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Growth Kinetics of the Endosymbiont *Buchnera aphidicola* in the Aphid *Schizaphis graminum*

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The aphid *Schizaphis graminum* is dependent on its prokaryotic endosymbiont, *Buchnera aphidicola*. As a means of determining *B. aphidicola* numbers during the growth cycle of the aphid we have used the quantitative PCR to measure the number of copies of *rrs* (the gene coding for 16S rRNA, which is present as one copy in the *B. aphidicola* genome). In addition we have measured the aphid wet weight and the DNA and protein content. The results indicate an approximately parallel (23- to 31-fold) increase of these properties during the period of aphid growth. A 1-day-old aphid (24 μg [wet weight]) has 0.2×10^6 copies of *rrs*, while a 9-day-old aphid (497 μg [wet weight]) has 5.6×10^6 copies. The coupling of endosymbiont and aphid growth is consistent with the requirement of the endosymbiont for growth and reproduction of the aphid.

Aphids are major pests of agriculturally important plants (17). Nearly all aphids harbor, in vesicles within specialized cells (mycetocytes), a prokaryotic endosymbiont which cannot be grown outside the aphid host (5, 8). The mycetocytes form a loose aggregate in the aphid body cavity known as the mycetome. Their number remains fairly constant during aphid growth but their volume increases (3). Aphids are dependent on endosymbionts; their elimination by antibiotics or other treatments leads to sterility and eventual death of the aphid (2, 8, 10, 16, 18). We have chosen for study the endosymbiont (*Buchnera aphidicola*) of the aphid *Schizaphis graminum* (1). This aphid is an important pest of cereals (17). Our results have indicated that *B. aphidicola* resembles free-living bacteria and not organelles in that it has genes coding for proteins involved in DNA synthesis, transcription, and translation, in protein secretion, and in energy-yielding metabolism (1). Isolated endosymbionts from *Acyrthosiphon pisum* have been shown to make over 210 different proteins (10). Evidence that among the functions of the endosymbiont are the overproduction of tryptophan, cysteine, and methionine for the aphid host has been presented (4, 11, 14). Sequence comparisons of endosymbiont 16S rRNA indicated that *Escherichia coli* is the nearest known relative (12, 15). Like other slowly growing bacteria, *B. aphidicola* has only one copy of *rrs*, *rrl*, and *rrf* (genes coding for 16S, 23S, and 5S rRNA, respectively) (13, 19, 25). *B. aphidicola* also differs from most bacteria in having *rrs* unlinked to *rrl* and *rrf* (13, 19).

There are several studies dealing with the growth kinetics of aphids during their most active reproductive stage, that is, females reproducing by parthenogenesis and giving birth to live young (22, 24). Since the mother aphid contains embryos in different stages of development, most of the results represent measurements of the constituents present in both the mother and the daughter embryos. Recently a method for the quantitation of mRNA in total RNA preparations (quantitative PCR) has been developed (6, 7, 21). This method can also be used for the estimation of the number of gene copies present in a DNA preparation. In the present study we determined the number of *B. aphidicola rrs* copies in the total DNA obtained from aphids at different times of their growth cycle. Since *rrs* is present as a single copy in the *B. aphidicola* genome (13), the results are an estimate of the number of endosymbiont cells.

Wet weight and protein content. *S. graminum* biotype E was grown on barley (CM72) at 24°C with a photoperiod of 16 h of light and 8 h of dark (14). Three aphids, in their reproductive stage of development, were placed by means of a thin camel's hair brush on the leaf of a 5-day-old barley seedling. After 16 to 18 h the mothers were removed and the incubation was continued. All plants were inspected daily. The birth of young was usually detected after 8 days, and the daughters were removed. At the specified times aphids were harvested, counted, and weighed with a Denver Instrument Co. (Denver, Colo.) model 100A balance. Depending of the age of the aphids, the weight of samples ranged from 4.2 to 36 mg. Samples were transferred to Kontes Scientific Glassware/Instrument Co. (Vineland, N.J.) microcentrifuge tubes, which were supplied with plastic pestles, and frozen at −70°C until use. Samples were made up to 2% (wt/wt) in 1 N NaOH, and the aphids were disrupted with the pestle and heated at 100°C for 10 min. After centrifugation, protein was determined by using the BCA Protein Assay Reagent (Pierce Co., Rockford, Ill.) with serum bovine albumin as the standard. The results are presented in Table 1 and Fig. 1. Aphids born within the initial period of 16 to 18 h had a weight of 24 μg. The wet weight of the aphids increased up to about 10 days; at 12 days the weight represented a 22.6-fold increase over the weight of the 1-day-old aphid. Protein increase paralleled that of the wet weight; a 12-day-old aphid had a 26.2-fold increase in protein over that of the 1-day-old aphid. In a 12-day-old aphid, protein constituted 8.7% of the wet weight and 32.9% of the dry weight (dry weight of the aphid was 26.4% ± 0.2% of the wet weight).

Determination of DNA content and *rrs* copy numbers. The methods used for the purification of DNA have been previously described (11, 20, 23). Only an outline and slight modification are given here. Aphids were suspended in lysis buffer and disrupted with a pestle. Following digestion with proteinase K and RNase, the preparation was extracted twice with phenol-chloroform and precipitated with sodium acetate-ethanol, and the precipitate was washed with 70% ethanol. The samples were resuspended in 10 μl of TE (20) for each milligram of the initial sample weight and heated for 10 min at 65°C. Aliquots were digested with *Xba*I, a treatment which helps dissolve the DNA and gives a 6.2-kb DNA fragment

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TABLE 1. Growth characteristics of *S. graminum* and its endosymbiont (*B. aphidicola*)

Day	No. of aphids counted	No. of samples	Aphid wt (μg)	Concn (μg/aphid) of:		No. of *rrs* copies/ pg of aphid DNA	No. of *rrs* copies (10^6)/aphid
				Protein	DNA		
1	1,530	8	24.0 ± 2.9	1.8	0.054	3.74 ± 0.12	0.20
2	322	3	44.5 ± 1.0		0.099	3.79 ± 0.42	0.38
3	863	8	68.6 ± 1.9	5.2	0.148	3.73 ± 0.21	0.55
4	191	3	111.3 ± 6.7	7.8	0.246	2.28 ± 0.21	0.56
5	271	5	178.0 ± 5.3	12.8	0.452	2.11 ± 0.27	0.95
6	157	3	242.7 ± 8.7		0.788	2.62 ± 0.15	2.07
7	232	5	324.2 ± 15.0	25.8	1.088	3.50 ± 0.25	3.81
8	155	3	437.0 ± 5.6	35.4	1.433	3.67 ± 0.20	5.26
9	143	3	497.3 ± 12.7	41.7	1.667	3.36 ± 0.21	5.60
10	137	3	535.0 ± 7.2	45.4			
11	188	3	541.0 ± 12.1	46.8	1.640	3.06 ± 0.17	5.02
12	162	4	541.3 ± 10.8	47.1	1.681	3.06 ± 0.08	5.14

containing *B. aphidicola rrs*. DNA was quantitated with a model 450 fluorometer (Sequoia-Turner, Mountain View, Calif.) with Hoechst dye 33258 (Polysciences, Inc., Warrington, Pa.) as described by the fluorometer manufacturer with λ bacteriophage as the standard. The results (Table 1 and Fig. 2) indicate that the increase in the DNA content per aphid approximately parallels the increase in wet weight and protein content. The maximum DNA content, observed at 9 days, corresponds to 0.34% of the aphid wet weight, or 1.27% of the dry weight. Using a different aphid and a different method of DNA determination, Srivastava et al. (22) found that in a 12-day-old aphid, DNA constituted 0.2% of the wet weight.

The principle and methodology of competitive PCR have been described (6, 7, 21). The *rrs* of the endosymbiont of *A. pisum*, which is closely related to the *rrs* from *B. aphidicola* (15), has two *Eco*RI sites 328 bp apart (23). This *Eco*RI fragment was removed from pBluescript (Stratagene, La Jolla, Calif.) containing an *rrs* insert, and the resulting construct was designated Δ*rrs*. Initially *rrs* was quantitated in an *S. graminum* DNA preparation obtained from a vigorously growing aphid population. The PCR mixtures (50 μl each) consisted of different amounts of *S. graminum* DNA, 5 pmol of the oligonucleotides described in reference 15 per μl, 1,190 molecules of Δ*rrs* per μl, 0.025 U of *Taq* polymerase per μl, and the components of the GeneAMP PCR Reagent Kit (Perkin Elmer Corp., Norwalk, Conn.). The procedures used are described by the manufacturer. The following controls were included in each experiment: (i) no aphid DNA, (ii) only Δ*rrs*, and (iii) only *rrs* (aphid DNA). DNA was denatured by exposure to 95°C for 5 min followed by 30 cycles of 1 min at

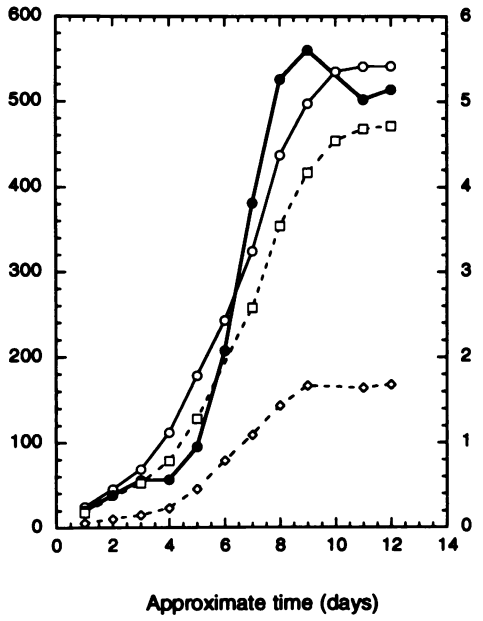

FIG. 1. Growth kinetics of *S. graminum* (aphid) and *B. aphidicola* (endosymbiont). Left axis, aphid weight (micrograms) (○); right axis, number of *rrs* copies (10^6) per aphid (●), micrograms of protein (10^1) per aphid (□), and micrograms of DNA per aphid (◇).

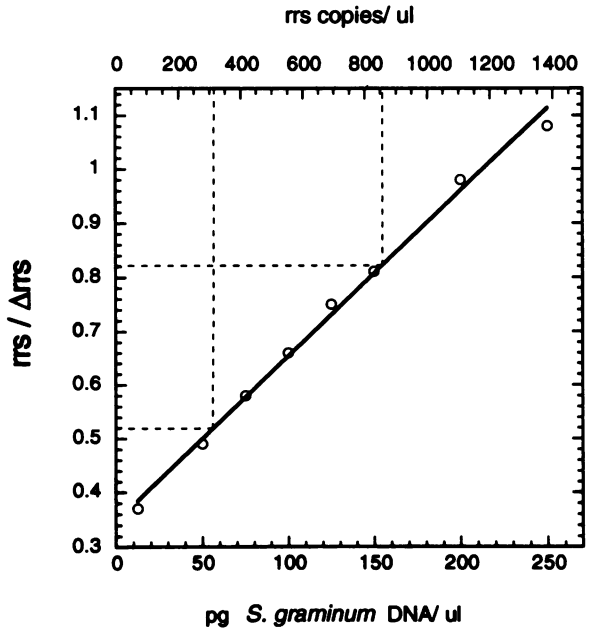

FIG. 2. Quantitation of *rrs* copies by competitive PCR. *rrs*/Δ*rrs*, ratio of peak heights of the complete 16S rRNA gene from *S. graminum* DNA (*rrs*) to the peak height of the 16S rRNA gene containing a deletion (Δ*rrs*). Bottom axis, concentrations *S. graminum* DNA obtained from a population containing aphids of different ages; top axis, *rrs* copy numbers in this DNA. Dashed lines enclose the range of *rrs*/Δ*rrs* ratios and *rrs* copy numbers found in DNA preparations obtained from aphids of different ages.

95°C, 2 min at 55°C, and 3 min at 70°C. After electrophoresis in 1% agarose (20-μl samples) and staining with ethidium bromide (20) the gels were photographed with Polaroid Corp. (Cambridge, Mass.) 665 film. The negatives were scanned by means of a Hoefer Scientific Instruments (San Francisco, Calif.) GS-300 scanning densitometer equipped with the GS-370 Data Analysis Systems, and the peak heights were determined. Following correction for size (*rrs* = 1,510 bp; Δ*rrs* = 1,182 bp), the ratio of the average peak heights (*rrs*/Δ*rrs*) was plotted against the concentration of aphid DNA (Fig. 2). At a ratio of 1.0 (equivalence), 215 pg of aphid DNA contained 1,090 copies of *rrs*. By using the latter value, the number of *rrs* copies per microliter was substituted for the amount (picograms) of aphid DNA per microliter (Fig. 2). The same aphid DNA preparation was tested at 200 pg/μl in a set of six replicate PCR experiments. From the ratios of *rrs* to Δ*rrs* and the plot presented in Fig. 2, a value of 4.79 ± 0.26 copies of *rrs* per pg of DNA was obtained. This is in agreement with the number of *trpB* copies in this preparation (5.1 ± 1.0/pg of DNA), previously determined by quantitation of bands in Southern blots (11).

The numbers of copies of *rrs* in the DNAs obtained from *S. graminum* at different stages of growth were determined with 200 μg of the DNA preparation per μl. A single set of experiments consisted of a replicate determination of each of the 11 time points; each set of experiments was performed three times. The *rrs*/Δ*rrs* ratios ranged from 0.56 to 0.82 (Fig. 2). There is some variation with respect to the number of *rrs* copies per picogram of aphid DNA (Table 1). At days 4 to 5 there is a drop followed by a gradual increase (6 to 9 days) and a subsequent slight drop (11 to 12 days). The significance (if any) of these changes is not known. Figure 1 presents a plot of the total number of *rrs* copies per aphid. At days 4 to 6 the increase in the number of *rrs* copies appears to lag behind the increase in aphid wet weight; subsequently the number of *rrs* copies increases faster than aphid weight. The maximum number of copies is observed at 9 days and corresponds to a 28-fold increase over the number of *rrs* copies in a 1-day-old aphid. The value 5.6×10^6 *rrs* copies per 9-day-old aphid (Table 1) is in agreement with the estimates of 5×10^6 endosymbionts for the adult *A. pisum* (4) and 1×10^6 endosymbionts per mg of aphid (wet weight) (24). By using the genome size of the *A. pisum* endosymbiont (9), it was calculated that 8.7% of the DNA of a 9-day-old aphid would be endosymbiont DNA.

The results of the present study are consistent with studies of growth of *A. pisum* (22) and its endosymbionts (24). Previously it has been established that during growth of two aphid species the number of mycetocytes does not change but their volume is increased (3); upon reaching maturity there is a decrease in the number of mycetocytes. Observations with the electron microscope have indicated that during the period of active growth the endosymbionts have a regular appearance characteristic of healthy cells but that they begin to disintegrate when the number of mycetocytes decreases (8, 10, 24). Our determination of *rrs* copy numbers does not distinguish between *B. aphidicola rrs* in the mother and that in the embryos. The rate of cell division in these two populations is different since in *A. pisum* a higher frequency of dividing endosymbionts was observed in embryonic than in maternal mycetocytes (24). The increase in *B. aphidicola rrs* copy number and the normal appearance of the bacterial cells suggests that during the growth of *S. graminum* there is an orderly increase in all of the *B. aphidicola* constituents. This is consistent with the demonstration that the endosymbiont has genes involved in DNA biosynthesis, transcription, and translation, in protein secretion, and in tryptophan biosynthesis (1, 11, 14).

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