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Cultivable Bacterial Diversity in the Gut of the Chagas Disease Vector *Triatoma dimidiata*: Identification of Possible Bacterial Candidates for a Paratransgenesis Approach

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Since bacterial symbionts play a vital role in the metabolism of hematophagous insect vectors the method known as paratrangenesis, which consists of the use of cultivable insect symbionts to interfere with the transmission of vector-transmitted pathogens has been shown to be effective in certain vector control oriented studies. In Chagas disease research a recent study introduced transgenes through a parastransgenic approach and prevented the development of a vector species for this disease. However this approach requires a previous characterization of the bacterial symbionts present in the species vector of interest, since a selection of the cultivable bacterial symbiont used is mandatory. In this study, we describe the gut bacterial diversity of *Triatoma dimidiata* specimens from Southern Mexico. Bacteria from both wild and laboratory-reared specimens were cultured, and resulting colonies were grown individually, harvested, and subsequently identified by 16S ribosomal loci sequencing. A total of five and three genera and a total of nine and six bacterial species were identified from field captured and laboratory reared *Triatoma dimidiata* specimens respectively. A majority of Gram positive bacteria were identified, which included the genera *Staphylococcus, Bacillus, Brevibacterium, Micrococcus,* and *Delftia*. Given previous studies we propose the use of *Staphylococcus saprophyticus, Micrococcus luteus,* and *Bacillus megaterium* as potential candidates for future paratransgenic efforts done with *Triatoma dimidiata*, which is one of the most important vectors of Chagas disease, in Central and South America. Given the vital association bacterial symbionts play in the metabolism of routes of hematophagous insect vectors Paratransgenesis consists of the use of cultivable insect symbionts to interfere with the transmission of vector-transmitted pathogens.

Keywords: paratransgenesis, symbionts, *Triatoma dimidiata*, *triatominae*, cultivable bacteria

INTRODUCTION

The World Health Organization has estimated Chagas disease affects about 6–7 million people worldwide, while 100 million people are at risk of acquiring the disease in the Americas. The main form of parasite transmission to humans is through the contact with the feces of blood-sucking triatomine bugs (WHO, 2015). Despite the successful use of insecticides through spraying campaigns in many endemic areas, triatomine bugs have not been completely controlled, in part due to a lack of sustained effort by health authorities and in part due to pesticide resistance (Vassena et al., 2000; Dias et al., 2002; Picollo et al., 2005).

The cost associated with the manufacture of environmentally damaging chemicals which can be used as insecticides is usually very high for developing countries. This high cost is one of the main reasons developing countries interrupt vector control programs, including those for Chagas disease. Therefore, new strategies for vector control are needed. Understanding some bionomic aspects of the triatomine insects can inform the design of better control programs to interrupt transmission of the parasite (Ramsey and Schofield, 2003).

Triatoma dimidiata (T. dimidiata) is one of the most important triatomine species involved in transmission of *Trypanosoma cruzi*, the etiological agent of Chagas disease, in Central and South America (Guhl, 2009). *Triatoma dimidiata* can be found within human dwellings, increasing the risk for Chagas disease transmission to humans (Dumonteil et al., 2002, 2013; Weeks et al., 2013; Zamora et al., 2015). A study in Colombia described tolerance to pyrethroid insecticides in specimens of *T. dimidiata* (Reyes et al., 2007), which confirms the importance of developing new vector control tools for this domesticated species.

Insects are inhabited by a wide diversity of microorganisms, most of which are mutualistic symbionts (Akman Gündüz and Douglas, 2009; Russell et al., 2009), since they can positively affect both nutrition and defense of their insect hosts. For example, protection against a virulent nematode (*Howardula aoronymphium)* by *Spiroplasma* through the production of a toxin has been observed in *Drosophila neotestacea*, illustrating the positive effect these interactions can have in the defense system of the hosts (Douglas, 2011; Hamilton et al., 2015). Another example is the bacteria in the digestive tract of insects that appear to be necessary for detoxification of plant material (Bugg et al., 2011). *Wolbachia* sp. in particular has been found to be responsible for: (1) the regulation of iron concentrations in order to protect its insect hosts from oxidative stress and (2) to provide B-complex vitamins in blood-fed insects (Kremer et al., 2009; Nikoh et al., 2014). A thorough literature review of insects or other arthropods bearing bacteria is shown in the Supplementary Material (Supplementary Table 1). In terms of bacteria associated with triatomine species, the first reports were in the mid-twentieth century. Ryckman and Blankenship (1984) describe 248 references that mentioned parasites, symbionts, and predators of triatomine bugs in a literature compilation covering studies from 1950 through 1984 (Ryckman and Blankenship, 1984).

Triatomine insects feed exclusively on blood throughout their developmental cycle, a diet that is deficient in certain vitamins and nutrients (Dasch and Weiss, 1984). The deficient vitamins are provided by extracellular bacterial symbionts located in the gut. Therefore these bacteria are essential for normal growth and development in Triatomine insects (Eichler and Schaub, 2002). The symbiotic bacteria are transmitted from adult triatomine to their offspring through coprophagy (Dasch and Weiss, 1984).

Studies on insect intestinal bacterial symbionts have provided important information on how to use these microorganisms to control parasites transmitted by insect vectors- a strategy known as paratransgenesis (Hurwitz et al., 2011). It is essential to characterize these symbionts in both wild and laboratory-reared insects, since information concerning the maintenance of symbionts in laboratory conditions is critical to the paratransgenic approach. The paratransgenic method consists of creating recombinant bacteria that express molecules that control the development of the parasite within the vector and are then taken by insects through their diet.

Several paratransgenic approaches have been reported for triatomines. In *Rhodnius prolixus*, one of the most important vectors in Central and South America, the symbiotic bacterium *Rhodococcus rhodnii*, has been efficiently transformed and stable expression of transgenes has been achieved (Dotson et al., 2003). Another example is a species of the *Corynebacterium* genus that was identified in *Triatoma infestans* as a cultivable symbiont and has been successfully used to express a single chain antibody within the insect (Durvasula et al., 2008). More recently, dsRNA expression in *Rhodnius prolixus* through transformed *Escherichia coli* was reported as a model system to control the populations of this vector (Taracena et al., 2015). Two other studies have addressed the paratransgenic approach for this important Chagas disease vector, describing the identity of possible bacterial symbionts (Pamela Pennington, personal communication) and the transformation of some of them for application of this control method in Guatemala (Pennington and Beard, unpublished data).

The main purpose of this study was to identify cultivable bacteria present in the intestine of wild and laboratory *T. dimidiata* from Southern Mexico, as a first step to a paratransgenic approach with this vector. Our study describes the particular bacterial species associated with this region of Mexico and compares our results to previous studies of the same vector in Central America.

MATERIALS AND METHODS

Laboratory Insects

Insects from a *T. dimidiata* colony that had been maintained at Centro Regional de Investigación en Salud Pública, Instituto Nacional de Salud Pública (CRISP/ INSP) (26.5 ± −1°C, 65 ± −5% Relative Humidity, given rabbit blood through feeders) for 5 years were used for this work. Five specimens from every developmental stage were dissected to test for bacteria.

Wild Insects

Twenty adults and one 4th instar nymph of *T. dimidiata* from five villages within three states in Mexico (Chiapas, Campeche and Oaxaca) were captured between March and August, 2010. In Campeche, collection sites were transition spaces between farming lands and wild areas. In Chiapas and Oaxaca, the collection sites were peridomestic.

Most of the insects' bodies were processed for diagnosis of *Trypanosoma cruzi* infection; their intestine (total gut) was aseptically dissected and divided to search for bacteria. None of the insects were positive for *T. cruzi* infection.

Dissection

Insects were anesthetized by incubating at low temperature (−20°C). They were superficial disinfected with 70% ethanol and placed on sterile Petri dishes; sterile forceps and needles were used to dissect organs. Digestive tract was exposed by cutting off the conecctive tissue around the abdomen and separating it the frontal and dorsal plaques. The small intestine or posterior midgut were cut and separated from the stomach and rectum (identification of organs according to Waniek et al., 2012) to be cultured. In laboratory-reared insects, fecal samples were taken by compression of the abdomen 5 days after the last blood meal, and then they were dissected following the same technique already described.

Isolation of Bacteria

Intestine macerates were cultivated on Brain Heart Infusion Agar, selected as an enriched medium to support growth of environmental bacteria. Once incubated at room temperature for 24 h, colonies that were morphologically different among each other were selected and grown in nutrient broth. Bacterial overnight cultures were harvested for DNA extraction.

Bacterial Identification

Bacterial species were identified by amplification of 16S Ribosomal gene using primers 63F: 5′-CAGGCCTAACACATGCAAGTC-3′ and 907R: 5′-CCGTCAATTCMTTTGAGTTT-3 (Marchesi et al., 1998; Muyzer and Smalla, 1998), using as a template the bacterial DNA extracted from bacterial cultures. PCR products were purified and sequenced bidirectionally in a 3730xl DNA Analyzer (Applied Biosystems). Chromas Lite v2.0 was used to edit all chromatograms sequenced and remove primer regions from the sequence. Once sequences were edited they were compared with those stored at Gene Bank through Nucleotide Blast V 2.2.28+. Using the criteria of best *e*-value, the best hits for each sequence were added to the phylogenetic analyses.

Phylogenetic Analysis

All sequences obtained from the study as well as those best hits described above were used for the phylogenetic study (**Table 1**). Each amplicon was used as a reference in Gen Bank through Nucleotide Blast V 2.2.28+ to search for its best hit. For every amplicon the best hit or hits (in cases when there was more than one best hit) were added to our symbiont bacteria data set and aligned using MUSCLE (Edgar, 2004) in Seaview version 4 (Gouy et al., 2010). Subsequently jModeltest2 was used to choose the best DNA substitution model for the data set (Darriba et al., 2012). A Bayesian phylogenetic reconstruction was performed in MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), with a chain length of 5 million generations, sampling every 1000 generations. The first 25% of trees were discarded as burn-in, and the final topology was edited in Figtree (Rambaut and Drummond, 2008).

RESULTS

From the field-collected insects only one type of colony was observed in every culture dish, so only one DNA isolation was made from each insect. For laboratory triatomine, a few morphologically different colonies were observed in every culture dish and some of them were unable to grow in liquid media.

We found 14 different isolates, GenBank accession numbers KX184211-KX184224, from all the *T. dimidiata* specimens examined (**Table 1**). The sequences from the 14 different isolates contained a total of 5 different genera (i.e., *Brevibacterium*, *Micrococcus*, *Bacillus*, *Staphylococcus* & *Delftia*) (**Figure 1**). Once all isolates were added to the phylogenetic analysis, we identified three distinct monophyletic bacteria. The phylum with the most number of bacteria was Firmicutes (9/14 isolates), followed by Actinobacteria (3/14 isolates), and Proteobacteria (2/14 isolates) (**Figure 1**). Of the Firmicutes phylum, the majority of isolates (6/9) had their most significant sequence identity hit to the *Staphylococcus* genera (i.e., *Staphylococcus.* sp. YX3, *S. saprophyticus*, *S. saprophyticus* subsp. bovis, *Staphylococcus nepalensis*, *Staphylococcus hominis,* and *Staphylococcus lentus*), while the other three Firmicutes isolates had their most significant sequence identity hit to the *Bacillus* genera (*Bacillus thuringiensis*, *B. sp.* Ult-816 and *B. megaterium*). The Actinobacteria phylum was formed by three isolates that could be grouped into two genera. Two of the isolates had their most significant sequence identity hit to *Brevibacterium avium* and *Brevibacterium iodinum*, while the other isolate had its most significant sequence identity hit in *Micrococcus luteus*. Finally, the two Proteobacteria isolates had their most significant sequence identity hit in *Delftia acidovorans* and *Delftia tsuruhatensis* (**Figure 1** and **Table 1**).

Field specimens were collected from very similar habitat type (i.e., rural areas), which were generally isolated areas far from roads and people. Most of the specimens were adults, so the comparison between mature and immature insects was limited. *Bacillus megaterium* was the only bacterial species present in triatomine insects from two distant collection sites (**Table 1**), whereas *Staphylococcus saprophyticus* and *S. sp.* YX3 were the only species isolated from both laboratory-reared and field-collected insects. In addition, *Staphylococcus saprophyticus* was the only species that was collected in different developmental stages, since it was present in two distinct nymphal stages (1st & 2nd) in both the intestinal tract and the feces as well as in the feces of the lab reared adults, the 4th nymphal stage and the intestine of an adult collected in Chiapas (**Table 1**). The subspecies identified as *S. saprophyticus* subsp. *bovis* was also

TABLE 1 | Bacterial species associated to 16S rRNA sequences obtained from field-collected and laboratory *Triatoma dimidiata* specimens.

Sequence_ID	Related taxa	Blast sequence percent identity	Accession number	Insect	Origin
CP-01	*Bacillus thuringiensis EGE-B-14.7i*	99%	KX184211	3rd instar nymph	Lab colony
CP-02	*Bacillus* sp. *Ult-816*	99%	KX184212	Adult male	Zohn Laguna, Campeche
CP-03	*Bacillus megaterium* IS07	99%	KX184213	Adult male	Calkini, Campeche
				Adult female	Berriozábal, Chiapas
CP-04	*Brevibacterium avium YPC11*	97%	KX184214	4th instar nymph	Lab colony
CP-05	*Brevibacterium iodinum* DSM 20626	99%	KX184215	Adult male	Salina Cruz, Oaxaca
CP-06	*Delftia acidovorans* 3VH-9	99%	KX184216	Adult female	Zohn Laguna, Campeche
CP-07	*Delftia tsuruhatensis* T7	99%	KX184217	Adult female	Zohn Laguna, Campeche
CP-08	*Micrococcus luteus* RMRCBF23	99%	KX184218	Adult female	Berriozábal, Chiapas
CP-09	*Staphylococcus* sp. YX3	99%	KX184219	3rd instar nymph	Lab colony
				4th instar nymph	Berriozábal, Chiapas
				Adult female	
CP-10	*Staphylococcus hominis* DM 122	99%	KX184220	Adult female	Berriozábal, Chiapas
CP-11	*Staphylococcus lentus* SZ1	99%	KX184221	4th instar nymph	Lab colony
CP-12	*Staphylococcus nepalensis* RCB1044	99%	KX184222	5th instar nymph	Lab colony
CP-13	*Staphylococcus saprophyticus* SPLN2	99%	KX184223	1st instar nymph	Lab colony
				2nd instar nymph	
				Adult male	
				Adult female	
				4th instar nymph	Berriozábal, Chiapas
				Adult female	
CP-14	*Staphylococcus saprophyticus* subsp. *bovis* GTC 843	99%	KX184224	1st instar nymph	Lab colony
				3rd instar nymph	
				4th instar nymph	
				5th instar nymph	
				Adult male	
				Adult female	

found in most nymphal stages (except for 2nd stage) and adults in both feces and intestinal tract of laboratory-reared specimens.

AVAILABILITY OF DATA AND MATERIAL

Sequences of isolates of rDNA have been uploaded to GenBank, accession numbers KX184211-KX184224.

DISCUSSION

Previous studies have reported a low diversity of bacteria associated with the microbiota of triatomine insects in comparison to other insect groups (Vallejo et al., 2009; da Mota et al., 2012; Gumiel et al., 2015). Our results agree with this observation since we found only 14 different 16S rRNA sequences among all field collecting sites, laboratory specimens, and life stages of the insects. The 14 isolates belong to 13 different species, all within the phyla Firmicutes, Actinobacteria, or Proteobacteria. We believe there are several reasons behind the low amount of bacterial species found in this haematophagous insect family. One of them being the existence of microbiological consortia, which is a group of bacteria that are interdependent among each other at the metabolic level. Therefore if one of the bacteria present within the consortia cannot grow in the simple microbiological culture media, due to a lack to fulfill the resource requirements of the bacterial species, then none of the bacteria of the consortia will be observed in the culture (Rosas et al., 2004). This has previously been observed with *Micrococcus luteus*, a species that has been used to "resuscitate" viable-but-not-cultivable bacteria, by the action of a cytokine produced by the former (Mukamolova et al., 1998; Su et al., 2014). Another potential factor influencing the low diversity observed could be the restricted diet of the triatomine and

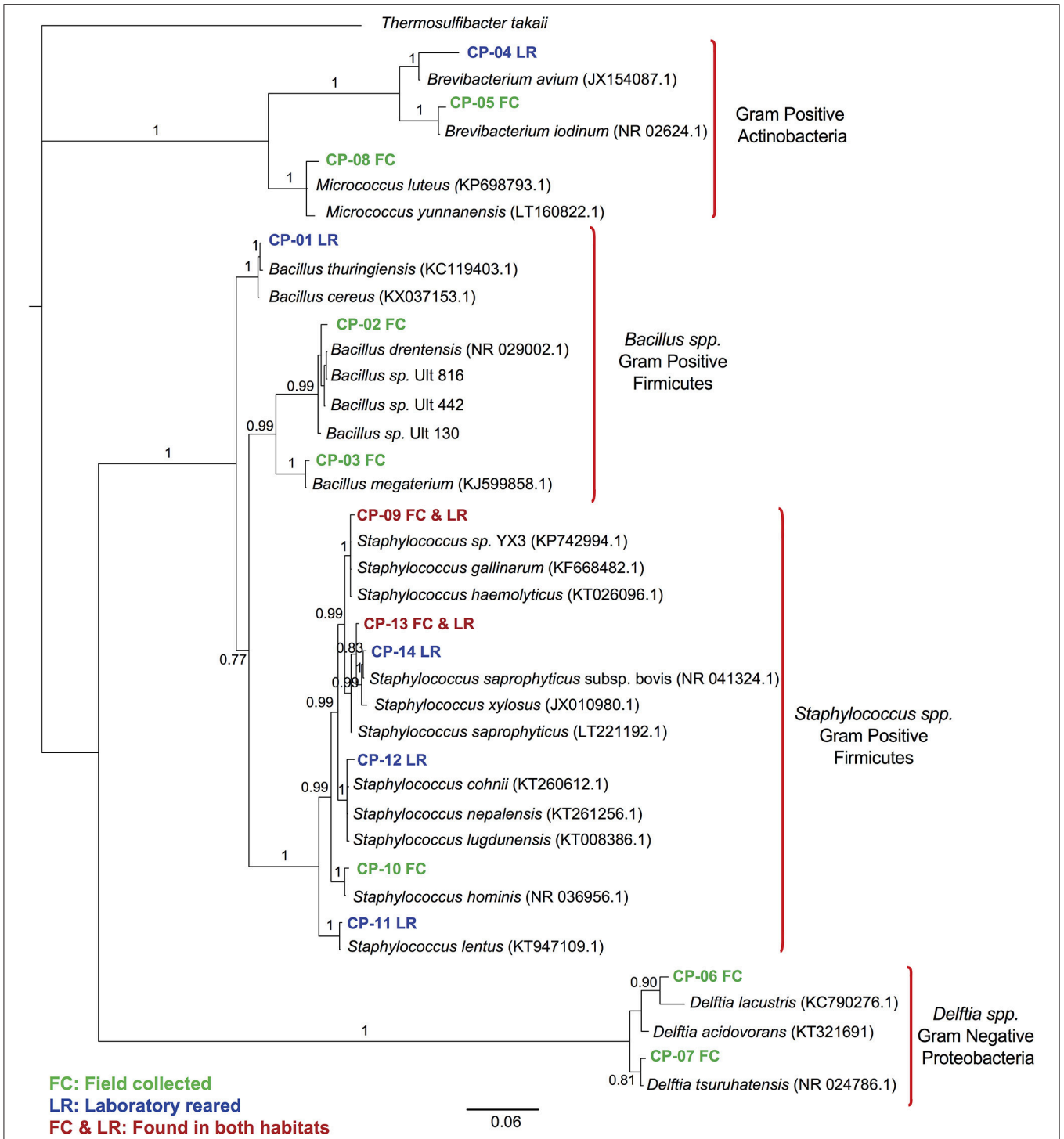

FIGURE 1 | 16S rRNA gene sequence phylogenetic tree of *Triatoma dimidiata* bacterial symbionts. Bayesian bacterial symbionts phylogeny from all 16S rRNA gene amplicons sequenced from field-collected and laboratory-reared *Triatoma dimidiata* specimens. Amplicons from our study are labeled CP, whereas species included in the tree are the most closely related bacterial species found on GeneBank. Numbers on branches represent posterior probabilities from the Bayesian analysis.

physiological structure of their intestine, as observed in another study that looked for bacteria by culture in *Rhodnius prolixus* (Nyirady, 1973).

Additionally, it has been observed that these haematophagous insects have low microbiota diversity due to the presence of several antimicrobial peptides. In *Rhodnius prolixus* (the

insect model species for Chagas disease), several studies have described the presence of these antimicrobial peptides which are trypanocidal in nature (Vallejo et al., 2009; Ursic-Bedoya et al., 2011). In *T. dimidiata* the presence of these antimicrobial peptides has not previously been studied, although the low genetic diversity of bacteria we found among all specimens (field-collected and laboratory-reared) and life stages could potentially point toward the presence of these antitrypanocidal peptides in *T. dimidiata*.

A greater bacterial diversity was found in field-collected insects of *T. dimidiata* compared to specimens that had been reared in a laboratory colony (**Figure 1** and **Table 1**). This finding is in line with previous studies of other insects (Rani et al., 2009; Belda et al., 2011; Rinke et al., 2011; Gumiel et al., 2015). The potential explanation behind this observation is that insects reared under laboratory conditions are not under the same selective pressure as wild specimens. As a result, there is less selective pressure to preserve the bacterial symbionts that might not be vital for their insect hosts, and therefore laboratory reared insects will have a poorer bacterial diversity. Additionally, insects reared under laboratory conditions have a more homogenous feeding source than in the wild. In our study the bacteria found in laboratory-reared insects were for the most part different than those found in field-collected insects, therefore both ideas could be contributing to the discrepancy in species. There were only two bacterial species of *Staphylococcus* (i.e., *S. saprophyticus* and *S. gallinarum*) present in both field captured and laboratory insects suggesting these species have kept a close association to their insect hosts despite the adaptation of insects to captivity.

These results are also in accordance with a study by da Mota et al. in 2012 that reported the characterization of the bacterial symbionts found in four triatomine genera, including two species of the *Triatoma* genus that inhabit South America (*Triatoma infestans* and *T. vitticeps*) (da Mota et al., 2012).

Concerning the bacterial species found in *T. dimidiata*, the 14 distinct isolates described were from five different genera (*Brevibacterium*, *Micrococcus*, *Bacillus*, *Staphylococcus*, and *Delftia*), where *Staphylococcus* was the most abundant genus (6/14 isolates). A comparison among the different life stages was only possible to do among the laboratory-reared specimens, since almost all field-collected specimens were adults. One of the most interesting results was the fact that *S. saprophyticus* was observed in both field-collected and laboratory-reared insects, as well as throughout the distinct life stages of the laboratory-reared insects, suggesting this species plays a very important role in the microbiota of *T. dimidiata*. The other five species reported from laboratory-reared insects were only observed in one of the life stages (*Bacillus thuringiensis/B. cereus, B. avium, S. gallinarum, S. lentus,* and *S. nepalensis*).

Two other studies have reported identification of bacteria as possible symbionts of *T. dimidiata* in Guatemala (Handler and James, 2000; Matthews et al., 2011). In Southern Mexico, *T. dimidiata* apparently only shares *Micrococcus luteus* as an intestinal symbiont with the Guatemalan specimens; in Mexico *Bacillus* and *Staphylococcus* spp. are also found and may correspond to the same species reported in Guatemala (*B. cereus, St. xilosus, St. equorum* and *St. simulans*). No members of the *Gordona* genus were found in our study, contrary to the three species found in Guatemala, among them *G. rubropertinctus*, which has evidence of being a symbiont (Matthews et al., 2011). Therefore, the same vector may have different bacterial populations due to differing environmental conditions.

In addition to the identification of bacterial taxa associated to *T. dimidiata,* a thorough literature review also allowed us to identify potential phenotypic associations of the reported bacteria with their arthropod hosts. Our analysis found that out of the 13 species identified in our study, *Bacillus megaterium* and *S. saprophyticus* appear to be the most common bacterial species described in other arthropods. A variety of studies globally have described both species in insects and mites (Aksoy and Ozman-Sullivan, 2008; Hillesland et al., 2008; Rani et al., 2009; Valiente Moro et al., 2009, 2013; Joyce et al., 2011; Zouache et al., 2011; Mukhopadhyay et al., 2012; Palavesam et al., 2012; Chandel et al., 2013; He et al., 2013; Tagliavia et al., 2014; Zhang et al., 2014; Maleki-Ravasan et al., 2015). Of these two species, *S. saprophyticus* was the bacterial species that was most reported, but had no potential phenotypic traits associated with its arthropod hosts. Only one study appeared to propose *S. saprophyticus* was an opportunistic pathogen of *Dermanyssus gallinae,* which is a haematophagus mite (Valiente Moro et al., 2009). On the other hand, *Bacillus megaterium* is a gram positive bacteria from the Firmicutes phylum that has been reported to have the enzymes necessary for the biosynthesis of some B vitamins (Eppinger et al., 2011). Given the lack of B vitamins in a blood diet, it is possible that triatomines obtain these nutrients from *Bacillus megaterium* (Baines, 1956). Transport and assimilation of iron are other possible functions for this microorganism, since solubilization agents for iron have been shown to be produced by *B. megaterium* (Santos et al., 2014). Therefore, it is unsurprising that previous studies have proposed *B. megaterium* for its use in paratransgenic studies of sand flies (which are vectors of Leishmaniasis) (Hillesland et al., 2008; Mukhopadhyay et al., 2012). *Bacillus thuringiensis* although well known for its insecticide use in GMO's, has few reports of being part of arthropod microbiotas (Chandel et al., 2013; Tagliavia et al., 2014), which makes sense given its insecticidal nature. In fact, an experiment by Nyirady (1973) showed that *Rhodnius prolixus,* also a vector of Chagas' disease, is killed when exposed to the subspecies *thuringiensis*. *Brevibacterium avium* and *B. iodinum* have only been reported in haematophagous arthropods and are described as a non-pathogenic bacteria with low abundance in their arthropod hosts (Hillesland et al., 2008; Valiente Moro et al., 2009; Palavesam et al., 2012). To our knowledge *Delftia tsuruhatensis* has not been previously reported in other arthropods, making this study the first report of its presence in an insect. *Delftia acidovorans* has been previously been reported in a omnivorous ground beetle, as well as in the haemtophagous mite *Dermanyssus gallinae* in which it is reported as a saprophyte (Valiente Moro et al., 2009; Lundgren and Lehman, 2010). *Micrococcus luteus*, is a gram positive bacteria from the Actinobacteria phylum that has been previously reported to play a role in regulating the growth of other bacteria (Mukamolova et al., 1998). Although in *Rhodnius prolixus* it was found to halt insect's development

when applied to nymphs, in our study it was found in one of the field-collected insects and may be part of the natural occurring bacteria in *T. dimidiata* (Gumpert, 1962; **Table 1**). *Staphylococcus homins*, *Staphylococcus lentus,* and *Staphylococcus nepalensis* have all been reported previously in mites and distinct insect orders, with no apparent phenotypic traits associated for *S. hominis* (Rani et al., 2009; Chandel et al., 2013; Pandey et al., 2013; Maleki-Ravasan et al., 2015). *Staphylococcus lentus* and *Staphylococcus nepalensis* have been reported to be opportunistic pathogens in the haematophagous mite *Dermanyssus gallinae* (Valiente Moro et al., 2009). Additionally in the common housefly *Musca domestica*, *S. lentus* is described to be beneficial to the development of its larvae (Zurek et al., 2000). All these findings reinforce the importance the *Staphylococcus* genus not only Triatominae, but also in a variety of other arthropods.

CONCLUSIONS

Current technological methods used for paratransgenic efforts still rely on the laboratory culture of the bacterial symbionts. This study identified cultivable bacterial symbionts in laboratory-reared specimens and in nature. As well as laying the groundwork for the development of future paratransgenic efforts for *T. dimidiata* (an important vector of Chagas disease in Mexico and Central America). Further studies, particularly assays of dependence, are needed to confirm the types of host-microbe association (mutualists, pathogens, commensals) and to describe the potential function of these bacteria. Finally, experiments on the influence of *Bacillus megaterium, Staphylococcus saprophyticus,* and *Micrococcus luteus* on the development of *T. dimidiata* are needed in order to evaluate the potential use of these bacterial species for paratransgenic efforts done with Chagas disease control programs.

AUTHOR CONTRIBUTIONS

TL-O designed the experiments and wrote the article; RM-L designed and performed the experiments; CF-L made the phylogenetic analysis and wrote the article; AC-H contributed to the data analyses and critically reviewed the manuscript; EC critically reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2017.00174/full#supplementary-material

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