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Retention Sites for *Xylella fastidiosa* in Four Sharpshooter Vectors (Hemiptera: Cicadellidae) Analyzed by Scanning Electron Microscopy

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Abstract *Xylella fastidiosa* is a xylem-limited bacterium that causes citrus variegated chlorosis (CVC), Pierce's disease of grapevine, and leaf scald of coffee and plum and many other plant species. This pathogen is vectored by sharpshooter leafhoppers (Hemiptera: Cicadellidae: Cicadellinae) and resides in the insect foregut. Scanning electron microscopy was used to determine the retention sites of *X. fastidiosa* for the most common vector species in Brazilian citrus groves, *Acrogonia citrina*, *Bucephalogonia xanthophis, Dilobopterus costalimai*, and *Oncometopia facialis.* After a 48-h acquisition access period on infected citrus or plum, adult sharpshooters were kept on healthy citrus seedlings for an incubation period of 2 weeks to allow for bacterial multiplication. Then the vector heads were incubated for 24 h in a fixative and transferred into a cryoprotector liquid. Bacterial rod cells exhibiting similar X. *fastidiosa* morphology were found laterally attached to different regions inside the cibarial pump chamber (longitudinal groove, lateral surface, cibarial diaphragm and apodemal groove) of *A. citrina*, *O. facialis*, and *D. costalimai*, and polarly attached to the precibarium channel of *O. facialis.* Polymerase chain reactions of vector's heads were positive for the presence of *X. fastidiosa.* No *X. fastidiosa*-like cells were detected in *B. xanthophis*. A different type of rod-shaped bacterium was found on *B. xanthophis* cibarium chamber and images suggest that the cibarium wall was degraded/digested by these bacteria. Colonization patterns of *X. fastidiosa* in their vectors are fundamental aspects to be explored toward understanding acquisition, adhesion, and transmission mechanisms for development of *X. fastidiosa* control strategies.

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Introduction

Xylella fastidiosa [25] is a xylem-limited bacterium that causes citrus variegated chlorosis (CVC), Pierce's disease (PD) of grapevine, phony peach disease, and scald or scorch diseases of numerous plant species [8, 18]. In Brazil, citrus [4, 5, 22], coffee [15], and plum [15] are greatly impacted. This pathogen is transmitted to plants exclusively by sharpshooter species (Hemiptera: Cicadellidae: Cicadellinae) [18, 19]. Sharpshooters from the tribe Cicadellini are usually more efficient vectors than those from the tribe Proconiini [9, 19]. Studies with the PD strain of *X. fastidiosa* have shown that the bacterium is foregut-borne (noncirculative) and propagative (multiply) inside the vector's foregut. Sharpshooter adults can retain infectivity for life [16]. Scanning electron microscopy (SEM) confirmed that bacterial cells acquired by sharpshooters

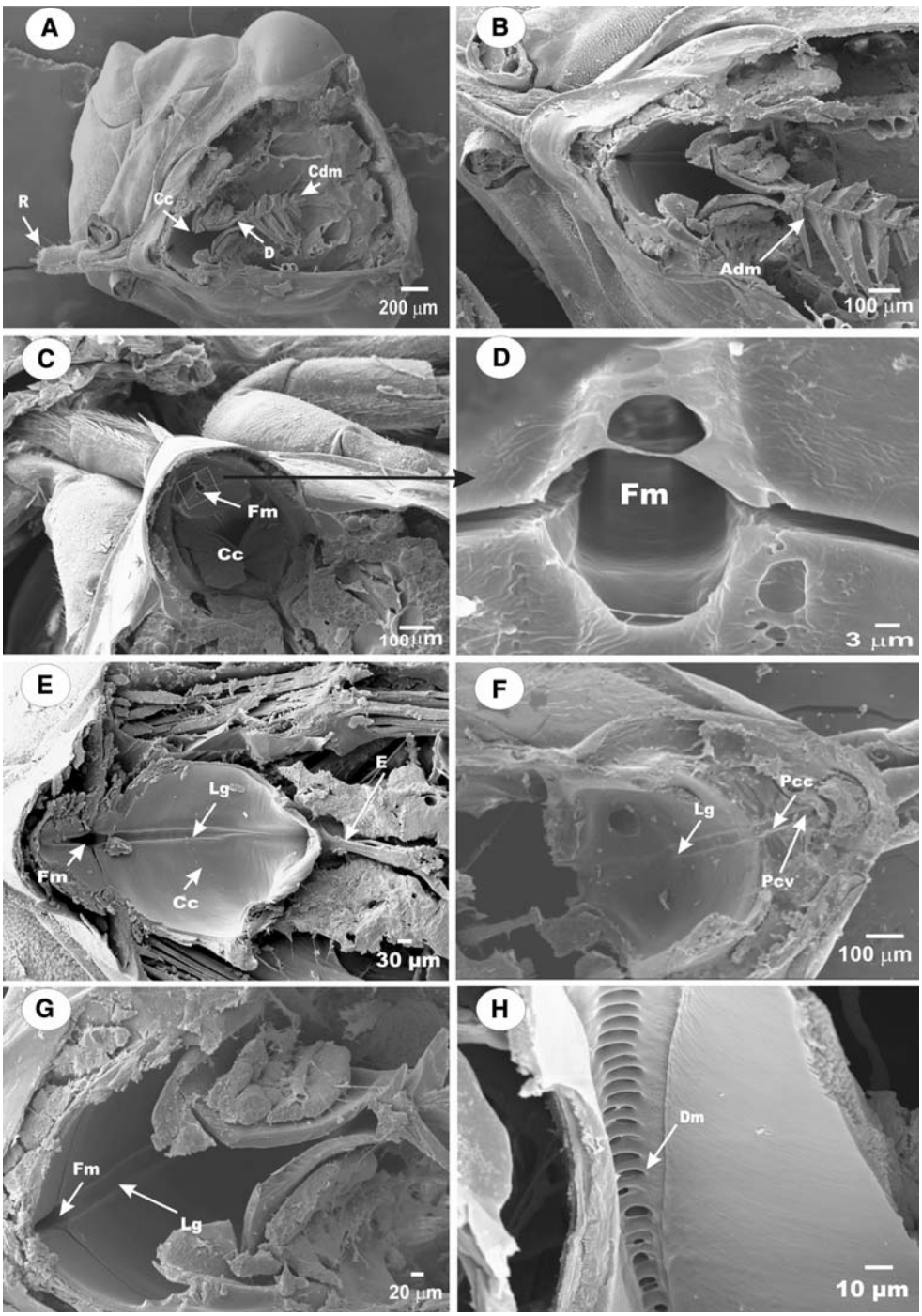

Fig. 1 General view and details of the several foregut parts of the species of *X. fastidiosa* vector sharpshooters and possible retention site. (**A**, **B**, **G**) *Acrogonia citrina*; (**C**, **D**, **E**) *Dilobopterus costalimai*; (**F**) *Oncometopia facialis*; (H) *Bucephalogonia xanthophis.* Adm, apodeme of dilator muscle; Cc, cibarium chamber; Cdm, clypeal dilator muscles; D, diaphram; Dm, diaphram membrane; Fm, food meatus; Lg, longitudinal groove; Pcc, precibarium channel; Pcv, precibarium valve; R, rostrum; E, esophagus

from infected plants adhere to the foregut cuticle, particularly in the anterior portion of the esophagus, cibarium (suction pump), and precibarium [16]. Nymphs lose infectivity after molting, suggesting that transmissible bacterial cells are limited to the vector's foregut which is shed during molting [16]. In Brazil, sharpshooters *Dilobopterus costalimai, Acrogonia terminalis*, *Acrogonia citrina* [15], and *Oncometopia facialis* were considered the most important vectors in orchards [20] and *Bucephalogonia xanthophis* in nurseries [21]. The sites of retention of the bacterium in insects from Brazil or from the CVC and plum leaf scald (PLS) strains of *X. fastidiosa* are not known.

In the present work, the retention sites of strains of *X. fastidiosa* were investigated in different regions of the foregut of four sharpshooter vectors commonly found in Brazilian citrus groves, *A. citrina*, *B. xanthophis*, *D. costalimai*, and *O. facialis*. Knowledge of retention sites is an

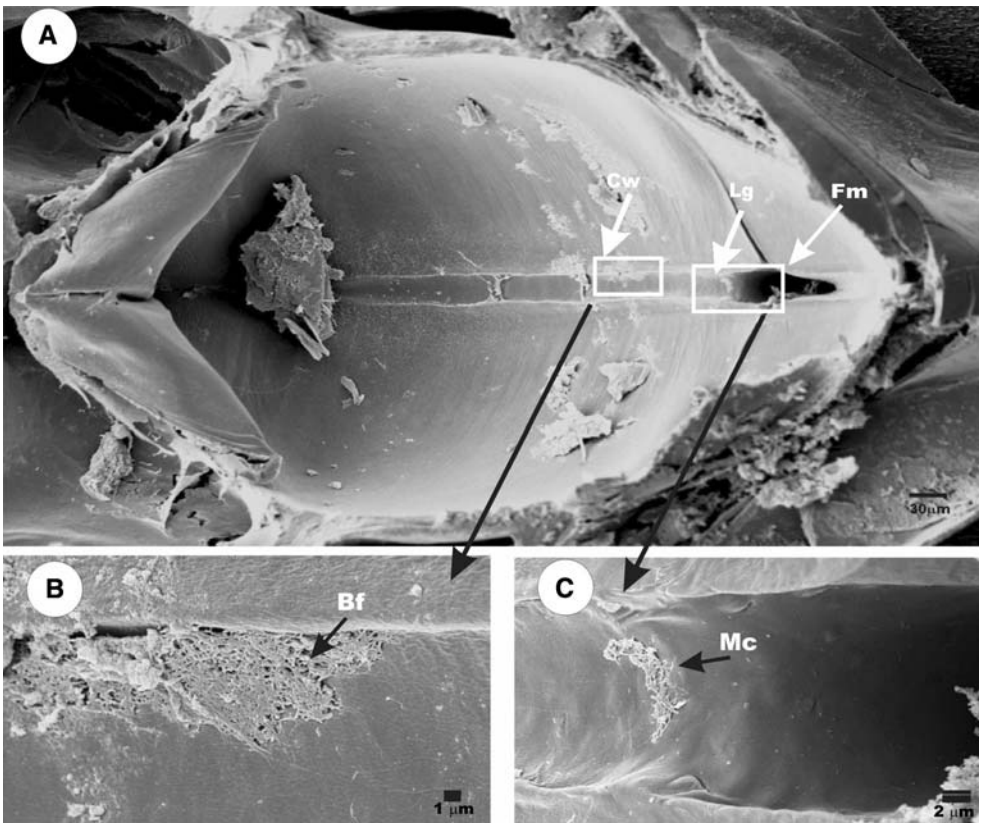

Fig. 2 (**A**) Dorsal view of the cibarium chamber (Cc) of *Acrogonia citrine*, (**B**) magnified view showing a bacterial biofilm (Bf); and (**C**) detail of bacterial cells forming a microcolony (Mc) on the precibarium. The insect was previously fed for 48 h on citrus trees infected with the citrus variegated chlorosis strain of *X. fastidiosa*

important step toward understanding the mechanisms of acquisition, incubation, and transmission of *X. fastidiosa* by insect vectors.

Materials and Methods

Rearing of healthy insect vectors Healthy sharpshooters were produced by rearing the nymphs on healthy and nonhost plants of *X. fastidiosa*. In order to obtain sharpshooter eggs, field-collected adults were confined for oviposition (7–14 days) on healthy plants of *Vernonia condensata* (*B. xanthophis* and *D. costalimai*) or *Citrus sinensis* cv. Pêra (*A. citrina* and *O. fascialis*). Plants of *V. condensata* containing eggs of *B. xanthophis* or *D. costalimai* were transferred directly to larger rearing cages (50 × 60 × 70 cm) for hatching and nymphal development. *Citrus sinensis* cv. Pêra was used as an oviposition host for *A. citrina* and *O. fascialis*. Egg-bearing leaves were detached from the plants and placed inside petri dishes. Citrus petioles were covered with moistened cotton to keep leaves in a turgid state. The petri dishes were kept in an incubator at 25°C and monitored daily for eclosion of nymphs. Soon after eclosion, first-instar nymphs were transferred to healthy seedlings of *V. condensata* inside the rearing cage [13].

Acquisition of bacterium inoculum by sharpshooters In order to obtain infective sharpshooters, adults of *A. citrina*, *B. xanthophis*, *D. costalimai*, and *O. facialis* were submitted to an acquisition access period (AAP) of 48 h on symptomatic citrus trees infected with a CVC isolate (CCT 6570) of *X. fastidiosa*. Thirty individuals were fed for 48 h on plum plants infected with a PLS strain (PLS1) of *X. fastidiosa.* Bacterial strains were obtained from the Tropical culture collection (André Tosello Fundation, Campinas, SP, Brazil). After a 48-h AAP on infected citrus or plum, sharpshooter adults were kept on healthy noninfective citrus seedlings for an incubation period of 2 weeks to allow for bacterial multiplication in the vector's foregut. The presence of *X. fastidiosa* was confirmed by (a) polymerase chain reaction (PCR), as described by Ciapina and Lemos [6], and (b) isolation of *X. fastidiosa* colonies in PW medium.

Preparation of sharpshooter specimens for scanning electron microscopy A total of 108 insects were used (50 of *A. citrina*, 12 of *B. xanthophis*, 28 of *D. costalimai*, and 18 of *O. facialis*). Ninety-one specimens of four sharpshooter species were allowed to feed on infected plants and 17 to

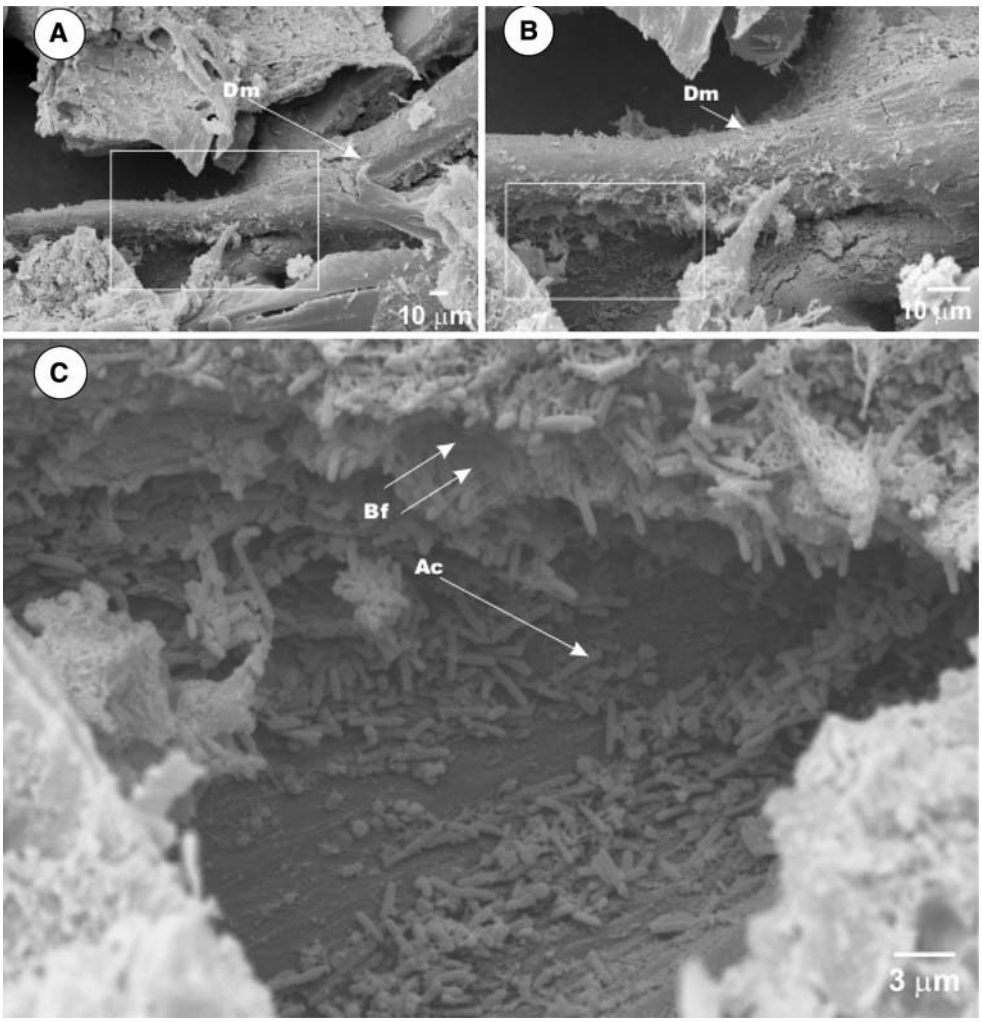

Fig. 3 Internal view of the base of the apodeme of clypeal dilator muscles of *Acrogonia citrine*: (**A**) diaphragm membrane with indication of a retention site; (**B**) bacteria adhered to the diaphragm membrane; (**C**) magnified view of bacterial aggregates

feed on healthy plants. Specimens were prepared for SEM. Sharpshooters heads were incubated at room temperature in a fixative solution of pH 7.2 (modified Karnovisky) for at least 24 h. Samples were washed three times in 0.005 M cacodylate buffer, transferred to a solution of 30% aqueous glycerol (cryoprotectant) for 30 min, and sectioned crosswise and longitudinally (dorsal and ventral sides) in liquid nitrogen**.** Postfixation was in aqueous 1% osmium tetroxide (OsO_4) for 1 h, washed three times in distilled water, dehydrated in an acetone series gradient (30, 50, 70, 90% [1×] and 100% [3×]) and critical point dried (CPD 050; Balzers).

These sectioned specimens were mounted on aluminum stubs, sputter covered with gold (Metalizer MED 010; Balzers) and observed in a LEO 435 VP SEM from NAP/MEPA-ESALQ/USP (Piracicaba, SP, Brazil). To better adjust the insect head position and to keep the parts assembled during the cutting, the sharpshooters were embedded in agar (0.5 g/10 mL) and kept in the refrigerator for 30 min before being sectioned in liquid nitrogen. Corel Draw image processing software was utilized to consolidate the picture mounts.

Results

Scanning electron microscopy of vector's foregut A total of 91 specimens of *A. citrina*, *B. xanthophis*, *D. costalimai*, and *O. facialis* that had been allowed to feed on citrus infected with the CVC strain of *X. fastidiosa* and *O. facialis* adults that were fed on plum trees infected with the PLS strain were dissected. Satisfactory exposure and visualization of the foregut were achieved for more than 50% of the individuals dissected, and attached cells, microcolonies, and biofilm of *X. fastidiosa* were found in 20 of 53 specimens that had their cibarium chambers exposed. No bacterial cells were found in those sharpshooters that had fed on healthy plants.

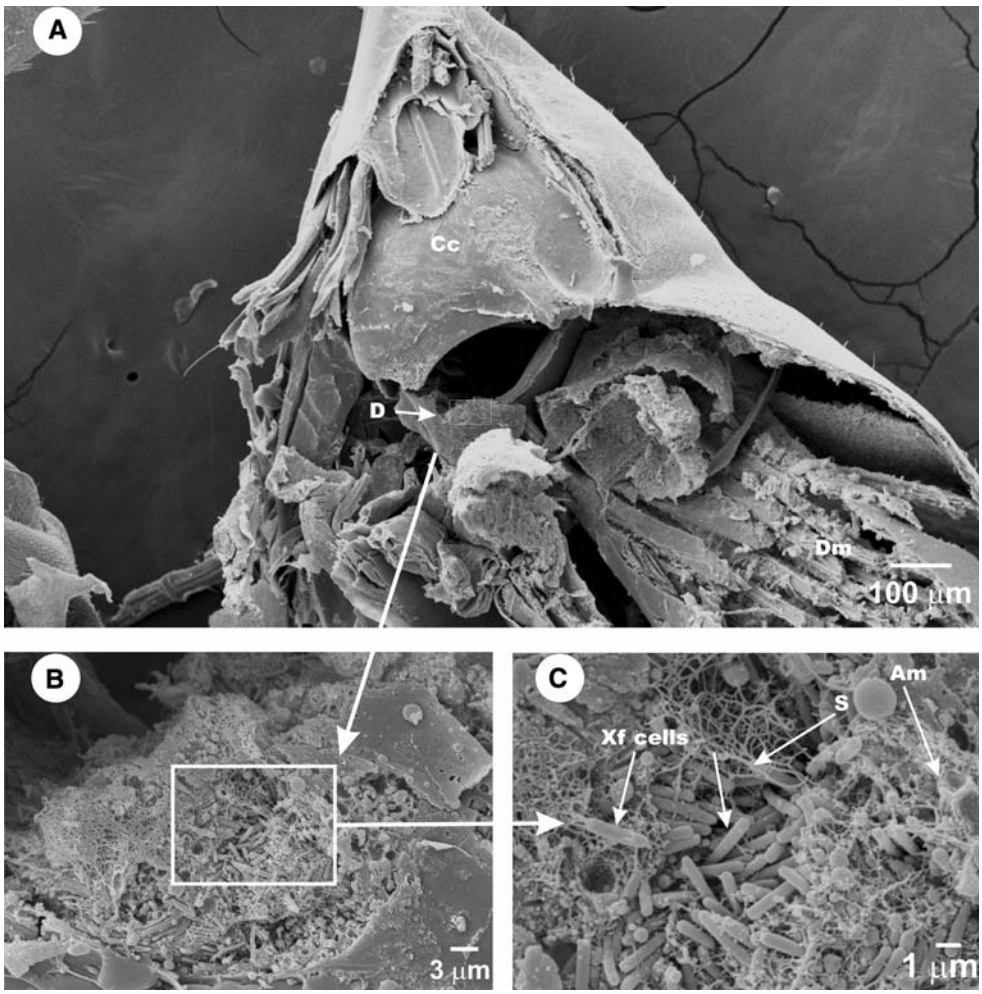

Fig. 4 Longitudinal and ventral cut of the head of *Acrogonia citrina*. (**A**) External view of the outer surface of the cibarium chamber (Cc) with detached portion of the diaphragm (D) membrane; (**B**) detail of inner surface of diaphragm showing evidence of *X. fastidiosa* biofilm formation, which is more complex than the biofilm formed in the cibarium chamber; (**C**) magnified view showing amorphous material (Am) and strands (s) surrounding *X. fastidiosa* cells

It was possible to recognize the main parts of the sharpshooter's foregut, including the rostrum (anterior portion that holds the stylets), precibarium (narrow channel linking the stylets to the cibarium), cibarium chamber (or suction pump), and opening to the esophagus. We also examined a narrow opening of the precibarium into the cibarium (the food meatus), the ventral and longitudinal groove leading to the esophagus, and the diaphragm membrane, which forms a dorsal groove (apodemal groove), and the clypeal dilator muscles were observed (Fig. 1). Several microcolonies were observed along the longitudinal groove and near the food meatus (Fig. 2). *X. fastidiosa* is known to form microcolonies in initial stages of colonization, consisting of a few layers of cells without deposition of fastidian gum as observed elsewhere [13]. In early stages of organization, cells may remain planktonic or aggregate. Adhesion, colonization, and cell division are important factors for acquisition and transmission of *X. fastidiosa*.

Retention sites of X. fastidiosa on sharpshooters PCRs were positive for presence of *X. fastidiosa* for the vector species *A. citrina*, *O. facialis*, and *D. costalimai*, indicating that the acquisition was successful. Scanning electron microscopy showed that numerous rod-shaped bacterial cells exhibiting a morphology similar to that of *X. fastidiosa* were laterally attached to various regions inside the cibarial pump chamber (longitudinal groove, lateral surface, cibarial diaphragm, and apodemal groove) of all three species as well as polarly attached to the precibarium channel of *O. facialis* (Figs. 2–5). *X. fastidiosa* cells were about 3 μm long and 0.3 μm wide.

No bacterial cells exhibiting a morphology similar to that of *X. fastidiosa* were detected in *B. xanthophis*. Interestingly, in this sharpshooter a different type of rod-shaped bacterium, measuring about 2 μm long × 1 μm wide, was found on the internal ventral surface of the cibarium chamber, near the food meatus and longitudinal groove (Fig. 6). PCR analyses were negative for *X. fastidiosa*. These bacteria appeared to be degrading the cuticular lining of the insect's cibarium (Fig. 6B).

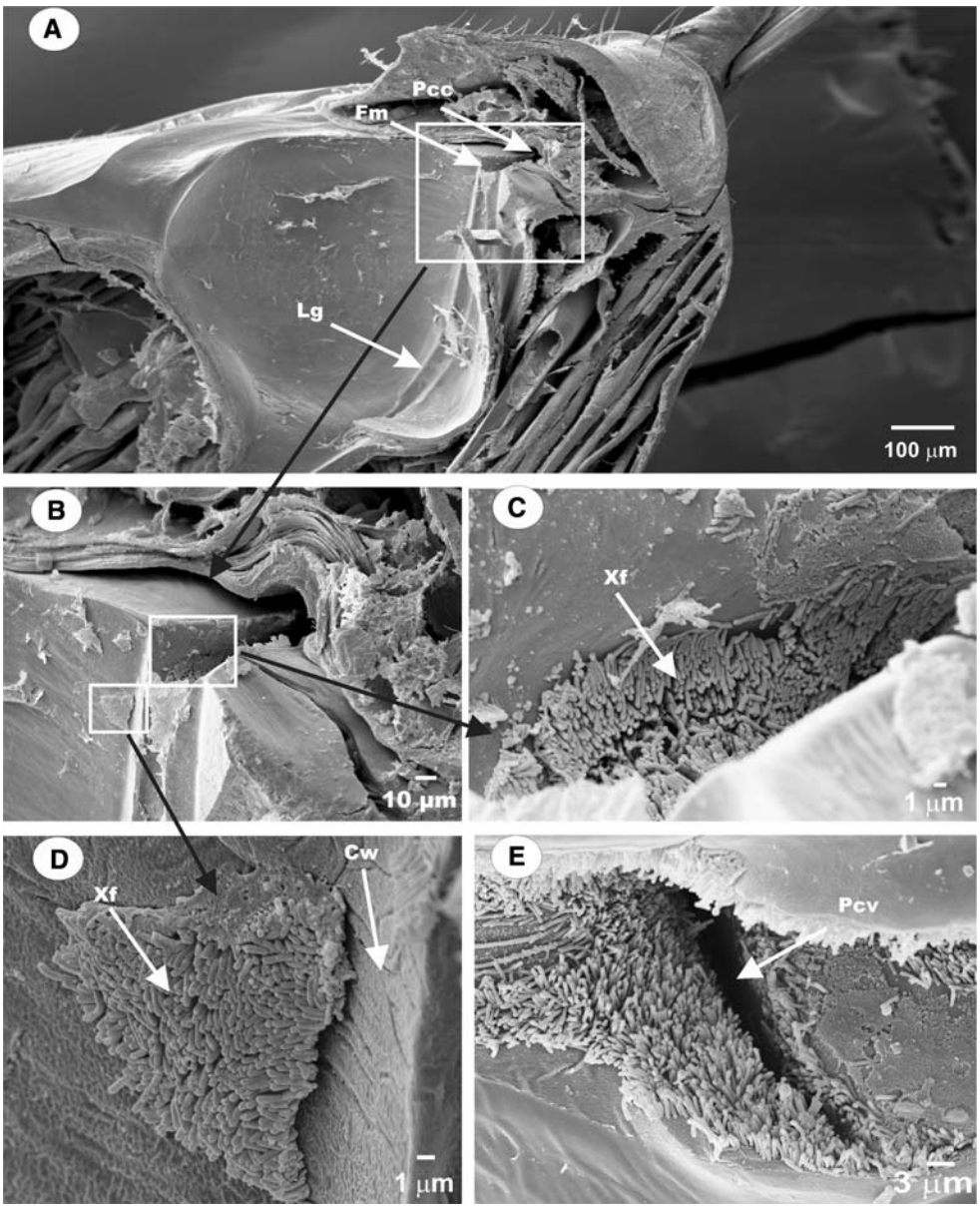

Fig. 5 (**A**) General view of the foregut of *Oncometopia facialis* with indication of retention sites of *X. fastidiosa* (longitudinal groove and precibarium channel); (**B**) detail of the precibarium channel with bacterial biofilms; (**C**) detail of bacterial cells attached to the cuticular lining of the precibarium; (**D**) bacterial aggregates on the precibarium wall and on the cibarium's ventral surface, near the food meatus; (**E**) detached piece of the foregut of the same specimen, showing a longer portion of the precibarium channel with attachment around the precibarium valve. The insect was previously fed for 48 h on plum trees infected with the plum leaf scald strain of *X. fastidiosa*

Discussion

Information about colonization patterns of *X. fastidiosa* inside vectors is a fundamental aspect to understanding adhesion and transmission mechanisms. Overall, three major bacterial retention sites were found in the sharpshooter vectors: (1) the longitudinal groove and the diaphragm/apodemal groove, (2) inside the cibarium chamber, and (3) the portion of the precibarium (including the food meatus) posterior to the precibarial valve (Figs. 2–5). These regions of the foregut have been reported as retention sites of the PD strain of *X. fastidiosa* in the blue-green sharpshooter, *Graphocephala atropunctata* [17], and of the phony peach disease strain in *Homalodisca vitripennis* and *Oncometopia nigricans* [3]. The location of different *X. fastidiosa* strains (CVC and PLS1) was similar to that reported for PD and phony peach strains.

Most bacterial cells found in the precibarium, precibarium valve, and wall near the precibarium (Fig. 5) showed a polar attachment, whereas those located on the longitudinal groove and diaphragm membrane were laterally attached (Figs. 2–4). A polar attachment of *X. fastidiosa* with biofilm of bacteria located in the cibarium and precibarium has been reported [13, 14]. These authors suggested that the polar attachment of the bacteria was via fimbria-like structures and the binding action of the extracellular material produced by bacteria. In other retention sites, bacteria were often attached laterally. This type of cell arrangement was considered by Purcell et al. [19] as a probable site of bacterial multiplication. The cells

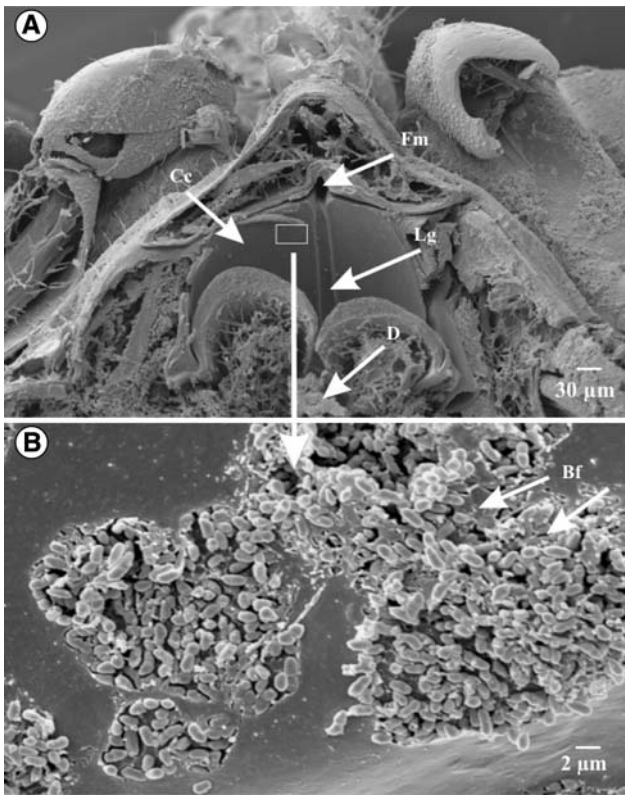

Fig. 6 Scanning electron micrographs. (**A**) Overview of a longitudinal and ventral cut of the head of *Bucephalogonia xanthophis* showed that *X. fastidiosa* cells could not be found. Bacterial cells with a distinct morphology were observed. However, other bacterial cells with a different morphology were found in great quantity. (**B**) Bacterial cell details. The insect was previously fed for 48 h on citrus trees infected with the CVC strain of *X. fastidiosa*

were surrounded by an amorphous material (Figs. 3 and 4), which is presumably fastidian gum [17]. That material is similar to that observed in xylem vessels of PD-infected grapevines [24]. Extracellular material may offer physical protection to the bacterial colony and this matrix also aids in the extraction of nutrients from the fluid stream since this material is negatively charged and thus can bind certain nutrients [7].

The one anomaly observed in the present study was the fact that *X. fastidiosa* was not detected in the foregut of *B. xanthopsis*, but instead it harbored another rod-shaped bacterium that seemed to degrade the cibarium wall cuticle. PCR was negative for the presence of *X. fastidiosa* and the bacteria were shorter and wider than *X. fastidiosa. B. xanthophis* has been shown to be a vector of CVC [9] and coffee leaf scorch [13] in Brazil. Additional work is required to establish the identity of the unknown bacterium species.

Commensal bacteria are long known to inhabit the cuticle surface and the alimentary tract of insects. These bacteria seem to be perfectly adapted to these conditions, as communities of bacteria are established. In particular, several Gram-negative bacteria are known to live within the body of other organisms without causing any harm. However some may turn pathogenic to the host insects if they access the hemocoel [2]. We hypothesize that bacteria established on the *B. xanthophis* cibarium surface compete with *X. fastidiosa* for the same retention site (Fig. 6). The foregut cuticle is a protein-chitinous material and cuticle degrading proteases are known to facilitate colonization and establishment of entomopathogens [23], which could help to explain the cuticle degradation (Fig. 6). In addition, *X. fastidiosa* was shown to be very vulnerable to environmental changes and specifically to the nutrient content of the xylem fluid [1]. *X. fastidiosa* is also affected by the balance of ions and the concentration of reducing agents and oxidizing agents [11].

Finally, this work extensively explored anatomic features of vectors' foreguts. Despite the obvious similarities, the cibarium wall surface may be exposed to bacterial cells going through the insect's gut with some key differences in surface chemical composition. Other bacteria occupied the putative retention site for *X. fastidiosa*, indicating that there is competition with several other organisms. This work is consistent with such competition; either *B. xanthophis* was already colonized or the conditions in the cibarium were not conducive for *X. fastidiosa* establishment.

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