The flagella of ‘Candidatus Liberibacter asiaticus’ and its movement in planta

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SUMMARY

Citrus huanglongbing (HLB) is the most devastating citrus disease worldwide. ‘Candidatus Liberibacter asiaticus’ (Las) is the most prevalent HLB causal agent that is yet to be cultured. Here, we analysed the flagellar genes of Las and Rhizobiaceae and observed two characteristics unique to the flagellar proteins of Las: (i) a shorter primary structure of the rod capping protein FlgJ than other Rhizobiaceae bacteria and (ii) Las contains only one flagellin-encoding gene flaA (CLIBASIA_02090), whereas other Rhizobiaceae species carry at least three flagellin-encoding genes. Only flgJmut but not flgJLas restored the swimming motility of Agrobacterium tumefaciens flgJ mutant. Pull-down assays demonstrated that FlgJLas interacts with FlgB but not with FliE. Ectopic expression of flaALas in A. tumefaciens mutants restored the swimming motility of ΔflaA mutant and ΔflaAD mutant, but not that of the null mutant ΔflaABCD. No flagellum was observed for Las in citrus and dodder. The expression of flagellar genes was higher in psyllids than in planta. In addition, western blotting using flagellin-specific antibody indicates that Las expresses flagellin protein in psyllids, but not in planta. The flagellar features of Las in planta suggest that Las movement in the phloem is not mediated by flagella. We also characterized the movement of Las after psyllid transmission into young flush. Our data support a model that Las remains inside young flush after psyllid transmission and before the flush matures. The delayed movement of Las out of young flush after psyllid transmission provides opportunities for targeted treatment of young flush for HLB control.

Keywords: citrus, flagella, HLB control, huanglongbing, Liberibacter, movement, psyllid.

INTRODUCTION

Citrus huanglongbing (HLB, also called citrus greening) is the most devastating citrus disease worldwide. The most prevailing HLB pathogen in the world is ‘Candidatus Liberibacter asiaticus’ (Las), an unculturable bacterium vectored by Asian citrus psyllid (ACP, Diaphorina citri), which is an invasive pest for citrus-producing areas outside of Asia (Bové, 2006; Wang and Trivedi, 2013). Currently, HLB management strategies mainly rely on psyllid control and numerous horticultural approaches (Blaustein et al., 2018; Li et al., 2016, 2017, 2018) since all commercial citrus varieties are susceptible to HLB (Folimonova et al., 2009). Current HLB and ACP management has not prevented HLB from spreading worldwide (Wang, 2019). For example, HLB has spread throughout Florida, to other major citrus-producing states (e.g. California and Texas) in the USA, and to neighbouring countries including Mexico and countries in the Caribbean and Central America (Wang et al., 2017). It is paramount to understand the biology and virulence mechanism of Las to design a suitable and efficient HLB control strategy. However, its unculturability has hampered our investigation of the biology and virulence mechanism of Las. Its close relatives, such as Liberibacter crescens, Agrobacterium and Sinorhizobium, have been used as surrogates in previous studies (Andrade and Wang, 2019; Jain et al., 2019; Naranjo et al., 2019; Vahling-Armstrong et al., 2012).

Las has been observed to be distributed throughout the whole plant, including leaf, stem, root, flower and seed coat (Tatineni et al., 2008). However, the details of Las movement in planta are still unknown. Most bacteria possess more than one organelle for motility. For most bacteria, flagella are the major motility organelles responsible for swimming and swarming, whereas type IV pili are responsible for the twitching motility. Las encodes the type IVc tight adherence-pili (Tad) that are usually not associated directly with motility. Instead, they are involved in adherence (Andrade and Wang, 2019). Flagellum-mediated motility allows bacterial cells to move toward and explore nutrient-rich habitats and move away from unfavourable environments (Anderson et al., 2010; Berry and Armitage, 1999;
Moens and Vanderleyden, 1996; Zhu et al., 2013). Flagella also play a key role in surface attachment and host–bacteria interactions (Heindl et al., 2014; Rossez et al., 2015). It has been known that some flagellated plant pathogenic bacteria suppress expression of flagella in the plant, probably to avoid triggering plant defence (Chatnaparat et al., 2016; Yu et al., 2013). Las contains 30 flagellar genes located in three clusters on the chromosome (Fig. 1) (Duan et al., 2009). However, it remains to be determined whether Las synthesizes flagella during within-plant movement. This is a fundamental question that has clear implications for the rate at which disease symptoms appear in both new flush and old leaves.

The flagellar machinery can be divided into five parts called the basal body, the hook, the hook–filament junction zone, the filament and the filament cap (DeRosier, 2006; Evans et al., 2014; Ghosh, 2004; Minamino and Namba, 2004). The basal body is composed of the cytoplasmatic C-ring (FliG, FliM and FliN), the inner or MS-ring (FliF), the periplasmatic P-ring (FlgI), the outer membrane L-ring (FlgH), the proximal rod (FlgB, FlgC, FlgF and FlgE) and the distal rod (FlgG) (Jones and Aizawa, 1991; Park et al., 2006). The basal body is embedded in the cell surface and plays a role in flagella rotation together with the Mot proteins (Minamino et al., 2008). The hook and filament, which are tubular structures composed of subunits of hook (FlgE) and flagellin (FliC), respectively, extend outwards from the cell (Macnab, 2003). The hook length and the switch of specificity from substrates hook-to-filament are tightly regulated by FliK (Erhardt et al., 2010; Waters et al., 2007). Deletion of fliK leads to formation of prolonged flagellar hooks (polyhooks), lack of filament structures and nonmotile phenotypes (Minamino et al., 2009). The hook–filament junction zone (FlgK and FlgJ) and the filament cap (FlhD) are located between the hook and filament and at the tip of the filament, respectively (Ikeda et al., 1996). Flagellar assembly begins with the basal body, proceeds with the hook and finishes with the filament. Capping proteins (FlgJ, FlgD and FlhD) are needed to permit and regulate the polymerization of rod, hook and filament, respectively, at different stages of flagellar assembly (Minamino and Namba, 2004). FlgJ, FlgD and FlhD exist at the tip of the growing rod, hook and filament structures, respectively (Evans et al., 2014).

The flagellar export apparatus is built into the central pore of the basal body MS-ring, and it is formed by interaction of six membrane proteins: FlhA, FlhB, FlgP, FlgQ, FlgR and FlgS, and three cytoplasmic proteins FlhD, FliA and FlhC. FliA is the ATPase that provides the energy for the translocation of proteins across the cytoplasmic membrane (Macnab, 2003). FlhC acts as an ATPase regulator, coupling the energy of ATP hydrolysis to flagellar protein export (Evans et al., 2014; Macnab, 2003). Also, the chaperone, FliJ, prevents premature aggregation of substrates in the cytoplasm, and FlgA, assists the P-ring assembly in the periplasm (Evans et al., 2006; Macnab, 2003).

Flagellins are the subunits that compose the flagellar filament and represent the major flagellar structural protein (Altegoer et al., 2014; Rossez et al., 2015). The number of flagellin-encoding genes in bacterial genomes varies and may range between one (Escherichia coli) and seven (Vibrio parahaemolyticus) (Fedorov and Kostyukova, 1984; Kim and McCarter, 2000; Stewart and McCarter, 2003). The flagellar filaments in E. coli K-12 are built up from only one flagellin protein (FlaC) (Turner et al., 2012), whereas Bdellovibrio bacteriovorus (Thomashow and Rittenberg, 1985), Campylobacter coli (Guerry et al., 1990), Caulobacter crescentus (Driks et al., 1989), Agrobacterium tumefaciens (Deakin et al., 1999; Mohari et al., 2018), Rhizobium lupini strain H13-3 and Sinorhizobium meliloti have at least two flagellin proteins forming their flagellar filaments (Scharf et al., 2001).

Young flush has been shown to be critical for ACP and Las infection. ACP preferentially feeds and exclusively reproduces on young, newly emerged flush shoots of citrus. ACP nymphs feed and complete their life stages on young flush shoots. This observation

![Fig. 1](image) Genetic organization of the three clusters encoding flagellar genes in the ‘Candidatus Liberibacter asiaticus’ genome. The names of the coding regions shown in the diagram are based on their homology with flagellar genes in Rhizobiaceae bacteria. Grey arrows, conserved hypothetical protein; white arrows, hypothetical proteins.
appears to be universal across different citrus-producing regions worldwide (Bové, 2006; Hall et al., 2013; Setamou et al., 2016; Sétamou and Bartels, 2015; Tomaseto et al., 2016). Not surprisingly, citrus trees are normally infected when new flush is present (Hall et al., 2016). In addition, ACPs that acquire Las as adults are poor vectors of Las compared with ACPs that acquire the pathogen as nymphs (Pelz-Stelinski et al., 2010). A threshold infection level (c. \(10^6\) Las/psyllid) is required for successful transmission to citrus plants (Ukuda-Hosokawa et al., 2015). How Las moves after psyllid transmission remains largely unexplored.

Here, we investigated the flagella of Las. Our results indicate that Las flagellar genes express at low levels in planta. Las moves to other sink tissues with phloem sap when young leaves mature into sources. This delayed Las movement after ACP transmission into young flush might present opportunities to develop new strategies for HLB management.

**RESULTS**

**Comparison of flagellar genes of ‘Ca. Liberibacter’ and other members of Rhizobiaceae**

The analysis of the flagellar components encoded by the Las genome (Fig. 1 and Table 1) showed the following characteristics that are shared by all bacterial species in Rhizobiaceae: (i) lack of cytoplasmic chaperone FilI and the components of export apparatus FilH/FilO; (ii) the function of FilK is performed by MotD (Eggenhofer et al., 2006); (iii) the rod-capping FlgJ protein lacks the muramidase domain in its carboxy-terminus (Herlihey et al., 2014); and (iv) lack of FilD, the filament cap (Table 1). It is important to note that all the traits cited above are shared by members of Rhizobiaceae that are known to contain flagellar apparatus and show swimming behaviour, such as A. tumefaciens and S. meliloti (Attmannspacher et al., 2005; Deakin et al., 1999; Mohari et al., 2018).

We observed two characteristics unique to the flagellar proteins of Las and Liberibacter crescens BT-1, a culturable bacterium, that may determine the fate of the Las flagellar machinery:

1. It has a shorter primary structure of the rod-capping protein FlgJ than other Rhizobiaceae bacteria (Fig. 2). In previous work, it was shown that the minimal functional region required of FlgJ for promoting the rod assembly consists of 151 residues in its amino terminus (Hirano et al., 2001). The primary structure of FlgJ in Las (CLIBASIA_01980) contains 112 amino acid residues, whereas in A. tumefaciens (Atu0584) and S. meliloti (SMc03071), FlgJ homologues consist of 175 and 184 residues, respectively.

2. Las contains only one flagellin-encoding gene flaA (CLIBASIA_02090), whereas other Rhizobiaceae species carry at least three flagellin-encoding genes (Fig. 1, Table 1). For example, Rhizobium lupini H13-3 and S. meliloti produce flagellar filaments composed of three and four flagellin subunits, respectively (Scharf et al., 2001). These flagellin-encoding genes are named flaA through flaD. Indeed, the complex flagellar filaments in Rhizobiaceae bacteria are composed of a majority flagellin and at least one secondary flagellin. Mutational analysis of flagellin genes revealed that, in both R. lupini and S. meliloti, FlaA is the principal flagellin and that FlaB, FlaC and FlaD are secondary (Deakin et al., 1999). In this way, FlaA and at least one secondary flagellin are required for assembling a functional flagellar filament in R. lupini and S. meliloti (Scharf et al., 2001). Similar results were demonstrated in A. tumefaciens (Mohari et al., 2018). Thus, the unique features of Las in FlgJ and FlaA are probably critical to functionality of its flagella.

**Characterization of the function of FlgJ and FlaA of Las using Agrobacterium as a surrogate**

We first evaluated whether FlgJ and FlaA of Las are functional by assessing whether they can complement corresponding mutants of A. tumefaciens (Atu). For this purpose, we constructed a nonpolar mutant of flgJ in A. tumefaciens. As expected, the flgJ mutant of A. tumefaciens became immobile, which is similar to the ∆flaABCD mutant with all four flagellin genes deleted (Fig. 3). Only flgJ<sub>Atu</sub> but not flgJ<sub>Las</sub> restored the swimming motility of A. tumefaciens flgJ mutant (Fig. 3A,B), indicating flgJ<sub>Las</sub> might not be functional or works differently in Las. To verify whether FlgJ<sub>Las</sub> can physically interact with the proximal rod proteins FlgB and FliE as shown in other well-studied systems (Hirano et al., 2001), we performed a pull-down experiment with those proteins. Interestingly, FlgJ<sub>Las</sub> interacted with FlgB but not with FliE (Fig. 4).

To test the function of flagellin protein FlaA<sub>Las</sub>, we generated mutant strains of A. tumefaciens carrying deletion in flaA alone (\(\Delta flaA\)), in flaA and flaD genes (\(\Delta flaAD\)), and in all flagellin-encoding genes (\(\Delta flaABCD\)). Interestingly, ectopic expression of flaA<sub>Las</sub> in A. tumefaciens mutants restored the swimming motility of ∆flaA mutant (Fig. 3C) and ∆flaAD mutant (Fig. 3D), but not that of the null mutant ∆flaABCD (Fig. 3D). The swimming halos for the tested strains were calculated and the means and standard deviation (\(n = 3\)) values are shown in Fig. S2. These results indicate that in the A. tumefaciens flagellar system FlaA<sub>Las</sub> functions as the majority flagellin interacting with other secondary flagellins such as FlaB or FlaC, which remain intact in the ∆flaAD mutant, to form a fully functional flagellar filament. However, FlaA<sub>Las</sub> alone could not form a fully functional flagellar filament in A. tumefaciens.

**Observation of Las flagellum in psyllids and in planta**

To verify whether Las forms a functional flagellum, we visualized Las cells isolated from Las-infected plants and psyllids using
Table 1  Identity of residues of flagellar proteins encoded by *Rhizobiaceae* bacteria (as percentage values)

| Gene               | Las | Atu | Sme | Lcc | Laf | Lso |
|--------------------|-----|-----|-----|-----|-----|-----|
| **Basal body**     |     |     |     |     |     |     |
| *FliG*             | +   | 46  | 45  | 54  | 81  | 82  |
| *FliM*             | +   | 30  | 30  | 42  | 70  | 66  |
| *FliN*             | +   | 47  | 50  | 50  | 77  | 74  |
| *FliF*             | +   | 49  | 50  | 56  | 78  | 75  |
| *FlgI*             | +   | 64  | 64  | 68  | 85  | 87  |
| *FlgH*             | +   | 54  | 56  | 63  | 80  | 76  |
| *FlgB*             | +   | 45  | 47  | 57  | 76  | 75  |
| *FlgC*             | +   | 59  | 62  | 68  | 79  | 85  |
| *FlgF*             | +   | 44  | 46  | 49  | 77  | 75  |
| *FlgE*             | +   | 37  | 37  | 42  | 67  | 63  |
| *FlgG*             | +   | 59  | 62  | 69  | 88  | 90  |
| *FlgJ*             | +*  | 29  | 32  | 37* | 61* | 68* |
| *MotA*             | +   | 62  | 58  | 64  | 60  | 63  |
| *MotB*             | +   | 31  | 32  | 36  | 65  | 75  |
| *MotC*             | +   | 29  | 27  | 34  | 65  | 60  |
| **Hook**           |     |     |     |     |     |     |
| *FlgE*             | +   | 50  | 41  | 58  | 75  | 74  |
| *FliK/MotE*        | +   | 32  | 28  | 29  | 44  | 48  |
| *FlgD*             | +   | 43  | 44  | 57  | 85  | 75  |
| **Junction zone**  |     |     |     |     |     |     |
| *FlgK*             | +   | 36  | 36  | 47  | 76  | 69  |
| *FlgL*             | +   | 27  | 30  | 39  | 61  | 57  |
| **Filament**       |     |     |     |     |     |     |
| *FlaA*             | +   | 50  | 42  | 49  | 67  | 61  |
| *FlaB*             | †   | +   | +   | −   | −   | −   |
| *FlaC*             | †   | +   | +   | −   | −   | −   |
| *FlaD*             | †   | +   | +   | −   | −   | −   |
| **Capping filament** |    |     |     |     |     |     |
| *FliD*             | †   | 1   | 1   | 1   | 1   | 1   |
| **Export apparatus** |   |     |     |     |     |     |
| *FliA*             | +   | 66  | 65  | 68  | 86  | 82  |
| *FliB*             | +   | 44  | 45  | 54  | 80  | 74  |
| *FliP*             | +   | 60  | 57  | 67  | 90  | 86  |
| *FliO*             | †   | 1   | 1   | 1   | 1   | 1   |
| *FliQ*             | +   | 57  | 61  | 65  | 90  | 90  |
| *FliR*             | +   | 40  | 43  | 51  | 81  | 78  |
| *FliH*             | †   | 1   | 1   | 1   | 1   | 1   |
| *FliI*             | +   | 62  | 63  | 68  | 87  | 84  |

(Continues)
Fig. 2  Sequence alignment of the rod cap proteins (FlgJ) encoded by Rhizobiaceae bacteria showing the rod-binding domain highlighted in green. Atu, ‘Candidatus Liberibacter asiaticus’ (CP001677); Laf, ‘Candidatus Liberibacter africanus’ (CP004021); Lso, ‘Candidatus Liberibacter solanacearum’ (CP002371); Lcc, Liberibacter crescens strain BT-1 (CP003789); Atu, Agrobacterium tumefaciens (AE007869); Sme, Sinorhizobium melliloti strain 1041 (AL561988).

*Indicates a shorter primary structure for FlgJ homologues when compared to A. tumefaciens and S. melliloti. †No homologues were identified. + and − indicate presence and absence of a homologue in the analysed genome, respectively.

Table 1 (Continued)

| Gene   | Las       | Atu       | Sme       | Lcc       | Laf       | Lso       |
|--------|-----------|-----------|-----------|-----------|-----------|-----------|
| Chaperones |          |           |           |           |           |           |
| FlgJ   | †††††     | ††        | ††††      | ††        | †††††     | ††        |
| FlgA   | +         | 42        | 46        | 53        | 70        | 69        |

Note: Comparative analysis of flagellar proteins encoded by different species of Rhizobiaceae bacteria. Las, ‘Candidatus Liberibacter asiaticus’ (CP001677); Laf, ‘Candidatus Liberibacter africanus’ (CP004021); Lso, ‘Candidatus Liberibacter solanacearum’ (CP002371); Lcc, Liberibacter crescens strain BT-1 (CP003789); Atu, Agrobacterium tumefaciens (AE007869); Sme, Sinorhizobium melliloti strain 1041 (AL561988).

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transmission electron microscopy (TEM). For the visualization, Las cells were confirmed using Las-specific antibody against Las OmpA and the second antibody anti-rabbit IgG-gold. Las cells isolated from grapefruit (Citrus paradisi) seed coats (Fig. 5A) and parasitic dodder stems (Cuscuta sp.) and psyllids are all filament-shaped or long-rod-shaped. Previous observation of Las under TEM suggested that it is polymorphic with Las cells being mostly filamentous in psyllids, but in different shapes in plants with diameter 0.33–1.5 μm and length 2.6–6.3 μm (Hartung et al., 2010). No flagellum was observed for Las cells isolated from grapefruit seed coats (Fig. 5A) and parasitic dodder stems (Fig. 5B). For Las isolated from psyllid guts, no flagellum was observed on most Las cells. A few Las cells showed thread-like structures from which we could not make a conclusive claim whether they are indeed flagella (Fig. 5C–H). However, L. crescens, which shares the same flagellar genes as Las (Table 1), was observed to contain flagella under TEM (Fig. 6).

**Expression of flagellar genes in psyllids and in planta**

To further assess whether Las synthesizes flagella in psyllids and plants, we tested the expression of flagellar genes at both transcription and translation levels using quantitative reverse transcription PCR (RT-qPCR). We found that flagellar genes were up-regulated in psyllids compared to in planta (Fig. 7). Specifically, the flagellin gene flaA is highly up-regulated in psyllids, which is consistent with our previous study (Yan et al., 2013). Likewise, flagellin protein was detected for Las inside psyllids, but not inside plants based on western blot (Fig. 8). Taken together, our data suggest Las lacks flagella in planta or flagellar genes express at low level in planta.

**Movement of Las in planta after ACP transmission of Las into young flush**

Las has been known to translocate through the sieve pore to move from the infection sites to tissues throughout the plants (Kim et al., 2009). Since Las does not produce flagella or its flagellar gene expression is low in planta, its movement to other parts (e.g. roots) of the plant from the infection site is unlikely to be mediated by flagella. Instead, we hypothesize that Las moves with phloem sap to sink tissues when young flush matures. To test this hypothesis, we caged ten Las-positive ACPs/flush (Valencia sweet orange) for each plant for different durations and tested for Las at 54 days post-psyllid feeding. For each treatment, we used five plants with one plant as a biological replicate. After 54 days, 40%, 100%, 100% and 80% of tested leaves were Las-positive following 6, 11, 18 and 29 days of ACP feeding, respectively. However, none of the root samples were Las-positive, indicating that Las had not moved to roots.

To further understand Las movement inside plants after ACP transmission, we trimmed nine citrus plants (Hamlin sweet orange...
Las flagella and movement

Las flagella have multiple important features. First, FlgJ of Las is not able to complement the flgJ mutant of A. tumefaciens. FlgJ plays an important role in flagellar assembly (Hirano et al., 2001). FlgJLas, as its homologues in several other bacterial phyla, including Alphaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria and Spirochaetes (Nambu et al., 2006), contains only the rod-capping domain involved in protein–protein interactions, but lacks the peptidoglycan hydrolase domain, which allows the elongating flagellar rod to penetrate through the peptidoglycan (PG) layer. The peptidoglycan hydrolase domain is commonly found in FlgJ from other bacteria but its function can be developed by other peptidoglycan hydrolase protein not related to flagellar proteins. Liberibacter genomes code for one peptidoglycan hydrolase, which contains a soluble lytic transglycosylase (SLT) domain and a periplasmic signal peptide (Fig. S1). In enteric bacteria such as Salmonella enterica serovar Typhimurium, flgJ null mutants fail to produce the flagellar rods, hooks and filaments, and are nonmotile (Hirano et al., 2001; Nambu et al., 1999). Instead, the flgJ mutant of Borrelia burgdorferi, which contains a single-domain FlgJ homologue as Las, can form intact flagellar basal bodies but had fewer and disoriented flagellar hooks and filaments (Zhang et al., 2012). Accordingly, the motility of the flgJ mutant of B. burgdorferi was partially deficient. In addition, FlgJ seems to be functional because it was able to interact with FlgB. Thus, Liberibacter spp. may have evolved some novel means involving FlgJ to assemble the rod. Second, even though Las encodes a functional FlaA that complements the corresponding mutant of A. tumefaciens, the complex flagellar filament in Rhizobiaceae bacteria is formed by the interaction of FlaA with at least one secondary flagellin, such as FlaB, C or D, that is absent in Las. Ectopic expression of FlaALas in ΔflaABCD did not restore the motility of the null mutant, suggesting that FlaALas is incapable of forming a fully functional filament. Alternatively, the observation of flagella in L. crescens suggests synthesis of Las flagella related to FlgJ and FlaA might involve some novel mechanisms different from known examples in Rhizobiaceae. Lastly, even though we could not totally rule out the possibility that Las might contain flagella in certain conditions (such as in psyllids, as implied in Fig. 8), expression of the flagellar genes is repressed in planta. Indeed, western blotting was not able to detect flagellin in planta.

The flagellar features of Las in planta might help Las avoid the elicitation of plant defence responses since flagellin is known to trigger plant immune responses as typical pathogen-associated molecular patterns (Thomma et al., 2011). Interestingly, mammalian cells use different receptors for recognition of extracellular flagellin or intracellular flagellin. For sensing flagellin outside the mammalian cell, the immune system uses Toll-like receptor TLR-5 (Yoon et al., 2012). If flagellin protein is detected within the cytosol of a cell, it is detected by the Nod-like receptor (NLR) Ipaf (Franchi et al., 2006). We reason that flagellin might activate some early immune responses when Las is transmitted into the phloem by psyllids, during which the flagellin expressed in psyllids remain available in planta. It is likely the recognition is not mediated by FLS2 (Gómez-Gómez and Boller, 2002), but instead by some unknown intracellular receptors. However, it is expected flagellar expression is turned off soon after transmission. Interestingly, multiple plant pathogenic bacteria, such as Pseudomonas syringae...
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(Cheng et al., 2018; Markel et al., 2016) and Xanthomonas axonopodis pv. glycines (Chatnaparat et al., 2016), have been known to use a similar strategy by reducing expression of flagellar genes and losing flagella once reaching their habitats inside the plant. On the other hand, the higher expression of flagellar genes in psyllids suggests flagella might be important for its movement inside the psyllid body to different organs and cell organelles (Ghanim et al., 2017). The induction of flagellar genes in psyllids probably results from differential physiochemical conditions inside psyllids from the phloem sap. In addition, the ACP genome encodes a deficient innate immune system lacking a number of genes that encode for antimicrobial peptides and the Imd pathway associated with defence against Gram-negative bacteria (Arp et al., 2017).

Fig. 5  Transmission electron microscopy analysis of ‘Candidatus Liberibacter asiaticus’ (Las). (A) Las cell isolated from Las-infected grapefruit seed coat. (B) Las cell isolated from dodder stem. (C)–(H) Las isolated from psyllid guts. (D), (F) and (H) are enlarged views of the indicated area in (C), (E) and (G), respectively. Las cells were visualized by using negative staining and antibody against Las OmpA and second antibody anti-rabbit IgG-gold. Arrows indicate thread-like structures. Size bars are indicated in the images.

Fig. 6  Visualization of the flagellar structures in Liberibacter crescens BT-1 cells. Negatively stained cells were analysed by transmission electron microscopy. Size bars are indicated in the images.
We have proposed the following movement model for Las after psyllid transmission into young flush (Fig. 9). In Phase 1 (lag phase), Las initially undergoes no or slow growth to adapt to the environmental changes from psyllids to phloem, then begins multiplication inside the young flush after an undefined period (Phase 2/multiplication phase). In the first two phases, Las stays inside the young flush before it turns into a source. With the maturation of the young flush, the infected leaf transits from a sink into a source. Meanwhile, Las begins to move from the infected leaf to other parts of the plant, such as roots, following phloem sap from source to sink (Phase 3/movement phase). The Las flagellar features probably suggest that Las moves with phloem sap rather than relying on flagella-mediated movement. This model is consistent with the data that newly infected young leaves become infectious within 10–15 days after an inoculum of Las from an adult psyllid (Lee et al., 2015). This movement pattern also promotes acquisition and transmission of Las by nymphs. Las has been suggested to move at approximately 2–3 cm/day in citrus after graft inoculation (S. Lopes, personal communication). However, we do not think that detecting Las in the leaves but not in the root results from the slow movement of Las. The average height (top to root) of the trimmed trees used for our Las movement assay is approximately 20 cm and we conducted the qPCR analysis at 54 days after psyllid feeding, which is supposed to be enough for Las to reach the root if moving right after feeding. In addition, microscopic analysis of Las in planta showed that Las cells float freely in the phloem sap without attaching to the sieve tube cell walls or each other (i.e. without forming biofilms) (Ding et al., 2018; Folimonova and Achor, 2010; Hartung et al., 2010; Hilf et al., 2013; Kim et al., 2009). A similar phenomenon has been observed for Lso and ‘Ca. Liberibacter americanus’ in planta (Liefing et al., 2009; Secor et al., 2009; Wulff et al., 2014). The bacteria move primarily in the vertical direction along the sieve tubes through the sieve pores, rather than horizontally to adjacent sieve tubes. This hypothesis was first suggested by observations of the uneven colonization of Las in dodder, where adjacent phloem vessel elements were observed to be completely full of Las or free of the pathogen (Hartung et al., 2010). Even though Las has delayed movement out of young flush, its population is highest in the seed coat compared to other tissues based on our experience. Dodder also hosts a high density of Las based on our previous experience. We used different tissues, including young flush, in our microscopy studies and could only observe Las when seed coats and dodder were used. Indeed, the procedure used to observe flagella under TEM is quite challenging for unculturable bacteria. More robust evidence for the Las movement model can be acquired by fluorescent microscopical analysis of green fluorescent protein (GFP)-labelled Las, as previously conducted for xylem-inhibiting Xylella (Newman et al., 2003), provided Las can be cultured or be labelled by GFP in vivo in the future.

Those unique features of the HLB pathosystem centring on young flush suggest it represents the Achilles’ heel for Las, and is thus the key point for HLB/ACP management. The psyllids preferentially feed and exclusively reproduce on young, newly emerged flush shoots of citrus, and their nymphs feed and complete their
life stages on young flush shoots. Young flush is critical for psyllids acquiring and transmitting Las (Bové, 2006; Hall et al., 2013; Setamou et al., 2016; Sétamou and Bartels, 2015; Tomaseto et al., 2016). The importance of controlling psyllids during flush stages has been recognized (Hall et al., 2016). The delayed movement of Las out of the young flush provides opportunities to develop novel control strategies. Interestingly, traditional citrus growers in China not only rely on high-frequency psyllid control of young flush using insecticides, but also remove flush (e.g. physical removal or spray with young flush killer) during summer or winter (for certain areas). This flush removal practice probably contributes to reducing the ACP population, preventing ACP from acquiring Las from young flush of HLB-positive trees and transmitting Las to young flush. The delayed movement of Las in young flush also helps the development of targeted early detection of Las after psyllid transmission and before HLB symptom appearance (Pandey and Wang, 2019). In addition, targeted treatment of young flush with bactericides might prevent Las from establishing in planta after psyllid transmission. The minimum concentration of oxytetracycline required to suppress Las populations in planta has been suggested to be 0.68 and 0.86 µg/g under greenhouse and field conditions, respectively (Li et al., 2019). Depending on application method, oxytetracycline concentrations in leaf tissues have been reported to range from 0 to 0.53 µg/g (Li et al., 2019; Vincent et al., 2019), below the concentrations required to suppress Las. It seems that successful prevention of Las establishment after psyllid transmission requires optimized delivery methods to increase bactericidal concentration to the minimum concentration of bactericides required to suppress Las populations in planta. The bactericidal effect needs to cover the whole flush period until leaf maturation. Additionally, it is interesting to know whether the targeted treatment effect can be obtained with non-antibiotic bactericides such as generally recognized as safe (GRAS) antimicrobial compounds.

In summary, the flagellar characteristics of Las support a delayed movement of Las from young flush after psyllid transmission. The movement pattern of Las provides unique opportunities for early diagnosis of HLB and targeted treatment of young flush to control HLB.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains, plasmids and growth conditions**

The plasmids, oligonucleotides and bacterial strains used in this study are listed in Tables S1 and S2. *Escherichia coli* cells were grown at 37 °C in Luria–Bertani (LB) medium. The *A. tumefaciens* strain C58 and mutant strains were grown in LB medium at 28 °C. Plasmids were introduced into *E. coli* by heat-shock at 42 °C and into *A. tumefaciens* by electroporation. *Agrobacterium tumefaciens* deletion mutants were generated using the suicide vectors pNPTS138 as described elsewhere (Hibbing and Fuqua, 2011). Cells of *L. crescens* strain BT-1 were grown at 28 °C at 150 rpm in BM7 medium. Antibiotics were used at the following concentrations: kanamycin 50 mg/mL, ampicillin 100 mg/mL, gentamicin 10 mg/mL.

**Deletion of flagellar genes and construction of complemented strains of *A. tumefaciens***

DNA manipulations and PCR were performed according to standard procedures (Sambrook et al., 2006). To construct the flaA, flaD and flgJ deletion mutants, approximately 1 kb of the upstream and downstream regions of those flagellar genes were amplified
by PCR from A. tumefaciens genomic DNA (oligonucleotides are listed in Table S2), and the two fragments were ligated to produce an in-frame deletion. The approximately 2 kb fragments that resulted were then cloned into the HindIII site of the pNPTS138 suicide vector, generating the plasmids pNPTS-FlaA, pNPTS-FlaD and pNPTS-FlgJ (Andrade and Wang, 2019; Andrade et al., 2014). These constructs were introduced into A. tumefaciens cells by electroporation, and the wild-type copies were replaced by the deleted version after two recombination events. For the first and second recombination events, A. tumefaciens cells were selected in LB medium without NaCl (LBON) with kanamycin and in LBON plus 5% sucrose, respectively. The single mutants ∆flaA, ∆flaD and ∆flgJ and the double mutant ∆flaAD were confirmed by PCR. To complement ∆flgJ, ∆flaA, the double mutant ∆flaAD and the null mutant strains, fragments including the coding region of flaA or flgJ genes were amplified by PCR from A. tumefaciens genomic DNA. Also, the coding region of flgJ was amplified from A. tumefaciens genome. Those three fragments were inserted into the BamHI site of the pTF53 vector that contains the constitutive promoter Trp (Andrade and Wang, 2019; De Feyter et al., 1993), creating the plasmids pF53-Las_FlaA, pF53-Las_FlgJ and pF53-Atu_FlgJ. The construct pF53-FlaA_Las was used to transform ∆flaA, ∆flaAD and ∆flaABCD mutant strains. ∆flgJ mutant strain was transformed with pF53-Las_FlgJ or pF53-Atu_FlgJ constructs. After electroporation, the transformed A. tumefaciens cells were selected on LB medium with gentamicin. All constructs were sequenced for confirmation.

**Motility assay**

The swimming motility of A. tumefaciens was assayed on semi-solid agar as described previously (Merritt et al., 2007). Briefly, bacteria cells grown in LB medium for 48 h at 28 °C were stabbed into the LB medium plus specific antibiotics (Table S1) at 37 °C and 200 rpm. Then, precultured cells were diluted 1:100 in 100 mL of LB medium and grown at 37 °C until OD600nm = 0.6. The expression of the recombinant proteins was induced by addition of 1 mM IPTG to the growth medium and the induction process occurred at 30 °C and 200 rpm for 6 h. After induction, E. coli cells were pelleted by centrifugation at 2348 g and 4 °C for 10 min. Pelleted E. coli cells expressing GST-FlgJLas protein were washed in phosphate-buffered saline (PBS, pH 7.4) and suspended into lysis buffer (PBS, 100 µg/mL lysozyme and 40 µg/mL DNAse I) and sonicated to generate the cell lysates. After centrifugation, the cell lysates were incubated with 0.3 mL of glutathione sepharose high-performance beads for 1 h then beads mixed with cell lysates were loaded on a 10 µL column, according to the manufacturer’s instructions (GE Healthcare, Pittsburgh, PA, USA). The beads were washed four times with PBS to remove the unbound proteins and incubated with E. coli cell lysates containing 6×HistEGFP, 6×HistFlgB or 6×HisFlIE for 2 h at 4 °C. After washing three times, the proteins were eluted with PBS plus reduced glutathione (7 mg/mL). Eluted samples were mixed with 4× SDS-PAGE loading buffer and boiled for 5 min. The controls (Inputs) and eluted samples were subjected to SDS 12% polyacrylamide gel electrophoresis and immunoblotting by using anti-GST (1:5000) and anti-6×His (1:3000) antibodies followed by secondary antibodies raised against rabbit (1:10 000) (Sigma).

**Las-infected psyllids and plants**

Las-infected psyllid colonies were reared in laboratory cultures maintained on Las-infected sweet orange trees at the University of Florida, Citrus Research and Education Center (CREC-UF, Lake Alfred, FL, USA). Psyllid colonies were maintained in insect-proof cages inside environmentally controlled growing chambers with a 12-h photoperiod. Las-infected seeds were obtained from lopsided fruits from HLB-positive grapefruit (Citrus paradisi). Las-infected dodder plants (Cuscuta sp) on HLB-positive pentwinkle (Catharanthus roseus) were used for TEM assay. Plants were grown in a greenhouse with a 12-h photoperiod and controlled temperature. Las-positive dodder tendrils were a gift from Dr Ed Etxeberria (University of Florida, CREC, Lake Alfred, FL, USA). Las in the insects, citrus plants, seed coats and dodder plants was verified by qPCR (Hartung et al., 2010; Hilf et al., 2013; Yan et al., 2013). A sleeve net (80 mesh) was used to cage Las-positive psyllids on young flush only. The caged plants were kept within a bigger cage to prevent interference from other psyllids.

**Isolation of Las from psyllid guts and plant materials**

About 80 Las-infected adult psyllids (Ct value = 26 to 32) were collected from the psyllid room (CREC-UF) and used to dissect midguts in sterile PBS under a dissecting stereomicroscope by using depressed glass wells and fine entomological needles. After
tissue samples were dissected, the tissues were washed three times with PBS and transferred to a sterile Eppendorf tube containing 0.3% bacitracin in PBS (Ghanim et al., 2016). Samples were pipetted up and down slowly to release the bacteria cells attached to the midgut.

Selected seeds isolated from infected and symptomatic citrus plants (Ct value about 27 to 29) were washed five times in sterile water, dried in filter paper and the coats were removed by using high-precision straight fine tweezers. Seed coats were divided into very small pieces and suspended with 0.3% bacitracin (Thermo Fisher, Waltham, MA, USA) in PBS.

Individual dodder tendrils positive for Las (Ct = 24 to 26) were removed intact from parasitized plants. A segment of 10 mm was removed from the centre of dodder stem pieces, washed five times in sterile water, dried in filter paper, transferred to 0.3% bacitracin in PBS and cut into very small pieces with razor blades. The mixtures were pipetted up and down slowly to release the bacteria. Solution containing Las was transferred to a sterile Eppendorf tube.

**Transmission electron microscopy and antibody-labelled Las cells**

Bacterial cultures of *L. crescens* were grown at 28 °C in BM7 medium antibody-labelled Las cells

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Las flagella and movement

program. We thank Dr Ed Etxeberria for the Las-positive dodder materials. This project was also supported by the National Key Research and Development Program of China (2018YFD0201500) to T.S.Y.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1 Sequence alignment of the peptidoglycan hydrolase encoded by ‘Candidatus Liberibacter’ species was generated by ClustalW. The signal peptide sequence found by PrediSi is highlighted in yellow. The SLT domain is indicated in green. CLas, ‘Ca. Liberibacter asiaticus’ (CLIBASIA_00965); CLaf, ‘Ca. Liberibacter africanus’ (G293_01215); CLam, ‘Ca. Liberibacter americanus’ (Lam_376); CLso, ‘Ca. Liberibacter solanacearum’ (CKC_02595); Lcc, Liberibacter crescens BT-1 (B488_10490).

Fig. S2 Swimming motility assay of the Agrobacterium tumefaciens wild-type, ΔflaAD and ΔflaABCD mutant strains, and the mutant strains complemented with ‘Candidatus Liberibacter asiaticus’ (Las) flaA. The mean values ± the standard deviations (n = 3) are plotted. Mean values were compared to the wild-type, * indicate statistically significant difference (P < 0.05, Student t test).

WT, A. tumefaciens wild-type strain carrying the empty vector. ΔflaAD and ΔflaABCD, A. tumefaciens wild-type strain carrying the empty vector. ΔflaAD + flaALas and ΔflaABCD + flaALas, mutant strains carrying the Las flaA gene.

Table S1 Bacterial strains and plasmids used in this study.

Table S2 Oligonucleotides used in this study.