Symbiosis and Insect Diversification: an Ancient Symbiont of Sap-Feeding Insects from the Bacterial Phylum *Bacteroidetes*

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Several insect groups have obligate, vertically transmitted bacterial symbionts that provision hosts with nutrients that are limiting in the diet. Some of these bacteria have been shown to descend from ancient infections. Here we show that the large group of related insects including cicadas, leafhoppers, treehoppers, spittlebugs, and planthoppers host a distinct clade of bacterial symbionts. This newly described symbiont lineage belongs to the phylum *Bacteroidetes*. Analyses of 16S rRNA genes indicate that the symbiont phylogeny is completely congruent with the phylogeny of insect hosts as currently known. These results support the ancient acquisition of a symbiont by a shared ancestor of these insects, dating the original infection to at least 260 million years ago. As visualized in a species of spittlebug (Cercopoidea) and in a species of sharpshooter (Cicadellinae), the symbionts have extraordinarily large cells with an elongate shape, often more than 30 μm in length; in situ hybridizations verify that these correspond to the phylum *Bacteroidetes*. “*Candidatus Sulcia muelleri*” is proposed as the name of the new symbiont.

Many invertebrates harbor obligate bacterial symbionts (5). Often the codependence is mutual, and, except for the brief period during which they are transferred to eggs or developing progeny, the bacteria are confined to the cytoplasm of specialized cells, called bacteriocytes. Buchner (5) proposed that the usual role of bacteriocyte-associated symbionts is the provisioning of nutrients to their hosts. He presented a general picture of such symbioses as widespread, important in the ecology and development of hosts, and originating deep in the evolutionary pasts of different invertebrate groups.

Buchner's picture has been upheld and expanded by more recent investigations in which molecular data have been used to document the histories and functional roles of symbionts, particularly in insects. Nucleotide sequence data have permitted unambiguous discrimination of symbiont types and the reconstruction of their evolutionary relationships to one another and to other bacteria. Among insects, many symbioses date to the origin of major taxonomic groups, such as whole insect families, thereby implying many millions of years of association. Among the groups shown to have ancient bacteriocyte-associated (or “primary”) symbionts are the aphids (34), tsetse flies (9), cockroaches (29), whiteflies (45), psyllids (46), mealybugs (2), weevils (28), and carpenter ants (15, 37). Together these studies yield a broad picture of the symbiotic origins and losses that have contributed to the evolutionary and ecological diversification of insects.

For several of these symbionts, experimental results have supported a nutritional role (see, e.g., references 5, 17, and 22). Sequence data have greatly extended our knowledge of symbiont contributions to their hosts. This approach, made possible by PCR, began with the sequencing of DNA fragments

from *Buchnera* (see, e.g., references 3 and 27) and has culminated in complete genome sequences of several insect symbionts (1, 19, 41, 44, 47). The selective retention of certain pathways for biosynthesis of nutrients needed by insect hosts is striking, in view of the extensive gene loss that is characteristic of the genomes of most obligate symbionts. For example, aphids feed on plant phloem sap, which has few essential amino acids, and *Buchnera* retains the needed biosynthetic pathways for making these compounds, despite having a highly reduced gene set (41). These observations from genomic contents have confirmed and elaborated Buchner's central thesis: symbiosis in animals is driven by nutritional needs of hosts and is thus especially common in hosts with restrictive feeding habits.

Among the most extraordinary systems of symbiosis in insects are those found in the sap-feeding insects often referred to as the suborder Auchenorrhyncha of the order Hemiptera; these include the singing cicadas (Cicadoidea), the spittlebugs or froghoppers (Cercopoidea), the clade containing leafhoppers plus treehoppers (Membracoidea), and the planthoppers (Fulgoroidea). The Auchenorrhyncha contains about 40,000 currently described species, including the majority of sap-feeding insect species and the vectors of many plant diseases. (The other main group of sap-feeding insects is the Sternorrhyncha, a related clade, which includes the aphids, whiteflies, psyllids, and scale insects, most of which also contain obligate bacterial symbionts.) Buchner (5) referred to the Auchenorrhyncha as the “fairyland of symbiosis” and described a great diversity of symbiotic associations in different host species. Buchner's student H. J. Müller devoted extensive study to the symbioses of this group and established that most species contain multiple symbiont types (33). Of 405 species that he examined, 348 had either two or three distinct symbionts based on features discernible by light microscopy. He proposed a scheme for the evolutionary history of these associations, hypothesizing a series of acquisitions and losses of particular symbiont lineages

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TABLE 1. Insect species studied

Species abbreviation used in figures	Insect species	Superfamily	Family	Location, collector ^a	No. of insects	Presence of <i>Bacteroidetes</i> symbiont	Symbiont sequence accession no.
Hv	<i>Hackeriella veitchi</i> (Hacker)	Coleorrhyncha	Peloriidiidae	Australia, 4	3	—	
Rt	<i>Rhacophysa taylora</i> (Evans)	Coleorrhyncha	Peloriidiidae	Australia, 4	1	—	
Pd	<i>Prokelisia dolus</i> (Wilson)	Fulgoroidea	Delphacidae	Arizona, 8	3	—	
Pm	<i>Peregrinus maidis</i> (Ashmead)	Fulgoroidea	Delphacidae	Arizona, 2	2	—	
Os	<i>Ormenis saucia</i> Van Duzee	Fulgoroidea	Flatidae	Arizona, 8	4	—	
Af	<i>Acanalonia fasciata</i> Metcalf	Fulgoroidea	Acanaloniidae	Arizona, 8	1	—	
Ss	<i>Scolops</i> sp.	Fulgoroidea	Dictyopharidae	Arizona, 8	1	+	DQ066627
Pfu	<i>Poblicia fuliginosa</i> Olivier	Fulgoroidea	Fulgoridae	Arizona, 2	1	+	DQ0666328
Mse	<i>Magiccada septendecim</i> (L.)	Cicadoidea	Cicadidae	Maryland, 7	2	+	DQ066625
Da	<i>Diceroprocta apache</i> (Davis)	Cicadoidea	Cicadidae	Arizona, 2	3	+	DQ066626
Hb	<i>Hindoloides bipunctatus</i> (Haupt)	Cercopoidea	Machaerotidae	Japan, 5	1	+	DQ066631
Cv	<i>Chaetophyes vicina</i> Lallemand	Cercopoidea	Machaerotidae	Australia, 6	1	+	DQ066633
Aq	<i>Aphrophora quadrinotata</i> Say	Cercopoidea	Aphrophoridae	New York, 3	1	+	DQ066629
Ps	<i>Philaenus spumarius</i> (Linnaeus)	Cercopoidea	Aphrophoridae	New York, 3	1	+	DQ066636
Msp	<i>Microsargene</i> sp.	Cercopoidea	Aphrophoridae	Costa Rica, 3	1	+	DQ066630
Mc	<i>Mahanarva costaricensis</i> (Distant)	Cercopoidea	Cercopidae	Costa Rica, 3	1	+	DQ066635
Ca	<i>Clastoptera arizonana</i> Doering	Cercopoidea	Clastopteridae	Arizona, 2	7	+	DQ066632
Co	<i>Clastoptera obtusa</i> (Say)	Cercopoidea	Clastopteridae	New York, 3	1	+	DQ066634
Hd	<i>Hamana dictatoria</i> (Gibson)	Membracoidea	Cicadellidae	Arizona, 2	1	+	DQ066638
En	<i>E. nigramericanus</i> Hamilton	Membracoidea	Cicadellidae	Arizona, 1	10	—	
Ei	<i>Evacanthus interruptus</i> (L.)	Membracoidea	Cicadellidae	Arizona, 1	2	—	
Hc	<i>Homalodisca coagulata</i> (Say)	Membracoidea	Cicadellidae	California, 2	2	+	DQ066646
Pi	<i>Paromenia isabellina</i> (Fowler)	Membracoidea	Cicadellidae	Arizona, 1	1	+	
Jo	<i>Jikradia olitoria</i> (Say)	Membracoidea	Cicadellidae	Arizona, 1	1	+	DQ066640
Ee	<i>Excultanus</i> nr. <i>excultus</i> (Uhler)	Membracoidea	Cicadellidae	Arizona, 2	1	+	DQ066645
As	<i>Acinopterus</i> sp.	Membracoidea	Cicadellidae	Arizona, 2	1	+	DQ066639
Es	<i>Excultanus</i> sp.	Membracoidea	Cicadellidae	Arizona, 2	1	+	DQ066644
Pfe	<i>Philya ferruginosa</i> (Goding)	Membracoidea	Membracidae	Arizona, 8	1	+	DQ066642
Pv	<i>Platycotis vittata</i> (Fabricius)	Membracoidea	Membracidae	Arizona, 8	1	+	DQ066643
Sf	<i>Spissistilus festinus</i> (Say)	Membracoidea	Membracidae	Arizona, 2	1	+	DQ066637
Pm	<i>Publilia modesta</i> Uhler	Membracoidea	Membracidae	Arizona, 8	1	+	DQ066641

^a Collectors: 1, R. Rakitov; 2, N. Moran and P. Tran; 3, J. Cryan; 4, G. Monteith; 5, K. Morishima; 6, M. Whiting; 7, J. Eisen; 8, same samples as described in reference 48.

as the Auchenorrhyncha diversified. The central player in this scheme was a very strange organism, called the “a-symbiont” by Müller and subsequent authors (see, e.g., references 5, 7, 8, 22, and 24). In different hosts, this symbiont type featured an extraordinarily large size and unusual morphology. Müller hypothesized that these were highly derived bacteria, descended from a lineage infecting an ancestor of all Auchenorrhyncha and subsequently retained through vertical transmission in some lineages but lost in others (33 [also reproduced in reference 5]). According to Müller, the a-symbiont was joined, and sometimes replaced, by one or more additional symbiont types in descendant host lineages, resulting in the current variety of associations.

Here we report the identity of the a-symbiont as a highly specialized member of the *Bacteroidetes* phylum of *Bacteria*, identified here from leafhoppers, treehoppers, cicadas, spittlebugs, and planthoppers.

MATERIALS AND METHODS

DNA isolation, PCR, and sequencing of 16S rRNA genes. The set of insect species studied is presented in Table 1. In most cases, the bacteriome was dissected from the abdomen of a live adult insect and placed in 100% ethanol.

DNA was extracted using methods as described by Moran et al. (31). The insect carcass was retained in 70% ethanol as a voucher specimen. In some cases, the intact insect was previously preserved in 95 to 100% ethanol and DNA was extracted from the entire insect. Each species was represented by 1 to 10 individual insects; for each, DNA was extracted, amplified, and, in some cases, sequenced separately. For the spittlebug *Clastoptera arizonana*, we dissected separately two portions of the bacteriome: a spherical or ovoid, yellow-orange structure about 0.5 mm in diameter and an associated dark-red, doughnut-shaped structure that is wrapped around the yellow-orange structure. Extractions, PCR, and sequencing were performed separately on the two structures for this species, in order to correlate sequences with locations as a further confirmation of in situ results (see below).

The PCR primers used previously for the 16S rRNA gene of the *Bacteroidetes* symbiont in *Homalodisca coagulata* (31) were used initially to amplify related symbionts in other species. These yielded only a portion of the 16S rRNA gene and gave no product for some of the insect species. To obtain a greater portion of the gene sequence, 16S rRNA gene sequences from full genomes of members of the *Bacteroidetes* were aligned and used to design primers that would amplify all known *Bacteroidetes*. These were 10_CFB_FF (5'-AGA GTT TGA TCA TGG CTC AGG ATG-3') and 1515_R (5'-GTA CGG CTA CCT TGT TAC GAC TTA G-3'). PCR conditions were similar to those described by Moran et al. (31). These primers were also used as sequencing primers for direct sequencing of PCR products following purification, using QIAGEN QIAquick PCR purification kits, as described by Moran et al. (31). The result was a near-full-length 16S rRNA gene sequence for each individual.

Phylogenetic analyses. We included all *Bacteroidetes* symbionts for which sequences were obtained except for *Paromenia isabellina* and *Clastoptera obtusa*. For *P. isabellina*, the *Bacteroidetes* symbiont was detected and partially sequenced but not included in the phylogenetic analysis due to inability to obtain a full sequence with our primers. For *C. obtusa*, the symbiont sequence was almost identical (>99.5%) to that for *C. arizonana*, so only the sequence from the latter species was included. Outgroups included members of *Blattabacterium* (the closest hits both in BLAST searches of GenBank and in comparisons to the Ribosomal Database Project [RDP] [11]) and other representative members of *Bacteroidetes*. A sequence reported from a bacterium associated with a coccinellid beetle (GenBank accession number Y13889) also appeared to be closely related to the auchenorrhynchan symbionts but was excluded from the phylogenetic analyses because it appeared to be of low quality.

Initial alignment of all 20 symbiont-derived DNA sequences was performed in ClustalW (<http://www.ebi.ac.uk/clustalw>) (10). Outgroup sequences, aligned taking into account secondary structure through the RDP (11), were downloaded from the RDP. Symbiont sequences were then merged into the outgroup alignment by using the *Blattabacterium* sp. (accession number Z35665) sequence as a reference so that secondary structure would be accounted for in the final symbiont-plus-outgroup alignment. Manual adjustments were then made in MacClade 4.06 (30) so as to minimize the number of changes across sites. Alignments were unambiguous for the ingroup (the set of auchenorrhynchan symbionts), but 99 of the 1,496 sites were excluded from analyses because they were ambiguous for the data set as a whole (ingroup plus outgroups). Of the 1,397 characters included, 368 were parsimony informative.

Parsimony analyses were conducted in PAUP* (version 4.0b10) (43), using a heuristic search with 10,000 random addition sequence replicates and tree bisection and reconnection branch swapping. Nodal support was assessed through nonparametric bootstrap analysis, using 5,000 bootstrap replicates with 10 random addition sequence replicates per bootstrap replicate.

The "general time reversible with proportion invariant plus gamma" model of evolution was estimated to be the most appropriate model via log-likelihood ratio tests using MrModeltest (version 2.2; distributed by the author, J. A. A. Nylander, Uppsala University). Likelihood analysis was conducted in PAUP* through successive iterations with starting parameters based on estimates from the previous search. Parameters for the first iteration were estimated from the most parsimonious tree with the best likelihood score. Iterations were continued until successive searches yielded identical trees. Four iterations were needed for convergence. Nodal support was assessed through nonparametric bootstrap analysis consisting of 1,000 bootstrap replicates, using neighbor joining to build the starting tree and tree bisection and reconnection branch swapping. Parameters were set to those estimated during the final successive iterations. Genetic distances, based on the maximum-likelihood model, were also determined by setting parameters to those estimated during the final successive iteration.

Six replicate Bayesian analyses were conducted with MrBayes 3.1.1b (23). Four Markov chains were used in each replicate, and the chain was sampled every 100 generations. The temperature parameter was set to 0.2. Analyses were allowed to run for five million generations. Trees from the first 50,000 generations of each replicate were excluded.

Phylogenetic information for the insect hosts of these symbionts was compiled from recent studies in the insect systematics literature. These studies relied on different gene sequences and, in a minority of cases, on morphological features, including those of fossils. In some cases, particular nodes are not resolved, including that defining Auchenorrhyncha as a clade. Results from this compilation are presented in Fig. 1, with relevant citations indicated for every resolved node of the tree. Nodes lacking substantial support, or having conflicting data, are collapsed.

Microscopy and in situ hybridizations. Both portions of the bacteriomes of late-stage nymphs of *C. arizonana* were dissected into PA buffer (50 mM Tris-HCl [pH 7.6], 100 mM EDTA, 250 mM sucrose), disrupted by manipulating gently under a coverslip, stained with SYBR-Gold, and examined using fluorescence at a 1,000-fold magnification. The same procedure was applied to the entire bacteriome of *Homalodisca lacerta*.

To determine the organisms corresponding to the *Bacteroidetes* rRNA gene sequences, in situ hybridization was performed. We used two probes, each 20 nucleotides long. One, CFB319 (5'-TGG TCC GTG TCT CAG TAC-3'), was a perfect match to positions 319 to 336 of the 16S rRNA of *Bacteroidetes* species, including all of our sequences from these symbionts; this probe shows two or more mismatches to known bacteria in other phyla. The other, Pro319 (5'-TGG ACC GTG TCT CAG TTC-3'), differed at two sites and was a perfect match to 16S rRNA sequences of *Buchnera*, "*Candidatus* Baumannia cicadellinicola", and some other *Gammaproteobacteria*. CFB319 was linked to 6-carboxytetramethylrhodamine (absorption/emission of 551/576 nm); Pro319 was linked to Alexa488

(absorption/emission of 499/520 nm). To determine the specificity of hybridization, we obtained a sample of bacteriocytes containing *Buchnera aphidicola* from pea aphids (*Acyrtosiphon pisum*) maintained as a colony in the lab. (*Buchnera* cells are easily recognized as regular spheres with a diameter of 3 μ m.) These, together with a portion of the dark-red bacteriome from one side of a nymph of *C. arizonana*, were fixed in 4% formaldehyde at 20°C for 4 h and centrifuged for 2 min at 3,000 rpm. The supernatant was then decanted, and the material was resuspended in water, transferred to a silane-coated slide, and air dried. Once samples adhered to slides, they were washed with hybridization buffer (0.9 M NaCl, 20 mM Tris-Cl, 5 mM EDTA, 0.1% sodium dodecyl sulfate, 10 $\times$ Denhardt's solution) and incubated with 80 μ l of hybridization buffer plus 10 μ l of each probe solution at 10 μ M. Incubation was carried out under a hybridization cover at 50°C for 4 h, under humid conditions and in the dark. Slides were then washed with SSC (0.15 M NaCl plus 0.015 M sodium citrate) and then with phosphate-buffered saline buffer and mounted in glycerol mounting buffer before examination with a Nikon Eclipse TE2000-U inverted microscope fitted with standard fluorescence filter sets and a digital imaging system.

The same fixation, mounting, and staining protocol was applied to the bacteriomes of *H. lacerta*, which was known to yield 16S rRNA gene sequences of "*Candidatus* Baumannia cicadellinicola" and of the *Bacteroidetes* symbiont.

RESULTS

Distribution of the *Bacteroidetes* symbionts in Auchenorrhyncha. *Bacteroidetes* symbionts were found in the majority of species of Auchenorrhyncha examined, including species from all four superfamilies (Table 1). Previously, the only symbiont sequence in the phylum *Bacteroidetes* and associated with a member of the Auchenorrhyncha was a partial 16S rRNA gene sequence from a bacteriome-associated symbiont of the glassy-winged sharpshooter, *Homalodisca coagulata* (GenBank accession number AY147399) (31). When our new sequences were used as queries in BLAST searches using the GenBank nucleotide database, this sequence invariably gave the highest bit score. The next BLAST "hits" were sequences from *Blattabacterium* (cockroach endosymbionts) and from a bacterium listed as a symbiont from the coccinellid beetle *Coleomegilla maculata* (GenBank accession number Y13889).

In cases in which symbionts were obtained for multiple individuals of a single host species, almost no polymorphism was found. The largest divergence within host species was for the isolate from *H. coagulata*, which showed four differences (0.3% divergence) with the previously deposited sequence (31). Four full sequences obtained for different individuals of *Clastoptera arizonana* were identical, indicating both a low error rate of amplification and sequencing and low polymorphism for this host species.

No symbionts of this type were found for Peloridiidae (two species), Flatidae (one species), Delphacidae (two species), and one species of Cicadellidae (*Evacanthus nigramericanus*), based on multiple primer sets and multiple individuals for each of these species. All of these insect species were observed to have bacterial symbionts, based on microscopic examination, on PCR amplification, and determination of 16S rRNA gene sequences corresponding to other bacterial phyla (data not presented).

Phylogenetics of symbionts and hosts. The results of parsimony, likelihood, and Bayesian analyses, both when outgroups were included and when outgroups were excluded, were highly concordant. Strongly supported clades (with support values of greater than 75) were consistent across

FIG. 1. Phylogenetic relationships of auchenorrhynchan lineages represented in the current study plus other major lineages of Hemiptera. The tree represents relationships with substantial support, as compiled from recent studies. Nodes for which no firm support is available or for which conflicting results have been reported are collapsed. Abbreviations in parentheses indicate study species as indicated in Table 1. Letters next to nodes refer to relevant studies giving support to particular clades, as follows (clade names are given for those with available nomenclature): a, Auchenorrhyncha-Sternorrhyncha-Coleorrhyncha-Heteroptera (36, 42, 48, 49); b, Fulgoromorpha (4, 6, 36, 42, 46); c, unnamed clade (4, 36); d, Dictyopharidae-Fulgoridae (reference 4 and references therein); e, Cicadomorpha (36, 46); f, Cicadoidea (16); g, Cercopoidea (and relationships within Cercopoidea) (14); h, Membracoidea (16, 46); i, Cicadellinae-Coelidiinae (and others) (16); j, Deltoccephalinae-Membracidae (and others) (16); k, Membracidae (and relationships within Membracidae) (12, 13, 16). Dates are minimum ages for the subsequent node, based on fossils assigned to the descendant clade (4, 20, 21, 27, 38, 39, 40). My, million years.

analyses. Exclusion of outgroups had only minor effects on support values.

Phylogenetic analyses consistently gave very strong support (100% bootstraps for all analyses) for a single clade corresponding to the symbionts of Auchenorrhyncha (Fig. 2). Their closest relatives include the obligate symbionts of cockroaches (*Blattabacterium*), with average divergences of 16.7% (13.3 to 20.4%) in the 16S rRNA sequence. Distances to all other outgroups were greater than 30%. The auchenorrhynchan symbionts are relatively distant, averaging 42% divergence, from "*Candidatus* Cardinium hertigii", another insect symbiont in *Bacteroidetes* that infects a wide variety of host species.

Parsimony analysis including outgroups of the symbiont clade yielded five most-parsimonious trees. Parsimony analysis excluding outgroups yielded 16 most-parsimonious trees. In all of these trees, symbionts formed clades corresponding to host higher classification.

The phylogeny of the symbiont clade is largely congruent

with the known phylogeny of the host insects as presented in Fig. 1 (aside from losses of symbionts in some insect lineages). More specifically, every one of the symbiont clades with greater than 75% bootstrap support for two of the three analyses corresponded to a known insect clade. There were no strongly supported nodes (by the same criterion) that showed conflict with known phylogeny for the hosts. The majority of nodes with substantial support from the published studies of insect phylogeny were also supported in the tree for the symbionts. These included nodes defining the following clades: Fulgoroidea, Cicadomorpha, Membracoidea, Cicadidae, Cercopoidea, Machaerotidae, Clastopteridae, Membracidae, and Deltoccephalinae (for the two species included here) (Fig. 2). Most other nodes are poorly resolved based on current knowledge of host relationships and in our analyses of symbionts; however, symbiont relationships were sometimes unresolved (by the above criterion) for some recognized insect clades, such as the subfamilies within Membracidae and families within

FIG. 2. Maximum-likelihood phylogram based on 16S rRNA genes of the symbionts (sym.) isolated from auchenorrhynchous insects and closely related members of the phylum *Bacteroidetes*. The topology of the likelihood tree is almost identical to that derived from Bayesian and parsimony analyses. Symbiont terminals are labeled with the host taxon, and outgroup terminals are labeled with the bacterial species name (see Table 1 for abbreviations) and GenBank accession number. Support values from analyses in which outgroups were included are indicated below the branch as nonparametric parsimony bootstrap value/nonparametric likelihood bootstrap value/Bayesian posterior probability, for branches on which all three support values are greater than 50. Asterisks indicate cases in which all three of the support values are 100.

Cercopoidea. This lack of resolution is not surprising given the very low levels of variation at these phylogenetic depths.

The largest pairwise divergences within the symbiont clade were between the symbionts of Fulgoromorpha and those of Cicadomorpha, consistent with the basal divergence of these insect groups in the symbiont and host trees. These values averaged 15.2% (range, 0.13.7 to 16.9%), using the maximum-likelihood model of substitution from the phylogenetic analysis. Within the symbionts of Cicadomorpha, the deepest divergences were between cicada symbionts and others, with an average divergence of 6.9% (6.6 to 7.2%).

Microscopy. In late-stage juveniles of *C. arizonana*, PCR results indicated that the *Bacteroidetes* organisms were located only in the dark-red portion of the bacteriome, a result agreeing with the observations of the a-symbiont by previous authors (5). Furthermore, we were able to amplify a second symbiont, in the *Betaproteobacteria*, only from the ovoid yellow-orange portion of the bacteriome and not from the dark-red portion (results not presented). Using fluorescent staining of DNA, we observed that the dark-red,

doughnut-shaped organs were filled with an organism resembling the a-symbiont, as depicted by Müller (33) and other authors (5, 7, 8, 24). Thus, in contrast to the bacteriomes of adult sharpshooters (31), the spittlebug bacteriomes appeared to contain a portion in which the a-symbiont resides exclusive of other organisms. We thus used these structures to determine if the organisms corresponded to the source of the *Bacteroidetes* sequences.

The in situ hybridizations of oligonucleotides matching the sequence of rRNA show clear specificity of the two probes, with Pro319 corresponding to the *Buchnera*, as expected (Fig. 3C), and CFB319 corresponding to the large strap-shaped cells in the dark-red bacteriomes of *C. arizonana* (Fig. 3B). Thus, the *Bacteroidetes* sequence corresponds to those depicted by Müller for the a-symbiont of another spittlebug species, *Philaenus spumarius* (5, 33) (Fig. 3A).

These organisms, in *C. arizonana*, are about 3 to 5 μm in width and of variable length, up to about 80 μm . They often appear to be coiled into balls that are enclosed within membranes, presumably of host origin. These membranes are

FIG. 3. Comparison of the a-symbiont as depicted by Müller (33) and FISH with symbionts from Auchenorrhyncha bacteriomes. (A) Reproduction of drawing (33) of a-symbionts dissected from the bacteriome of *Philaenus spumarius* (Cercopoidea). (B and C) FISH of the *Bacteroidetes* symbiont ("Candidatus *Sulcia muelleri*") [Sm] dissected from the bacteriomes of *Clastoptera arizonana* (Cercopoidea) intermixed with round *Buchnera aphidicola* (Ba) cells from a pea aphid as controls. (B) FISH results from a *Bacteroidetes* diagnostic rRNA probe with 6-carboxytetramethylrhodamine (red); (C) FISH results with a *Gammaproteobacteria*-diagnostic rRNA probe with Alexa488 (green). (D) Superimposed FISH images of bacteriome contents of the sharpshooter *Homalodisca lacerta*, showing "Candidatus *Sulcia muelleri*" (Sm) (red) and "Candidatus *Baumannia cicadellinicola*" (Bc) (green).

readily disrupted so that most symbionts spill out, making their length and overall shape more apparent.

To confirm the general shape and appearance of the *Bacteroidetes* symbiont and *Baumannia cicadellinicola* in the same host species within Cicadellinae, we used fluorescent in situ hybridization (FISH) with probes specific to *Bacteroidetes* and to *Gammaproteobacteria* and confirmed as matching the two symbiont sequences in *Homalodisca lacerta* (a close relative of the glassy-winged sharpshooter, *H. coagulata*). The *Bacteroidetes* probe hybridized with a large strap-shaped organism (Fig. 3D) with a general appearance very similar to that found in *C. arizonana* (Fig. 3B) and to the a-symbionts described by Müller (Fig. 3A) and others (7, 8, 24, 33). The *Gammaproteobacteria* probe corresponded to spherical cells of about 2 μm in diameter (Fig. 3D), as described previously for "Candidatus *Baumannia cicadellinicola*" (31).

DISCUSSION

Our phylogenetic results indicate that this well-defined *Bacteroidetes* symbiont clade is characteristic of many insects within the Auchenorrhyncha and that it was likely acquired by a shared ancestor of these insects. This diversification dates at least to the Permian, 260 to 280 million years ago, based on fossils of Fulgoromorpha from that time and on fossils of all four major host groups from the Triassic (20, 38, 39, 40) (Fig. 1). Lineages of Auchenorrhyncha colonized a broad spectrum of higher plants on all continents, moving from older groups of vascular plants to newly diversifying ones, such as angiosperms, as these became dominant (25, 26). These insects now display a huge variety of lifestyles, morphologies, and feeding habits, with different species feeding on phloem sap, xylem sap, or contents of cells and on roots, leaves, or shoots. Auchenorrhynchan species are a significant component of most modern terrestrial ecosystems, consuming plant-derived nutrients directly and acting as major vectors of phytopathogens. Our results suggest that this symbiont has been present throughout the diversification of this major insect group. It probably resides in more host species than any known bacteriome associ-

ate, and it is one of the oldest, comparable in age to *Blattabacterium*, the symbiont of many cockroaches (29). The overall sequence divergence, averaging about 15% for the basal divergence (of Cicadomorpha from Fulgoromorpha), is consistent with an ancient divergence of >260 million years; these values would require a rate of substitution of ~3% per 100 million years (i.e., 6% divergence per 100 million years). An even deeper history, of continuous vertical transmission since the time of the shared ancestor of cockroaches and Auchenorrhyncha, is highly speculative; this possibility would imply a large number of losses of symbionts, since the split between these two insect groups corresponds to the ancestor of the Neoptera, which includes most insect orders.

An additional symbiont, "Candidatus *Baumannia cicadellinicola*", present in species of sharpshooter (Cicadellinae), falls within the *Gammaproteobacteria*, near other insect symbionts such as *Buchnera aphidicola*. "Candidatus *Baumannia cicadellinicola*" was previously suggested to correspond to the a-symbiont of Müller (31). That proposal was incorrect, based on the further information reported here. Both morphology and distribution among hosts indicate that the large *Bacteroidetes* symbiont presented here corresponds to the a-symbiont and has similar shape and size in both sharpshooters (Cicadellinae) and spittlebugs (Cercopoidea) (Fig. 4), which are divergent host groups. "Candidatus *Baumannia cicadellinicola*" probably corresponds to the t-symbiont of Müller (33) and exemplifies one of the numerous instances proposed by Müller in which a second symbiont has been recruited in addition to the a-symbiont. "Candidatus *Baumannia cicadellinicola*" shows a more limited phylogenetic distribution, with hosts corresponding roughly to the leafhopper subfamily Cicadellinae (sharpshooters). Based on the fossil record for these insects, the origin of "Candidatus *Baumannia cicadellinicola*" as a symbiont was probably in the late Cretaceous (about 70 to 100 million years ago).

The Auchenorrhyncha itself is possibly paraphyletic (42); under this scenario, the Cicadomorpha (cicadas, spittlebugs, leafhoppers, and treehoppers) is a sister group to the Fulgoroida-Heteroptera-Coleorrhyncha. The Heteroptera is a large

FIG. 4. Proposed history of the association between the *Bacteroidetes* symbiont and its insect hosts, as reconstructed from the insect phylogeny presented in Fig. 1. Abbreviations in parentheses indicate study species as indicated in Table 1. Heavy lines indicate lineages that were continuously associated with the *Bacteroidetes* symbiont, assuming a single initial infection in an ancestral lineage. Depending on the as-yet-unknown resolution of basal branches within Hemiptera, heteropteran or coleorrhynchan lineages either have lost ancestral infections, have never been infected, or retain undetected infections. Phylogenetic analyses of the symbionts provide strong support for clades defined by the marked nodes and do not conflict with the remaining nodes in the host tree.

group with species showing diverse feeding habits, including plant parts, other invertebrates, and blood of vertebrates. If Auchenorrhyncha is paraphyletic as proposed, our results would imply either that the *Bacteroidetes* symbiont infected Fulgoromorpha and Cicadomorpha independently or that this symbiont was present in the shared ancestor but later lost in most or all Heteroptera. The latter conclusion is based on the facts that most Heteroptera appear not to have bacteriome-associated symbionts (5) and that, of those Heteroptera that do contain symbionts, the few for which DNA sequences have been obtained do not fall within *Bacteroidetes* (18). Some morphological evidence supports the Fulgoromorpha as the sister

group of the Cicadomorpha, making the Auchenorrhyncha monophyletic (see, e.g., reference 50). In this case, our results are explained more readily; the distribution of the *Bacteroidetes* symbiont would suggest that the auchenorrhynchan ancestor was infected by an ancestral symbiont after the split from Heteroptera. In Cicadomorpha, the symbionts are located in distinctive paired, lateral, abdominal bacteriomes. These are apparently homologous across the included insect groups, consistent with a single origin and continued persistence in the same organ. Fulgoroidea lack these paired bacteriomes and house the symbionts in structures that do not appear to be evolutionarily homologous (5). This difference is consistent with either an independent origin in Fulgoromorpha or an evolutionary change in anatomical location subsequent to a single infection of an auchenorrhynchan ancestor.

Müller (33) examined material of one species of Coleorrhyncha (family Peloridiidae), a basally branching Gondwanan group living on mosses in the southern hemisphere, and reported that it too possessed the a-symbiont. We examined several specimens each of two species of the Peloridiidae (Coleorrhyncha), using several pairs of PCR primers that should have amplified this organism (Table 1). Neither species yielded sequences corresponding to this symbiont, although other 16S rRNA sequences were obtained. Coleorrhyncha is now tentatively placed as the sister group of Heteroptera and not at the base of an auchenorrhynchan clade as in Müller's scheme (36), so the absence of the symbiont is consistent with the proposal of infection of Auchenorrhyncha after its divergence from Coleorrhyncha. Because Müller's report was based on microscopy only, using preserved material shipped to him, it is possible that he observed a different bacterial lineage. We were able to amplify and sequence 16S rRNA gene sequences from bacteria in our specimens of Coleorrhyncha, but these corresponded to an apparent symbiont from the *Betaproteobacteria* plus sequences corresponding to *Microbacteriaceae*, plant-pathogenic bacteria probably present in the gut lumen of the specimens. The question of whether any Coleorrhyncha contain the *Bacteroidetes* symbiont remains open; our results indicate that some species lack it.

In other cases, we found that some groups of Auchenorrhyncha lacked the *Bacteroidetes* symbiont, based on repeated attempts to amplify the 16S rRNA gene with different primer sets. We were able to amplify other apparently symbiotic bacteria, mostly in the *Betaproteobacteria* (unpublished data). Species for which this symbiont appears to be absent include members of Flatidae and Delphacidae among the planthoppers, a result that corresponds to Müller's conclusion that these families lack the a-symbiont. We also found that a species of Cicadellidae, *E. nigramericanus*, lacks the symbiont. These absences are most readily interpreted as losses of an ancestral symbiont, and all except *E. nigramericanus* correspond to proposals of losses made by Müller. (He did not report on *Evacanthus*.)

The symbiont group described here is an extreme case of a phenomenon that is widespread in obligate symbionts inhabiting bacteriomes and showing long-term codiversification with hosts. In such organisms, cells are often spherical or irregular in shape and greatly enlarged. For example, *Buchnera* cells are spheres of 3 μm in diameter with ~ 15 -fold more cytoplasm than related rod-shaped bacteria such as *Escherichia coli*. *Car-*

sonella ruddii, the obligate symbiont of psyllids, shows an irregular amoeboid cell shape and even larger dimensions (46), and *Nardonella* of certain weevils (28) has dimensions similar to those of the *Bacteroidetes* symbiont we have described for Auchenorrhyncha. The unusually large cell size of the a-symbiont was described by Müller (33) and later authors (7, 8, 24) for several host lineages. Cells may be smaller during some parts of the life cycle, such as the infectious stages (5). Most other known *Bacteroidetes* have a typical rod shape and dimensions from 0.5 to 3 μm .

Thus, our results provide strong support for Müller's (33) hypothesis that these symbionts consist of a highly derived bacterial type that originated in the deep evolutionary past of this major insect group. In several other cases, molecular results have verified hypotheses of Buchner and his associates. For example, Buchner wrote extensively on the symbionts of aphids and recognized the primary symbiont (later named *Buchnera aphidicola*, after him) as well as so-called "secondary" symbionts that are more scattered in distribution within and among aphid species. Microscopy alone is often insufficient for discriminating among symbiont types; genetically and ecologically distinct bacteria can have effectively identical morphologies, and the same bacterium can have different shapes and sizes depending on environmental conditions or life cycle stage. Molecular sequence data have helped to resolve many of these issues. For example, sequence data revealed that the aphid secondary symbionts described by Buchner as one entity instead comprise at least three independent lineages (32).

According to Müller and Buchner (5, 33), and consistent with our findings, all Auchenorrhyncha examined that possess the *Bacteroidetes* symbiont also contain at least one other bacterial symbiont. Except in Fulgoroidea, the different symbionts live in the same bilaterally paired organ in the abdomen, although there may be some spatial separation within that organ. Outstanding questions about this symbiont clade include the nature of its metabolic contributions to hosts and the basis for its apparent dependence on its host and one or more additional symbiont partners. This mutual interdependence of the insects and multiple symbiont types might drive the "hunger for symbionts" that Buchner noted as characteristic of the Auchenorrhyncha (5).

The phylogenetic analysis defines a novel clade of symbionts that live in a distinctive set of hosts, consisting of species within the Auchenorrhyncha. We propose the designation "*Candidatus Sulcia muelleri*", in keeping with the procedure for naming species that have not been cultivated in laboratory media (35). The generic name honors Karel Sulc, a Moravian embryologist at University of Brno who, while studying cicadas in 1909, was one of the first biologists to recognize the bacteriome of an insect as an organ containing microorganisms (5). The species name refers to H. J. Müller, a student of Buchner who conducted extensive studies of auchenorrhynchan symbionts and who proposed a scheme of succession of symbionts within insect lineages, with the original colonizer being the a-symbiont lineage that corresponds to "*Candidatus Sulcia muelleri*" (5, 33). Distinctive features of "*Candidatus Sulcia muelleri*" include residence within bacteriomes of auchenorrhynchan insects, a large cell size during part of the life cycle with a distinctive strap-like shape from 2 to 5 μm in width and from 5 to 100 μm in length, and unique 16S rRNA gene sequences,

as follows (positions correspond to homologous *E. coli* positions): TAA TAT ACG AAT AAG TAT C (positions 486 to 504), ACG AAT AAA TTG GAA A (positions 1001 to 1016), and AGT TGG AAG TAC CT (positions 1418 to 1431).

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