University of South Bohemia in České Budějovice

Faculty of Science

Localization and functional characterization of a mealybug rRNA methyltransferase of bacterial origin

Master‘s thesis

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Annotation: The aim of this thesis was to study the localization and function of the rRNA large subunit methyltransferase I in the mealybug *Planococcus citri* and its endosymbiont *Tremblaya princeps* by bisulfite sequencing, immunohistochemical analysis, and fluorescence *in situ* hybridization.

Affirmation

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Helmut Stanzl

Abstract

Pseudococcinae mealybugs harbor a bacterium-within-bacterium-within-insect symbiotic system. Interestingly, the outermost bacterium (*Tremblaya princeps*) was shown to have the lowest number of genes reported for a bacterium, not called an organelle. This system probably is complemented by horizontally transferred bacterial genes (HTGs) encoded on the insect genome. In *Planococcus citri*, one of these HTGs is a 23S rRNA methyltransferase (*rlmI*) of γ-proteobacterial origin. In this thesis, the function and localization of RlmI was elucidated with immunohistochemistry, FISH and bisulfite sequencing. The localization and function of RlmI in *P.citri* and its endosymbionts could provide evidence that further blurs the line between endosymbionts and organelles.

Acknowledgment

First, I would like to thank my supervisor Zdeněk Paris, for the opportunity to work in his laboratory and for the overall guidance throughout this project. Further, I want to express my gratitude to Filip Husnik who supported me with his seemingly limitless knowledge about mealybugs and endosymbionts. I am very grateful to Hana Sehadova for teaching and introducing me, in her heartwarming way, into the fascinating world of microscopy. Further I want to express my gratitude to Julius Lukes and Alena Zikova for allowing me to use their laboratory equipment and facilities. I want to acknowledge Eva Doleželová and Lucie Pauchová for their help with experiments (Äkta chromatography and section preparation, respectively). My special thanks go to Sneha Kulkarni with who I had limitless scientific (and not so scientific) discussions. She does not miss a single opportunity to help by sharing her experiences and suggesting essential troubleshooting. I want to thank all the other people in the labs where I was working in, for the nice working atmosphere. Last but not least, I want to thank all my friends and family for supporting me throughout my years of study.

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List of Abbreviations

DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
FISH	Fluorescence *in situ* hybridization
HGT	Horizontal gene transfer
HTG	Horizontally transferred gene
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kan	Kanamycin
Kb	Kilobase pairs
kD	Kilo Dalton
LB	Lysogeny broth media
m^5C	5-methylcytidine
M. endobia	*Candidatus* Moranella endobia
MS	Mass spectrometry
NaCl	Sodium Chloride
PB	Phosphate buffer
PUA	Pseudouridine synthase and archaeosine transglycosylase domain
P. citri	*Planococcus citri*
rlmI	Ribosomal RNA large subunit methyltransferase gene I
RlmI	Ribosomal RNA large subunit methyltransferase I
RNA	ribonucleic acid
rRNA	Ribosomal ribonucleic acid
T. princeps	*Candidatus* Tremblaya princeps
TEM	Transmission electron microscope
tRNA	Transfer RNA

Table of content

1. Introduction

1.1. The thin line between organelles and endosymbionts

Many organisms require intracellular bacteria for survival. The oldest and best-known examples are organelles in eukaryotes, most importantly mitochondria and chloroplasts [1]. Organelles are defined as intracellular membrane-bound structures in eukaryotic cells, usually specialized for a particular function [2]. Organelles are named after the diminutive analogs to organs because under the microscope, their subcellular structures remind of those in multicellular organisms. At the time of their discovery, the term had no connection to their evolutionary origin [3–6]. However, nowadays it is well established that mitochondria and plastids (chloroplasts) arose through the endosymbiotic uptake of an α-proteobacterium and cyanobacterium, respectively [7–10]. Nevertheless, it is still unclear how bacteria transformed into highly integrated organelles [11].

More than a decade ago the sequencing of the complete genome of a recently evolved photosynthetic body in *Paulinella chromatophora* raised questions on the distinction between 'endosymbiont' and 'organelle'. It pointed out how vague the boundaries between these terms are [12]. Keeling and Archibald proposed three criteria to evaluate the status of an endosymbiont/ organelle: (i) Genetic integration, (ii) Cellular integration, and (iii) Metabolic integration. Genetic integration describes if and how many genes are targeted to the candidate organelle and how many have been lost from the endosymbiont. Cellular integration characterizes how synchronized the partners are in their cell cycles, how the endosymbionts are transmitted to the progeny and how stable they transfer them to their daughter cells. Metabolic integration defines the complementation of metabolic products between the host and the endosymbiont [3,12]. Nowack and Grossman, on the other hand, define the "moment when the endosymbiont – as a consequence of gene loss – becomes dependent for survival and proliferation on the import of nuclear-encoded proteins", combined with vertical inheritance and benefit to the host, as the criteria which the endosymbiont has to fulfill to gain the status of an organelle [3,13].

In the last few years, many findings started to challenge our perception of where to draw the line between endosymbionts and organelles. In sap-feeding insects and their essential endosymbionts, cases of extensive genome reduction in the symbiont, horizontal gene transfer (HGT) from various sources to the host genome, and the targeting of protein products from host to symbiont have been described [11,13–15]. The bacteriocytes of the citrus mealybug

(*Planococcus citri*) are examples of cells where the complexity of bacterial integration into host cells rivals that of organelles [15–18].

1.2. Pseudococcidae

The symbiosis between insects and bacteria may be traced back to the origins of this lineage 100-250 million years ago [19–21]. It is considered as one of the main reasons for the evolutionary success of insects, as many of them have a mutualistic symbiotic relationship with intracellular bacteria [22]. Almost all the members of the large hemipteran suborder Sternorrhyncha (including e.g. aphids, whiteflies, psyllids, and mealybugs), suck plant sap, which is low in nutrients. They depend on the metabolic products of their bacterial symbionts to compensate for their nutrient-deficient diet [23–28].

Mealybugs (Insecta: Hemiptera: Pseudococcidae) are the second largest family of scale insects with approximately 2000 identified species in more than 270 genera. They can feed on plants from many different families but prefer grasses and legumes. Even though they are distributed worldwide; they are found predominantly in the tropics. Several species of mealybugs are considered agricultural pests of economic importance [29]. With their thin, long mouthparts they penetrate through the plant tissue to feed on the phloem sap. Male mealybugs have a short life span, are hard to find or are even absent in some species. The females have a soft, often elongated or oval body shape. Usually, they are attached to the surface of plants, regularly covered with a ‘mealy or cottony’ wax secretion.

Many mealybugs are oviparous with sexual or parthenogenetic reproduction [19,30-34]. Female mealybugs possess a large organ called bacteriome inside their body cavity. This organ is composed of bacteriocytes, specialized highly polyploid cells that harbor bacterial endosymbionts [23,30,35–37]. These bacterial endosymbionts compensate for the nutritional deficiency of the mealybugs by supplying essential amino acids and vitamins, which are limited in their host’s phloem diet [38,39].

Phylogenetic analyses have identified two subgroups of mealybugs, the subfamilies Pseudococcinae and Phenacoccinae [29]. Phenacoccinae possesses an endosymbiotic system inside their bacteriocytes, in which they harbor a single β-proteobacterial endosymbiont called *Candidatus* Tremblaya phenacola [40]. On the other hand, Pseudococcinae harbor an exceptional Matryoshka-like arrangement in which their β-proteobacterial endosymbiont *Candidatus* Tremblaya princeps (from now on *T. princeps* or *Tremblaya*) houses its own γ-

proteobacterial endosymbionts. In the mealybug *Planococcus citri,* this γ-proteobacterium is called *Candidatus* Moranella endobia (from now on *M. endobia* or *Moranella*) [19,22,41].

Figure 1: A mature female adult of Planococcus citri with a second instar nymph. The white wax layer covers the mealybug. Author: Alexander Wild [42].

1.3. Tremblaya princeps

The longtime dependence of endosymbionts on their hosts leads to gene loss and extremely reduced genomes in the bacteria, approaching that of organellar genomes [15,16,18]. An extreme example is *Candidatus* Tremblaya princeps which possesses, with only 139 kilobase pairs (kb), one of the smallest prokaryotic genomes reported so far in terms of gene number and genome size. The designation "*Candidatus"* indicates that *T. princeps* has not been isolated and cultivated under laboratory conditions yet. *T. princeps* are pleomorphic bacteria with a gram-negative cell wall without peptidoglycan, which is enclosed by an additional host-derived membrane [30,35,37,43]. Usually, bacteria with strongly reduced genomes have an extremely high gene density. However, the genome of *T. princeps* has a coding density of only 73% and additionally, it contains approximately 19 detectable pseudogenes. The rest of the approximately 120 protein-coding genes are mostly devoted to the expression of almost complete ribosomes, the assembly of iron-sulfur clusters and the partial synthesis of several essential amino acids. Surprisingly, the genome was found to be missing essential translation-related genes, such as aminoacyl-tRNA synthetases, bacterial translational release factors, bacterial elongation factors, ribosome recycling factors and peptide deformylase [22,39,44,45].

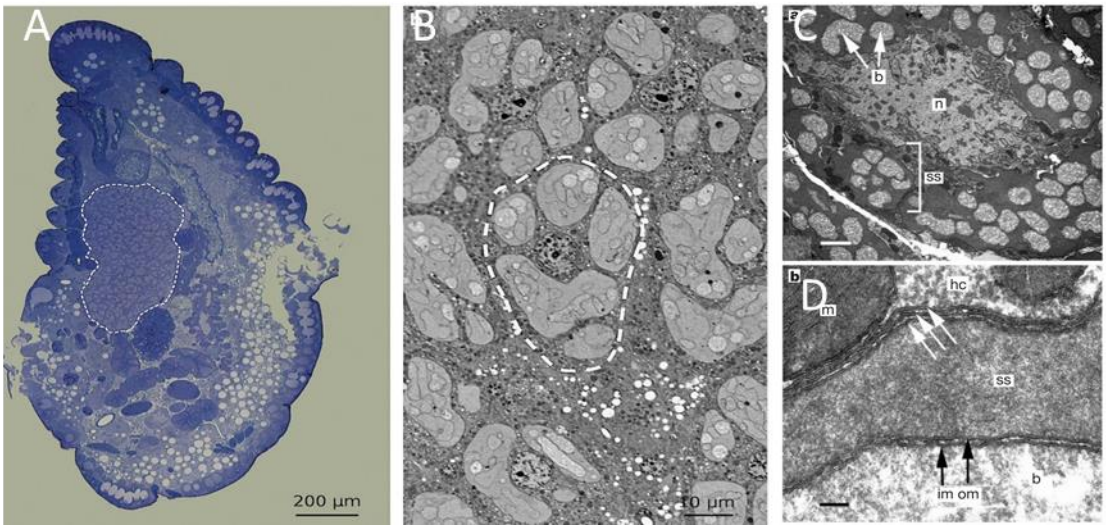

Figure 2: Brightfield and transmission electron microscopy (TEM) pictures from mealybug and bacteriocytes. **A)** Light microscopic image showing a longitudinal section of a mealybug. The bacteriome, outlined in white, is in the abdomen of the insect. Image adapted from Szabo *et al.* [38]. **B)** TEM images depicting one bacteriocyte outlined by a white dashed line. Individual bacteriocytes contain up to seven enlarged bacterial symbionts, which themselves host several bacteria. Images adapted from Szabo *et al.* [38]. **C)** TEM image showing the structure of a bacteriocyte, where the central host nucleus is surrounded by approximately seven *T. princeps* cells containing *M. endobia*. Scale bar is 2.33 µm; b, *M. endobia* n, nucleus ss, symbiotic sphere (*T. princeps*). Image adapted from von Dohlen *et al.* [19]. **D)** TEM image displays the three membranes of *T. princeps* (white arrows) and two membranes of *M. endobia* (black arrows) at high magnification. Scale bar is ~0.07 µm; hc, host cell cytoplasm; im, inner membrane; m, mitochondrion; om, outer membrane. Image adapted from von Dohlen *et al.* [19].

1.4. Moranella endobia

In general, bacteria with the smallest genomes (<500 kb) are often missing essential genes, including those involved in DNA (Deoxyribonucleic acid) and RNA (Ribonucleic acid) synthesis, DNA repair, transcription, translation, and transfer RNA (tRNA) aminoacylation. How these organisms are still able to function, replicate, and express genes regardless of the absence of important genes for the cellular machinery remains an unanswered question [16,46].

In the case of *T. princeps,* the genomic erosion might be explained by the presence of *M. endobia* in its cytoplasm [44]. The genome of *M. endobia* is with 538 kb almost four times larger than the genome of *T. princeps*. Its 406 protein-coding genes contain all the critical translation-related genes missing or pseudogenized in *T. princeps* [39,44]. Therefore, it is hypothesized that many metabolites, proteins, and tRNAs essential for replication, transcription, and translation, are transported either from *M. endobia* or from the host cell to *T. princeps* in order to substitute the missing genes [22].

Regardless of many *in silico* predictions, the way in which *M. endobia* supplies *T. princeps* is still unknown. Husnik *et al.* [44] reported that *P. citri* obtained more than 20 genes of diverse bacterial origin (other than its own endosymbionts) via HGT. Many of these

genes play a role in peptidoglycan production and recycling. It has been hypothesized that those genes are involved in the controlled cell lysis of *M. endobia.* In this way, *T. princeps* could "harvest" gene products from its endosymbiont [22,44]. A recent publication analyzed the acquired complex metabolic patchwork for the formation of the peptidoglycan layer in *M. endobia*. Although no new evidence for the regulation of cell lysis was published, the study revealed that horizontally transferred gene (HTG) products from the insect genome are targeted and functional in the endosymbiont. [15]

1.5. Horizontal gene transfer

Horizontal gene transfer is the movement of one gene from one to the genome of another organism in a different way than vertical inheritance [15]. In general, HGT is possible between any two DNA-based life-forms. Although genes are frequently transferred between microorganisms, they are only relatively rarely transferred from microorganisms to multicellular hosts with a separated germline. Little is known about the importance of HGTs in the evolution of classical organelles. However, there are many traces of genes from external bacteria in eukaryotic genomes. The search for HTG in multiple analogous endosymbiotic systems has revealed numerous examples. Usually, the HTGs are involved in nutrition or protection from predators, pathogens and environmental stress [11,15,47–51].

Contrary to classical organelles, there are no documented gene transfers from *T. princeps* to the host genome. At least 22 HTGs were uncovered from heterologous bacterial sources (not from the current endosymbionts) in the genome of *P. citri*, which are expressed in bacteriocytes. All these genes are absent in the genomes of *T. princeps* and *M. endobia.* HTGs are involved in vitamin and amino acid biosynthesis as well as in bacterial cell wall maintenance, by complementing biosynthetic pathways of both *T. princeps* and *M. endobia.* Eukaryotic signal sequences were identified in five HTGs, which are overexpressed in the bacteriome. Four of them play a role in peptidoglycan formation and the fifth was identified as ribosomal RNA (rRNA) large subunit methyltransferase gene I *(rlmI).* This gene, which originates from the Enterobacteriaceae family (γ-proteobacteria), was found to be 26.4 times more abundant in the transcriptome of the bacteriome than in the rest of the insect [44].

1.6. rRNA large subunit methyltransferase I

If RlmI would have been a standard eukaryotic protein, its name would be in all capitals "RLMI", but since it has a bacterial origin it is named "RlmI". In prokaryotes, a range of rRNA-modifying enzymes can be found, which recognize their target nucleotides without a

guide RNA. Especially, methyltransferases are very stringent in modifying their substrate; each methylation site has its own specific enzyme [52]. The methylated nucleotides are involved in the assembly, maturation, and regulation of the ribosomes. It was reported, they can even provide antibiotic resistance [53–58].

In prokaryotes, methylations at the 5-position of cytosine (m^5C or 5-methylcytidine) are present only in rRNA, where they are hypothesized to improve translational fidelity [58,59]. Ribosomal RNA of *Escherichia coli (E. coli)* contains three m^5C, which were biochemically and structurally extensively investigated [60]. Those methylations are at the positions C967 and C1407 of 16S rRNA and at C1962 of 23S rRNA. The corresponding methyltransferases are RsmB (formerly Fmu/Fmv), RsmF (formerly YebU) and RlmI (formerly YccW), respectively [52,60].

Interestingly, the sequence of *rlmI* is more closely related to known 5-methyluridin methyltransferases than to the two other methyltransferases in *E. coli* [60]. It consists of three domains: The N-terminal pseudouridine synthase and archaeosine transglycosylase (PUA) domain, the central domain, which has a similar motif present in other 5-methyluridine methyltransferases, and the C-terminal catalytic domain which has an S-Adenosylmethionine-binding site. The last two domains are connected by a β-hairpin structure [53].

In bacteria, the 23S rRNA forms together with the 5S rRNA the 50S rRNA also called large ribosomal subunit, which assembles together with the 16S rRNA and additional ribosome proteins to the complete ribosomes [61]. RlmI only modifies the unassembled 23S rRNA. It does not modify 50S ribosomal subunits, even though, the position m^5C1962 is located at the subunit interface and should, therefore, be rather accessible (Figure 3) [60]. It was observed that all three m^5C methylations are equiplanar to the tRNA at the P-site. The methylation at C1962 is conserved in bacteria and its absence leads to a marginally observable decrease in fitness [52]. There might be a more prominent effect if bacteria are exposed to stress conditions. 5-methylcytidine at the position 1962 is part of a phylogenetically highly conserved structure of helix 70. Therefore the 5-methyl group might aid the coaxial helical and cross-strand stacking interactions which is thought to stabilize 23S rRNA. Other studies connect m^5C methylations with the initiator tRNA selection (m^5C1407) and stabilization of the tRNA interaction in the P site (m^5C967) [52,54,62–65].

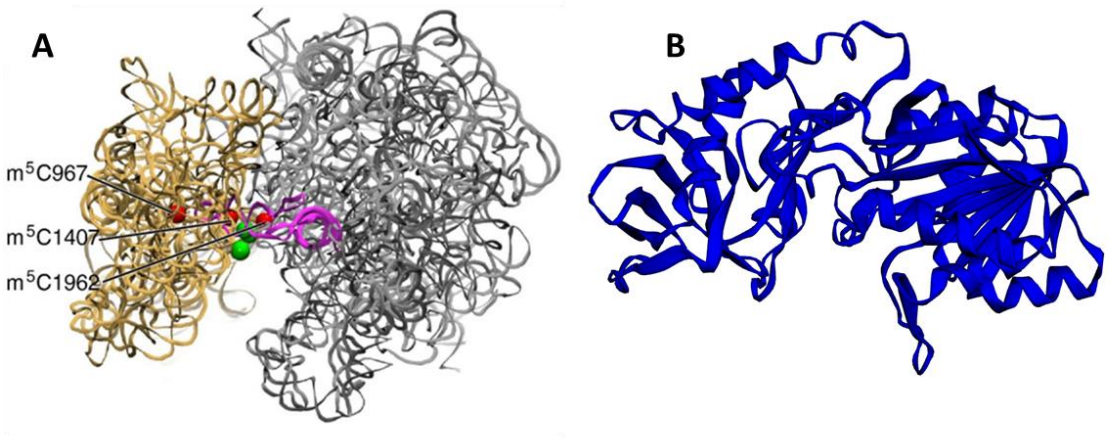

Figure 3: Models of 23S rRNA and RlmI. A) A model of 16S (yellow) and 23S (grey) rRNA without ribosome proteins, showing the locations of the m^5C modifications, indicated by red spheres. Pseudouridine modifications at positions indicated with green spheres and the ribosomal P site are represented by the bound tRNA (magenta) [52]. **B)** Protein homology model of RlmI created with Phyre2 [66,67].

The following passage starting from page 7-10, in total 4 pages, (referring to the original version) contains classified information which is available only in the archived original of the graduation thesis deposited at the Faculty of Science USB.

2. Material and Methods

2.1. Cultivation of the mealybugs

Samples of the mealybug species *P. citri* (citrus mealybug; collection location: winter garden of the Faculty of Science, University of South Bohemia) were identified and provided by Filip Husnik, Biodiversity Research Centre & Department of Botany, Vancouver, Canada. The mealybugs were cultivated on sprouting potatoes in a terrarium at room temperature.

2.2. Bisulfite sequencing

Bisulfite treatment of endosymbiotic rRNA was performed with the EpiTect Bisulfite Kit (Qiagen), according to Schaefer *et al.* [71]. Reverse transcription of 1 µg of bisulfite-treated RNA was performed by Superscript IV reverse transcriptase (Thermo Fisher) with random hexamers. Regions of interest were amplified by PCR with primers specific for the converted rRNA sequence (Table 1). Gel extracted PCR amplicons were cloned into a pGEM T-Easy vector (Promega). Plasmids of 10 clones were sequenced by Sanger sequencing (Eurofins).

Table 1: List of the primers used to amplify the bisulfite-treated regions of interest.

The following passage in the scope of one table contains classified information which is available only in the archived original of the graduation thesis deposited at the Faculty of Science USB.

2.3. Preparation of the overexpression vector

The construct for the overexpression of the Rlm1 was created by PCR. The designed primers (Table 2) were specific for the exon 3 of *rlm1* (**Error! Reference source not found.**), flanked with the restriction sites at 5‘ and 3‘ for HindIII and BamHI, respectively. The resulting PCR product was cloned into pGEM, transfected into XL – Blue Super competent cells (Agilent Technologies), and sequenced. The verified construct and the overexpression vector pSKB3 (Addgene) were digested with BamHI HF and HindIII HF (New England Biolabs), and the obtained gel-purified fragments were ligated with T4 Ligase (New England Biolabs). Next, 50 ng of the plasmid was transfected into 50 µl of OverExpress™ C41 *E. coli* cells, followed by selection with 30 µg/ml kanamycin (Kan).

Table 2: List of primers used to amplify the exon 3 of the *rlmI* gene.

Forward	5'- CGCGGATCCGGCAGCATTCCCAAAACACCC -3'
Reverse	5'- CCCAAGCTTTTACTCGACCCGACAAGCGAATC -3'

2.4. Solubility test of the overexpressed protein

5 ml of overnight *E. coli* culture was transferred into 100 ml lysogeny broth (LB) media with 30 µg/ml Kan and grown under constant shaking for 2 h at 37°C. The overexpression was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), cells were incubated for another 4 h, and harvested at 4000 g for 20 min. The pellet was resuspended in lysis buffer (50 mM sodium dihydrogen phosphate, 300 mM sodium chloride (NaCl), 10 mM imidazole, pH8), frozen and then sonicated. Soluble and insoluble fractions were separated by centrifugation at 10,000 g for 30 min. Proteins were visualized by Coomassie-stained SDS/PAGE and by western blot analysis using mouse α-His (1:2000) as primary and goat α-mouse HRP (1:2000) as secondary antibodies.

2.5. Purification of the recombinant protein and generation of polyclonal antibodies

The recombinant protein was affinity purified using an ÄKTA prime plus chromatography systems (GE Healthcare Life science) equipped with a His Trap column (GE Healthcare Life science), eluted in elution buffer (200 mM NaCl, 400 mM imidazole, 0.5% sodium lauroyl sarcosinate (Sarkosyl), 20 mM phosphate buffer (PB), pH 7) and dialyzed against the storage buffer (200 mM NaCl, 125 mM imidazole, 0.5% Sarkosyl, 20 mM PB, 10% glycerol, 2 mM Dithiothreitol (DTT), pH 7). The protein concentration was determined with Bradford assay (Biorad), the amino acid sequence was validated by Mass spectrometry analysis (MS) and the protein was sent to "Davids Biotechnologie GmbH" for antibody production. Obtained polyclonal antibodies, from recombinant proteins and synthesized oligopeptides, were partially purified by the company using affinity chromatography against Rlm1. Another batch of polyclonal antibodies was raised in the animal facility of the Biology Center of the Czech Academy of Sciences in Budweis. The homemade sera were affinity purified with Cyanogen Bromide-Activated Matrices (Sigma) against the recombinant protein. Western blot analysis was performed to evaluate the specificity of generated antibodies against the recombinant protein.

2.6. Giemsa staining

Smears of bacteriomes were fixed with methanol and stained with Giemsa (Sigma Aldrich). Slides were observed under fluorescence microscope AxioPlan 2 (Carl Zeiss Microscopy GmbH, Jena, Germany) equipped with CCD DP73 camera (Olympus Japan).

2.7. Mallory staining

Mealybugs were fixed overnight at 4°C in Bouin-Hollande solution without acetic acid supplemented with 0.7% mercuric chloride [72]. Standard techniques were used for tissue dehydration, embedding in paraplast, sectioning to 7 μm, deparaffinization, and rehydration. The sections were treated with Lugol's iodine followed by a 7.5% solution of sodium thiosulphate to remove residual heavy metal ions and then washed in distilled water. Staining was performed with Trichrome stain (Masson) kit (Sigma) according to the manufacturer's protocol. Stained sections were dehydrated, mounted in DPX mounting medium (Fluka) and viewed and imaged under BX51 (Olympus, Japan) equipped with CCD DP camera (Olympus, Japan).

2.8. Immunohistochemical analyses

Whole *P. citri* and dissected bacteriomes were fixed in 4% paraformaldehyde at 4°C for 8 h, washed (3 times, 15 min each) in PB and blocked with 5% normal goat serum in PB supplemented by 0.5% Triton X-100 (PBT) at room temperature for 2 h. The samples were incubated with rabbit α-RlmI antibody diluted 1:100 in the blocking solution at 4°C for 2 days, washed (6 times, 10 min each) in PBT and incubated with goat α-rabbit IgG (H+L) Alexa Fluor 488 (Life Technologies) diluted 1:200 in the blocking solution at 4°C for 8 h. Pre-immune serum was used as a negative control. After washing (6 times, 10 min each) in PBT, the samples were mounted in FluoroShield^{TM} (Sigma Aldrich) containing 4',6-diamidino-2-phenylindole (DAPI) on glass slides and observed and imaged under FluoView FV3000 confocal laser scanning microscope (Olympus, Japan). Image processing was done with FV31S-SW (Olympus), Photoshop CS4 Version 11 (Adobe Systems) and Imaris 6.3.1 (Bitplane).

2.9. Fluorescence *in situ* hybridization

Dissected bacteriomes were fixed in 4% paraformaldehyde and dehydrated by 2x 10 min incubations in 70%, 96%, and 100% ethanol. Hybridization was performed according to the work by Pernthaler *et al*. [73]. Probes were generously provided by Filip Husnik.

No-probe controls were used to assess insect tissue autofluorescence. The fluorophore-labeled oligonucleotide probes targeting 16S rRNA, which were used for endosymbiont *in situ* hybridization are listed in Table 3. Otherwise, the samples were mounted, visualized and processed in the same way as mentioned above (Chapter 2.8)

Table 3: List of the probes used for the 16S rRNA FISH.

T. princeps (b886)	5′-Cy3-TCAGGCGGTCGACTTCAT-3′
M. endobia (g630)	5′-Cy5-CGAGACTCTAGCCTATCAGTTTC-3′

The following passage starting from page 15-27, in total 13 pages, (referring to the original version) contains classified information which is available only in the archived original of the graduation thesis deposited at the Faculty of Science USB

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