

ORIGINAL ARTICLE

Convergent patterns in the evolution of mealybug symbioses involving different intrabacterial symbionts

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Mealybugs (Insecta: Hemiptera: Pseudococcidae) maintain obligatory relationships with bacterial symbionts, which provide essential nutrients to their insect hosts. Most pseudococcinae mealybugs harbor a unique symbiosis setup with enlarged betaproteobacterial symbionts ('*Candidatus Tremblaya princeps*'), which themselves contain gammaproteobacterial symbionts. Here we investigated the symbiosis of the manna mealybug, *Trabutina manipara*, using a metagenomic approach. Phylogenetic analyses revealed that the intrabacterial symbiont of *T. manipara* represents a novel lineage within the *Gammaproteobacteria*, for which we propose the tentative name '*Candidatus Trabutinella endobia*'. Combining our results with previous data available for the nested symbiosis of the citrus mealybug *Planococcus citri*, we show that synthesis of essential amino acids and vitamins and translation-related functions partition between the symbiotic partners in a highly similar manner in the two systems, despite the distinct evolutionary origin of the intrabacterial symbionts. Bacterial genes found in both mealybug genomes and complementing missing functions in both symbioses were likely integrated in ancestral mealybugs before *T. manipara* and *P. citri* diversified. The high level of correspondence between the two mealybug systems and their highly intertwined metabolic pathways are unprecedented. Our work contributes to a better understanding of the only known intracellular symbiosis between two bacteria and suggests that the evolution of this unique symbiosis included the replacement of intrabacterial symbionts in ancestral mealybugs.

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Introduction

Mealybugs (Insecta: Hemiptera: Pseudococcidae) are phloem sap-feeding insects, which harbor a unique intrabacterial symbiotic system within their bacteriome, a large specialized organ in the abdomen. Bacteriocytes of most pseudococcinae mealybugs (Hardy *et al.*, 2008) contain betaproteobacterial symbionts, which themselves host gammaproteobacterial symbionts within their cytoplasm (von Dohlen *et al.*, 2001). Similar to other plant sap-feeding

insects (Baumann, 2005; Moran *et al.*, 2008), these bacterial symbionts provide essential amino acids (and vitamins) scarce in the phloem diet of the mealybugs (McCutcheon and von Dohlen, 2011). The monophyletic betaproteobacterial lineage, named *Tremblaya*, and the insect hosts show congruent phylogenies, indicating a single infection followed by long-term co-speciation in ancestral mealybugs (Fukatsu and Nikoh, 2000; Thao *et al.*, 2002; Baumann and Baumann, 2005; Downie and Gullan, 2005; Gruwell *et al.*, 2010). However, *Tremblaya* was likely infected by different enterobacterial precursors in different mealybug lineages, as suggested by phylogenetic analyses of the 16S and 23S ribosomal RNA genes of these secondary symbionts (Fukatsu and Nikoh, 2000; Thao *et al.*, 2002). Thus the unique nested symbiosis of mealybugs, also referred to as 'a bug in a bug in a bug', likely emerged multiple times during evolution (Thao *et al.*, 2002).

To date, genome sequences of the symbiont pair of the citrus mealybug *Planococcus citri* ('*Candidatus Tremblaya princeps*' and '*Candidatus Moranella*

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endobia') (McCutcheon and von Dohlen, 2011; López-Madriral *et al.*, 2013) and the sole symbiont of the mealybug *Phenacoccus avenae* ('*Ca. T. phenacola*') that does not contain an intrabacterial symbiont have been determined (Husnik *et al.*, 2013). These revealed an extreme reduction of *Tremblaya* genomes making them one of the smallest known (Moran and Bennett, 2014). More than one-fifth of the 139 kb chromosome of '*Ca. T. phenacola*' in *P. citri* is devoted to essential amino-acid biosynthesis, which is the central role of the symbiosis. However, *Tremblaya* in *P. citri* has lost several genes even in functions key for the maintenance of the symbiosis (such as genes involved in translation and essential amino-acid synthesis), and its genome is still in the process of reduction. Its intrabacterial partner, *Moranella* is metabolically more versatile and can complement some of these missing functions, but operation of the symbiosis requires a substantial supply of metabolites and gene products from the mealybug host (McCutcheon and von Dohlen, 2011; Husnik *et al.*, 2013; López-Madriral *et al.*, 2013).

Recent studies demonstrated a heterogeneous nature of contribution of the insect hosts in maintaining the symbiosis in aphids, psyllids and mealybugs, which is mediated by eukaryotic genes as well as genes of bacterial origin encoded in the insect genomes (Nikoh *et al.*, 2010; Hansen and Moran, 2011; Husnik *et al.*, 2013; Sloan *et al.*, 2014). It was suggested that bacterial genes were acquired by *P. citri* through ancient horizontal gene transfer events from a surprisingly diverse set of putative facultative bacterial symbionts. Genes of bacterial origin encoded in the mealybug genome seem to be overexpressed in the bacteriome and can bypass key reactions, for instance, in essential amino-acid and vitamin synthesis (Husnik *et al.*, 2013). Thus the *P. citri* symbiosis requires a complex interplay between the insect host and its two bacterial symbionts, which also involves gene products of horizontally acquired bacterial genes encoded in the mealybug genome (McCutcheon and von Dohlen, 2011; Husnik *et al.*, 2013).

Here we investigated the symbiosis of the manna mealybug *Trabutina manipara* (Ben-Dov, 1988), inhabiting the leaves and branches of the salt-secreting desert tree tamarisk (*Tamarix nilotica*; Waisel, 1961; Qvit-Raz *et al.*, 2008; Finkel *et al.*, 2011). *Tremblaya* cells in *T. manipara* harbor an apparently novel gammaproteobacterial symbiont. Using a metagenomic approach to investigate the *T. manipara* system and comparing our data with those available for *P. citri*, we asked whether the nested bacterial symbioses involving different intrabacterial symbionts in the two mealybug species display convergent patterns of evolution. Our results demonstrate that metabolic functions are partitioned in a highly complex yet surprisingly similar manner between the three symbiotic partners despite the polyphyletic origin of the intrabacterial symbionts in

the two consortia. Moreover, bacterial genes involved in essential amino-acid and vitamin production are present in both mealybug genomes and were likely laterally acquired before *T. manipara* and *P. citri* diversified.

Materials and methods

Sample collection and microscopy

Egg sacs of the mealybug *T. manipara* were collected from tamarisk (*T. nilotica*; Waisel, 1961; Qvit-Raz *et al.*, 2008; Finkel *et al.*, 2011) in the Dead Sea area in Israel (31.271980°N, 35.215690°E). Mealybugs were transferred to Vienna while still attached to the tamarisk branches at ambient temperature. Egg sacs were stored at -80°C for genomic DNA isolation.

We used fluorescence *in situ* hybridization, including an oligonucleotide probe specific for the 16S rRNA of the gammaproteobacterial symbiont of *T. manipara* (Trabutina-300: 5'-CAGTGTGGC TGTATCC-3'). Details on the fluorescence *in situ* hybridization procedure are given in Supplementary Information together with the light and transmission electron microscopic methods used for the visualization of the symbionts on semithin and ultrathin sections of the mealybug bacteriome, respectively.

Genome sequencing and assembly

Insects were dissected in 96% ethanol. Ventral sides of the mealybugs were opened and bacteriomes—usually showing up instantly as large and compact organs—were isolated by using fine forceps. Bacteriomes of 15–20 individuals were collected by pipetting, pooled, washed twice and homogenized in $1\times\text{TE}$ using a plastic pestle prior to DNA isolation. High molecular weight DNA was extracted by a CTAB-based isolation method (Zhou *et al.*, 1996) using 1.5 v/v% polyvinylpyrrolidone in the extraction buffer.

Sequencing of a paired end library was performed on a HiSeq 2000 Illumina platform (Illumina Inc., San Diego, CA, USA). Nearly 177 million reads were obtained in both directions. Sequencing reads were filtered and trimmed by PRINSEQ (Schmieder and Edwards, 2011) and assembled with the SPAdes v3.1 genome assembler (Bankevich *et al.*, 2012). In the initial assembly, using a subset of reads, three groups of contigs could be clearly separated by their G+C content and coverage. Assemblies were subsequently refined by recursive mapping of the reads and performing novel assemblies using the set of reads not mapped on non-target genomes, that is, contigs belonging to the insect host or one of the bacterial symbionts. Reads were mapped by the Burrows–Wheeler Alignment tool using the BWA-MEM algorithm (Li and Durbin, 2009). As a result, a single contig representing the circular chromosome of the gammaproteobacterial symbiont could be obtained

with 1216-fold coverage. The genome of the outer symbiont *Tremblaya* is currently represented by four contigs with an average 592-fold coverage. Further bacterial contigs were not detected with reasonable coverage. Excluding those sequences that could be mapped back on the genomes of the bacterial symbionts, we generated a draft genome of *T. mannipara* with a coverage of ~11-fold. Only contigs >1000 bp were used for further analysis. Genome sequences have been submitted to the European Nucleotide Archive and are available under the accession numbers LT594522, FLRF01000001–FLRF01000004 and FKYK01000001–FKYK01037035.

Annotation, functional and comparative analysis of the symbiont genomes

The putative origin of replication was set with GenSkew (<http://genskew.csb.univie.ac.at/>). Synteny and rearrangements between genomes were visualized by Mauve (Darling *et al.*, 2010).

We used an in-house pipeline, called ConsPred (Weinmaier *et al.*, unpublished; available at <https://sourceforge.net/p/conspred/>) for gene prediction and annotation. Detailed description of the annotation method is available in the Supplementary Information. Genome annotations were manually curated using the UniPro UGENE tool (Okonechnikov *et al.*, 2012). In order to identify potential pseudogenes, intergenic regions and hypothetical proteins were submitted to blastx searches against the non-redundant protein database (nr) of the National Center for Biotechnology Information and the UniProt Swiss-Prot databases. Protein domains were predicted according to the Conserved Domain Database (Marchler-Bauer *et al.*, 2011) of National Center for Biotechnology Information. Pseudogenes were identified as remnants of genes, which are truncated or interrupted by internal stop codons and/or frameshifts showing high confidence (e -value < $1e^{-3}$) blast hits to proteins with defined functions. Regions with premature stop codons were identified as pseudogenes if their length were < 80% of the length of the reference sequences. Pseudogene coordinates were set according to the best blast hit against the Uniprot Swiss-Prot database.

Mobile genetic elements were checked by ISfinder (Siguier *et al.*, 2006). Metabolic pathways were manually inspected using the Pathway Tools software (Karp *et al.*, 2010) and the Ecocyc, Biocyc and Metacyc resources (Caspi *et al.*, 2010). Clusters of orthologous groups shared by the relevant genomes or unique to the symbionts were identified by OrthoMCL using an $1e^{-5}$ e -value threshold (Li *et al.*, 2003).

Phylogenetic placement of the symbionts

The relationship of the intrabacterial symbiont of *T. mannipara* with other symbionts of mealybugs was inferred by 16S rRNA-based phylogenetic

analysis. A Bayesian tree was calculated with MrBayes v3.2.6 (Ronquist *et al.*, 2012), using the GTR+I+G model with 4gamma categories. Two runs each with four chains were performed. The proportion of invariable sites and the gamma shape parameter were estimated from the data. Chains were stopped when convergence diagnostics fell < 0.03; 50% majority consensus trees were generated with a relative burn-in of 25%. Tree calculations were performed on the CIPRES Science Gateway v.3.3. web interface (Miller *et al.*, 2010).

A phylogenomic approach was also employed to characterize the relationship of the gammaproteobacterial symbiont of *T. mannipara* to other insect symbionts and free-living bacteria within the Enterobacteriaceae. For this, single copy marker genes were selected and aligned to the gammaproteobacterial reference data set integrated in Phyla-AMPHORA (Wang and Wu, 2013). Poorly aligned positions were excluded by Gblocks 0.91b (Castresana, 2000) using the default settings apart from the followings: minimum length of a block: 5, allowed gap positions: with half. A Bayesian phylogenetic tree was reconstructed from the concatenated alignment of 34 ribosomal proteins. We employed the CAT+GTR models (Lartillot and Philippe, 2004) as implemented in PhyloBayes MPI v1.6j (Lartillot *et al.*, 2009, 2013) together with the Dayhoff 6 amino-acid recoding scheme, as suggested for phylogenetic reconstruction of symbiont lineages by Husník *et al.* (2011). Two chains were run until all discrepancies between the chains fell < 0.1 and effective sample sizes were < 100. The first 20% of the trees were discarded prior to generation of a majority consensus tree.

Phylogenetic trees were visualized with FigTree v1.4.1 (<http://tree.bio.ed.ac.uk/software/figtree/>) and iTOL (Letunic and Bork, 2011).

Identification of contribution of the insect host to selected bacterial pathways

We investigated the role of the insect host in the *T. mannipara* symbiosis based on previous results by Husník *et al.* (2013) on the symbiosis of the mealybug *P. citri*. Protein/DNA sequences already available for eukaryotic genes as well as genes of bacterial origin in the *P. citri* genome were used as queries in tblastn/blastn searches against the draft genome of *T. mannipara*, respectively. Genomic regions corresponding to the subject sequences of the best significant blast hits (e -value $\geq 1e^{-3}$) were extracted together with their flanking regions and were submitted for a blastx search against nr. Top hits (up to 100) were collected for each query and were used for phylogenetic analysis. Individual protein alignments were generated by the MAFFT L-INS-i algorithm (Kato and Standley, 2013). Ambiguous positions were filtered with Gblocks as described above. Bayesian trees were generated in MrBayes v3.2.6 (Ronquist *et al.*, 2012) as described for the 16S

rRNA-based analysis above, except that we applied the WAG+I+G model with 4gamma categories.

Results and Discussion

T. manipara contains a novel intrabacterial symbiont
The 16S rRNA of the betaproteobacterial symbiont of *T. manipara* was most similar (97%) to ‘*Ca. T. phenacola*’ strains in other mealybugs, and its affiliation with the *Tremblaya* lineage was highly supported in the 16S rRNA-based phylogenetic analysis (data not shown). In contrast to the monophyletic origin of the primary symbionts of mealybugs, previous phylogenetic analyses of 16S and 23S rRNA genes suggested a polyphyletic origin of their secondary symbionts among the Enterobacteriaceae (Fukatsu and Nikoh, 2000; Thao et al., 2002). Consistent with this, our 16S rRNA-based analysis (Supplementary Figure S1) placed the inner symbiont of *T. manipara* in a well-supported cluster with those of other mealybugs (*Melanococcus albizziae*, *Australicoccus grevilleae*, *Amonostherium lichensoides* and *Antonina pretiosa*) from the tribe Trabutinini (Hardy et al., 2008) with which it shared 90.3–92.1% sequence similarity. This group clustered with other insect symbionts and the inner symbionts of other Trabutinini mealybugs (*Paracoccus nothofagicola* and *Cyphonococcus alpinus*) (Thao et al., 2002), although the exact relationships among these clades remain unresolved. *Moranella endobia*, the intrabacterial symbiont of *P. citri*, formed a relatively closely related yet distinct cluster together with the secondary symbiont of *Planococcus ficus*, in agreement with previous reports (Thao et al., 2002). Given its low degree of sequence similarity to known gammaproteobacterial species, the intrabacterial symbiont of *T. manipara* represents a novel lineage within the Enterobacteriaceae. We thus propose to tentatively classify these symbionts as ‘*Candidatus* *Trabutinella endobia*’ (according to Murray and Stackebrandt, 1995); ‘*Trabutinella*’ alluding to the mealybug host, *T. manipara*; ‘endo-bia’ (‘inside-living’) referring to the intrabacterial lifestyle of this bacterium).

Fluorescence *in situ* hybridization using 16S rRNA-targeted-specific probes demonstrated that *Trabutinella* is present within the cells of *Tremblaya*, which in turn are located in the bacteriocytes of *T. manipara* (Figure 1d). Light and electron microscopic observations on sections of *T. manipara* bacteriomes showed that the intrabacterial *Trabutinella* are polymorphic and have a size of 2–7 µm in diameter (Figures 1a–c). Large (10–20 µm in diameter) cells of the outer bacterial symbiont *Tremblaya* contain up to 20 *Trabutinella* cells within their cytoplasm.

The genomes of bacterial symbionts are often characterized by skewed nucleotide composition and rapid sequence evolution, which hampers phylogenetic analysis and may result in grouping

of unrelated taxa. Thus, in order to get further support for the relationship of *Trabutinella* and *Moranella* to other insect symbionts and free-living bacteria, we employed multilocus sequence data and advanced phylogenetic methods to overcome these biases, as suggested earlier (Husník et al., 2011). Phylogenetic analysis of a concatenated set of 34 ribosomal proteins (Figure 2, Supplementary Figure S2) showed that, although *Trabutinella* fell within the same monophyletic group as *Moranella*, they did not appear as sister taxa. This is consistent with our 16S rRNA-based analysis and confirms that the intrabacterial symbiosis in *T. manipara* is a result of an infection of the *Tremblaya* cells independent from the one in *P. citri*. *Trabutinella* and *Moranella* were interleaved in a large group containing other facultative and obligate insect symbionts, such as *Sodalis* species, described as closest relative of *Moranella* (McCutcheon and von Dohlen, 2011), *Baumannia* in sharpshooters (Wu et al., 2006), *Wigglesworthia* in tsetse flies (Akman et al., 2002) and *Blochmannia* in carpenter ants (Gil et al., 2003; Degnan et al., 2005; Williams and Wernegreen, 2010). Although a common origin of this diverse set of symbionts is still debated, this group is consistent with one of the major symbiont lineages among the Enterobacteriaceae, which could be distinguished in recent comprehensive phylogenomic analyses (Husník et al., 2011; Manzano-Marín et al., 2015). Taken together, our data showed that phylogenetically different bacterial symbionts have independently infected *Tremblaya* during the evolution of *T. manipara* and *P. citri*.

A highly conserved Tremblaya genome and loss of functions in the intrabacterial symbiont from T. manipara

We contrasted our results on the *T. manipara* consortium to those reported for *P. citri* (McCutcheon and von Dohlen, 2011; Husník et al., 2013; López-Madrugal et al., 2013). This analysis revealed that genomic sequences of *Tremblaya* were highly syntenic and harbored nearly identical sets of genes in the two systems in accordance with a monophyletic origin of the outer symbionts among mealybugs. Currently, three large contigs and a short fragment represent the genome of *Tremblaya* from *T. manipara*. The latter corresponds to the 16S–23S rRNA and the 30S ribosomal protein S15 (rpsO) gene sequences, all duplicated in other *Tremblaya* strains. The draft genome of *Tremblaya* from *T. manipara* (137 475 bp) was similar in size to the *Tremblaya* genome from *P. citri* (138 927 bp) (McCutcheon and von Dohlen, 2011). Only two genes were unique to *Tremblaya* from *T. manipara* compared with *Tremblaya* from *P. citri* (McCutcheon and von Dohlen, 2011; Husník et al., 2013; López-Madrugal et al., 2013). These encode an argininosuccinate lyase (*argH*), the final enzyme in the arginine synthesis, and a 16S rRNA methyltransferase (*rsmH*).

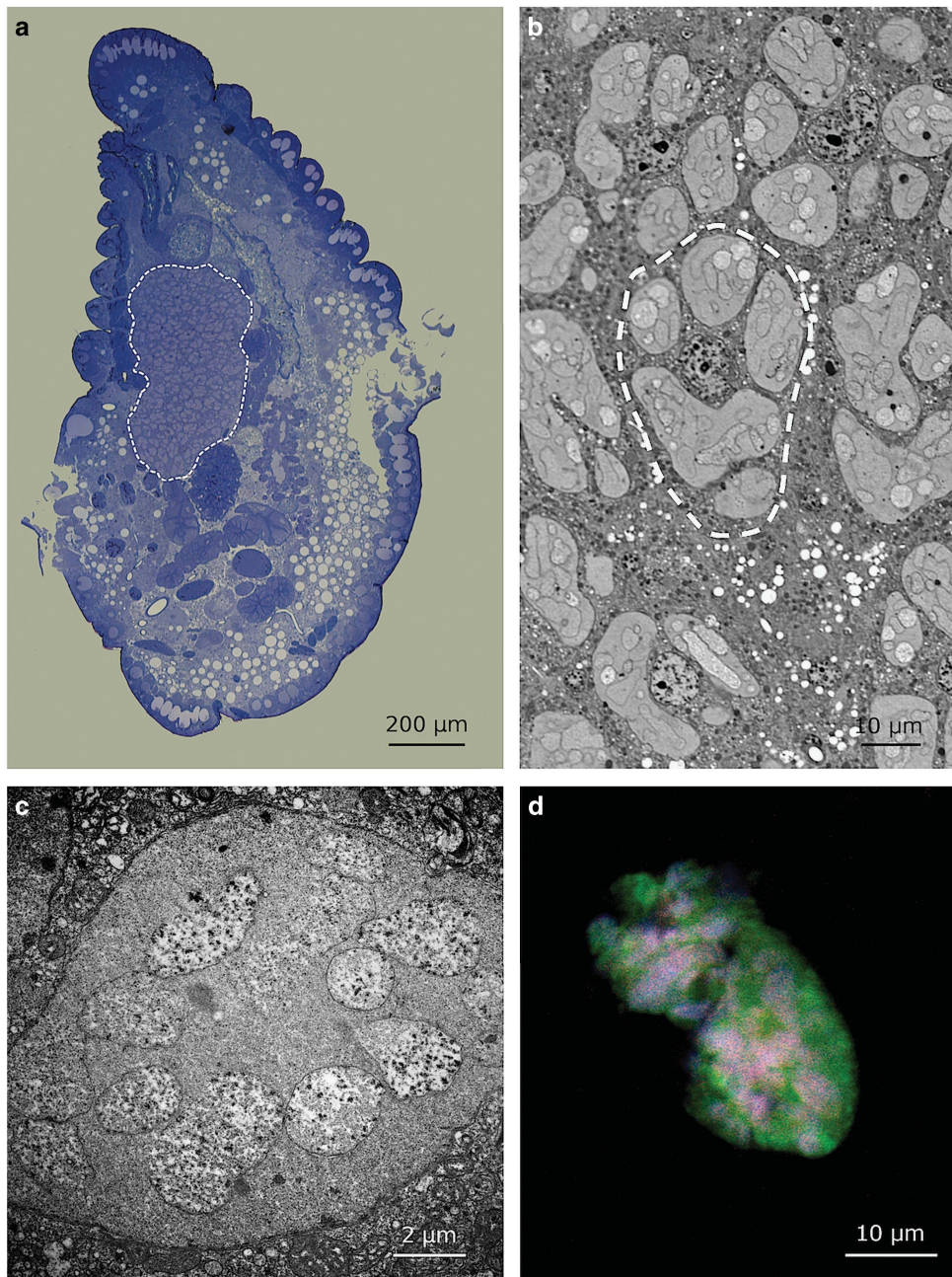


Figure 1 (a) Light microscopic micrograph at low magnification of a longitudinal section of the mealybug *T. mannipara*. The bacteriome (outlined in white) occupies a considerable part of the insect abdomen. (b) Light microscopic micrograph at higher magnification of the bacteriome. One of the bacteriocytes is outlined in white. The bacteriocytes present central nuclei with large nucleoli, indicating high cellular activity. Individual bacteriocytes contain up to seven enlarged bacterial symbionts (*Tremblaya*) in highly amorphous shapes, which themselves harbor several polymorphic bacteria (*Trabutinella*). (c) Transmission electron microscopic observation of a single host bacterium (*Tremblaya*) containing several bacteria (*Trabutinella*) within its cytoplasm. (d) Localization of ‘*Candidatus* *Trabutinella* endobia’ within ‘*Ca. T. phenacola*’ cells as seen with fluorescence *in situ* hybridization. A general bacterial probe (in green), a gammaproteobacterial probe (in red) and a *Trabutinella*-specific probe (in blue) were used; cells of *Trabutinella* appear white–purple, *Tremblaya* green.

Five genes (*leuB*, *leuC*, *leuD*, *iscS* and *fdx* (putative 2Fe-2S ferredoxin)) were present in the genome of *Tremblaya* from *P. citri* but were missing from the genome of the outer symbiont of *T. mannipara*. Among these, the leucine synthesis gene (*leu*) and the cysteine desulfurase

gene (*iscS*) were pseudogenized and thus were likely recently inactivated in *Tremblaya* from *T. mannipara*. This is further supported by the observation that a functional homolog for each of these genes was found in the *Trabutinella* genome.

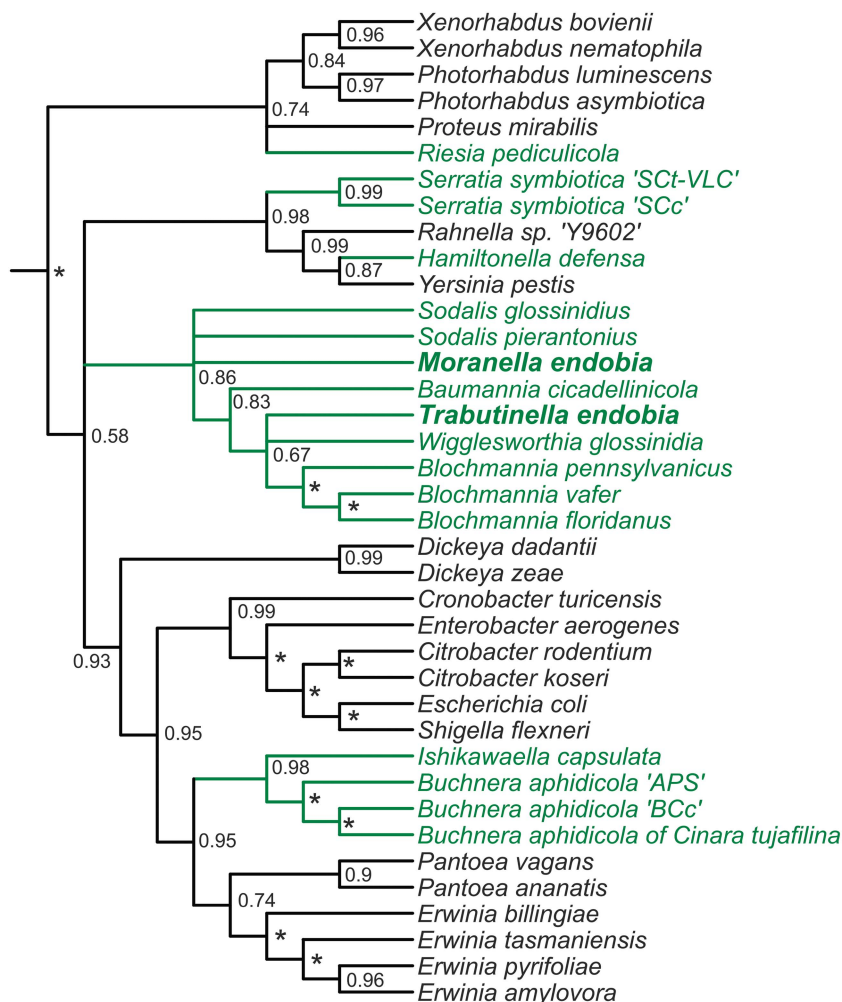


Figure 2 Phylobayes cladogram showing the affiliation of ‘*Candidatus Trabutinella endobia*’, intrabacterial symbiont of the mealybug *T. manipara*, among Enterobacteriaceae. Insect symbiont lineages are colored in green. The complete phylogenetic tree with the original branch lengths is given in Supplementary Material (Supplementary Figure S2). The analysis is based on a concatenated set of 34 ribosomal proteins. Posterior probabilities are indicated on the internal nodes. Asterisks stand for posterior probabilities equal to 1. Nodes with a support of ≤ 0.5 are collapsed.

The genome of the intrabacterial symbiont *Trabutinella* showed only minimal synteny with that of *Moranella* from *P. citri*, which is consistent with the distinct evolutionary origin of these symbionts. Remarkably though, the *Trabutinella* genome was substantially more reduced having a size of 298 471 bp compared with the 538 294 bp genome of *Moranella* (McCutcheon and von Dohlen, 2011). The chromosome of *Trabutinella* had a G+C content of 32.6%: encoded for 237 proteins, one rRNA operon, and 41 tRNA genes. This means a >40% reduction in the number of protein-coding genes compared with *Moranella*. Moreover, the *Trabutinella* genome is likely still in the process of reduction as indicated by the presence of 27 pseudo-genes (a feature typically not seen in tiny genomes of obligate symbionts) (Moran and Bennett, 2014).

Similarly to other obligate insect symbionts with small genomes (Moran *et al.*, 2008; McCutcheon and Moran, 2012), most of the *Trabutinella* genome is devoted to replication, transcription, translation and to its symbiotic role, which—based on pathway reconstructions—is the complementation of *Tremblaya* with respect to essential amino-acid synthesis and vitamin provision of the insect host. In comparison to *Moranella*, >50% decrease in the percentage of genes involved in the central metabolism, coenzyme and lipid production, inorganic ion metabolism, transport mechanisms in general, cell envelope synthesis and cell division was observed (Supplementary Figure S3). For instance, while *Moranella* is still capable of glycolysis and can convert pyruvate to acetate and build up its own NAD⁺ pool (López-Madrigal *et al.*, 2013), *Trabutinella* lacks all these capabilities except for the last two glycolytic enzymes (*eno*, *pykF*). Unlike *Moranella*, *Trabutinella* has lost

the genes for the SUF machinery of the [Fe-S] cluster biosynthesis (only *sufA* is present). However, [Fe-S] clusters might be still synthesized by the combined action of both symbionts, with the help of the products of *iscS* (encoded in *Trabutinnella*), *iscU* and *erpA* (in *Tremblaya*) (Vinella *et al.*, 2009). Moreover, *Trabutinnella* has apparently also lost its transporters and maintained only a putative mechanosensitive ion channel. This type of transporter, generally involved in the osmotic stress response, was also identified in *Moranella* among others. It has been proposed that mechanosensitive ion channels might facilitate the release of metabolites, perhaps even small proteins from *Moranella*, as skewed metabolic activity of the inner and outer symbionts might result in osmotic stress for the inner symbiont (López-Madrugal *et al.*, 2013; López-Madrugal *et al.*, 2013).

Surprisingly, *Trabutinnella* does not encode a single known gene responsible for generation of the cell envelope, while *Moranella* still harbors most of the genes required for fatty acid and lipopolysaccharide synthesis, some genes for peptidoglycan synthesis and two rod shape-determining proteins (McCutcheon and von Dohlen, 2011; López-Madrugal *et al.*, 2013). As *Tremblaya* does not possess any of these functions either, synthesis of the cell envelopes of both bacterial symbionts likely fully relies on metabolites derived from the insect host in the *T. mannipara* system. A similar situation was described in mealybugs containing solely *Tremblaya* in psyllids, in whiteflies and in most members of the Auchenorrhyncha (Nakabachi *et al.*, 2006; McCutcheon and Moran, 2010; Husnik *et al.*, 2013; Jiang *et al.*, 2013). However, how the process of insect host-mediated cell envelope synthesis is accomplished remains yet to be determined.

In accordance with the absence of cell wall synthesis genes, *Trabutinnella* cells were polymorphic and appeared often in highly amorphous shapes (Figure 1). On the contrary, the cells of *Moranella* are typically rod shaped (von Dohlen *et al.*, 2001), although apparently degenerative forms were also observed in mealybugs closely related to *P. citri* (Koga *et al.*, 2013). It has been proposed that *P. citri* might complement missing functions in the peptidoglycan synthesis and degradation in *Moranella* owing to products of nine genes of bacterial origin present in the insect genome (Husnik *et al.*, 2013). Differential expression of those genes might serve to regulate the integrity of *Moranella* cells and thereby the accessibility of *Moranella* products for the other symbiosis partners. In the absence of genes for peptidoglycan synthesis in both the bacterial symbionts and the *T. mannipara* draft genome, such a control mechanism seems unlikely to be at work in the *T. mannipara* symbiosis.

Bacterial genes in the *T. mannipara* genome

Out of the 22 genes of bacterial origin previously identified in the genome of the mealybug *P. citri*

(Husnik *et al.*, 2013), a set of nine genes was found in the draft genome of *T. mannipara*. Eight genes showed high similarity (>86% on the protein level) to their respective counterpart in *P. citri*, suggesting that their function is conserved and that they might be active in *T. mannipara*, as well. These genes are involved in essential amino-acid (*cysK*, *dapF*, *lysA*), biotin (*bioA*, *bioB*, *bioD*) and riboflavin (*ribA*, *ribD*) production (Figure 3). Function, putative origin and distribution of individual genes among the symbiosis partners in the *T. mannipara* versus *P. citri* consortia are shown in Figure 3 (genes of bacterial origin encoded in the insect genomes are highlighted by asterisks).

One gene, *tms1* (tryptophan 2-monooxygenase oxidoreductase), shared only moderate similarity (amino-acid sequence identity = 42.6%, similarity = 60.4%) with its equivalent in *P. citri*, and its FAD-binding domain seemed to be disrupted. This gene was found in two copies in the *P. citri* genome, while only one was identified in *T. mannipara*. According to Husnik *et al.* (2013), *tms1* was slightly over-expressed in the bacteriome. Its product is known to function in the bacterial synthesis of the phytohormone auxin, which is considered an important virulence factor in several plant-associated bacteria (Valls *et al.*, 2006; Chen *et al.*, 2007). The role of *tms1* in mealybug symbioses remains unclear; however, the lower copy number and the disrupted functional domain found in *T. mannipara* suggest that this gene rather represents a non-functional relic of an ancient horizontal gene transfer event.

In addition, a *ddlB* (D-alanine-D-alanine ligase) pseudogene was found in the *T. mannipara* genome, which suggests that this bacterial gene has recently lost its function in this mealybug species. The *ddlB* pseudogene in *T. mannipara* contains frameshift mutations and internal stop codons, while *ddlB* was intact and highly active in the bacteriome of *P. citri* (Husnik *et al.*, 2013). Degeneration of this gene, which is generally involved in peptidoglycan synthesis, is consistent with the absence of other genes accountable for bacterial cell envelope synthesis in the *T. mannipara* consortium.

Interestingly, all of the laterally acquired genes present in *T. mannipara* and *P. citri* and investigated here appeared as sister taxa in phylogenetic trees (Supplementary Figure S4). This indicates that they share a common origin and were present in ancestral mealybugs before *P. citri* and *T. mannipara* diversified. In accordance with previous findings (Husnik *et al.*, 2013), our analyses suggested known symbionts of insects as potential donors, including *Alphaproteobacteria* (*lysA* from *Ehrlichia*, *dapF* from *Rickettsia*, *ribD* from *Wolbachia*) and *Gamma-proteobacteria* (*cysK* from *Sodalis*, *ribA* from *Arsenophonus*). The affiliation of *bioABD* in *T. mannipara* could not be clearly determined, as they grouped both with *Cardinium* and members of the Rickettsiales (similarly to *P. citri*). *Tms1* was putatively acquired from *Pseudomonas*, *Burkholderia* or

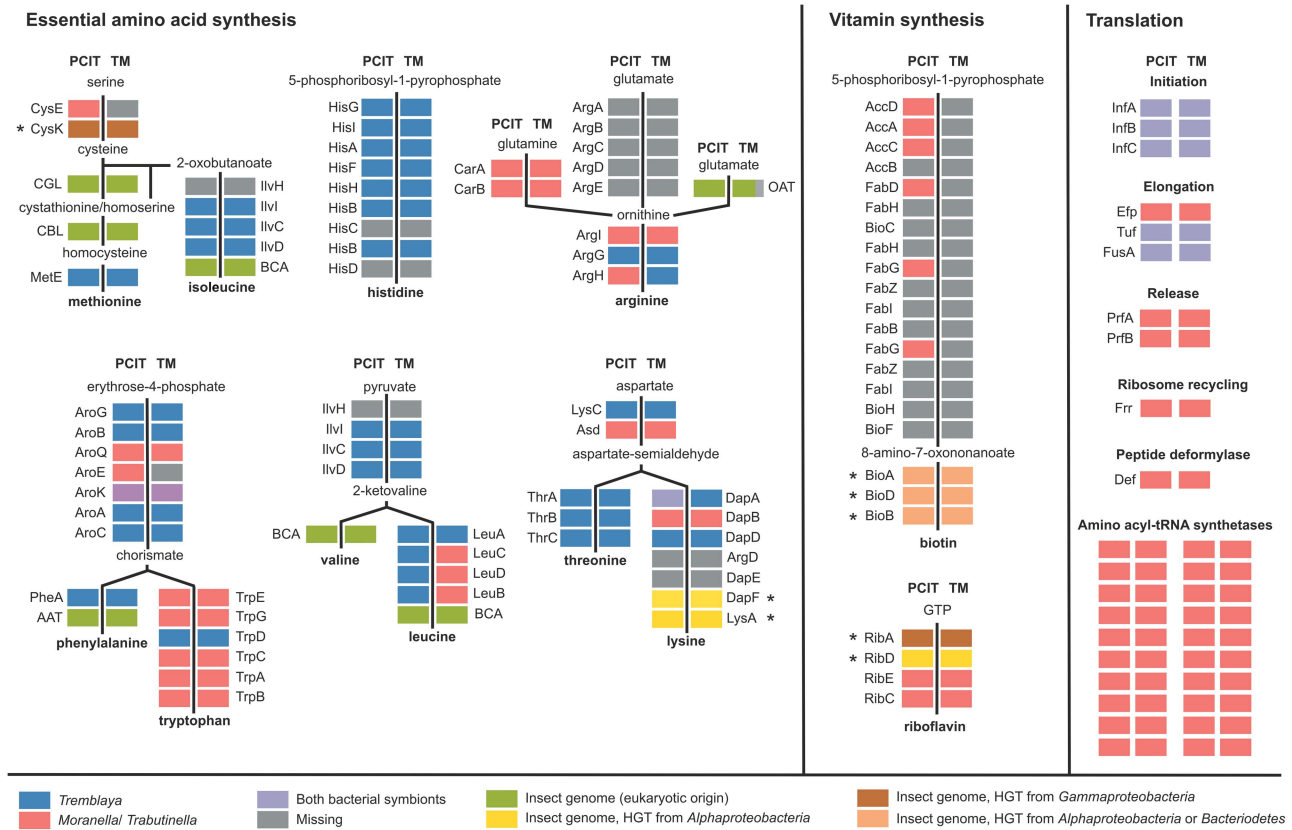


Figure 3 Convergent evolution of symbiotic roles in essential amino-acid synthesis, vitamin production and translation in *T. mannipara* (TM) and *P. citri* (PCIT). Colored boxes show the distribution of individual genes among the three symbiotic partners in the *T. mannipara* and *P. citri* consortia. Data for *P. citri* were taken from Husnik *et al.* (2013). Asterisks highlight genes of bacterial origin encoded in the *T. mannipara* and *P. citri* insect genomes. These genes were likely obtained by horizontal gene transfer during ancient associations of mealybugs with bacterial symbionts affiliated with the *Alphaproteobacteria*, *Gammaproteobacteria* or *Bacteroidetes*.

Ralstonia species, microbes typically not found as symbionts of insects but encompassing auxin-producing plant-associated bacteria (Valls *et al.*, 2006; Chen *et al.*, 2007; Compant *et al.*, 2008). Horizontal gene transfer from plant-associated bacteria to insects has recently also been proposed for the silkworm *Bombyx mori* (Li *et al.*, 2011).

High degree of convergence between *T. mannipara* and *P. citri* symbioses

Symbiotic partners of the *T. mannipara* and *P. citri* systems partition the synthesis of essential amino acids in a highly similar manner (Figure 3). Although the majority of genes responsible for essential amino-acid synthesis are encoded in the *Tremblaya* genomes, only histidine might be produced by the outer symbionts alone in both consortia. The bacterial partners together can produce tryptophan and threonine, while synthesis of other essential amino acids likely requires additional gene products from the mealybug hosts.

In detail, although the majority of genes for histidine synthesis are present in all *Tremblaya* strains, the pathway is still incomplete in both symbiotic systems. Notably, *Tremblaya* of *T. mannipara* lacks exactly the same histidine-

synthesizing genes as *Tremblaya* of *P. citri* and *Tremblaya phenacola* in *P. avenae*. Our observations thus further support that this pathway is indeed active but functions by a yet unknown mechanism in mealybug symbioses (Husnik *et al.*, 2013).

The production of methionine, isoleucine, phenylalanine, arginine, valine and leucine likely requires products of eukaryotic genes (cystathionine beta (CBL)/cystathionine gamma (CGL), AAT, OAT, BCA), which were found in the *T. mannipara* genome, similarly to *P. citri*. These genes have also been identified in other insects, such as aphids (Hansen and Moran, 2011) and psyllids (Sloan *et al.*, 2014), where they are typically overexpressed in the bacteriome compared with the rest of the insect body.

The synthesis of lysine and methionine seems to involve genes of bacterial origin present in both mealybug genomes (*cysK*, *dapF*, *lysA*). Similar to the synthesis of histidine, production of lysine is incomplete in both mealybug symbioses and might operate by a yet undetermined manner. The respective genes are also missing in members of Auchenorrhyncha (*dapE*) (McCutcheon and Moran, 2010) and in psyllids (*argD*) (Sloan *et al.*, 2014), while the rest

of the lysine synthesis pathway is encoded in the *Sulcia* and *Carsonella* symbionts, respectively.

It is striking that, in most of the essential amino-acid production pathways, exactly the same steps are carried out by the inner or the outer symbiont in *T. mannipara* and *P. citri*, despite the independent origin of the intrabacterial symbionts. For instance, intermediate genes in the chorismate synthesis are encoded in the inner symbionts, while the rest is present in *Tremblaya* in both systems (Figure 3). Moreover, the same genes are present exclusively in the inner symbiont in case of the synthesis of threonine and lysine. Altogether we found only five differences between the biosynthesis pathways for essential amino acids in the two consortia. (1) *AroE* in the chorismate (phenylalanine and tryptophan) synthesis is missing in the *T. mannipara* system, while it is present in *P. citri*. Lack of the shikimate dehydrogenase *AroE* despite the presence of all other genes in the chorismate synthesis has also been reported for psyllids, suggesting an alternate route which can bypass this gap in the pathway (Sloan *et al.*, 2014). (2) The final enzyme of the arginine synthesis (*argH*) is encoded in *Moranella* in the *P. citri* consortium while it was identified in *Tremblaya* of *T. mannipara*. (3) Unlike in *P. citri*, *dapA* in the lysine synthesis does not exist in both symbionts but is only present in *Tremblaya* in *T. mannipara*. This is consistent with an increased reduction of functional redundancy in long-term symbioses. (4) *Trabutinella* carries out three steps of leucine synthesis (*leuCDB*), which are encoded by the *Tremblaya* genome in *P. citri*; and (5) *cysE* in the cysteine (and methionine) synthesis seems to be missing from both symbionts of *T. mannipara*, while it was found in *Moranella* in *P. citri*. As the bacterial cysteine synthase *CysK* encoded in the *T. mannipara* genome seems to be intact, it might contribute to the metabolism of cysteine if the necessary precursors were present. Sequences similar to CGL and CBL lyases were also found in the draft genome of *T. mannipara*, similar to *P. citri*, aphids and psyllids (Hansen and Moran, 2011; Husnik *et al.*, 2013; Sloan *et al.*, 2014). Thus cysteine might also be produced by the CGL activity from exogenous cystathionine, if that was available. Cystathionine, rather than cysteine might also be the substrate of the synthesis of methionine, as demonstrated by enzymatic assays for pea aphids (Russell *et al.*, 2013). In this scenario, CBL activity of the host converts cystathionine into homocysteine, which can be converted to methionine by the homocysteine trans-methylase *MetE* encoded in *Tremblaya* in case of both mealybugs. (Note that the insect host might also contribute to the production of methionine by the activity of a homocysteine S-methyltransferase, which was found in the genome of both *P. citri* (Husnik *et al.*, 2013) and *T. mannipara*.) In addition, CGL might also have a role in the synthesis of isoleucine using homoserine, an alternative of cystathionine for 2-oxobutanoate production (Husnik *et al.*, 2013; Russell *et al.*, 2013).

In addition to essential amino acids, the cofactor riboflavin can also be produced in both mealybug symbiotic associations (Figure 3). Its synthesis is apparently performed in a similar manner in both consortia as it involves gene products from the gammaproteobacterial symbionts (*ribEC*) as well as insect host genes of bacterial origin (*ribAD*). The synthesis of biotin is carried out through *Moranella* and horizontally transferred genes in the *P. citri* genome, although several steps are missing from that pathway. Interestingly, symbionts of *T. mannipara* do not encode any of the genes involved in this pathway, although remnants of *fabGZI* are present as pseudogenes in the *Trabutinella* genome. Only the last three steps of the pathway might be carried out by bacterial genes encoded in the *T. mannipara* genome (*bioADB*), which could be used to build up biotin from 8-amino-7-oxononanoate, if that substrate would be available. This pathway is thus further reduced in the *T. mannipara* consortium compared with *P. citri*.

Similar to *P. citri*, the intrabacterial symbiont took over most of the translation-related functions in *T. mannipara*, including the ribosome-recycling factor, the peptide deformylase, the two release factors and all amino acyl tRNA synthetases. Only the three translation initiation factors (IF1–IF3) and two elongation factors (EF-Tu, EF-G) have been retained in *Tremblaya* as well as the inner symbiont in *T. mannipara*, exactly similar to *P. citri* (Figure 3). As translation-related genes were present in the genome of *T. phenacola*, which does not contain an intrabacterial symbiont, loss of these essential genes in *Tremblaya* of both *T. mannipara* and *P. citri* was likely facilitated by the acquisition of the inner symbionts (McCutcheon and von Dohlen, 2011; Husnik *et al.*, 2013). However, our data imply that genes required for the initiation and elongation phase of translation are not subject of further reduction but instead are maintained in *Tremblaya* to perhaps provide some sort of independent control over protein production between the two symbionts.

Conclusions

In most plant sap-feeding insects, usually one of the bacterial symbionts is responsible for complete or nearly complete synthesis of individual metabolites. The high degree of metabolic complementarity, that is, the step by step division of labor between the nested symbionts in mealybugs is unusual, and is likely the consequence of their intimate intrabacterial association (McCutcheon and von Dohlen, 2011). Gradual degradation of the inner symbionts and release of their metabolites might facilitate the exchange of metabolites between the symbionts, thus allowing for a more complex partition of individual pathways between the partners in this association (McCutcheon and von Dohlen, 2011; Husnik *et al.*, 2013; Koga *et al.*, 2013). The highly

congruent patterns of gene loss and metabolic complementarity observed in the *T. manipara* and *P. citri* symbioses show that partition of the pathways is apparently not random as certain steps are carried out by the same symbiont in the two systems—despite the different evolutionary origin of the intrabacterial symbiont. A similar, yet far less complex situation has been observed among members of Auchenorrhyncha, where *Sulcia* synthesizes eight or seven essential amino acids while the remaining two or three are produced by different co-symbionts in different lineages, for instance, by *Baumannia* in sharpshooters, *Hodgkinia* in cicadas and *Zinderia* in spittlebugs (McCutcheon and Moran, 2010; Bennett and Moran, 2013).

A conceivable scenario explaining the observed similarities between the *P. citri* and *T. manipara* symbioses would be that the *Tremblaya* ancestor was already infected by an (intra-)bacterial symbiont in ancestral mealybugs before *P. citri* and *T. manipara* diverged. This ancient association would have facilitated reduction of the *Tremblaya* genome and has shaped its gene repertoire. We favor this scenario over alternative scenarios such as ancient co-obligatory or facultative associations of *Tremblaya* with another bacteriocyte-associated symbiont because of the high level of congruence between the loss of genes in different *Tremblaya* strains at intermediate steps of essential amino-acid synthesis pathways (such as in the synthesis of chorismate, lysine and threonine). These suggest that the primordial bacterial symbiont likely had an obligatory status and was already located within the cytoplasm of *Tremblaya* (facilitating exchange of metabolites and gene products) in the progenitor of the two mealybug species. The inner symbiont might have been subsequently replaced in the ancestor of *P. citri* and/or *T. manipara*, with the new symbiont taking over the functions required by *Tremblaya* and at the same time allowing for loss of further genes from the *Tremblaya* genome, which would account for the observed differences between the two systems.

In summary, the identification of novel bacterial symbionts of *Tremblaya* in the mealybug *T. manipara* and the genomic analysis of this consortium demonstrated an unexpected high degree of convergent evolution in these unique symbioses, in which—despite of the different evolutionary origin of one of the partners—both bacterial symbionts and the insect host act in concert in a highly complex manner. We also show that further reduction of essential functions in the already extremely deteriorated *Tremblaya* genome is possible.

Note

A paper by Husnik and McCutcheon (Husnik and McCutcheon, 2016) that appeared during the revision/proof stage of this article demonstrated that symbiont replacement also occurred in other mealybug lineages.

Conflict of Interest

The authors declare no conflict of interest.

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