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Bacterial endosymbionts in field-collected samples of *Trialeurodes* sp. nr. *abutiloneus* (Hemiptera: Aleyrodidae)

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Abstract

Facultative bacterial endosymbionts are common, influential associates of arthropods, yet their movement among host species has not been well documented. Plant-mediated transmission of *Rickettsia* has been shown for the whitefly *Bemisia tabaci*. *Bemisia tabaci* in USA cotton fields harbors the secondary symbionts *Rickettsia* and *Hamiltonella*, and co-occurs with *Trialeurodes* sp. nr. *abutiloneus* whiteflies. To determine whether symbionts may be shared, the microbial diversity of these whiteflies on cotton across the USA was analyzed. *Trialeurodes* sp. nr. *abutiloneus* bore *Portiera*, *Pseudomonas*, *Serratia*, *Arsenophonus* and *Wolbachia*. No *Rickettsia* or *Hamiltonella* were detected. These results provide no evidence for horizontal transmission of symbionts between these whitefly genera.

Keywords: Bacteria; Distribution pattern; Symbiosis; Transmission routes

1. Introduction

Facultative, maternally inherited endosymbionts are common and important components of terrestrial arthropod biology and ecology. Symbionts may spread in host populations by contributing to host nutrition, mitigating the effects of abiotic factors or natural enemies on their hosts, and/or manipulating host reproduction in ways that enhance symbiont transmission (reviewed in Ref. [26]). The presence of closely related symbionts in evolutionarily distant arthropod hosts indicates a means of horizontal transmission among host taxa, and phylogenetic analyses have provided evidence for horizontal transmission among interacting, unrelated species [17]. Recently we demonstrated that plants can serve as a reservoir for within-species horizontal transmission of the bacterium *Rickettsia* sp. nr. *bellii* among individuals of the sweetpotato whitefly *Bemisia tabaci* (Aleyrodidae) [4]. Additionally, Ref. [14], provided evidence that *Rickettsia* swept into *B. tabaci* populations in the southwestern USA between the years 2000 and 2006, and showed that the bacterium is now virtually fixed in that insect host, together with the facultative symbiont *Hamiltonella*, which has been detected in all field populations of *B. tabaci* tested in the USA. Compared with uninfected whiteflies, Rickettsia-infected *B. tabaci* can cause marked fitness benefits [14].

The name *B. tabaci* refers to a large, diverse group of cryptic sibling species in warm temperate and tropical regions worldwide [8]. The species provisionally known as the "B biotype", or "MEAM1", of the *Bemisia* complex is the only member of the complex found in field populations in the USA. In many cases, this *B. tabaci* co-occurs on the same host plant with the

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banded-winged whitefly *Trialeurodes* sp. nr. *abutiloneus* (Aleyrodidae), a North American polyphagous insect that is known as an occasional economic pest of cotton [18]. After a recent survey of *B. tabaci* collected in twenty-two sites across the southern USA showed *Rickettsia* at high frequencies (Cass et al., unpubl. data), the current study was initiated in order to test the hypothesis that *Rickettsia* or other endosymbionts may be horizontally transferred among whitefly genera that share a host plant. Whiteflies in the genus *Trialeurodes* with a dusky banded-wing pattern have been recorded on Malvaceae throughout the USA [18] and have all been considered *T. abutiloneus*. In the current study, we characterized the symbionts of this whitefly across the southern United States. Heterogeneity discovered in sequences of the whitefly mitochondrial gene cytochrome oxidase I (COI) suggested that more than one *T.* sp. nr. *abutiloneus* species of similar morphology was sampled. For the purposes of this study then, all whiteflies will be referred to as *T.* sp. nr. *abutiloneus*. While all aleyrodids examined to date bear the obligate nutritional symbiont *Portiera aleyrodidarum* [1], sympatric populations of *B. tabaci* sibling species do not generally share facultative symbionts, suggesting barriers to interspecific horizontal transmission [5]. One symbiont that appears to be different in this respect is *Rickettsia*. In addition to the experimental evidence of intraspecific transmission of *Rickettsia* mentioned above, *Rickettsia* is the only symbiont shared between the B and the "Q"(="MED") species in Israel [5]. Lastly, *Rickettsia* in *B. tabaci* has been shown to infect the parasitoid, *Eretmocerus eremicus*, when this wasp develops on infected whiteflies [6].

2. Materials and methods

2.1. Whitefly sampling

Individual adult *T.* sp. nr. *abutiloneus* was collected from cotton plants in 18 fields located in eight different states throughout the USA in the summer of 2011 (Fig. 1), many from the same plants and some from the same leaves as *B. tabaci*. Upon collection, the whiteflies were briefly chilled, preserved in 100% ethanol and kept at −20 °C until processing.

2.2. Characterization of T. sp. nr. abutiloneus bacterial diversity

Denaturing gradient gel electrophoresis (DGGE) and bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) were used to assess the diversity of bacteria in *T.* sp. nr. *abutiloneus*.

DGGE was performed on one or two *T.* sp. nr. *abutiloneus* from each site (a total of 38 insects). Whiteflies were crushed and their DNA extracted as previously described [12]. PCR was performed using general 16S rRNA bacterial primers (Table 1), and product separation was conducted on 6% (wt/vol) acrylamide gel with a denaturing gradient ranging from 20% to 60% urea–formamide by electrophoresis at 70 V and 60 °C for 20 h. The resulting gels were incubated in ethidium bromide solution (250 ng/ml) for 10 min and bands representing bacteria were eluted, cloned and sequenced [12]. The

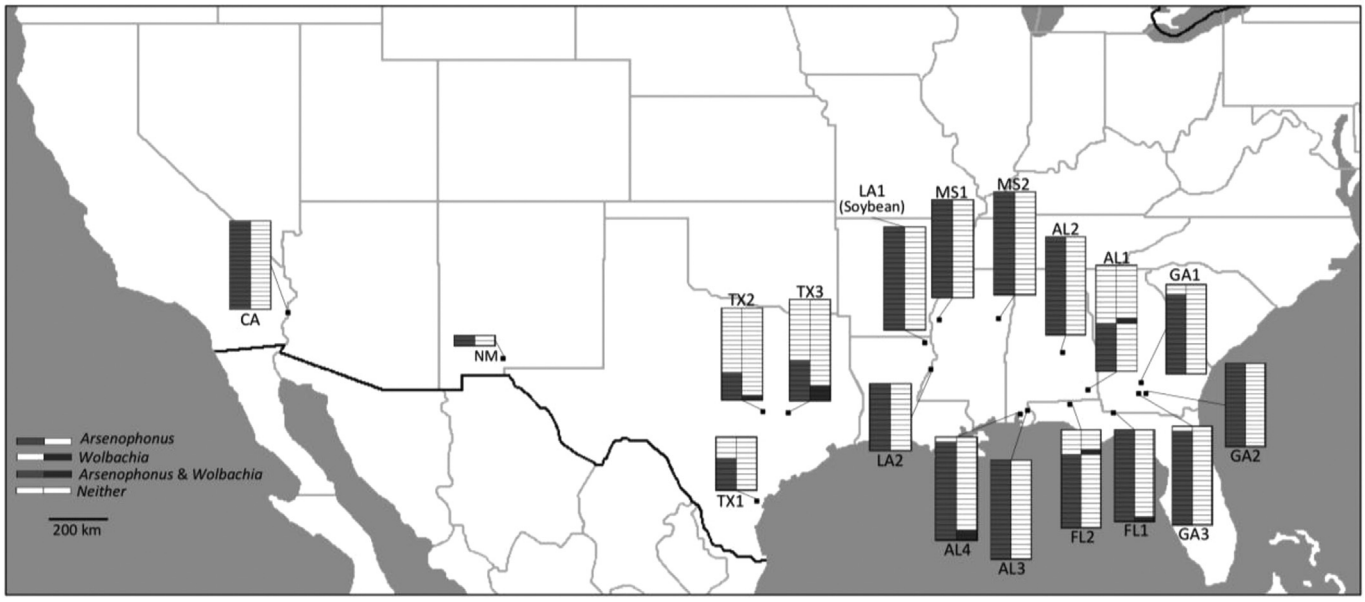

Fig. 1. Map showing collection sites for *T.* sp. nr. *abutiloneus* and infection frequencies of the symbionts *Arsenophonus* and *Wolbachia* at each site. All whiteflies were collected on cotton except for the one soybean site in Darnell, LA. For each rectangular stack, a row (two boxes) represents an individual. A filled-in box in the left column means the individual carried *Arsenophonus*, while a filled-in box in the right column means the individual carried *Wolbachia*. A row in which both boxes are filled represents an individual with both symbionts. The location code incorporates the state two-letter abbreviation and a number representing localities as follows: CA = Blythe, NM = Las Cruces, TX1 = Corpus Christi, TX2 = Thrall, TX3 = College Station, LA1 = Darnell, LA2 = St. Joseph, MS1 = Stoneville, MS2 = Starkville, AL1 = Dothan, AL2 = Montgomery, AL3 = Fairhope, AL4 = Loxley, FL1 = Quincy, FL2 = DeFuniak Springs, GA1 = Tifton, GA2 = Ellenton, GA3 = Sparks.

Table 1

Targeted gene	Primer name	Primer sequence (5′→3′)	Reference	Annealing temp. (°C)/product size (bp)
Arsenophonus 23S rDNA	Ars23S-1	CGTTTGATGAATTCATAGTCAAA	[24]	60/~600
	Ars23S-2	GGTCCTCCAGTTAGTGTTACCCAAC		
General bacteria 16S rDNA (for bTEFAP)	28F	GAGTTTGATCNTGGCTCAG	Research and Testing Laboratory (Lubbock, TX).	
	519R	GTNTTACNGCGGCKGCTG		
General bacteria 16S rDNA (for DGGE)	968F	CGCCCGGGGCGCGCCCCGGGC GGGGCGGGGGCACGGGGG GAACGCGAAGAACCTTAC	[13]	64/~433
	1401R	CGGTGTGTACAAGGCCCGGGAACG		
Wolbachia outer surface protein	WSPF	TGGTCCAATAAGTGATGAAGAAAC	[2]	58/~600
	WSPR	AAAAATTAAACGCTACTCCA		
Rickettsia 16S rDNA	528F	ACTAATCTAGAGTGTAGTAGGGGATGATGG	[6]	60/~500
	1044R	GTTTTCTTATAGTTCCTGGCATTACCC		

results obtained were compared to known sequences using BLAST in the NCBI database.

bTEFAP was performed on DNA from a pooled sample of 60 *T.* sp. nr. *abutiloneus* adults, including 3–4 individuals from all but the two western sites. For this analysis, DNA was extracted with an SDS phenol chloroform method [7] and resuspended in TE buffer pH 8.0. The DNA (conc. 66.5 ng/μL) was split into four 20 μl aliquots; three were sent for 16S rDNA microbial diversity screening at Research and Testing Laboratory (Lubbock, TX) and one was kept at −20 °C as a reference. Pyrosequencing was performed using the Roche 454 sequencing system as previously described [9] for ~3000 reads per sample with the primer sets 28F–519R targeting the 16S rRNA gene (Table 1).

Retrieved sequences were analyzed using mothur software (http://www.mothur.org/). Sequences shorter than 200 bp, as well as those of low quality (multiple N, chimeras, etc.), were omitted. Sequences were aligned using the Silva-compatible alignment database, and a distance matrix was calculated. Sequences were grouped into operational taxonomic units (OTUs) at a 97% sequence similarity threshold (i.e., sequences that differ by 3% were clustered in the same OTU). Representatives of each OTU were classified with mothur, and their affiliation, down to the genus level, was verified by NCBI GenBank databases. Coverage of species richness was calculated by the equation (1 − (No. singletons/No. sequences)) × 100. Diversity indices were calculated using PAST software with 95% bootstrap support (http://nhm2.uio.no/norlex/past/download.html).

All sequences obtained from the 454 analyses were deposited in the Sequencing Read Archive database with the project accession PRJEB4985 (Sample unique names BW1-ERS371202; BW2-ERS371203; BW3-ERS371204).

2.3. Determination of secondary symbiont prevalence at the population level in the field

The frequencies of *Arsenophonus* and *Wolbachia* (identified by DGGE and bTEFAP), and *Rickettsia* were assessed in individual whiteflies from across the USA. The presence of the three symbionts was determined using PCR with symbiont-specific diagnostic primers (Table 1). PCRs were conducted in 13 μl volumes each containing 2 μl of the template DNA lysate, 10 pM of each primer and 0.5 unit of 2× RedTaq Mix (APEX). The PCR program followed the one described in Ref. [6] with primer-specific annealing temperatures (Table 1). In order to verify the product identity, bands were eluted, inserted into the pGEM T-Easy plasmid vector and transformed into *Escherichia coli*. For each cloned product, two colonies were randomly picked and sequenced. For samples that failed to amplify, an additional PCR was performed using primers targeting *Portiera* as a positive control for DNA quality (Table 1). Additionally, sequencing of *Portiera* and *Arsenophonus* was performed on several samples (Table 1). Sequences were deposited at the NCBI database under accession numbers KF803281–KF803283.

3. Results and discussion

3.1. T. sp. nr. abutiloneus bacterial diversity

Both bTEFAP and DGGE analyses showed a number of common bacterial taxa associated with *T.* sp. nr. *abutiloneus* in the USA (Fig. 2). Pyrosequencing resulted in more than 10,000 high-quality bacterial 16S rRNA gene fragment sequences, assigned into 68 OTUs. Over 99% of the sequences were shared by all three samples and diversity indices showed no significant differences among the replicates (Table S1). The dominant bacterial taxon from bTEFAP was the primary symbiont *Portiera* (Fig. 2A), which was also present in all DGGE samples (Fig. 2B). *Arsenophonus* and *Wolbachia* were detected by both the bTEFAP and DGGE, but neither of the facultative symbionts of the *B. tabaci* B species (*R.* sp. nr. *bellii* and *Hamiltonella*) were found in *T.* sp. nr. *abutiloneus* with either method (Fig. 2). Another common bacterium identified by bTEFAP was *Serratia* sp., but its low frequency in the DGGE survey (1 of 38 individuals, Fig. 2B) and the greater similarity of the sequence to the opportunistic pathogen *Serratia marcescens* (100% similar) than to the endosymbiotic *Serratia symbiotica* (95–98% similar) make it unlikely that this bacterium is an endosymbiont. *Pseudomonas* sp. was also present in the pyrosequencing reads. Members of this genus are not known as intracellular symbionts of insects, but some are common epiphytes of the phyllosphere, and could be colonists in the whitefly gut. *Pseudomonas* strains may be commensal or pathogens of plants as well as insects [22].

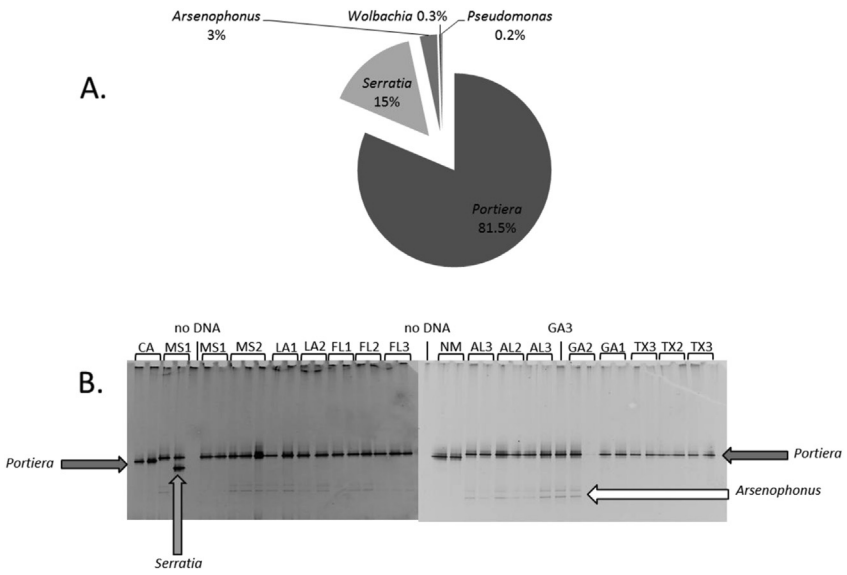

Fig. 2. A) Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) of 60 pooled whiteflies. Most of the sequences recovered belong to the obligate primary whitefly symbiont *Portiera*, followed by *Serratia*, *Arsenophonus*, *Wolbachia* and *Pseudomonas*. The infrequency of *Serratia* in individual samples, and the greater similarity of the sequence to the insect pathogenic species *S. marcescens* than to the symbiont *S. symbiotica*, suggests that the *Serratia* recovered may be a pathogen. B) DGGE results from each collection locality. Sequencing indicated that the bands marked with dark gray arrows are *Portiera*, the two bands marked with a white arrow are *Arsenophonus*, and the one sample marked with a light gray arrow contained *Serratia*. Lane labels correspond to location codes as per Fig. 1.

3.2. Symbiont diversity across geographic regions

The DGGE survey across geographic regions showed a high frequency of *Arsenophonus* in *T.* sp. nr. *abutiloneus*, but no *Rickettsia*. Similarly, species-specific diagnostic PCR recovered no *Rickettsia* in any populations screened, consistent with bTEFAP and DGGE results. *Arsenophonus* was detected in all populations screened, generally with high within-population frequencies (30%–100%), and was fixed in 10 collection sites. *Wolbachia* was absent from 12 out of the 18 populations screened and exhibited much lower within-population frequencies (0–15%, Fig. 1). A high frequency of *Arsenophonus* infection has also been found in the congeneric greenhouse whitefly, *T. vaporariorum* [23]. Taken together, our results provide no evidence of symbionts that are shared between *B. tabaci* B species and *T.* sp. nr. *abutiloneus* in the USA, despite the frequent close physical association of these whiteflies in the field.

Possible routes for movement of bacteria among host taxa are via feeding on a common host plant [3,4], via generalist parasitoid ovipositors [11] or, among parasitoids, exploitation of a common host [10,15]. However, empirical evidence of movement within species is scarce [4,15,16,19], and almost non-existent for interspecific transmission that results in vertical transmission of the symbiont in the recipient species [27]. The mixed results of attempts to transfect species by microinjection also suggest that gaining entrance to a host does not ensure symbiont establishment [21]. In spite of the difficulty of observing it in real time, evidence of dynamic exchange of facultative symbionts among lineages abounds. While, among obligate nutritional symbionts, phylogenies indicate that cospeciation of host and symbiont is the rule [17], the lack of congruence in phylogenies of arthropods and facultative symbionts has long been noted, and horizontal transmission of secondary symbionts has been inferred from clustering of related symbionts with interacting species (e.g. Refs. [20,25]). Generally, these results underline the complexity of patterns of transmission of different intracellular bacteria in herbivores, and the low frequency of horizontal transmission events even among related species.

4. Conclusions

The results presented provide the first characterization of the symbionts of *T.* sp. nr. *abutiloneus*, common whiteflies and occasional pests of cotton in the USA. In addition to *Portiera*, the common facultative endosymbiont lineages of whiteflies *Arsenophonus* and *Wolbachia* were found. The role of another bacterium, *Pseudomonas*, is unclear, but this common plant epiphyte may be ingested in the process of feeding. Lastly, we found no evidence of shared facultative symbiont lineages between *T.* sp. nr. *abutiloneus* populations and the B species of the *B. tabaci* complex, although these whiteflies could be found feeding on the same leaves of the same plant. In contrast, in Israel, *Rickettsia* is shared between two members of the *B. tabaci* complex, and laboratory experiments indicate a mechanism for potential transfer among whiteflies [4]. More work is necessary to understand the interactions of ecological and physiological barriers that determine the complex patterns of interspecific transmission we see in phylogenetic reconstructions of symbionts and their arthropod hosts.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2014.01.005.

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