Biology of Bacteriocyte-Associated Endosymbionts of Plant Sap-Sucking Insects

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Key Words

Buchnera, *Carsonella*, *Portiera*, *Trembalya*, endosymbiotic associations

Abstract

Psyllids, whiteflies, aphids, and mealybugs are members of the suborder Sternorrhyncha and share a common property, namely the utilization of plant sap as their food source. Each of these insect groups has an obligatory association with a different prokaryotic endosymbiont, and the association is the result of a single infection followed by maternal, vertical transmission of the endosymbionts. The result of this association is the domestication of the free-living bacterium to serve the purposes of the host, namely the synthesis of essential amino acids. This domestication is probably in all cases accompanied by a major reduction in genome size. The different properties of the genomes and fragments of the genomes of these endosymbionts suggest that there are different constraints on the permissible evolutionary changes that are probably a function of the gene repertoire of the endosymbiont ancestor and the gene losses that occurred during the reduction of genome size.

Pel piacer di porle in lista.
Leporello
Because Annushka has already bought the sunflower oil, and not only bought it, but spilled it too.
Master and Margarita

Contents

INTRODUCTION

P: primary

S: secondary

Associations between insects and intracellular bacteria are quite common in nature. It is estimated that as many as 15% of all insect species harbor such endosymbionts (39). Early work on such associations involved a natural history approach to the insect host coupled with histological studies. In 1965 Paul Buchner (21) published a massive treatise, part of which summarized these studies. Buchner noted an association between a restricted diet and the presence of endosymbionts in insects and suggested that the function of the endosymbionts was the synthesis of missing nutrients for the host. Buchner recognized the presence of one morphological type within all (or most) of the insects of a taxonomic group, which was designated the P-endosymbiont. This attribute suggested that the association was a result of a single ancient infection of an insect and that the P-endosymbiont was essential for the survival of the host. Buchner also recognized the presence of other morphologically diverse bacteria in insect cells, usually called the S-endosymbionts, but also sometimes called facultative endosymbionts, guest bacteria, or accessory bacteria [here designated S-endosymbionts if their location is clearly established or S-symbionts if their location is not always certain (102)]. Because there are a variety of morphological types of S-symbionts and because some insects have the P-endosymbiont but lack the S-symbiont(s), it was suggested that these organisms do not perform an essential function required for the survival of the host. These conclusions have been recently validated by the application of molecular genetic methods to the study of insect endosymbionts. Since 1989, when studies were initiated on the evolutionary relationships of insect endosymbionts (89, 138), followed by initiation of sequence analysis of P-endosymbiont DNA (64), calibration of the rate of sequence change (84), and a review on the molecular biology of the endosymbionts (11), this field has attracted an increasing number of investigators (12, 17, 81, 146, and references therein). Recently, a great deal of new information has been added by the DNA sequence determination of several endosymbiont genomes (112, 124, 140).

Symbiotic associations span a spectrum of types that differ with respect to the effect of the symbiont on the host (mutualism, commensalism, parasitism) (74, 116, 152). This review focuses primarily on the association of plant sap-sucking insects and their P-endosymbionts. The characteristic of these associations is the phylogenetic congruence between the P-endosymbiont and the host. This is illustrated in **Figure 1**, in which the phylogenetic tree based on mealybug *18S*–28S* ribosomal DNA* (*rDNA*, asterisks are used to designate host mitochondrial or nuclear *rDNA*) is similar to that based on P-endosymbiont (*Tremblaya*) *16S-23S rDNA*. These results have been interpreted as indicative of a single ancient infection of an insect ancestor followed by cospeciation (or

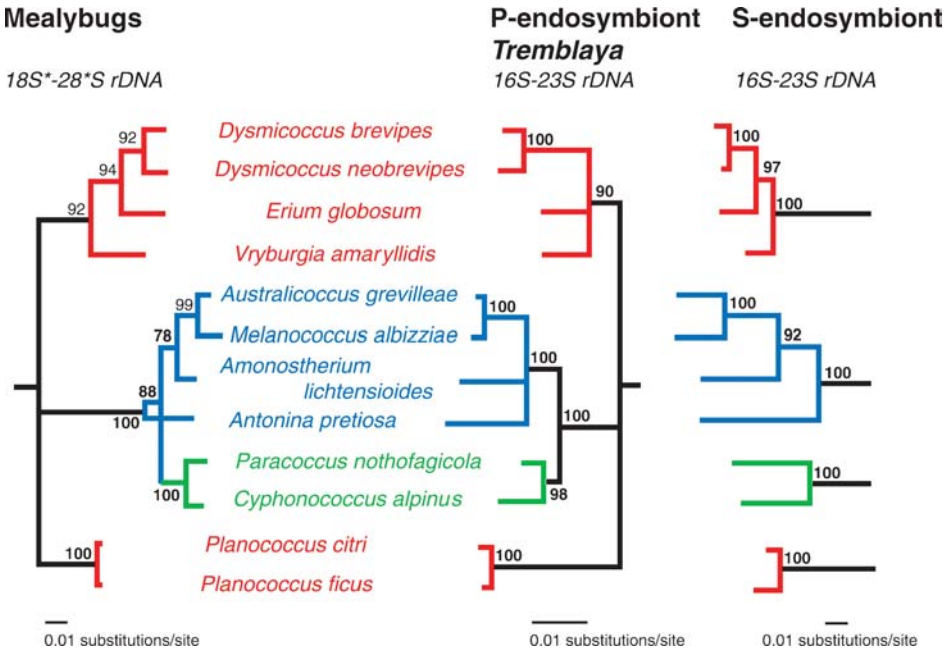

Figure 1

Comparisons of the phylogeny of mealybugs with *Tremblaya* (P-endosymbionts) and the *Tremblaya*-contained S-endosymbionts. *Tremblaya* is monophyletic, suggesting a single infection of a mealybug ancestor followed by cospeciation with the insect host. The S-endosymbionts are polyphyletic, the different clusters are more closely related to a variety of sternorrhynchal insect S-endosymbionts than to each other. Maximum likelihood analysis, numbers at nodes represent % bootstrap values after 500 replicates; only nodes supported by 70% or greater are shown. Host sequences are from Reference 40; *Tremblaya* and S-endosymbiont sequences are from Reference 134.

cocladogenesis) of the insect and the host. This is consistent with the maternal transmission of the endosymbionts (established by histological studies) and the lack of exchange of endosymbionts between insects (vertical evolution). An additional property of these associations is the location of the P-endosymbiont in specialized host cells (bacteriocytes) that may constitute a larger structure called a bacteriome. The associations considered in this review involve insects that use a diet deficient in essential nutrients, and there is evidence that the P-endosymbionts upgrade the diet by synthesizing the missing essential nutrients (39). These associations would consequently appear to be mutualistic. Although the advantage for the host is readily perceived, the advantage for the endosymbiont is frequently not evident. It is perhaps more meaningful to regard this association as a domestication of the bacterium by the host for the provision of essential nutrients absent in its diet (116, 152).

In contrast to this relatively well-defined association between the P-endosymbionts and insects, the association between S-symbionts and insects is currently not amenable to a simple definition, since these organisms form a heterogeneous group with respect to location in the insect and possibly in their function. As in the case of the P-endosymbionts, the S-symbionts are also maternally inherited. A property of the associations of the host and the S-symbionts is that phylogenetic trees based on S-symbiont genes and host genes show no similarity. This is illustrated in **Figure 2**, in which the phylogeny of whiteflies, based on mitochondrial genes and cospeciating P-endosymbionts, is compared with the

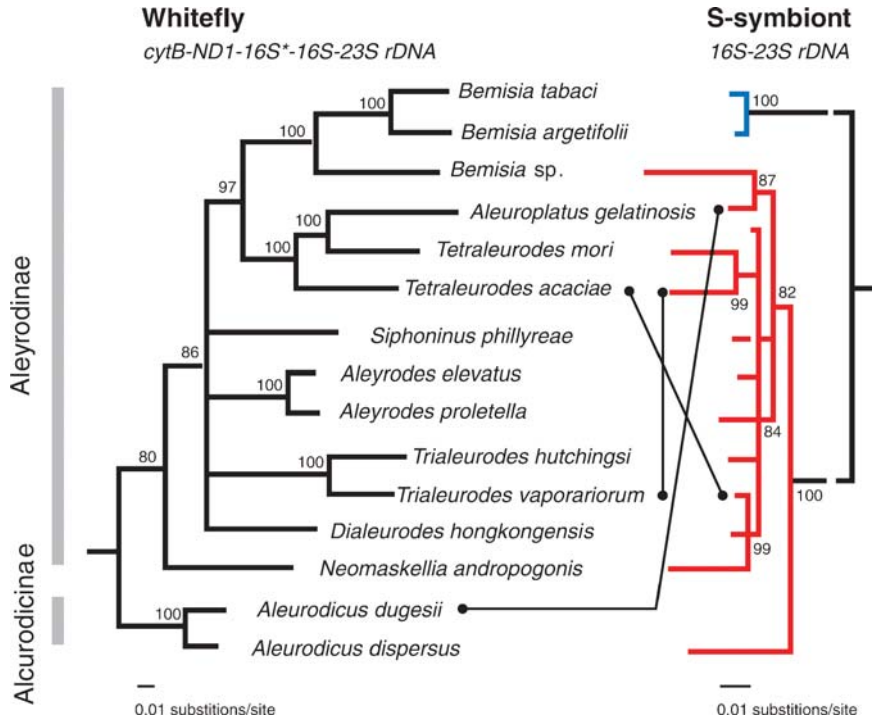

Figure 2

Comparisons of the phylogeny of whiteflies and their S-symbionts. The whitefly tree is based on composite sequences from their mitochondria (*cytB-ND1-16S* rDNA*) and cospeciating *Portiera* (P-endosymbiont, *16S-23S rDNA*). Red lines represent *Arsenophonus*-like S-endosymbionts; blue lines, T-type symbionts. S-endosymbionts that could not be placed adjacent to their hosts are joined to their host by lines. Maximum likelihood analysis, numbers at nodes are % bootstrap values after 500 replicates; only nodes supported by 70% or greater are shown. Data are from References 130 and 131 (includes a correction).

phylogeny of the S-symbionts. These results have been interpreted to indicate multiple infections and/or horizontal transmission of the S-symbionts (a partial exception in the case of mealybugs is discussed below).

In this review I attempt to provide an update of the current status of our knowledge on the P-endosymbionts of plant sap-sucking insects. Because many aspects of the genomics of *Buchnera* and related insect endosymbionts have been extensively reviewed, I consider this topic only briefly and concentrate primarily on the diversity of the P-endosymbionts. (An extensive review of bacteriocyte-associated insect endosymbionts, together with citations to the earlier literature, is found in Reference 12. For additional recent reviews see References 39, 57, 68, 81, 142, 146, and 156). Other P-endosymbionts of insects with different nutritional needs that have also been extensively studied are *Wigglesworthia* (tsetse fly) (3), *Blochmannia* (carpenter ants) (110, 111), *Blattabacterium* (cockroaches, termites) (72), and SOPE (weevil) (56). The horizontally transmitted pathogen *Wolbachia*, which causes a variety of reproductive disorders of insects, is outside the scope of this review (120, 152). Some members of this genus may, however, enter into an endosymbiotic association with nematodes that has some resemblance to the associations considered in this review. The endosymbionts become essential for the survival of the nematode, and phylogenetic trees based on host and endosymbiont properties are

congruent (37). It should be kept in mind that because the P-endosymbionts considered here have not been cultured on laboratory media, most of the conclusions concerning these organisms are derived from nucleotide sequence data, which places major constraints on interpretations of functional properties.

P-ENDOSYMBIONTS OF PLANT SAP-SUCKERS

Overview of the P-Endosymbionts and their Hosts

Table 1 summarizes some of the properties of the P-endosymbionts considered in this review together with some attributes of their hosts. All these insects belong to the order Hemiptera. The evolutionary relationships of species representative of three superfamilies (psyllids, whiteflies, and aphids) and one family (mealybugs) within the fourth superfamily of the suborder Sternorrhyncha are presented in **Figure 3**. The host phylogeny is based on mitochondrial genes (*cytB-ND1-16S*rDNA*) and host *18S* rDNA*. Due to the rapid rate of sequence change, mitochondrial genes were not able to resolve the order of branching between the major insect groups, such that for this purpose the results of the nuclear *18S* rDNA* phylogeny are included in **Figure 3** (22, 144). The relationships of the P-endosymbionts to each other, to related P-endosymbionts of other insects, and to related free-living bacteria are also presented in **Figure 3**. These studies are based on endosymbiont *16S-23S rDNA*. As previously mentioned, there is general agreement between the phylogeny of each insect group and its constituent P-endosymbionts. The lengths of the P-endosymbiont branches are somewhat longer than those of their free-living relatives, a finding consistent with the accelerated rate of P-endosymbiont sequence change relative to free-living organisms (80, 84).

In our studies of the evolutionary relationships of insect hosts based on mitochondrial DNA fragments containing *cytB*, *ND1*, and *16S* rDNA*, we found considerable variation in the G + C content of the DNA (125, 127, 131). The moles % G + C content of this mitochondrial DNA fragment from whiteflies, psyllids, and aphids was 17 to 31 moles % (**Table 1**). The lowest G + C content was 12 to 13 moles % and was found in mealybugs. Within each insect group the maximal divergence in the sequence of the nucleic acids also varied considerably (**Table 1**). Because the approximate date of the establishment of the endosymbiontic associations is roughly the same in each insect group and because in our studies we probably have encompassed the species diversity within each group, the internal mitochondrial sequence divergence is a reasonable indication of the relative rate of sequence change. From **Table 1** and **Figure 3** it can be seen that the most rapid rate of mitochondrial sequence change occurred in whiteflies and the slowest in aphids. Within the P-endosymbionts (**Table 1**), the maximal *16S-23S rDNA* divergence is 8% to 10%. This is considerably greater than what is generally considered the divergence characteristic of a bacterial species (3%) (12, 119).

The members of the Sternorrhyncha share a number of common nutritional and structural properties because they are usually plant phloem feeders (55, 99). Feeding is accomplished by means of needle-like stylets that probe plant tissues between plant cells until they enter the phloem-sieve elements. Due to this mode of nutrient uptake, some species are of major agricultural importance in that they vector plant pathogens. In high numbers they may also cause plant debilitation due to excessive nutrient consumption, leaf curling, and gall formation (12, 55). Because phloem constitutes an unbalanced diet having a high content of carbohydrates relative to free amino acids and is deficient in essential amino acids, the excess carbohydrate is excreted by the insects in the form of honeydew (39, 106). The phloem composition of the host plants used by these insects is probably similar, and therefore it is probable that in all of these organisms the principal function of the P-endosymbionts is

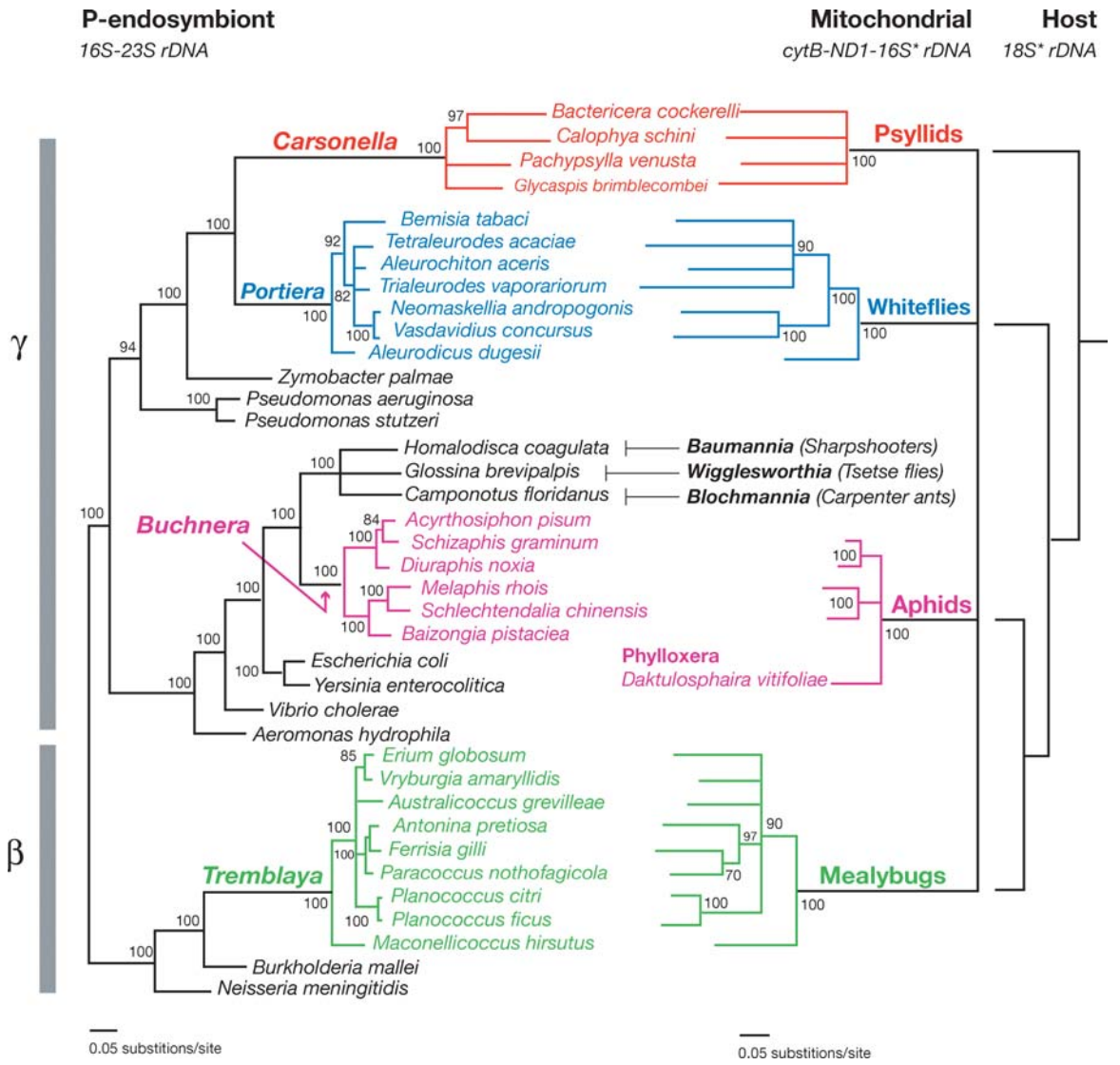

Figure 3

Comparisons of the evolutionary relationships of the P-endosymbionts of plant sap-sucking insects to the phylogeny of their hosts as well as to other related P-endosymbionts and free-living bacteria. Greek letters refer to proteobacterial subdivisions. Bacterial phylogeny is based on *16S-23S rDNA*; host phylogeny of plant sap-sucking insects is based on mitochondrial *cytB-ND1-16S* rDNA*. The maximum likelihood method was used; numbers at nodes represent % bootstrap values after 500 replicates; only nodes supported by 70% or greater are shown. References to host mitochondrial data and P-endosymbionts are given in text. The relationships among psyllids, whiteflies, aphids, and mealybugs could not be resolved on the basis of mitochondrial genes because of their rapid rate of sequence change. These relationships were established on the basis of host *18S* rDNA* (22, 144).

Table 1 Some properties of the P-endosymbionts and their host mitochondria considered in this review[a]

Endosymbiont genus	*Carsonella*[g]	*Portiera*[g]	*Buchnera*	*Tremblaya*[g]	*Baumannia*[g]
Endosymbiont species	*rudii*	*aleyrodidarum*	*aphidicola*	*princeps*	*cicadellinicola*
Insect host (common name)	Psyllids	Whiteflies	Aphids	Mealybugs	Sharpshooters
Suborder	Sternorrhyncha	Sternorrhyncha	Sternorrhyncha	Sternorrhyncha	Auchenorrhyncha
Superfamily	Psylloidea	Aleyrodoidea	Aphidoidea	Coccoidea	Cicadoidae
Family				Pseudococcidae	Cicadellidae
Principal host food source	Phloem sap	Phloem sap	Phloem sap	Phloem sap	Xylem sap
Maximal mito-DNA diference (%)[b]	31	35	14	23	
Moles % G + C content of mito-DNA[b]	25–31	17–30	18–20	12–13	28
P-endosymbiont morphology[c]	P	P	S	S	S
Gram-negative cell wall[d]	+	–	+	+	+
Age of the association (mya)	100–250	100–200[h]	115–230[h]	100–200[h]	90–180[h]
Proteobacterial subgroup	γ	γ	γ	β	γ
Free-living relative[e]	Pa	Pa	Ec	Bm	Ec
Genome sequenced	–	–	+	–	–
Genome size (kb)	ND	ND	450–641	ND[i]	678
Size of analyzed DNA (kb)	37	33	Genome	64	ND
Moles % G + C content of DNA	20	30	25–26	57	ND
% coding	>99	71	83–88	82	ND
% G + C of intergenic spaces	–	24	18–19	57	ND
Protein coding genes	32	33	504–545	37	ND
Pseudogenes	0	0	9–38	0	ND
% G + C of *16S-23S rDNA*	33–36	45–47	46–49	56–58	46
Maximal *16S-23S rDNA* difference (%)	10.1	8.4	9.3	8.0	
Order of rRNA genes 16S-23S-5S	+	+	–[j]	+	+
tRNA between *16S* and *23S rDNA*	–	+	–	–	+
Number of rRNA operons	1	1	1	2	ND
16S rDNA 3′-end contains Shine-Dalgarno complement	–	+	+	+	+
Essentially no intergenic space	+	–	–	–	ND
Translational coupling the norm[f]	+	–	–	–	ND

(*Continued*)

Table 1 (*Continued*)

Increased A + T content in poorly conserved genes	3+	+	+	–	ND
Some have plasmids	ND	ND	+	ND	ND

[a] For references see text.
[b] Based on a 1.7-kb mitochondrial DNA fragment containing partial *cytB-ND1-16S*rDNA*.
[c] P, pleiomorphic; S, spherical.
[d] +, outer and cytoplasmic membranes; –, only cytoplasmic membrane.
[e] Pa, *Pseudomonas aeruginosa*; Ec, *Escherichia coli*; Bm, *Burkholderia mallei*.
[f] Suggested from large number of gene pairs in which the initiation codon and the stop codon overlap.
[g] *Candidatus* status.
[h] Based on deepest divergence within clade and a rate of 2% to 4% sequence change per 100 million years (93).
[i] ND, not determined.
[j] In *Buchnera* the 16S and 23S-5S rRNA genes are on different transcription units.

the synthesis of essential amino acids, which are required by the host (12, 39). Thus psyllids, whiteflies, aphids, and mealybugs have four different P-endosymbionts, which represent the infection of the four different insect hosts each with a different bacterium that became domesticated for the same or similar purpose.

Figure 3 contains a cluster consisting of the P-endosymbionts of aphids (*Buchnera*), sharpshooters (*Baumannia*), carpenter ants (*Blochmannia*), and tsetse flies (*Wigglesworthia*) (3, 82, 111). This cluster is supported by a phylogenetic analysis involving many additional genes (53, 70). Consequently, a single unknown (unculturable?) free-living bacterial type may have had a predisposition for entering into endosymbiotic associations and may have infected the ancestor of aphids, sharpshooters, carpenter ants, and tsetse flies, insects that belong to three different orders. The G + C content of the *rDNAs* of these endosymbionts is somewhat low and the possibility has been suggested that this cluster represents sequence convergence of *16S-23S rDNA*. This interpretation appears to be excluded by the fact that the omission of high AT codons from the phylogenetic analyses of protein encoding genes still results in the clustering of these endosymbionts (70). The nutrient source of sharpshooters is xylem sap, which is nutrient poor and differs in composition from phloem (the nutrient source of aphids) (20, 106). The nutrient source of tsetse flies is vertebrate blood (deficient in B-complex vitamins) (3), and carpenter ants have a varied diet (110, 111). Consequently, it is possible that from the ancestor of this endosymbiont cluster emerged four types of P-endosymbionts, each of which, depending on the host, became domesticated for different purposes.

Psyllids-*Carsonella*

Host properties and endosymbiont location. There are about 2500 described species of psyllids, also commonly known as jumping plant lice because of the ability of adults to jump backwards when disturbed (55). Psyllids feed by sucking sap, mostly of woody dicotyledonous plants. The adults are generally small, ranging from 0.2 to 8.0 mm in length. All psyllids have obligate sexual reproduction (**Figure 4*a***). They develop from eggs and as nymphs are relatively sessile (i.e., attached to plants while feeding). As nymphs they have five instars; the adults are winged and relatively active. Within psyllids is a bacteriome that has some structural variability (21) (**Figure 4*c*,*d***). The most common bacteriome described consists of a large multinucleate syncytium within which are uninucleate bacteriocytes (21). The latter contain a polymorphic bacterium called *Carsonella ruddii* (135) (**Figure 5**). This organism has a

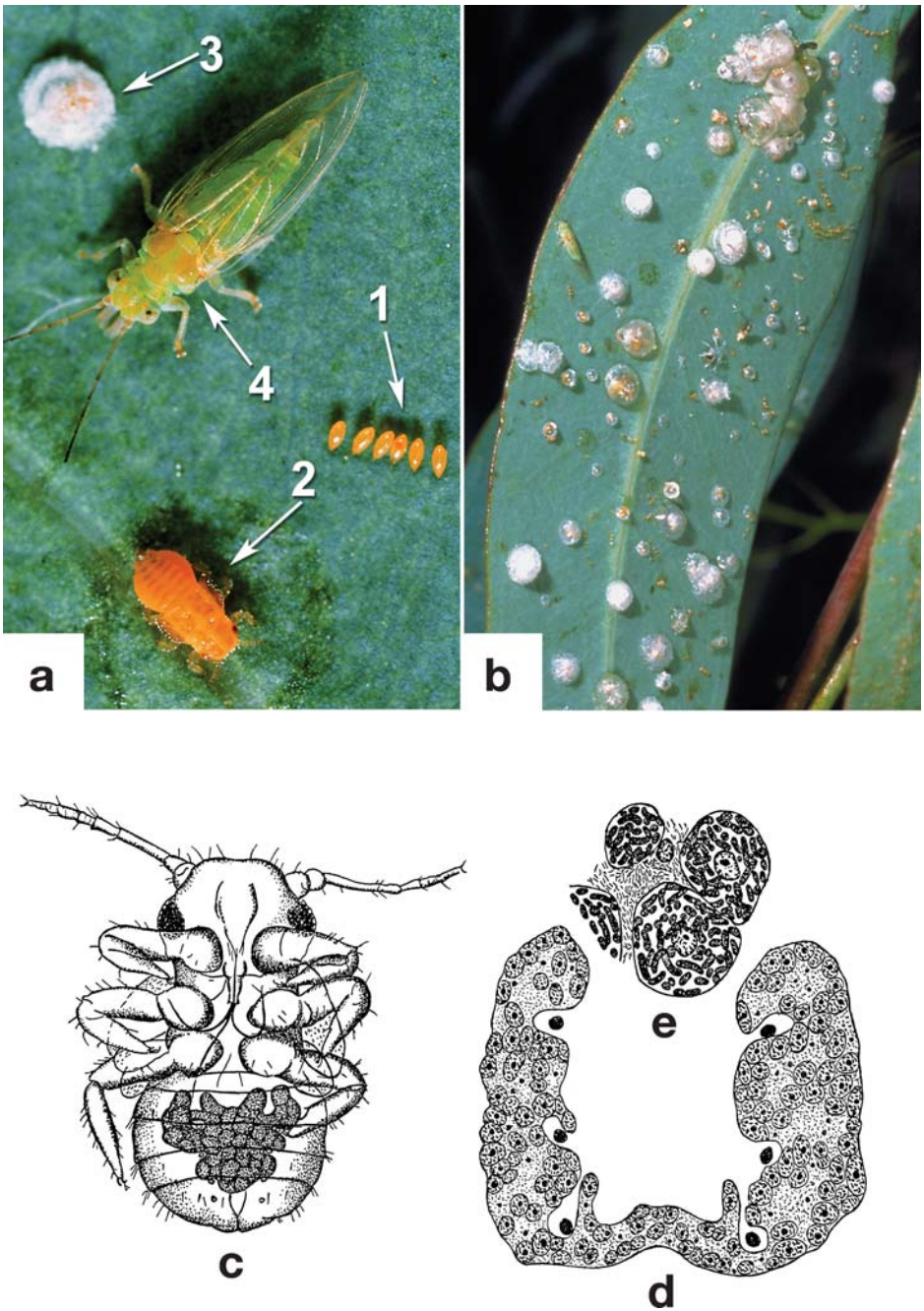

Figure 4

Morphological properties of psyllids and their endosymbionts. (*a*) *Glycaspis brimblecombei* eggs (1), nymph (2), lerp (3, tent-like structure harboring nymph), and adult (4). (*b*) Eucalyptus leaf showing infestation with *G. brimblecombei*. (*c*) Nymph showing location of the bacteriome in the body cavity. (*d*) Syncytial bacteriome containing bacteriocytes. (*e*) Bacteriocytes containing *Carsonella* within the syncytial bacteriome containing rod-shaped S-endosymbionts. Panels *a* and *b* used with permission of John Clark and The Regents of the University of California. Panels *c–e* are from Reference 21, reprinted with permission of Birkhäuser-Verlag AG.

gram-negative cell wall and is enclosed within host-derived vesicles (135). The syncytial region of the bacteriome may also harbor a morphologically distinct S-endosymbiont (21) (**Figure 4*e***). Using oligonucleotide probes specific to the *16S rDNA* of *Carsonella* and the S-endosymbiont, it has been shown, in two psyllid species, that *Carsonella* is present in the bacteriocyte while the S-endosymbiont is present in the syncytial region (45, 121).

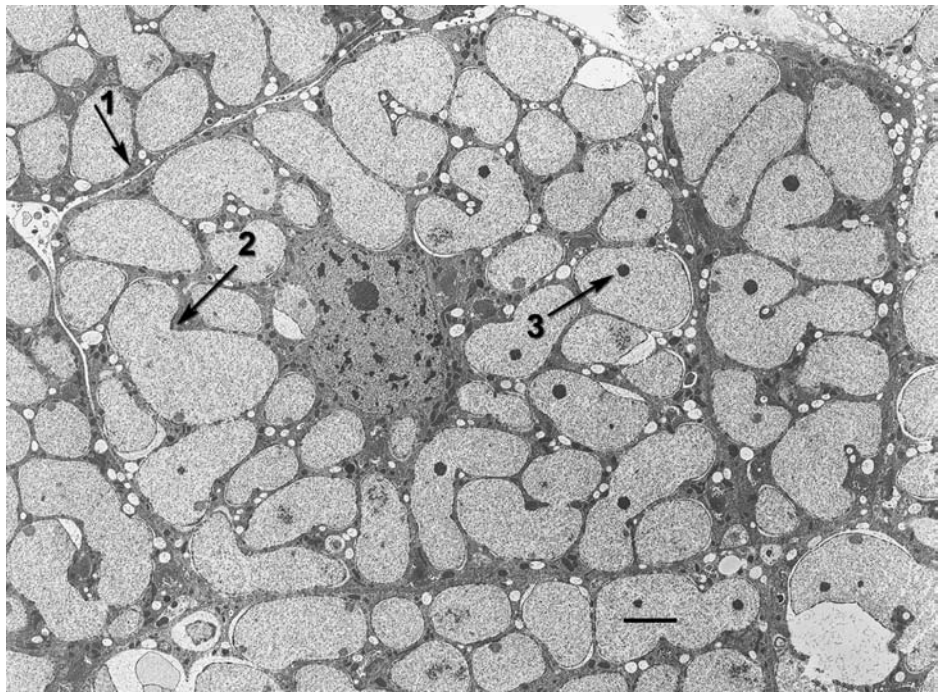

Figure 5

Transmission electron micrograph of a bacteriocyte from the psyllid *Pachypsylla venusta*. (1) bacteriocyte, (2) *Carsonella*, (3) unidentified electron dense aggregate. Bar is 2 μm. Figure is reprinted from Reference 135, with permission from the American Society for Microbiology.

Carsonella is maternally transmitted through the eggs (21).

Phylogeny. The initial studies of the *16S rDNA* of the P-endosymbionts of psyllids (*Carsonella*), which involved only a few species, established that they were a distinct monophyletic group (45, 117). These organisms appeared to be related to the P-endosymbionts of whiteflies (*Portiera*) and to *Zymobacter palmae*, a bacterium isolated from palm sap that is related to members of the genus *Pseudomonas* (**Figure 3**). In these studies it was also noted that *Carsonella* had the lowest moles % G + C content of any *16S rDNA* of the domain Bacteria. Subsequently, these studies were extended to a characterization of the *16S-23S rDNA* of *Carsonella* from 32 species of psyllids (135). A phylogenetic analysis of the data indicated good agreement with the assignments of species into subfamilies on the basis of morphology. Comparisons of the *atpAGD* genes of *Carsonella* from 31 psyllid species indicated good agreement with the phylogeny derived from the *16S-23S rDNA* (133). Similarly, congruence was observed with the tree derived from the P-endosymbiont *rpoBC* of 15 psyllid species (133). Comparisons of the phylogeny based on *Carsonella 16S-23S rDNA* and the host "wingless" gene also did not indicate any discrepancies (135). Currently this is the largest data set that indicates congruence between endosymbionts and their insect hosts, an observation supporting the monophyletic origin of *Carsonella* and subsequent cospeciation of the host and the endosymbiont (vertical evolution). The corollary of this conclusion is that that *Carsonella* is not exchanged between psyllid species.

DNA analysis. Nucleotide sequence determination of three DNA fragments from

Carsonella (37 kb in total) indicated that this P-endosymbiont has some unique genetic properties that distinguish it from other characterized P-endosymbionts (**Table 1**) as well as other prokaryotes (27). The DNA of *Carsonella* has a G + C content of 20 moles %, the lowest of any known prokaryote. In addition, *Carsonella* essentially lacks intergenic spaces; most of the adjacent genes overlap using the sequence ATGA, in which the last triplet (underline) is the stop codon of the upstream gene and the first triplet (overline) is the start codon of the downstream gene. These observations suggest that long mRNAs are made and translational coupling occurs (27). The latter is consistent with the finding that *Carsonella* is unique among prokaryotes in that it lacks the complement of the ribosome binding site (Shine-Dalgarno sequence) at the 3′ end of *16S rDNA* (27). An additional attribute of *Carsonella* is operon fusion. Ribosomal protein operons that are separate in various Bacteria appear to be fused in *Carsonella*, resulting in a single transcriptional unit. In *Carsonella* there is also a strong correlation between the moles % G + C content of genes and the extent of protein sequence conservation; the greater the amino acid sequence conservation of the protein the higher the G + C content of the gene (27) (**Figure 6**). *Carsonella* also has proteins that on the average are 9% shorter than homologous proteins from related organisms (27). The nucleotide sequence of the *Carsonella* genome is currently being determined (N.A. Moran, personal communication).

Whiteflies-*Portiera*

Host properties and endosymbiont location. The whiteflies get their name because they usually make a powdery, white waxy secretion spread over the body and wings of the adult (55). There are approximately 1450 species of whiteflies (superfamily Aleyrodoidea) that are divided into two subfamilies, Aleyrodinae (to which most of the species belong) and Aleurodicinae (55). Reproduction of whiteflies is usually sexual: Following emergence from the egg, the first instar (crawler) is capable of movement, whereas the second, third, and fourth instars are sessile. By convention the fourth instar is called the pupa, and its morphology is generally used as the basis of whitefly classification. The pupa are 0.5 to 2.0 mm in length. There are problems associated with classifying species by their pupal stage, since pupal appearance may be influenced by environmental factors. Winged adults emerge from the pupa and reproduce. Some species of whiteflies, such as *Bemisia tabaci* and *Aleurodicus dugesii*, are major agricultural pests that cause plant debilitation and transmit plant viruses (55).

All whiteflies have a relatively small, paired, and roundish or oval bacteriome that is usually orange in color (21). Studies using the electron microscope have indicated that whitefly bacteriocytes contain a pleiomorphic bacterium called *Portiera aleyrodidarum* within host-derived vesicles (131). Unlike other described P-endosymbionts, this organism lacks the outer membrane of the gram-negative cell wall (32, 33, 122). Bacteriocytes may contain

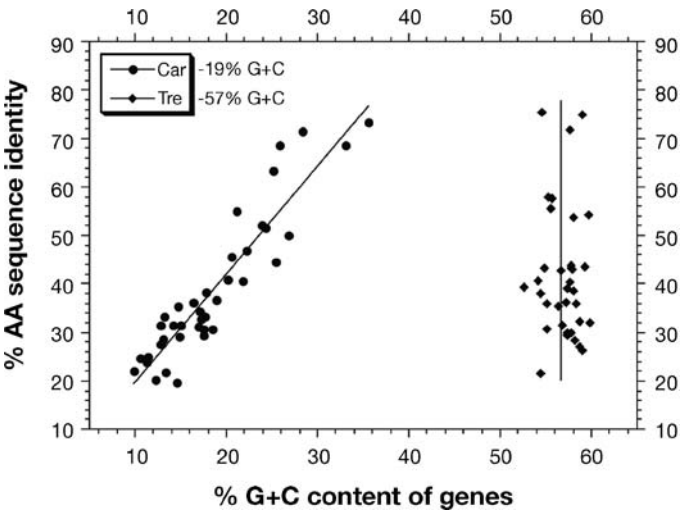

Figure 6

Comparisons of the amino acid (AA) sequence identity of homologous proteins of *Carsonella* (Car) and *Escherichia coli* (γ-subdivision) and *Tremblaya* (Tre) and *Neisseria meningitidis* (β-subdivision) with the moles % G + C content of the P-endosymbiont genes. Redrawn from Reference 9.

additional morphologically distinct bacteria such as S-endosymbionts and chlamydia (32, 33, 41, 122, 129). Whiteflies have a unique method of transmitting endosymbionts to progeny. In other members of the Sternorrhyncha the endosymbionts leave the bacteriocytes and enter the germ cells, in whiteflies the intact bacteriocytes migrate to the ovaries and enter the eggs (21, 31).

Phylogeny. Initially, studies on the phylogeny of *Portiera*, using a few whitefly species and *16S rDNA*, have indicated that these P-endosymbionts constitute a lineage distinct from that of other P-endosymbionts (26) (**Figure 3**). *Portiera* appears to be related to *Carsonella* and *Z. palmae* (94). *Z. palmae* was isolated from palm sap, raising the possibility that *Portiera* was derived from a relative of this organism. Recently a more extensive study was performed using *16S-23S rDNA* from 22 whitefly species, and the phylogeny was compared with that of the mitochondrial *cytB-ND1-16S* rDNA* of the hosts (131). The resulting phylogenies are similar, consistent with a single infection of the host and its cospeciation with the P-endosymbiont. The results from both host and P-endosymbiont genes support the separation of the whiteflies into two subfamilies: Aleurodicinae (containing the species *A. dugesii* and *A. dipersus*) and Aleyrodinae (containing the remaining species) (55, 131) (**Figure 2**).

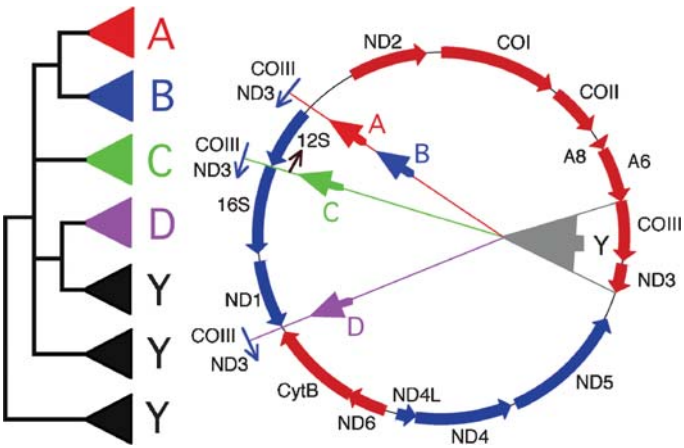

Figure 7

Relation of the whitefly mitochondrial gene arrangement type to the phylogeny of whiteflies. The whitefly tree is based on composite sequences from their mitochondria (*cytB-ND1-16S* rDNA*) and cospeciating *Portiera* (P-endosymbiont, *16S-23S rDNA*). (*Left*) A, B, C, and D indicate transposition types, and Y indicates the ancestral mitochondrial gene arrangement. (*Right*) Arrowhead Y (*gray*) in mitochondrial genome indicates the original position of the transposed genes. Arrowheads A, B, C, and D (red, blue, green, and purple, respectively) indicate the position of the insertion of the genes. Arrows outside the circle indicate the direction of the transcription of the transposed genes. Black arrow adjacent to arrowhead C indicates the changed direction of transcription of the *12S rDNA.* tRNAs have been omitted. A6 (ATP synthase, subunit 6), A8 (ATP synthase, subunit 8), COI (cytochrome oxidase, subunit I), COII (cytochrome oxidase, subunit II), COIII (cytochrome oxidase, subunit III), ND1 (NADH dehydrogenase, subunit 1), ND2 (NADH dehydrogenase, subunit 2), ND3 (NADH dehydrogenase, subunit 3), ND4 (NADH dehydrogenase, subunit 4), ND4L (NADH dehydrogenase, subunit 4L), ND5 (NADH dehydrogenase, subunit 5), ND6 (NADH dehydrogenase, subunit 6), 12S (small subunit of mitochondrial *rDNA*), *16S* (large subunit of mitochondrial rDNA). Adapted from Reference 127.

While studying whitefly host mitochondria (131) we noted that *B. tabaci* had a mitochondrial gene order that differed from the highly conserved gene order found in most insect groups (15, 16). This led us to sequence additional whitefly mitochondria and to develop PCR-based diagnostic procedures to detect alterations to the mitochondrial gene order (127). Our results are summarized in **Figure 7**. Many whiteflies contain a mitochondrial gene order nearly identical to the proposed ancestral gene order for insects (designated Y in **Figure 7**). In some species of whiteflies the genes for *COIII-ND3* and adjacent tRNAs are "restless," i.e., they are excised (**Figure 7**, arrowhead Y) from their usual position and transposed to four different locations, resulting in four types of gene arrangements (arrowheads A, B, C, and D). These gene arrangement types correlate with the phylogenetic clusters obtained with whitefly mitochondrial and cospeciating *Portiera* genes (**Figure 7**). In one case the cluster containing the D type gene arrangement is related to a cluster containing the ancestral (Y) gene arrangement. From the divergence of *Portiera* in these two clusters and the estimated endosymbiont rate of sequence change (84, 93), it can be inferred that the transposition occurred,

at the most, from 30 to 60 million years ago.

DNA Analysis. The nucleotide sequence of two DNA fragments (33 kb in total) from *Portiera* of *B. tabaci* was determined (8). The G + C content of the DNA was 30.2 moles %. *Portiera* was different from the other P-endosymbionts in that it contains $tRNA^{Ile}$ between the *16S* and *23S rDNA* (**Table 1**). The results also suggested the presence of one *16S-23S-5S rDNA* copy per *Portiera* genome. The arrangement of *trpB* and *trpA* on one of the DNA fragments was similar to that of members of the genus *Pseudomonas*. A relationship to this genus was also indicated on the basis of phylogenetic analysis of TrpB, an observation consistent with the analysis using *16S-23S rDNA* (131) (**Figure 3**).

Aphids-*Buchnera*

Host properties and endosymbiont location. There are approximately 4400 species of aphids, which are small soft-bodied insects, the adults ranging in size from 1 to 8 mm in length (55). The reproductive cycle of aphids includes parthenogenetic (asexual) reproduction and frequently sexual reproduction. In aphids, parthenogenetic reproduction entails "telescoping of generations," in which the mother aphid contains embryos, within which are additional embryos. This property allows aphids to reach large population sizes within short periods. Sexual reproduction involves eggs. Following the egg, or embryonic, stage are four instars, followed by the adult. Adults may be winged, which allows them to spread from overcrowded plants. Within the body cavity of aphids is a bilobed bacteriome, containing 60 to 80 bacteriocytes. The bacteriocytes contain vesicles enclosing *Buchnera aphidicola*, the P-endosymbiont of aphids that has a gram-negative cell wall (11). Most aphids appear to contain *Buchnera*; in some cases it is replaced by a yeast-like symbiont located within the body cavity (44). In sexual reproduction *Buchnera* is transmitted to the egg, and in parthenogenetic reproduction it is transmitted to the embryo. In *Acyrthosiphon pisum*, the first instar nymph (about 0.1 mg) was found to contain 1.2×10^5 *Buchnera* cells and the egg 1.9×10^3 cells (76). These numbers were obtained from direct counts of cells from electron-micrograph-thin sections. Aphids are important pests of agricultural crops because they transmit viruses; in large numbers they can cause plant debilitation due to nutrient consumption (55).

Phylogeny. Extensive studies on the phylogenetic relationships of *Buchnera* and its aphid hosts involve a variety of endosymbiont chromosomal, endosymbiont plasmid, and host genes [12 (and see references therein), 50, 75, 88, 149]. These studies indicate congruence between the phylogenies obtained from different *Buchnera* and host genes, a result consistent with a single infection of an aphid ancestor and subsequent cospeciation and the lack of genetic exchange of *Buchnera* between different aphid species. In addition, studies within a single species or closely related species also indicated the lack of horizontal exchange of *Buchnera* or its plasmids (1, 29, 49, 50). The divergence of phylloxera (a major pest of grapes), which lacks endosymbionts, occurred at about the same time as the divergence of the two major *Buchnera*-containing clusters of aphids (**Figure 3**).

Genome. The size of the *Buchnera* genomes ranges from 450 to 642 Mb (52, 112, 150). The genome is present in multiple copies in the cell (10 to 600) and varies according to the age of the aphid (60, 61). The full sequence of genomes (616 to 642 Mb) was initially obtained for *Buchnera* of the aphid *A. pisum* (112), followed by *Buchnera* of the aphids *Schizaphis graminum* (124) and *Baizongia pistaciae* (140). The first two species have been estimated to diverge 50 to 70 mya, and a common ancestor of both diverged from the third species 150 to 200 mya (124, 140). The G + C content of these genomes are 25.3 to 26.3 moles %. A total of 504

to 560 protein-encoding genes and 9 to 38 pseudogenes were detected. Almost all the genes had homologs in *Escherichia coli*. There was only 1 copy of the 16S, 23S, and 5S rRNA genes, and 32 tRNA genes. *Buchnera* has a full complement of "housekeeping" genes necessary for transcription and translation. One of the most striking attributes of these findings is that *Buchnera* from the three species of aphids has virtually the same gene complement, and the genes are arranged in essentially the same order on the endosymbiont chromosome. This indicates that in the last 150 to 200 million years *Buchnera* from these three species of aphids had little change in gene content and essentially no change in gene order. Some possible reasons for genome stability are discussed below. The fact that some *Buchnera* have a genome size of 450 Mb indicates that at least in some aphid lineages further gene loss was still possible (52). In *Buchnera*, as in the case of *Carsonella*, genes encoding for proteins that were poorly conserved had a reduction in their G + C contents. This is evident by comparing the number of nonsynonymous substitutions (a measure of protein sequence divergence) of genes from the P-endosymbiont genomes of two closely related aphid species with the G + C contents of the compared genes (23, 124). The greater the number of nonsynonymous substitutions the lower the G + C content of the genes.

With respect to *Buchnera* function and integration into the host metabolism (domestication), the results are of great interest. The biosynthetic pathways necessary for the synthesis of essential amino acids are virtually complete, whereas those for the synthesis of nonessential amino acids are almost completely missing (112). These results are consistent with *Buchnera* providing the host with essential amino acids and obtaining nonessential amino acids from the host. Another potential example of integration with the host's metabolism is biosynthesis of pantothenate-coenzyme A (112). *Buchnera* has the genes to synthesize pantothenate from pyruvate but not to convert it to pantothenate-coenzyme A, a function that may be provided by the host.

The gene complement of *Buchnera* also indicates that it is capable of aerobic respiration and has the complete glycolytic pathway, the pentose phosphate pathway, but not the tricarboxylic acid cycle. It also has an ATP synthase, which indicates that it may be capable of producing ATP from the proton motive force (112). *Buchnera* has few potential transport systems (glucose, mannitol) and is not able to synthesize lipopolysaccharides or phospholipids. The source of the phospholipid in the *Buchnera* membrane is presumably the host.

The domestication of a free-living organism to perform a useful function for the host requires an alternation of its regulatory properties from energy conservation in the presence of endproducts to overproduction of these metabolites (11, 12). This would have to be the case with essential amino acid biosynthetic pathways. In free-living organisms they are reduced or turned off in the presence of the endproduct, whereas for overproduction they must be active even under conditions where endproducts may accumulate. Consistent with this is the lack of transcriptional regulators or transcriptional attenuation in regulation of amino acid biosynthesis in *Buchnera* (112).

Previous to obtaining the full sequence of the *A. pisum Buchnera* genome (112), 130 kb (encoding for 126 proteins) were sequenced from *Buchnera* of *S. graminum*. A number of the conclusions concerning properties of the *Buchnera* genome, the presence of some amino acid biosynthetic pathways, and the absence of regulatory factors were previously derived from this partial sequence (12).

Mealybugs-*Tremblaya*

Host properties and endosymbiont location. Mealybugs are plant sap-sucking insects that constitute the family Pseudococcidae of about 2000 species (55). The name is derived from the fact that they are usually covered with a mealy or cottony wax secretion.

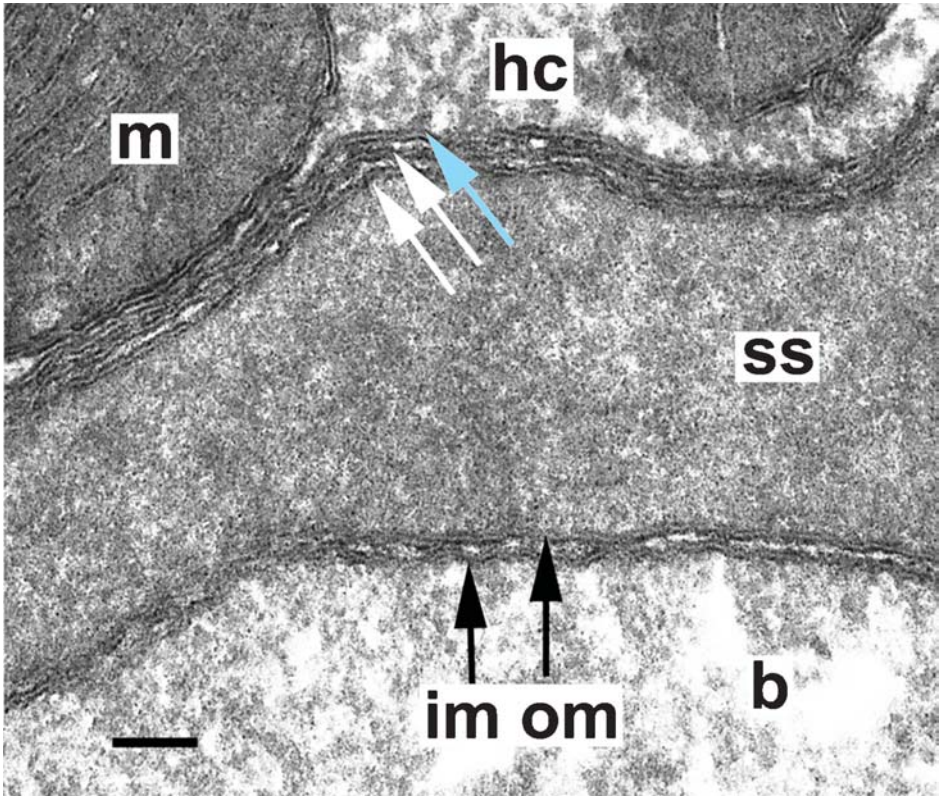

Figure 8

Transmission electron micrograph of a bacteriocyte of a mealybug with *Tremblaya* (SS) containing the S-endosymbiont (*b*). Light blue arrow: vesicle membrane; white arrows: gram-negative-type cell wall of *Tremblaya*; right, black arrow: om, outer membrane of the S-endosymbiont; left, black arrow: im, inner membrane of the S-endosymbiont; m, mitochondrion; hc, host cell cytoplasm. Bar is 0.0706 μm. Reprinted from Reference 143, with permission from C.D. von Dohlen and the Nature Publishing Group.

Most mealybug species lay eggs, and most have sexual reproduction, although some are parthenogenetic. The appearance and development of males is different from that of females. The latter are soft, often elongate or oval, usually attached to plant surfaces, and generally are long-lived (months). Males are smaller, and the final stage is winged and short-lived (few days), such that males are rarely seen. Within the body cavity of a female mealybug is a single large bacteriome composed of large bacteriocytes (21). The bacteriocytes contain vesicles within which is the P-endosymbiont, *Tremblaya princeps*, that has a gram-negative cell wall. Remarkably, *Tremblaya* may contain within its cells an S-endosymbiont that also has a gram-negative cell wall (143) (**Figure 8**). Previously, one case of a prokaryote containing another prokaryote has been described in a free-living bacterium (54). Several mealybug species such as *Planococcus citri* and *Maconellicoccus hirsutus* are important agricultural pests (55).

Phylogeny. Initial studies of *16S rDNA* of the P-endosymbionts of mealybugs established that they were in the β-subdivision of the Proteobacteria (90) (**Figure 3**). Subsequently it was also found that they might have S-endosymbionts that belong in the

γ-subdivision of the *Proteobacteria* (46). A more extensive phylogenetic analysis of *16S-23S rDNA* from 22 mealybug species indicated that they were grouped into six clusters (134). A more limited analysis of S-endosymbiont *16S-23S rDNA* from 12 mealybug species indicated that they formed four different clusters corresponding to the clusters based on *16S-23S rDNA* of *Tremblaya* (**Figure 1**). Unlike *Tremblaya*, which is monophyletic, the four clusters of S-endosymbionts were related more to other sternorrhynchal S-symbionts than to each other. These results suggested that *Tremblaya*, within a number of different mealybug lineages, was infected with different S-endosymbiont precursors. Once the association between *Tremblaya* and the S-endosymbiont was established, the two were inherited as a unit resulting in cospeciation (134). Recently an extensive study of phylogenetic relationships of mealybugs has been performed using several host genes (40). Many of the DNA samples used were also used in the studies of mealybug endosymbionts (134). A phylogenetic analysis of the mealybug *18S*-28S*rDNA* genes from the species common to both studies indicated good congruence of the host and *Tremblaya* phylogeny (**Figure 1**), a result consistent with a single infection of a mealybug ancestor with the *Tremblaya* precursor followed by cospeciation.

DNA analysis. Two DNA fragments of 30 and 35 kb from *Tremblaya* have been sequenced (9). This DNA differed from the other P-endosymbionts in that it has a considerably higher G + C content (57 moles %) (**Table 1**). Both fragments contained a 5.7-kb region of sequence identity that included the rRNA operon (16S-23S-5S). Sequence analysis of the duplicated region from four additional mealybug species suggested that in an ancestor of these mealybugs *Tremblaya* underwent a duplication of a DNA fragment containing part of *leuA-rps15-16S-23S-5S-yabC*, and that this fragment was inserted between *prs* and *dnaQ* in another portion of the endosymbiont genome (9). In this gene duplication of the rRNA operon, *Tremblaya* differs from the other P-endosymbionts of plant sap-sucking insects that have only one copy of the rRNA operon (**Table 1**). Unlike *Carsonella* and *Buchnera*, in *Tremblaya* there is no decrease in the G + C content of poorly conserved genes (**Figure 5**), nor is there a decrease in the G + C content of intergenic spaces, which is usually observed in most bacteria (9) (**Table 1**).

Sharpshooters-*Baumannia*

Host properties and endosymbiont location. Sharpshooters are members of the suborder Auchenorrhyncha (**Table 1**) (38). Sharpshooters reproduce sexually from eggs. Unlike the members of the Sternorrhyncha, which probe between cells and feed on phloem, sharpshooters pierce cells and feed on xylem. Within the body of sharpshooters is a bilobed bacteriome. In *Homalodisca coagulata*, a species studied in some detail, the bacteriome has a red-pigmented portion and a yellow-pigmented portion (82). The red portion contains bacteriocytes with vesicles enclosing the spherical P-endosymbiont, *Baumannia cicadenillicola*, which has a gram-negative-type cell wall (82). The yellow portion also contains a rod-shaped endosymbiont (82). The composition of xylem (low in sugars and amino acids) is different from that of phloem, so that the function of the endosymbionts is probably different from that of the members of the Sternorrhyncha (20, 82, 106). Several species of leafhoppers are important agricultural pests in part owing to their ability to transmit plant pathogens such as *Xyllela fastidiosa*. The mitochondrion of *H. coagulata* has been recently shown to have the same order of protein-encoding genes and rRNA genes as the mitochondria of most Sternorrhyncha and most other insects (127) (L. Baumann, personal communication).

Phylogeny. Studies of *16S rDNA* showed that *Baumannia* is a member of the γ-subdivision of the *Proteobacteria* (82). These

as well as additional studies also indicated a closer relationship to *Blochmannia* and *Wigglesworthia* and a more distant relationship to *Buchnera* (52, 70) (**Figure 3**). There was evidence of cospeciation between the host and *Baumannia* (82). The rod-shaped endosymbiont, present in the yellow portion, is a member of the *Bacteroidetes* phylum (*Flavobacterium-Bacteroides* rRNA subgroup) (82).

Genome size. The genome size of *Baumannia* from the sharpshooter *H. coagulata* is 680 kb, similar to that of several other insect endosymbionts (**Table 1**). The G + C content of a 3-kb endosymbiont DNA fragment encoding for *rpoBC* is 37 moles %, somewhat higher than that of the related endosymbionts (82) (**Table 1**). The nucleotide sequence of the *Baumannia* genome is currently being determined (N.A. Moran, personal communication).

EVOLUTIONARY DYNAMICS OF P-ENDOSYMBIONTS

Increase in the Rate of Sequence Change

Most studies of the evolutionary dynamics of endosymbionts of plant sap-sucking insects have been performed in *Buchnera* and were initiated by analysis of the rates of sequence change in this and other endosymbionts (80). In the initial study it was found that the rate of nucleotide sequence change of *16S rDNA* (1% to 2% per 50 million years) was about twice that of free-living bacteria (80, 84). Studies involving protein-encoding genes have suggested that an increase in the rate of sequence change probably applies to the whole genome (80, 147, 149, 151). The finding that the rate of sequence change in endosymbiont lineages is higher than that of free-living bacteria is a well-established observation. Attempts to provide an explanation for this and other possibly related observations has led to the formulation of an interpretation that has as its principal basis the population structure of *Buchnera* and the functioning of Muller's ratchet (80). It is stated that the relatively small number of *Buchnera* transmitted to progeny creates bottlenecks, so that there is reduced purifying selection and the accumulation of slightly deleterious mutations. This process is irreversible (ratchet-like) and would result in the loss of gene function and the degeneration of the endosymbiont genome. An additional factor that may account for the increase in the rate of sequence change is the major reduction in recombination and repair genes in endosymbiont genomes compared with free-living bacteria (35, 86, 115). With some exceptions the endosymbionts studied have a G + C content of 20 to 30 moles % (**Table 1**). This "AT enrichment" is taken as evidence of an increase in deleterious mutations and is said to be characteristic of endosymbiotic associations (80, 100, 101). Also cited as a major support for this argument is the increase in nonsynonymous substitutions relative to synonymous substitutions in the same genes of *Buchnera* compared with *E. coli* and *Salmonella enterica* serovar Typhimurium (28, 80), which is consistent with a reduced purifying selection. An additional observation, cited in support of accumulations of deleterious mutations, is an increase in the destabilizing mutations in *16S rDNA* of endosymbionts (67, 118). This genomic degeneration may be counteracted by several factors. Endosymbionts, like many intracellular organisms, have an elevated, constitutive level of GroEL (10, 59). This chaperone is involved in protein folding and recovery from environmental stress, and it is proposed that high levels may result in the proper folding of proteins that contain deleterious mutations (80, 103). Another factor that may counteract the accumulation and transmission of deleterious mutations is the high polyploidy of *Buchnera* (60, 61, 123). The hypothesis that Muller's ratchet and the accumulation of deleterious mutations explain the accelerated evolutionary rate in endosymbionts has achieved the authority that repeated citation bestows.

The validity of some of its features, which are said to be unique to endosymbiotic associations, is diminished by comparisons with free-living bacteria and other endosymbionts. Recently the applicability of this hypothesis to endosymbiotic associations and the much more extreme case of mitochondria has been questioned and the results observed explained simply on the basis of a higher mutation rate (58).

Although from a reconstruction of the past evolutionary history it is probable that the ancestor of *Buchnera* had a higher G + C content (83, 114), there is no reason to suppose that in the context of its habitat (the bacteriocyte) or its functional attributes (synthesis of essential nutrients) *Buchnera* has degenerated or that the lowered G + C content (25 to 26 moles %) is a result of deleterious mutations. *Tremblaya* has a G + C content of 57 moles %, so that a lower G + C content cannot be considered a characteristic of all endosymbiotic associations (9). The G + C content of prokaryotes is about 25 to 75 moles % (9). There is no reason to imply that a G + C content of near 50 moles % (such as in *E. coli*) represents normalcy or that lower (or higher?) G + C contents are deleterious. In fact, vigorous free-living organisms are found at both ends of the G + C spectrum and there is no evidence that organisms with extreme G + C contents have functionally deficient proteins. Perhaps the uncertainty of this point is reflected in the statements that *Buchnera* has mutations that are "slightly deleterious" (80) or that *Buchnera* has "great accumulation of deleterious mutations" (52). Currently there is no direct evidence to indicate that endosymbiont proteins are in any way deficient, and conclusions that "functions and conformations of *Buchnera* proteins have been seriously impaired or strongly modified" are based solely on sequence comparisons (113). It has been stated that one of the consequences of an endosymbiotic association, consistent with Muller's ratchet and the accumulation of deleterious mutations, is the lower thermal stability of the secondary structure of endosymbiont 16S rRNA (67, 118). However, the comparisons made mostly include *16S rDNA* from endosymbiotic organisms with a lower G + C content and selected free-living bacteria with *rDNAs* having a higher G + C content. This cannot be taken as an indication that the lowered thermal stability is a specific result of the endosymbiotic association, since comparisons with free-living organisms having a G + C content comparable to that of the endosymbionts were not performed. A replot of the data from Reference 67 shows a reasonable correlation (R = 0.75) between thermal stability and G + C content of the 16S rRNAs. It has also been frequently stated that the major increase in nonsynonymous substitutions relative to synonymous substitutions for the same genes in *Buchnera* compared with *E. coli* and *S. enterica* serovar Typhimurium is a major support for the decrease of purifying selection due to bottlenecks in the transmission of *Buchnera* and the operation of Muller's ratchet (28, 80). As pointed out by Itoh et al. (58) and Ochman et al. (93), this interpretation is not tenable because it is not the *Buchnera* ratio but that of *E. coli/S. enterica* serovar Typhimurium that is atypical. This anomalous ratio appears to be due to a low rate of nonsynonymous substitutions in the comparison involving the latter pair of organisms (28, 93). The *Buchnera* ratio of nonsynonymous substitutions to synonymous substitutions is similar to that of other bacteria (93). Perhaps as a further indication of the interpretive uncertainty surrounding this topic is the opposite suggestion that many of the mutations that arise in endosymbiotic associations are extremely deleterious and not just slightly deleterious. Deleterious mutations would lead to a regular loss of individuals, whereas entire host lineages would not be imperiled by gradual and cumulative mutation accumulation (73). Such a scenario suggests that the longevity of these endosymbiotic associations is due to their extreme sensitivity to mutations and not to their relative invulnerability.

An attempt was made to mimic the conditions of endosymbiont propagation and

transmission using *E. coli* (42). A mutator and a nonmutator strain of this organism were repeatedly propagated from single colonies (bottlenecks in transmission), and their ability to compete against the ancestral strain was tested in a minimal medium (apparently deficient in a variety of inorganic constituents). In both strains there was a similar reduction of fitness. (A comparison with a strain propagated in a similar manner but differing in the use of large inocula was not performed.) Upon introduction by transduction of a constitutively expressed GroESL and cultivation in complex but not in minimal media, most of the fitness was regained. These results were explained as being due to the accumulation of deleterious mutations and their sparing by GroEL accumulation. Essentially no difference was noted between nonmutator and mutator strains. *E. coli* grown on a comparable medium contains 1.35% of its proteins as GroEL (96). In the constitutive GroESL-producing strain, the amount of GroEL was said to be increased by about 86-fold (42), a value higher than is possible. Assuming that either the strain makes a lower than usual amount of GroEL or that there is an error in the determination of its increase, the high expression may result in most of the GroESL being found in insoluble intracellular inclusions, as is the case with many overproduced proteins. For this and other reasons the interpretation of these results is difficult and questionable.

Genome Reduction

The nearest neighbors of *Portiera*, *Carsonella*, or *Tremblaya* are quite distant, whereas *Buchnera*, *Baumannia*, *Blochmannia*, and *Wigglesworthia* can be readily placed within the *Enterobacteriaceae* (53, 70) (**Figure 3**). Studies of evolutionary relationships have provided strong evidence that *Buchnera*, *Baumannia*, *Blochmannia*, and *Wigglesworthia* arose from a related organism(s) that established itself in these four insect lineages and subsequently underwent a major reduction in its genome size (2, 53, 83, 112, 124, 140). That these are independent events is suggested from the different gene complements in these endosymbionts, each potentially reflecting its function within the endosymbiotic association.

The phylogenetic position of *Buchnera* has allowed plausible reconstruction of the evolutionary events that led to the formation of the reduced endosymbiont genome from the genome of a reconstructed common ancestor (83, 114). Comparisons of *Buchnera* with such a genome suggest that a major part of the genome reduction occurred through large deletions accompanied by chromosomal rearrangements (83). In addition, some reduction occurred via pseudogene formation followed by gene deletion (83, 114). Because the free-living relatives of *Buchnera* have G + C contents of 38 to 62 moles %, the ancestor probably had a G + C content in this range and there was a lowering of the G + C content during the process of genome adaptation. It is suggested that genome reduction occurred early in the establishment of the endosymbiotic association, since the chromosomes of *Buchnera* from three aphid species, which diverged at least 150 mya, are virtually identical with respect to gene complement and gene order (112, 124, 140). Part of the reason for gene stability is postulated to be a major reduction in DNA repair and recombination systems in *Buchnera*, including the lack of *recA* and *recF* (86, 112, 115). Another factor is the absence of repeated sequences and insertion elements. In an extensive comparison of sequenced genomes of prokaryotes, it was concluded that large deletions are common events and major factors in the evolution of these organisms (77). In spite of this deletional bias, intergenic spaces are maintained in all the genomes, including those of *Buchnera*, *Wigglesworthia*, and *Blochmannia* (77). An exception to this is *Carsonella*, which virtually had no intergenic spaces (27). *Buchnera*, *Wigglesworthia*, and *Blochmannia*, like most Bacteria, have a decreased G + C content in the intergenic spaces compared with the structural genes. Because there would appear to be less

OIS: obligate intracellular symbiont

OIP: obligate intracellular pathogen

FsEP: fastidious extracellular pathogen

FcIP: facultative intracellular pathogen

Ec/Pa: *Escherichia coli*/*Pseudomonas aeruginosa*

selective pressure for sequence conservation in intergenic spaces, a reduction in G + C content is consistent with "AT" pressure. *Tremblaya* is an exception to this in that the moles % G + C content of the intergenic spaces was slightly higher than that of the structural genes (9).

A comparison of some genome properties of P-endosymbionts with selected bacterial types is presented in **Figure 9**. We have compared *Buchnera*, *Wigglesworthia*, and *Blochmannia* (OIS) with OIP, FsEP, FcIP, and Ec/Pa. The gene categories and data are from the TIGR website (**http://www.tigr.org/**) and the organisms are grouped according to Cossart & Lecuit (30). In general the number of genes encoding core functions such as transcription and translation is similar in all organisms (data not shown). In most of the compared properties Ec/Pa and FcIPs resemble each other and have many more genes than the other organismal types. The P-endosymbionts have a major reduction of genes in most categories and resemble the OIPs and the FsEPs. *Buchnera* and *Blochmannia* differ from *Wigglesworthia*, OIPs, and FsEPs in that they have the most genes for the synthesis of essential amino acids, and *Wigglesworthia* differs from *Buchnera*, FsEPs, and most OIPs in that it has more genes for the synthesis of cofactors (2, 112). Compared to FcIPs and Ec/Pa, all of these organisms have a major reduction in genes associated with regulatory functions, an observation consistent with their relatively stable habitat, either intracellularly or extracellularly, in the body of animals (30). The limited responsiveness to environmental changes due to lack of regulatory mechanisms has been illustrated in a recent study of the heat shock response using *Buchnera* microarrays (153). Only a modest increase in transcriptional activity was noted for 5 of 20 *E. coli* heat shock orthologs that were retained by *Buchnera*. The increase included *groEL* and *groES*; the former is known to be overexpressed in *Buchnera* (10, 59). Under somewhat different conditions, an increase in temperature did not appear to increase the amount of GroEL (10, 59). Although the amount of this protein in *Buchnera* is generally considered high, it has not been adequately quantified. Our estimate of ~10% (10) is incorrect because the number of cells was equated with the number of genome copies and *Buchnera* is now known to be polyploid (60, 61). In addition, *Buchnera* GroEL is found in substantial amounts in the aphid hemolymph (139), such that immunological estimates of this molecule in extracts of whole aphids probably do not correspond to the amount found only in *Buchnera*.

Pseudogenes

Examination of the genomic sequences of *Buchnera* from *A. pisum* and *S. graminum* indicated that the former had 13 and the latter 38 pseudogenes (112, 124). *Buchnera* from *S. graminum* had five pseudogenes in the cysteine biosynthetic pathway and six pseudogenes in the pathway of peptidoglycan biosynthesis. *Buchnera* from *A. pisum* had good copies of all these genes but in addition had one pseudogene in a different gene for peptidoglycan biosynthesis. Many of these changes involved deletions of single nucleotides in a homopolymeric stretch of As or Ts (124, 125). In an evolutionary study of the genes of peptidoglycan biosynthesis, it was found that many of these pseudogenes were acquired 30 to 50 mya and persisted in a variety of species (125). One interpretation of these findings is that, due to its position within vesicles inside bacteriocytes, *Buchnera* no longer needs an intact peptidoglycan layer and, consequently, these enzymes are not necessary for its synthesis. If this is the case these genes probably would be inactivated or deleted in all endosymbionts, since there is no reason to believe that the intravesicular environment varies among aphid species. An alternative, suggested explanation is that these genes are expressed at a lower efficiency via translational frameshifting, transcriptional slippage, or both (5, 125). In addition, the homopolymeric stretches of As and Ts resemble some of the signals used for

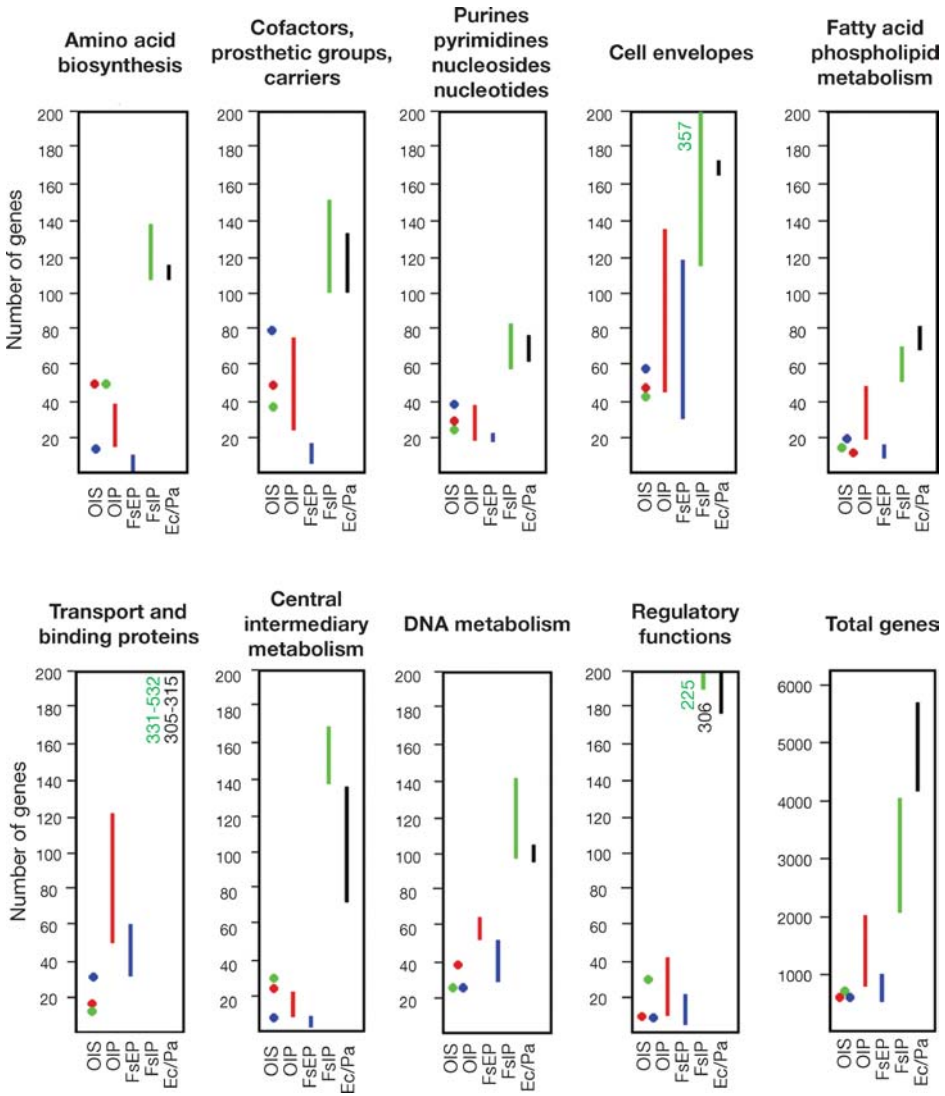

Figure 9

Comparisons of selected gene categories present in OIS [*Buchnera* (*red dot*), *Wigglesworthia* (*blue dot*), and *Blochmannia* (*green dot*)]; OIP, red line (*Chlamydia pneumoniae*, *Coxiella burnetii*, *Rickettsia prowazekii*, and *Wolbachia pipientis*); FsEP, blue line (*Borrelia burgdorferi*, *Mycoplasma genitalium*, and *Treponema pallidum*); FcIP, green line (*Brucella melitensis*, *Listeria monocytogenes*, and *Shigella flexneri*); and Ec/Pa, black line. Numbers indicate off-scale gene range. The compilation is from the TIGR website (**http://www.tigr.org/**).

translational recoding in a variety of bacteria (6). One indirect method to distinguish between these alternatives is to determine the synonymous/nonsynonymous substitution ratios between pairs of complete genes and between complete genes and pseudogenes. If the pseudogenes are not transcribed into functional proteins, there are reduced constraints on nonsynonymous substitutions and the ratios should decrease. The results indicate that the ratios are the same for comparisons involving complete genes

and pseudogenes, a result consistent with the translation of pseudogenes into functional proteins (125). Similarly, it has been suggested that the presence of pseudogenes in the pathway of cysteine biosynthesis in *Buchnera* of *S. graminum* results from the aphid gaining sufficient cysteine by ingestion, and consequently this pathway is dispensable (107, 124, 125). However, a comparison of the synonymous/nonsynonymous substitutions indicates that with one exception the ratios are similar to those observed in the evolutionary changes of functional genes (124, 125). The demonstration that these pseudogenes are translated into functional proteins requires the purification and characterization of the proteins.

PLASMIDS IN *BUCHNERA*

Many species of aphids have *Buchnera* with plasmids encoding for anthranilate synthase, the first enzyme of tryptophan biosynthesis (pTrpEG) (63), as well as plasmids encoding for four enzymes of leucine biosynthesis (pLeu) (18). There is considerable variation in the organization of these plasmids and they appear to constitute two or three different replicon types (12, 18, 63, 141). Many of the pTrpEG plasmids consist of tandem repeats of a *trpEG*-containing unit. In *Buchnera* from several species of aphids *trpEG* and *leuABCD* are chromosomal (12, 65, 104, 105). In the initial study, which involved the aphid *S. graminum*, the number of copies of *trpEG* (plasmid genes) was approximately 16 times greater than that of *trpB* (a chromosomal gene) (63). Compared with other systems, it was suggested that amplification of *trpEG* was a means of increasing the amount of this usually limiting enzyme and, therefore, overproducing tryptophan for the host. Similarly, pLeu was amplified about 24-fold in *Buchnera* of *S. graminum* (128). Subsequently it was found that *Buchnera* from the aphid *Diuraphis noxia* contains a plasmid consisting of one good copy of *trpEG* and about seven tandem repeats of *trpEG* pseudogenes (Ψ*trpEG*) (66). There were numerous differences between *trpEG* and Ψ*trpEG* involving frameshifts and premature termination; most of the changes were in the N-terminal region of the *trpE* and many were also in the upstream putative promoter region. In addition, the *trpEG/trpB* ratio was about 2 and the pLeu/*trpB* ratio was about 1 (128). Based on the fact that *D. noxia* caused considerable damage to its host, it was suggested that this resulted in an increase of free amino acids available to the aphid. Energy was presumably conserved by silencing most of the *trpEG* copies and also by reducing the plasmid copy number, thereby decreasing the amount of TrpEG. Subsequently it was shown that populations of this species from different geographical areas have plasmids with 6 to 9 Ψ*trpEG* for every *trpEG* (148). In *Buchnera* from *D. noxia*, Ψ*trpEG* can be distinguished from *trpEG* by the presence of an *EcoRI* site in the latter. Pseudogene formation or deletion of part or a whole gene of *trpE* or *trpG* is also found in *Buchnera* from a variety of aphid species (7, 12, 141, 151). In *D. frequence* only Ψ*trpEG* was detected (148). Pseudogenes have not been detected in pLeu (12).

To obtain an explanation for gene amplification as well as pseudogene formation, attempts were made to determine the ratios of plasmid *trpEG* and *leuB* to chromosomal *trpB* and to investigate whether there is a correlation between these properties and some physiological, nutritional, or ecological parameters. The methods used to obtain the ratios were quantitative PCR and quantitative dot-blot hybridization. Neither method can distinguish *trpEG* from a relatively intact Ψ*trpEG*. Using quantitative dot-blot hybridization, Birkle et al. (14) found that in *Buchnera* of 12 *A. pisum* clones the *trpEG* (±Ψ*trpEG*)/*trpB* ratio was between 2 and 16, and there was no correlation of this ratio with several aphid growth parameters. In another study of *trpEG* and *leuABCD* amplification in *Buchnera*, in different clones of the aphid *Uroleucon ambrosiae* the *trpEG* (±Ψ*trpEG*)/*trpB* ratio was 0.3 to 1.9 and the

pLeu/*trpB* ratio was 0.5 to 2.8 (97). Owing to the polyploidy of *Buchnera* cells, the values below 1.0 were interpreted to indicate that plasmid association of these genes may be a mechanism to reduce the rate of tryptophan and leucine synthesis. When comparing *D. noxia* (*trpEG* and Ψ*trpEG*) with the related *D. mexicana* (only *trpEG*Ψ), it was found that the body fluids of the former contained a reduced level of tryptophan and, consequently, the presence of Ψ*trpEG* was said to constitute a "decay in mutualistic potential" (148). A table summarizing the ratios of plasmid-borne biosynthetic genes to chromosomal genes suggests an unexplained discrepancy between the results obtained by quantitative dot-blot hybridization and quantitative PCR (85).

Another approach which might give results explaining plasmid amplification is to examine the composition of phloem of plants on which aphids are feeding and relate it to the rate of ingestion of amino acids and their requirement for aphid growth (107). Surprisingly, the calculations indicated that for both *S. graminum* and *D. noxia* growing on wheat, more tryptophan was taken up than is required for growth. Less leucine was taken up by *D. noxia*, whereas more than necessary was taken up by *S. graminum*. These studies question the role of *Buchnera* in provisioning tryptophan and leucine for the aphid under these conditions of cultivation (107, 109). A study of *U. ambrosiae* on a suboptimal host suggested that tryptophan was limiting in the phloem and *Buchnera* made a contribution to its synthesis (13). The *trpEG/trpB* ratios, however, were not determined for *Buchnera* of *U. ambrosiae* grown on this suboptimal host.

Taken together these findings indicate that we do not have an explanation for *trpEG* or *leuABCD* amplification/reduction or for the formation and persistence of Ψ*trpEG* in *Buchnera*. Perhaps the association of *Buchnera* and its host is too complex to expect a simple direct association between the biosynthetic activities of the endosymbiont and the phloem composition of its host. *Buchnera* is in host vesicles within bacteriocytes, and its nutritional environment is a reflection of the activities of the aphid host, such as its ability to obtain nutrients from the plant and the demand of the host and the endosymbiont for these nutrients. Therefore, host properties, such as efficiency of nutrient uptake from the plant and their transformation and delivery, may determine the nutritional parameters within bacteriocyte vesicles and impose the selective pressure resulting in *Buchnera* adaptation to an endosymbiotic association. Alternatively, the contributions of the endosymbiont may be variable and dependent on the nature of the host plant and its physiological state.

S-SYMBIONTS

In initial studies of the endosymbionts of the aphid *A. pisum*, the rod-shaped, bacteriome sheath-associated S-endosymbiont was found to be related to the "classical" members of the *Enterobacteriaceae* (138). Subsequently, it has been shown that there is a variety of bacterial symbionts present in many different insect species (**Table 2**). The location of these organisms in the insects is not always known, and in such cases they are called S-symbionts (102); the term S-endosymbionts is used when their intracellular location has been established. Because some insects may contain only the P-endosymbiont, the S-symbionts do not appear to perform an essential function required by the host under all growth conditions. The S-symbionts of aphids have been subdivided into several groups (**Table 2**), of which the R-type is one of the better studied (47, 102, 108, 137, 138). By means of electron microscopy and in situ DNA hybridization with specific 16S rRNA probes, the R-type endosymbionts have been localized to the sheath cells that surround the bacteriome, to special bacteriocytes that contain only the R-type endosymbionts, or to bacteriocytes that have both *Buchnera* and the R-type endosymbionts (47). Similar studies have been performed with S-endosymbionts of psyllids and mealybugs (45, 46). Some

Table 2 Bacterial S-symbionts associated with insects of the suborder Sternorrhyncha and sharpshooters

Designation	Other designations	Evolutionary affiliation	Insect hosts	Some grown in laboratory media	Some detected in bacteriocytes	References
Arsenophonus-type		*Proteus*, γ-Proteobacteria	Aphids, mealybugs, psyllids, whiteflies	+	–	51, 102, 121, 130, 132, 154
Sodalis-type	So-So	*Enterobacteriaceae*, γ-Proteobacteria	Psyllids, tsetse	+		3, 34, 102
R-type	PASS	*Serratia*, γ-Proteobacteria	Aphids		+	47, 102, 108, 137, 138
T-type	PABS	*Enterobacteriaceae*, γ-Proteobacteria	Aphids, psyllids, whiteflies		–	36, 102, 108
U-type	PAUS	*Enterobacteriaceae*, γ-Proteobacteria	Aphids		–	102, 108, 137
V-type	*Enterobacteriaceae*, γ-Proteobacteria	Aphids			–	102
YSMS		*Enterobacteriaceae*, γ-Proteobacteria	Aphids		+	43
Other *Enterobacteriaceae*			Psyllids, mealybugs			46, 118, 132, 134, 143
Fritschea[a]		*Chlamydia*	Whiteflies		+	41, 129
Rickettsia	PAR	α-Proteobacteria	Aphids		–	25, 137
Sharpshooter S-endosymbiont		*Bacteroidetes*	Sharpshooters		+	82
Wolbachia		α-Proteobacteria	Psyllids, whiteflies, sharpshooters		–	82, 92, 121, 154
Cardinium[a] relative	CFB-BP	*Bacteroidetes*	Whiteflies			8, 145, 155
Spiroplasma		*Mycoplasma*	Aphids, mealybugs		–	46, 48, 137

[a] *Candidatus* status.

S-symbionts have not been localized to specific cells, and histochemical studies suggest that they are not located in a specific host cell type (121). For many endosymbionts localization has not been attempted. There is some degree of certainty that they have an association with insects because similar types are repeatedly isolated from different insects and they form a unique group different from common free-living bacteria or, in some cases, other insect S-symbionts (102, 108, 130, 132, 137). The state of the biological samples used as the source of DNA for PCR is important with respect to the potential contamination with a variety of saprophytic bacteria. Laboratory-grown

aphids on young plants contain virtually no cultivable bacteria (12). As the plant becomes overcrowded the bacterial population on the aphid or within the aphid may increase. As would be expected, compromised insects develop a varied bacterial flora (91). The problems associated with PCR amplification and cloning of PCR products as an indication of diverse types in a natural DNA sample have been reviewed (4). Using this approach, the potential for missing a substantial number of bacterial types is illustrated in our studies with the whitefly *B. tabaci* (8). PCR products consistently gave *Portiera* and the S-symbiont. In Southern blot analyses of the DNA using *rDNA* probes derived from *Portiera*, multiple bands were obtained, two of which consisted of a chlamydial-type bacterium and a relative of *Cardinum* (*Encarsia* bacterial-type) (8, 129, 155). From the intensity of the bands these organisms would appear to be relatively abundant but they were missed in PCR-amplified DNA.

Evolutionary studies have indicated that the phylogeny of S-symbionts differs from that of the host or the P-endosymbionts (**Figure 2**), a fact that has been interpreted to indicate multiple infections of the hosts as well as horizontal transmission. In the case of mealybugs, it was found that the S-endosymbionts are within *Tremblaya*, the P-endosymbiont (143) (**Figure 8**). There were still multiple infections of different *Tremblaya* with different S-endosymbiont precursors, but once the infection was established there was cospeciation of the S-endosymbiont with *Tremblaya* (134) (**Figure 1**).

Table 2 provides a summary of the different types of S-symbionts and the insect types with which they are associated. It is possible that some of these bacteria can be cultivated on laboratory media, as is the case with the initial isolate of *Arsenophonus nasoniae* (51) and with *Sodalis glossinidius* (34). The function(s) of the S-symbionts (if any) is not known in most cases. Recent evidence has shown that they have an effect on host plant preference (136, but see 69) and may make the aphid more resistant to thermal stress as well as to infection with parasitoids (79, 95). GroEL is found in the hemolymph of aphids and whiteflies (87, 139). This chaperonin plays a major role in protecting viruses and allowing their persistence in the insect (139). In the case of the whitefly *B. tabaci* GroEL appears to originate from the S-endosymbiont, whereas in the case of the aphid GroEL originates from *Buchnera* (87, 139).

SPECULATIONS AND FUTURE DIRECTIONS

The properties of the P-endosymbionts and their association with plant sap-sucking insects described in this review are in accordance with the "Mark Principle" (New Testament, Mark 4:25), which, when applied to these systems, predicts that "interactions across a boundary (in this case membranes) favor the side with the higher variety which feeds upon the other side" (24, 126). The result of this interaction can be said to be the domestication of a free-living bacterium to serve the purposes of the host. This domestication resulted in a major reduction of the genome size and this generalization probably holds for all of these ancient endosymbiotic associations. The different properties of the DNAs of *Carsonella*, *Buchnera*, and *Tremblaya* (**Table 1**) suggest that different constraints on the permissible evolutionary changes are probably a function of the gene repertoire of the endosymbiont ancestor and the gene losses that occur during reduction of the genome size (83). During this process there are also changes that result in the integration of the endosymbiont gene products with the metabolism of the host. The endosymbionts retain the functions necessary for their perpetuation and also the special functions that the host lacks (synthesis of essential amino acids). The latter attribute makes the host dependent on the endosymbiont. This domestication also necessitates the modification or elimination of regulatory mechanisms that, in free-living organisms, are geared toward

maintaining the cell as an independent self-replicating entity, conserving energy when structural building blocks are present in the environment. In the case of the endosymbionts the modification or elimination of these regulatory mechanisms must allow for overproduction of essential nutrients in a relatively constant intracellular environment (12).

The P-endosymbionts are found within host-derived vesicles and appear to lack genes necessary for infection and entry into cells (112). It would seem that the P-endosymbiont is a rather passive entity under the control of the host. Consequently, the host must have undergone substantial adaptations for governing the activities of the endosymbionts as well as their vertical transmission (78, 136). One site of host regulation could be the vesicular membrane, which may have uptake and exit systems for endosymbiont substrates and products and thereby regulate their metabolic activities. In addition, either the host or the endosymbiont could create a proton motive force in the vesicular space that could be harvested to transport nutrients or generate ATP.

In the process of adapting to an endosymbiotic association, the P-endosymbionts and their genomes are often stated to have undergone "decay," "degeneration," or "degradation" or are approaching "genomic meltdown" (27, 58, 68, 80, 140, 142). These changes are thought to be analogous to a terminal disease, as illustrated by the term "symbiotic syndrome" (101). This may be a useful viewpoint if one takes as a reference an organism that grows on a minimal medium and thereby has all the biosynthetic pathways needed for the synthesis of cell material as well as a G + C content of about 50 moles % (*E. coli*). However, such comparisons, although informative, are misleading, because the entity of biological interest is the composite organism, the insect-endosymbiont, in which such changes represent a new functional integration that has greatly expanded its capabilities (74, 116). The arguments used for the degradation of P-endosymbionts have also been previously applied to the degradation mitochondria (58). Aphids, whiteflies, and psyllids have endosymbionts with a G + C content of 20 to 30 moles % and mitochondria with G + C contents of 16 to 26 moles %. Both endosymbionts (about 200 million years old) and mitochondria (about 1.5 billion years old) are essential for these insects. By this line of reasoning both can be said to be undergoing degradation, with the mitochondria being more degraded than the endosymbionts. The amazing reproductive potential of aphids, whiteflies, or psyllids can, however, be readily observed by visiting an infested field (populations of up to 2×10^9/acre). It would appear that the symbiotic syndrome and the degeneration have little or no effect on their reproductive potential. Perhaps "Theory is all gray but the golden tree of life is green" (Goethe).

Reconstructions of the ancestry of P-endosymbionts as well as many of the comparisons of P-endosymbionts and free-living bacteria involve a comparison of nonculturable and culturable organisms. As is known from extensive studies in many environments, more than 95% of prokaryotes are not culturable on common laboratory media (4, 98). This lack of laboratory cultivation, a major inconvenience for the investigator, should not imply any deficiency in the organism, since their success is evident from their widespread distribution in nature. It is possible that cultivation on laboratory media requires the presence of a set of properties (such as a reasonably rapid growth rate) absent in nonculturable organisms and that a more meaningful comparison would be between the P-endosymbionts and their unknown and probably slow growing, nonculturable, free-living relatives.

In the case of mitochondria and plastids some of the genetic information from the ancestral genome resides in the host nuclear genome (71). Recently it has been shown that a fragment of the genome of *Wolbachia* has been transferred to an insect host (62). *Wolbachia* may be associated with germ line cells,

and its DNA would then presumably have an opportunity of entering the genome of these cells and being transmitted to progeny (120). P-endosymbionts are sequestered in somatic cells separate from the germ line, and this may have limited opportunities for the transfer of genes from the P-endosymbiont to the germ line. Therefore it would appear unlikely that the P-endosymbiont genome would become integrated into the host cells.

This review points to the diversity of the endosymbiotic associations of sap-sucking insects. The information obtained suggests that, at this stage of this ancient association, the endosymbiont has a relatively passive role and is controlled by the host. Future advances in this field will be from studies that elucidate the mechanisms and the adaptations of the host required for the control and perpetuation of this association. Such studies have been recently initiated (19, 78).

NOTE ADDED IN PROOF

The survey of the literature for this review ended in July 2004. Since then several papers of direct relevance to this topic have been published. These include Baumann L, Baumann P. 2005. Cospeciation between the primary endosymbionts of mealybugs and their hosts. *Curr. Microbiol.* 50:84–87; Downie DA, Gullan PJ. 2004. Phylogenetic congruence of mealybugs and their primary endosymbionts. *J. Evol. Biol.* 18:315–24; and Zientz E, Dandekar T, Gross R. 2004. Metabolic interdependence of obligate intracellular bacteria and their insect hosts. *Microbiol. Mol. Biol. Rev.* 68:745–70.

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