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Identification of the saprophytic endosymbionts in the whitefly (*Bemisia tabaci*) colonizing cotton and eggplant

FATEMEH RAHIMI[1], ALIDAD AMIRI BEHZADI[2] and V G MALATHI[3]

Indian Agricultural Research Institute, New Delhi 110 012

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ABSTRACT

Whitefly transmitted virus diseases are a major challenge in improving productivity of agricultural and horticultural crops. The survival or increased fitness of the vector *Bemisia tabaci* is attributed to bacterial endosymbiont present in their body parts. In recent year, the bacteria are also shown to play a vital role in facilitating transmission of viruses. In the present study, an attempt was made to characterize the obligate and saprophytic endosymbionts in two populations of whitefly, one occurring on cotton plants in the field and the other of vector reared on egg plant in the laboratory. The bacteria were identified by characterization of 16S r RNA genes. Results showed that the whitefly population from cotton field contains *Bacillus thuringiensis*, *Bacillus altitudinis*, *Bacillus pumillus*, *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus cereus* and *Bacillus* spp., *Staphyloccus haemolyticus* and *Ralstonia* spp. in all developmental stages, egg, four instars and in adults. However, in the case of glasshouse reared population, the predominant bacteria were *Ralstonia pickettii* besides *Bacillus pumilus* present in instars only.

Key word: *Bemisia tabaci,* Cotton, Endosymbiont,Whitefly

Presence of endosymbionts microorganism has been recorded in about 10% of insect species (Douglas 1989). The contribution of endosymbionts to biological fitness of the insect is especially vital in the case of insects with restricted diet, as in the case of phloem-feeders which have diet rich in carbohydrates but deficient in amino acids (Baumann 2005). In whitefly, *Bemisia tabaci*, two types of endosymbionts are present organized in specialized cells called mycetocytes or bacteriocytes originating from mesoderm. These mycetocytes contain primary (P) and secondary (S) endosymbionts. The primary symbiont is *Candidatus protiera aleurodidarum* which is present in all the whitefly populations of the world, whereas, S-endosymbiont differs in the different populations of the world.

The S-endosymbiont so far identified in *Bemisia tabaci* are *Arsenophonus, Cardinium, Rickettsia, Hamiltonella* (Gottlieb *et al.* 2006). The peculiarity of endosymbionts in whitefly is the vertical transmission of intact mycetocytes into ovaries, from there to eggs (Costa *et al*. 1997, Szklarzewic and Moskal 2001). This peculiar vertical transfer feature of endosymbionts of whitefly makes it an interesting subject to study. Vertical transmission is a widespread feature of symbiotic interactions (Bright and Bulgheresi 2010). As these symbionts are passed directly from parents to offspring, they have the potential to affect hosts during all developmental stages and can often be experimentally manipulated within host populations without contagious spread. In recent years endosymbionts are also considered to contribute to the efficiency of virus transmission by whiteflies. Though there are lot of studies on transmission characteristics of the whitefly, till date no information is available on the type of obligate and saprophytic endosymbionts present in the whitefly population in India. The objective of the present study is to examine and identify such bacteria in whiteflies, and to characterize them.

MATERIALS AND METHODS

Two whitefly populations were studied to analyze the microbial colonization. Bacterial isolation were attempted from these populations. One population reared in field on cotton and another population reared in glasshouse on eggplant. These two different populations were taken to see whether bacterial profile of the whitefly maintained in glasshouse for more than five years, is distinct compared to the one occurring in the field. A total of 25 individuals for every stage, egg, first, second, third, fourth instar and adult were examined. Five individuals (egg / nymph (1^{st}, 2^{nd}, 3^{rd} and 4^{th} instar)/adult) were put in one eppendorf tube and

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[1] M Sc (Plant Pathology) (email: rahimi.science@gmail.com), [2] M Sc (Genetics), [3] Principal Scientist (Plant Pathology), Division of Plant Pathology

treated as one sample. Likewise five samples were made for each stage for two whitefly populations. They were separated, labeled and preserved in 70% alcohol. These individuals (five eggs, five nymphs 1^{st}, 2^{nd}, 3^{rd} and 4^{th} instar) and five adults) were kept in an eppendorf tube and added 50µl of DNA extraction buffer. The samples were ground in 50µl of DNA extraction buffer (50 mM Tris. HCl pH = 8, 0.5% Tween 20, 0.25 mg/ml Proteinase K) in eppendorf tube with the help of crushing tube and were incubated at 65ºC for 2 hours. After incubation 5µl of the extract kept in boiling water for 5 minutes. After boiling, the tube was kept at –20ºC for 5 minutes. The suspension is centrifuged at 8000 rpm 4ºC for 5 minutes. The supernatant solution contains the DNA which was stored at –20ºC or used for PCR. The egg, nymphs, and adult whiteflies were disinfected by suspending in 0.5% streptomycin for 1 hour. After completion the time, samples were washed in distilled water three times for 20, 10 and 5 minutes. The samples were dissected in sterile saline in cavity slides. The mycetome observed under stereomicroscope were transferred to 20µl saline, the suspension was vortexed and from this stock, 1:10, 1:100 and 1: 1000 dilutions were made. 200 µl of each dilution were placed on nutrient agar. The bacterial colony were individually picked up and streaked on nutrient agar. To study the morphology of bacteria, the bacterial colonies were suspended in saline and 5 µl of suspension were viewed under electron microscope (JEM-1011). The bacteria isolated were subjected to gram staining essentially following the protocol as described by Hans Christian Joachim Gram (Gram 1884). In order to identify the saprophytic endosymbiont, the bacteria isolated from mycetome were separately inoculated onto Luria broth. The genomic DNA was prepared using Quiagen genome isolation kit following manufactures protocol. The DNA concentration was estimated by measuring absorption at 260nm in a spectrophotometer. Using the formula 1 OD_{260}= dsDNA 50 µg/ml DNA, concentration was estimated. In order to identify the bacteria based on 16S r RNA gene, PCR reaction was performed using the primers, forward primer, 8F: 5´ GGATCCAGACTTTGATYMTGGCTI 3´ (Felske *et al.* 1997) and reverse primer, 907R: 5´ CCGTCAATTCMTTTGAGTTT 3´ (Lane *et al.* 1985). The PCR was performed in a thermocycler (Biometra). PCR conditions are as given below: Denaturation: 94ºC for 3 min; 94ºC for 1 min (denaturation), 55ºC for 1 min (annealing) and 72ºC for 1 min and 30 second (extension); Amplification: 30 cycles and final extension at 72ºC for 10 min 4 ºC. PCR mixture: Taq DNA polymerase buffer (10X) 2.5 µl (1X), DNA 2 µl (100 ng), $MgCl_2$ (25 mM) 2 µl (2mM), dNTP mix (10mM each) 1 µl (200nM), Forward primer (100ng) 0.5 µl (1ng), Reverse primer (100ng) 0.5 µl (1ng), Taq DNA polymerase (5 units/µl) 0.5 µl (5units), d.dH2O volume up to 25 µl. The PCR amplicons were purified and cloned in pGEMT easy vector following the protocol outlined in Sambook and Russel (2001). The presence of insert in the colony was confirmed by restriction of the miniprep plasmid DNA by EcoRI. Alkaline lysis method (Birnboim and Doly 1979) was followed to isolate plasmid DNA from *E.coli.* Exposure of bacterial suspension to the strongly anionic detergent like sodium dodecyl sulphate (SDS), at high pH breaks the cell wall; denature chromosomal DNA and proteins and releases plasmid DNA into the supernatant (Sambrook and Russell 2001). All plasmid DNA isolated were subjected to restriction using specific endonuclease. Fragments were separated by agarose gel electrophoresis to determine the correct recombinant clones. The single restriction reaction mixture was 20 µl as follows, 5 µl Plasmid DNA (100ng), 2µl ECORI Buffer (10X), 1 µl ECORI Enzyme (20 units/µL) and 12 µl d.dH 20, 1 µl. The restriction reaction mixtures were incubated at 37ºC for 5 hours. The reaction was stopped by 6X loading dye, and electrophoresed on 1% agarose gel.

The recombinant plasmid DNA that were screened earlier by rapid analysis colony PCR and restriction digestion with endonucleases, were further confirmed by sequencing in an automatic DNA sequencer (at Delhi university, South Campus, New Delhi and Ansul Biotechnology company). Sequence were edited and analyzed in BLAST, an online tool available for sequences identify analysis. The test bacterium was identified as belonging to the specific species with which it shows more than 95% sequence identity in16S r DNA region.

RESULTS AND DISCUSSION

Saprophytic endosymbiont

Bacteria were cultured from mycetome, isolated from whitefly adults, nymphs and eggs collected from cotton plants in field and egg plant in the glasshouse. In all the cases, distinct bacterial colonies appeared within 18 hr at 37ºC. The type of bacterial colony obtained and their frequency among the population of bacteria are given in Table 1.

Approximately 42 colonies from glasshouse samples and 65 colonies from field samples were seen in stock, and 621 colonies from glasshouse reared and 982 colonies from field in 1/10, 1/100 and 1/1000 dilutions. Each colony that differed in morphology and colour were separately streaked. Tables 2 and 3 give number of colonies obtained in five replicates for each stage.

From the bacterial population isolated, two bacteria were selected based on similarity in colony morphology for further identification. They were given name indicating whether they are from glasshouse or cotton field, the developmental stage from which isolated and the specific number of the colony. For example: Adult G1 refers to one bacteria selected from glasshouse reared adult whitefly.

Microscopic observation of bacteria showed that the round colony of bacteria seen in different developmental stages (egg, 1st, 2nd, 3rd instar and adult) with white colour had three type of flagellation such as monotrichous, peritrichous, amphitrichous. Bacteria having coccoid morphology did not have flagella. Gram‘s staining of these bacteria were done to group them, some of them were gram positive and some were gram negative (Table 2 and 3).

From the results Tables 2 and 3, it is clear that even within one developmental stage, different bacterial population may be present. For example both non flagellate and monotrichous bacteria are seen within the isolates from eggs. Likewise in 3rd instar population, both Gram positive and negative bacteria were present. Between egg, instars and adults, again there is difference in the type of bacteria harbored by them.

The bacterial colonies were for further identified using 16S r DNA marker. The genomic DNA concentration extracted from all the bacteria used for study ranged from 820.7 ng/µl to 1.1 µg/µl. Agarose gel electrophoresis of the DNA revealed a single band of non sheared good quality, which could serve as PCR compatible template. Agarose gel electrophoresis of PCR amplicons using bacterial genomic DNA as template revealed 1.5 kb band uniformly in all the bacteria taken for study. Size of PCR amplification is estimated to be 1.5 kb length.The amplicon bands were eluted and cleaned product was ligated with pGEMT easy vector. Transformation with the recombinants gave approximately 130 white colonies.

Miniprep plasmid DNA when restricted with *EcoRI* gave rise to a vector band of 3kb and insert band of 1.5kb size in restricted clones got with amplicon of bacteria isolated from glasshouse adult whitefly sample. However the clones got with amplicon of bacteria Adult G there were vector band of 3kb length and insert band divided into 0.75Kb and 0.6Kb sizes due to presence of *EcoRI* site. The 16S r DNA primer was further authenticated by sequences. Sequence

Table 1 Color and morphology of bacterial colony isolated from whitefly occuring in cotton field (F) and the population reared in glasshouse (G) in 1/10, 1/100 and 1/1000 dilutions

Development stage	Colony morphology	Colony colour obtained	Number of colonies
Egg F	Round shaped	White	76
Ist instars F	Round shaped	White	98
2nd instars F	Round shaped	White	150
3rd instars F	Round shaped	White	240
4th instars F	Round shaped	White, Yellow	220
Adult F	Round shaped	White, Yellow	198
Egg G	Round shaped	White	30
Ist instars G	Round shaped	White	33
2nd instars G	Round shaped	White	87
3rd instars G	Round shaped	White	120
4th instars G	Round shaped	White	143
Adult G	Round shaped	White	208

Table 2 Morphology, Gram’s staining and flagellation of bacteria isolated from whitefly reared in glasshouse

Bacterial colony no	Development stage	Gram staining	Flagellation	Morpho-logy
Adult G 1	Adult	Gram negative	Monotrichous	Rod shaped
Adult G 2	Adult	Gram negative	Monotrichous	Rod shaped
4th G1	4th instars	Gram positive	Non flagellate	Coccoid shaped
4th G2	4th instars	Gram positive	Monotrichous	Rod shaped
3rd G1	3rd instars	Gram negative	Monotrichous	Rod shaped
3rd G2	3rd instars	Gram positive	Non flagellate	Rod shaped
2nd GI	2nd instars	Gram negative	Non flagellate	Rod shaped
2nd G2	2nd instars	Gram negative	Non flagellate	Rod shaped
Ist G1	Ist instars	Gram negatives	Non flagellate	Rod shaped
Ist G2	Ist instars	Gram negative	Non flagellate	Rod shaped
Egg G1	Egg	Gram negative	Non flagellate	Rod shaped
Egg G2	Egg	Gram negative	Monotrichous	Rod shaped

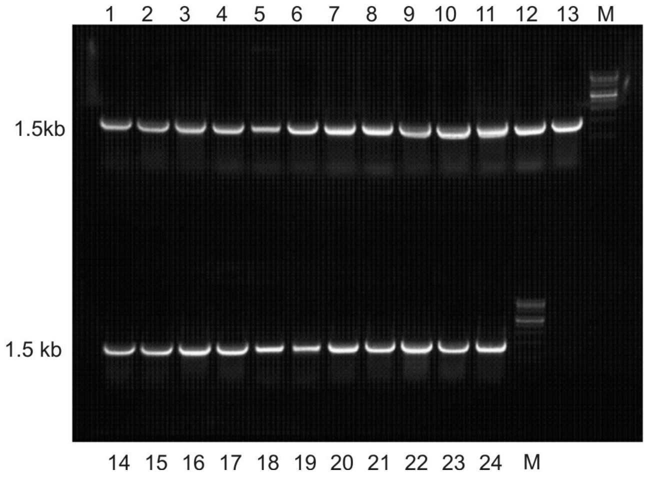

Fig 1 Agaroses gel electrophoresis of 16S r DNA amplicons of bacteria isolated from all stages of whitefly (egg, instars, adult) population on cotton plants and eggplants in glasshouse. Lane M=1kb ladder. Lanes 1= Adult G1, lane 2= Adult G2, lane 3= 4th G1, lane 4= 4th G2, lane 5= 3rd G1, lane 6= 3rdG2, lane 7= 2nd G1 ,lane 8 =G2, lane 9= 1stG1, lane 10=1st G2, lane 11=eggG1, lane 12=eggG2 , lane 13=Adult F 1, lane 14=Adult F2, lane 15= 4th F1, lane 16= 4th F2, lane 17= 3rd F1, lane 18= 3rdF2, lane 19= 2ndF1 ,lane 12= 2ndF2, lane 21= 1stF1, lane 22=1st F2, lane 23=eggF1, lane 24=eggG2. PCR amplicon of 1.5 Kb length.

Table 3 Morphology, Gram's staining and flagellation of bacteria isolated from collected from cotton field.

Bacterial colony no	Development stage	Gram staining	Flagellation	Morpho-logy
Adult F1	Adult	Gram positive	Non flagellate	Rod shaped
Adult F2	Adult	Gram positive	Non flagellate	Rod shaped
4th F1	4th instars	Gram positive	Non flagellate	Rod shaped
4th F2	4th instars	Gram positive	Non flagellate	Rod shaped
3rd F1	3rd instars	Gram positive	Peritrichous	Rod shaped
3rd F2	3rd instars	Gram positive	Monotrichous	Rod shaped
2nd FI	2nd instars	Gram positive	Non flagellate	Rod shaped
2nd F2	2nd instars	Gram negative	Peritrichous	Rod shaped
Ist F1	Ist instars	Gram positive	Peritrichous	Rod shaped
Ist F2	Ist instars	Gram negative	Peritrichous	Rod shaped
Egg F1	Egg	Gram negative	Monotrichous	Rod shaped
Egg F2	Egg	Gram negative	Amphitrichous	Rod shaped

analysis of 1.5 kb amplicon was done in BLAST to identify the bacterium. For every amplicon characterized, the nearest BLAST hits are shown in Table 4 and 5. Based on the BLAST hits which represents the sequence identity in the 16S r DNA region between the test bacterium and the Database entries, the bacteria were identified which are given in Table 4 and 5. It is evident from the Table 4 and Table 5 that, bacterium harbored at the egg stage in both field and glasshouse reared population is *Ralstonia* species. However in later stage, different species of a *Bacillus* dominated in cotton field population contrasting to dominance of *Ralstonia* species in glasshouse reared population. Significantly there was only one coccoid shape *Staphyloccus haemolyticus* identified in glasshouse population the bacteria in the field population are typically *Bacillus* species, except the second instar samples which was a *Ralstonia* species. Attempts were made to study the localization of bacterium in different body parts of the adult insect in glasshouse population. The body parts were approximately divided into anterior, middle and posterior portion. And after surface sterilization, mycetome was isolated. In the case of body parts, anterior portion and middle portion did not give rise to any bacterial colony. Bacterial colonies were obtained only in the posterior portion which was identified as *Ralstonia* species based on colony morphology and colour. In order to study the role of saprophytic endosymbionts, in transmission. It is necessary to raise bacterial free population of whitefly to achieve this, the adult whiteflies in glasshouse were allowed to oviposit on the leaf suspended on sterilized B5 medium. It was expected that eggs will be laid and adults will emerge which can be used for transmission. Unexpectedly, there were lot of bacterial and fungal contamination that proper experimentation could not conducted.

Whiteflies are small homopteran that feed as nymphs and adults on the phloem sap of plants. The hatching crawler settles near the hatching site where it goes through four immobile nymphal instars before developing into an adult. Like other phloem feeding insects whiteflies require bacteria for supplementing their unbalanced diet. There symbionts are housed in specialized organs called bacteriomes which are composed of bacteriocytes. The primary symbiont (P) of

Table 4 Identification of bacteria from whitefly on cotton field based on BLAST analysis

Bacterial isoation no.	Name and accession number of bacteria, first hit in BLAST		Percentage sequence identity	Bacteria in the present study identified as
Adult F1	*Bacillus* spp.	GU217692.1	97%	*Bacillus* spp.
Adult F2	*Bacillus thuringiensis*	GQ201999.1	81%	Unidentified
4th F1	*Bacillus altitudinis*	FJ174G23.1	98%	*Bacillus altitudinis*
4th F2	*Bacillus pumillus*	GU980767.1	87%	Unidentified
3rd F1	*Bacillus megaterium*	GQ181059.1	99%	*Bacillus megaterium*
3rd F2	*Bacillus subtilis*	GQ926882.1	99%	*Bacillus subtilis*
2nd FI	*Bacillus cereus*	HM067839.1	98%	*Bacillus cereus*
2nd F2	*Ralstonia* spp.	GU936705.1	98%	*Ralstonia* spp.
Ist F1	*Bacillus megaterium*	HM027880.1	99%	*Bacillus megaterium*
Ist F2	*Ralstonia* spp.	GU936705.1	99%	*Ralstonia* spp.
Egg F1	*Ralstonia* spp.	FM958426.1	98%	*Ralstonia* spp.
Egg F2	*Ralstonia* spp.	GU936705.1	98%	*Ralstonia* spp.

More than 95% identity is considered for identification

Table 5 Identification of bacteria from whitefly in glasshouse samples based on BLAST analysis

Bacterial isoation no.	Name and accession number of bacteria, first hit in BLAST		Percentage sequence identity	Bacteria in the present study identified as
Adult G1	*Ralstonia pickettii*	GQ169785.1	97%	*Ralstonia pickettii*
Adult G2	*Ralstonia* spp.	GU936705.1	97%	*Ralstonia* spp.
4th G1	*Staphyloccus haemolyticus*	EU659857.1	96%	*Staphyloccus haemolyticus*
4th G2	*Bacillus pumillus*	GU980767.1	99%	*Bacillus pumillus*
3rd G1	*Ralstonia* spp.	GU980767.1	95%	*Ralstonia* spp.
3rd G2	*Bacillus pumillus*	GU936705.1	98%	*Bacillus pumillus*
2nd GI	*Ralstonia pickettii*	GQ169785.1	98%	*Ralstonia pickettii*
2nd G2	*Ralstonia* spp.	GU936705.1	99%	*Ralstonia* spp.
Ist G1	*Ralstonia* spp.	GU936705.1	98%	*Ralstonia* spp.
Ist G2	*Ralstonia* spp.	AF280433.1	96%	*Ralstonia* spp.
Egg G1	*Ralstonia pickettii*	GQ389626.1	97%	*Ralstonia pickettii*
Egg G2	*Ralstonia* spp.	GU936705.1	98%	*Ralstonia* spp.
Adult A1	*Ralstonia pickettii*	GQ169785.1	97%	*Ralstonia pickettii*
Adult B2	*Ralstonia* spp.	GU936705.1	97%	*Ralstonia* spp.

More than 95% identity is considered for identification

whitefly is a unculturable bacterium *Candidatus Portiera aleyrodidarum.* There are secondary symbionts like *Candidatus Cardinium hertigii, Fritchea bemisiae, Candidatus Hamiltonella defense.* Beside their obligate endosymbionts, many of saprophytic endosymbionts are also reported to be present (Zchori-Fein *et al.* 2004, Gottlieb *et al.* 2006). In the present study attempts were made to identify the obligate and saprophytic endosymbiont.

Among the saprophytic endosymbionts cultured, *Bacillus* species dominated in field population and *Ralstonia* species in glasshouse population. These bacteria are definitely endosymbiont is evident from the fact that the same bacterium was obtained in more than 25 samples analyzed. The bacterial population within one stage especially eggs differed. It is interesting to note that the bacteria identified in cotton field population are *Bacillus megaterium*, *Bacillus subtilis, Bacillus altitudinis, Bacillus cereus, Ralstonia* spp., and the ones identified in glasshouse reared population are mainly *Ralstonia.*

The bacteria may contribute in two ways, they may contribute to digestion of metabolites and nutrition as whitefly mainly feed on carbohydrate rich phloem sap. *Bacillus subtilis* and *Bacillus megaterium* isolates are known to produce medium lengths oligomers from sucrose. The oligomers may be precursors for several metabolites and contribute to the nutrition of the insect. In addition to carbohydrate metabolism, these bacteria may also contribute to stickiness of honeydew of the insect. Besides obligate endosymbionts presence of these saprophytic bacteria in the mycetocytes is very vital, as they may affect the physiology and fitness of the insect. Bacterial profile of the field population occurring on cotton is very distinct from the one that occur on eggplant in glasshouse. This variation needs to be verified from field populations of these two crops. Bacteria occurring on phylloplane might have been infested by the bacteria which may be different from host to host. It will be necessary to extend these studies to more number of fields to see whether these bacteria are specific for cotton and eggplant population. The study has indicated the presence of *Bacillus megaterium*, *Bacillus subtilis* which are all successfully used as biocontrol agent. Potential of using this bacterium with engineered Bt toxin constructs or RNAi constructs targeting the viruses or the vector genes are some promising lines of work of that need to be explored.

REFERENCES

Baumann P. 2005. Bacteriocyte-associated endosymbionts of plant sap sucking insect. *Biology of Bacteriocyte* **59**: 155–89.

Birnboim H C and Doley J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic *Acids Research* **7**: 1 513–23.

Bright M and Bulgheresi S. 2010. A complex journey: transmission of microbial symbionts *Nat. Rev. Microbiol.* **8**: 218–30.

Costa H S, Henneberry T J and Toscano N C. 1997. Effects of antibacterial materials on *Bemisia argentifolii* (Homoptera: Aleyrodidae) oviposition, growth, survival, and sex ratio. *Annals of Entomology* **90:** 333–9.

Douglas A E. 1989. Mycetome symbiosis in insect. *Biological Review* **67**: 409–34.

Felske A, Douglas A E. 1989. Mycetone symbiosis in insect. Biological Review **67:** 409-34. Rheims H, Wolterink A, Stackebrandt E and Akkermans A D L. 1997. Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. *Microbiology* **143**: 2 983–9.

Gottlieb Y, Ghanim M, Chiel E, Gerling, D, Portnoy V, Steinberg S, Tzuri G, Horowitz, A, Eduard, R, Belausov, D, Kontsedalov, N, Gershon S, Gal M, Katzir, S N and Zchori-Fein E. 2006. Identification and localization of a *Rickettsia* sp. in *Bemisia tabaci* (*Homoptera: Aleyrodidae*). *Microbiology* **72**: 3 646–52.

Gram H C. 1884. Uber die isolierte Färbung der Schizomyceten in Schnitt- und Trockenpräparaten. Fortschritte der Medizin (in German) **2**: 185–9. English translation in: Brock, T.D. 1999. Milestones in Microbiology 1546-1940. *ASM Press* **2**: 215–8.

Lane D J, Pace B, Olsen G J, Stahl D A, Sogin M L and Pace N R. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of National Academy of Science USA* **82**: 6 955–9.

Sambrook J and Russel D W. 2001. *Molecular Cloning, a Laboratory Manual,* 3rd edn. Cold Spring Harbor Laboratory Press, New York.

Szklarzewicz T and Moskal A. 2001. Ultrastructure, distribution, and transmission ofendosymbionts in the whitefly *Aleurochiton aceris* Modeer (Insecta, *Hemiptera, Aleyrodinea*). *Journal of Microbiology* **29**: 755–62.

Zchori-Fein E, Perlman S J, Kelly S E, Katzir N and Hunter M S. 2004. Characterization of a, *Bacteroidetes* symbiont in *Encarsia* wasps 82 (Hymenoptera: Aphelinidae): proposal of *Candidatus* Cardinium hertigii?. *Int. J. Syst. Evol. Microbiol* **54**: 961–8.