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Evidence for the Establishment of Aphid-Eubacterium Endosymbiosis in an Ancestor of Four Aphid Families†

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Aphids (superfamily Aphidoidea) contain eubacterial endosymbionts localized within specialized cells (mycetocytes). The endosymbionts are essential for the survival of the aphid hosts. Sequence analyses of the 16S rRNAs from endosymbionts of 11 aphid species from seven tribes and four families have indicated that the endosymbionts are monophyletic. Furthermore, phylogenetic relationships within the symbiont clade parallel the relationships of the corresponding aphid hosts. Our findings suggest that this endocytobiotic association was established in a common ancestor of the four aphid families with subsequent diversification into the present species of aphids and their endosymbionts.

Mutualistic associations between insects and intracellular bacteria (endosymbionts) are widespread among several insect orders (2–4). One such association which has been well studied is found in the aphids (superfamily Aphidoidea) (6, 12, 13). Aphids are a monophyletic group originating in the Jurassic; they comprise about 4,000 modern species of plant sap-feeding insects and are diverse with respect to their food sources and life cycles (5, 19). Some species live on the surfaces of plants, and others colonize the roots; a number of species form galls. It is generally thought that all aphids contain endosymbionts (2).

The body cavity of the aphid contains mycetomes, which are aggregates of specialized cells designated mycetocytes (12, 13, 22). The mycetocytes contain large numbers of a round or oval bacterium which has a gram-negative cell wall and is enclosed in a vacuole derived from the host membrane (11, 12). This organism has not been cultured outside the aphid host. In the cases of two species of aphids, it has been found that the mycetome consists of 60 to 90 mycetocytes, each of which contains 50,000 to 60,000 endosymbionts (7, 8). The endosymbionts are essential to the lives of their hosts: aphids lose their ability to reproduce when they are experimentally deprived of their endosymbionts by treatment with antibiotics (6, 12, 13). Aphids have evolved complex mechanisms to ensure that their progeny are infected transovarially (1, 6, 12). The essential function(s) performed by the endosymbionts has not been definitively established (3, 6, 13).

Some aphid species, such as *Acyrthosiphon pisum* (pea aphid), contain two endosymbionts, the predominant of which is the round or primary (P-) endosymbiont which is found in the mycetocytes (11, 18). Smaller numbers of a rod-shaped or secondary (S-) endosymbiont are found within the sheath cells which partially surround the mycetome. The genes coding for the 16S rRNAs of the P- and S-endosymbionts of *A. pisum* have been cloned and sequenced (29, 30). On the basis of sequence comparisons, it was found that the S-endosymbiont is a member of the family *Enterobacteriaceae*, while the P-endosymbiont constitutes a distinct lineage within the gamma-3 subdivision of the class *Proteobacteria* (26, 32).

In this article, we present the results of analysis of the genes coding for 16S rRNAs (16S rDNAs) from the P-endosymbionts of 10 additional species of aphids which are representative of seven tribes and 4 of the 10 families of the superfamily Aphidoidea (9). Our results are consistent with the establishment of successful endosymbiosis in an ancestral insect and with the subsequent diversification of the endosymbiont and the host into the present species of aphids.

MATERIALS AND METHODS

Source of aphids. Apterous parthenogenic aphids (Table 1) were collected, placed on ice, and subsequently stored at −70°C. Samples were shipped on dry ice to University of California, Davis. *Mindarus victoria* was the gift of L. E. Ehler, and *Uroleucon sonchi* was the gift of D. E. Ullman. The remaining aphids were provided by the authors (N.A.M., D.J.V., and B.C.C.).

Purification of DNA. The micromethods used are based on those of Weeks et al. (31); general procedures are as described by Maniatis et al. (17). An aphid sample of 200 to 500 mg (wet weight) was placed in a small mortar, cooled, kept on powdered dry ice, and ground with a precooled pestle. The material was transferred to a tube that had also been cooled on dry ice, and 1.5 ml of lysis buffer was added (400 mM NaCl–40 mM EDTA–100 mM Tris-HCl [pH 7.6]–2% [wt/vol] sodium dodecyl sulfate). The mixture was incubated at 50°C for 30 min. The sample was extracted with phenol, phenol-chloroform, and chloroform (17), and the volume was brought to 1.6 ml with water. CsCl and ethidium bromide were added to final concentrations of 1.05 g/ml and 10 mg/ml, respectively. The sample was centrifuged in a TL-100 Ultracentrifuge with a Vti100 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 80,000 rpm, for 5 h. The DNA band was removed, and the ethidium bromide was extracted with butanol (17). Two volumes of Tris-EDTA (TE) buffer (17) were added, and the DNA was precipitated with 95% ethanol (17). After an incubation of 10 min at room

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TABLE 1. Names and taxonomic placements of aphid hosts containing the endosymbionts for which 16S rDNA sequences were obtained[a]

Species	Tribe	Family
Schizaphis graminum (Rondani)	Aphidini	Aphididae
Rhopalosiphum maidis (Fitch)	Aphidini	Aphididae
Rhopalosiphum padi (Linnaeus)	Aphidini	Aphididae
Myzus persicae (Sulzer)	Macrosiphini	Aphididae
Uroleucon sonchi (Linneaus)	Macrosiphini	Aphdidae
Acyrthosiphon pisum (Harris)[b]	Macrosiphini	Aphididae
Diuraphis noxia (Mordvilko)	Macrosiphini	Aphididae
Chaitophorus viminalis (Monell)	Chaitophorini	Drepanosiphidae
Mindarus victoria (Essig)	Mindarini	Mindaridae
Pemphigus betae (Doane)	Pemphigini	Pemphigidae
Melaphis rhois (Fitch)	Fordini	Pemphigidae

[a] Higher taxonomic categories according to Heie (9).
[b] 16S rDNA previously sequenced.

temperature, the DNA was pelleted by centrifugation, resuspended in 0.5 ml of 300 mM sodium acetate, and reprecipitated by the addition of 95% ethanol, and the pellet was washed with 70% ethanol (17). After drying under a vacuum, the pellet was suspended in 100 μl of TE buffer. The final concentration of the DNA ranged from 400 to 1,000 ng/μl as determined by fluorometry. This preparation contained both the endosymbiont DNA and the host DNA. Previous estimations suggest that in the case of *A. pisum* about 5% of the total purified DNA is from the endosymbiont (29).

Amplification, cloning, and sequencing of the 16S rDNA. The Perkin-Elmer Cetus (Norwalk, Conn.) polymerase chain reaction kit was used according to directions given by the manufacturer. The 16S rDNA was amplified by using the polymerase chain reaction and two primers (Fig. 1, line a), each containing a *Bam*HI site (numerals following the oligonucleotide sequence designate *Escherichia coli* rRNA nucleotide [nt] numbers) [primer 1: 5′-GGA TCC AGA GTT TGA TCA TGG CTC AGA TTG-3′ (nt 8 to 30); primer 2: 5′-GGA TCC TAC CTT GTT ACG ACT TCA CCC CAG-3′ (nt 1507 to 1484)]. The reaction mixture consisted of 1 μg of the DNA

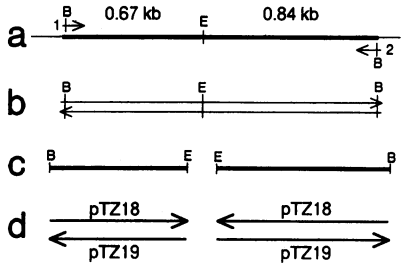

FIG. 1. Strategy used for the amplification, cloning, and sequencing of 16S rDNAs from different species of aphids. (a) Thick line, DNA coding for 16S rDNA; numbers, primers; arrow, direction of DNA synthesis; B, *Bam*HI site; E, *Eco*RI site. (b) Polymerase chain reaction-amplified product. (c) Amplified product digested by *Bam*HI and *Eco*RI. (d) Templates and directions of sequencing prepared from the phagemid vectors pTZ18 and pTZ19.

preparation and 1 μM (each) primers 1 and 2. The cycle consisted of 1 min at 95°C, 2 min at 55°C, and 5 min at 70°C; a total of 25 cycles were performed (Fig. 1, line b). The DNA was extracted and dried as described above and was suspended in 100 μl of TE buffer. An aliquot was digested with *Bam*HI and *Eco*RI, and the initial sample and the digested sample were electrophoresed in 1% agarose (17). In all cases, the amplified sample contained a single band of about 1.5 kb (Fig. 1, line b), while the digested sample contained two bands of about 0.67 and 0.84 kb (Fig. 1, line c). The DNA was cloned into the phagemid vectors pTZ18 and pTZ19 (15) (Fig. 1, line d).

Single-stranded template DNA was purified as described previously (15) and sequenced by the dideoxy chain termination method (24). The three primers of Lane et al. (16) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and the following primers were used: forward direction, 5′-ACT CCT ACG GGA GGC AGC AGT-3′ (nt 338 to 358), 5′-AAA CTC AAA TGA ATT GAC GGG-3′ (nt 907 to 927), 5′-GAG GAA GGT GGG GAT GAC GTC-3′ (nt 1174 to 1194), and 5′-TCT GCA ACT CGA CTC CAC-3′ (nt 1313 to 1330); reverse direction, 5′-TTA CTC TAC CAA CAA GCT AA-3′ (nt 263 to 244), 5′-ACT GCT GCC TCC CGT AGG AGT-3′ (nt 358 to 338), and 5′-GGG TTG CGC TCG TTG CGG GAC-3′ (nt 1114 to 1094). In the case of the endosymbiont of *Diuraphis noxia*, only one strand of each of the 0.67- and 0.84-kb inserts was sequenced; both strands of all the inserts of the remaining endosymbionts were sequenced. In no case was there a difference in the sequences of the two strands.

Restriction enzyme and Southern blot hybridization analyses. The methods used have been described elsewhere (30). The hybridization probe was p8Z2-9, which contains a 0.84-kb insert of the second half of the *Schizaphis graminum* 16S rDNA (Fig. 1). The restriction enzymes used were *Bgl*II, *Eco*RI, *Hin*dII, *Pvu*II, and *Sca*I.

Analysis of the data. Analysis of the sequence data was carried out with the MacIntosh version 3.0L of PAUP (27, 28). Following alignment, constant and uninformative positions were deleted, yielding 278 informative characters. Bases that had been scored as multistate, unordered characters, and deletions were treated as missing data. Analysis was divided into two stages. First, to determine the monophyly of the endosymbionts and their placement relative to other bacteria, an analysis with the 11 endosymbionts plus three other bacterial species from the gamma-3 subdivision (*E. coli*, *Proteus vulgaris*, and *Ruminobacter amylophilus* [21]) was performed. The last species was set as the outgroup. To obtain an index of confidence for particular nodes on the tree, the bootstrapping protocol was utilized in the initial analysis. Under both 1:1 and 1:10 ratios of transitions to transversions, 100% of the bootstrap runs placed all endosymbionts in a single clade for which the sister clade contained *E. coli* and *P. vulgaris*. Since results for the two ratios were essentially identical, only the 1:1 ratio was used in subsequent analysis.

Since initial analysis strongly supported a monophyly of the endosymbionts, relationships among them were analyzed separately with *P. vulgaris* as an outgroup. By using the Branch and Bound procedure of PAUP (27), the three shortest trees were found; these varied only in the positions of their terminal taxa within the Aphididae. Figure 2 shows the strict consensus of these three trees. The consistency index (an index of support of the tree by the data) for the tree in Fig. 2 is 0.62, which is near the average for studies with this number of taxa, on the basis of analysis of published

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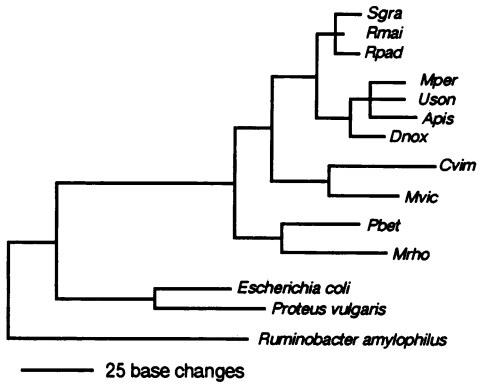

FIG. 2. Phylogenetic tree based on the 16S rDNA sequences of endosymbionts from species of aphids as well as those of bacteria representative of the gamma-3 subdivision of the class *Proteobacteria*. For full names of aphid species, tribes, and families, see Table 1.

consistency indices (23) for molecular data sets (predicted index = 0.61) or for morphological and molecular data sets (predicted index = 0.62).

Nucleotide sequence accession numbers. The sequences were deposited in GenBank under accession numbers M63246 to M63255.

RESULTS AND DISCUSSION

A phylogenetic tree based on the 16S rDNAs of the endosymbionts from 11 species of aphids and constructed with a maximum-parsimony computer program is presented in Fig. 2. The results indicate that all the endosymbionts are related and constitute a distinct lineage within the gamma-3 subdivision of the class *Proteobacteria* (21, 26, 32). The longer length of the endosymbiont tree relative to that of the other bacteria shown in Fig. 2 raises the possibility that the endosymbionts evolved at a more rapid rate. The phylogenetic tree (Fig. 2) suggests that a successful endosymbiotic association was established in a common ancestor of the 11 aphid species and that subsequent diversification of insects and bacteria occurred in parallel to yield the present species of aphids and their endosymbionts. A prediction of this scenario is that endosymbiont and aphid phylogenies should show the same order of branching. Although phylogenetic relationships within the Aphidoidea are not firmly established, two facts suggest that the aphid and bacterial phylogenies match as predicted. First, the relationships established by the endosymbiont 16S rDNAs (Fig. 2) agree closely with accepted taxonomic classification of the aphids (Table 1). Second, the order of branching in the endosymbiont phylogeny in Fig. 2 is identical to that of the corresponding aphid hosts in the phylogeny proposed by Heie (10). This support for a single infection with subsequent parallel radiation suggests a minimum age of 80 million years for the aphid-endosymbiont association, since fossils from Cretaceous amber show that Mindaridae, Aphididae, and Drepanosiphidae were distinct lineages at that time (10). A similar conclusion of a single ancient infection was derived from a study of the DNA divergence of cockroaches and their endosymbionts, although that study did not compare insect and bacterial phylogenies (33). The lineage represented by the aphid endosymbionts (Fig. 2) has been given the generic and specific designation *Buchnera aphidicola* (20), with the endosymbiont of *S. graminum* designated as the type strain of the species.

Restriction enzyme and Southern blot analyses indicated that in all cases the probe containing a fragment of the 16S rDNA from the endosymbiont of *S. graminum* hybridized with only one band (results not shown), a finding consistent with the presence of a single copy of the 16S rDNA in the endosymbiont genome. Previous studies with the endosymbionts of *S. graminum*, *Myzus persicae*, and *A. pisum* led to an identical conclusion (29, 30). The presence of only one or two copies of the rRNA operon appears to be restricted to bacteria with very slow growth (14). This is probably also true for the endosymbionts which should approximate the growth rate of the aphid host (25).

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