Secondary (γ-Proteobacteria) Endosymbionts Infect the Primary (β-Proteobacteria) Endosymbionts of Mealybugs Multiple Times and Coevolve with Their Hosts

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Mealybugs (Hemiptera, Coccoidea, Pseudococcidae) are plant sap-sucking insects that have within their body cavities specialized cells containing prokaryotic primary endosymbionts (P-endosymbionts). The P-endosymbionts have the unusual property of containing within their cytoplasm prokaryotic secondary endosymbionts (S-endosymbionts) [C. D. von Dohlen, S. Kohler, S. T. Alsop, and W. R. McManus, Nature (London) 412:433-436, 2001]. Four-kilobase fragments containing 16S-23S ribosomal DNA (rDNA) were obtained from the P-endosymbionts of 22 mealybug species and the S-endosymbionts of 12 representative species. Phylogenetic analyses of the P-endosymbionts indicated that they have a monophyletic origin and are members of the β-subdivision of the Proteobacteria; these organisms were subdivided into five different clusters. The S-endosymbionts were members of the γ-subdivision of the Proteobacteria and were grouped into clusters similar to those observed with the P-endosymbionts. The S-endosymbiont clusters were distinct from each other and from other insect-associated bacteria. The similarity of the clusters formed by the P- and S-endosymbionts suggests that the P-endosymbionts of mealybugs were infected multiple times with different precursors of the S-endosymbionts and once the association was established, the P- and S-endosymbionts were transmitted together. The lineage consisting of the P-endosymbionts of mealybugs was given the designation “Candidatus Tremblaya” gen. nov., with a single species, “Candidatus Tremblaya princeps” sp. nov. The results of phylogenetic analyses of mitochondrial DNA fragments encoding cytochrome oxidase subunits I and II from four representative mealybug species were in agreement with the results of 16S-23S rDNA analyses, suggesting that relationships among strains of “Candidatus T. princeps” are useful in inferring the phylogeeny of their mealybug hosts.

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Mealybugs (Hemiptera, Coccoidea, Pseudococcidae) are plant sap-sucking insects that constitute a family with about 2,000 species, some of which are major pests of agricultural plants (7, 8, 21, 32, 40, 52). They feed by inserting their thin, long mouthparts through the plant tissue to suck up phloem sap. Female mealybugs are soft, often elongate or oval, and usually attached to plant surfaces. Frequently they are covered with a mealy or cottony wax secretion. Males are short lived and rarely seen or, in some species, are absent. Most mealybug species lay eggs, and most have sexual reproduction, although some are parthenogenetic (31, 37, 51). Within the body cavity of a female mealybug is a large structure called a bacteriome, composed of large cells called bacteriocytes (9, 19, 26, 50). In the older literature, there are descriptions of a variety of different bacterial types embedded within “mucous spherules,” and it has been stressed that this is a morphologically unique type of insect endosymbiosis (9, 26, 50). Electron microscopy studies showed that the spherules were bounded by three unit membranes and that the enclosed bacterium contained two unit membranes, which is characteristic of gram-negative bacteria (26, 50). Initially, it was established that mealybugs contain primary endosymbionts (P-endosymbionts) that are members of the β-subdivision of the Proteobacteria, in marked contrast to most other described insect endosymbionts, which are in the γ-subdivision (5, 34, 38). Subsequently, it was found that mealybugs also contain secondary endosymbionts (S-endosymbionts) that are in the γ-subdivision (19, 27). Both types of endosymbionts have been localized to the mealybug bacteriome by means of in situ hybridization with specific probes complementary to their rRNA (19, 51). von Dohlen et al. (51) have recently made the remarkable observation that the mealybug endosymbiotic association has a unique organization involving a hitherto unknown property of prokaryotes. Using the species Planococcus citri and a combination of electron microscopy, confocal light microscopy, and in situ hybridization, these investigators found that the mucous spherules found within the bacteriocytes are in fact the β-proteobacterial P-endosymbionts and that the γ-proteobacterial S-endosymbionts reside within the β-proteobacterial P-endosymbionts. The three unit membranes which surround the mucous spherule are the host-derived vesicle membrane and the gram-negative cell wall of the P-endosymbiont. Previously, there were no known cases in which one prokaryote harbored another prokaryote within its cytoplasm.

Mealybugs are members of the suborder Sternorrhyncha, which also includes other scale insects, aphids, psyllids, and whiteflies (22). All of these insects feed on plant sap, a diet containing excess carbohydrate but limited amounts of essential amino acids, and all of these insects contain bacterial endosymbionts (5, 34). The association of the P-endosymbiont...
Buchnera aphidicola with aphids has been the most extensively studied association (5, 35, 53). From nutritional studies and from the sequence of the B. aphidicola genome, there is good evidence which indicates that one of the functions of the endosymbionts is synthesis of essential amino acids for the aphid host. It is probable that P-endosymbionts play a similar function in mealybugs, psyllids, and whiteflies (5, 6, 16, 28, 42).

The associations between the P-endosymbionts and aphids and psyllids, as well as several other insects groups, are a consequence of single infections of insect ancestors with different free-living bacteria and subsequent cospeciation of the endosymbionts and the hosts (3, 5, 12, 34, 44, 45, 48). The similar evolutionary trees obtained for the endosymbionts and hosts are consistent with vertical evolution of the endosymbionts (that is, no exchange of endosymbionts between different insect lineages). All of these endosymbionts are maternally inherited, and none have been cultivated in laboratory media.

It appears that one of the characteristics of these associations is acceleration of the rate of endosymbiont DNA sequence change compared to the rate of DNA sequence change in free-living bacteria (33). This has been explained as a consequence of endosymbiont population structure, in which bottlenecks in transmission to progeny result in reduced purifying selection and a possible accumulation of deleterious mutations (Muller’s ratchet) (33). Many of these insects can also have bacteriocyte-associated S-endosymbionts that are not always present in all species. Evolutionary studies have indicated that in marked contrast to P-endosymbionts, S-endosymbionts may be quite different and can become established as a result of multiple infections of insect hosts, as well as horizontal transmission (2, 18, 20, 45, 49). The function of the S-endosymbionts is not clear; under some host cultivation conditions they may have a beneficial effect, while under other conditions they may be deleterious (11). When some S-endosymbionts are injected into closely related species, they are pathogenic and result in death of the insect host (2, 10). Sodalis glossinidius, the S-endosymbiont of tsetse fly, has been grown in laboratory media (13). It has been shown that this organism requires a functional type III secretion system to establish itself in its insect host (14).

In the present study, we established the evolutionary relationships of the P-endosymbionts of 22 mealybug species based on a sequence analysis of 16S-23S ribosomal DNA (rDNA). In addition, we compared the results with the data for S-endosymbionts from 12 mealybug species. Our results suggest that the P-endosymbionts of mealybugs were infected multiple times with different precursors of the S-endosymbionts and that this association is stable, resulting in cospeciation.

**Materials and Methods**

Collection and preservation of biological material. Table 1 presents the sources of the mealybugs, together with information on the host plants and locations and dates of collection. All of the mealybugs were stored in 100% ethanol and delivered to the Davis laboratory, where they were stored at 4°C. Each sample contained between 10 and 200 mealybugs. In this paper, we use the designation P- or S-followed by the host mealybug species name to indicate P- or S-endosymbiont origin.

Molecular biology methods. The methods used are identical to those used in a study of evolutionary relationships of psyllid endosymbionts and have been described in detail (48). This includes methods for purification of DNA, amplification of the 16S-23S rDNA genes by PCR, cloning into pBluescript (Stratagene, La Jolla, Calif.), and determination of the DNA sequence. Besides the T3 and T7 primers, the following primers were used for the sequencing reactions involving P-endosymbionts (the positions of the primers are given as positions in the 16S-23S rDNA fragment of P-Dysmicoccus brevipes): 5’–3’ direction, nucleotides (nt) 486 to 507, 1028 to 1048, 1459 to 1483, 2018 to 2049, 2080 to 2028, 2397 to 2419, 2936 to 2956, 2956 to 3462, 3462 to 3465, and 3795 to 3813; and 3’–5’ direction, nt 507 to 486, 1048 to 1028, 1483 to 1459, 3462 to 3465, and 3813 to 3795. The following additional primers were also used (position in 16S-23S rDNA of the P-endosymbionts): for P-Maccelloccus australensis (5’–3’ direction), nt 2009 to 2028; and for P-Maccelloccus hirsutus (5’–3’ direction), nt 1890 to 1906. For the S-endosymbionts most of the primers used for the P-endosymbionts were used in the sequencing reactions. The exceptions were the P-endosymbiont primers at nt 2008 to 2033, 2936 to 2956, and 3795 to 3813, which were not used. The following additional primers were used for sequencing the S-endosymbionts (positions in the S-D brevipes 16S-23S rDNA sequence): 3’–5’ direction, nt 1963 to 1985, 2191 to 2208, 2914 to 2931, and 3919 to 3936; and 5’–3’ direction, nt 2511 to 2489, 2831 to 2914, and 3936 to 3919. In the case of S-Cyphonococcus alpinus, an additional primer was also used (3’–5’ direction, nt 1866 to 1882 [position in S-C. alpinus sequence]).

Similar methods were used to clone and sequence a 1.5-kb DNA fragment containing a portion of mitochondrial cytochrome oxidase subunit I (COI), tRNA^A^5^-, and a portion of cytochrome oxidase subunit II (COII) from four representative species of mealybugs (Table 1). The primers were used mtD-8 and mtD-18 described by Moran et al. (37) extended at the 5’ end by addition of a BamHI site and an EcoRI site, respectively.

**Analysis of the sequence data.** The methods used for analysis of the sequence data were described previously (23, 48, 49). The intergenic space between 16S and 23S rDNA was removed, and the resulting sequences were aligned by using Pileup of SeqWeb, version 2 (Genetics Computer Group, Madison, Wis.). Phylogenies were reconstructed by using maximum-likelihood and neighbor-joining methods and parsimony of PAUP 4.1b (46). Either 300 or 1,000 bootstrap replicates were used to assess support for the individual nodes.

**Nucleotide sequence accession numbers.** All the 16S-23S rDNA sequences were deposited in the GenBank database. The accession numbers are given in Table 1. The 16S rDNA sequences of the following organisms were also used in the analyses (accession numbers are given in parentheses): Acetobacter internedius (Y146694, Y14680), Bordetella pertussis (AF142327, X68323), Burkholderia cepacia (U96927, X16368), Burkholderia mallei (AF110188, Y17183), Neisseria meningitidis (AE002988), Ralstonia solanacearum (NC003295), and Thiomonas caprinus (U67162, X97929). Figure 4 shows the positions of the 16S rDNA sequences of the following organisms, listed in the same order as in the figure: S-Antonia cravii (AB030020), S-Pyella pyrillae (AF286125), S-Amonotoma mori (AB013087), S-Troiaz magnesium (AF077607), S-Heteroprycola cubana (AF286126), S-Yamatacollacis tokyoensis (AB064513), Blomchannia (X92550, X92552), Sodalis glossinidius (M99060), S-Pitophorus oryzae (AF005235), S-Pyella floccosa (AF286128), Arsenophonus nasoniae (M98081), Arsenophonus tratoomanurum (U91786), Proteus vulgaris (X07652), Xenobabals beidingii (X82254), T-type S-endosymbiont of aphids (AF293616), S-Bemisia argentifolii (Z11920), U-type S-endosymbiont of aphids (AF293618), Escherichia coli (AE000474), Salmonella enterica (X80681), Citobacter freundii (U77928), Klebsiella pneumoniae (AJ233420), Erwinia carotovora (Z96091), Serratia entomophila (AF133427), Serratia ficaria (AB004745), R-type S-endosymbiont of aphids (M27049), Yersinia enterocolitica (Z94828), Aeromonas hydrophila (AF090922, X67943), and Ruminobacter amylophilus (AB004908). In analyses of COI and COII, the sequence of Schizaphis graminum (accession number AF059608) was used as the outgroup.

**Results**

**General properties of the 16S-23S rDNA.** Figure 1 presents the results of PCR amplification of 16S-23S rDNA from three species of mealybugs. The bands corresponding to undigested PCR product are shown in lanes 2, 3, and 4. The major observed band is at about 4.1 kb, and in lane 3 there is an additional lighter band at 4.6 kb. Treatment with XhoI resulted in digestion of P-D. brevipes and P-P. citri, yielding fragments at about 2.8, 1.2, and 0.1 kb (lanes 5 and 6), while digestion of P-M. hirsutus resulted in fragments at 2.7, 1.3, and 0.1 kb (lane 7) (the 0.1-kb fragments are not seen on the gel). The 16S-23S rDNA of S-D. brevipes was reduced to fragments 4.0 and 0.2 kb.
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<th>Location</th>
<th>Date (mo/yr)</th>
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* The number in parentheses is the accession number for the host mitochondrial DNA fragment containing COI and COII.

b See reference 38.

**TABLE 1.** Information concerning the mealybugs used in this study and the accession numbers of the 16S-23S rDNA of the P-endosymbionts and the S-endosymbionts.
long (lane 5), while the 16S-23S rDNA of *S. P. citri* was reduced to 4.4-kb and 0.2-kb fragments (lane 6) (the 0.2-kb fragments are not visible on the gel). Treatment with *Apa*I resulted in digestion of *S. D. brevipes* to 3.3-kb and 0.9-kb fragments (lane 8, lighter bands), while the *P*-endosymbiont was not affected. In the case of *P. P. citri*, *Apa*I treatment resulted in formation of bands at 2.1 and 1.0 kb and digestion of the *S*-endosymbiont 16S-23S rDNA that yielded bands at 3.6 and 1.0 kb (lane 9). *M. hirsutus* had only a *P*-endosymbiont (lane 10). The patterns in Fig. 1 are representative of the results obtained upon amplification and digestion of 16S-23S rDNA from 22 species of mealybugs. With the exception of *Ferrisia* and *Maconellicoccus* species, which had only *P*-endosymbionts, all the remaining mealybug species had both *P*- and *S*-endosymbionts.

The lengths of the cloned 16S-23S rDNA fragments corresponding to the *P*-endosymbionts were 4,103 to 4,118 nt, and the G+C contents were 56.1 to 57.9 mol%. The lengths of the 16S rDNA portions were 1,513 to 1,522 nt, and the lengths of the 23S rDNA portions were 2,527 to 2,534 nt. The G+C contents of the 16S and 23S rDNA portions were similar (55.6 to 57.4 and 56.4 to 58.3 mol%, respectively). The nucleotide sequence of the intergenic spaces between 16S and 23S rDNA was highly conserved, and these spaces were 62 to 64 nt long; with one exception, the G+C contents were 55.6 to 60.3 mol%. The exception, the intergenic space of *C. alpinus*, had a G+C content of 49.2 mol%. Compared to the similar sizes and G+C contents of the 16S-23S rDNA-containing fragments of the *P*-endosymbionts, the sizes and G+C contents of the 16S-23S rDNA-containing fragments of the *S*-endosymbionts were heterogeneous. The latter fragments were 4,233 to 4,808 nt long, and their G+C contents were 42.7 to 49.8 mol%. The 16S rDNA portions were 1,504 to 1,528 nt long and had G+C contents ranging from 46.1 to 51.5 mol %, while the 23S rDNA portions were 2,564 to 2,628 nt long and had G+C contents ranging from 43.4 to 51.0 mol%. There were major differences in the intergenic regions, which were 142 to 671 nt long and had G+C contents of 16.9 to 38.8 mol%.

**Phylogenetic analyses.** Phylogenetic analyses of the combined 16S-23S rDNA sequences of the *P*-endosymbionts from 22 mealybug species and other members of the β-subdivision of the Proteobacteria (*B. cepacia*, *Burkholderia mallei*, *B. pertussis*, *N. meningitidis*, *R. solanacearum*, and *T. cuprinus*), as well as *A. intermedius* (an α-subdivision species) as an outgroup, strongly supported the previous conclusion (38) that the *P*-endosymbionts form a monophyletic group (100% bootstrap support in neighbor-joining and parsimony trees). Since the branch leading to the mealybugs was of considerable length, we do not show the complete tree but instead present only the portion which contains the mealybug *P*-endosymbionts in Fig. 2. As the tree shows, the *P*-endosymbionts form six clusters (clusters A to F). The major divergence is between clusters A to E and cluster F. The latter cluster consists of two species in the genus *Maconellicoccus*; one of these is native to Australia, and the other is a widespread pest. Clusters A to E contain species collected from the United States, Australia, and New...
organisms are presented in Fig. 4. This S-endosymbionts, other insect S-endosymbionts, and related letonic. The results of analyses of the 16S rDNA of mealybug endosymbionts are also formed on the basis of analyses of the 16S-23S rDNA of P- endosymbiont. The results indicate that clusters A, C, D, and E formed on the basis of the phylogenetic analyses of 16S-23S rDNA of P- and S-endosymbionts from these 12 mealybug species is presented at the nodes are bootstrap percentages based on 300 replicates; only nodes supported by bootstrap values of 70% or greater are shown. The names are the names of the mealybug hosts, and the brackets indicate major clusters.

Zealand (Table 1). The same clusters and the same order of branching were obtained with parsimony analyses. Similar results were also obtained with neighbor-joining analyses; the sole minor difference is the joining of cluster E with clusters A and B.

We also determined the sequences of the 16S-23S rDNA fragments of the S-endosymbionts from 12 species of mealybugs. A comparison of the phylogenetic trees for the P- and S-endosymbionts from these 12 mealybug species is presented in Fig. 3.

Identical or very similar trees were formed when the data were analyzed by parsimony or nearest-neighbor methods. The results indicate that clusters A, C, D, and E formed on the basis of the phylogenetic analyses of 16S-23S rDNA of P- endosymbionts are also formed on the basis of analyses of the 16S-23S rDNA of S-endosymbionts. The previous phylogenetic analyses indicated that the P-endosymbionts were monophyletic. The results of analyses of the 16S rDNA of mealybug S-endosymbionts, other insect S-endosymbionts, and related organisms are presented in Fig. 4. This figure indicates that mealybug S-endosymbiont clusters A, C, D, and E each remains coherent and distinct from the S-endosymbionts of psyllids, aphids, and other related bacteria. Similar results were also obtained in parsimony analyses, which formed the same major clusters without resolving interrelationships among them. It appears that although each of the mealybug S-endosymbiont clusters is monophyletic, these clusters have distinct origins within the large and heterogeneous group consisting of many insect-associated bacteria related to the classical Enterobacteriae. It should be noted that cluster C (Fig. 4) also contains the S-endosymbiont of A. craniit, whose 16S rDNA has been characterized previously (19). The P-endosymbiont of this insect also belongs in cluster C of Fig. 2, closely related to the P-endosymbiont of Antonina pretiosa.

Comparisons of the percentage differences in the nucleotide sequences for the 16S-23S rDNA of pairs of P-endosymbionts with the percentage differences in the nucleotide sequences for the 16S-23S rDNA of pairs of S-endosymbionts within the same cluster suggested that the rate of sequence change of some S-endosymbionts is greater than the rate of sequence change of P-endosymbionts. In cluster A, the ratio of the differences between pairs of S-endosymbionts to the differences between pairs of P-endosymbionts ranged from 1.20 to 1.94 (mean ± standard deviation, 1.46 ± 0.29), while in cluster C the range was 3.2 to 4.0 (mean ± standard deviation, 3.54 ± 0.34). In both of these comparisons, the value for the most closely related pairs (the D. brevipes-D. neobrevipes and A. brevipes-A. neobrevipes) was omitted; the small sequence differences resulted in ratios that differed from the ratios for the remaining comparisons.

Phylogenetic analyses of the mitochondrial COI and COII DNA fragments from D. brevipes (representative of clusters A and B), M. albizziae (clusters C and D), P. citri (cluster E), and M. hirsutus (cluster F) gave results consistent with the results of the analysis of 16S-23S rDNA of the P-endosymbionts (Fig. 2).

**DISCUSSION**

The results of the phylogenetic analyses indicate that on the basis of 16S-23S rDNA relationships, the P-endosymbionts of mealybugs have a monophyletic origin and can be subdivided into a number of clusters (Fig. 1). The major divergence is between clusters A to E and cluster F. If we use the previously proposed rate of sequence change of insect endosymbionts (36, 41), then the divergence between these two groups occurred 100 to 200 million years ago. This is similar to the estimated time of origin of the endosymbiotic association of other plant sap-sucking insects (41, 48).

In past studies of aphids and psyllids there was general agreement between the results of a phylogenetic analysis of endosymbionts and the taxonomy of the host (36, 48), suggesting that there was cospeciation of the host and the endosymbiont. Currently, there is no satisfactory classification of mealybug genera into higher categories (for a review see reference 7), and the only estimate of relationships is a phenetic analysis based on morphology of adult male mealybugs belonging mostly to one subfamily (1). Thus, a detailed comparison between our clusters and higher taxonomic groups is not possible. However, some interesting relationships are suggested in Fig. 2 and can be compared with ideas on mealybug classification derived from morphological studies. These studies indicate that the genera Amonos theorem, Australicoccus, Melanococcus, and Nipaeococcus all belong to the so-called blue-green or blue-black mealybugs (32, 52), which are placed either in the Trabimentinae (29, 30, 47, 52) (the definitions and compositions of this subfamily proposed by different authors are different) or in the Phenacoccinae (31). Furthermore, Antonina and related legless mealybugs are usually placed in their own subfamily, the Sphaerooccocinae (25, 47), or in their own tribe, the Sphaerococcini (15), but Koteja (31) hypothesized that they were closely related to the Phenacoccinae, a suggestion which is consistent with the endosymbiont relationships shown in Fig. 2 (i.e., cluster C). Two of the three genera (Cyphonococcus, Paraococcus, and Sarococcus) whose members contain the P-endosymbionts in cluster D have never been assigned formally.
to a subfamily or tribe, but *Paracoccus* (as *Allococcus*) has been placed in one concept of the Trabutininae and has been allied with *Planococcus* (47). Our data do not support a relationship between *Paracoccus* and *Planococcus*; however, we have sampled only the P-endosymbiont of the New Zealand species *Paracoccus nothofagicola*. Clusters A, B, and E in Fig. 2 contain P-endosymbionts from mealybug taxa that either have been referred to or inferred to belong to the Pseudococcinae (as defined by Koteja [29, 30, 31] and Williams [52]) or the Pseudococcini (15) or have not been formally allocated to a higher taxon.

*Pseudococcus* and *Dysmicoccus* are closely allied in all classifications except that of Tang (47). *Planococcus* (cluster E) and related genera (not studied here) usually are treated as a separate group, the *Planococcus* group (32) or the Planococcini (17, 52) within the Pseudococcinae. The P-endosymbionts of *Maconellicoccus* form a distinct cluster (cluster F) in Fig. 2, but based on morphology this mealybug genus has been allied either with the Planococcini of the Pseudococcinae (52), the *Planococcus* group as defined broadly by Afifi (1), or the Phenacoccinae as defined by Tang (47). The phylogenetic tree based on mitochondrial COI and COII from four representative mealybug species is consistent with the tree derived from P-endosymbiont 16S-23S rDNA (Fig. 2). From this observation, as well as past studies of the P-endosymbionts of aphids (*Buchnera*), psyllids (*Carsonella*), and other insects, we expect the P-endosymbionts to cospeciate with their mealybug hosts (5, 34, 36, 48). Thus, we consider it justifiable to form hypotheses concerning the relationships of the mealybugs based on the relationships of their P-endosymbionts. Phylogenetic analysis of the P-endosymbionts from a broader sample of mealybugs, especially members of the subfamily Rhizoecinae that were not available to us, may provide valuable insights into mealybug relationships and hence higher classification.

Previously, it has been established from relative rate tests that like other endosymbionts of plant sap-sucking insects, the mealybug P-endosymbionts have an accelerated rate of sequence change compared to that of free-living bacteria (36, 48). We confirmed this by using additional mealybug P-endos-
symbionts, as well as other related, free-living bacteria within the β-subdivision (results not shown).

von Dohlen et al. have shown that the P-endosymbionts of mealybugs are unique in that they have the potential for enclosing S-endosymbionts within their cytoplasm (51). It appears that once an infection of a P-endosymbiont with an S-endosymbiont is established, it is stable, as indicated from the cospeciation of the P- and S-endosymbionts (Fig. 3). In our collection of mealybugs, infection of the P-endosymbiont lineages probably occurred four times, each time with a different precursor of the S-endosymbiont (Fig. 2 and 3). This is consistent with the previously noted morphological diversity of what are now considered to be the S-endosymbionts (9, 26, 50). We have no explanation for the accelerated rate of sequence change observed in the S-endosymbionts of some clusters compared to the rate of sequence change of the P-endosymbionts.

Past studies of S-endosymbionts in aphids, psyllids, and tsetse flies have indicated that the association between these organisms and their insect hosts may have arisen through multiple independent infections, as well as horizontal transmission between insects (2, 18, 20, 43, 49). From Fig. 3 it is clear that members of a rather heterogeneous group related to the classical Enterobacteriaceae (γ-subdivision) have a propensity to enter into associations with insects. In some cases, such as the association between weevils and their endosymbionts (24), the association is beneficial to the host, whereas in other cases it is not clear if the association has any effect. In one study of aphid S-endosymbionts it was found that the presence of the S-endosymbionts may be beneficial or deleterious depending on the conditions of cultivation (11). In the case of one psyllid, the S-endosymbiont was a member of the β-subdivision of the Proteobacteria (46).

Currently, partially characterized bacteria that have not been cultivated on laboratory media are given the designation Candidatus (39). We propose to name the lineage corresponding to the P-endosymbionts of mealybugs “Candidatus Tremblayia” (Trembla'y.n. L. fem. n.) in honor of Ermengildo Tremblay, an Italian entomologist who has made extensive contributions to our knowledge of endosymbionts of plant sap-sucking insects. “Candidatus Tremblayia” consists of pleomorphic bacteria having a gram-negative cell wall that are enclosed in host-derived vesicles found within bacteriocytes of mealybugs (26, 50, 51). They have the unusual property of potentially harboring other bacteria within their cytoplasm. The 16S rRNA gene of these organisms is directly upstream of the 23S rRNA gene. The G+C contents of the 16S rRNA and 23S rRNA genes are 55.6 to 57.4 and 56.4 to 58.3 mol%, respectively. Based on the sequence of 16S-23S rRNA these organisms are assigned to the β-subdivision of the Proteobacteria.

“Candidatus Tremblayia” contains a single species, “Candidatus Tremblayia princeps” (princeps. N. adj., first in rank). The P-endosymbiont of D. brevipes is proposed as the type strain (GenBank accession no. AF47082). The G+C content of 64 kb of “Candidatus T. princeps” DNA is 57.1 mol% (4). The following sequences are unique to “Candidatus T. princeps”: 16S rDNA, 5′-CG(G/A) AGA TTC T(G/A)C (C/T)GA GAG GCG GAA; and 23S rDNA, 5′-CGG CCT CAG GAA ACA CCC CTA AGC AC-3′.

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