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Intracellular symbionts of sharpshooters (Insecta: Hemiptera: Cicadellinae) form a distinct clade with a small genome

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Summary

The leafhoppers (Insecta: Hemiptera: Cicadellidae) are the most species-rich group of invertebrates in which intracellular symbionts are usual. Here we present the first molecular characterization of bacteriome-associates in the leafhoppers, with focus on the subfamily Cicadellinae (sharpshooters). Phylogenetic analyses of 16S rDNA sequences from intracellular symbionts residing in the bacteriomes of five host species indicate that these symbionts form a well-defined clade within the γ-3 Proteobacteria, consistent with an ancient colonization and strict vertical transmission. More extensive gene sequence information is reported for the symbiont of *Homalodisca coagulata* (Say). The genome size, as determined by pulsed field gel electrophoresis, is approximately 680 kb. This finding, when combined with published results for symbionts of aphids, ants, psyllids and tsetse flies, adds to an emerging pattern which suggests that bacteriome associates often descend from ancient infections by γ Proteobacteria, and that these lineages have undergone pronounced genome reduction. A new genus and species name, 'Candidatus *Baumannia cicadellinicola*' (sp. *nov.*) is proposed for this newly characterized clade of symbiotic bacteria.

Introduction

Many insects contain obligate bacterial endosymbionts that live intracellularly within specialized organs called bacteriomes and that are transmitted from mother to progeny through eggs (Buchner, 1965; Moran and Baumann, 2000). Such symbionts are found in virtually all insects that feed primarily on plant sap, including phloem and xylem (Buchner, 1965). For example, most aphids (Insecta: Hemiptera: Aphidoidea) ingest phloem sap and contain the symbiotic bacterium *Buchnera aphidicola* in specialized large cells (bacteriocytes) within the body cavity. Both physiological and molecular studies, including full genome sequencing, have shown that *B. aphidicola* provisions its hosts with essential amino acids, which are present in insufficient concentrations in the phloem sap diet (Sandström and Moran, 1999;Shigenobu *et al*., 2000; Tamas *et al*., 2002). This fits with Buchner's (1965) proposal that these symbioses have a nutritional role, providing hosts with nutrients not present in their restricted diets.

Leafhoppers (order Hemiptera, suborder Auchenorrhyncha, family Cicadellidae) include over 20 000 described species, and are the largest family of sap-feeding insects and the largest insect family in which obligate intracellular symbionts are usual (Dietrich *et al*., 2001). As in other sap-feeding insects, these symbionts are vertically transmitted to progeny and are likely to play a role in host nutrition (Buchner, 1965). Leafhopper symbionts present a particularly interesting case because different leafhopper groups utilize nutritionally distinct diets consisting of either phloem sap, which is rich in sugars and contains nitrogen almost exclusively in the form of free amino acids, or xylem sap, which contains little or no sugar and contains sources of inorganic nitrogen. Comparative studies of leafhopper symbionts could reveal how symbiotic relationships reflect changes in dietary habits of a particular insect group. In addition, leafhoppers include many plant pests, and some are the sole vectors of economically important plant-pathogens.

Bacteriome-associated symbionts are present in all leafhopper subfamilies examined so far, with the exception of the Typhlocybinae (Buchner, 1965). In most of these groups, adult hosts contain bilaterally paired bacteriomes positioned in the lateral margins of the anterior abdomen. Based on microscopy, Buchner believed that different subfamilies of leafhoppers possess different symbiont types and that individual insects may contain two or

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more distinct symbionts. Because symbiotic bacteria can exhibit different morphology depending on conditions and life cycle stage, such premolecular characterizations must be regarded as tentative and, in any case, do not reveal the phylogenetic affinities of the symbionts. In this paper we provide the first molecular characterization of leafhopper bacteriome-associates. We found a consistent symbiont type located in the bacteriomes of adults of the subfamily Cicadellinae (with English common name of 'sharpshooters'). All species we examined have a two-part bacteriome consisting of a yellow and red portion (Fig. 1); the main symbiont we characterized is present in both parts. In addition, we detected a second organism for one of the insect species and confined to the yellow section of the bacteriome.

Some Cicadellinae have a major impact on agriculture as vectors of bacterial diseases of woody plants. The host species upon which we have focussed is *Homalodisca coagulata* (Say) commonly called the 'Glassy-Winged Sharpshooter'. This insect inflicts major damage on grapes and is a potential threat to other crops because of its role as an efficient vector of the bacterial disease agent, *Xylella fastidiosa*, one strain of which causes Pierce's disease of grapevines (Purcell and Hopkins, 1996; Purcell *et al.*, 1999; Hendson *et al.*, 2001).

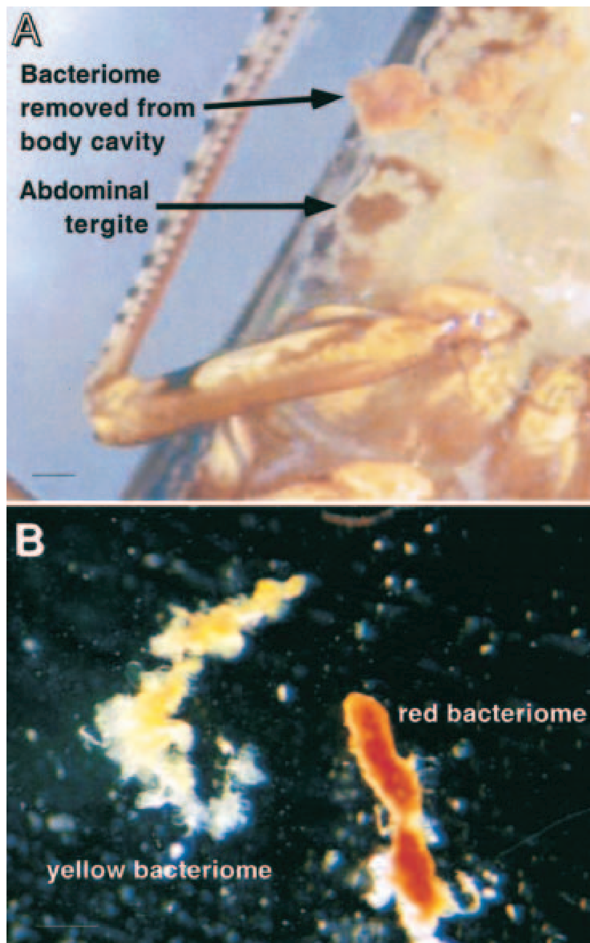

Fig. 1. A. Ventral view of anterior abdomen of *H. coagulata*, showing the red bacteriome removed from the body cavity.
B. Red and yellow bacteriomes removed from one side of an adult female. Scale bars = 250 μm.

Results

Characterization of endosymbiont gene sequences

Five species of Cicadellinae were studied: *H. coagulata*, *Homalodisca lacerta* (Fowler), *Graphocephala cythura* (Baker), *Graphocephala hieroglyphica* (Say), and *Graphocephala aurora* (Baker). For every species, polymerase chain reaction (PCR) amplifications of 16S rDNA, using 'universal' eubacterial primers 10F and 1507R yielded single products, and each amplification gave a single sequence, both for direct sequencing from PCR products (performed for all host species) and for sequencing from multiple clones of PCR products (performed for *H. coagulata*). For *H. coagulata*, 19 sequences were obtained from one individual and single sequences for four other individuals; these included sequences derived from both the yellow and red portions of the bacteriome. Identical sequences were obtained from both portions of the bacteriome and from different individuals for these PCR products. The insect from Florida gave identical sequence to that obtained from California hosts.

In addition, the 16S-23S rDNA fragment was amplified and sequenced as a single longer fragment from the red bacteriome of another Riverside individual of *H. coagulata*. Five fragments were cloned from PCR product, and all were of identical length. Because the amplified fragment spans the internal transcribed spacer that is highly variable in length among bacterial species, this result again suggested that only one product was amplified. Furthermore, the sequenced 16S-23S fragment was identical, in the overlapping region, to the previously obtained sequence of the 16S rDNA fragment recovered using different primers and direct sequencing.

Using the same primers on dissected bacteriomes, symbiont 16S rDNA sequences were obtained for two individuals of *H. lacerta*, three of *G. cythura*, and one each of *G. hieroglyphica* and *G. aurora*. In cases of multiple hosts, sequences were identical for symbionts of the same host species. In addition, diagnostic restriction digests of the 16S rDNA PCR products from each host species yielded only those fragment lengths expected on the basis of the sequence. These results indicate that the 'universal' primers (10F and 1507R) amplify the 16S rDNA of only one bacteriome-associated symbiont and that this organism is present in both red and yellow bacteriomes.

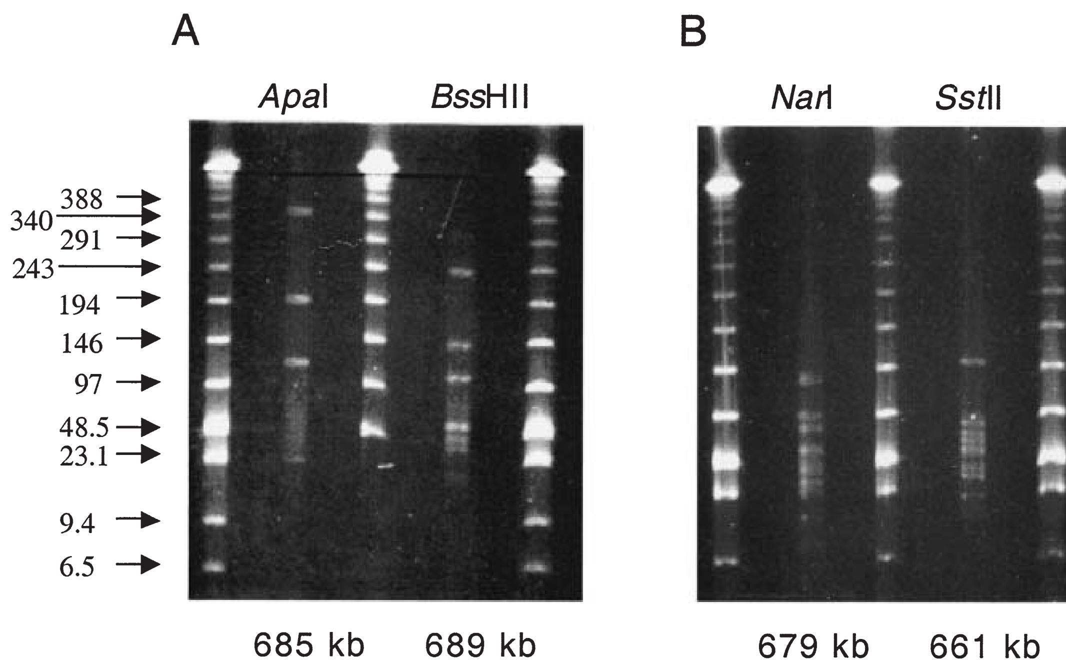

Fig. 2. Estimation of the genome size of 'Candidatus *Baumannia cicadellinicola*'. Sizes of molecular weight standards (in kilobases) are indicated on the left. Restriction enzymes and estimates of genome size are written at the top and bottom, respectively, of the lane corresponding to each digestion. The mean estimated genome size is 678 kb.

The nucleotide base composition for a 2956 bp DNA sequence encoding *rpoBC* was 36.7% GC. This is more A + T-biased than most free-living members of the family Enterobacteriaceae, but not as extreme as that of some other endosymbionts, such as *Carsonella ruddii* (19% GC; Clark *et al*., 2001) or *B. aphidicola* (26% GC; Shigenobu *et al*., 2000).

Genome size

Based on pulsed-field gel electrophoretic (PFGE) separation of digested chromosomes isolated from the red bacteriome, the genome of the *H. coagulata* bacteriome-associate was estimated to be 678 kb. This size is based on the lengths of fragments obtained after digestion with four different restriction enzymes, yielding genome size estimates ranging from 661 kb to 689 kb (Fig. 2). No additional bands were present on the gels, indicating that no other bacterial species was abundant in the red bacteriome.

Phylogenetic analyses

Analyses were based on the nearly complete 16S rDNA sequences that we obtained using primers 10F and 1507R with DNA from the five host species together with sequences for other γ Proteobacteria. Alignment length was 1480 nucleotides. The symbionts of Cicadellinae form a strongly supported clade within the γ-3 Proteobacteria (Fig. 3). This clade is supported by 100% bootstrap values in both Maximum Parsimony and Neighbour-joining analyses (1000 replicates). The closest relatives currently in the database are the carpenter ant symbionts ['Candidatus *Blochmannia camponotii*' (Sauer *et al*., 2000) and the related symbiont of pseudococcids (Fukatsu and Nikoh, 2000; von Dohlen *et al*., 2001)]. Very similar trees were obtained using Maximum Parsimony and Distance search options; in particular, both methods gave very strong support for monophyly of the symbionts of Cicadellinae and for a relationship with the pseudococcid and ant symbionts.

Within the clade of five endosymbionts, there are two strongly supported subclades, corresponding to the two host genera, *Homalodisca* and *Graphocephala*. The tree topology for the species considered is consistent with the generic classification and with the topology based on a mitochondrial sequence of the host (Fig. 4). The order of branching within the host genus *Graphocephala* cannot be fully resolved with the present mitochondrial sequence data.

Microscopy

Electron microscopy revealed that the red-pigmented bacteriomes of *H. coagulata* were filled with irregularly spherical bacteria approximately 1.5–2.0 μm in diameter

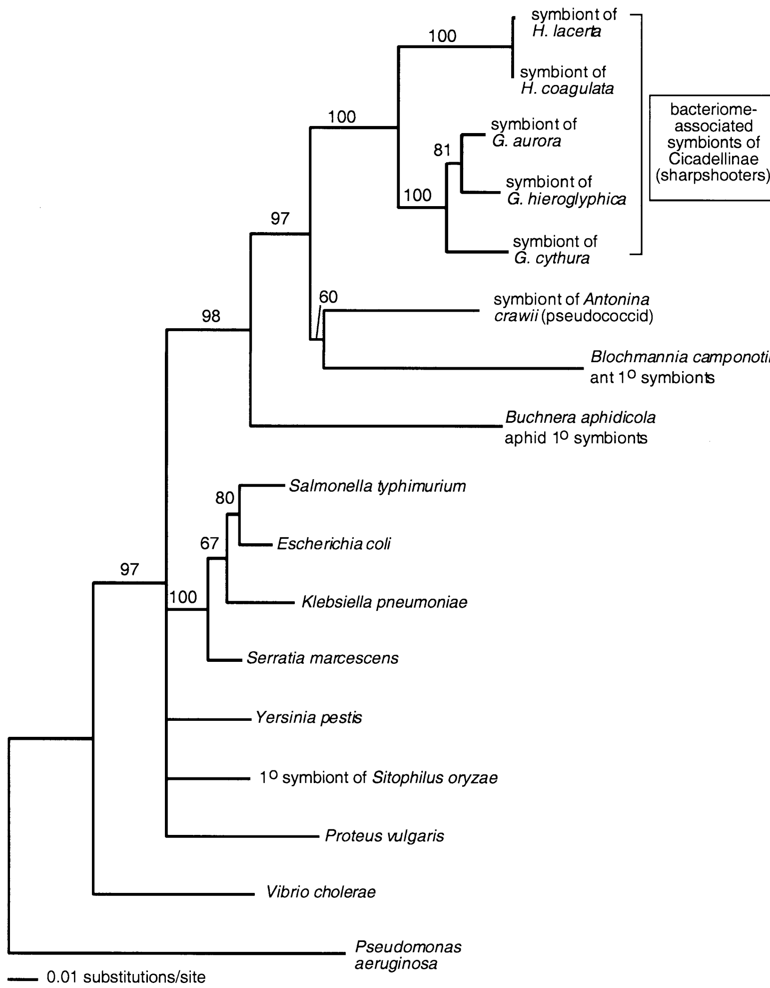

Fig. 3. Phylogeny of representative γ-3 Proteobacteria showing the relationships of the symbionts of Cicadellinae, based on 16S rDNA sequences. Numbers above nodes represent bootstrap values; relationships with less than 50% bootstrap support are collapsed.

(Fig. 5). No other organisms were observed within the red portion of the bacteriome. This same tissue was the source of the DNA sequences that we report for the main symbiont type, and no other 16S rDNA sequences could be amplified from this tissue using universal primers for eubacteria. The red bacteriomes were also the source for the genome size determinations, which also support the presence of only one abundant organism. Together, these

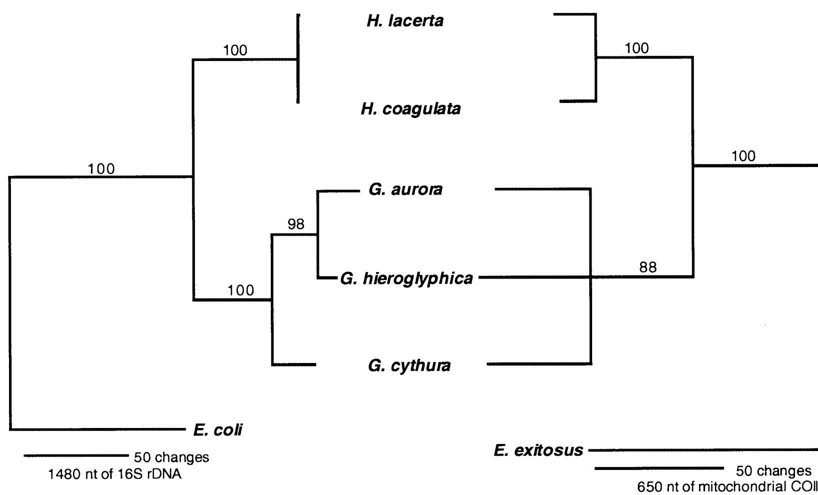

Fig. 4. Phylogenetic trees based on 16S rDNA of symbionts and on a 650-bp fragment mitochondrial cytochrome oxidase II from the corresponding host insect species.

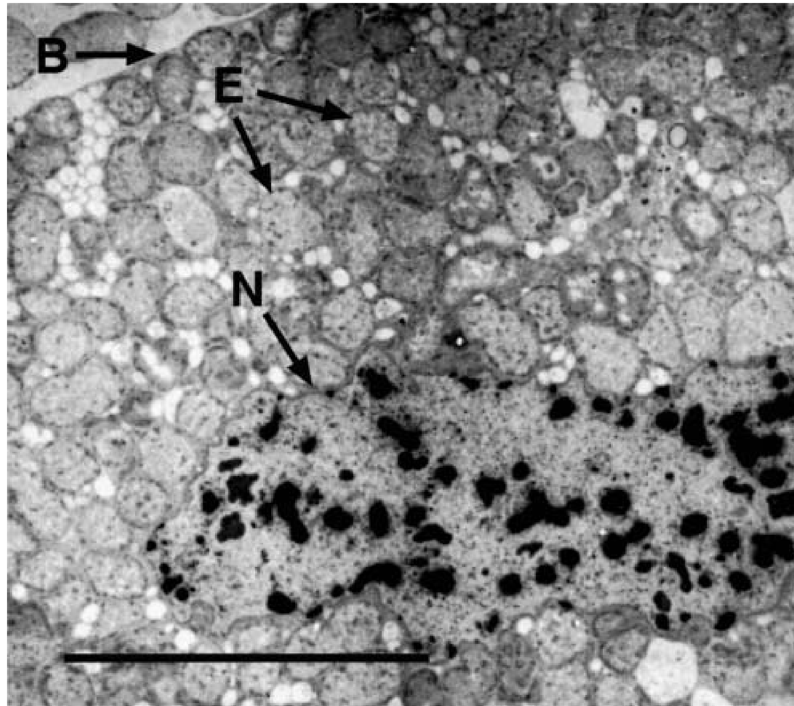

Fig. 5. Electron micrograph of a section of the red portion of the bacteriome of *H. coagulata* (see Fig. 1B), showing the irregular spheroid symbionts, designated 'Candidatus *Baumannia cicadellinicola*'. B, bacteriocyte boundary; N, nucleus of host bacteriocyte; E, endosymbiont. Scale bar = 10.

observations provide strong evidence that the organisms in Fig. 5 correspond to the reported sequences and genome size.

Wolbachia observations

Using light microscopy to examine testes squashed under a cover slip in bacteriological saline, we observed abundant rod-shaped bacteria in the testes of an adult male *H. coagulata* from Riverside. DNA extracted from the examined material and from testes dissected from other males in the same collection gave positive PCR amplifications with *Wolbachia*-specific primers. The 896 bp nucleotide sequence of one such PCR product was obtained (GenBank accession AF501664). The most closely related sequences in databases are the 16S rDNA of *W. pipientis* from other insect species, which show up to 99% identity to the new sequence.

Organism inhabiting yellow bacteriome of H. coagulata

Although commonly used 'universal' eubacterial primers consistently amplified a single rDNA sequence for both 16S and 16S–23S amplifications, we did not rule out the possibility that another bacterium could be present but not amplified. We suspected that the second symbiont might be a eubacterium with an unusual 16S rDNA sequence in the region of either 10F or 1507R. Thus, we attempted to isolate sequences using alternative primers complementary to other highly conserved regions, with template DNA extracted from red or yellow bacteriomes of *H. coagulata*. The red bacteriome yielded no alternative sequences using several combinations of primers expected to amplify eubacterial 16S and/or 23S rDNA sequences. Archaeal 16S rDNA primers also yielded no product for either bacteriome. However, amplifications from the yellow bacteriome with primers 84F and 1370R gave a product that, after cloning and sequencing, was found to correspond to a second, distinctive 16S rDNA sequence (GenBank accession # AY147399), falling within the phylum Bacterioidetes. The finding of a second sequence in the yellow bacteriome is consistent with microscopic observations of at least two bacteria-like organisms in the yellow bacteriome, one being similar in appearance to that observed in the red bacteriome (R. Rosell and H. Costa, personal communication). This sequence was reliably obtained from the yellow bacteriome in successive amplifications from over 25 individual insects. In contrast, multiple attempts to amplify this sequence from the red bacteriome were unsuccessful.

BLAST searches and phylogenetic reconstruction indicated that this second bacteriome-associated organism

falls within the Flavobacteria and that the closest relatives are *Blattabacterium* species endosymbiotic in cockroaches (Dictyoptera: Blattidae and Cryptocercidae; represented by GenBank accessions AF322471 and X75626) and Flavobacteria species endosymbiotic in ladybeetles (Coleoptera: Coccinellidae; accessions AJ009687 and Y13889). Based on its consistent presence in all individuals tested, and on its relationship to previously characterized symbionts of insects, this sequence probably represents a symbiont that is restricted to the yellow bacteriome of this host.

Discussion

Based on previous molecular phylogenetic studies, symbionts associated with animal bacteriomes sometimes reflect an ancient infection that is maintained through strict vertical transmission over millions of years (Moran and Baumann, 2000). This history is implied by the concordance between symbiont and host phylogenies, as is now documented for several associations, including aphids and *B. aphidicola* (Munson *et al*., 1991; Funk *et al*., 2000), tsetse flies (Diptera: Glossinidae: *Glossina*) and *Wigglesworthia glossinidia* (Chen *et al*., 1999), psyllids (Hemiptera: Psylloidea) and Candidatus *Carsonella ruddii* (Thao *et al*., 2000b, 2001; Spaulding and von Dohlen, 2001), carpenter ants (Hymenoptera: Formicidae: *Camponotus*) and Candidatus *Blochmannia camponotii* (Sauer *et al*., 2000) and clams (Mollusca; Vesicomyidae) and their symbionts (Peek *et al*., 1998). The evolution of these symbiotic bacteria may be accompanied by massive reduction of the genome, as evidenced by *B. aphidicola* (450–641 kb: Charles and Ishikawa, 1999; Wernegreen *et al*., 2000; Gil *et al*., 2002), *W. glossinidia* (770 kb: Akman and Aksoy, 2001), and *Wolbachia pipientis* (Sun *et al*., 2001), the only other chronic animal symbionts for which genome sizes have been resolved.

The symbionts associated with the conspicuous bilateral paired bacteriomes of the five sharpshooters that we examined form a distinctive bacterial clade, with 16S rDNA divergence from the closest relatives greater than 10%. Furthermore, this clade appears to be ancient, based on the substantial divergence (~7%) between symbionts of the sampled host species. Members of this clade show several traits in common with previously studied insect endosymbionts: as in *B. aphidicola* and *W. glossinidia*, the base composition is A + T-biased and the genome size is extremely small. Phylogenetic relationships of the species examined here (Fig. 4) are consistent with a single infection of a common ancestor of the Cicadellinae with subsequent vertical transmission and co-divergence. Similar findings and conclusions have been reported for *B. aphidicola*, *W. glossinidia* and *Blochmannia*, as discussed above. Together these represent all of the bacteriome associates of insects for which phylogenetic congruence with hosts have been assessed. These findings contrast with those for facultative associations with symbionts that live in various insect tissues, such as *Wolbachia pipientis* and the so-called ‘accessory’ or ‘secondary’ symbionts of tsetse (Dale and Maudlin, 1999), aphids (Sandström *et al*., 2001) and psyllids (Thao *et al*., 2000a).

Furthermore, this clade of sharpshooter symbionts appears to be ancient, based on the substantial divergence (~7%) between symbionts of the sampled host species (corresponding to the node at the split between symbionts of *Graphocephala* and *Homalodisca* in Fig. 3). *Buchnera aphidicola*, the bacteriome associate of aphids, shows a per lineage rate of nucleotide base substitution for 16S rDNA of about 2–4% per 100 million years (Moran *et al*., 1993). If the same rate applies to the symbionts of Cicadellinae, then the observed 7% divergence of the two lineages leading to symbionts of *Graphocephala* and *Homalodisca*. (Table 1) would imply an age of 80–175 million years for the shared ancestor. This date is consistent with the general time scale of divergence of the host insects based on the fossil record for leafhoppers. Although the fossil record for Cicadellinae is fragmentary for the late Cretaceous, most modern leafhopper genera were established between the lower Cretaceous and the early Tertiary (Dietrich and Vega, 1995).

Based on electron microscopy studies, Kaiser (1980)

Table 1. Distances among 16S rDNA sequences of Cicadellinae symbionts (‘Candidatus *Baumannia cicadellinicola*’ sp. *nov.*) and related bacteria. Values are estimated number of substitutions per 100 bases (Jukes–Cantor correction for multiple changes).

			‘Candidatus *Baumannia cicadellinicola*’ sp. nov.				
	E. coli	*Blochmannia*	(*H. coag.*)	(*H. lac.*)	(*G. Hier.*)	(*G. auro*)	(*G. cyth*)
Buchnera	11.04	15.61	15.95	15.39	14.94	14.35	15.26
E. coli	0.00	12.46	13.94	13.39	13.82	12.40	13.59
Blochmannia		0.00	10.60	10.86	12.11	10.87	12.45
Baumannia (*H.coag.*)			0.00	0.07	7.31	6.77	7.57
Baumannia (*H.lac.*)				0.00	7.10	6.92	7.29
Baumannia (*G.hier.*)					0.00	2.16	3.96
Baumannia (*G.auro.*)						0.00	4.12

reported that two morphologically distinct symbiont types were present in bacteriomes of *Graphocephala coccinea*. Following terms used by Buchner and predecessors (Buchner, 1965), these were designated the 'a-symbionts', which were irregularly spherical and about 2 μm diameter and the 't-symbionts', which were smaller, irregularly rod-shaped forms surrounded by an electron-transparent region. The symbionts upon which we have focussed correspond to the a-symbionts. Using light microscopy of crushed bacteriomes, we observed huge numbers of organisms that resemble those present in the EM sections (Fig. 5) and depict organisms that closely resemble those designated as the a-symbionts by Kaiser. She showed that, in adult *G. coccinea*, the t-symbionts are present in a separate region that is internal to the bacteriome containing the a-symbionts, which is just beneath the epidermis. The second symbiont we report, in the Flavobacteria and characterized only from the yellow bacteriome, may corresond to this t-symbiont or to another symbiont. Buchner (1965) described several combinations of symbiont types in leafhopper species, including some that he believed to be fungi. Ours is the first report on the bacteriome-associates of *Homalodisca* species. Other sap-feeding insect families often have multiple symbiont types with characteristic positions within the insect body; the function and significance of these organisms are generally not known (e.g. Fukatsu and Nikoh, 2000; Thao *et al*., 2000a; Sandström *et al*., 2001; von Dohlen *et al*., 2001). Although we have identified one characteristic symbiont within the bacteriome and a suspected second one present in at least *H. coagulata*, there is still the possibility that other symbiotic bacteria inhabit the bacteriome in low densities or other tissues in larger numbers. Specifically, we have not excluded the possibility of a fungal symbiont, although none was apparent in electron microscopy of the red bacteriomes of *H. coagulata*.

Buchner (1965) hypothesized that the a-symbiont represented an ancient infection predating the divergence of leafhopper subfamilies and of the related families Cercopidae ('spittlebugs'), Cicadidae ('cicadas'), and Membracidae ('treehoppers'). He proposed that the a-symbiont persists in many of these related insect groups, sometimes in combination with additional symbiont types. This hypothesis remains to be tested with molecular data from symbionts of these insect groups.

The primary sharpshooter symbionts are almost certainly obligate mutualists of their hosts. As in the case of *B. aphidicola* (Shigenobu *et al*., 2000), their genome may encode enzymes involved in the production of amino acids or other nutrients that are lacking or rare in the host diet. Species in the Cicadellinae feed on xylem sap, a diet that is unbalanced from the perspective of animals (Brodbeck *et al*., 1999). In light of nutritional differences between xylem and phloem, there are likely to be differences between *B. aphidicola* and the sharpshooter symbionts in terms of the set of biosynthetic pathways that have been retained. For example, *B. aphidicola* strains vary in which biosynthetic pathways are retained, and these differences correspond to differences in nutritional requirements of their respective hosts (Tamas *et al*., 2002). Potentially, knowledge of the genome content of these symbionts could be used to design specific control measures for use against pest insects such as *H. coagulata*.

Nomenclature

These symbionts are reliably found in the bacteriome structures of examined members of the Cicadellinae and have not been cultivated on laboratory media. Therefore, we propose to name this newly characterized group of bacteria using the designation 'Candidatus *Baumannia*', following the recommendation of Murray and Schleifer (1994) that non-cultivable organisms be named under 'Candidatus'. The genus is named for Paul Baumann and Linda Baumann, who were first to apply PCR, gene cloning, and DNA sequencing to characterize endosymbionts of insects (e.g. Unterman *et al*., 1989; Munson *et al*., 1991; Baumann *et al*., 1995; Baumann *et al*., 2002); these approaches have resulted in dramatic progress in our understanding of this vast but little known category of organisms. 'Candidatus *Baumannia*' contains the single species 'Candidatus *Baumannia cicadellinicola*'. The species epithet means 'lover of Cicadellinae', referring to the subfamily of leafhoppers commonly called sharpshooters in English. The bacteriome-associate from *H. coagulata* is proposed as the type strain. Because *Graphocephala* and *Homalodisca* represent divergent lineages within Cicadellinae (Dietrich *et al*., 2001) yet harbour the same symbiont type, we expect other species in this subfamily to contain closely related bacteria residing in similar bacteriome structures. These would be included as strains of the species 'Candidatus *Baumannia cicadellinicola*', and the host species should be noted in any studies of these symbionts. Among the distinctive traits of these organisms are their location within the coloured bilateral abdominal bacteriomes of the subfamily Cicadellinae (Fig. 1) as well as several distinctive 16S rDNA sequences corresponding to stem-loop structures. These include the following: 5′-AGT AGG TGA GAT AAT AGC TCA CCT AGG CAA CGA TCT CT-3′ (corresponding to *E. coli* positions 250–287) and 5′-CGA TAC AAC GCG AAA AAC CTT ACC TAC TCT TGA CAT CCA GAG TAT AAA GCA GAA AAG-3′ (*E. coli* positions 982–1038). The small genome size and the irregular spherical shape (Fig. 5) observed for the symbiont of *H. coagulata* are probably general traits of this clade.

Experimental procedures

Insect collections and identification

Insect samples were collected outdoors by the authors (N.M., C.D., W.S.) and identified by C. Dietrich (Illinois Natural History Survey). In addition to *H. coagulata*, we dissected bacteriomes and obtained symbiont sequences for *Homalodisca lacerta* (Fowler), *Graphocephala cythura* (Baker), *Graphocephala hieroglyphica* (Say), *Graphocephala aurora* (Baker). We also collected *Exitianus exitiosus* (Uhler), a member of the subfamily Deltocephaline (family Cicadellidae), for use as an outgroup in the mitochondrial analysis. Insects were collected in Tucson, Arizona except for *H. coagulata*, collected in both Riverside, California and Quincy, Florida.

Dissection for isolation of bacteriomes

The bacteriomes of *H. coagulata* are paired structures positioned laterally and just below the insect tergites within the first three abdominal segments (Buchner, 1965; Kaiser, 1980; Fig. 1). They are coloured yellow, orange or red and are supplied with tracheae; bacteriomes of *Homalodisca* adults consist of distinct yellow and orange-red portions (referred to as 'yellow' and 'red' bacteriomes) (Fig. 1B). Bacteriomes were removed by immersing the insect in saline under a dissecting microscope, slitting the cuticle and teasing out the structure using insect pins. The red and yellow portions of the bacteriomes were teased apart and separately placed into 95% ethanol until DNA was extracted. Each extraction for obtaining DNA for PCR and sequencing was performed on the bacteriome from a single insect.

DNA preparation

DNA extractions of dissected bacteriomes were performed using methods modified from Bender *et al*. (1983). An individual bacteriome was placed in a 1.7 ml microfuge tube, frozen by immersion in liquid nitrogen and crushed by grinding with a disposable pestle. Following addition of 200 μl of homogenization buffer [100 mM NaCl, 200 mM Sucrose, 100 mM Tris/HCl (pH 9.1), 50 mM EDTA, 0.5% w/v SDS, pH 9.2], the tissue was homogenized further by grinding. Tubes were centrifuged briefly to collect material and incubated at 65°C for 30 min. To precipitate proteins, potassium acetate was added to a final concentration of 1 M, and tubes were placed on ice for at least 30 min. Samples were centrifuged for 15 min at 20000 *g* and the resulting supernatants transferred to fresh tubes. The DNA was precipitated by addition of 200 μl of absolute ethanol to sample supernatants, followed by centrifugation for 15 min at 20000 *g*. After removing the ethanol, DNA pellets were washed by resuspension in 200 μl of cold 70% w/v ethanol and DNA collected by centrifugation for 5 min at 20000 *g*. To dehydrate samples, the DNA pellet was washed in 200 μl cold absolute ethanol, collected by centrifugation and dried under vacuum. Purified DNA samples were resuspended in 50 μl TE [1 mM Tris/Hcl (pH 8.0), 0.1 mM EDTA (pH 8.0)] and stored at 4°C until use.

PCR and sequencing of mitochondrial and symbiont genes

Universal eubacterial primers 10F (5′-AGT TTG ATC ATG GCT CAG ATT G-3′), a forward primer from the 5′ end of the 16S rDNA gene, and 1507R (5′-TAC CTT GTT ACG ACT TCA CCC CAG-3′), a reverse primer from the 3′ end of the 16S rDNA gene were used to amplify approximately 1500 bp of 16S rDNA, using DNA samples purified from the bacteriomes as templates. Reactions contained 2 μl of the template DNA suspension (from extraction described above), 25 pmol of each primer and 2.5 units of *Taq* DNA Polymerase (Promega, Madison, WI) with a final $MgCl_2$ concentration of 1.5 mM in a total volume of 50 μl. The PCR cycle included a denaturation step (94°C for 2 min); followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min; and a final extension of 72°C for 6 min For *H. coagulata,* DNA was extracted separately from four individuals from California and one individual from Florida. These templates were amplified individually. All PCR products were purified with the QIAquick PCR purification Kit (Qiagen) and eluted in 50 μl water, and the nucleotide sequences of purified products were determined on an ABI 377 automated sequencer. A total of 24 sequencing reads were obtained, with 10-fold coverage of the 16S rDNA fragment. No discrepancies were present among any of these reads.

Cloning and sequencing of genes from symbionts of H. coagulata

A 3048 bp fragment spanning the *rpoB* and *rpoC* genes was amplified by PCR from DNA extracted from the red portion of the bacteriomes of *H. coagulata* using degenerate oligonucleotide primers: 5′-CGC GGA TCC GGW GAA ATG GAA GTW TGG GCW-3′ and 5′-CGC CTG CAG TCG ACC WGG TTC WCC WAT WSW TTG WGC WGC-3′. The PCR reaction mixes were the same as described above for amplification of 16S rDNA except that they contained 50–100 ng of template DNA and 2.5 mM $MgCl_2$. Cycling conditions consisted of an initial denaturation step (5 min at 95°C) followed by 40 cycles of denaturation (1 min at 95°C), annealing (1 min at 55°C), and extension (3 min at 72°C), followed by a final extension (5 min at 72°C) to promote A-tailing. The PCR products were purified and cloned into pTOPO-XL (Invitrogen), following the manufacturer's instructions. Identical sequences were obtained from three recombinant clones. Internal oligonucleotide sequencing primers were designed and synthesized as sequence data became available.

A 4400 bp fragment containing the 16S-23S genes and intergenic spacer (containing tRNA-Glu) was amplified and cloned from the red bacteriome of *H. coagulata* (forward primer: 5′-GCA CTG CAG GAT CCA GAG TTT GAT CAT GGC TCA GAT TG-3′, reverse primer: 5′-GCA GGT ACC GCG GCC GCG CTC GCG TAC CAC TTT AAA TGG CG-3′, internal sequencing primers as in Thao *et al*. (2000b). Five fragments were cloned from the reaction product.

In order to check for additional symbionts within the bacteriomes, we used additional primer pairs applied to DNA templates extracted separately from both the yellow and red portions of *H. coagulata.* Using primers 84F (5′-CGG CGS AYG GGT GCG TAA C-3′) and 1370R (5′-CGT ATT CAC

CGG ATC ATG GC-3′) and reaction conditions as above except that annealing temperature was 50°C and reaction mix contained 45 mM $MgCl_2$, we amplified a partial sequence of the 16S rDNA from templates consisting of DNA extracted from the yellow bacteriome of *H. coagulata*. This was performed for more than twenty-five individuals. The resulting products consisted of two bands, one of the expected length (~1300 bp) and one shorter one (around 900 bp). The mixed product was cloned as described above, inserts were run out on agarose gels to size, and sequences were determined.

Universal insect mitochondrial primers 'mtd 13′ (5′-AAT ATG GCA GAT TAG TGC A-3′) from Liu and Beckenbach (1992) and 'mtd 18′ (5′-CCA CAA ATT TCT GAA CAT TGA CCA-3′) from Simon *et al.* (1994) were used to amplify an approximately 650 bp region of mitochondrial DNA containing a portion of cytochrome oxidase subunit II and a fragment of the adjacent tRNA gene. Polymerase chain reactions proceeded as above except that the annealing temperature was 55°C.

Genome size determination by pulsed field gel electrophoresis (PFGE)

For isolation of symbionts, collections of the host species *H. coagulata* were made in a lemon grove in Riverside, California. Red-pigmented bacteriomes were removed from 100 live adult *H. coagulata* by dissection and placed in 5 ml ice-cold isolation buffer (90 mM KCl, 55 mM $CaCl_2$, 15 mM $MgSO_4$, 25 mM NaCl, 200 mM sucrose). These dissections were performed immediately after collection, and bacteriomes were transported on ice to Tucson for subsequent isolation steps, which were carried out within 48 h of dissection.

To promote lysis of host cells and the liberation of intracellular bacteria, isolated bacteriomes were transferred to 5 ml fresh isolation buffer containing 0.5% (w/v) sodium deoxycholate. Bacteriomes were homogenized by crushing between microscope slides, and the homogenate was recovered by rinsing slides with isolation buffer. Homogenates were filtered successively through 11 μm, 8 μm and 5 μm PVDF filters (Millipore, Bedford, MA), and host cell nuclei removed from the filtrate by centrifugation at 250 *g* for 5 min at 4°C. Bacteria were collected by centrifugation at 3000 *g* for 5 min at 4°C and resuspended in 1 ml of isolation buffer. The bacterial suspension was loaded onto a continuous Percoll density gradient (20–90% Percoll, 5% PEG 6000, 1% bovine serum albumin and 1% Ficoll in isolation buffer) and centrifuged at 11 000 *g* for 20 min at 4°C. The bacterial band in these gradients was visualized under white light and removed by aspiration. The aspirate was diluted 10-fold with isolation buffer, and bacteria were collected by centrifugation at 3000 *g* for 5 min at 4°C. The pellet was resuspended in 200 μl of buffer PA [50 mM Tris-HCl (pH 7.6), 100 mM EDTA, 250 mM sucrose] to yield approximately 5×10^8 bacteria ml^{-1}.

Symbiont chromosomes were prepared for PFGE in agarose plugs for digestion by restriction enzymes. The agarose plugs were cast in plastic moulds after combining 200 μl of bacterial suspension in buffer PA with 180 μl of molten (55) 1% InCert agarose (FMC) in 0.25 M EDTA and 20 μl of 4 mg ml^{-1} lysozyme. Following solidification, agarose plugs were immersed in 20 volumes of buffer PB (1% *N*-lauroylsarcosine, 0.2 mg ml^{-1} proteinase K in 0.5 M EDTA) for 48 h, refreshing buffer PB after 24 h. Agarose plugs were washed three times for 2 h at room temperature in 20 volumes of 50 mM EDTA. Plugs were stored at 4°C in 10 volumes of 50 mM EDTA.

For restriction digests, we selected enzymes with recognition sites that cut AT-rich DNA infrequently (*Apa*I, *Bss*HII, *Nar*I and *Sst*II). Agarose plugs were sliced into 20 μl portions and equilibrated in 20 volumes of TE at room temperature for 3 h. Agarose plugs were then equilibrated overnight in 500 μl of 1 ×_restriction buffer. Restriction buffers were replaced, and plugs were digested according to the enzyme supplier's recommendations (New England Biolabs). The PFGE was carried out using a CHEF DR-II unit (Bio-Rad) through a 1% agarose gel at 200 V, 14°C, with a linear switch time ramp (5–40 s) for 26 h.

Phylogenetic analyses

We used PAUP* version 4.0b10 (PPC) (Swofford, 1998) to reconstruct evolutionary relationships of the symbionts present in the red bacteriomes of the five host species based on the 16S rDNA sequences. Following initial BLAST searches to determine the general group of bacteria to which the symbionts belonged, we selected representative bacteria from the γ-3 subdivision of the Proteobacteria to include in the phylogeny. These included the following species (GenBank accession #): *E. coli* (NC_000913), *Salmonella enterica* (NC_003197), *Serratia marcescens* (AF286873), *Yersinia pestis* (NC_003143), *Klebsiella pneumoniae* (AF453251), *Proteus vulgaris* (AJ301683), *Vibrio cholerae* (NC_002505), *Xylella fastidiosa* (NC_002488), *Pseudomonas aerugninosa* (as outgroup, NC_002516), and the symbionts Candidatus *Blochmannia camponotii* (X92552), Candidatus *Carsonella ruddii* (AF211143), the 'gamma symbiont' of *Antonina crawii* (AB030020), *Buchnera aphidicola* (NC_002528), and the 'primary symbiont of *Sitophilus oryzae*' (AF005235). We aligned sequences using Pileup of GCG (Genetics Computer Group, 2001). Phylogenetic analyses were carried out using Maximum Parsimony and Distance (with Tamura-Nei distances) options in PAUP*, and 1000 bootstrap replicates were run for both methods.

Additional analyses were performed on the sequence obtained from the yellow bacteriome of *H. coagulata* in order to determine its relationship to previously characterized organisms. BLAST searches indicated relationship to symbiotic members of the Flavobacteria within the eubacteria. Using Pileup, an alignment was made containing the new sequence and representative sequences from the closest BLAST hits plus outgroups within the same group of bacteria (Bacteroidetes). Accession numbers of sequences used in the phylogenetic analysis were: AB050106, AB078047, AF133536, AF150713, U41346, AF289153, U87106, AF322471, X75626, AF368756, L16493, Y13889, AJ009687, L16498.

Microscopy

Red-pigmented bacteriomes were dissected from the same Riverside samples of *H. coagulata* used for DNA sequence studies. Procedures and equipment used for fixing, sectioning, viewing and photographing were the same as those

described in Thao *et al*. (2000b) for the bacteriome-associated symbionts of psyllids. These procedures were carried out at the University of Arizona Imaging Facility.

Wolbachia observations

During dissections, we examined the testes of adult male *H. coagulata*. Using light microscopy, we observed rod-shaped bacteria intermixed with the sperm. We removed the testes from one male, extracted DNA as above, and amplified a fragment of approximately 900 bp, using primers designed for diagnostic amplification of 16S rDNA from *Wolbachia pipientis*. Both the primer pair and the reaction conditions were the same as those described by O'Neill *et al*. (1992) for diagnostic PCR of *W. pipientis* 16S rDNA.

Genbank accession numbers

GenBank accession numbers are as follows AF489427 (16S-23S) and AY075156 (*rpoBC*) for *H. coagulata*, AF465794-7 for 16S rDNA genes of the other symbionts inhabiting the red bacteriomes of other Cicadellinae, AY147399 for the 16S rDNA of the organism obtained from the yellow bacteriome of *H. coagulata*, and AF456139-44 for mitochondrial COII of the insect hosts.

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