generations and the absence of any other facultative symbionts were confirmed by PCR assay and observation by light microscopy. The nutritional condition of the insects was altered by rearing cohorts of genetically identical aphids for the first 7 days of nymphal development on chemically defined artificial diets containing final total nitrogen concentrations of 25, 50, 100, and 150 mM. Diet preparation and composition were identical to those described previously (21), with 50 mol percent essential amino acid content and 500 mM sucrose.

**Insect performance.** Both aphid clones IS and AIST$^{IS}$ settled and fed on the diets, as indicated by the excretion of honeydew and regular production of exuviae. After 7 days feeding on the diets a small number of aphids on the 150 mM nitrogen diet had molted to the adult stadium. The other aphids feeding on the 150 mM nitrogen diet and all aphids reared on the 100 mM nitrogen diet were in the fourth nymphal stadium. Aphids on the 25 mM and 50 mM nitrogen diets were noticeably smaller and had not molted beyond the third nymphal stadium. The weight gain of the insects positively correlated with the total nitrogen concentration in the diet (Fig. 1) such that aphids reared on the 150 mM diet were approximately threefold larger than aphids reared on the 25 mM diet. In addition, clone AIST$^{IS}$ was slightly larger than clone IS at all nitrogen concentrations (see "clone" data in the legend to Fig. 1). These data highlight the importance of nitrogen as a limiting factor in the nutrition of aphids and other phytophagous insects (2, 12, 18). The remainder of this paper examines how the nutritional condition of the host, as influenced by dietary nitrogen, impacts populations of obligate and facultative endosymbiotic bacteria.

**Symbiont populations in the aphids.** The bacteria in the aphids were quantified by quantitative real-time PCR using a TaqMan PCR core reagent kit and an ABI 7700 system (Applied Biosystems) as previously described (10), and the results are reported in Table 1. This approach quantifies the number of bacterial genomes within an insect rather than the absolute number of bacteria: the *B. aphidicola* genome (but not the *S.*

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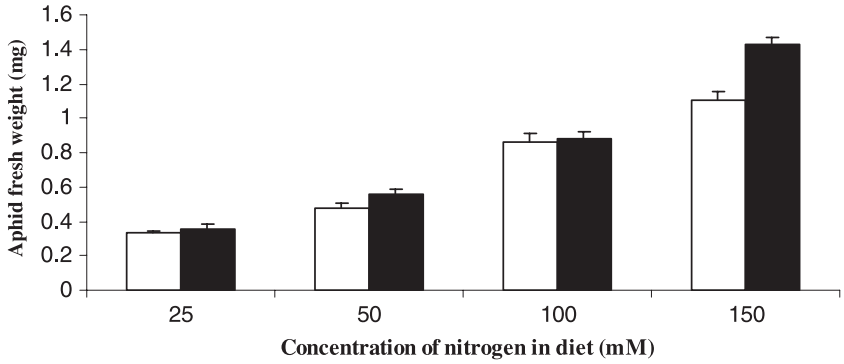


FIG. 1. The impact of total dietary nitrogen on the body weight of fresh aphids reared on chemically defined diets for 7 days. Open bars, clone IS; closed bars, clone AIST$^{IS}$; all data represent means ± standard errors ($n = 5$). ANOVA results: clone, $F_{(1,32)} = 7.8$ ($0.01 > P > 0.001$); diet, $F_{(3,32)} = 175.8$ ($P < 0.001$); interaction, $F_{(3,32)} = 1.4$ ($P > 0.05$).

*symbiotica* genome) is thought to be remarkably polyploid (11). The microbiota were quantified from aphids of the same chronological age (7 days) to limit such confounding effects of ploidy and developmental time on the different diets (see above), and individual aphid fresh weight was used to convert absolute numbers of bacterial genomes to genome densities (preliminary results indicated that insect cell number, as quantified in terms of elongation factor 1α, was not a reliable indicator of aphid size, probably due to differences in the rate of ovariole development on the different diets).

*B. aphidicola* genome number was quantified in terms of *groEL* gene copies by use of primers BuchGroEL-AF1 (5′-CAGCAACATTATTAGCACAATCTATAGTAAAT-3′) and BuchGroEL-AR1 (5′-TGATAACAGCTTTATCAATTCCACGT-3′) in combination with the fluorescently labeled probe BuchGroEL-TP1 (5′-AAGCAGTAGCAGCTGGTATGAATCCAATGG-3′). The titers of *B. aphidicola* were not significantly different in the aphid clones and positively correlated with the total nitrogen concentration in the diet (see "clone" and "diet" data, respectively, in analysis of variance [ANOVA] results), although a clone-dependent difference was observed in aphids reared on the 150 mM diet. The general consistency of results obtained for the aphid clones indicates that variability in the degree of ploidy of the *B. aphidicola* genome is unlikely to be the underlying cause of the observed patterns, although this possibility cannot be excluded. The genome density of *B. aphidicola* in both clones IS and AIST$^{IS}$ also tended to increase with dietary nitrogen concentration, but the significant interaction term in the ANOVA again highlights the reduction in *B. aphidicola* density in clone IS on the 150 mM diet. Although little is known about the regulatory mechanisms that maintain *B. aphidicola* densities within aphids, these results demonstrate that (i) the proliferation of *B. aphidicola* is linked to the availability of nitrogen and (ii) the density of *B. aphidicola* is not maintained at a fixed level and can vary, possibly to meet the nutritional demands of the host. In particular, there is likely to be a high demand for the metabolic repertoire of the bacteria in the rapidly growing aphids reared on diets containing 150 mM nitrogen compared to the results seen with aphids reared on diets containing 25 mM nitrogen that grow very slowly. An additional factor is the increasing contribution of the reproductive tissues to aphid size with both developmental and chronological age. The proliferation of *B. aphidicola* is linked to reproductive condition—the density of

TABLE 1. Bacterial populations in 7-day-old pea aphids reared on chemically-defined diets of different total nitrogen concentrations[a]

| Clone and concentration of nitrogen in diet (mM) | *Buchnera* titer (no. of *groEL* gene copies per insect × $10^7$)[b] | *Serratia* titer (no. of *groEL* gene copies per insect × $10^7$)[c] | Contribution of *Serratia* to total bacterial load (% gene copies) | *Buchnera* density (no. of *groEL* gene copies per mg aphid wt × $10^7$)[d] | *Serratia* density (no. of *groEL* gene copies per mg aphid wt × $10^7$)[e] |
|---|---|---|---|---|---|
| IS | | | | | |
| 25 | 4.96 ± 0.228 | 0.22 ± 0.066 | 4.2 | 15.1 ± 0.70 | 0.7 ± 0.19 |
| 50 | 9.80 ± 0.732 | 0.39 ± 0.070 | 4.0 | 19.6 ± 1.47 | 0.8 ± 0.14 |
| 100 | 27.76 ± 2.482 | 0.30 ± 0.080 | 1.1 | 28.5 ± 2.85 | 0.3 ± 0.09 |
| 150 | 19.01 ± 1.948 | 0.26 ± 0.079 | 1.3 | 17.1 ± 1.76 | 0.2 ± 0.07 |
| AIST$^{IS}$ | | | | | |
| 25 | 4.54 ± 0.371 | 0.44 ± 0.170 | 8.2 | 12.6 ± 1.03 | 1.2 ± 0.47 |
| 50 | 8.65 ± 0.794 | 0.79 ± 0.156 | 8.3 | 15.5 ± 1.42 | 1.4 ± 0.28 |
| 100 | 18.56 ± 1.608 | 0.21 ± 0.054 | 1.1 | 21.1 ± 1.83 | 0.2 ± 0.06 |
| 150 | 46.74 ± 3.592 | 0.70 ± 0.107 | 1.5 | 32.9 ± 2.53 | 0.5 ± 0.07 |

[a] All data, except nitrogen concentration and percent contribution, represent means ± standard errors ($n = 5$).
[b] ANOVA results: $F_{(1,32)}$ for clone, 2.7 ($P$ not significant); $F_{(3,32)}$ for diet, 187.8 ($P < 0.001$); $F_{(3,32)}$ for interaction, 20.8 ($P < 0.001$). (All ANOVA variables were ln transformed to obtain normality and homogenous variance prior to analysis.)
[c] ANOVA results: $F_{(1,32)}$ for clone, 7.9 ($0.01 > P > 0.001$); $F_{(3,32)}$ for diet, 4.2 ($0.05 > P > 0.01$); $F_{(3,32)}$ for interaction, 2.5 ($P$ not significant).
[d] ANOVA results: $F_{(1,32)}$ for clone, 0.1 ($P$ not significant); $F_{(3,32)}$ for diet, 19.9 ($P < 0.001$); $F_{(3,32)}$ for interaction, 14.3 ($P < 0.001$).
[e] ANOVA results: $F_{(1,32)}$ for clone, 4.9 ($0.05 > P > 0.01$); $F_{(3,32)}$ for diet, 11.4 ($P < 0.001$); $F_{(3,32)}$ for interaction, 1.8 ($P$ not significant).

TABLE 2. Bacterial populations in 7-day-old pea aphids *Acyrthosiphon pisum* reared from birth on the host plant *Vicia faba* cv. The Sutton[a]

| Aphid clone | Fresh wt (mg)[b] | *Buchnera* titer (no. of *groEL* gene copies per insect × $10^8$)[c] | *Serratia* titer (no. of *groEL* gene copies per insect × $10^8$)[d] | *Buchnera* density (no. of *groEL* gene copies per mg of body wt × $10^8$)[e] | *Serratia* density (no. of *groEL* gene copies per mg of body wt × $10^8$)[f] |
|---|---|---|---|---|---|
| IS | 2.1 ± 0.12 | 12.3 ± 1.21 | 0.3 ± 0.033 | 3.3 ± 0.36 | 0.2 ± 0.02 |
| $AIST^{IS}$ | 3.7 ± 0.40 | 13.3 ± 0.97 | 1.7 ± 0.58 | 3.5 ± 0.26 | 0.5 ± 0.16 |

[a] All data represent means ± standard errors ($n = 5$).
[b] Two-tailed $t$ test result: $t_8 = 3.9$ ($0.01 > P > 0.001$).
[c] Two-tailed $t$ test result: $t_8 = 0.7$ ($P$ not significant).
[d] Two-tailed $t$ test result: $t_8 = 2.3$ ($0.05 > P > 0.01$).
[e] Two-tailed $t$ test result: $t_8 = 0.5$ ($P$ not significant).
[f] Two-tailed $t$ test result: $t_8 = 2.6$ ($0.05 > P > 0.01$).

*B. aphidicola* reaches a peak in actively reproducing young adults and declines in postreproductive adults (10). This relationship may explain the decrease in *B. aphidicola* density in clone IS reared on the 150 mM diet, although degrees of reproductive investment did not differ significantly between aphids reared on the different diets (T. L. Wilkinson, unpublished results). The symbiosis is known to disintegrate in post-adult aphids through a reduction in the number of *B. aphidicola* cells and in the number of bacteriocytes (1, 8) and a decrease in the bacterial division rate in fourth instar larvae (20). Whatever the mechanisms involved, the regulation of *B. aphidicola* density on a day-to-day basis appears to be fine tuned to meet the metabolic demands of the host, as would be expected given the long evolutionary history of the host-symbiont association (15).

*S. symbiotica* is considered a facultative bacterial symbiont because its presence is not required for the survival and reproduction of the host insect (16). The population dynamics of *S. symbiotica* follow a simple logistic growth pattern during aphid development (10), suggesting a lack of strict control over the proliferation of the facultative symbiont. In the present study, *S. symbiotica* genome numbers were quantified in terms of *groEL* gene copies by use of primers PASSGroE-AF1 (5′-CC TCAAGGCTGTGGCCG-3′) and PASSGroE-AR1 (5′-GAG TTTGCAGAGATGGTGCCTA-3′) in combination with the fluorescently labeled probe PASSGroE-TP1 (5′-AAGCAGTT GTTGCGGCGGTTGAA-3′). The titer of *S. symbiotica* in the insects was an order of magnitude lower than that of *B. aphidicola*, reflecting both the numerical dominance and the genome polyploidy of *B. aphidicola* (11). While *S. symbiotica* titers were variable, particularly in clone $AIST^{IS}$, there was no consistent pattern with respect to total nitrogen concentration in the diet. However, the number of *S. symbiotica* in clone $AIST^{IS}$ was significantly higher than in clone IS (see main effect "clone" in ANOVA). The population density of *S. symbiotica* in clone $AIST^{IS}$ was also significantly higher than in clone IS, and in both clones the density of *S. symbiotica* tended to decrease as dietary nitrogen concentration was elevated. The high population density of *S. symbiotica* in aphids reared on diets containing low concentrations of nitrogen was reflected in the contribution of *S. symbiotica* to total bacterial load. In clone IS, *S. symbiotica* represented approximately 4% of the total endosymbiont population when the aphids were reared on diets containing 25 mM total nitrogen, equivalent to a 3.2-fold increase compared to aphids reared on the 150 mM diet. Similarly, *S. symbiotica* contributed over 8% of the endosymbiont population in clone $AIST^{IS}$ reared on a diet containing 25 mM total nitrogen, a 5.5-fold increase over aphids reared on the 150 mM diet. The increase in *S. symbiotica* when aphid performance is limited by dietary nitrogen is surprising, since intuitively the proliferation of *S. symbiotica* must be dependent on nutritional resources from the aphid, the availability of which will be strongly affected by the quality of the diet. The most plausible explanation is that the proliferation rate of *S. symbiotica* is constant and independent of insect body size or nutritional condition, such that the density of *S. symbiotica* is higher in smaller insects of the same chronological age reared on the nutritionally restricted diets, as was observed. Similarly, one would expect newly emerged adult aphids (i.e., of the same developmental age) reared on the 25 mM diet to contain larger populations of *S. symbiotica* than aphids reared on the 150 mM diet, since development time is extended by approximately 6 days on the nitrogen-restricted diet. However, this was not observed in supplemental experiments (data not shown) in which there was no significant difference between the titers of *S. symbiotica* from adult aphids from either clone reared on the 25 mM and 150 mM nitrogen diets, suggesting that the proliferation of *S. symbiotica* is not independent of regulatory controls. Further experiments are required to determine (i) whether the response of other *S. symbiotica* strains to dietary nitrogen, from *A. pisum* and from other aphid species, is the same as reported here and (ii) whether other factors linked to nutritional stress, such as an impaired immune response, may be responsible for the proliferation of *S. symbiotica*.

**Endosymbiont transmission.** The density of *S. symbiotica*, but not that of *B. aphidicola*, was higher in the aphid clone $AIST^{IS}$ than in clone IS. The difference between the clones was consistent in aphids reared on the chemically defined diets (Table 1) and was also observed in 7-day-old aphids reared on the host plant *Vicia faba* (Table 2), indicating that the higher density of *S. symbiotica* arises from a difference between the aphid clones. To explore possible mechanisms that might give rise to this interclonal variation, the transmission of *S. symbiotica* to the parthenogenetic embryo was investigated. Early embryos were dissected from adult aphids reared on the plant and subjected to whole-mount fluorescent in situ hybridization as previously described (16). *B. aphidicola* and *S. symbiotica* were detected by specific probes targeting their 16S rRNA: Cy5-ApisP2a (Cy5-5′-CCTCTTTTGGGTAGATCC-3′) and Cy3-PASSisR (Cy3-5′-CCCGACTTTATCGCTGGC-3′), respectively. Host cell nuclei were stained with SYTOX Green (Molecular Probes). The specimens were mounted in SlowFade antifade solution (Molecular Probes) and observed under a laser confocal microscope (Pascal 5; Carl Zeiss). The local-

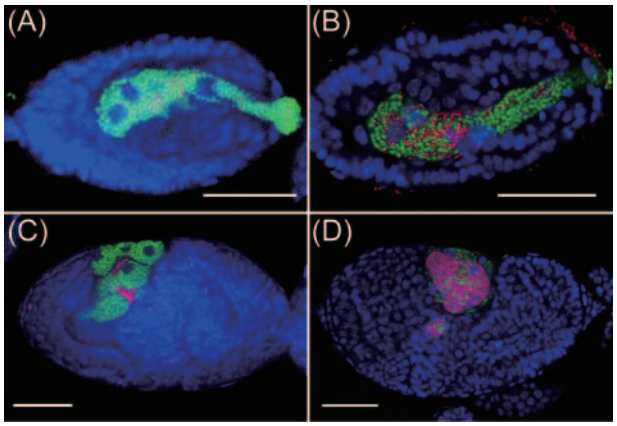


FIG. 2. Symbiont infection in early embryos of the clones IS and AIST$^{IS}$. (A) A stage 8 embryo of clone IS; (B) a stage 8 embryo of clone AIST$^{IS}$; (C) a stage 12 embryo of clone IS; (D) a stage 12 embryo of clone AIST$^{IS}$. Green, red, and blue signals indicate *B. aphidicola*, *S. symbiotica*, and nuclei of host cells, respectively. Bars, 50 μm. At stage 8, embryos are infected with the symbionts, and at stage 12, a large syncytial cytoplasm harboring the symbionts cellularizes into many bacteriocytes. Developmental staging of *A. pisum* is according to reference 14.

izations and densities of *B. aphidicola* were generally similar between the aphid clones (see Fig. 2), but in contrast, the number of *S. symbiotica* cells infecting embryos of the clone AIST$^{IS}$ was much greater than that infecting clone IS. At later stages of embryonic development, the *S. symbiotica* infection in clone IS remained localized to the interstitial areas between bacteriocytes and was at a much lower density than the infection in clone AIST$^{IS}$, in which *S. symbiotica* occupied the same tissue location as *B. aphidicola*. These findings suggest that efficient transmission and rapid proliferation of *S. symbiotica* in the clone AIST$^{IS}$ may be the cause of the higher bacterial density observed in this study, a result which is probably linked to the history of the symbiotic association and/or the genetic background of the host insect.

In this study, we demonstrated that, although cohabiting in the same insect, the obligate symbiont *B. aphidicola* and the facultative symbiont *S. symbiotica* respond to nutritional stress imposed on the host in different ways. This finding highlights the complex host-symbiont and symbiont-symbiont interactions in this endosymbiotic ecosystem and indicates the nutritional, physiological, and ecological relevance of endosymbiotic associates in natural insect populations. The natural diet of aphids is phloem sap, the availability and quality of which may vary temporally from diurnal changes through to seasonal changes and spatially between different parts of the plant and even different sieve elements (see reference 7 for a review). In polyphagous aphids, host plants of different taxonomic affiliation provide further variations in the quality and quantity of the food source. Under natural conditions, therefore, the endosymbiotic microbiota in aphids must be influenced by these nutritional fluctuations, possibly as observed in this study. Of particular relevance is the observation that the infection density of an unidentified facultative symbiont in the polyphagous aphid *Aphis fabae* was significantly higher on a host plant on which the aphid exhibited the poorest performance (22).

The biological consequences of such up- or down-regulation of endosymbiont populations are difficult to predict a priori, since a number of factors are affected simultaneously. For example, increasing the population density of a facultative symbiont could enhance both negative and positive aspects of the symbiont infection by acting directly on the host insect or at the same time indirectly by influencing the host via *B. aphidicola* or other microbial associates. These considerations are relevant to virtually all aphid species; *B. aphidicola* is ubiquitous in aphids that feed on phloem sap, and many aphids, but not all, possess facultative secondary bacteria (16). Indeed, there is also relevance for the approximately 10% of insect species that utilize the metabolic capabilities of symbiotic microorganisms in their nutrition. In summary, to improve our understanding of the physiology and ecology of aphids and other insects, we must take into account the complex interplay between the host insect, obligate and facultative symbionts, and the environment.

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