CULTURABLE BACTERIA ASSOCIATED WITH THE WHITEFLY, *BEMISIA ARGENTIFOLII* (HOMOPTERA: ALEYRODIDAE)

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ABSTRACT

Several different types of bacteria were cultured from surface-sterilized *Bemisia argentifolii* Bellows, Perring, Gill and Hedrick 1994 (Homoptera: Aleyrodidae) adults and nymphs, including *Bacillus* spp., Gram-variable pleomorphic rods and Gram-positive cocci. Two of the isolates were capable of being ingested by adults and passed into the honeydew. One of these, *Enterobacter cloacae*, was found within the gut cells of adult whiteflies and was mildly pathogenic. This isolate represents the first bacterium with potential as a pathogen of whiteflies. Bacteria which were not capable of being ingested, may have been located in structures which were protected from surface sterilization, such as the lingula or the female reproductive tract.

Key Words: *Bemisia tabaci* B-biotype, *Enterobacter cloacae*, *Bacillus* sp., symbiotic bacteria

RESUMEN

Diferentes tipos de bacterias fueron aisladas de adultos y ninfas de *Bemisia argentifolii* Bellows et al., 1994 (Homoptera: Aleyrodidae) esterilizados superficialmente. Entre las bacterias aisladas se encontró *Bacillus* spp., bacilos pleomórficos Gram-variables y cocos Gram-positivos. Los adultos fueron capaces de ingerir a dos de los organismos aislados y de transferirlos a la mielecilla que secretan. Uno de éstos, *Enterobacter cloacae*, fué encontrado dentro de células intestinales de mosquitas blancas adultas y fué moderadamente patogénico. Esta bacteria representa el primer organismo aislado que posee potencial patogénico para el control de la mosquita blanca. Otras bacterias aisladas no fueron capaces de ser ingeridas, lo cual se atribuyó a que se ubicaron en estructuras protegidas de la esterilización superficial, como la lígula o el tracto reproductivo femenino.

The silverleaf whitefly, *Bemisia argentifolii* (= *B. tabaci* B biotype) (Bellows, Perring, Gill and Hedrick, 1994), is one of the most damaging insects to agriculture in the southern United States and in warmer regions of many other countries. *B. argentifolii* feeds on a wide variety of plant species, including cotton, melons, brassicas, tomato, peppers, and ornamentals such as poinsettia (Cock 1986, De Barro 1995). This insect causes economic damage in four ways: by removing photosynthetic products during phloem feeding; by contaminating cotton fiber and ornamental plants with sticky excreted honeydew; by inducing physiological responses in the host such as squash silverleaf and tomato irregular ripening; and by vectoring viruses (Byrne & Bellows 1991).

Bemisia argentifolii possesses pleomorphic and coccoid obligate intracellular bacterial endosymbionts, housed in mycetomes, which are transmitted from the female to

her eggs (Costa et al. 1993a). Growth and development of the nymph and induction of the squash silverleaf disorder, for which the species is named, are retarded if the symbiotic bacteria are reduced or eliminated by feeding antibiotics to the adult female before oviposition or if fed to the nymph via the leaf (Costa et al. 1993b, 1997). These endosymbionts have not reportedly been cultured on laboratory media.

The objectives of this study were to investigate whether other bacteria are present in whiteflies, and modes of entry of these bacteria into the whiteflies. We have isolated a variety of bacteria from surface-sterilized *B. argentifolii* adults and nymphs, some of which have previously been implicated in the production of medium-length oligosaccharides in the honeydew (Davidson et al. 1994). Two of the isolated bacteria were shown to be ingested by the whitefly and one of these was mildly pathogenic.

Materials and Methods

Whiteflies

Whitefly adults, nymphs and eggs were collected from cotton, cabbage, cucumber, squash, lantana, pepper or melon plants and surface sterilized with ethanol and household chlorine bleach as described previously (Davidson et al. 1994). Because of the small size of these insects, samples were processed in groups of ca. 50-200. Adults, nymphs and eggs were processed separately. Surface sterilized insects were inoculated into liquid nutrient broth-yeast extract-salts medium (Myers and Yousten 1978) or brain heart infusion broth, either whole or homogenized in sterile 0.9% saline. Homogenized insects were also plated directly on microbiological media including nutrient agar, brain heart infusion agar, tryptose agar, Luria agar, purple agar, and chocolate agar (Sigma, St. Louis, MO) and incubated aerobically at 25°C.

Bacterial Cultures

When bacterial growth was observed in liquid or agar bacterial media, cultures were streaked for purity on agar media of the same type on which positive growth had been observed. Cultures were preserved by freezing at -70°C in 20% glycerol in liquid medium which best supported growth of each culture.

Bacteria were identified according to microscopic appearance, Gram's stain, anaerobic growth, catalase production, colony morphology, and reactions on API 20E and API CH identification strips (bioMerieux Vitek, Hazelwood, MO). Two isolates, designated WFA73 and WFN29, were also identified by gas chromatography (GC) fatty acid profiles using the MIDI identification system, by Dr. Joel Siegel, Illinois Natural History Survey.

Whitefly Ingestion of Bacteria

Eight different bacterial strains, representative of the morphological and Gram-stain groups isolated from whiteflies (Table 3), were suspended in 30% sucrose and green food coloring at ca. 10^9 cells/ml, and fed to adult whiteflies through parafilm sachets. Control whiteflies were fed on sucrose alone. After 48 hr, mortality was recorded, and all insects were surface sterilized, homogenized, and plated to appropriate agar medium. Sterile petri dishes were used to collect honeydew from control whiteflies and those fed bacteria. The dishes were rinsed with sterile saline and the saline plated to agar medium. Bacterial colonies recovered were compared microscopically and in colony morphology to those originally fed to the whiteflies. Bio-

assays of strains WFA73 and WFN29 were repeated twice and % mortality reported as the mean of two replicates; at least 100 insects were included in each treatment.

Electron Microscopy

Adult whiteflies were fed strain WFA73, which had been found to be ingested and mildly pathogenic in experiments described above, in green sucrose solution at ca. 10^9 cells/ml in parafilm sachets. Control insects were fed on green sucrose only. After 24 hr, insects with green digestive tracts were prepared for electron microscopy. Insects were gently pierced in the thorax region while immersed in fixative, which consisted of 4% glutaraldehyde in 0.05M cacodylate buffer. Insects were fixed for 4 hr, postfixed in 0.5% osmium tetroxide in 0.05M cacodylate buffer for 1-2 hr, dehydrated in ethanol, and embedded in Spurr's resin or LR White resin. Thin sections from 2 control and 4 experimental whiteflies were observed by transmission electron microscopy using a Philips EM 200 TEM (Eindhoven, Netherlands).

Results

Bacteria from Whiteflies

Bacteria were cultured from surface-sterilized whitefly adults and nymphs collected from all host plant species and from all groups of whiteflies collected at 30 different times over a period of five years. A greater variety of bacteria was recovered from adult whiteflies than from nymphs, however none were cultured from surface-sterilized eggs. A total of 80 isolates were preserved from adults and 29 from nymphs; representative examples are shown in Tables 1 and 2. There was no relationship between the host plant species and the type of bacteria isolated from the whiteflies (Tables 1 and 2).

Four major types of bacteria were cultured from *B. argentifolii* using standard microbiological media. These included: 1. Gram-positive sporeforming aerobic rods, *Bacillus* spp.; 2. Gram-positive cocci; 3. Gram-variable short pleomorphic rods producing very short rods to cocci in older cultures; 4. Gram-variable long, thin, highly pleomorphic rods forming cocci in older cultures (Tables 1 and 2).

There was a strong correlation between the insect life stage and the types of bacteria isolated. Most isolates of Gram-positive or Gram-variable rod-shaped bacteria were obtained from adult whiteflies, whereas most isolates of cocci were produced from nymphs (Tables 1 and 2).

Bacterial Identification

Sporeforming aerobic bacteria were identified as *Bacillus licheniformis* (Weigman), *B. megaterium* deBary, *B. amyloliquefasciens* Fukumoto, and *B. subtilis* (Ehrenberg), based upon microscopic examination and the results of API diagnostic tests. The *B. subtilis*, *B. licheniformis* and *B. megaterium* isolates were found to produce medium-length sugars from sucrose in an earlier study (Davidson et al. 1994).

Spherical cells, forming diads and tetrads and occasionally chains, were frequently isolated from *B. argentifolii* nymphs. Isolates which were Gram-positive, catalase positive, facultatively anaerobic, and capable of growing in the presence of 10% NaCl, were identified as *Staphylococcus* spp., close to *S. aureus* Rosenbach, *S. sciuri* Kloos, Schleifer & Smith and *S. epidermidis* (Winslow & Winslow). Larger gram-positive cocci forming diads and tetrads resembled *Sporosarcina* spp. (Orla-Jensen) although spores were not observed in these cultures (Tables 1 and 2).

TABLE 1. MORPHOLOGY, HOST AND IDENTIFICATION OF BACTERIA RECOVERED FROM ADULT *B. ARGENTIFOLII*. *IDENTIFICATION BY MIDI; ALL OTHERS IDENTIFIED BY API. ND = NOT DETERMINED.

Designation	Description	Host	Identification
WFA1	sporeforming rod	cotton	*Bacillus amyloliquefasciens*
WFA2	spheres in diads	cotton	ND
WFA3	sporeforming rod	cucumber	*B. licheniformis*
WFA5	sporeforming rod	cucumber	*B. megaterium*
WFA8	sporeforming rod	cucumber	*B. licheniformis*
WFA9, 10, 11, 12	sporeforming rod	melon	*Bacillus* spp.
WFA12, 14, 35, 36, 40, 45, 46	sporeforming rod	cotton	*Bacillus* spp.
WFA37	short pleomorphic rod, yellow colony	cotton	ND
WFA41	cocci, diads	cotton	ND
WFA43,44	small pleomorphic rods, clear colonies	cotton	*Chryseomonas luteola*
WFA52	diplococci, tetrads	cotton	ND
WFA56, 59	very short rods, thick at one end	cotton	*Acinetobacter lwoffsii*
WFA67	short rods, yellow colonies	cotton	*Citrobacter sp.*
WFA68	short pointed rods	cotton	*Flavomonas oryzihabitans*
WFA69, 70	short curved rods	cotton	*Acinetobacter baumanii?*
WFA71	sporeforming rod	cucumber	*Bacillus* sp.
WFA 73	short pleomorphic rods	cotton	*Enterobacter cloacae**
WFA74	short rod with inclusions	cotton	ND
WFA80	short pleomorphic rods	cotton	*Flavomonas oryzihabitans*

On the basis of API tests, short, Gram-negative or Gram-variable pleomorphic rods were identified as *Enterobacter cloacae* (Jordan), *Flavimonas oryzihabitans* Holmes et al., *Citrobacter* sp. Workman and Gillen, *Cellulomonas* sp. Bergey et al., *Chryseomonas luteola* Holmes et al., *Acinetobacter lwoffsii* Brisou & Prevot or *A. baumanni* (Deacon). Some strains produced no reaction on API tests and therefore remain unindentified (Tables 1 and 2). Two strains which were found to be ingested by adult whiteflies (below) were further identified by MIDI-GC.

Whitefly Ingestion of Bacteria

To determine whether bacteria isolated from whiteflies could have entered the insects during feeding, we fed eight different bacterial strains to adults (Table 3). Bacteria strains designated WFA73 and WFN29 were recovered in large quantity from surface-sterilized adults and their honeydew following feeding on these bacteria. Bacterial colonies, morphologically and microscopically identical to WFA73 or WFN29, were not found in homogenates or honeydew of control insects. WFA73 is a short pleomorphic Gram-variable rod, identified by GC analysis of fatty acids and API analysis

TABLE 2. BACTERIA ISOLATED FROM *B. ARGENTIFOLII* NYMPHS. MORPHOLOGY, HOST AND IDENTIFICATION OF BACTERIA RECOVERED FROM ADULT *B. ARGENTIFOLII*. *IDENTIFICATION BY MIDI; ALL OTHERS IDENTIFIED BY API. ND = NOT DETERMINED.

Designation	Description	Host	Identification
WFN1	spheres, diads	cotton	ND
WFN3	sporeforming rod	cotton	*Bacillus* sp.
WFN4	large diplococcus	cotton	*Sporosarcina* sp.?
WFN6	sporeforming rod	cabbage	*Bacillus* sp.
WFN7	coccus	cabbage	*Staphylococcus epidermidis*
WFN10, 11, 13	cocci	cabbage	*Staphylococcus epidermidis*
WFN12	cocci	cabbage	*Staphylococcus aureus*
WFN14	large cocci	cabbage	*Sporosarcina* sp.?
WFN15	short pleomorphic rods	cabbage	ND
WFN17A	very thin rods	cabbage	*Agromonas* sp.
WFN28	sporeforming rod	cabbage	*Bacillus licheniformis*
WFN29	pleomorphic rods forming cocci	cabbage	*Cellulomonas turbata**

as *E. cloacae* (0.70 agreement). Isolate WFN29, which formed bent rods in young cultures and cocci in older cultures, was identified by GC and API analysis as *Cellulomonas (Oerskovia) turbata* (Erikson) (0.78 agreement). *Bacillus* spp., Gram-positive cocci and the other Gram-negative or Gram-variable isolates were not recovered from adults fed these isolates (Table 3).

TABLE 3. MORPHOLOGY, IDENTIFICATION AND RECOVERY OF BACTERIA FED TO ADULT *B. ARGENTIFOLII*.

Strain designation	Morphology	Identification	Recovery from insect or honeydew
WFA5	Gram-positive Bacillus	*Bacillus megaterium*	no
WFA11	Gram-positive Bacillus	*Bacillus subtilis*	no
WFN12	Gram-positive Coccus	*Staphylococcus aureus*	no
WFN7	Gram-positive Coccus	*Staphylococcus epidermidis*	no
WFA73	Short pleomorphic Gram-variable rod	*Enterobacter cloacae*	yes
WFN29	Pleomorphic Gram-negative rod	*Cellulomonas turbata*	yes
WFA69	Short curved or branched rod	*Acinetobacter baumanii*?	no
WFA74	Short rod with inclusions	No API reaction, Gram-variable	no

Bioassay Results

Strain WFA73 (*E. cloacae*) produced an average 34% mortality (s.d. = 1.41) of adult whiteflies at ca. 10^9 bacterial cells/ml after 24 hr in two experiments (control mortality = 4.0%; s.d. = 2.83). Mortality increased to 75% (s.d. = 0) after 48 hr in adult whiteflies fed WFA73 (control mortality = 9.5%; s.d.= 6.36). Bacteria identical to *E. cloacae* were also recovered from honeydew of adults fed these bacteria but not from the honeydew of control adults fed only on sucrose. Strain WFN29 (*C. turbata*) produced only 4.5% mortality (s.d. = 2.12) of adult *B. argentifolii* at 10^9 cells/ml after 48 hr, similar to control mortality (4.0%; s.d. = 1.41), but was recovered from homogenized adults fed this strain and from their honeydew.

Electron Microscopy

Bacteria were not observed in the digestive system of control whiteflies in these studies (not shown).

In adults fed strain WFA73 (*E. cloacae*), large numbers of bacteria were seen throughout the digestive tract (Fig. 1). The lumen of the entire midgut was filled with rod-shaped bacteria (WFA73). Bacteria adhered to the apical portion of the descend-

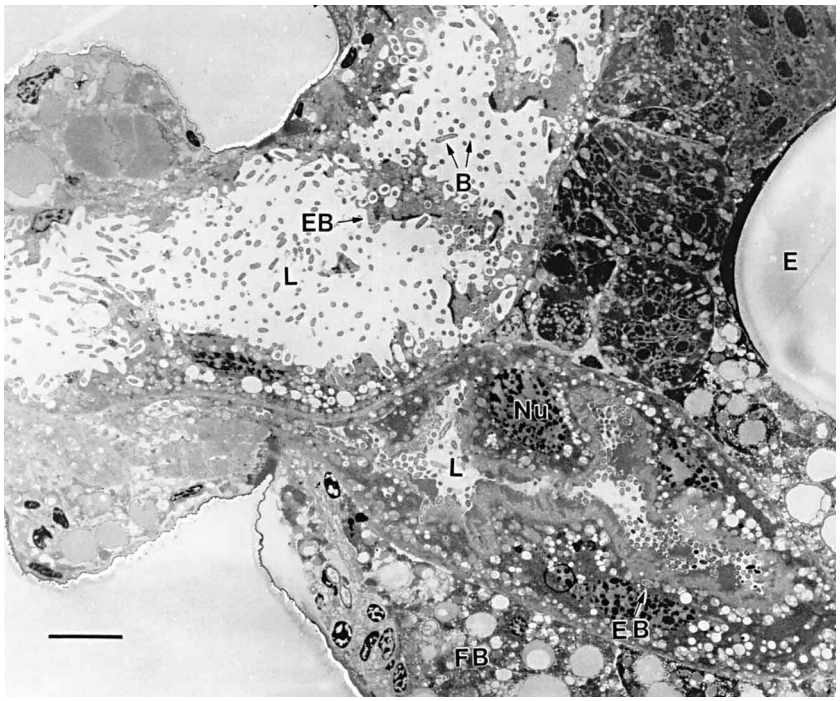

Fig. 1. After feeding on bacteria, both the ascending and descending portions of the midgut of *B. argentifolii* contained high numbers of WFA73 (*E. cloacae*) bacteria (B) in the lumina (L). Thorax appears to the left and abdomen to the right. Note the presence of engulfed bacteria (EB) in the apical portion of the epithelial cells. Nu, nucleus of midgut cell; FB, fat body; E, egg. Bar = 10 μm.

ing and ascending midgut epithelial cells and were also found within the epithelial cell cytoplasm in membrane bound vesicles (Figs. 2 and 3). Cells in the descending midgut appeared to be taking up the bacteria by phagocytosis as evidenced by bacteria in various stages of engulfment (Figs. 3 and 4). Descending midgut cells of infected insects also exhibited poorly developed microvilli, numerous spherical vesicles, each with a small amount of electron dense material, many large electron dense lysosomal-like vesicles, and small electron dense residual bodies (Fig. 4). Vacuolation of mitochondria was observed both in control and bacteria-fed whiteflies, probably a result of slow fixative penetration.

We observed bacteria-like organisms in the reproductive tracts of two female whiteflies (Fig. 5). The bacteria in the female reproductive tracts were readily distinguishable from sperm, which were observed in the spermatheca and appeared as electron-dense structures which lacked notable internal structure, and were aligned in packets (not shown). The identity of the bacteria found in the female reproductive tracts is not currently known.

Discussion

Some of the bacteria cultured from whiteflies may be involved in a mutualistic relationship with the insects, contributing to the digestion and nutrition of the insect, while obtaining access to the high sugar content of phloem sap in the gut of the insect and honeydew. As described earlier (Davidson et al. 1994), *Bacillus spp*. associated with *B. argentifolii* may produce long-chain sugars which contribute to the stickiness of the honeydew of this insect. During a study of digestive tract ultrastructure (Rosell et al., unpubl.), bacteria were observed in the esophagus of adult *Bemisia spp.* taken from a laboratory colony reared on cotton.

Similar gut bacteria have been isolated from other Homoptera. *Staphylococcus sciuri* and *S. epidermidis*, and Gram-negative rods, close to *Pseudomonas fluorescens* Migula, have been isolated from the pea aphid, *Acythosiphon pisum* (Harris) (Grenier et al. 1994). The flora in the aphid gut was assumed to have been acquired during probing on the leaf surface, as is probably the case with most of the bacteria isolated from whiteflies as well. Srivastata and Rouatt (1963) isolated *Sarcina, Micrococcus, Achromobacter* and *Flavobacterium* from aphids. Bacteria have also been reported in the hemocoel of aphids and leafhoppers (Grenier et al. 1994, Purcell et al. 1986). At this time we cannot rule out the possibility that some of the bacteria isolated from *B. argentifolii* are also occasional residents in the hemocoel.

We do not currently know the significance of bacteria present in the female reproductive tract. Morphologically, they resemble the short rod-shaped bacteria commonly isolated from adult whiteflies (Table 1). Following electron microscope observation of bacteria in the female reproductive tract, two collections of whiteflies were separated into males and females, surface sterilized and processed separately for bacterial isolation. Bacteria were cultured only from females. Their presence suggests possible transmission of these bacteria to offspring, however bacteria were not cultured from surface-sterilized eggs.

The results presented here confirm that *B. argentifolii* adults and nymphs can ingest certain culturable bacteria and contain these bacteria in their digestive tracts. As these bacteria were ingested through Parafilm, and were recovered from honeydew after feeding, the bacteria were truly ingested and not simply resident on the external surface or mouthparts of the insect. Our results are in agreement with those of Zeidan and Czosnek (1994) who found that *B. tabaci* could ingest *Agrobacterium*. The failure to culture similar bacteria from surface sterilized whitefly eggs, suggests that these

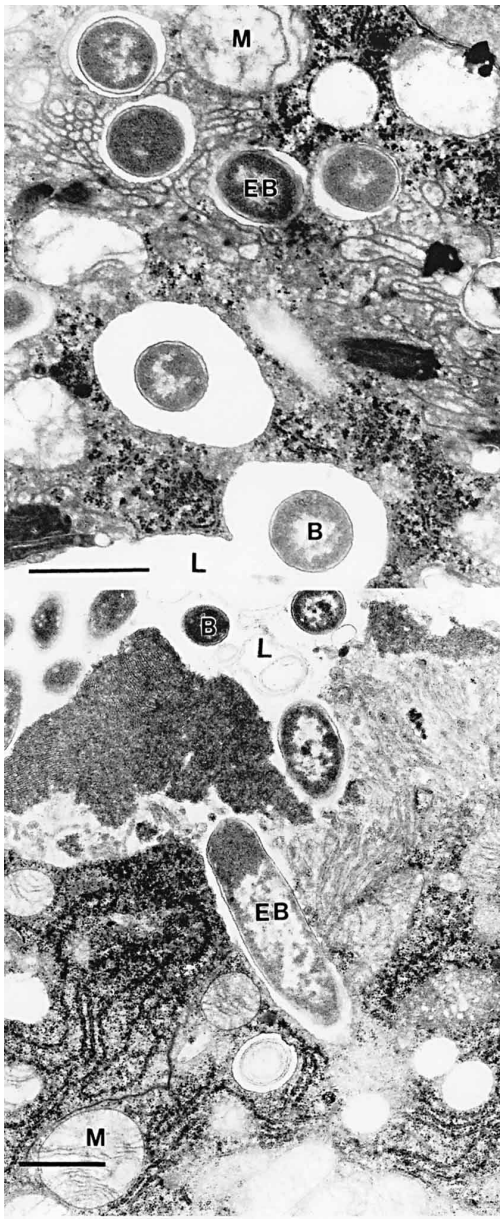

Fig. 2. Descending midgut from whitefly fed WFA73 bacteria. Note bacterium (B) present in the lumen (L) that is being engulfed and bacteria (EB) present in membrane bound vesicles. M, mitochondrion. Bar = 1 μm.

Fig. 3. WFA73 bacterial cell (EB) actively being engulfed at apical surface of descending midgut epithelia in this whitefly fed the bacteria. Extracellular bacteria (B) are present in the lumen (L). M, mitochondrion. Bar = 1 μm.

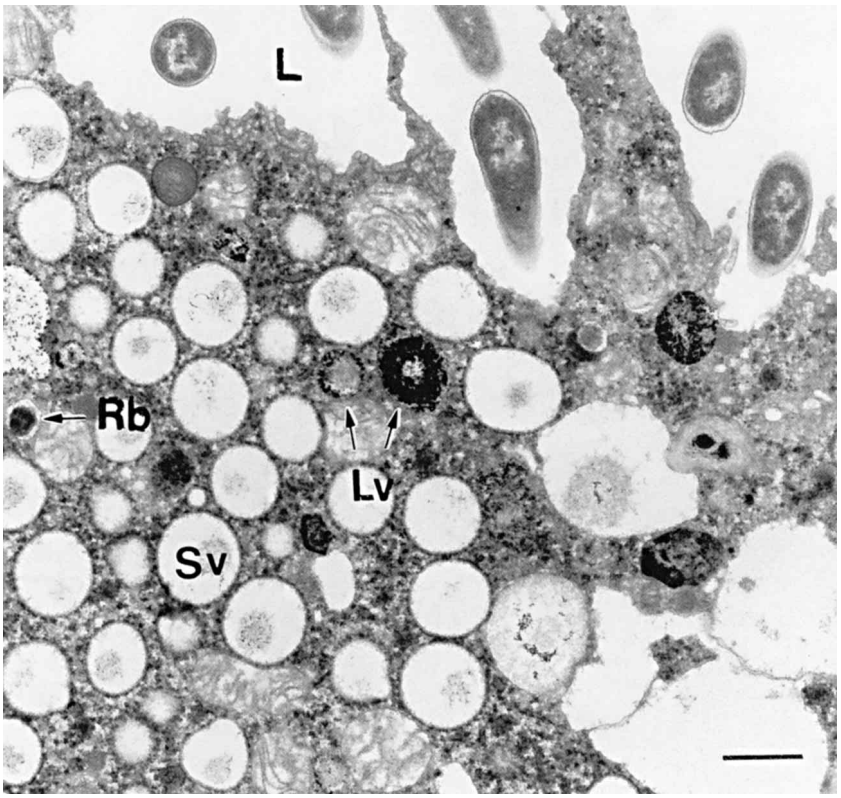

Fig. 4. Descending midgut from whitefly fed WFA73 showing electron lucent spherical vesicles (Sv), electron dense lysosomal-like vesicles (Lv) and small dense residual bodies (Rb). Bacteria are present in the lumen (L). Bar = 1 (m.

bacteria were obtained from the leaf surface during probing prior to feeding. These bacteria are not transovarially transmitted, as the same bacteria were not isolated from all samples and bacteria were not isolated from homogenized surface-sterilized eggs. These culturable bacteria are therefore not obligate symbionts.

Rosell et al. (1995) demonstrated ultrastructurally that the adult *B. tabaci* stylet food canal is 0.65 μm in diameter. Using fluorescent beads, we have recently shown that 0.2 μm beads are ingested and pass in honeydew, but 0.5 μm beads do not enter the insect (Rosell et al., in prep). Therefore in order for bacteria to enter the food canal, they must be less than 0.5 μm in diameter, or be pleomorphic with some members of the population smaller than 0.5 μm. The bacteria which successfully entered the food canal, strains WFA73 and WFN29, fulfilled these requirements. In contrast, Gram-positive bacteria including *Bacillus* spp. and *Staphylococcus* spp., which have generally larger diameters, entered the food canal poorly or not at all. As several *Bacillus* spp. and *Staphylococcus* spp. were isolated from surface-sterilized whiteflies, these organisms may have been located under the lingula at the posterior of the gut, where honeydew accumulates prior to excretion, or in the female reproductive tracts as seen in electron microscopy (Fig. 5).

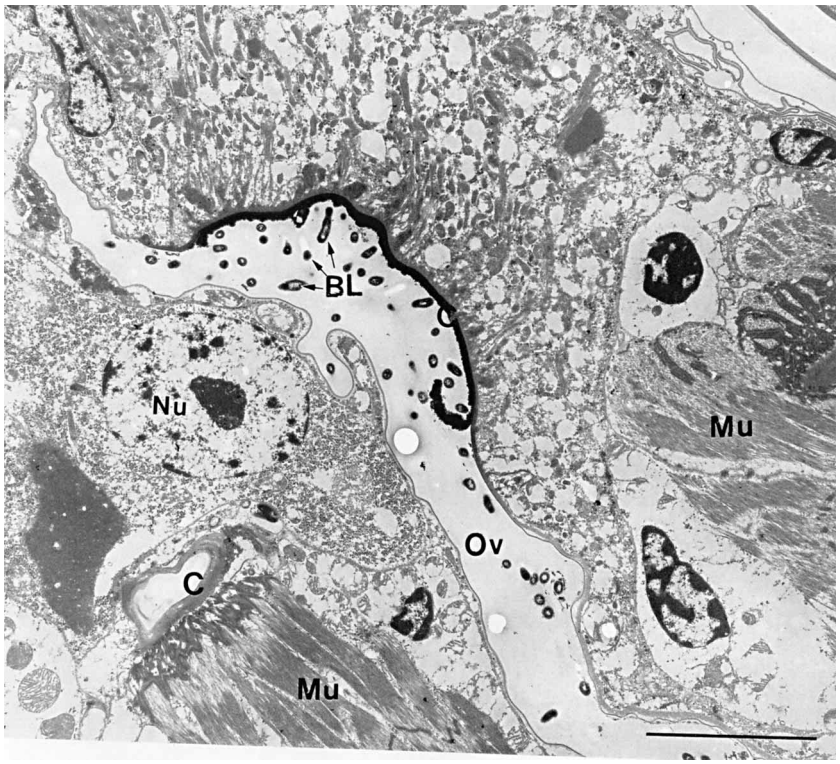

Fig. 5. Bacteria-like organisms (BL) are found in the female reproductive tract which is convergent with the ovipositor canal (OV). C, cuticle; Mu, muscle; nu, nucleus of epidermal cell. Posterior of the whitefly is to the right. Bar = 5 μm.

Isolate WFA73 (*E. cloacae*) appears to be mildly pathogenic to *B. argentifolii* adults, and represents the first bacterial pathogen reported from whiteflies. In specimens fed this bacterium, midgut cells were massively invaded by bacteria, likely leading to loss of part or all of gut function (Fig. 1). Phagocytosis of bacteria by insect midgut cells has been observed during the pathogenesis of both American foulbrood disease of honey bees (Davidson 1973), and milky disease of beetles (Kawanishi et al. 1978, Splittstoesser et al. 1978). *Enterobacter cloacae* was described as a pathogen of grasshoppers under its original name, *Coccobacillus acridiorum. Enterobacter (Aerobacter) aerogenes* is a pathogen of lepidoptera in association with *Proteus mirabilis* (Tanada and Kaya 1993, Wysoki and Raccah 1980) and occurs in the gut flora of the New Zealand grass grub (Stucki et al. 1984).

Isolate WFA73 (*E. cloacae*), which was readily ingested by adults, originated from whiteflies with enhanced resistance to the insecticide Danitol® (Valent, USA) and is capable of precipitating Danitol *in vitro* (E. Davidson, L. Williams and D. Alexander, unpubl. results). Therefore culturable bacteria associated with *B. argentifolii* may be important to insecticide degradation in the phylloplane, and perhaps in the insect as well.

The relationship of *E. cloacae* to *B. argentifolii* is similar in several aspects to the bacterium designated BEV in leafhoppers. BEV can be cultured on bacteriological me-

dia, is mildly pathogenic to its normal host, *Euscelidius variegatus* Kirshbaum, and penetrates gut cells in a manner ultrastructurally similar to *E. cloacae* in the whitefly. However BEV is both transmitted transovarially and acquired from the plant (Purcell et al. 1984, Purcell and Suslow 1987, Cheung and Purcell 1993).

Clark et al. (1992) examined the endosymbionts of *B. argentifolii* and *B. tabaci* using 16S rDNA analysis, and found the secondary endosymbiont of *Bemisia* is related to Enterobacteriaceae. Results presented here confirm that Enterobacteriaceae are commonly present in *B. argentifolii*, and *E. cloacae* can enter the cells of the insect, suggesting that such bacteria could have been ancestors of whitefly endosymbiotic bacteria, as suggested by Harada et al. (1996) for an endosymbiont of the pea aphid. Gut bacteria also present contaminating foreign bacterial DNA which may confuse genetic analysis of endosymbionts, as pointed out by Grenier et al. (1994) for aphids.

While it is clear that mycetome endosymbionts are critical to the development of *B. argentifolii* (e.g. Costa et al. 1993b), the presence of other bacteria must be taken into consideration in studies of the physiology of this insect. Although WFA73 (*E. cloacae*) is only mildly pathogenic to *B. argentifolii*, its ability to penetrate whitefly gut cells suggests that this microorganism could be genetically modified to enhance its effectiveness as a biological control agent. Transformation of a cotton phyllosphere bacterium, *B. megaterium*, with *Bacillus thuringiensis* Berliner toxin genes for control of lepidoptera (Bora et al. 1994) is an example of such manipulation. Finally, genetic modification of gut symbionts of *Rhodnius prolixus* to interfere with vectoring of Chagas disease has been reported (Beard et al. 1992, 1993). Similar modification of gut bacteria in other insects, such as whiteflies, to alter their ability to vector plant viruses or other characteristics of these insects, is an intriguing possibility (Richards 1993).

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