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Molecular and Histological Characterization of Primary (*Betaproteobacteria*) and Secondary (*Gammaproteobacteria*) Endosymbionts of Three Mealybug Species

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Microscopic localization of endosymbiotic bacteria in three species of mealybug (*Pseudococcus longispinus*, the long-tailed mealybug; *Pseudococcus calceolariae*, the citrophilus mealybug; and *Pseudococcus viburni*, the obscure mealybug) showed these organisms were confined to bacteriocyte cells within a bacteriome centrally located within the hemocoel. Two species of bacteria were present, with the secondary endosymbiont, in all cases, living within the primary endosymbiont. DNA from the dissected bacteriomes of all three species of mealybug was extracted for analysis. Sequence data from selected 16S rRNA genes confirmed identification of the primary endosymbiont as "*Candidatus* Tremblaya princeps," a betaproteobacterium, and the secondary endosymbionts as gammaproteobacteria closely related to *Sodalis glossinidius*. A single 16S rRNA sequence of the primary endosymbiont was found in all individuals of each mealybug species. In contrast, the presence of multiple divergent strains of secondary endosymbionts in each individual mealybug suggests different evolutionary and transmission histories of the two endosymbionts. Mealybugs are known vectors of the plant pathogen *Grapevine leafroll-associated virus 3*. To examine the possible role of either endosymbiont in virus transmission, an extension of the model for interaction of proteins with bacterial chaperonins, i.e., GroEL protein homologs, based on mobile-loop amino acid sequences of their GroES homologs, was developed and used for analyses of viral coat protein interactions. The data from this model are consistent with a role for the primary endosymbiont in mealybug transmission of *Grapevine leafroll-associated virus 3*.

Mealybugs, aphids, psyllids, and whiteflies are all plant sap-sucking insects that have cultivated intimate relationships with mutualistic bacteria since their early evolutionary history (14, 15, 22, 35). Mealybugs (Pseudococcoidae, Hemiptera) have an obligate association with prokaryotic primary endosymbionts (P-endosymbionts) of the *Betaproteobacteria* (23, 33), whose major function appears to be the synthesis of essential amino acids that are lacking in plant sap (16, 29). They are acquired through vertical maternal transmission (6, 7) and are stored within specialized cells called bacteriocytes that form well-defined organs in the mealybug's body cavity (bacteriomes) (5). Each group of these insects has its own coevolved primary endosymbionts, and phylogenetic analyses are consistent with an infection of an ancestor with a precursor of the endosymbiont, followed by a coevolutionary history of vertical transmission of the endosymbiont to progeny (5). Mealybugs are unusual in having betaproteobacterial endosymbionts; the P-endosymbionts of the other, related insects noted above are all gammaproteobacteria. In mealybugs, there can be a further layer of bacterial symbiosis, with the P-endosymbionts themselves harboring secondary endosymbiotic bacteria (S-endosymbionts) of gammaproteobacteria (45). These bacteria are present in most, but not all, mealybugs and form distinct clades, suggesting multiple evolutionary origins, and their transmission mechanism is unknown (24, 41). Gammaproteobacterial endosymbionts are known in many insect species (8), but this arrangement within the P-endosymbiont is thought to be unique to mealybugs. Thus, betaproteobacteria exist as free-living bacteria or P-endosymbionts of eukaryotes, whereas gammaproteobacteria exist as free-living bacteria, P-endosymbionts of eukaryotes, and S-endosymbionts of both prokaryotes and eukaryotes.

Mealybugs are the principal vectors of *Grapevine leafroll-associated virus 3* (GLRaV-3), an ampelovirus (26) that causes grapevine leafroll disease (9, 10; J. G. Charles and D. T. Jordan, presented at the New Zealand Grape and Wine Symposium, Auckland, New Zealand, 1993). In New Zealand, three species of mealybugs (*Pseudococcus longispinus*, the long-tailed mealybug; *Pseudococcus calceolariae*, the citrophilus mealybug; and *Pseudococcus viburni*, the obscure mealybug) are known to transmit GLRaV-3 (37). In California, two additional species, the grape mealybug (*Pseudococcus maritimus*) and the citrus mealybug (*Planococcus citri*), have also been shown to transmit the virus (20). In France, two additional species of mealybug, *Heliococcus bohemicus* and *Phenacoccus aceris* (Pseudococcidae), and the soft scale insect, *Parthenolecanium corni* (Coccidae), were shown to transmit GLRaV-3 (39). The disease is present in grapevines around the world but is a particular issue in New Zealand (J. G. Charles and D. T. Jordan, presented at the New Zealand Grape and Wine Symposium, Auckland, New Zealand, 1993), where it is usually sufficiently warm during spring and summer for mealybug populations to become very large yet not warm enough in autumn for diseased grapevines to ripen fruit adequately. GLRaV-3 is recognized by the wine industry as the biggest production threat to their economic future (11). Even if vineyards are initially mealy-

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TABLE 1 PCR primers used in this study[a]

Primer name	Organism(s) used	Specificity	Orientation	Sequence (5′–3′)
Ec8	*E. coli*	Conserved general	Forward	AGAGTTTGATCATGGCTCAGATTG
Ec1507	*E. coli*	Conserved general	Reverse	TACCTTGTTACGACTTCACCCCAG
Bpsf	Betaproteobacteria	Selective	Forward	CACATGCAAGTCGTACGGCAGCAC
Gpsf	Gammaproteobacteria	Selective	Forward	CAGRCCTAACACATGCAAGTCGAG
BGpsr	Beta- and gammaproteobacteria	Selective	Reverse	TTGTTACGACTTCACCCCAGTCAT
BGDf	Beta- and gammaproteobacteria	Conserved sequencing	Forward	CGTGCCAGCAGCCGCGGTAATACG
BGDr	Beta- and gammaproteobacteria	Conserved sequencing	Reverse	CGTATTACCGCGGCTGCTGGCACG
BGMf	Beta- and gammaproteobacteria	Conserved sequencing	Forward	ACAGGTGCTGCATGGCTGTCGTCA
BGMr	Beta- and gammaproteobacteria	Conserved sequencing	Reverse	TGACGACAGCCATGCAGCACCTGT

[a] All primers were designed against bacterial 16S rRNA genes.

bug free, it is impossible to prevent infection from airborne, dispersing young crawlers over time (12).

It has been proposed (1) that GroEL homologs (i.e., proteins homologous to the *Escherichia coli* GroEL protein) produced by endosymbionts (for mealybugs, the β-endosymbiotic bacteria, which are the primary endosymbiont and always present, were suggested), are involved in virus transmission by insect vectors. GroEL homologs might bind to the virion (in the midgut or as it is being transported into the midgut epithelium) and protect it against degradation in the hemolymph while it is being transported (specifically) to the salivary glands (1). This interaction is partly specific and may exert some control over which viruses a given host can transmit. Thus, GroEL may be essential for circulative transmission of many viruses. Since GLRaV-3 is probably transmitted circularly (13), the GroEL hypothesis is potentially applicable to GLRaV-3. Further, a recent report (18) showed that expression of the relevant endosymbiont GroEL protein in tobacco confers tolerance for a virus with a viral load decreased 1,000-fold, and the plants are essentially asymptomatic.

GroEL is a chaperonin/heat shock-induced protein in *E. coli* and functions as a complex with another protein, GroES (eukaryotic proteins Hsp60 and Hsp10 are structurally and functionally nearly identical) (25, 27). Because it was found that *E. coli* GroEL did not bind GLRaV-3 (1), these data have been interpreted as showing that the GroEL mechanism is not relevant for GLRVa3 in mealybugs (13). Stan and coworkers have developed a theoretical model to predict the binding of proteins to *E. coli* GroEL (40) using the sequence properties of the GroES mobile loop, which also fits the GroEL binding site. We have extended their model to predict potential interactions using the observed ability of various insects to transmit the differing viruses based on the viral coat proteins and the putative binding specificities of the GroEL proteins of their primary endosymbionts (based on the relevant GroES sequences).

In this study, we present data that extend previous observations of multiple evolutionary origins for secondary endosymbiotic bacteria in mealybugs to multiple origins within individual insects of two distinct species, indicating a high degree of mobility for these endosymbionts. Further, we predict interaction between GLRaV-3 coat protein and mealybug betaproteobacterial GroEL (and not with gammaproteobacterial GroEL, which has an *E. coli*-like GroES) and discuss the evolutionary consequences of these observations.

MATERIALS AND METHODS

Material. *P. longispinus*, *P. calceolariae*, and *P. viburni* mealybugs were obtained from colonies maintained on potatoes sprouting shoot and root buds at Plant and Food Research in Auckland, New Zealand, or from various plants in orchards around New Zealand.

Microscopy. Initially, whole immature adult female insects were prepared using two fixation methods: either in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 under vacuum for 1 h or in acidified 2,2-dimethoxypropane (DMP) (32). DMP rapidly dehydrates tissue by converting water to ethanol. Fixed whole insects from each species were then dehydrated and embedded in LR White resin (London Resin, Reading, United Kingdom). Serial sections (1 μm thick) were then dried onto slides, stained with 0.05% toluidine blue in benzoate buffer (pH 4.4), dried, and mounted in Shurmount (Triangle Biomedical Sciences, Durham, NC). Sections were examined by light microscopy using an Olympus Vanox AHT3 microscope (Olympus Optical, Tokyo, Japan). Both fixation methods were successful in retaining structural detail.

Once the location of the bacteriome within the insect body was understood, bacteriomes were dissected from mealybugs, isolated, and fixed as isolated organs. The isolated bacteriome, although very small, was fixed and embedded more successfully than when it was part of a whole mealybug. Isolated bacteriomes were also fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 under vacuum for 1 h, washed in buffer, postfixed in 1% osmium tetroxide, dehydrated in an ethanol series, and embedded in Spurr's resin. Sections 100 nm thick were collected on Formvar-coated copper grids and stained with 1% (vol/vol) uranyl acetate and lead citrate (38). The sections were viewed in a JEOL (Tokyo, Japan) JEM-1200EX II transmission electron microscope operating at 80 kV.

Isolation of bacteriomes and bacterial DNA. Bacteriomes were dissected from adult *P. longispinus*, *P. calceolariae*, and *P. viburni* in insect Ringer's solution (10× stock: 1,280 mM NaCl, 15 mM $CaCl_2$, 50 mM KCl, pH 7.4), dried, frozen with liquid nitrogen, and then ground separately in 1.5-ml microcentrifuge tubes prior to the extraction of DNA. Extraction of genomic DNA from ground bacteriomes was carried out following standard protocols for the DNeasy Tissue Kit (Qiagen Inc., Santa Clara, CA), except for the use of 100 μl elution buffer incubated for 5 min prior to the eluate and DNA being centrifuged through the mini-column membrane.

PCR. All PCRs were carried out using Platinum *Taq* HiFi (Invitrogen) according to the manufacturer's directions under the following conditions: 40 cycles of melting at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 68°C for 100 s, with a final extension of 5 min. The initial PCR was carried out on DNA isolated from individual bacteriomes using a universal bacterial primer pair designed from the conserved regions of the 16S rRNA gene for *E. coli* and other species (Table 1). These primers were designed to amplify an ~1,500-bp 16S sequence from alpha-, beta-, and gammaproteobacteria, i.e., no assumptions as to the nature of the endosymbionts was made. PCR products were of the expected size and were shotgun cloned into the pCR 2.1 TOPO Vector (Invitrogen) according to the manufacturer's instructions and sequenced using M13 standard sequencing primers and conserved internal sequencing primers (Table 1).

Subsequently, a more detailed study of the endosymbiont populations

TABLE 2 Bioinformatic prediction of plant virus coat proteins interacting with *E. coli* GroEL and "*Ca.* Tremblaya princeps" GroEL[a]

Genus	Virus name (acronym), accession no.	Binding of intact virus to *E. coli* GroEL (1)	Virion shape	Properties of coat protein (M_w, pI, charge, and Arg [%])	No. of *E. coli* GroES patterns[c]			*E. coli* GroEL binding prediction[b]	No. of "*Ca.* Tremblaya princeps" GroES patterns[c]			"*Ca.* Tremblaya princeps" GroEL binding prediction[b]
					4	5	6		4	5	6	
Begomovirus	Tomato yellow leaf curl virus (TYLCV), X15656	Yes	Geminate	30,285; 10.4; 22.7; 12.9	4	2	2	Yes	4	3	2	Yes
Begomovirus	African cassava mosaic virus (ACMV), AF366902	Yes	Geminate	30,129; 10.3; 22.8; 12.5	3	1	0	Yes	2	2	1	Yes
Cucumovirus	Cucumber mosaic virus (CMV), D10538	Yes	Globular	24,113; 10.3; 12.2; 13.0	2	1	1	Yes	2	2	1	Yes
Luteovirus	Bean leafroll virus (BLRV), NC 003369	Yes	Globular	21,966; 11.2; 22.3; 13	3	0	0	Yes	3	0	0	Yes
Luteovirus	Barley yellow dwarf virus (BYDV), NC 002160	Yes	Globular	21,930; 12.1; 23.2; 15.0	4	2	0	Yes	1	0	0	No
Luteovirus	Soybean dwarf virus (SbDV), NC_001747	Yes	Globular	22,201; 11.3; 22.3; 13.5	3	1	0	Yes	1	0	0	No
Luteovirus (*Enamovirus*)	Pea enation mosaic virus (PEMV), NC_003629	Yes	Globular	21,104; 11.2; 19.3; 15.6	3	2	1	Yes	3	1	1	Yes
Luteovirus (*Polerovirus*)	Potato leafroll virus (PLRV), NC_001747	Yes	Globular	23,127; 11.6; 24.2; 15.5	3	2	1	Yes	1	0	0	No
Luteovirus (*Polerovirus*)	Beet western yellows virus (BWYV), NC_003743	Yes	Globular	22,459; 11.7; 22.2; 16.0	2	1	0	Yes	1	1	1	No
Potexvirus	Potato virus X (PVX), AF260641	No	Filamentous	25,111; 7.0; 0.06; 5.3	3	2	0	Yes	3	2	1	Yes
Potyvirus	Potato virus Y M95491	No	Filamentous	29,879; 5.9; 3.5; 7.0	1	0	0	No	0	0	0	No
Tricovirus	Grapevine virus A (GVA), NC_003604	No	Filamentous	21,624; 8.4; 1.2; 6.9	3	2	0	Yes	3	2	1	Yes
Ampelovirus (*Closteroviridae*)	Grapevine leafroll virus 3 (GLRVa 3), NC_004667	No	Isometric	4,802.8; 6.78; 0.4; 3.0	4	2	1	Yes	4	2	2	Yes
Llavirus (*Bromoviridae*)	Prune dwarf virus (PDV), U31310	Yes	Isometric	23,922.2; 10.0; 9.0; 6.26	4	2	1	Yes	3	0	0	Yes
Nepovirus (*Comoviradae*)	Tobacco ringspot virus (TSRV), AF461164	No	Isometric	57,177.8; 7.25; 1.78; 6.03	6	2	2	Yes	7	0	0	Yes
Tobamovirus	Tobacco mosaic virus (TMV), NC_001367	No	Filamentous	17,620.0; 4.83; 2.04; 9.36	2	2	1	Yes	2	0	0	Yes

[a] Adapted from reference 1 with permission of H. Czosnek and Springer-Verlag Wien.

[b] In the yes/no scoring, "yes" means that the number of GroES patterns is at least 2 and that the number of contacts at each site is at least 4 in the (major) coat protein sequence (40).

[c] Pattern match scores are as follows: 4, P_HHH and H_HHH; 5, P_HHH_P and H_HHH_−; or 6, P_HHH_P_H and H_HHH_−_H for *E. coli* and "*Ca.* Tremblaya princeps," respectively (see Materials and Methods for further explanation).

was carried out on individual bacteria of each species (7 from *P. calceolariae*, 6 from *P. viburni*, and 6 from *P. longispinus*) using selective primer pairs (Table 1). Selective PCR was carried out using primer pairs designed to selectively amplify ~1,500-bp sequences from beta- and gammaproteobacteria (Table 1). These reactions used a common reverse primer that would select against alphaproteobacteria and forward primers selective for either beta- or gammaproteobacteria. PCR products of the expected size were obtained and were initially sequenced directly using the amplification primers and conserved internal sequencing primers. DNA sequence data were obtained from all individual mealybug bacteriome samples (7 from *P. calceolariae*, 6 from *P. viburni*, and 6 from *P. longispinus*). PCR products from a total of 6 of the gammaproteobacterial reactions were shotgun cloned into pCR 2.1 as described above and sequenced. All reported sequences were fully sequenced in both directions. The search for variant sequences in individuals was not exhaustive, and those sequences found for each species were used to construct the phylogenetic tree (see Fig. 5).

Phylogenetic analysis. Alignments were constructed using ClustalX (v.1.83) with the default settings (42). Manual trimming, if required, was carried out using GeneDoc (34). Phylogenetic analysis was carried out using the PHYLIP suite of software (19). The phylogenetic trees (see Fig. 4 and 5) were constructed using the neighbor-joining method. Bootstrap values (percent) were calculated from 1,000 bootstrap replicates. The trees were rooted with a distinct outgroup. TreeView (v.1.6.6) was used to display the resulting trees (36).

The GroEL binding model. GroEL is the equivalent of the eukaryotic 60-kDa chaperonin/heat shock protein, and its associated protein, GroES, is the bacterial equivalent of the eukaryotic 10-kDa chaperonin/heat shock protein. GroEL binds proteins by means of a mainly hydrophobic groove and is actively involved in their correct folding.

A model using a sequence-based approach (40) that identifies natural substrates, including viral coat proteins, for these chaperonins has been developed. The authors hypothesized that natural substrate proteins of GroEL contain two or more patterns of residues similar to that of the GroES mobile loop, i.e., G_IVL_G_A (where "_" represents an arbitrary residue).

The GroES pattern was translated into residue chemical types, hydrophobic (H), hydrophilic (P), and positively (+) and negatively (−) charged. The four classes are H (C, F, I, L, W, V, M, Y, and A), P (G, P, N, T, S, Q, and H), + (R and K), and − (D and E). A pattern may contain 4 (P_HHH), 5 (P_HHH_P), or 6 (P_HHH_P_H) GroES-like contacts. The minimum sequence separation between consecutive patterns in each sequence is 23 residues. The authors showed that *E. coli* GroEL preferentially binds to sequences similar to that of the mobile loop of GroES (P_HHH_P_H). The consensus pattern for the mobile-loop region of GroES in bacteria, including *Sodalis* spp. (see Fig. 6), generally fits the *E. coli* model.

However, in this study, we observed that eukaryotic sequences (human, zebrafish, chicken, insect [*Tribolium*], plant [*Arabidopsis*]), red alga, green alga, yeast, and fungus) consistently fit a different pattern (P_HHH_−_H) and that the sequence of "*Candidatus* Tremblaya princeps" GroES (Q8KTR9; AAM75979) is H_HHH_−_H, which nearly fits the eukaryotic pattern. The latter is the pattern searched for in Table 2 under the name of "*Ca.* Tremblaya princeps."

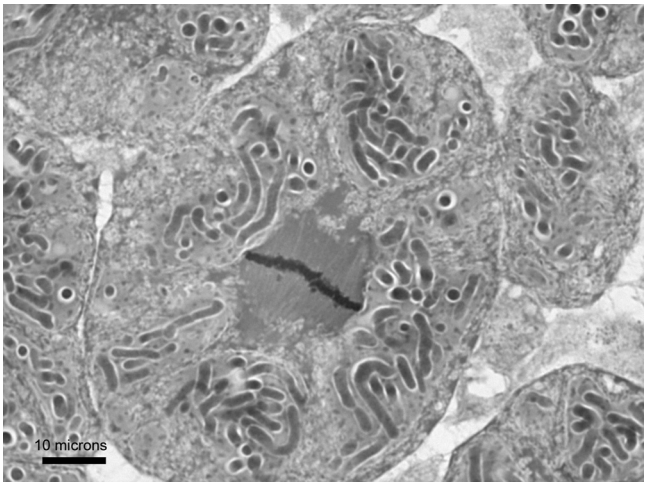

FIG 1 Light micrograph of a *P. calceolariae* bacteriocyte (shown in mitosis) containing six P-endosymbionts, each containing a number of rod-like S-endosymbionts.

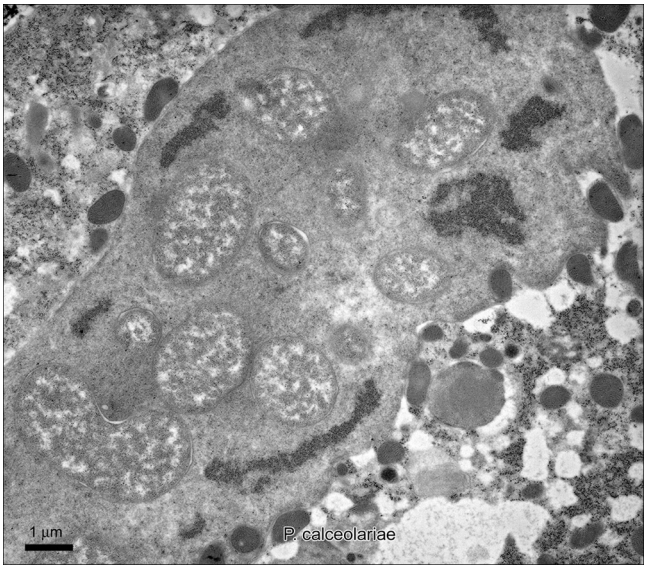

FIG 3 Transmission electron microscopy (TEM) of multiple S-endosymbiont within a P-endosymbiont in a bacteriocyte of *P. calceolariae*.

Nucleotide sequence accession numbers. The accession numbers of the three species' consensus betaproteobacterial sequences are as follows: *P. longispinus*, JN182336; *P. viburni*, JN182337; and *P. calceolariae*, JN182335.

RESULTS

Microscopic localization of endosymbionts. Bacteriomes were identified in a number of individuals from each species of mealybug. The basic structures of the bacteriomes from all three species, as observed using light microscopy (Fig. 1 and 2; *P. viburni* not shown), were similar. The bacteriome (approximately 300 to 500 by 150 to 200 μm) was located centrally, and each bacteriome contained ~100 bacteriocytes. Each bacteriocyte was an insect cell containing 8 to 10 globular primary bacteria. Each primary bacterium contained a number (on the order of 10 to 20) of rod-like secondary bacteria.

The bacteriomes of two species, *P. calceolariae* and *P. viburni*, were examined by electron microscopy (Fig. 3; *P. viburni* not shown). Secondary bacteria, in particular, have good structure and a clearly visible membrane structure. The structures in the two species were very similar.

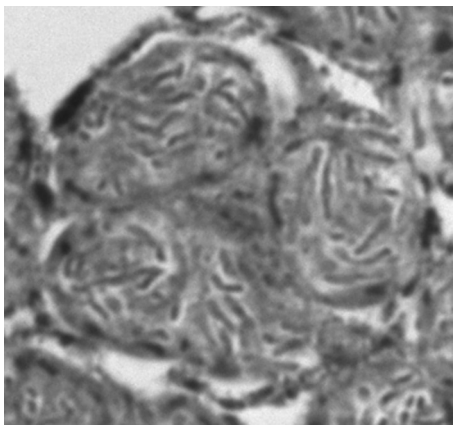

FIG 2 Light micrograph of a *P. longispinus* bacteriocyte containing four P-endosymbionts, each containing a number of rod-like S-endosymbionts.

Initial PCR-based identification of endosymbionts. Initial PCR was performed using the general primer pair based on *E. coli* (Table 1). These primers were designed to amplify any proteobacterium within the bacteriome. PCR products of the expected length (1,500 bp) were obtained from each of two individuals of each species. Twelve random clones from each individual bacterium obtained by shotgun cloning of these products were selected for sequencing.

From these data, we observed that the sequences comprised sequences homologous to those of betaproteobacteria, multiple sequences homologous to those of gammaproteobacteria, and two sequences from a single individual closely homologous to those of alphaproteobacterial soil bacteria and presumed to be contaminants. For the betaproteobacterial type, within any one species, the sequence was invariant, apart from occasional single base changes in the clones consistent with PCR errors and single/double base calls consistent with sequencing compressions (with the exceptions of a C-G and a G-T base change, each in a single clone). The gammaproteobacterial sequences obtained by this method showed variability: *P. calceolariae* yielded a single sequence and *P. viburni* a number of closely homologous but nonidentical sequences.

Analysis of the three species' consensus betaproteobacterial sequences using annotated BLAST (Fig. 4) showed that the P-endosymbionts were "*Candidatus* Tremblaya princeps." The sequences across the three species were highly homologous (~98%) to each other and to the already-published sequences for the P-endosymbionts from *P. viburni* (AF476095) and *P. longispinus* (M68889 and AF476093). Comparison of our sequences with those published showed that all differences were single base changes or single-base indels. The three published sequences referred to are identical to each other in the region where they overlap, despite being from two different host insect species, and identical to our *P. viburni* sequence, though different from our sequence from *P. longispinus*.

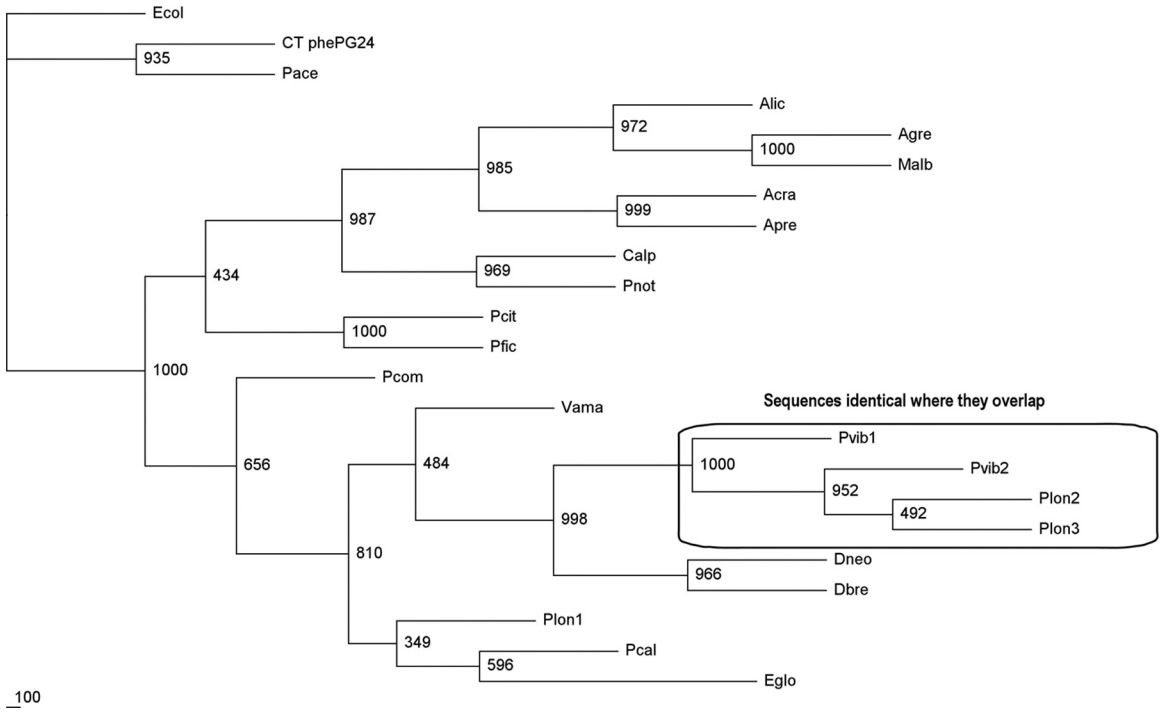

FIG 4 Primary (betaproteobacterial) endosymbiont 16S ribosomal sequence phylogenetic tree. All sequences are those of the 16S RNA genes isolated from the betaproteobacterial symbionts of the named pseudococcid mealybug species. The sequence for "*Candidatus* Tremblaya princeps" from *P. calceolariae* is a first publication and supports a recent mealybug phylogeny study (21). The 16S ribosomal sequence from the free-living gammaproteobacterium *Escherichia coli* W3110 (NCBI accession no. AP009048, annotated Ecol) was used as the outgroup.

Possible explanations for these observations include variability between geographically isolated mealybug populations, species misidentification, cross-contamination, or even problems in mealybug taxonomy. In our study, we did not observe any differences in P-endosymbiont sequences among those obtained from endosymbionts isolated from a single individual mealybug that could not be interpreted as PCR errors in individual clones and none in the total PCR product sequences (see below). Our data represent the first sequences for the P-endosymbiont from *P. calceolariae.*

Based on annotated BLAST, the S-endosymbiont (gammaproteobacterial) sequences are most similar to those of strains of *Sodalis glossinidius* and are homologous to each other and to other bacteria from this group (Fig. 5). which are P-endosymbionts in some insects and S-endosymbionts in others (46). They are highly homologous to gammaproteobacterial sequences from the mealybug *Pseudococcus comstocki* (AB374418) (24). The name "*Candidatus* Moranella endobia" has very recently been proposed for these bacteria (28). We did not find sequences suggesting S-endosymbionts in any individual *P. longispinus* mealybug by this method, although the search was not exhaustive.

Selective PCR analysis of identified endosymbionts. The PCR products using the selective primers for betaproteobacteria were sequenced directly, and the sequences were identical to those obtained initially using the universal primer pair (see above). These consensus sequences were used for phylogenetic analysis (Fig. 4) and NCBI submission. The description of sequences used for phylogenetic analysis are shown in Table 3.

For the gammaproteobacteria, sequencing of the PCR products from the selective primers yielded consistent "mass average" sequences but with clear evidence of sequence variability that was consistent for each of the mealybug species. However, the consensus sequences for the gammaproteobacteria were not identical to those of the relevant clones obtained initially. In order to resolve this problem, PCR products from three individual insects, obtained using the selective primers for gammaproteobacteria, were cloned and sequenced (3 each from two individual *P. viburni* mealybugs and 2 from a single *P. calceolariae* mealybug). In all three individual bacteria, the gammaproteobacterial sequences in a given sample bacteriome showed the presence of a population comprising up to three distinct sequences, with only 96% identity between the two most divergent sequences. This represented sequence variation that was greater than that observed between the primary β-endosymbionts from any two of the mealybug species. A phylogenetic analysis of the gammaproteobacteria is shown in Fig. 5, and the sequences used are shown in Table 4.

Also detected under these conditions were sequences closely homologous to those of *Pseudomonas* and *Acinetobacter* species; both are gammaproteobacteria common in soil and marine samples and not known to include endosymbiont species. They were presumed to be contaminants from the diet, environment, gut, or possibly the hemolymph and are not further discussed. No gammaproteobacteria closely homologous to *S. glossinidius* were detected in *P. longispinus* by the above-mentioned molecular methods, but S-endosymbionts were clearly visible within the P-endosymbionts by light microscopy (Fig. 2).

Predicted endosymbiont GroEL-virus interactions. An alignment of GroES and HSP10 proteins is shown in Fig. 6, and the sequences used are described in Table 5. The bacterial consensus GXIVLXGXA (P_HHH_P_H) is shared by the free-living gammaproteobacterium *E. coli* and the other gammaproteobacterial

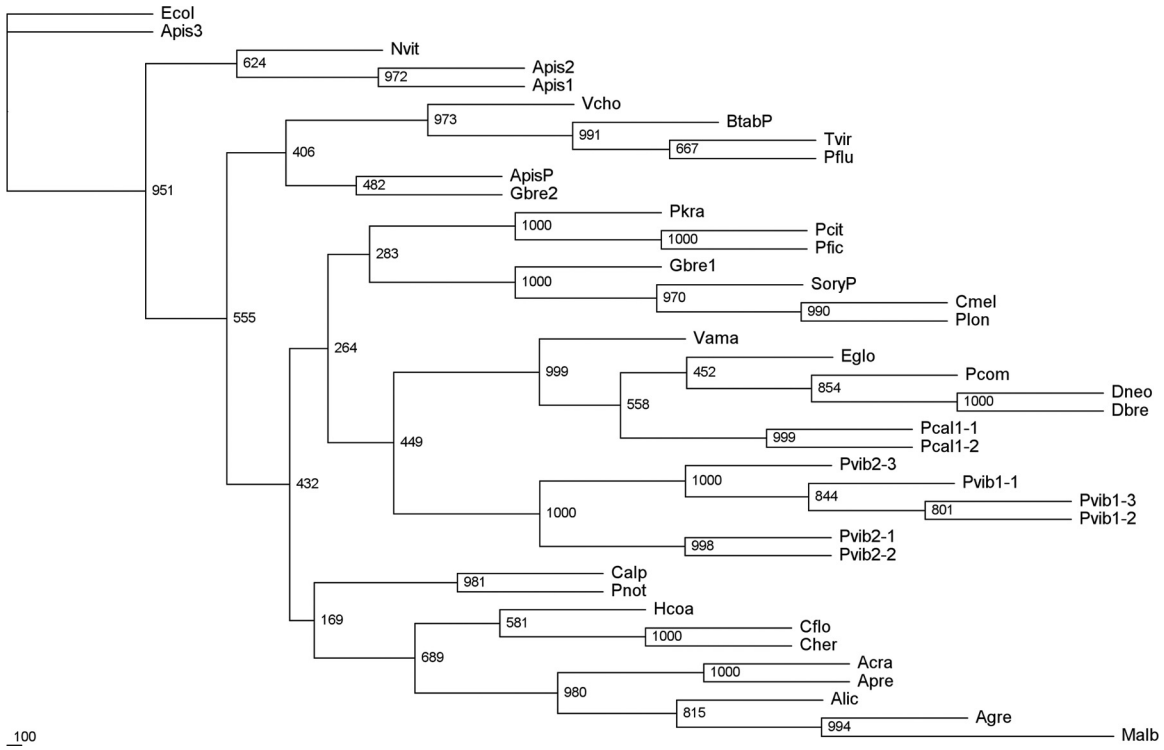

FIG 5 Secondary (gammaproteobacterial) endosymbiont 16S ribosomal sequence phylogenetic tree. All sequences, except three free-living gammaproteobacterial species, are those of the 16S RNA genes from gammaproteobacterial symbionts of the named insect species. Known primary endosymbionts have "P" suffixed. The 16S ribosomal sequence from the free-living gammaproteobacterium *E. coli* W3110 (NCBI accession no. AP009048, annotated Ecol) was used as the outgroup.

TABLE 3 Primary (betaproteobacterial) endosymbiont 16S ribosomal sequences used in Fig. 4[a]

Species used for isolation	16S Beta accession no.	Tree annotation
Amonostherium lichtensioides	AF476078	Alic
Antonina crawii	AB030021	Acra
Antonina pretiosa	AF476079	Apre
Australicoccus grevilleae	AF476077	Agre
Cyphonococcus alpines	AF476081	Calp
Dysmicoccus brevipes	AF476082	Dbre
Dysmicoccus neobrevipes	AF476083	Dneo
Erium globosum	AF476084	Eglo
Melanococcus albizziae	AF476087	Malb
Paracoccus nothofagicola	AF476094	Pnot
Phenacoccus aceris	HM449982	Pace
Phenacoccus solani	HM449979	Psol
Planococcus citri	AF322017	Pcit
Planococcus ficus	AF476092	Pfic
Pseudococcus calceolariae[b]	JN182335	Pcal
Pseudococcus comstocki	AB374416	Pcom
Pseudococcus longispinus[b]	JN182336	Plon1
Pseudococcus longispinus	AF476093	Plon2
Pseudococcus longispinus	M68889	Plon3
Pseudococcus viburni[b]	JN182337	Pvib1
Pseudococcus viburni	AF476095	Pvib2
Vryburgia amaryllidis	AF476097	Vama

[a] All sequences are those of the 16S RNA gene from the betaproteobacterial symbiont isolated from the named pseudococcid mealybug species. The 16S ribosomal sequence from the free-living gammaproteobacterium *E. coli* W3110 (NCBI accession no. AP009048, annotated Ecol) was used as the outgroup.
[b] Consensus sequence.

symbionts except "*Candidatus* Carsonella ruddii," which has GSI FLPFND, which is P_HHH_H_− in terms of hydrophobicity. The free-living betaproteobacterium *Neisseria gonorrhoeae* also fits the P_HHH_P_H consensus. The protist amoeba is the divergent eukaryote GGIFIPTNK/P_HHH_P_+ (as might be expected), and the *Arabidopsis thaliana* chloroplast has diverged from its (presumed) ancestral bacterial sequence to an apparently novel sequence, GGVLLPKAA/P_HHH_+_H, with a lysine at the 7th position. Species of the mealybug S-endosymbiont have the typical prokaryotic sequence for the mobile loop of the GroES protein. However, unexpectedly, the GroES mobile loop of "*Ca.* Tremblaya princeps" (the P-endosymbiont in mealybugs; Q8KTR9; AAM75979) has a binding pattern of CGIVIPDSA/H_HHH_−_H, which closely resembles the eukaryotic consensus sequence GGIVLPEKA/P_HHH_−_P and is quite distinct from the prokaryotic consensus sequence present in other known endosymbionts (Fig. 6). The seventh position is aspartic acid, similar to eukaryotic sequences, which have glutamic acid at this position, and quite distinct from other prokaryotic sequences, which have glycine at the seventh position. Matching the charged residue in the "*Ca.* Tremblaya princeps" pattern is thought to be the crucial point of difference from the *E. coli* prediction (40). The *E. coli* experimental and model results are in fair agreement, considering that the experimental results (Table 2) show binding of GroEL to whole intact viral particles and the model predicts binding to the denatured major viral coat protein. Further, in a personal communication, G. Lorimer (University of Maryland) noted that "your experimental analysis of plant viral coat proteins and their interaction with GroEL, based only on sequence, seems to work

TABLE 4 Secondary (gammaproteobacterial) endosymbiont 16S ribosomal sequences used in Fig. 5[a]

Common name	Species used for isolation	16S Gamma NCBI accession no.	Tree annotation[b]	Gammaproteobacterium
Aphid	*Acyrthosiphon pisum*	M27039	ApisP	*Buchnera aphidicola*
Aphid	*Acyrthosiphon pisum*	AF293616	Apis1	"*Candidatus* Hamiltonella defensa"
Aphid	*Acyrthosiphon pisum*	AF293618	Apis2	"*Candidatus* Regiella insecticola"
Aphid	*Acyrthosiphon pisum*	AB033777	Apis3	"*Candidatus* Serratia" symbiotic
Bloodsucking fly	*Craterina melbae*	EF174495	Cmel	*Sodalis* sp.
Carpenter ant	*Camponotus floridanus*	X92549	Cflo	"*Candidatus* Blochmannia floridanus"
Carpenter ant	*Camponotus herculeanus*	X92550	Cher	"*Candidatus* Blochmannia herculeanus"
	Free-living bacterium	NC_012660	Pflu	*Pseudomonas fluorescens*
	Free-living bacterium	AP009048	Ecol	*Escherichia coli* W3110
	Free-living bacterium	AJ294747	Tvir	*Thalassomonas viridians*
	Free-iving bacterium	X74694	Vcho	*Vibrio cholerae*
Jewel wasp	*Nasonia vitripennis*	M90801	Nvit	*Arsenophonus nasoniae*
Mealybug	*Antonina pretiosa*	AF476101	Apre	
Mealybug	*Amonostherium lichtensioides*	AF476100	Alic	
Mealybug	*Antonina crawii*	AB030020	Acra	
Mealybug	*Australicoccus grevilleae*	AF476099	Agre	
Mealybug	*Cyphonococcus alpines*	AF476102	Calp	
Mealybug	*Dysmicoccus brevipes*	AF476103	Dbre	
Mealybug	*Dysmicoccus neobrevipes*	AF476104	Dneo	
Mealybug	*Erium globosum*	AF476105	Eglo	
Mealybug	*Melanococcus albizziae*	AF476106	Malb	
Mealybug	*Paracoccus nothofagicola*	AF476109	Pnot	
Mealybug	*Planococcus citri*	AF322016	Pcit	
Mealybug	*Planococcus ficus*	AF476108	Pfic	
Mealybug	*Planococcus kraunhiae*	AB374417	Pkra	
Mealybug	*Pseudococcus calceolariae*	JN182338	Pcal1-1	
Mealybug	*Pseudococcus calceolariae*	JN182339	Pcal1-2	
Mealybug	*Pseudococcus comstocki*	AB374418	Pcom	
Mealybug	*Pseudococcus longispinus*	EU727120	Plon	
Mealybug	*Pseudococcus viburni*	JN182340	Pvib1-3	
Mealybug	*Pseudococcus viburni*	JN182341	Pvib1-2	
Mealybug	*Pseudococcus viburni*	JN182342	Pvib1-1	
Mealybug	*Pseudococcus viburni*	JN182343	Pvib2-3	
Mealybug	*Pseudococcus viburni*	JN182344	Pvib2-1	
Mealybug	*Pseudococcus viburni*	JN182345	Pvib2-2	
Mealybug	*Vryburgia amaryllidis*	AF476110	Vama	
Sharpshooter	*Homalodisca coagulata*	AF465793	Hcoa	"*Candidatus* Baumannia cicadellinicola"
Tsetse fly	*Glossina brevipalpis*	AP008232	Gbre1	*Sodalis glossinidius* strain Morsitans
Tsetse fly	*Glossina brevipalpis*	BA000021	Gbre2	*Wigglesworthia glossinidia*
Weevil	*Sitophilus oryzae*	AF005235	SoryP	
Whitefly	*Bemisia tabaci*	Z11925	BtabP	"*Candidatus* Portiera aleyrodidarum"

[a] All sequences except three from free-living gammaproteobacteria are those of the 16S RNA gene from the gammaproteobacterial symbiont isolated from the named insect species. The 16S ribosomal sequence from the free-living gammaproteobacterium *E. coli* (NCBI W3110) was used as the outgroup.

[b] Known P-endosymbionts are annotated with the suffix "P." Sequences from individual insects (this study) are annotated as *x-y*, where *x* is individual insect and *y* is independent sequence.

quite well with highly charged plant viral coat proteins but fails with proteins that are only slightly charged. Obviously we are missing an electrostatic component. A similar conclusion was reached based on analysis of barnase mutants with GroEL" (4).

Using our variation (see Materials and Methods) of the existing binding model (40), GLRaV-3 has two perfect binding sites for a "*Ca.* Tremblaya princeps" GroEL homolog in its coat protein but only imperfect sites for the prokaryotic GroEL pattern (Table 2). This predicts that the "*Ca.* Tremblaya princeps" GroEL homolog is able to bind GLRaV-3 coat protein whereas *E. coli* GroEL cannot, the latter observation having been confirmed (1). The data suggest a role for "*Ca.* Tremblaya princeps" GroEL in viral transmission in mealybugs. Predictions by computational studies of hydrophobic sites that remain exposed on the surfaces of assembled viral particles and hence available for binding GroEL are possible, given X-ray crystal structures of assembled viral coats or their equivalent, but are beyond this investigation.

DISCUSSION

Previous studies have used 16S RNA sequences of mealybugs to link the sequence variation of P-endosymbionts with the evolutionary histories of their insect hosts and proposed cospeciation (5). Our data are consistent with this hypothesis. In the single case of the divergent

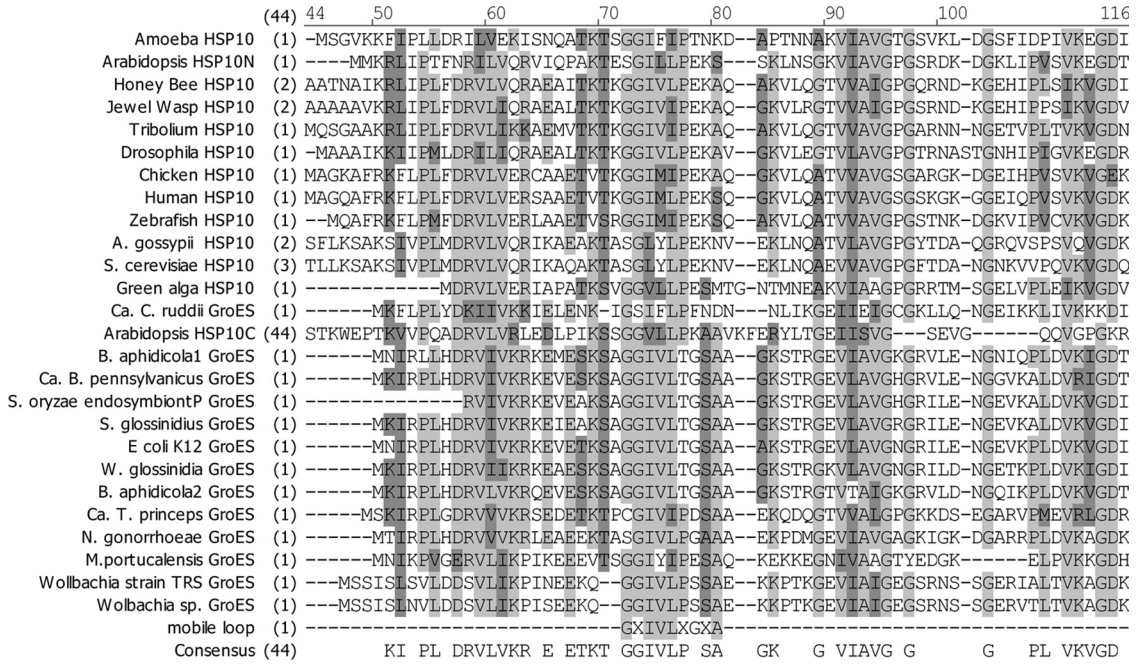

FIG 6 Clustal alignment of HSP10 and GroES sequences from diverse organisms. Light gray, conserved sequence; dark gray, block of similar sequence, as defined in AlignX (Vector NTI, Invitrogen).

sequence we have identified from *P. longispinus*, it is possible that some sequence drift occurs within these essentially clonally replicating species. However, the alternative explanations (species misidentification and cross-contamination) seem more likely.

The situation with the S-endosymbionts is quite different and complex, showing multiple and divergent sequences within individual mealybugs. The possibility exists that at least some of these divergent ribosomal sequences are chimeric in nature and hence artifactual. Chimera formation in PCR amplification is the most frequent 16S rRNA gene artifact currently displayed by new submissions to the public repositories (3). Chimeras tend also to be the most insidious artifacts, since if undetected they can be responsible for spurious phylogenetic reconstructions, inaccurate taxonomic identifications, and overestimations of microbial diversity. However, the nature of the variations seen in the data (short indels and base changes inconsistent with simple PCR errors) and the close homology with published *S. glossinidius* sequences over their entire lengths are inconsistent with the six *P. viburni* sequences being gross cross-species chimeras. While it is still possible for them to represent chimeras between close (*S. glossinidius*-like) species, this would not invalidate the observation of multiple strains of S-endosymbionts in an individual mealybug, as multiple strains would still need to be present to create the hypothetical chimeras. The two *P. calceolariae* sequences present insufficient data to draw conclusions in this matter.

Sequence differences in the regions of the PCR primers may explain our failure to detect S-endosymbionts in *P. longispinus*, as may the *P. longispinus* S-endosymbionts being from some more divergent group of bacteria. Another explanation is the possibility that the *S. glossinidius*-like sequences from the other two mealybug species were contaminants, independent endosymbionts, or exosymbionts. This is unlikely, because *S. glossinidius*-related species have been detected in related mealybugs (5, 24, 28, 41). Furthermore, other bacteria were not seen in the photomicrographs, meaning that, if present, they would be at very low concentrations and hence unlikely to be detected by the initial molecular method employed. Finally, there is a report of a partial sequence for a *P. longispinus* gammaproteobacterium endosymbiont 16S gene (17) that groups closely with the *Sodalis* clade and only more loosely with the other mealybug S-endosymbiont clades (Fig. 5).

Thao et al. (41) found S-endosymbionts in many mealybug species, including *P. citri*, *Planococcus ficus*, and *Paracoccus nothofagus* (a New Zealand species), but did not detect S-endosymbionts in *Pseudococcus* species. The sequences for these mealybug species are closely homologous (Fig. 1, AF476107, AF476108, and AF476109) to the *S. glossinidius* sequences and our individual sequences from *Pseudococcus* species. They concluded that the S-endosymbionts had infected their host P-endosymbionts multiple times and coevolved with them, i.e., across a wide range of mealybug species, independent infections occurred, events that then resulted in coevolution. A recent study (24) supports this conclusion, which is in contrast with the results from tsetse flies (2), where no evidence for coevolution of the S-endosymbiont was found. Our data extend previous studies on mealybug S-endosymbiont sequences through the identification of multiple sequences isolated from each individual insect examined where an S-symbiont was detected. This was achieved by sequencing clones from PCR products rather than sequencing PCR products directly, where we had also observed only consensus sequences. It is possible that the sequences previously reported are consensus sequences similar to those we obtained by direct sequencing of PCR products and that multiple sequences also occur in these other mealybug species but were not detected by the methods used in the earlier studies.

The reason for this variability is unknown. The mealybug S-endosymbiont is closely related phylogenetically to *S. glossinidius*, an S-endosymbiont of tsetse flies that is able to live both

TABLE 5 HSP10 and GroES sequences from diverse organisms[a]

Species	NCBI accession no.	Alignment annotation	Organism	Note
Wolbachia strain TRS	YP_198180	*Wolbachia* strain TRS GroES	Alphaproteobacterium	Endosymbiont of nematode *Brugia malay*
Wolbachia sp.	NP_966108	*Wolbachia* sp. GroES	Alphaproteobacterium	Symbiont of fruitfly *Drosophila melanogaster*
"*Candidatus* Tremblaya princeps"	Q8KTR9	"*Ca.* Tremblaya princeps" GroES	Betaproteobacterium	P-endosymbiont of mealybugs
Neisseria gonorrhoeae	YP_002003183	*N. gonorrhoeae* GroES	Betaproteobacterium	Free living
Buchnera aphidicola	Q9F4E4	B. aphidicola1 GroES	Gammaproteobacterium	P-symbiont of aphids
Buchnera aphidicola	AAC04236	B. aphidicola2 GroES	Gammaproteobacterium	P-endosymbiont of aphid *Myzus persicae*
"*Candidatus* Blochmannia pennsylvanicus"	YP_277588	"*Ca.* Blochmannia pennsylvanicus" GroES	Gammaproteobacterium	P-symbiont of the carpenter ant
"*Candidatus* Carsonella ruddii"	YP_802449	"*Ca.* Carsonella ruddii" GroES	Gammaproteobacterium	P-endosymbiont of psyllids
Escherichia coli	NP_418566	*E. coli* K12 GroES	Gammaproteobacterium	Free living
Unnamed	AAB97669.1	*S. oryzae* P-endosymbiont GroES	Gammaproteobacterium	Endosymbiont of aphid *Sitophilus oryzae*
Sodalis glossinidius	YP_453985	*S. glossinidius* GroES	Gammaproteobacterium	S-endosymbiont of tsetse fly *Glossina morsitans morsitans*
Wigglesworthia glossinidia	NP_871262	*W. glossinidia* GroES	Gammaproteobacterium	P-endosymbiont of tsetse fly *Glossina brevipalpis*
Methanohalophilus portucalensis	ABO16620	*M. portucalensis* GroES	Archaeon	
Dictyostelium discoideum	XP_636819	Amoeba HSP10	Protist	
Saccharomyces cerevisiae	NP_014663	*S. cerevisiae* HSP10	Yeast	
Ashbya gossypii	NP_984626	*A. gossypii* HSP10	Fungus	
Apis mellifera	XP_624910	Honey bee HSP10	Bee	
Tribolium castaneum	XP_975179	Tribolium HSP10	Beetle	
Drosophila melanogaster	NP_648622	Drosophila HSP10	Fruitfly	
Nasonia vitripennis	XP_00159999	Jewel wasp HSP10	Wasp	
Ostreococcus lucimarinus	XP_001421602	Green alga HSP10	Alga	
Arabidopsis thaliana	NP_563961	Arabidopsis HSP10N	Dicot	Nuclear gene
Arabidopsis thaliana	NP_566022	Arabidopsis HSP10C	Dicot	Chloroplast gene
Gallus gallus	NP_990398	Chicken HSP10	Bird	
Danio rerio	NP_571601	Zebrafish HSP10	Fish	
Homo sapiens	NP_002148.1	Human HSP10	Mammal	

[a] These sequences were used in the alignment in Fig. 6.

inter- and intracellularly in various host tissues, including the midgut and hemolymph. Phylogenetic studies have not indicated a correlation between the evolution of *Sodalis* spp. and tsetse flies, unlike the tsetse fly P-endosymbiont, *Wigglesworthia* (2), and the mealybug endosymbiont "*Ca.* Tremblaya princeps" (5). It seems that *S. glossinidius*-related species are more parasitic than endosymbiotic and that it is routinely and frequently acquired and may be able to be passed from one individual insect to another. *Sodalis* species have been cultured *in vitro* in insect cell lines from species widely diverged from their normal hosts (46), and members of the genus have a genome about 50% the size of the *E. coli* genome (43), as opposed to that of "*Ca.* Tremblaya princeps" at 10%. Erosion of the genome is a characteristic of symbionts. *S. glossinidius*-related species therefore may even have characteristics suitable for maintaining a nonsymbiont life stage in nature, although this has not been observed to our knowledge. It certainly appears to be able to pass from insect to insect with its ability to grow in nonhost cells, whereas normal endosymbionts are unable to survive outside the cells of their usual host. Certainly, individuals of an essentially clonally replicating species carrying multiple strains of an endosymbiotic bacterium strongly suggests multiple acquisitions over time.

The GroES mobile-loop sequence convergence of "*Ca.* Tremblaya princeps" with the eukaryotic HSP10 mobile loop is a novel observation in this study and provides an explanation for a role for "*Ca.* Tremblaya princeps" in GLRaV-3 transmission. A role in viral transmission for S-endosymbionts is correspondingly remote. The convergence is, however, unlikely to be a selection to bind the virus coat, since no apparent advantage to the endosymbiont results from the interaction. The convergence might be better explained as a presumed convergence of "*Ca.* Tremblaya princeps" GroEL with eukaryotic HSP60. However, because the binding site on the GroEL homologs contains contacts from several parts of the GroEL molecule, this can only be determined from the GroES mobile-loop sequence. We hypothesize that "*Ca.* Tremblaya princeps" has a selective advantage in the ability of its GroEL to recognize, bind, and fold eukaryotic proteins, *viz.*, the proteins of its host mealybug. It seems probable that this ability is a key reason why the endosymbiont has been able to substitute eukaryotic proteins for its own in a folded and hence active form following transport of unfolded protein across the bacterial membrane from the host. Thus, we believe that GroEL convergence to the specificity of the host of this primary endosymbiont is a major factor in allowing the observed genome erosion to occur.

Is convergence of prokaryotic GroEL with eukaryotic GroEL a common feature of endosymbionts and other degraded prokaryotic genomes, such as mitochondria and chloroplasts? If this is likely, we should observe typical prokaryotic GroES mobile loops in free-living bacteria and in the S-endosymbiont of mealybugs that lives within a bacterium rather than a eukaryote but converged GroES mobile loops in P-endosymbionts. Figure 6 shows some tantalizing hints, but further studies are required to explore this hypothesis.

Of particular relevance to the research on the mitigation of grapevine leafroll disease is a study that has implicated the endosymbiotic bacteria of the whitefly *Bemisa tabaci* in that insect's ability to transmit tomato yellow leaf curl virus (31). At first glance, this may not seem relevant to mealybugs, because the received wisdom is that GLRaV-3 is transmitted via a semipersistent mechanism whereby the virus is restricted to the fore- and/or midgut. If this is the case, then it is difficult to see what effects the endosymbionts, which are restricted to their bacteriocyte on the other side of the gut wall, might have on the ability of a mealybug to transmit a plant virus. However, very recent research suggests that GLRaV-3 may, in fact, be circulatively transmitted in mealybugs (13). This means that the virus is transported from the gut to the salivary glands and hence must cross the gut wall into the hemocoel. This transmission mechanism is considerably more complex and allows much greater opportunities for the endosymbionts to have an impact.

However, the mealybug species that was studied (13) transmits GLRaV-3 much more efficiently than GLRaV-1, and hence, there is a specific and selective step in the process that transports (and protects) the virus from the gut through the hemolymph to the salivary glands. Others (1, 30, 31, 41) have found that (for other insect-transmitted viruses in sucking insects) a specific region of the intact virus (i.e., the assembled viral coat) binds to the endosymbiont GroEL and that mutant viruses do not persist in the insect. For the virus to be specifically transported from the gut to the salivary glands without being degraded there must be some protein specifically binding to the virus. If this protein is the endosymbiont GroEL homolog, which is not identical in sequence to *E. coli* GroEL, then the fact that the virus does not bind to *E. coli* GroEL does not conflict with or invalidate the hypothesis.

After initial submission of this paper, details of a novel inversion in the genome of "*Ca.* Tremblaya princeps" were published, as well as a sequence and proposed name ("*Candidatus* Moranella endobia") for the secondary endosymbionts (28). These data raise similar questions about the evolution and maintenance of multiple genetic elements in linked genomes where no compelling case for a selective advantage can readily be made.

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