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Partitioning of Symbiotic Bacteria between Generations of an Insect: a Quantitative Study of a *Buchnera* sp. in the Pea Aphid (*Acyrthosiphon pisum*) Reared at Different Temperatures

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The population of symbiotic *Buchnera* bacteria in parthenogenetic females of the pea aphid *Acyrthosiphon pisum* was determined by quantitative hybridization of a DNA probe (*groESL*) to aphid homogenates. The aphids bore 1×10^7 to 2×10^7 bacterial cells per mg (fresh weight). In teneral aphids (i.e., aphids that had moulted to adulthood but that had not initiated reproduction), >75% of the bacteria were in the embryos, and the density of bacteria in the embryos was consistently greater than that in the maternal tissues. The bacterial density in teneral aphids increased from 1.3×10^7 to 2.0×10^7 cells mg (fresh weight) of aphids^{-1} with temperature between 15 and 25°C. This variation could be attributed to a temperature-dependent increase in both the density of bacteria in the embryos and embryo content of the aphids.

A variety of insects possess symbiotic microorganisms from which they derive specific nutrients, e.g., vitamins or essential amino acids (2, 3, 7). This nutritional benefit is, however, dependent on the insect's capacity to regulate the population size and growth rates of the microorganisms, which, due to their small size, may have a higher intrinsic growth rate than their insect hosts (8).

Two complementary approaches are commonly adopted to study the regulation of microorganisms in symbiosis with animal hosts: quantification of the microbial density, including how it varies with environmental conditions and developmental age of the host, and direct investigation of the mechanisms by which a stable microbial density is maintained, e.g., limitation of growth and division rates, expulsion, and lysis of "excess" microbial cells (8). There is, however, very limited information on the regulation of symbiotic microorganisms in insects. Two aspects of the insect symbioses contribute to this situation.

The first is that most microbial symbioses in insects are spatially complex (3). This reflects the anatomical complexity of the insect host, including the restriction of the microorganisms to particular insect organs and the morphologically complex processes mediating vertical transmission of the bacteria from mother to offspring. In aphids, the subject of this paper, the symbiotic bacteria, *Buchnera*, are in the cytoplasm of specific insect cells, called bacteriocytes (also known as mycetocytes), located in the abdominal hemocoel (body cavity) of the insect (3). The symbiosis is particularly complex in parthenogenetic aphids, which are viviparous (i.e., produce live young) and initiate embryo production as young larvae or even as embryos, such that an adult female may have both daughters and granddaughters in her ovaries. The bacteria are transmitted to young embryos (14), and a single parthenogenetic aphid, therefore, bears multiple, spatially separate symbioses: one in the hemocoel of the mother and one in each of up to 50 daughter embryos and up to 10 granddaughter embryos. The symbiosis can be addressed at multiple spatial scales: the individual bacteriocyte, the generations (most simply, maternal versus embryo symbioses), and the whole insect (1, 10, 15, 25).

The second factor contributing to the paucity of research on the regulation of insect symbioses is that the bacterial symbionts of insects are generally intractable to quantification. In particular, they cannot be enumerated by standard microbiological techniques because they are unculturable. Molecular techniques, however, are not subject to these limitations. Baumann and Baumann (1) have used competitive PCR to quantify the unculturable *Buchnera* bacteria in aphids. They demonstrated that the aphid *Schizaphis graminum* bears between 5×10^6 and 12×10^6 *Buchnera* cells per mg (fresh weight), varying in a predictable fashion with aphid developmental stage. An alternative molecular technique, quantitative DNA hybridization, has been used with success to quantify the symbiotic bacteria of other animals, e.g., the luminescent bacteria *Vibrio fischeri* in *Euprymna* (17, 18). This methodology is sensitive, requiring small amounts of material.

The study described in this paper used quantitative DNA hybridization to explore the density of *Buchnera* in aphids reared at different temperatures. It is well established that temperature is a major determinant of aphid size and population increase (5). The response of the aphid-*Buchnera* symbiosis to temperature has not, however, been investigated, beyond the demonstration that the symbiosis breaks down at extremes of high (37°C) and low (−10°C) temperatures (20, 22). We specifically selected temperatures (15 to 25°C) that are not overtly deleterious to the insect.

The experiments were conducted on long-term cultures of the parthenogenetic pea aphid *Acyrthosiphon pisum* clone Ox2, reared over multiple generations at 15, 20, and 25°C on *Vicia faba* The Sutton under a 16-h light:8-h dark regimen. All experimental aphids were weighed to the nearest microgram on a Mettler MT5 microbalance, surface-sterilized with 5% HCl followed by 70% ethanol, and then homogenized in an ice-cold buffer, comprising 50 mM Tris-HCl (pH 7.5), 10 mM KCl, 0.25 M sucrose, 5 mM dithiothreitol, and 5 mM phenylthiourea. Subsamples were removed for protein assay, and the remainder was used for quantification of *Buchnera* (see below). Aphid protein content was determined by the protein assay kit of the Bio-Rad Chemical Co., according to the manufacturer's instructions for the microassay, with bovine serum albumin as

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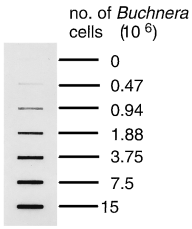

FIG. 1. Autoradiograph showing hybridization of a bacterial *groESL* probe to a dilution series of *Buchnera* cells.

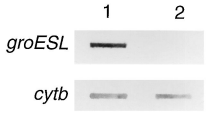

FIG. 2. Autoradiograph illustrating the hybridization of the bacterial *groESL* probe and a mitochondrial DNA probe, 0.45-kb cytochrome *b* (*cytb*) sequence, to *A. pisum*. Lanes: 1, aphids containing symbiotic bacteria; 2, aphids treated with chlortetracycline, which disrupts the symbiotic bacteria. Each slot contained the equivalent of 10 μg of aphid protein. (The cytochrome *b* probe was a PCR-generated sequence, obtained from a template of *A. pisum* DNA with primers L15302 and H15709 [19].)

the standard. Some experiments concerned the bacteria in aphid embryos, which were dissected from surface-sterilized insects with fine pins (9). Particular attention was paid to separating the embryos from the maternal bacteriocytes that are associated with the germarium of each aphid ovary (11). The isolated embryos were then homogenized for quantification of protein and bacterial content.

The DNA probe used for quantitative hybridization experiments was a 375-bp sequence of the *Buchnera groESL* operon, obtained by PCR amplification of DNA isolated from *Buchnera* preparations, as described in reference 24. The primers were 5′-ATCGACTCGAGGAACAGTGA-3′ and 5′-GGCTA CGGATACACCATCTT-3′, and the PCR mix for amplification included 2.5 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates, and *Taq* polymerase (Promega) at 0.05 U μl^{-1}. The amplification conditions were 30 cycles of 90°C for 1 min, 48°C for 1.5 min, and 72°C for 2 min. The sequence of the product was 98% identical to nucleotides 351 to 724 of the published *groESL* sequence of *Buchnera* (21) (data not shown). The *groESL* probe was labelled with chemiluminescence by the ECL direct nucleic acid labelling and detection system (Amersham), according to the manufacturer's instructions. The samples used for quantification of *Buchnera* were homogenates of aphids (obtained as described above) comprising 10 μg of protein, applied to a Zeta-probe GT blotting membrane (Bio-Rad) in a slot blot apparatus, according to the Zeta-probe instruction manual. The membrane was hybridized with the ECL-labelled *groESL* probe by the procedure described in the ECL booklet of Amersham. The blotting membrane was then exposed to Hyperfilm-ECL X-ray film, and the absorbance in each band was quantified with an LKB Ultroscan XL enhanced laser densitometer. Each blot included a dilution series of *Buchnera* cells (Fig. 1) that was used as a standard for the conversion of absorbance values to number of *Buchnera* cells per milligram of aphid protein and per aphid. The bacteria were isolated from *A. pisum* as described in reference 24 and quantified by hemocytometer counts at a ×1,000 magnification. Preliminary experiments (16) demonstrated that the hybridization signal from *Buchnera* cells was not affected by an aphid homogenate containing 2 to 20 μg of protein and that the relationship between absorbance and log number of *Buchnera* cells was linear over the range 0.4×10^6 to 15×10^6 cells.

The specificity of the method was indicated by >99% reduction in the hybridization signal when the probe was hybridized to homogenates of aphids treated with the antibiotic chlortetracycline (by the procedure described in reference 6) to eliminate the bacteria (Fig. 2). The evidence that the probe hybridized to *Buchnera* in the homogenates is twofold. First, *Buchnera* is the principal microorganism associated with *A. pisum* clone Ox2. Microscopical and molecular studies of this aphid clone have not revealed microorganisms in the insect gut or tissues other than *Buchnera* and a small population of "secondary symbionts" (2, 3, 7) associated with the bacteriocytes (4a). (This is in contrast with some clones of *A. pisum* that possess microorganisms in the gut and hemolymph [4, 13].) Second, subsequent to this study, experiments using a *Buchnera*-specific DNA probe (1.1-kb probe spanning *aroE-rrl*) (23) have obtained estimates of *Buchnera* populations per aphid comparable to the values obtained with the *groESL* probe (26).

The first set of experiments quantified the *Buchnera* in homogenates obtained from insect samples (ca. 50 mg) of mixed ages. Aphids at all temperatures contained 1×10^7 to 2×10^7 *Buchnera* cells per mg (fresh weight). These results are consistent with the published values for *Buchnera* in the aphid *S. graminum* (1), but they are an order of magnitude greater than estimates derived from hemocytometer counts of bacteria isolated from homogenates of *A. pisum* (13). It is very likely that the estimate in reference 13 is in error.

The density of symbiotic bacteria in aphids varies with insect developmental stage (1, 16). Insects of uniform age were, therefore, used for the detailed analysis of variation in bacterial density with temperature. We selected teneral apterae, i.e., insects of the wingless morph that had just moulted to adulthood but that had not initiated reproduction, and each sample was derived from the homogenate of a single aphid. The numbers of *Buchnera* cells per teneral aphid are listed in Table 1. They did not vary significantly with temperature ($F_{2,15} = 0.066$; $P > 0.05$). However, the size of the aphids declined progressively with increased rearing temperature, and linked to this, the density of bacteria in the aphids increased with temperature (Table 1). The quantitative effect of temperature on bacterial density depended on the index of aphid biomass adopted, because aphid protein content decreased more rapidly than fresh weight with increasing temperature. Correlated

TABLE 1. *Buchnera* in teneral *A. pisum* tissues[a]

Temp (°C)	No. of *Buchnera* cells per aphid (10^7)	mg of protein per aphid	No. of *Buchnera* cells mg of aphid protein^{-1} (10^7)	mg (fresh wt) per aphid	No. of *Buchnera* cells mg (fresh wt) of aphids^{-1}(10^7)
15	4.3 ± 0.7	0.26 ± 0.014	17 ± 2.6	3.4 ± 0.27	1.3 ± 0.22
20	4.0 ± 0.9	0.16 ± 0.018	26 ± 4.1	2.6 ± 0.20	1.5 ± 0.31
25	3.9 ± 0.6	0.11 ± 0.004	35 ± 4.9	2.0 ± 0.12	1.9 ± 0.30

[a] Each sample (10 μg of protein) was a subsample of the crude homogenate of a single aphid. Means ± standard errors for six replicates are shown.

TABLE 2. *Buchnera* in teneral *A. pisum* embryos[a]

Temp (°C)	No. of *Buchnera* cells [10^7] (%[b])	mg of embryo protein (%[b])	No. of *Buchnera* cells mg of embryo protein^{-1} (10^7)
15	3.3 ± 0.5 (77)	0.11 ± 0.005 (42)	29 ± 4.9
20	3.1 ± 0.4 (78)	0.09 ± 0.006 (56)	34 ± 3.5
25	3.7 ± 0.4 (95)	0.08 ± 0.007 (73)	46 ± 7.0

[a] Each sample (10 μg of protein) was a subsample of the crude homogenate of the embryo complement of a single aphid. Means ± standard errors for six replicates are shown.
[b] Of the corresponding value for total aphid tissues.

with this, the mean number of bacteria per unit of aphid weight increased with temperature by 50% from 1.3×10^7 to 1.9×10^7 mg^{-1} (fresh weight), and the number per unit of protein doubled from 17×10^7 to 35×10^7 cells mg of protein^{-1}.

A parallel analysis of the bacterial content of embryos in teneral adults revealed the processes underlying the relationship between temperature and bacterial density. As Table 2 shows, 77 to 95% of the bacteria in these aphids were recovered from the embryos. The bacterial density in embryos was quantified exclusively in terms of aphid protein content because reliable estimates of embryo fresh weight cannot be obtained (9). The mean number of bacteria per unit of embryo protein increased by nearly 60% with temperature between 15 and 25°C (Table 2), and at each temperature the bacterial density was higher in the embryos than in the total tissues. The maternal tissues were estimated, by subtraction, to bear 12×10^7, 8×10^7, and 11×10^7 bacteria per mg of protein at 15, 20, and 25°C, respectively, without any clear-cut variation with temperature.

One implication of these data is that the increase in density of bacteria in the embryos, but not the maternal tissues, can account for much of the variation in total bacterial density in aphids with temperature. This effect is compounded by a temperature-dependent increase in embryo content, as a proportion of total aphid protein (Table 2).

The demonstration that the bacterial density is higher in the embryos than in the maternal tissues could be interpreted as evidence for a greater nutritional requirement for the symbiosis in embryos than in the maternal tissues of aphids. Although this possibility has not been investigated directly, it is consistent that elimination of the bacteria has a greater deleterious effect on the growth of embryos than that of maternal tissues of *A. pisum* (9). The contributions of differences in the numbers of bacteria transmitted to each embryo and proliferation rates of the bacteria in embryos to the temperature-dependent variation in bacterial density in embryos also remain to be studied.

These results also have implications for the study of regulation of the symbiotic bacteria in aphids: the number of *Buchnera* cells in an aphid depends on the insect's embryo content. An aphid with high embryo content would, for example, have a disproportionately high bacterial content because of the greater bacterial density in embryos than in the maternal tissues. The increase in bacterial density with larval age of aphids (1, 16) can, for example, be accounted for by the developmental increase in embryo content (9). More generally, any study of the populations of *Buchnera* in aphids should consider the symbioses in the maternal and embryo generations separately, so that the direct effects of factors on the symbiosis can be teased apart from their effects on embryo content.

As this study illustrates, some aspects of the regulation of microbial symbionts at the level of the whole host organism can best be understood in terms of processes at a finer spatial scale.

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