Chapter 5

The Symbiome of *Llaveia* Cochineals (Hemiptera: Coccoidea: Monophlebidae) Includes a Gammaproteobacterial Cosymbiont *Sodalis* TME1 and the Known *Candidatus* Walczuchella monophlebidarum

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Abstract

The genome and transcriptome of the endosymbiotic flavobacterium *Candidatus* Walczuchella monophlebidarum revealed its role in the synthesis of essential amino acids for its host, the wax cochineal *Llaveia axin axin*. There were, however, missing genes in the endosymbiont for some biosynthetic pathways. Here, we characterized TME1, another cochineal symbiont that may metabolically complement *Walczuchella*. TME1 was ascribed to the gammaproteobacterial genus *Sodalis* on a phylogenomic basis using gene sequences from 143 proteins core genome sequences and the core average nucleotide identity (ANI) confirmed its position. Additionally, we describe *Sodalis* as a coherent genus. TME1 genome is around 3.4 Mb and has complete gene sequences for the biosynthesis of 10 essential amino acids, for polyamines, flagella, nitrate respiration, and detoxification among many others. Transcripts from ovaries and bacteriomes allowed the identification of differentially transcribed genes from the endosymbionts and host. Highly transcribed genes were identified in TME1 and transcripts involved in amino acid biosynthesis were found. We review here that cosymbionts that derived from different bacterial classes and genera seem to be advantageous for insects that have Flavobacteria as the primary endosymbionts.

Keywords: endosymbionts, scale insect, Gammaproteobacteria, *Sodalis*-like, Alphaproteobacteria, fungi

1. Introduction

All organisms are inhabited by microbes that exert different effects on their hosts. In insects, there are many examples of beneficial associations with symbiotic microbes that have been linked to the insect ecological success. Symbionts that are vertically transmitted from mother to offspring and with an intrinsic interdependence with the insect host are considered as primary endosymbionts and they have reduced genomes [1, 2]; they do not grow on standard laboratory media. In theory, endosymbionts evolved from gut bacteria [3] that are largely more complex and may be determined by the diet and the environment. Primary endosymbionts may reside inside insect cells called bacteriocytes that may be found in specialized host structures called bacteriomes. Bacteriomes may be equivalent to plant-root nodules considering that they are host structures harboring particular bacterial species with specific roles [4]. But even in plants, cosymbionts have been encountered; for example, the slow-growing actinobacteria *Micromonospora* is found in nodules formed by *Bradyrhizobium, Rhizobium*, or *Frankia* in several legumes or actinorhizal roots, although *Micromonospora* is unable to form nodules [5]. *Micromonospora* has been reported to enhance nodulation and promote plant growth, may enhance plant defense responses, or inhibit pathogens [6].

In insects, cosymbiosis is not uncommon and there are cases in which two or more bacterial symbionts are found in the bacteriome [7, 8]. Additionally, other microbes including fungi may be found in the hemolymph or in different insect tissues [9–11]. Fungal symbionts may be found as well in specialized insect structures known as mycangia [12] or inside insect cells called mycetocytes [13].

In insects, primary bacterial endosymbionts synthesize essential amino acids or vitamins for their hosts and reside intracellularly in bacteriomes. In some cases, complementation of metabolic pathways seems to occur among different insect symbionts [14–17]. Additionally, cosymbionts may have different roles, and some have been implicated in defense [18–21], tolerance to stress [22], resistance to high temperatures [23–25], to virus [26–28], or may manipulate sex differentiation [29]. There is an example in which a secondary endosymbiont substituted a lost primary *Buchnera* symbiont in an aphid [30]. Among others, alpha, gamma and beta-proteobacteria have been found as cosymbionts; for example, the primary endosymbiont *Candidatus* Sulcia muelleri ("*Sulcia*" from here on) (phylum Bacteroidetes, class Flavobacteria) with a highly reduced genome has betaproteobacteria as cosymbionts found in green rice leafhoppers [7], stinkbugs [31], and spittlebugs [32, 33]. In leafhoppers, the symbionts occupy different types of bacteriocytes that constitute the outer or inner regions of the bacteriome [7]. The *Sulcia* cosymbionts are *Hodgkinia, Zinderia, Nasuia* [34, 35] with very small genomes, and the gammaproteobacteria *Baumannia, Arsenophonus*, or *Sodalis*, the latter considered as a new acquisition. Surprisingly a gammaproteobacterium may be found inside *Sulcia* cells and be transmitted to the next generation [36].

Scale insects (Hemiptera: Coccoidea) feed on plant sap, which is a nutritionally poor diet that lacks most of the essential amino acids. Therefore, these insects have built up symbiotic associations with bacteria that can synthesize them. Most of the scale insect families that have been analyzed, such as Monophlebidae, Coelostomidiidae, Orthezidae, Phenacoccinae from Pseudococcidae,

Coccidae, Lecanodiaspididae, Diaspididae, and a clade of Eriococcidae, harbor flavobacteria as primary symbionts and enterobacteria as secondary symbionts. [37–39]. It has been reported that the families from scale insects Dactylopiidae, some Eriococcidae, and Pseudococcinae from Pseudococcidae harbor different endosymbionts, which could indicate that they lost their flavobacteria and enterobacteria and acquired other endosymbionts [39]. Flavobacteria seem to be very ancient symbionts, perhaps starting symbiosis before the divergence of scale insects [39] (150–250 mya [40]). Although it has been suggested that Flavobacteria have cospeciated only within Monophlebidae, Coelostomidiidae, Ortheziidae, and Diaspididae [38–41], and host switches seem to have occurred in the other families [39]. Otherwise, enterobacteria have undergone more evolutionary events (losses, duplications, and host switches). Some scale insects have enterobacteria closely related to *Sodalis* endosymbionts (*Sodalis*-like). But others may have symbionts closely related to *Pantoea* and *Klebsiella* [39].

Sodalis cosymbionts have been identified mainly by their 16S rRNA but also by other gene sequences. They have been found within various insect orders including Diptera, Coleoptera, Phthiraptera, and Hemiptera [42–45]. The first described was *S. glossinidius*, the secondary symbiont of tsetse flies [46]. Later, bacteria with related gene sequences were referred as *Sodalis*-like [47] or *Sodalis*-affiliated but more recently several "*Sodalis*-like" bacteria and SOPE [48] are classified as *Sodalis*, others have been assigned to different genera. Still, scientists are in the process of making correct adscriptions for some of these bacteria [49].

The flavobacteria endosymbiont *Candidatus* Walczuchella monophlebidarum ("*Walczuchella*" from here on) was sequenced from the giant wax cochineal *Llaveia axin axin* (Llave) (Coccoidea: Monophlebidae) [50]. This insect has been used to obtain a lacquer to coat traditional art crafts by native people in Mexico and Guatemala since pre-Hispanic times [51]. The flavobacterial genome revealed that the endosymbiont's major role is to synthesize and provide amino acids to the insect host [50]. The Flavobacteria genome was obtained from the analysis of a metagenome of *L. axin axin*. From this metagenome, we could also ensemble sequences from other microorganisms. Here, we present the draft genome of another cosymbiont of *Walczuchella*, a *Sodalis*-like bacteria that is designated here as *Sodalis* TME1. We also present a comparison to the genomes of five other *Sodalis*, as well as preliminary data of a metatranscriptome performed in the bacteriome of *L. axin axin* adults and in the ovaries of senescent adults.

2. Materials and methods

DNA, sequencing, and assembly were performed from bacteriomes (Illumina HiSeq 2000) and from the homogenized of female adults (pyrosequencing) of *L. axin axin* collected in the state of Chiapas, Mexico, as described [50]. A photograph from *L. axin axin* female adults is shown in **Figure 1**. RAST and GosthKOALA from KEGG [52] were used for genomic and metabolic pathway annotation of the metagenomic data that was previously reported when we obtained the *Walczuchella* genome [50]. *Sodalis* TME1 genome sequence has been deposited at DDBJ/ENA/GenBank under the accession MNBX00000000. The version described in this chapter is MNBX01000000.

Figure 1. *L. axin axin* adult females on a *Jatropha curcas* plant.

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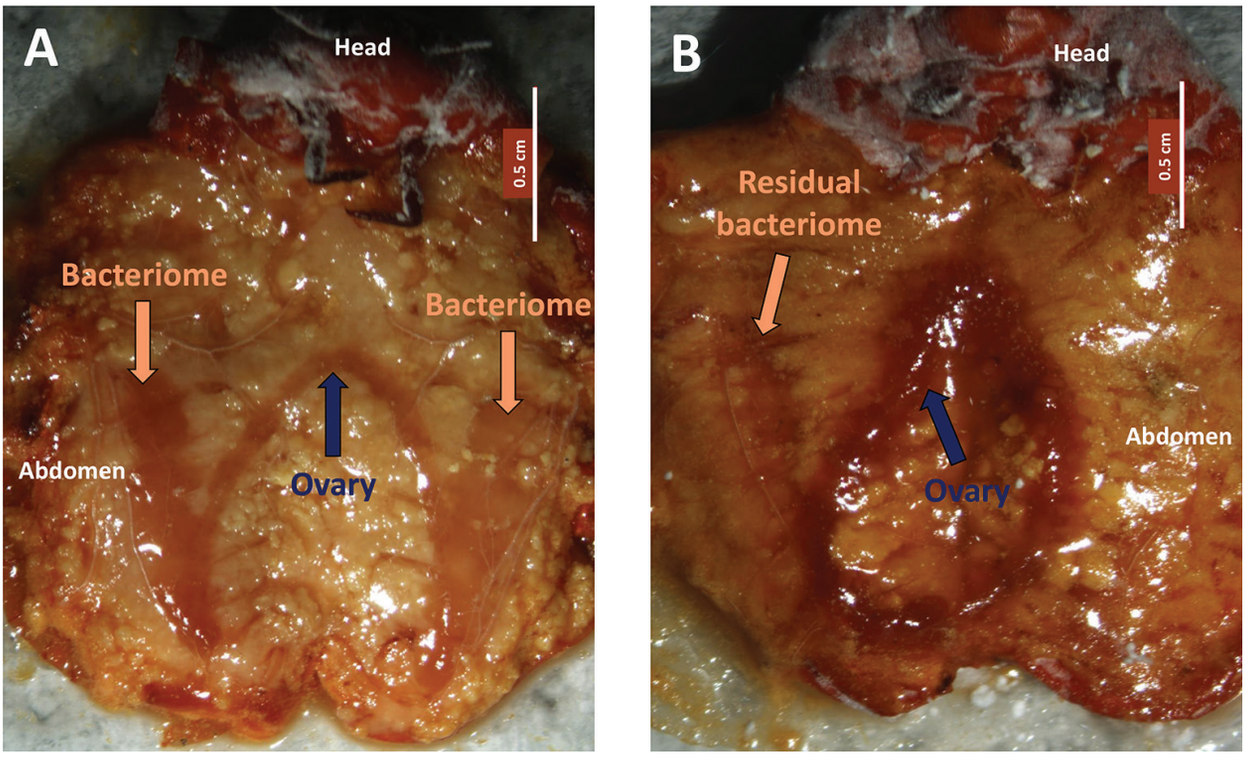

Figure 2. Dissected *L. axin axin* adult females used for the metatranscriptome analysis. (A) early stage and (B) late stage or senescent adults.

Comparative phylogenomic analysis was performed with 20 genomes of gammaproteobacteria from GeneBank. Gene calling of all genomes was performed using GeneMark version 2.5 [53]. The pangenome and core genome from orthologous genes of all strains were obtained by GET_HOMOLOGUES version 2.0 software [54] with -A -c -t 0 -M -n 35 and -A -c -t 0 -G -n 35 parameters. We selected a set of 143 unique single-copy orthologous genes from the core genome. Translated coding sequences of each gene were concatenated using BioEdit Version 7.2.5 and aligned with Clustal Omega version 1.2.1 [55]. Prottest3 version 3.4.2 [56] was used to select the best amino acid substitution model using the AICc correction. The edited alignment contained 47,803 amino acid positions. Maximum likelihood phylogeny was performed by PhyML software version 3.1 [57] using the CpREV model with the Shimodaira–Hasegawa-like procedure for internal branch support [58]. The genome of *Escherichia coli* K-12 MG1655 was used as outgroup.

Comparative genomics was carried out with the following *Sodalis* genomes: *S. glossinidius* morsitans from tsetse fly, *Sodalis*-like endosymbiont from the blood-feeding lice *Proechinophthirus fluctus* (an obligate ectoparasite of fur seals), *S. pierantonus* SOPE from rice weevils *Sitophilus oryzae*, the free-living *S. praecaptivus*, and *Sodalis*-like symbiont of the meadow spittlebug *Philaenus spumarius*. Orthologous genes and the core genomes were obtained by GET_HOMOLOGUES as described above. Core genome matrix was parsed from GET_HOMOLOGUES result, using the parsing_pangenome_matrix.pl script. Shared genes between *Sodalis*-like TME1 and all other strains were retrieved by parsing the core matrix

using custom perl scripts. Annotation of each gene cluster was carried out by BLASTp 2.2.30+ [59] searches against Uniref100 database. Furthermore, average nucleotide identity (ANI) was determined for all *Sodalis* genomes described above using the ANIcalculator software described by Varghese et al. [60] with the default parameters.

RNA was extracted from the bacteriome of *L. axin axin* female adults and from the ovaries of senescent female adults that do not possess the structure of the bacteriomes (bacteriomes degrade in senescent adults) (**Figure 2**). Sequencing of cDNA was performed by SOLID technology. The sequences were mapped to the genomes of *Walczuchella, Sodalis*-like TME1, and two insect reference genomes, *Drosophila melanogaster* and to the aphid *Acyrthosiphon pisum*. Differentially expressed genes were identified by comparing expression values between samples and using Kal's *Z*-test of proportions [61]. Genes with a change in the expression more than twofold and a *p*-value of <0.01 in the *Z*-test were considered as differentially expressed genes.

To determine the uric acid and uricase activity, *L. axin axin* adult females were individually dissected under sterile conditions. Guts including the Malpighian tubules were extracted and metabolic activities were detected as described [62].

3. Results

We found gene sequences of an enterobacterium (gammaproteobacterium) related to *Sodalis* in the metagenome of the wax cochineal *L. axin axin* [50]. The phylogeny with a set of 143 conserved genes shows that the enterobacterium of *L. axin axin* is closely related to other *Sodalis*-like endo-

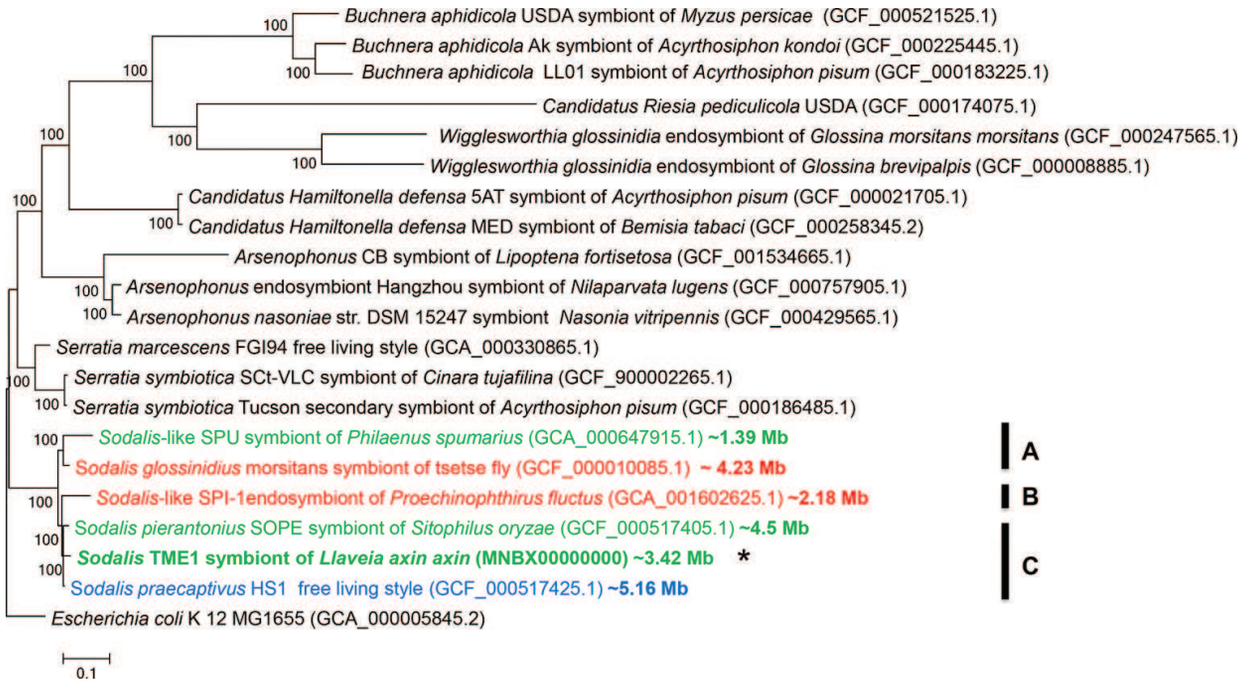

Figure 3. Maximum likelihood phylogeny of sequenced enterobacterial endosymbionts performed with 143 conserved genes. *Sodalis* endosymbionts of plant feeding host: green; blood feeding host: red; free-living style: blue. * : *Sodalis* TME1 used in this study. Scale bar indicates 1 % estimated sequence divergence. SH-aLRT values > 50 are indicated.

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symbionts, especially close to the free-living *S. praecaptivus* [63] (**Figure 3**). The small branches in the *Sodalis* group may indicate that they have recently diverged while the large differences found in genome sizes among these endosymbionts indicate that evolution may be occurring mainly by genome reduction when compared to the larger genome of the free-living *Sodalis* (**Figure 3**).

TME1 was compared with the ANI (average nucleotide identity) metric to other *Sodalis* using the same core genome used in the phylogenomic analysis. TME1 showed ANIs well over 95% that is used to delineate species with *S. pierantonius* SOPE and *S. praecaptivus* HS1, but lower than 95% with *S. glossinidius* morsitans, *Sodalis*-like SPU, and *Sodalis*-like SPI-1 (**Table 1**). There was a good correlation of the ANI values obtained and phylogenetic positions that allowed the identification of three groups within *Sodalis* (**Figure 3** and **Table 1**).

The draft assembly of the enterobacterial endosymbiont *Sodalis* TME1 genome consisted of 679 scaffolds with an N50 of 7713 and an average G + C content of 55.6%. The scaffolds sum 3.4 Mb [50]. A total of 3067 genes were identified to which a functional annotation was assigned. The functional categories more represented by the annotated genes were catabolic and cellular process as well as carbohydrate, amino acid and transcription DNA dependent metabolism (**Figure 4**). Interestingly, many phage-related sequences were found as well as genes for different multidrug efflux pumps and type III and IV secretion systems. TME1 has genes for polyamine biosynthesis and excretion as well as Ankyrin repeat domains and for a lactoyl-glutathione lyase that is a detoxifying enzyme [64]. Among the conserved genes in the core genome of *Sodalis* TME1, *S. pierantonius* str. SOPE and *S. praecaptivus* str. HS1 are genes for the synthesis of flagella and for nitrate reduction (*nar*GHI) and nitrite reduction (*nfr*ABCD). Maybe nitrate serves in *Sodalis* as an electron acceptor in anaerobiosis as occurs in bacterial symbionts of marine bivalves *Lucinoma aequizonata* [65]. *Sodalis* TME1 genome has genes for uric acid utilization such as uricase (*ua*Z), allantoinase (*all*B), allantoate deiminase (*all*C), and urease (*ure*C and *ure*D). Comparative genomics with all *Sodalis* strains show that *all*C and the alpha subunit for urease gene (*ure*C) orthologous were only present in *Sodalis* TME1. Experimentally, uric acid and uricase activity were quantified in *L. axin axin* female adults. We detected 5.86 ± 0.77 ng of uric acid per tissue μg^{-1} and 32.87 ± 5. 25 mU of uricase per tissue μg^{-1} in female cochineals.

	Sodalis str. TME1	*Sodalis pierantonius* SOPE	*Sodalis praecaptivus* HS1	*Sodalis glossinidius* str. morsitans	*Sodalis*-like str. PSPU	*Sodalis*-like str. SPI-1
***Sodalis* str. TME1**						
Sodalis pierantonius str. SOPE	**98.45**					
Sodalis praecaptivus str. HS1	**98.54**	**98.46**				
Sodalis glossinidius str. morsitans	91.24	91.04	91.27			
Sodalis-like str. PSPU	89.81	89.67	89.87	**95.48**		
Sodalis-like str. SPI-1	92.25	91.94	92.15	89.91	85.86	

Table 1. Average nucleotide identity (ANI) percentage among *Sodalis* strains. Values in bold are >95%. Colors correspond to green, plant-feeding host; red, blood-feeding host; blue, free-living style..

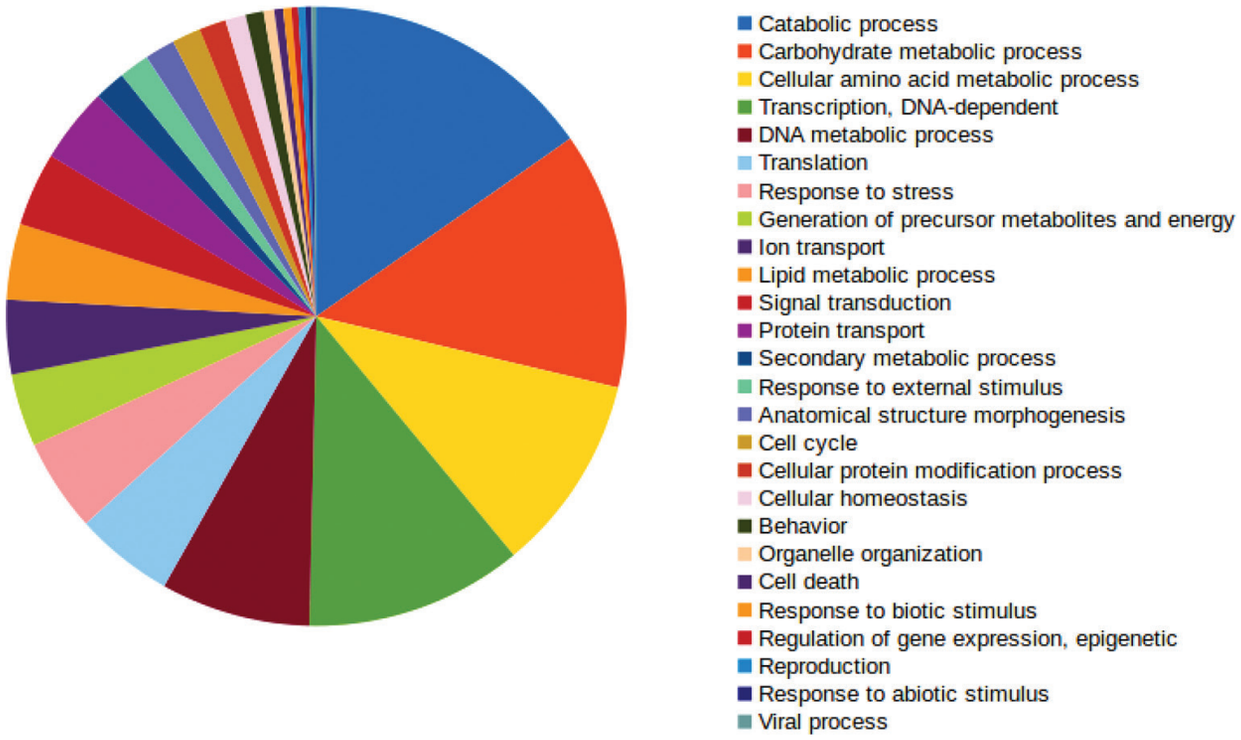

Figure 4. Gene functional categories of *Sodalis* TME1.

We obtained 11,042,037 and 11,042,428 reads from the cDNA sequence of the bacteriome and the ovaries, respectively. These two organs were selected for studying the differentially expressed genes because endosymbionts are transferred from bacteriomes to the ovaries for vertical transmission to their offspring. It was expected to find genes related to the migration of the endosymbionts from the bacteriome and the colonization of the ovaries. Reads mapped to the reference genomes are shown in **Table 2**. The number of genes that were statistically differentially expressed is shown in **Table 3**.

Walczuchella in the bacteriome tissue showed only two genes that exhibited differential expression, a putative hydrolase and the chaperone GroEL. Other genes showed a change in expression less than twofold compared to their expression in the ovary. The chaperonin GroES is almost at the limit for differential expression with 1.86-fold (**Table 4**).

From the ovary tissue, we found differential expression of *Walczuchella* genes that code for some ATP synthase subunits (some of them annotated previously as pseudogenes), cyto-

Reference genome	Bacteriome	Ovaries
Drosophila melanogaster (exons)	2,019,585	2,008,381
Acyrthosiphon pisum (mRNA refseq)	3,082,319	2,912,207
Walczuchella	1,052,077	87,502
Sodalis TME1	409,128	483,601

Table 2. Number of reads mapped to the reference genomes.

Reference genome	Bacteriome	Ovaries
Drosophila melanogaster (exons)	494	680
Acyrthosiphon pisum (mRNA refseq)	244	280
Walczuchella	2	89
Sodalis TME1	66	50

Table 3. Number of genes differentially expressed according to *Z*-test ($p < 0.01$).

	Walczuchella	*Sodalis* TME1	Insect
Bacteriome (differential expression) (high RPKM)	Putative hydrolase	T3SS-secreted effector	Chaperon Hsp70
	Chaperones GroEL, GroES	Allantoinase	ABC transporters
	Hypothetical proteins	Hypothetical proteins	Antiparasitic-like peptide
	ATP synthase B subunit	NAD biosynthesis	Asparaginase
	Amino acids biosynthesis genes	FtsE cell division gene	Unknown genes
		Transcriptional regulation	Extracellular glutamate receptor channel
		Flagellum synthesis	Phospholipids synthesis
			Transcriptional regulation
Ovary (differential expression) (high RPKM)	ATP synthase B and A subunits (pseudogenes)	Hypothetical proteins	ATPase subunit
	AhpC oxidative stress gene	NAD biosynthesis	Transmembrane transporters of sugars and amino acids
	Glycoprotease	Flagellum synthesis	Peptidoglycan-binding protein
	Amino acids biosynthesis genes	FtsE cell division gene	Lysozyme
	Cytochrome c oxydase	Glycolysis	Unknown genes
	SecY translocase	Phage lysozyme	Transcriptional regulation
	Hypothetical proteins	Transcriptional regulation	Phospholipids synthesis

Table 4. Highly expressed and differentially expressed genes in the bacteriome and the ovaries in the endosymbionts *Walczuchella* and *Sodalis* TME1 and the host *L. axin axin*.

chrome c oxidase, also some genes of protein translocation systems, tryptophan, histidine and chorismate biosynthesis, one gene related to oxidative stress, and a gene that encodes a possible component of an ABC transporter (**Table 4**).

In the bacteriome, the enterobacterium TME1 showed very strong overexpression of a gene that codes an effector protein possibly secreted by the type III secretion system (TTSS), expressed 66.8-fold compared to its expression in the ovaries. Also, a gene that codes an allantoinase that participates in uric acid metabolism is highly overexpressed in the bacteriome, showing

a 50-fold change. Other genes with overexpression in the bacteriome are four ABC transporters, a peroxidase, the heme synthase, two genes related to nucleotides biosynthesis, two genes related to lipid A biosynthesis, and two genes of the type III secretion system (**Table 4**).

In the ovary, TME1-overexpressed genes were related to NAD synthesis, carbohydrate metabolism, stress response, and some transporters and transcriptional regulators (**Table 4**).

Among the insect differentially expressed genes in the bacteriome there were 19 putative transporters (for amino acids, carbohydrates, vitamins, drugs, or unknown substrates), five genes related to defense systems including an antiparasitic peptide with identity to Drosomycin, three from *D. melanogaster*, two genes related to heat-shock response, an oxidative stress response gene, seven genes related to amino acid metabolism, and some genes related to lipid, carbohydrate, and vitamin metabolism (**Table 4**).

On the other hand, we found that in the insect, in the ovaries there was overexpression of 15 transporters, 17 immune response genes, some genes related to heat shock, desiccation, oxidative stress, and hypoxia response, and genes related to lipids, vitamins, carbohydrates, nucleotides, amino acids, and chitin synthesis and metabolism (**Table 4**).

4. Discussion

Due to the annual cycle of the wax cochineal, we are only able to collect insects once a year during the rainy season. It is worth mentioning that in 2015 and 2016, we did not find cochineals in many of the places where we had collected previously. Considering the menace of mosquitoes transmitting Zika, or Chikungunya, extensive fumigations with chemical insecticides have been carried out in many places in Mexico, especially in Chiapas. The relation to the diminished populations of cochineals remains to be established.

A previous survey of symbiotic bacteria from scale insects in Mexico revealed the prevalence of Flavobacteria and Gammaproteobacteria [39]. Some of the Gammaproteobacteria had 16S ribosomal gene sequences closely related to those of TME1, and thus they may be considered as *Sodalis* as well. They were obtained from different scale insects such as *Insignorthezia* sp. and *I. insignia, Icerya purchasi, Cripticerya* sp., and *Pseudococcus longispinus* that together with *Llaveia* would be hosts for *Sodalis*.

While Flavobacteria and insects showed a co-divergent pattern of evolution, the phylogenetic relationships of the Gammaproteobacteria and insects were not parallel, indicating multiple enterobacterial transfers among the different hosts, and a more recent and less dependent symbiosis. In agreement, the genome size of the gammaproteobacterium TME1 is much larger than that from the primary endosymbiont from wax cochineals, the Flavobacteria *Walczuchella*, and also larger than those from other cosymbionts as the Betaproteobacteria that accompany the bacteroidete *Sulcia* found in some insects.

The genome from the gammaproteobacterium TME1 (3.4 Mb) is within the range of those from other *Sodalis* (1.4–4.7 Mb, **Figure 3**). There are very few genomes available from *Sodalis*,

namely those from *Sodalis* found in blood-sucking insects as in lice [42] and tsetse flies [66], in plant-feeding insects as the rice weevils [44], in spittlebugs [45], and from a free-living bacterium [67]. The average nucleotide identity (ANI [68] being used for global genomic comparisons and considered now as a gold standard in prokaryote taxonomy [69]) was estimated for the *Sodalis* with available genomes. ANI values and the phylogenomic analysis performed showed *Sodalis* as a defined and coherent genus with three groups A–C. These groups could represent at least three different species according to the global standards [69]. Two of these groups were identified as different lineages by Lo et al. [49]. The phylogenetic groups that we described here have a 100 SH-like value support, group A contains *S. glossinidius* from tsetse flies and *Sodalis* from the meadow spittlebug *P. spumarius*, group B is constituted by *Sodalis* from the fur seal *P. fluctus*, and group C contains the closely related TME1, the free-living *S. praecaptivus* and *S. pierantonius* SOPE. The nucleotide sequence conservation among the group A symbiotic and free-living *Sodalis* may reflect that the former were recent acquisitions in insects without enough time for sequence divergence in their hosts. The presence of very similar *Sodalis* in distinct insect isolates reinforces the reports that indicate that they may frequently be transferred among hosts [39, 47].

TME1 has biosynthetic pathways for all essential amino acids and may supply the needs of the wax cochineal and of *Walczuchella* that does not have complete pathways for the biosynthesis of all essential amino acids. Since *Sodalis* TME1 has all enzymes for TCA it may complement this pathway in *Walczuchella*. It is worth noting that the flavobacterium *Candidatus* Uzinura diaspidicola, an endosymbiont from the armored scale insect *Aphytis melinus* that feed on parenchyma which may provide more nutrients than sap, supplies its host with all nutrients without the need of a cosymbiont [70]. Other armored scale insects have been reported to have a *Sodalis*-like endosymbiont [39].

In *S. glossinidius* that is a secondary symbiont of tsetse flies, a type III secretion system was found implicated in cell invasion and maybe required for colonizing the insect bacteriocytes [71]. Genes encoding for a similar system were found in TME1. Notably, genes that code for the type III secretion system (TTSS) as well as a gene coding for an effector protein that may be secreted by this system were among the most highly induced in the bacteriome of TME1. In *Salmonella enterica*, polyamines are required for full expression of TTSS and for some effector coding genes. Mutants in polyamine biosynthesis are affected in intracellular colonization and survival and may be complemented by adding polyamines to the medium [72]. Furthermore, the modulation of a TTSS by a spermidine transporter has been reported in *Pseudomonas aeruginosa*. Exogenous addition of spermidine to the wild *P. aeruginosa* strain increased the expression of genes that produce effector proteins [73]. TME1 has all genes for spermidine and putrescine biosynthesis as well as for the excretion of spermidine. Polyamines may regulate host defense responses as do some effectors secreted by TTSS. This remains to be tested.

Uric acid and uricase activity were detected in *L. axin axin* females. Uric acid is the final product of purine metabolism. Only few insects are capable of degrading uric acid into other products. In plant-feeding insects, bacterial and fungal symbionts are capable of recycling uric acid into other nitrogen sources [74–76]. *Sodalis* TME1 has uricase and allantoinase-codifying

genes, and the latter was highly expressed in bacteriomes suggesting that *Sodalis* TME1 could participate in providing nitrogen to the host by uric acid recycling.

By reverse transcriptase-polymerase chain reaction (RT-PCR) using primers targeted to *Sodalis*, we found sequences from *Sodalis* in the bacteriome (our own unpublished results), thus we may suppose that *Sodalis* are localized in bacteriomes as *Walczuchella*. In *Llaveia*, in addition to *Walczuchella* and *Sodalis* we found sequences of alphaproteobacteria that are related to Rickettsiales and several fungi that are reported elsewhere (Vera Ponce de León, submitted). Coincidently, the seal lice with a *Sodalis* endosymbiont also harbor a *Rickettsia* that is very abundant. The role of the very little abundant *Rickettsia*-like bacterium in *Llaveia* is unknown. *Wolbachia* is found in members of the Coelostomidiidae family [37] that is closely related to Monophlebidae insect family that contains the Mexican wax cochineals.

Here, we used the term symbiome [27] to refer to the group of primary and secondary (cosymbionts) endosymbionts (and/or their genomes), residing in a host. We consider that the term symbiome is more adequate than the terms endosymbiotic community or consortium that are sometimes used instead.

The cosymbionts of different Flavobacteria in scale insects are diverse lineages of related Gammaproteobacteria [39]. Similarly, the cosymbionts of *Sulcia* (a flavobacterium as *Walczuchella*) are varied and may be different even in related hosts [7, 36]. *Sulcia* cosymbionts may belong to alpha, beta, or gammaproteobacteria, with alpha and betaproteobacteria looking like the oldest symbionts. It was reported that *Candidatus* Zinderia insecticola, the Betaproteobacteria of spittelbugs was probably substituted by a *Sodalis*-like symbiont in members of the Philaenini tribe of the spittelbugs [33, 45]. The displacement of betaproteobacterial cosymbionts by the gammaproteobacterium *Sodalis* seems recent and was described as an event "*in statu nascendi*" (in the stage of being born) in *Cicadella viridis* [77]. There are other examples where one endosymbiont may substitute another one or is on the way toward displacement of a highly reduced-genome endosymbiont [33, 77–79]. Distinct (apparently replaceable) cosymbionts may fulfill the different needs of insects that may change overtime and conditions specially if the insect changes habit [22], otherwise there may be cosymbiont redundancy, with different bacteria performing the same or very similar role (e.g., the synthesis of essential amino acids). The *Sodalis* cosymbiont in the wax cochineals seems to be recently acquired as in *C. viridis*. The insect symbiome seems plastic or dynamic with cosymbionts playing a key role in this plasticity. Here, we enlarged the list of putative functions of *Sodalis* that may include uric acid recycling, polyamine biosynthesis, or detoxification.

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