Colonization and Intrusive Invasion of Potato Psyllid by ‘*Candidatus Liberibacter solanacearum*’

Joseph M. Cicero, Tonja W. Fisher, Jawwad A. Qureshi, Philip A. Stansly, and Judith K. Brown

First, second, and fifth authors: School of Plant Sciences, 303 Forbes Bld., University of Arizona, Tucson 85721; and third and fourth authors: Southwest Florida Research & Education Center, University of Florida IFAS, 2685 State Road 29 North, Immokalee 34142.
Current address of J. M. Cicero: University of Florida, Entomology and Nematology, 1881 Natural Area Dr., Steinmetz Hall, Gainesville 32611.
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ABSTRACT

Previous studies have shown that the fastidious bacterial plant pathogen ‘*Candidatus Liberibacter solanacearum*’ (CLso) is transmitted circulative and propagatively by the potato psyllid (PoP) *Bactericera cockerelli*. In this study, the temporal and spatial interrelationships between CLso PoP were investigated by scanning electron microscopy of the digestive system of PoP immature and adult instars and salivary glands of adults post CLso ingestion. CLso biofilms were not detectable on the outer midgut surface of the first and second instars; however, for third to fifth instars and teneral and mature adults, biofilms were observed in increasing numbers in each

successive developmental stage. In adult PoP midguts, CLso cells were observed between the basal lamina and basal epithelial cell membranes; in basal laminar perforations, on the outer basal laminar surface, and in the ventricular lumen, epithelial cytosol, and filter chamber periventricular space. CLso were also abundantly visible in the salivary gland pericellular spaces and in the epidermal cell cytosol of the head. Collectively, these results point to an intrusive, systemic invasion of PoP by CLso that employs an endo/exocytosis-like mechanism, in the context of a propagative, circulative mode of transmission.

Insects placed in the order Hemiptera are the predominant type of arthropod vector for plant-infecting viruses and fastidious bacteria, the latter including members of the bacterial genus ‘*Candidatus Liberibacter*’. Among the most common are aphids, leafhoppers, mealybugs, psyllids, planthoppers, and whiteflies (Nault 1997). Most hemipterans feed in the plant phloem, whereas others specialize in the xylem, the respective locations in which the plant pathogens they transmit also reside. Some plant pathogens carry out their life cycle in the insect vector or plant host but not both, whereas others multiply in both the insect vector and plant host, thus having the ability to infect members representing two evolutionarily divergent kingdoms, Plantae and Animalia (Ammar et al. 2009; Hogenhout et al. 2003, 2008; Weintraub and Beanland 2006; Whitfield et al. 2005).

The two main modes by which insects transmit viral and bacterial pathogens to plants are noncirculative and circulative transmission (Nault 1997). In the noncirculative mode, the pathogen most often interacts with the cuticles lining the preoral and foregut lumina and is delivered to the plant by way of regurgitation during feeding (Harris 1977), simultaneously combined with salivation (Backus et al. 2015). Noncirculative viral and fastidious bacterial plant pathogens are characteristically associated with the inner cuticular walls of the oral region lumina, specifically that of the stylet bundle canals or the preoral section, and do not pass into the midgut or beyond (Killiny et al. 2010; Kwon et al. 1999; Ng and Falk 2006; Pirone and Perry 2002; Uzest et al. 2007). In contrast, plant viruses that utilize a circulative mode of transmission, such as some of those transmitted by certain aphids and whiteflies (Brown and Czosnek 2002; Gildow 1993; Cicero and Brown 2011) and leafhoppers and planthoppers (Ammar 1986; Hogenhout et al. 2008; Nault 1997), among others, are translocated across the gut at various specific locations, including the filter chamber, ventriculus, or hindgut wall, to reach the hemolymph, from where they transit to and enter the

salivary glands, prior to their inoculation to the host plant during feeding.

In certain virus- and fastidious bacteria-insect vector systems, the pathogen circulates and multiplies in the insect vector, while also multiplying in and causing disease of the host plant (Ammar et al. 2009; Nadarasah and Stavrinides 2011; Whitfield et al. 2005). In the latter mode, most often, the insect vector is thought to serve as an alternate host of the pathogen (Nadarasah and Stavrinides 2011). The circulative-propagative mode of transmission has unique layers of complexity owing to diverse anatomical, biochemical, and molecular host-pathogen interactions, including specific pathogen effectors that mediate host-pathogen or -parasite symbioses, thus creating a number of unique challenges to elucidating these different transmission pathways. Further, additional routes can involve transovarial (from mother to offspring) or transstadial vertical transmission but also, at times, horizontal (sexual) transmission, thereby expanding the types of effectors involved, collectively complicating the search for determinants of transmission and the basis for transmission specificity (Alves et al. 2008; Hogenhout et al. 2008; Nault 1997; Weintraub and Beanland 2006; Whitfield et al. 2005).

Liberibacter spp. are gram-negative, phloem-limited bacteria in the Phylum α -Proteobacteria and, except for *Liberibacter crescens*, are unculturable to date (Fagen et al. 2014). The combined results from psyllid- and graft-mediated transmission studies (Crosslin et al. 2010; Hansen et al. 2008; Secor et al. 2009), polymerase chain reaction (PCR) amplification and detection in plants (Hansen et al. 2008; Li et al. 2009), transmission and scanning electron microscopy (TEM and SEM, respectively) in plants (Secor et al. 2009), and identification of ‘*Candidatus Liberibacter solanacearum*’ (CLso) by colloidal gold in situ hybridization (Cicero et al. 2016) have provided several lines of evidence that CLso is consistently associated with zebra chip disease of potato (Munyanza et al. 2007) and vein-greening disease of tomato (Brown et al. 2010) and, therefore, its role as the causal agent of these diseases has been generally accepted.

The CLso bacterium and its potato psyllid (PoP) vector, *Bactericera cockerelli* (Sulc), are endemic to the U.S. western

Corresponding author: J. K. Brown; E-mail address: jbrown@ag.arizona.edu

states, Mexico, Canada, and Central America (Crosslin et al. 2010), whereas '*Ca. L. asiaticus*', the causal agent of citrus greening disease, also known as huanglongbing (HLB) or yellow dragon disease, and its Asian citrus psyllid vector *Diaphorina citri* Kuwayama (ACP), are endemic to Asia, making it an exotic pathogen to the Americas, where recent HLB outbreaks have occurred (Gottwald et al. 2007).

Despite the different familial designations of PoP and ACP (Triozidae and Liviidae), the external and internal structural morphology of their digestive systems was found to be indistinguishable using available imaging technology (Cicero et al. 2009). Specifically, both species have a looped, appendaged ventriculus and similarly structured filter chamber, which allows excess water to be shunted into the hindgut so that nutrients are not diluted by direct passage into the midgut. Use of the term "ventricular appendage" herein, instead of Malpighian tubule (Ammar et al. 2011b), requires clarification. In psyllids, the appendages are short, wide in diameter, and positioned along the length of the ventricular loop, in comparison with those that are characteristically long, thin, and positioned at the apex of the hindgut as in other taxa (Cicero et al. 2009). This unique positioning in psyllids along the midgut, which is endodermal in origin, brings their function into question because Malpighian tubules are ectodermal in origin (Beyenbach et al. 2010). In psyllids, they may be ventricular outpockets, as are the gastric caecae of the general insect anatomy; therefore, herein the term ventricular appendage is adopted until more information becomes available. Also, throughout, the "outer ventriculus" refers to the loop and "inner ventriculus" refers to the section of midgut within the filter chamber and enclosed by a sheath, as described by Cicero et al. (2009). The term midgut "component" is used throughout to refer to either the filter chamber or the midgut loop when it was not possible to differentiate them from each other.

Using fluorescence in situ hybridization (FISH), '*Ca. L. asiaticus*' and CLso have been localized to the vector inner and outer ventriculus, the primary salivary glands, and several other structures (Ammar et al. 2011a; Cooper et al. 2014). Cicero et al. (2016) localized a characteristic, rod-shaped form of CLso cells in biofilms in the salivary gland pericellular space, the tentorium, and on the outer midgut surface of PoP adults. In the latter study, CLso cells also were observed to have a motile form, with apparent flagella and pili attached.

The objective of this study was to investigate the interstadial relationships between CLso and PoP digestive system anatomy at the histological and cytological levels in relation to the CLso infection cycle. To accomplish this, eggs, whole bodies, and midguts of first to fifth instars and adults plus the adult head, including the esophagus and salivary glands, were studied for the PoP born and reared on CLso-infected tomato plants compared with those reared on uninfected tomato plants.

MATERIALS AND METHODS

Psyllid colonies. One laboratory colony was established using infected adult PoP collected from naturally infested tomato plants grown in a commercial greenhouse in Snowflake, AZ during 2004. Two other colonies were established from CLso-uninfected adult PoP collected from naturally infested tomato plants grown in a commercial greenhouse in Willcox, AZ or provided from PoP colonies established in the United States Department of Agriculture–Agricultural Research Service Laboratory in Wapato, WA. All PoP colonies were maintained and haplotyped (Lin et al. 2012; Swisher et al. 2012; Wen et al. 2013) as previously described by Cicero et al. (2016). Herein, 'CLso⁺' refers to psyllids that were collected from the CLso-infected PoP colony and 'CLso⁻' to psyllids collected from the uninfected or Liberibacter-free colony.

CLso detection in PoP life stages by polymerase chain reaction. Eggs, nymphal first to fifth instars, and teneral adults (10 each) were collected from CLso⁺ colonies and individually

assayed using CLso 16S ribosomal RNA gene primers and 12 ng of genomic DNA per reaction, with the cycling parameters as previously described (Liefting et al. 2009).

Specimen preparation for SEM. Eggs from the CLso⁻ colony were collected from their CLso-free (uninfected) tomato plants by trimming off the leaf margin upon which they were oviposited, and affixing them in groups to the upper surfaces of leaves of PoP-free and CLso-free tomato plants (30 to 40 cm tall). After hatch, the plants were periodically placed horizontally under a dissecting microscope to monitor and collect age-synchronized cohorts based on changes in size, contour, and length of the apicolateral pronotal angle that are characteristic of each consecutive instar.

CLso⁻ nymphal instars were collected directly from these cohorts to use as negative controls. CLso⁺ nymphal instars were collected after the following isolation process. Plants from the CLso⁺ PoP colony, heavily populated with all instars, were isolated in separate cages so that all adults could be removed and returned to their respective colony. Instars from these plants were collected using the same method described above. The pharate nymphal stages were avoided if separation of cuticle (apolysis) could be seen through the transparent integument of the wingpads. Specimens were glued, dorsum down, using Loctite Quick Set epoxy (Henkel Corporation, Westlake, OH), to a thumbtack embedded in a paraffin-filled microwatch glass and submerged in distilled water (dH₂O). Air bubbles were eliminated on contact with solid ducosate sodium (Aerosol OT, a wetting agent [sodium 1,2-bis (2-ethylhexoxycarbonyl)]; Sigma-Aldrich, St. Louis) on the tip of an insect pin. After rinsing with dH₂O, the watch glass was filled with 0.01 M Na⁺ K⁺ phosphate-buffered saline (PBS), pH 7.75 (Sambrook et al. 1989). For first-instar nymphs, one lateral abdominal margin of each was cut away. For older nymphs, the cuts were made transversely, across the meso-metathorax area. For both, the midguts were exposed by applying gentle pressure to the abdomen with tweezers. For teneral adults and adults, midguts were exposed by tearing the ventral abdominal cuticle from base toward apex with tweezers.

These nymphs, teneral adults, and adults were transferred to aldehydes for overnight fixation (4% formaldehyde, 0.5% glutaraldehyde, and 0.1% Tween-20 in PBS). Specimens were rinsed in PBS, dehydrated in an extended ethanol series (12.5% unit increments to 87.5%) to minimize turbulence, placed in 95% overnight and then twice in 100% for 3 h, and critical point dried (SPI-DRY; Structure Probe, Inc. West Chester, PA).

A second group of adults only was transferred to Carnoy's fixative (a coagulant fixative, 6:3:1 [vol/vol] chloroform-ethanol-acetic acid) for at least 3 h at room temperature after exposing the abdomen, passed through an ethanol series (33, 66, 95, and 100%), then critical point dried and mounted. A third group of adults only (CLso⁺ PoP, *n* = 5; and CLso⁻ PoP, *n* = 10), was dissected in PBS to expose their salivary glands, fixed overnight in aldehydes, dehydrated as above, and critical point dried. All groups were viewed with a Hitachi S3400 Scanning Electron Microscope.

Tabulation of CLso detected by SEM. Tabulation of the presence or absence of CLso in PoP instars was carried out using SEM images of the aldehyde-fixed whole mounts. A magnification of approximately ×3,000 was determined to be the minimum required to detect CLso cells while scanning midguts at a reasonably efficient speed. Magnification was raised to approximately ×7,000 for verification and further, as needed, for high-resolution micrographs. The CLso bacterial colonies were studied at the higher magnifications for ultrastructural characterization and to visualize other bacteria-like forms, if present. Because midguts were variably obscured by the surrounding milieu, CLso⁺ and CLso⁻ midguts were tabulated as CLso absent only when most of the outer ventriculus was visible and CLso cells were not observed.

Identification of CLso cells by SEM (and TEM) was based on the findings from TEM colloidal gold in situ hybridization studies that characterized CLso as the only rod-shaped bacterium of approximately 2.5 μm in diameter and of variable length, having a rough, granular

cytosol and, at times, forming biofilms on the external midgut, esophageal, and salivary gland surfaces (Cicero et al. 2016).

CLso⁺ specimens were recorded as CLso present if the midgut possessed bacterial cells with the above characteristics, applicable to SEM, and met any of the following four criteria: (i) present in uncountable numbers anywhere on the midgut basal lamina, whether as a localized cluster or as profuse clusters; (ii) present in countable, relatively high numbers that were partially lysed through the basal lamina; (iii) visible in relatively high numbers through damaged tissues (e.g., ruptures, lesions, exfoliations, and so on, collectively referred to as “compromised”), in the basal lamina; or (iv) visible in at least small, countable numbers through several different compromised areas of the basal lamina.

The midguts from CLso⁺ PoP and CLso⁻ PoP were counted as CLso absent if (i) no morphotypic bacteria were observed on the exposed (and viewable) portion of the midgut, (ii) only a few solitary bacteria were present and surface-bound but not associated with a lysis of the basal lamina, (iii) only a few solitary bacteria were present and partially protruded through holes in the basal lamina, or (iv) the bacteria observed were on the adjacent, nonalimentary tissues.

Specimen preparation for TEM. The CLso⁺ and CLso⁻ adult psyllids ($n = 12$ and 12 , respectively) were glued to thumbtacks and processed as above for SEM, except that body sections were kept intact. The tip of the abdomen was cut off and hind coxae, profemora, mesofemora, and antennae were removed. After aldehyde fixation and dehydration, specimens were infiltrated with LR White (Electron Microscopy Supplies, Hatfield, PA) (25, 75, and 100%) and polymerized at 52°C. Ultrathin sections from the abdomen and head were cut with an LKB Ultratome 5 and mounted on grids coated with pioloform support film (Electron Microscopy Supplies).

RESULTS

CLso detection in PoP life stages by polymerase chain reaction. The frequency of CLso detection by PCR in the first instars was 33%, compared with 50 to 53% detection in the eggs and second and third instars (Table 1). PCR detection of CLso increased with each successive instar stage by developmental age, ranging from 60 to 100% for the fourth to fifth instars and teneral adults, respectively.

SEM. Results using aqueous (aldehyde) fixation of CLso⁺ PoP midguts indicated that the number of individuals tabulated as CLso present increased while the number of individuals tabulated as CLso not seen decreased in samples of successively older CLso⁺ nymphal instars (Table 2). No CLso-like forms or biofilms were associated with the first and second instar midguts. The earliest appearance of CLso biofilms occurred in 39% of third instars and increased, consecutively, to a peak in the fifth instar at 91%; thereafter, 12 to 15% fewer teneral and adults met the tabulation criteria.

Representative CLso⁺ instars (Table 2) that were examined using the criteria established above for tabulating CLso presence are shown in Figures 1, 2, 3, and 4. Based on the criteria used to document CLso presence, bacterial cells of the CLso morphotype

(rod-shaped) were the predominant type of bacteria observed in CLso⁺ psyllid instars, and rarely or not at all were bacteria-like forms of other morphologies observed in psyllids. On the rare occasions when putative bacteria-like forms were observed (Figs. 1Cf and 3Ce, Gh), they were readily distinguishable by size, shape, and numbers from the CLso morphotype.

With aldehyde fixation, only an occasional clump of external biofilm was retained and, therefore, it was not possible to determine whether more extensive biofilms were present at the time of fixation. However, the condition of the underlying basal lamina could be assessed (Fig. 1 and elsewhere in the Results). In the case of Carnoy’s fixed tissue (Fig. 2), the integrity of the epithelium could not be viewed except where the overlying CLso biofilms did not occur or were sloughed off (Fig. 2D). In one Carnoy’s fixed specimen, two crater-like indentations in CLso-associated biofilms were preserved (Fig. 2A and B). It was not possible to determine whether these anomalies represented lesions in the underlying tissue. A sheet of minute, filamentous structures resembling flagella or pili that appeared to cover, in part, the biofilm layer is shown in Figure 2Bd (inset). In most specimens, thick CLso biofilms were observed, seemingly intact, along with, perhaps, adherent hemocytes and other unidentifiable material. In some specimens, the CLso biofilms almost completely obscured the epithelium (Fig. 2C).

TABLE 2. Tabulation of ‘*Candidatus Liberibacter solanacearum*’ (CLso) presence in the midgut of five nymphal, teneral adult, and adult life stages of the potato psyllid (PoP) *Bactericera cockerelli* following aldehyde fixation and examination by scanning electron microscopy

		CLso			
		Observed ^c		Not observed ^d	
PoP colony ^a , life stage	N ^b	Infected (n)	N (%)	Uninfected (n)	N (%)
Uninfected					
First instar	24	0	0	24	100
Second instar	19	0	0	19	100
Third instar	72	0	0	72	100
Fourth instar	28	0	0	28	100
Fifth instar	13	0	0	13	100
Teneral adult	24	0	0	24	100
Adult	15	0	0	15	100
Total	195	0	...	195	...
Infected					
First instar	9	0	0	9	100
Second instar	17	0	0	17	100
Third instar	36	14	39	22	61
Fourth instar	29	15	52	14	48
Fifth instar	11	10	91	1	9
Teneral adult	17	13	76	4	24
Adult	19	15	79	4	21
Total	138	67	...	71	...
Overall total	333	67	...	266	...

^a PoP colonies uninfected or infected by CLso.

^b N = number of individuals at each stage.

^c Of PoP examined, Infected (n) = number infected, and N (%) = n as a percentage of N.

^d Of PoP examined, Uninfected (n) = number uninfected, and N (%) = n as a percentage of N.

TABLE 1. Frequency of detection of ‘*Candidatus Liberibacter solanacearum*’ (CLso) by polymerase chain reaction in the egg and the life stages of the potato psyllid *Bactericera cockerelli* from the first instar to the teneral adult

Life stage	Replicate 1 (%)	Replicate 2 (%)	Replicate 3 (%)	Replicate 4 (%)	Infection (%)
Egg	4 of 10 (40)	5 of 10 (50)	6 of 10 (60)	NA ^a	50
First instar	2 of 10 (20)	2 of 10 (20)	5 of 10 (50)	4 of 10 (40)	33
Second instar	5 of 10 (50)	5 of 10 (50)	7 of 10 (70)	4 of 10 (40)	53
Third instar	3 of 10 (30)	3 of 10 (30)	7 of 10 (70)	7 of 10 (70)	50
Fourth instar	6 of 10 (60)	6 of 10 (60)	6 of 10 (60)	6 of 10 (60)	60
Fifth instar	6 of 10 (60)	6 of 10 (60)	9 of 10 (90)	7 of 10 (70)	70
Teneral adult	10 of 10 (100)	10 of 10 (100)	10 of 10 (100)	NA	100

^a NA = not available.

With aldehyde processing, CLso cells were commonly seen through compromised basal laminae and within mechanical break-ages. There were no bulges or discontinuities in otherwise smooth, uncompromised basal laminar surfaces that might indicate a sublaminal presence by CLso. In Figure 1A and B, for example, extremely wide areas of intact basal lamina were observed even though the underlying infection was extreme. In many examples, CLso cells were observed through compromised aspects of the outer ventriculus (Fig. 1C) and, in several of these instances, the surface of the epithelial basal membrane underneath the CLso cells was visible (Fig. 3Df and Ff). CLso cells were also observed through compromised filter chamber sheaths (Fig. 4A), and were distributed in the periventricular space around and ventral to the V₁ bulb (Fig. 1A). Both CLso^o and CLso⁺ specimens showed varying degrees of exfoliation (Figs. 3B, D, and F and 4A). Also, CLso cells were often positioned vertically in openings slightly wider than their diameter (Fig. 3Eg).

Representative micrographs (Figs. 3A and 4B) show examples of CLso^o instars (Table 2) that were examined using the established criteria for tabulating CLso presence, illustrating that in no instance were the criteria fulfilled such that CLso would be tabulated as being present. Only one fourth-instar specimen had indications of a somewhat compromised basal lamina that might have been interpreted as a CLso presence (Fig. 3Gh); however, the criteria for tabulating them as CLso were not fulfilled.

CLso cells were not detectable by SEM in the salivary glands of CLso^o or CLso⁺ PoP, whether fixed with aldehydes or with Carnoy's, nor were compromised tissues observed, such as those seen in the midgut basal lamina, regardless of the preparation method. Aldehyde fixation preserved the shape of the salivary glands, as indicated by their torpid and inflated appearance (Fig. 4C). Each had three lobes but neither had a digitate appendage or "accessory gland". Two other paired organs of unknown identity occurred between the glands, one granular and the other smooth-walled (Fig. 4Ce and f).

TEM. In CLso⁺ specimens, large numbers of CLso cells occurred in clusters that adhered to the outer basal laminar surfaces of the midgut components (Fig. 5). Relatively fewer CLso cells were observed inside the basal (Figs. 6Ae and 7Aa and Ba), mesal (Fig. 6Af), and apical (Fig. 6Bg) cytoplasm. Only a few were located between microvilli (Fig. 6Bh), and inside the lumen (Fig. 6Co). Also, CLso cells were associated with the many different tissue types in the head (data not shown) and inside epidermal cells adjacent to the head exoskeleton, at considerable distances from the alimentary canal (Fig. 8B). The midgut and esophagus from the CLso^o colony (Fig. 8C and D), and heads from the same colony (data not shown) were negative for CLso presence.

CLso cells in all locations (external midgut surface, epithelial cell cytosol, head tissue, and salivary glands) were surrounded an electron transparent material (Figs. 5, 6A and B, and 8B). Biofilms on the external midgut surface were composed of groups of CLso cells compartmentalized together with this surrounding material into vesicle-like structures with an outer envelope (Fig. 5Ah, Bh,

Ch, upper inset, o). These structures were stacked into wide (approximately 5 to 10 μ m) layers such that, in some cases, they spanned the full distance between closely spaced midgut components (Fig. 5Ah and Bh). Similar but somewhat disrupted structures were discernable between other midgut components further away from each other (Fig. 5Cm). In these disrupted structures, individual CLso cells appeared to have been freed (Fig. 5Cn) into the hemolymph (Fig. 5Cf and lower inset).

Some CLso cells were confined between tightly appressed midgut component surfaces (Figs. 5Ac and Bi and 6Ab). Those in Figure 5Ac were of particular interest because they were interpolated between the V₁ bulb of the filter chamber and another component (Fig. 5Ae) located in the native position of the inner hindgut, the latter of which is known in uninfected psyllids to be spindle shaped and firmly adnate to V₁ to facilitate the filtration process. Other CLso cells occurred inside vesicle-like structures within the cytosol near basal epithelial cell membranes, and some were open to the hemolymph (Fig. 5Ck, lower inset).

Also, CLso cells were present in small numbers between the laminae of the basal lamina (Figs. 6Ac and 7Ab and Bb). A breach in the basal lamina was evident in Figure 7A (see arrow).

The cross-sections of epithelial cells in Figure 6Cj and Dj were nearly filled with CLso cells but all four sides were still intact. In Figure 6C, the brush-border (l) ended abruptly, and the lumen contained mitochondria (n), CLso cells (o), and a crenate-shaped residue (m). A singular, putative, endocytotic event was observed on the luminal side (Fig. 6Dp, inset).

The esophagus (Fig. 8D) and salivary glands of CLso^o PoP showed no evidence of CLso-like bacterial cells of any form. In CLso⁺ PoP, large numbers of CLso cells were observed around the periphery of and within the cytosol of the esophagus (Fig. 8A), and in the pericellular space of the salivary glands (Fig. 9Ab, Cb, and Db). CLso cells were not observed in the lumen of the salivary ductule (Fig. 9Dg). Bacteria and bacterial forms were located in the salivary gland secretory cell cytosol that did not fulfill the criteria for identification as CLso (Fig. 9Ac). In some specimens, CLso cells surrounding the esophagus were continuous with those around the salivary gland periphery, intruding into the salivary gland pericellular space and reaching the core, where the ductule was positioned. The nominal CLso cell diameter was approximately 0.5 $\times$ to 1.0 $\times$ of either the duct or ductule lumina (Fig. 9Bd, e and Db, g, respectively). Potentially "loadable" sections of the ductule system (lacking a cuticular lining) were not observed in the TEM cross-sectioned samples; therefore, the potential for CLso cells to access the ductule lumen through them could not be ascertained.

DISCUSSION

In this study, CLso was detected by PCR amplification in the eggs, first to fifth instars, and teneral and mature adults when the PoP were born and reared on CLso-infected tomato plants, indicating

Fig. 1. CLso⁺ potato psyllid adult midguts after aldehyde fixation. **A**, Interior of the filter chamber. Size bar = 20 μ m. **B**, Inset from A. Interior of the third ventriculus. Size bar = 5 μ m. **C**, Exterior of third ventriculus revealing sublaminal presence of many CLso cells. Size bar = 5 μ m. **a**, Filter chamber (intentionally) rolled over the scanning electron microscopy stub adhesive to remove the sheath and reveal the interior harboring CLso cells. **b**, Bulb of inner ventriculus. Large numbers of CLso cells occur in the periventricular space. **c**, Large numbers of CLso cells between d and e. **d**, Luminal material in which CLso was not observed. **e**, Basal lamina. **f**, Example of a putative bacteria-like form not characteristic of the CLso morphotype.

that CLso was present in a percentage of the eggs and in all developmental stages of PoP. In contrast, for PoP instars experiencing the same rearing regimen, SEM analysis revealed a sequentially increasing presence of CLso biofilms on the midgut of only the third to fifth instars and in the teneral and mature adults. Further, TEM observations revealed CLso cells in the head, esophagus, and salivary glands of PoP adults. Regardless of the developmental stage or methods, CLso was undetected in psyllids reared on CLso-free tomato plants. These results provide the first documentation by molecular detection combined with direct observation of CLso cells or biofilms in developmental stages of PoP that fully supports the hypothesis for a circulative, propagative mode of CLso transmission.

For SEM, the use of coagulant (Carnoy's) fixative instead of aqueous (aldehyde) fixative resulted in different appearances of the

midgut surfaces, the most notable of which was the retention of external midgut biofilms. In contrast, the absence of external midgut biofilms in aldehyde-fixed midguts indicated that any external biofilms that may have been present at fixation were largely rinsed off during the processing steps which, nonetheless, allowed the outer midgut surface to be studied. Biofilm removal during processing revealed distinctly compromised basal laminae. CLso cells were retained despite processing when they were wedged underneath the shredded lamina or in vertical positions, filling punctures of the basal laminae. However, when the CLso cells were highly abundant sublaminae, it was not possible to determine whether the compromised basal laminae were due to artifacts caused during processing or to interstitial expansion, possibly associated with invasion of CLso. Based on the likelihood that the sublaminae

Fig. 2. CLso[®] potato psyllid adult midguts after fixation with Carnoy's reagent. **A**, Entire midgut. Upper inset, location of crater-shaped indentation in **B**. Lower inset shows the location of a second, similar crater-shape. Size bar = 200 μ m. **B**, Upper inset from **A**. Third ventriculus (midgut loop) with anomalous, crater-shaped indentation in the CLso biofilm. Inset = zoom-out of material also observed in **d**. Size bar = 5 μ m. **C**, Entire midgut. Epithelium covered with CLso biofilm. Size bar = 100 μ m. **D**, Third ventriculus. Apparent sluffing of the CLso biofilm allowing a view of sublaminae CLso. Size bar = 1.5 μ m. **a**, Third ventriculus. **b**, Thick CLso biofilm. **c**, Filter chamber. **d**, Filamentous material that may represent CLso surface appendages detached from the bacteria. **e**, Esophagus. **f**, Thick, rough clumps of CLso biofilms. **g**, Thick, smooth-surfaced CLso biofilms.

presence of CLso could weaken the integrity of the basal lamina, as has been reported for *Spiroplasma citri* in its leafhopper vector (Kwon et al. 1999), the latter hypothesis is favored.

By analogy to most well-studied hemipteran-transmitted phyto-bacterial pathogens utilizing a circulative, propagative mode of transmission, CLso cells must pass through the midgut epithelium

into the hemocoel (Ham et al. 2011; Hendricks and Bomberger 2014) and disperse in the hemolymph so that some reach and enter the salivary glands prior to inoculation into the plant host (Nault 1997). In the PoP outer ventriculus, the apical epithelial membrane, cytosol, and the basal membrane/basal lamina complex constitute three consecutive barriers that must be overcome. In this study,

Fig. 3. Aldehyde-fixed potato psyllid. Midguts of various instars. **A**, CLso⁺ first instar containing no observable CLso. Size bar = 20 µm. **B**, CLso⁺ second instar. Midgut with filter chamber containing no observable CLso. Size bar = 25 µm. **C**, CLso⁺ second instar. Size bar = 2.5 µm. **D**, CLso⁺ third instar. CLso seen through the compromised ventricular basal lamina. Size bar = 5 µm. **E and F**, CLso⁺ fourth instars. **E**, Size bar = 5 µm. **F**, CLso seen through the compromised ventricular basal lamina. Size bar = 5 µm. **G**, CLso⁺ fourth instar containing possible bacteria-like structures that were not tabulated as CLso. Size bar = 2.5 µm. **a**, Possible neuronal track on the ventriculus. **b**, Ventriculus. **c**, Exfoliation of the filter chamber sheath. **d**, Exfoliation revealing the periventricular space within (inset). **e**, Non-CLso bacteria-like forms. **f**, Epithelial cell basal membrane. **g**, One of many CLso cells penetrating vertically through the holes in the basal lamina of approximately the same diameter. **h**, Bacteria-like structures appear to extend vertically through holes.

CLso cells were observed in multiple locations, suggesting that they interact with all three barriers, but their directionality of movement from the gut to the hemolymph side of the epithelium or vice versa could not be interpreted solely by SEM or TEM imaging. Given the invasive nature of CLso infection of the psyllid host, the directionality of transit across apical and basal midgut epithelial membranes could theoretically proceed in one direction or bidirectionally.

The presence of CLso cells in the esophageal cytosol suggests that they are capable of entering esophageal cells from the basal laminar side which, in turn, suggests that they are also capable of entering midgut cells in the same direction. This is a plausible hypothesis because the lumen of the foregut is lined with a cuticular intima, which acts as a barrier preventing CLso from exiting the lumen into the cytosol, unless otherwise assisted by enzymatic or other unknown factors (Killiny et al. 2010). However, entry into cells of these tissues from the basal laminar side is not known to facilitate circulation that leads to transmission, making entry of CLso from the luminal side into epithelial cells essential instead. In this study, this directionality is supported by an apparent endocytic event occurring at the apical epithelial cell membrane, and the presence of CLso cells in the brush-border as well as in the apical, mesal, and basal epithelial cytosols. Also, this direction of transit is inferred by the presence of CLso cells between laminae of the basal lamina, in the basal cytosol associated with vesicles that open into the hemolymph, and in intact, stacked, vesicle-like structures on the outer basal laminar surface. In the context of an exocytosis scenario, these latter structures appear to be most likely composed of and to have originated from the basal lamina. Thus, in the PoP circulative propagative transmission scenario, CLso cells access the basal lamina, postingestion, by utilizing endocytosis and active intracellular transport, followed by exocytosis to arrive at the external midgut basal laminar surfaces.

The abundance of CLso cells on the external surfaces of the midgut suggests that this is an optimal location for multiplication and formation of biofilms, from which sliding and escape into the

hemolymph is thought to occur, followed by CLso systemic invasion, with some cells routing to and entering the salivary glands. The term “sliding” is used to refer to the expansion by multiplication of biofilm-incorporated bacteria, which may not necessarily involve active movement (Harshey 2003) and, thus, appears to be an apt term for the process used by CLso to circulate between the psyllid vector organs involved in the transmission pathway.

Here, TEM studies provided numerous direct observations of individual CLso cells present in the psyllid vector hemolymph, and evidence of hemolymph transport of CLso based on documentation of the proximity of CLso adjacent to the head exoskeleton, sufficiently far from the alimentary canal to suggest that they were founded by hemolymph-bound, motile CLso cells. This scenario is further supported by previous reports of *Liberibacter* presence in hemolymph smears of *Liberibacter*-infected psyllids (Ammar et al. 2011a; Cooper et al. 2014), and by CLso genomic sequence and transcriptome analyses that have identified CLso genes encoding pilin and flagellar proteins (Cicero et al. 2016; Fisher et al. 2014). Therefore, it is hypothesized that the liberation of CLso from outer midgut surface biofilms into the hemolymph and its subsequent motility are facilitated by bacterial effectors or by psyllid protein–CLso effector interactions (Fisher et al. 2014) and, perhaps, during disruption of the stacked, vesicle-like structures caused by transposition (movement by twisting or bunching up, then straightening out) of the midgut loop within the abdominal hemocoel, as described by Cicero et al. (2009).

Entry into the hemolymph is an exit strategy utilized by several other intracellular bacterial pathogens of eukaryotic organisms (Hybiske and Stephens 2015; Kwon et al. 1999). Further support for this hypothesis is provided by comparative gene expression studies of CLso⁺ and CLso[−] adult and nymph PoP, which have revealed gene products with predicted endo-exocytosis and endotoxin activity, respectively, in response to CLso invasion (Fisher et al. 2014; Vyas et al. 2015).

Two types of surface appendages, flagella and pili, are commonly employed by well-studied bacterial pathogens that have a motile

Fig. 4. Aldehyde-fixed CLso⁺ potato psyllid. **A and B**, Teneral adults. **A**, Filter chamber with V₁ bulb exposed by exfoliation of the sheath. Size bar = 5 μ m. **B**, Full view of midgut containing no observable CLso. Size bar = 100 μ m. **C**, Adult head. No observable CLso. Size bar = 100 μ m. **a**, Large numbers of CLso cells exposed by the exfoliation. **b**, Filter chamber. **c**, Midgut loop. **d**, Lobes. **e**, Paired, granular organs of unknown identity. **f**, Paired, smooth-walled organs of unknown identity.

Fig. 5. Adult potato psyllid midguts, CLso[±]. Size bars = 10 μ m. **A**, Filter chamber. **B**, Filter chamber. **C**, Midgut components. Upper inset, different specimen. Biofilms on the external midgut surface are partitioned into colonies. Size bar = 1 μ m. Lower inset, close-up for locants k, l, and m. **a**, Lumen and cytoplasm of the V₁ bulb. No bacteria identified. **b**, Lumina. No bacteria seen within. **c**, CLso within an extracellular niche that divots into both the basal cytoplasm of the V₁ bulb and the opposed, closely appressed, midgut component. **d**, Locant points to a very narrow section of V₁ bulb cytoplasm, apparently caused by pressure from **e**. **e**, Lumen of an alimentary component. Cannot be identified as the midgut loop or the inner hindgut because its shape is rounded and the sheath is too faintly indicated. **f**, Hemolymph. **g**, Filter chamber sheath is faintly indicated in this specimen. **h**, CLso not confined between closely appressed midgut components form thick biofilms of individually partitioned colonies (cf. o). These can be broken open when the midgut loop moves, as in m. **i**, CLso tightly confined between closely appressed filter chamber components. **j**, Lateral cell-cell membranes. **k**, CLso appear to be inside basal lamina- or basal membrane-encased vesicles. **l**, Position of CLso uncertain, whether as in k or actually inside basal epithelial cytoplasm. **m**, Partitions comparable to h and o were broken open, allowing CLso to enter the hemolymph. **n**, Individual CLso cells freely suspended in hemolymph. **o**, Two vesicular envelopes indicate partitioning and stacking of colonies. **p**, Extracellular polymeric substance-like matrix.

stage (Harshey 2003). Herein, CLso was found to occur in crater-shaped patterns on the outer midgut surface and to be associated with mesh-like layers of fibrous material that were similar in appearance to the flagella of *Methanococcus maripaludis* (Jarrell et al. 2011) based on its curved, threadlike appearance, although it also could possibly be a pilus cluster (pili). These observations are consistent with the report of CLso-associated pilus- and flagellum-like structures observed in midguts extirpated from CLso⁺ PoP adults (Cicero et al. 2016; Fisher et al. 2014). The observations that flagella are attached to CLso in the psyllid host indicate that the bacterium is motile during at least part of its life cycle in the psyllid host. Although CLso motility is expected to be essential for navigating in the host hemolymph, precisely when flagella develop, whether within mature biofilms or as cells slough away from biofilm matrices to disperse systemically (circulatively), is not known.

An electron-transparent matrix was observed that had an appearance similar to extracellular polymeric substances (EPS), known to be responsible for the structural and functional integrity of biofilms (Wingender et al. 1999). The EPS-like matrix surrounded or partially covered individuals and clusters of CLso cells. Polysaccharides, the major component of EPS matrices, are typically associated with microbial cell surfaces as lipopolysaccharides (LPS). Data mining of the annotated genome sequence of '*Ca. L. asiaticus*' (a relative of CLso) and other available *Liberibacter* spp. for relevant predicted gene sequences, and a KEGG analysis of the '*Ca. L. asiaticus*' genome sequence, predicted the presence of LPS-related genes (http://www.genome.jp/kegg-bin/show_pathway?las00540). Notably absent are several of the genes that are classically used to encode enzymes for synthesizing canonical LPS O-antigen sugars (Kuykendall et al. 2012; Wulff et al. 2014). However, genes

Fig. 6. Adult potato psyllid midguts, CLso⁺. Intracellular and luminal CLso. **A**, CLso between appressed midgut components. Size bar = 1 μ m. **B**, CLso in brush border. Size bar = 1 μ m. **C**, Ventriculus with residual material from lysis of an epithelial cell. CLso are in lumen and intact epithelial cells. Circle references the same locus for comparing with D. Size bar = 2 μ m. **D**, Low magnification zoom-out of ventriculus in C. Inset, close-up for locant p. Size bar = 10 μ m. **a**, CLso in an apparent compartment of the basal cytoplasm. **b**, CLso between respective basal lamina of opposed midgut components. **c**, CLso between laminae of the basal lamina of cell at right. **d**, Circular muscle. **e**, CLso in basal cytoplasm. **f**, CLso in mesal cytoplasm. **g**, CLso in apical cytoplasm. **h**, CLso in brush-border. **i**, Intact epithelial cells with cytosol. **j**, Intact epithelial cells consumed by CLso. **k**, Lateral cell membrane. **l**, Intact brush-borders. **m**, Residual brush-border from lysed epithelial cell. **n**, Mitochondria. **o**, CLso in lumen. **p**, Event of, putatively, endocytosis.

encoding enzymes essential for lipidA biosynthesis, a critical outer-membrane component of the outer-leaflet in most gram-negative bacteria, and for the synthesis of inner-core low molecular weight KDO sugars, were identified in the annotated '*Ca. L. asiaticus*' genome sequence (National Center for Biotechnology Information Ref Seq NC_014774.1), and suggest that '*Ca. L. asiaticus*' could synthesize a truncated form of LPS, referred to as lipooligosaccharide (LOS). This scenario is reminiscent of the LOS produced by species of *Chlamydia* (Kosma 1999), itself an obligate intracellular pathogen of animals that encases itself within protective, replicative vacuoles or "inclusions" after internalizing itself inside the host cell (Hybiske and Stephens 2015). Further, LipidA has been reported to function in pathogen-induced host toxicity (Needham and Trent 2013) and, thus, despite the absence of (detectable) canonical O-antigen polysaccharides, '*Ca. L. asiaticus*' lipidA may possibly contribute potent endotoxin activity. Further, CLso genes were identified that encode enzymes known to be involved in the biosynthesis of surface polysaccharides, glycosyl transferase, dTDP-4-dehydrorhamnose epimerase, and dTDP-4-dehydrorhamnose reductase, potential components of a carbohydrate-containing EPS layer or capsule. This genetic evidence, when taken together with SEM observations of an EPS-like matrix, suggests that *Liberibacter* spp. synthesize a rhamnose-containing EPS structure. An alternative scenario is that traditional LPS sugars are instead substituted by rhamnose, particularly if other noncanonical molecules are used in its construction. Also, genes were identified that encode glycan biosynthesis, including UDP- α -D-galactose; therefore, these, too, are possible candidates as constituents of EPS. The well-characterized roles of capsular or other extracellular polysaccharide matrices for better-studied bacterial pathogens include antiphagocytic activity, biofilm formation, host defense evasion, and signaling (Kawaharada et al. 2015; Nguyen et al. 2011; Wingender et al. 1999), and are feasibly consistent with the invasive, circulative scenario reported herein for CLso-*psyllid* host interactions.

In this study, observations of the outer salivary gland cell surfaces revealed that CLso cells move pericellularly to the core, where the ductules are known to be located. The capsular-shaped salivary glands of PoP (and whiteflies) have a basal lamina surrounding groups of secretory cells (Cicero and Brown 2011; Cicero et al. 2009). Although direct evidence that CLso enters the ductule lumen was not obtained herein, the transmission model for other circulative, propagative, fastidious bacterial-hemipteran study systems

indicates that entry into the salivary gland lumen is essential for subsequent psyllid vector-mediated CLso transmission to the plant host. Once CLso cells have entered the glands, how they enter the lumen remains to be determined. Perhaps, endo-exocytosis or endotoxin activity, as is proposed herein for CLso-midgut epithelial interactions, is similarly applicable to crossing this barrier. One caveat to positing a similar hypothesis for both is that ductule cells are ectodermal whereas midgut cells are endodermal and have different histological characteristics. Also, in the adult, the salivary pump, afferent duct, and ductule lumina are approximately 1 $\times$ to 2 $\times$ the nominal diameter of the adjacent CLso cells, suggesting difficulty with flow of CLso through these conduits during transmission. The hypodermal cells of the latter two have apical membranes that exhibit microvillar-like folds (Ammar 1986; Cicero et al. 2009) which may permit expansion during secretion or subsequent transmission of CLso cells. However, the salivary pump, as it is currently understood (Cicero and Brown 2012), has no provision for expansion.

For the immature psyllid instars, particularly third and younger, it is not known whether the luminal diameters of the salivary duct system could represent a size constraint to the flow of CLso cells into the stylet bundle during salivation; neither is it known whether they are involved in the acquisition phase of transmission. The diameters of the different nymphal instar stylet bundle food canals are expected to become consecutively larger with each (increasing) developmental stage. The diameter of the adult stylet food canal has been estimated by TEM as approximately 0.818 μ m (Ammar et al. 2013), 0.6 μ m (Cicero et al. 2015), and 0.9 μ m (Garzo et al. 2012). Given this range in size, a predicted minimum threshold diameter by which CLso ingestion could occur would seem possible after stylet biogenesis during the pharate third instar.

Detection of CLso by PCR confirmed the presence of *Liberibacter* spp. in the two youngest PoP instar stages (first and second) (Table 1), despite the inability to detect CLso in midguts of the same aged instars by SEM. One hypothesis is that CLso has not yet multiplied to levels that are sufficient for SEM-detection in these two instars. However, both detection approaches showed CLso at the third instar stage onward and, taken together with that of CLso systemic distribution in the host, are indicative of propagation. The evidence for a differential accumulation of CLso in the midgut of the various PoP developmental stages may be explained on the basis of results from functional genomics studies of CLso⁺ and CLso⁻ immature and adult instars (Fisher et al. 2014; Vyas et al. 2015) that

Fig. 7. Adult potato psyllid midguts, CLso⁺. Intracellular, intrabasal laminar, and extraventricular CLso. Size bars = 1 μ m. **A**, Midgut component appressed to a muscle mass. Arrow, apparent breach in basal lamina. **B**, CLso between laminae of the basal lamina. **a**, CLso in basal cytoplasm. **b**, CLso between laminae of basal lamina. **c**, CLso between muscle and basal lamina. **d**, Hemolymph.

have demonstrated differences in expression levels of gene products with predicted involvements in developmental processes, immune responsiveness, and nutrition, depending on physiological age. CLso titer may also be expected based on anatomical differences occurring during instar development, including that of the adults.

Indirect labeling of '*Ca. L. asiaticus*' and CLso cells by FISH has been used to detect '*Ca. Liberibacter* spp.' in dissected organs and in hemolymph smears, and has demonstrated their near-systemic distribution in adult ACP and PoP, that includes bacteriosomes, fat tissues, midgut appendages, midguts, muscle tissues, ovaries, salivary glands, and testes (Ammar et al. 2011a,b; Cooper et al. 2014). TEM imaging of direct labeling of CLso supports these observations for the midgut, salivary glands, and oral region (Cicero et al. 2016). Results from additional TEM studies indicate that CLso distribution in PoP is extensive, including within the interior of the brain and tentorial muscle masses (J. M. Cicero and J. K. Brown,

unpublished data), and in the cytosol of hypodermal cells, underscoring the intrusive, systemic nature of *Liberibacter* psyllid host invasion. Robust evidence indicates that *Liberibacter* spp. proliferate in at least most of the anatomical compartments and organs of the psyllid host, assuring that at least some CLso cells reach a portal of exit for inoculation (transmission) of the plant host.

In summary, indirect and direct evidence is provided for PoP presence in all psyllid instars and, for adults, direct evidence of a distribution of CLso cells throughout the esophagus, gut, and salivary glands. The latter results raise the possibility that motile bacteria circulating in the hemolymph also could serve as founders of new infection sites. In addition, *Liberibacter* pilar- and flagellar-like surface appendages were visualized that are candidates for CLso attachment to organs (midgut and others) and for motility in the hemolymph, respectively. Also, transcriptome profiling has revealed differentially expressed genes in *Liberibacter* spp.-infected

Fig. 8. Transmission electron microscopy of adult potato psyllid. **A**, CLso⁺. Esophagus. Size bar = 5 μ m. **B**, CLso⁺. Epidermal cells of the head, located at considerable distance from the alimentary canal. Size bar = 10 μ m. **C**, CLso⁻. Filter chamber. No CLso cells are present. Size bar = 10 μ m. **D**, CLso⁻. Esophagus. No CLso cells are present. Size bar = 2 μ m. **a**, Midgut lumen. **b**, Midgut epithelial cell. CLso is not indicated. **c**, CLso between esophagus and midgut. **d**, CLso inside cells of esophageal pleats exterior to circumferential muscle band. **e**, Circumferential muscle band. **f**, Biofilm surrounding esophagus. **g**, CLso inside esophageal cells interior to circumferential muscle band. **h**, CLso inside a compartment of unknown identity, possibly a sinus. **i**, CLso inside epidermis. **j**, Airspace outside the body. **k**, Cruminal cuticle. **l**, Cruminal hollow.

PoP and ACP adults and nymphs (Fisher et al. 2014; Vyas et al. 2015), among which are transcripts implicated in microfilament dynamics (e.g., actin, annexin, fibrillin, integrin, and tubulin), collectively suggesting that membrane remodeling is an inherent feature of '*Ca. L. asiaticus*' or CLso psyllid gut invasion. Results herein and elsewhere (Fisher et al. 2014; Vyas et al. 2015) suggest that CLso-induced cytoskeletal rearrangement is necessary for systemic psyllid invasion or infection. A similar invasion strategy is used by bacterial pathogens of mammalian (Lee et al. 2014) and phytophagous insect (Duret et al. 2014; Hogenhout et al. 2008) hosts, and also by circulative, propagative plant viruses in their insect hosts (Badillo-Vargas et al. 2012; Hogenhout et al. 2003; Whitfield et al. 2005). Finally, the expression of psyllid-encoded clathrin, profilin, Rac 1, talin, and vinculin gene products previously implicated as *Liberibacter*–PoP interactors with

predicted involvement in systemic invasion (Fisher et al. 2014; Vyas et al. 2015) is consistent with the observations reported herein during different stages of PoP infection by '*Ca. Liberibacter*', particularly for the fourth and fifth nymphal instars and also in the teneral and mature adults. Validation of in-silico-identified psyllid host–*Liberibacter* effector protein partners by reverse genetics experiments is expected to identify lucrative knockdown targets that, if effectively silenced by RNA-interference (RNAi) (Huvenne and Smaghe 2010), could disrupt *Liberibacter* invasion of the psyllid host and abate psyllid-mediated transmission to the host plant. Further, RNAi, if effective, could reduce or eliminate altogether the (putative) sexual transmission of *Liberibacter* spp. between adult psyllids and (putative) transovarial (vertical) transmission of *Liberibacter* spp. to psyllid offspring.

Fig. 9. Adult potato psyllid, CLso[®]. **A**, Salivary gland. Size bar = 10 μ m. **B**, Salivary duct, external to the gland, with CLso nearby. Diameter of duct lumen and that of CLso can be directly compared. Size bar = 5 μ m. **C**, CLso in pericellular space of the salivary gland core. Size bar = 10 μ m. **D**, Close-up of the internal duct (ductule) in C, showing CLso reaching its periphery. Diameter of ductule lumen is approximately 2 $\times$ that of CLso. Size bar = 2 μ m. **a**, Salivary gland secretory cells. **b**, CLso inside salivary gland pericellular space. **c**, Bacteria appear to be CLso but they do not fulfill the criteria currently established for identification as CLso. **d**, CLso near salivary duct. **e**, Salivary duct intima. **f**, Salivary duct cell apical membrane infoldings. **g**, Lining of salivary ductule lumen.

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