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Article

Microbial Communities in the Developmental Stages of B and Q Biotypes of Sweetpotato Whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae)

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Whiteflies are sap-sucking insects belonging to the hemipteran order. They are well known for their menace to agriculture, as pests and vectors, and are reported for their bacterial and *Rickettsia* association in B biotype. In the present investigation, culture-dependent and -independent methods were used to reveal the bacterial phylotypes associated with B and Q biotypes. Cultivable bacterial phylotypes varied with respect to growth media and biotypes. Twenty different bacterial genera, including 31 species belong to Actinobacteria, 'alpha'-, 'beta'-, 'gamma'- Proteobacteria, and Firmicutes were isolated from both the biotypes. Of the seventeen phylotypes, *Bacillus*, *Kocuria*, *Moraxellla*, *Micrococcus*, *Sphingomonas* and *Staphylococcus* were common to both B and Q biotypes. Moreover, B biotype was associated with *Acinetobacter*, *Deinococcus*, *Modestobacter*, *Microbacterium*, and *Pseudomonas*, whereas Q biotype was associated with *Arthrobacter*, *Bradyrhizobium*, *Janibacter*, *Morganella*, *Naxibacter*, and *Streptomyces*. Application of a culture-independent method revealed the presence of additional symbiotic bacteria: *Rickettsia* in B biotype and *Halomonas* in Q biotype, as well as primary endosymbiont in both biotypes, which could not be obtained through culture-dependent method. Presence of *Staphylococcus*, *Micrococcus* (in both B and Q biotypes), and *Bacillus* (only in B biotype) in all developmental stages of *B. tabaci* indicated their close association with host insect.

Key words: bacterial phylotypes, *Bemisia tabaci*, biotype, developmental stage, insect gut bacteria

It has long been known that the largest classes of invertebrates, Insecta, were involved in several types of symbiosis, mainly with bacteria. A high diversity of bacteria has been reported from different types of insects. The bacterial association with insects plays a significant role in the host insect morphogenesis, food digestion, nutrition, antifungal toxin production, pheromone production, regulation of pH, synthesis of vitamins, temperature tolerance, resistance against parasitoid development, and detoxification of noxious compounds [Dillon and Dillon, 2004; Genta *et al.,* 2006]. Growth and development of the nymph and induction of the disease vectored by the host insect are retarded when its symbiotic bacteria are reduced or eliminated through feeding antibiotics [Costa *et al.,* 1993; Costa *et al.,* 1997]. Several studies have reported the importance of gut bacteria to the host insect processes with direct evidences [Davidson *et al.,* 2000; Dillon *et al.,* 2002]. At the onset of the 21st century, silver leaf whitefly, *Bemisia argentifoli* (B biotype of *B. tabaci*) has been reported for its possession of pleomorphic and coccoid obligate intracellular bacterial endosymbionts, which are transmitted from the female to eggs, and the bacterial strains isolated from the *B. argentifoli* were ingested by the host insect and passed into the honeydew [Davidson *et al.,* 2000].

In general, the development of resistance to insecticides is a major constraint in insect pest management programs, which has been mediated through detoxification enzymes such as glutathione-S-transferase [Mohan and Gujar, 2003; Kim *et al.,* 2007]. Recently, our laboratory strains

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B and Q biotypes were tested for their insecticide susceptibility, along with their glutathione-S-transferase (GST) activity. Q biotype exhibits lower susceptibility to the tested insecticides including neonicotinoids than B biotype. In addition, the Q biotype showed higher GST activity and prefererence to feed on red pepper and tomato than the B biotype, which feed only on tomato [Kim *et al.,* 2007; Seo *et al.,* 2007].

Cotton boll worm, *Helicoverpa armigera* and insecticide resistant, susceptible and field populations of diamondback moth, *Plutella xylostella* were documented for their variation in gut bacterial association [Xiang *et al.,* 2006; Indiragandhi *et al.,* 2007]. Some of the earlier studies indicated that the toxic substances ingested by the insect species were detoxified with the help of their associated bacteria [Lauzon *et al.,* 2003; Genta *et al.,* 2006] and suggested that bacterial enzymes such as GST may contribute to the detoxification process, along with the host tissue-derived enzymes. In addition, several species of the facultative bacterial phylotypes play important biological roles in the host insect. For instance, in pea aphid, *Acyrthosiphon pisum* symbionts confer tolerance to high temperature, resistance to parasitic wasps and pathogenic fungi, and broadening of the host plant range [Hosokawa *et al.,* 2007].

The present study was carried out to describe the bacterial communities inhabiting surface-sterilized whitefly, including all developmental stages (egg through adult) of B and Q biotypes using both culture-based and PCR-DGGE techniques. Knowledge on the microorganisms associated with each biotype and developmental stage may pave a way for controlling the pest insect species, either through using the bacterial symbionts as biocontrol agent or manipulating/eliminating their association with the host insect.

Materials and Methods

Whitefly-*Bemisia tabaci*. The whitefly population was collected from the greenhouse rose at Iwol and Jincheon, and red pepper field in Miryang, Korea. Based on mitochondrial cytochrome oxidase I (mtCOI) PCR product, whitefly population collected from greenhouse rose and red pepper were identified as B and Q biotypes, respectively [Kim *et al.,* 2007]. Because these two biotypes showed variation in their host plant selection, B and Q biotypes were maintained in tomato (*Lycopersicon esculentum*) and red pepper (*Capsicum annuum*) plants, respectively, at 16 h light and 8 h dark photoperiod with 50-60% RH and temperature at 25±1°C. The wild type of *B. tabaci* was also maintained in red pepper.

Isolation of bacteria by culture-dependent method. B and Q biotypes eggs, nymphs (I, II, III, and IV instars), and adults of *B. tabaci* were collected from tomato and red pepper plants, respectively. Because of the small size, the insect samples were processed in groups of about 150-200 [Davidson *et al.,* 2000]. Samples were sterilized on the surface with 90% ethanol for 30 min, followed by six 5 min washes with sterile physiological water (0.85% NaCl). Eggs and different stages of nymphs and adults were processed separately. Surface-sterilized samples were triturated in 2 mL saline with a sterile micro pestle and mortar. Subsequently, 100 μL of the homogenized sample was plated directly on the microbiological media such as brain heart infusion (BHI), Chocolate, Luria Bertani (LB), MacConkey (Mc), Nutrient (NA), Purple (P), Pseudomonas (Ps), R2A and Tryptose (T) agar media (Difco, St. Louis, MO), and incubated at 28±2°C and surveyed every 24 h for development of new colonies. Because the growth requirements of the whitefly gut bacteria were unknown, homogenates were plated on ten different bacterial media as reported previously [Davidson *et al.,* 2000; de Vries *et al.,* 2001]. The colonies were differentiated based on their size, color, and morphology, and a single representative isolate of each morphotype was transferred to new plates. After five to six successive streaking, the culture purity was ascertained by examination under a light microscope. The purified strains were maintained in 50% glycerol at −80°C.

Molecular characterization of cultivable bacteria. For the molecular characterization, 56 and 61 isolates from B and Q biotypes, respectively, were selected based on their colony morphology and growth at different media. The selected bacterial strains were subjected to DNA extraction according to the method of Sambrook *et al.* [1989]. The 16S rRNA genes were amplified by PCR using the forward primer 27f-AGAGTTTGATCCTGGC TCAG and reverse primer 1492r-GGTTACCTTGTTAC GACTT. The 16S rRNA nucleotide sequences were identified by PCR-direct sequencing using the fluorescent dye terminator method (ABI prism™ Bigdye™ Terminator cycle sequencing ready reaction kit V.3.1; Applied Biosystems, Foster, CA), and the products were purified with a Millipore-Montage dye removal kit; Applied Biosystems, Foster, CA). Finally, the products were run in an ABI 3730XL capillary DNA sequencer (50 cm capillary). The nucleotide sequences obtained were submitted to the National Center for Biotechnology Information (NCBI) database, and their accession numbers are available in the GenBank for B biotype egg through fourth instar nymph FJ357579-FJ357621 and for adults FJ217179-FJ217188; for Q biotype egg through fourth

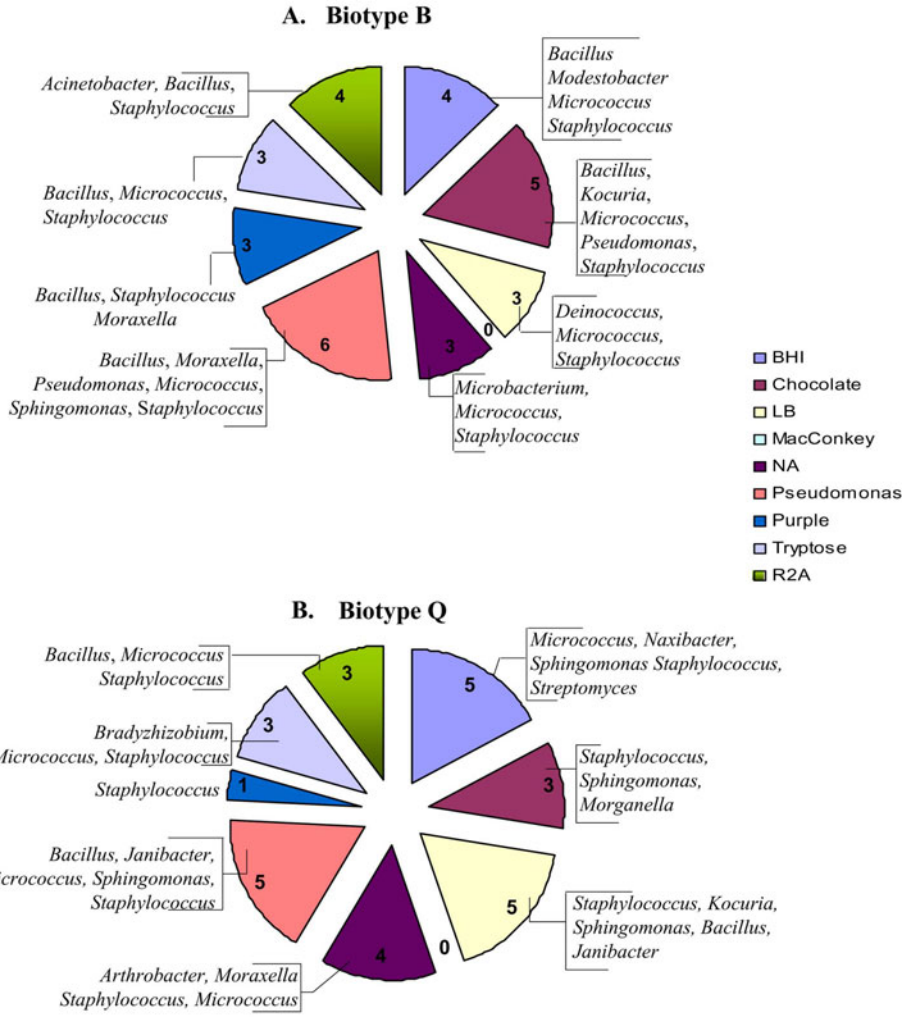


Fig. 1. Relative abundance of bacterial genera in whitefly *Bemisia tabaci* biotype B (A) and Q (B) with reference to different growth media used for isolation. Numbers in circles indicates no. of genera isolated from each medium.

instar nymph FJ380952 to FJ380997 and for adults FJ217189 - FJ217203.

Phylogenetic analysis. The complete 16S rDNA sequences of all isolates from different developmental stages of biotypes B and Q were combined individually and aligned with Clustal X Ver 1.83 [Thompson *et al.,* 1994] and adjusted manually with Chromas Ver. 2.23. Multiple alignments were based on CLUSTAL X or MAFFT Ver. 6.5. A tree was constructed based on the Euclidian distance using the neighbor-joining algorithm [Saitou and Nei, 1987] and Kimura 2-parameter corrections [Kimura, 1980]. The statistical confidence of the nodes was estimated by bootstrapping, using 1000 replications [Felsenstein, 1985].

Polymerase chain reaction (PCR). PCR for DGGE was conducted using the primer set GC338f ACGGGGG GACTCCTACGGGAGGCAGCAG and 518r ATTACC GCGGCTGCTGG [Muyzer *et al.,* 1993]. The DNAs extracted from *B. tabaci* biotypes B and Q, and the wild type adults were subjected to PCR amplification, followed by DGGE. A GC clamp (5'-CGCCCGCCGCG CGGCGGGCGGGGCGGGGGC-3') was added to the forward primer (338f) to prevent strand dissociation during DGGE and reflect heterogeneity of amplified sequences. PCR amplification was performed at a total volume of 50 µL containing 1 µL (50 ng/µL) template, 10 mM Tris HCl (pH 9.0), 40 mM KCl, 250 µM deoxynucleoside triphosphates (dNTPs), 2.5 U Taq polymerase, 1.5 mM $MgCl_2$, and 20 pmole of each primer. The reactions were performed in a PTC-220 DNA Engine Dyad MJ Research thermalcycler (Watertown, MA). The PCR cycle profile consisted of initial denaturation for 3 min at 94ºC, followed by 32 cycles consisting of 30 s at 94ºC, 30 s at the annealing temperature at 60ºC, 45 s at 72ºC for elongation, and 5 min at 72ºC for the final cycle.

Denaturing gradient gel electrophoresis (DGGE). DGGE analysis of the PCR products was performed using a Bio-Rad Dcode universal mutation system with

Table 1. Classification of bacterial strains isolated from the tobacco whitefly *tabaci* biotypes B and Q

Group	Family	Genera	Species	Host biotype
Firmicutes	Bacillaceae	*Bacillus*	*Bacillus* sp.	B,Q
			Bacillus arbutinivorans	Q
			Bacillus firmus	Q
			Bacillus cereus	B
			Bacillus thuringiensis	B
			Bacillus ginsengihumi	B
Alphaproteobacteria	Bradyrhizobiaceae	*Bradyrhizobium*	*Bradyrhizobium* sp.	Q
	Deinococcaceae	*Deinococcus*	*Deinococcus* sp.	B
Gamaproteobacteria	Enterobacteriaceae	*Morganella*	*Morganella. morgani*	Q
	Geodermatophilaceae	*Modestobacter*	*Modestobacter* sp	B
Actinobacteria	Intrasporangiaceae	*Janibacter*	*Janibacter* sp	Q
			Janibacter sanguinis	Q
	Microbacteriaceae	*Microbacterium*	*Microbacterium paraoxydans*	B
Actinobacteria	Micrococcaceae	*Micrococcus*	*Micrococcus* sp.	B, Q
			Micrococcus aquilis	Q
			Micrococcus indicus	Q
			Micrococcus luteus	B, Q
			Kocuria sp.	Q
			Arthrobacter sp.	Q
Gamaproteobacteria	Moraxellaceae	*Moraxella*	*Moraxella* sp.	B,Q
		Acinetobacter	*Acinetobacter* sp.	B
Betaproteobacteria	Oxalobacteraceae	*Naxibacter*	*Naxibacter haematophilus*	Q
Gamaproteobacteria	Pseudomonadaceae	*Pseudomonas*	*Pseudomonas* sp.	B
			Pseudomonas oryzihabitans	B
			Pseudomonas plecoglossicida	B
			Pseudomonas putida	B
Alphaproteobacteria	Sphingomonadaceae	*Sphingomonas*	*Sphingomonas azotifigens*	Q
Firmicutes	Staphylococcaceae	*Staphylococcus*	*Staphylococcus capitis*	B, Q
			Staphylococcus epidermidis	B, Q
			Staphylococcus gallinarum	B
			Staphylococcus haemolyticus	B, Q
			Staphylococcus pasteuri	Q
			Staphylococcus subs. *saprophyticus*	Q
			Staphylococcus saprophyticus	B,Q
			Staphylococcus sp.	Q
Actinobacteria	Streptomycetaceae	*Streptomyces*	*Streptomyces* sp.	Q
			Streptomyces griseoaurantiacus	Q

8% (w/v) acrylamide (acrylamide/bis-acrylamide 37.5:1 w/w) gels containing a linear chemical gradient ranging from 30 to 60% [100% denaturant contains 7 M urea and 40% (v/v) deionized formamide]. PCR products were electrophoresed in a running buffer containing 1×TAE [20 mM Tris, 10 mM acetate, 0.5 mM EDTA (pH 8.3)] at a constant temperature of 60°C for 14 h at 80 V. After electrophoresis, the gels were stained with the SYBR Green I nucleic acid stain (Bioneer, Seoul, Korea) and photographed under UV transillumination. Sterile blades were used to excise bands from the gels, which were then mixed with 20 μL of 0.1×TE buffer solution and incubated overnight at 4°C. One microliter of each solution was then PCR-reamplified with the appropriate primer set and directly sequenced. The sequences were taxonomically assigned by using BLAST on the GenBank nucleotide database (NCBI).

Table 2. Cultivable bacterial strains of tobacco whitefly *Bemisia tabaci* B biotype

Biostage	Bacterial strain	Accession no. of isolate	Closest match in Genbank	Accession no. of match	Seqence length (bp)	Similarity (%)
Egg	BBEB-03d	FJ357579	*Staphylococcus epidermidis*	AY741152	1436	100
	BBEC-01d	FJ357580	*Staphylococcus capitis*	L37599	1439	99
	BBEC-02d	FJ357581	*S. capitis* ssp. *Urealyticus*	AB233325	1300	99
	BBEN1-01	FJ357582	*Staphylococcus* sp.	EU195954	1432	99
	BBEN-01d	FJ357583	*Staphylococcus epidermidis*	AY030340	1432	99
	BBEP1-01	FJ357584	*Staphylococcus haemolyticus*	AP006716	1250	99
	BBER-01d	J357585	*Staphylococcus capitis*	L37599	1434	100
I instar	BBN1B3-02	FJ357586	*Staphylococcus epidermidis*	AY030340	1443	99
	BBN1B3-03	FJ357622	*Modestobacter* sp.	EF451683	862	90
	BBN1B-03d	FJ357587	*Staphylococcus epidermidis*	AY741152	1441	99
	BBN1L2-01	FJ357588	*Micrococcus luteus*	AJ409096	1390	99
	$BBN1L_d$-02_a	FJ357589	*Staphylococcus epidermidis*	AY741152	1440	99
	BBN1R2-01	FJ357590	*Bacillus ginsengihumi*	AB245378	1439	99
II instar	BBN2C-01d	FJ357591	*Bacillus* sp.	EU584540	1433	100
	BBN2C-02d	FJ357623	*Kocuria* sp.	EU070410	1406	99
	BBN2C-03d	FJ357592	*Micrococcus* sp.	EF522836	1416	99
	BBN2L-03d	FJ357593	*Micrococcus* sp.	EF522836	1390	99
	BBN2N-01d	FJ357594	Micrococcus luteus	EU438932	1394	99
	BBN2N-02d	FJ357595	*Microbacterium paraoxydans*	EU714377	1404	99
	BBN2P-01d	FJ357596	*Staphylococcus capitis*	L37599	1436	99
	BBN2P-02d	FJ357597	*Moraxella* sp.	AY162144	1414	99
	$BBN2P_S$-02_d	FJ357598	*Bacillus* sp.	EU584535	1441	99
	BBN2R-02d	FJ357599	*Staphylococcus capitis*	L37599	1437	99
	BBN2T-01d	FJ357600	*Bacillus thuringiensis*	EU440975	944	100
III instar	BBN3B-01d	FJ357601	*Micrococcus luteus*	DQ490458	1411	99
	BBN3C-02d	FJ357602	*Pseudomonas plecoglossicida*	DQ140383	1422	99
	BBN3C-04d	FJ357603	*Bacillus* sp.	EU719665	922	99
	BBN3L-01d	FJ357604	*Deinococcus* sp.	AM900794	929	99
	BBN3L-04d	FJ357605	*Micrococcus* sp.	AB188213	1389	100
	BBN3N-02d	FJ357606	*Micrococcus* sp.	EF522836	1407	99
	BBN3N-03d	FJ357607	*Staphylococcus epidermidis*	AY741152	1430	99
	BBN3P-01d	FJ357608	*Staphylococcus capitis*	L37599	1432	99
	BBN3P-04d	FJ357609	*Bacillus cereus*	EU621383	909	100
	$BBN3P_s$-02d	FJ357610	*Sphingomonas* sp.	AY749436	1371	100
	$BBN3P_S$-03d	FJ357611	*Moraxella* sp.	AY162144	1413	99
	BBN3R-01d	FJ357612	*Acinetobacter* sp.	AY043369	1419	99
	BBN3T-03d	FJ357613	*Micrococcus* sp.	EF522836	1405	99
	BBN3T-04d	FJ357614	*Staphylococcus capitis*	L37599	1436	99
IV instar	BBN4B-01d	FJ357615	*Micrococcus luteus*	EU438932	1410	99
	BBN4B-02d	FJ357616	*Bacillus cereus*	EU048539	919	99
	BBN4C-01d	FJ357617	*Bacillus cereus*	EU621383	921	99
	BBN4N-01d	FJ357618	*Micrococcus luteus*	EU438932	1250	99
	BBN4P-01d	FJ357619	*Staphylococcus saprophyticus*	D83371	1442	99
	BBN4P-02d	FJ357620	*Bacillus cereus*	EU794727	924	99
	BBN4Ps-01d	FJ357621	*Micrococcus luteus*	EU438932	1404	99

Table 2. Continued

Biostage	Bacterial strain	Accession no. of isolate	Closest match in Genbank	Accession no. of match	Seqence length (bp)	Similarity (%)
Adult	BBAL-01d	FJ217179	*Staphylococcus gallinarum*	DQ350835	1471	99
	$BBAL_5$ -01	FJ217180	*Pseudomonas putidae*	DQ288952	1451	99
	BBAL-03d	FJ217181	*Pseudomonas oryzihabitans*	AM262973	1435	99
	BBAL-02d	FJ217182	*Pseudomonas* sp.	AJ575816	1437	99
	BBAN-03d	FJ217183	*Micrococcus luteus*	EU438932	1483	99
	BBAPs-01	FJ217184	*Pseudomonas* sp.	EU883660	1441	99
	BBAPs-01d	FJ217185	*Micrococcus* sp.	AM990848	1436	99
	BBAPs-02	FJ217186	*Staphylococcus saprophyticus*	AP008934	1455	99
	BBAR-10d	FJ217187	*Bacillus* sp.	EU584539	1451	99
	$BBAR_7$ -13d	FJ217188	*Staphylococcus epidermis*	AY741152	1466	99

Results

The use of a cultivable method revealed several genera of bacteria as colonizers of *Bemisia tabaci* biotypes B and Q. The maximum number of bacterial genera was obtained from *Pseudomonas* agar, followed by BHI, Chocolate, R2A, LB, Nutrient, Purple agar, and Tryptose for B biotype (Fig. 1A). In the case of Q biotype, BHI, LB, and *Pseudomonas* agar supported five phylotypes, followed by Nutrient, Chocolate, R2A, Tryptose, and Purple agar (Fig. 1B). Interestingly, irrespective to the biotypes and developmental stages, MacConkey medium did not support the growth of any bacterial genera.

In total, 31 species of bacterial strains belonging to 13 different families were obtained in the present study (Table 1). With reference to different biotypes (B and Q), there was a difference in their bacterial phylotypes. Only six genera *viz*., *Bacillus*, *Kocuria*, *Micrococcus*, *Moraxella*, *Sphingomonas*, and *Staphylococcus* were common to both B and Q biotypes. In addition, the former was associated with five additional genera *viz*., *Acinetobacter*, *Deinococcus*, *Modestobacter*, *Microbacterium*, and *Pseudomonas*; the latter was associated with six additional genera including *Arthrobacter*, *Bradyrhizobium*, *Janibacter*, *Morganella*, *Naxibacter*, and *Streptomyces*.

Bacterial strains of *B*. *tabaci* B and Q biotypes at all developmental stages of the insect are given in Tables 2 and 3, respectively, with GenBank accession number and similarity to known species. With the exception of BBN1B3-03, all isolates from B biotype showed 99-100% 16S rRNA similarity to their closest relatives retrieved from the GenBank database, whereas isolate BBN1B3-03 showed only 90% 16S rRNA gene sequence similarity to *Modestobacter* sp. (Table 2). In the case of Q biotype, isolates BQN1B-05d, BQN1C-02d, BQN2T-03d, BQN3C1-03d, BQN3C2-03d, BQN4T-01d, and BQAPs-02d showed 98% similarity with their respective close relatives (Table 3). Phylogenetic relatedness among the gut bacterial isolates of *B*. *tabaci* showed that Q biotype was associated with 'Alpha'-, 'Beta'-, and 'Gamma'- subclasses of Proteobacteria, and in B biotype 'Gamma'- subclass of Proteobacteria was absent (Figs. 2 and 3).

Abundance of bacterial phylotypes in B and Q biotypes was determined upto the genus level (Data not shown). Results revealed that *Staphylococcus* (33.9%) was predominant in biotype B, followed by *Micrococcus* (23.2%), *Bacillus* (17.8%), *Pseudomonas* (8.9%), *Moraxella* (3.5%), and other phylotypes, each contributing 1.7%. In Q biotype, the predominant phylotype was also *Staphylococcus* (32.8%), followed by *Micrococcus* (18.0%) and *Sphingomonas* (13.1%). Furthermore, *Bacillus*, *Janibacter*, and *Streptomyces* each contribute 5% of the total phylotypes. The other phylotypes including *Arthrobacter*, *Bradyrhizobium*, *Bacterium*, *Naxibacter*, *Kocuria*, *Moraxella*, *Morganella*, and *Micrococcaceae* contributed 1.6% each in biotype Q. Considering the different developmental stages of both B and Q whitefly populations, the genus *Staphylococcus* and *Micrococcus* were present in the egg through adult stage. The genus *Bacillus* was associated with all (I, II, III, and IV instars) nymphal stages of biotype Q and only in biotype B adult (Fig. 1).

Furthermore, B and Q biotypes of *B*. *tabaci* adults and the wild type adults were analyzed by PCR-DGGE to reveal the differences among different populations in terms of their gut bacterial phylotypes. The results revealed that bacterial phylotypes, which could not be isolated through culture-dependent method, can be obtained through culture-independent analysis. The phylotypes of PCR-DGGE differed from those of culture-dependent methods. Results of PCR-DGGE analysis on adult insects are shown in Fig. 4; though the primary endosymbiont of *B*. *tabaci* (band 1) was common to both

Table 3. Cultivable bacterial strains of the tobacco whitefly *Bemisia tabaci* Q biotype

Biostage	Bacterial strain	Accesson no. of isolates	Closest match in Genbank	Accession no. of closest match	Sequence length (bp)	Similarity (%)
Egg	$BQEL_1$-01b	FJ380952	*Staphylococcus epidermidis*	AY741152	1426	99
	BQEN3-01	FJ380953	*Micrococcus luteus*	AJ717369	1399	99
	BQEN3-02	FJ380954	*Moraxella* sp.	FJ006859	1408	99
	$BQEN_3$-03	FJ380955	*Staphylococcus capitis*	L37599	1433	99
	$BQEP_2$-01d	FJ380956	*Staphylococcus capitis*	L37599	1433	99
	$BQER_2$-01	FJ380957	*Staphylococcus epidermidis*	AY741152	1430	99
I instar	BQN1B-04d	FJ380958	*Micrococcus luteus*	EU438932	1405	99
	BQN1B-05d	FJ380959	*Naxibacter haematophilus*	EU554441	1410	98
	BQN1C-02d	FJ380960	*Sphingomonas azotifigens*	AB217473	1094	98
	BQN1L-01d	FJ380961	*Staphylococcus haemolyticus*	AP006716	1434	99
	BQN1L-02d	FJ380962	*Kocuria* sp.	EU070410	1408	99
	BQN1N-01d	FJ380963	*Moraxella* sp.	FJ006859	1404	99
	BQN1N-02d	FJ380964	*Staphylococcus epidermidis*	D83362	1439	99
	BQN1N-03d	FJ380965	*Micrococcus* sp.	AM990780	1407	99
	BQN1P-01d	FJ380966	*Staphylococcus epidermidis*	AY741152	1437	99
	BQN1P-02d	FJ380967	*Staphylococcus* sp.	EU554434	1439	99
	BQN1R-01d	FJ380968	*Staphylococcus epidermidis*	AY030340	1436	99
	BQN1R-02d	FJ380969	*Micrococcus luteus*	DQ490458	1397	99
	BQN1T-01d	FJ380970	*S.saprophyticus.* ssp. *sprophyticus*	AP008934	1439	99
	BQN1T-03d	FJ380971	*Micrococcus luteus*	EU438932	1399	99
II instar	BQN2B-01d	FJ380972	*Bacillus arsenicus*	AY745842	1429	99
	BQN2B-02d	FJ380973	*Sphingomonas azotifigens*	AB217473	1369	96
	BQN2C-02d	FJ380974	*Sphingomonas azotifigens*	AB217473	1014	99
	BQN2L-01d	FJ380975	*Arthrobacter* sp.	EU439407	1405	99
	BQN2N-01d	FJ380976	*Staphylococcus hominis*	EU520332	1428	99
	BQN2N-02d	FJ380977	*Sphingomonas azotifigens*	AB217473	1036	99
	BQN2Ps-04d	FJ380978	*Staphylococcus* sp.	EU520332	1437	100
	BQN2P-01d	FJ380979	*Staphylococcus* sp.	EU554434	1437	99
	BQN2T-01d	FJ380980	*Staphylococcus haemolyticus*	AP006716	1421	99
	BQN2T-03d	FJ380981	*Sphingomonas azotifigens*	AB217473	1120	98
III instar	BQN3C-03d	FJ380984	*Sphingomonas azotifigens*	AB217473	1094	99
	BQN3C1-03d	FJ380985	*Morganella morganii*	AB244432	1408	98
	BQN3C2-03d	FJ380986	*Sphingomonas azotifigens*	AB217473	1094	98
	BQN3L-01d	FJ380987	*Staphylococcus* sp.	DQ356499	1432	99
	BQN3L-01d	FJ380987	*Staphylococcus* sp.	DQ356499	1432	99
	BQN3L-02d	FJ380988	*Bacillus arbutinivorans*	AF519469	1414	99
	BQN3N-02d	FJ380989	*Staphylococcus pasteuri*	AF532917	1440	99
	BQN3N-03d	FJ380990	*Staphylococcus epidermidis*	AY741152	1439	99
	BQN3T-01d	FJ380991	*Sphingomonas azotifigens*	AB217473	914	99
	BQN3T-02d	FJ380992	*Staphylococcus pasteur*i	AF532917	1440	99
IV instar	BQN4N-02d	FJ380993	*Micrococcus luteus*	EU438932	1404	99
	BQN4Ps-01d	FJ380994	*Staphylococcus saprophyticus*	D83371	1436	99
	BQN4Ps-02d	FJ380995	*Janibacte*r sp.	AJ717360	1407	99
	BQN4T-01d	FJ380996	*Bradyrhizobium* sp.	EU256461	1366	98
	BQN4T-04	FJ380997	*Staphylococcus* sp.	FJ006896	1430	99

Table 3. Continued

Biostage	Bacterial strain	Accesson no. of isolates	Closest match in Genbank	Accession no. of closest match	Sequence length (bp)	Similarity (%)
Adult	BQAB-01d	FJ217189	*Micrococcus luteus*	EU440972	1423	99
	BQAB-04d	FJ217192	*Streptomyces griseoaurantiacus*	AB184676	1423	99
	BQAB-06d	FJ217194	*Micrococcus* sp.	EU086818	1424	99
	BQAB-06	FJ217195	*Staphylococcus pasteuri*	AF532917	1449	99
	$BQAL_7$-01	FJ217196	*Bacillus* sp.	EU863836	1424	99
	$BQAL_7$-02d	FJ217197	*Janibacter sanguinis*	AY383745	1420	99
	BQAPs-02d	FJ217199	*Micrococcus aquilus*	EU005372	1415	98
	BQAPs-03d	FJ217200	*Bacillus firmus*	AJ717383	1450	99
	BQAPs-04d	FJ217201	*Micrococcus indicus*	EU440972	1463	99
	BQAR-01d	FJ217203	*Bacillus cereus*	EF472263	1492	99

B and Q biotypes, the former was abundant with *Rickettsia bellii* (bands 5 and 6), whereas the latter was with *Halomonas* sp. (bands 8 and 9).

Discussion

The importance of bacterial association with arthropod has been addressed, for many insects often reveal a range of parasitic associations from the pathogenic to the mutualistic. These associations have been used to develop novel control strategies of arthropods of agricultural, medical, and veterinary importance [Costa and Brown, 1991; Broderick *et al.,* 2004]. As an essential initial step to seeking a new strategy for whitefly management, the bacterial communities associated with the two biotypes B and Q were explored. The present investigation showed the differences in the bacterial communities between the two biotypes by both methods used. Variations in the numbers and types of bacterial phylotypes obtained through different media suggested that the selection of medium is an important criterion, by playing a major role in the cultivable bacterial population from a target insect [Davidson *et al.,* 2000].

Absence of Beta-proteobacteria in B biotype and the different bacterial phylotypes found in the two biotypes indicated that bacterial genera would be variable with the different biotypes of whitefly (Table 2). Based on similarity percentage, novel genus and species in the whitefly gut bacteria were anticipated, since a similarity value below 97% and 98% between 16S rRNA gene sequences is acceptable for new species level differentiation by genomic relatedness. Hence, it could be possible that the isolate BBN1B3-03 is not associated with already known genus and may belong to a novel geno-species; BQN1B-05d, BQN1C-02d, BQN2T-03d, BQN3C1-03d, BQN3C2-03d, BQN4T-01d, and BQAPs-02d probably belong to a new phylotype, because their similarity value of 16S rRNA is low. Although it is not a new species, it is just recognized as a new phylotype such as subspecies [Stackebrandt and Goebel, 1994]. However, whether they are a new phylotype can be validated through detailed polyphasic taxonomic characterization.

Molecular characterization identifying more bacterial phylotypes at nymphal stage rather than adult stage indicated that the young and developing stages would have more different bacteria [Davidson *et al.,* 2000; Lacava *et al.,* 2007]. Irrespective to the stage and biotype, the existence of *Staphylococcus*, right from egg to adult stage, suggested that the bacterium may enter the oocyte together with the bacteriocytes (symbiont housing cells) when the egg is laid; the bacterium now multiplies, spreads throughout the egg during embryogenesis, and disperses throughout the body of the hatching nymph, excluding the bacteriomes [Gottlieb *et al.,* 2006]. A similar report was documented for the Israel population of *B. tabaci* and its association with *Rickettsia* [Gottlieb *et al.,* 2006]. Furthermore, association of bacterial phylotypes such as *Pseudomonas* (=*Flavomonas*) *oryzihabitans*, *Staphylococcus epidermidis*, and *Bacillus* were documented for *B. argentifoli* [Davidson *et al.,* 2000].

Studies have shown that the presence of bacterial populations, which could not be detected when only culture-based method was applied, could be detected by the use of culture-independent methods such as 16S rDNA library sequence phylogenetic analysis, 16S random fragment length polymorphism (RFLP)-related techniques, and 16S rDNA DGGE [Broderick *et al.,* 2004; Xiang *et al.,* 2006; Lacava *et al.,* 2007]. Similarly, in the present study we also obtained different bacterial phylotypes, which did not appear in the cultivable method. So far, *Rickettsia* has been reported only from a pea aphid, *A. pisum*, and a collembolan insect [Chen *et al.,* 1996; Czarnetzki and Tebbe, 2004]. Although the role of *Rickettsia* in the biology of herbivorous insects is

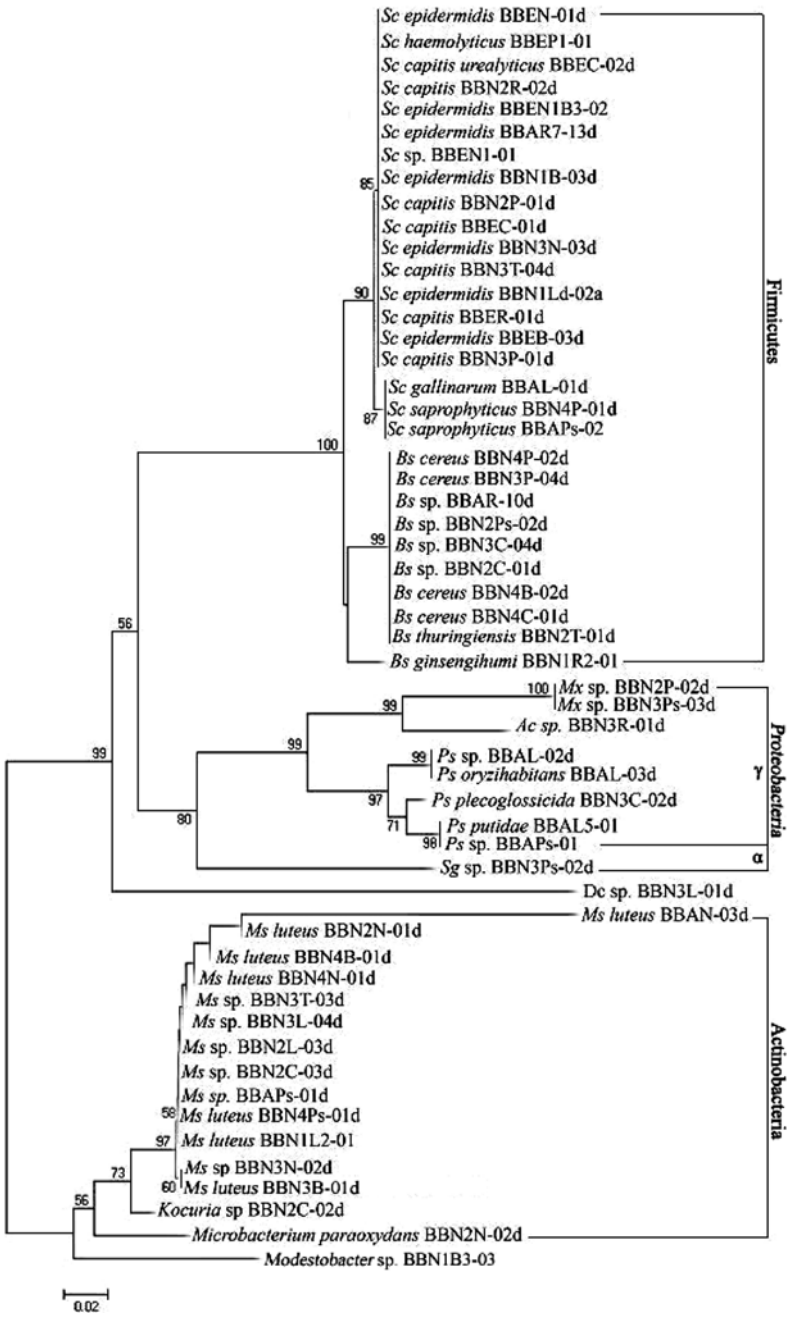


Fig. 2. Phylogenetic tree for bacterial strains isolated from different biostages of the sweetpotato whitefly *Bemisia tabaci* B biotype within Firmicutes, *Alphaproteobacteria*, *Gamaproteobacteria*, and Actinobacteria based on 16S rRNA gene sequence analysis. Bootstrap values (from 1000 replicates) greater than 50% are shown at the branch points. Bar 2% nucleotide sequence difference. *Ac*, *Acinetobacter*; *Bs*, *Bacillus*; *Dc*, *Deinococcus*; *Kc*, *Kocuria*; *Sc*, *Staphylococcus*; *Sg*, *Sphingomonas*; *Mx*, *Moraxella*; *Mb*, *Microbacterium*; *Mg*, *Morganella*; *Ms*, *Micrococcus*; *Ps*, *Pseudomonas*.

unknown [Gottlieb *et al*., 2006], the present study also confirmed the presence of *Rickettsia* in the biotype B next to Israel population of *B. tabaci* rather than biotype Q. Thus, the application of both methods would reveal most of the bacterial phylotype-association with its host insect. However, there may well be minor species not detected by either 16S rRNA sequence analysis or DGGE. Therefore, *Rickettsia* and *Halomonas* may exist in the gut dominantly, because DGGE can detect only the major species over 10% in the gut. Other species detected by other methods were recognized as minor species.

From the variation in the bacterial phylotypes of *B. tabaci*, different biotypes differ in terms of their gut bacterial association. Thus, testing the functional roles of individual phylotypes, may give a clear picture concerning the significance of these associations and differences

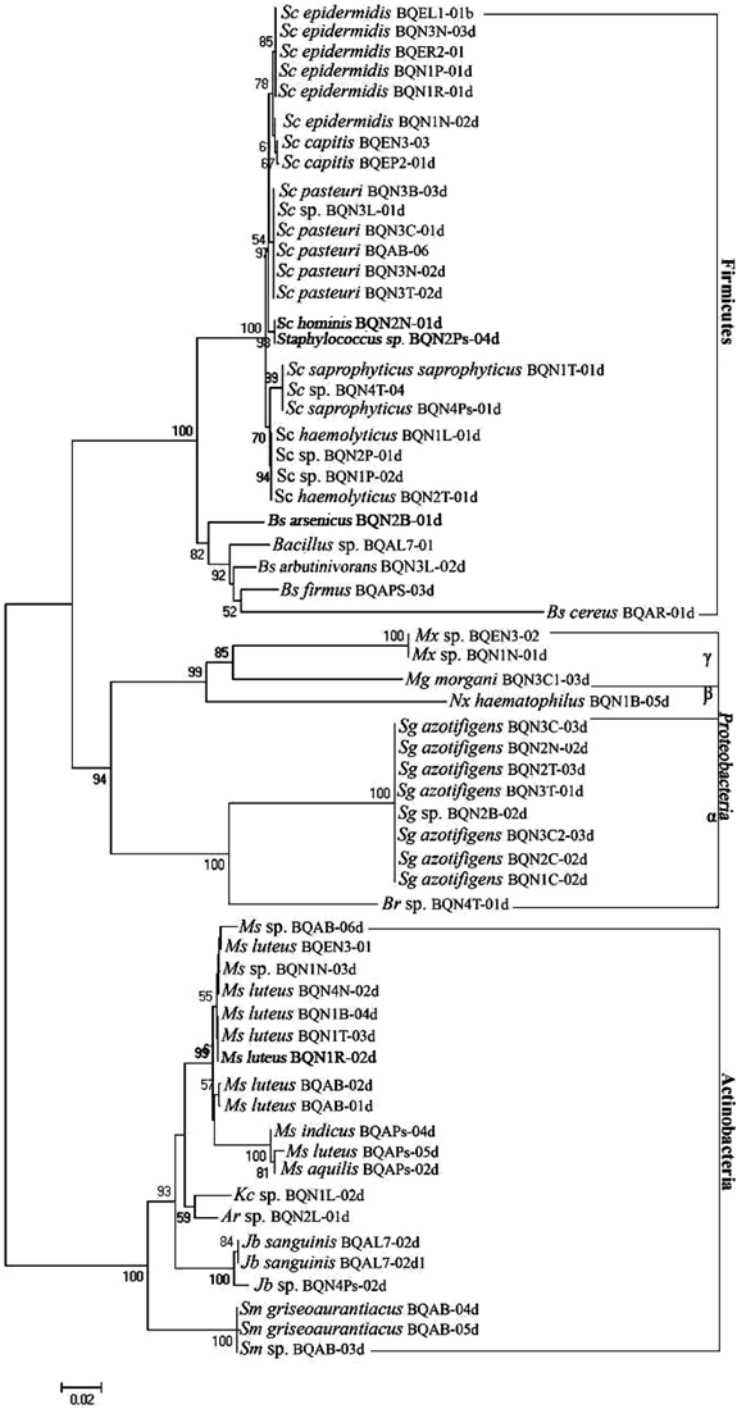
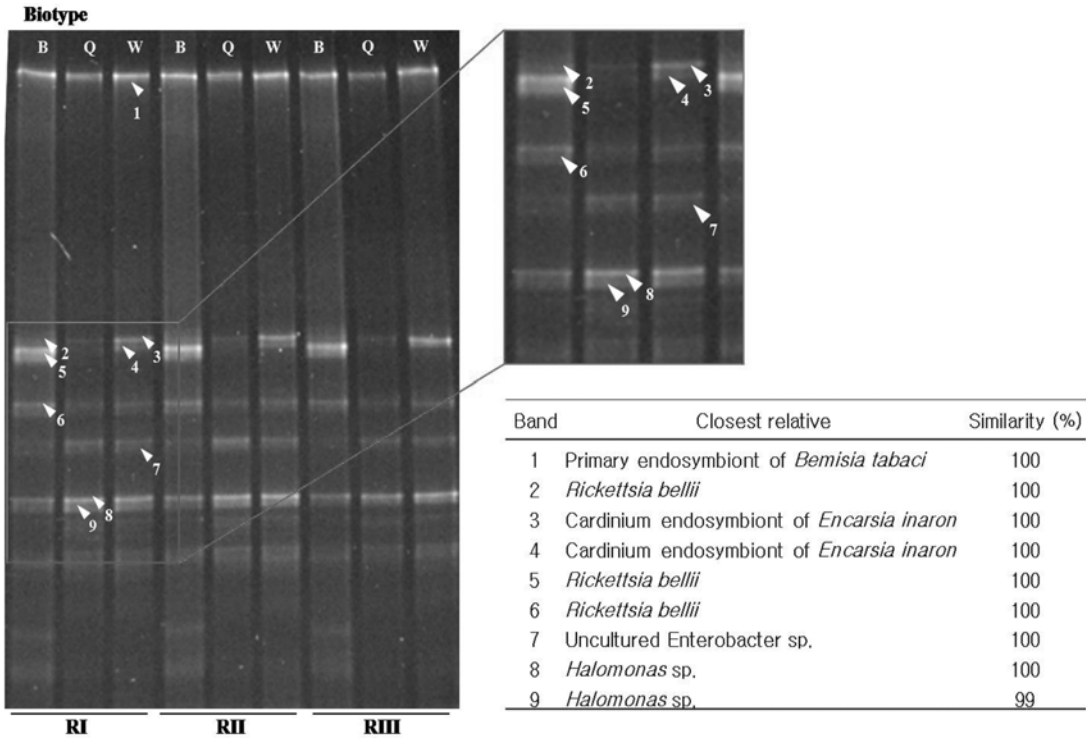


Fig. 3. Phylogenetic tree of bacterial strains associated with the different biostages of sweetpotato whitefly *Bemisia tabaci* Q biotype in Firmicutes, *Alphaproteobacteria*, *Betaproteobacteria*, *Gamaproteobacteria*, and Actinobacteria based on 16S rRNA gene sequence analysis. Bootstrap values (from 1000 replicates) higher than 50% are shown at the branch points. Bar 2% nucleotide sequence difference. *Ar*: *Arthrobacter*; *Bs, Bacillus*; *Br*, *Bradyrhizobium*; *Jb*, *Janibacter*; *Kc*, *Kocuria*; *Sc*, *Staphylococcus*; *Sg*, *Sphingomonas*; *Sm*, *Streptomyces*; *Mx*, *Moraxella*; *Mg*, *Morganella*; *Ms*, *Micrococcus*; *Nx*, *Naxibacter*.

between host insect bacteria and biotypes. Previously, the genus *Bacillus* and *Staphylococcus* from the whitefly were reported for their potential to produce medium-length sugars from sucrose and contribute to the stickiness of the honeydew secreted by the host insect [Davidson *et al.,* 2000]. Compared to B biotype, abundance of *Bacillus* sp. was low in biotype Q; however, it should be noted that low abundance does not necessarily imply that these bacteria are functionally unimportant [de Vries *et al.,* 2001]. There is a possibility



Band	Closest relative	Similarity (%)
1	Primary endosymbiont of *Bemisia tabaci*	100
2	*Rickettsia bellii*	100
3	Cardinium endosymbiont of *Encarsia inaron*	100
4	Cardinium endosymbiont of *Encarsia inaron*	100
5	*Rickettsia bellii*	100
6	*Rickettsia bellii*	100
7	Uncultured Enterobacter sp.	100
8	*Halomonas* sp.	100
9	*Halomonas* sp.	99

Fig. 4. DGGE profiles of PCR amplified DNA from bacteria present in the sweetpotato whitefly-*Bemisia tabaci* biotypes B, Q, and wild type adults. RI, RII, and RIII indicate three replications.

that the density and diversity of different bacterial community may reflect the broad host plant range and survival by exhibiting less susceptibility to insecticides [Broderick *et al.,* 2004; Xiang *et al.,* 2006]. Similarly, Q biotype has been reported for its broad host range (redpepper and tomato) and resistance to different insecticides [Kim *et al.,* 2007; Seo *et al.,* 2007]. There could be a correlation between the host plant selection/ preference and gut bacteria, as documented for the hemipteran insect pest species aphid, *Acyrthopisum sativum* [Tsuchida *et al.,* 2004], which has a feeding pattern and habit similar to those of whiteflies.

The bacterial phylotypes *Staphylococcus*, *Micrococcus*, and *Bacillus* found in all developmental stages of the whitefly were common members of the gut microbial communities in insects such as mulberry longicorn beetle and *Apriona germari*, and were reported for their role in reducing the pathogenic gut microbe population by maintaining the gut pH [Takatsuka and Kunimi, 2000]. Thus, these bacterial genera could be passed from egg to further developmental stages through vertical transmission [de Vries *et al.,* 2001; Gottlieb *et al.,* 2006]. Similar findings were reported for other insect species such as thrips, *Erwinia*, and bean bug *Burkholderia* [de Vries *et al.,* 2001; Kikuchi *et al.,* 2005]. The close relationship of bacterial isolates from different developmental stages of *B. tabaci* with nitrogen-fixing *Staphylococcus* strain supports the hypothesis that nutritionally imbalanced diet ingested by the host insect could be balanced by the gut symbionts [Nardi *et al.,* 2002; Behar *et al.,* 2005]. Hence, association of *Bacillus*, *Staphylococcus*, and *Micrococcus* at all *B. tabaci* developmental stages indicates a symbiosis and, indeed, suggests a nutritional interdependence and detoxification of toxic substances [Iverson *et al.,* 1984; Lauzon *et al.,* 2003; Dillon and Dillon, 2004; Genta *et al.,* 2006].

Studies exploring the influence of secondary symbionts in other homopteran insects revealed numerous roles of these tenants in the biology of host, including conferring resistance to heat, parasitoid, and influencing host plant preferences [Dillon and Dillon, 2004; Tsuchida *et al.,* 2004; Gottlieb *et al.,* 2006]. Accordingly, in the present investigation B biotype also recorded fewer bacterial phylotypes than Q biotype and preferred to feed and survive only on tomato. On the other hand, the Q biotype is able to feed on both red pepper and tomato and is less susceptible to different classes of tested insecticides than the B biotype [Kim *et al.,* 2007; Seo *et al.,* 2007]. Hence, the differences in the host plant preference and the susceptibility to insecticide by these two biotypes could be influenced by the bacterial phylotypes associated with their respective host insects.

It is intriguing how the host insects bear the effect of pathogenic bacteria; for instance, BQN3C1-03d, which

showed 98% similarity with the insecticidal strain *Morganella morgani* isolated from the antlion gut, caused 80% mortality to its prey, common cutworm [Nishiwaki *et al.,* 2007]. It could be explained that other bacterial phylotypes may be responsible for avoiding the deleterious effect caused by insecticidal strain of bacteria, through maintaining the population level of other bacterial communities and physiological factors (e.g. gut pH), which are essential for the growth and development of the pathogenic bacteria present in the gut of the host insect [Takatsuka and Kunimi, 2000; Broderick *et al.,* 2004; Indiragandhi *et al.,* 2008].

In the present study the dominance of bacteria belonging to *Staphylococcus* and *Micrococcus* at all stages of both biotypes suggested that the bacteria of these two genera could be common members of the gut microflora. The interaction between gut microbes and insect host may either be simply regarded as nutrition assistance, withdrawing the insect pathogen or more complicated multitrophic interaction between insects and plants [Moran *et al.,* 2003; Dillon and Dillon, 2004]. The presence of different bacterial phylotypes in *B. tabaci* indicates that each phylotypes may have different roles on their biological processes of the host insect, supporting the hypothesis that insect gut bacteria are essential for the survival of their host insects. However, whether they have a functional significance with regards to the physiology and nutrition of whitefly remains to be further studied. The elimination of bacterial phylotypes associated with each developmental stage or use of specific concentration of pathogenic bacteria isolated from the same host insect pest may serve as novel method for managing the insect pest.

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