The Intracellular Citrus Huanglongbing Bacterium, ‘Candidatus Liberibacter asiaticus’ Encodes Two Novel Autotransporters

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Abstract

Proteins secreted by the type V secretion system (T5SS), known as autotransporters, are large extracellular virulence proteins localized to the bacterial poles. In this study, we characterized two novel autotransporter proteins of ‘Candidatus Liberibacter asiaticus’ (Las), and redesignated them as LasA1 and LasA2 in lieu of the previous names HyvL and HyvR. As a phloem-limited, intracellular bacterial pathogen, Las has a significantly reduced genome and causes huanglongbing (HLB), a devastating disease of citrus worldwide. Bioinformatic analyses revealed that LasA1 and LasA2 share the structural features of an autotransporter family containing large repeats of a passenger domain and a unique C-terminal translocator domain. When fused to the GFP gene and expressed in E. coli, the LasA1 C-terminus and the full length LasA2 were localized to the bacterial poles, similar to other members of autotransporter family. Despite the absence of a typical signal peptide, LasA2 was found to localize at the cell surface by immuno-dot blot using a monoclonal antibody against the partial LasA2 protein. Its surface localization was also confirmed by the removal of the LasA2 antigen using a proteinase K treatment of the intact bacterial cells. When co-inoculated with a P19 gene silencing suppressor and transiently expressed in tobacco leaves, the GFP-LasA2 translocator targeted to the mitochondria. This is the first report that Las encodes novel autotransporters that target to mitochondria when expressed in the plants. These findings may lead to a better understanding of the pathogenesis of this intracellular bacterium.

Introduction

Autotransporters are large multi-domain virulence factors encoded by genomes of diverse gram-negative bacteria. A typical autotransporter consists of three functional domains: a Sec-dependent N-terminal signal peptide, a secreted passenger domain (α-domain) and a conserved C-terminal translocator domain (β-domain) [1]. The central passenger domain will ultimately be either attached to the cell surface or secreted. This type of self-transporting protein system is referred to as a type V secretion system (T5SS). Known virulence factors secreted by T5SS have been shown to be cytotoxic, contain protease activities, or functions such as adhesions. Based on structural features, autotransporters have recently been classified into three sub-types: classical autotransporters (T5aSS), two-partner secretion system (T5bSS) and trimeric autotransporters (T5cSS) [2]. The signal peptide directs export of the precursor protein across the inner membrane using the Sec machinery and then is cleaved by peptidase. Subsequently, the β-domain inserts into the outer membrane and forms a pore with 12 transmembrane β-strands through which the passenger domain is presumed to be exported [3]. Once the passenger domain is translocated to the cell surface, it is usually cleaved from the translocator domain and released extracellularly. In some cases the passenger domain is not cleaved and remains tightly associated with the cell membrane [4]. The trimeric autotransporters (T5cSS) known as AT-2 are exemplified by the oligomeric coiled-coil adhesions from various pathogenic bacteria, such as YadA of Yersinia [5], Hia of Haemophilus [6], and Hap of Haemophilus [7]. Compared with the conventional translocator domain that typically contains about 300 amino acids, AT-2 contains a short translocator domain of about 70 amino acids that is sufficient for translocation of the passenger domains [8,9]. Deletion of...
the YadA translocator domain abolishes the ability to insert into the outer membrane [9]. Many AT-2 passenger domains contain large repeat units of about 70 residues. Phylogenetic clustering of these repeat units revealed that they share striking clustering patterns in which some of the repeats are almost identical in sequence [10]. It has been reported that autotransporters from a variety of rod-shaped pathogenic bacteria, including IcsA and SepA of *Shigella flexneri*, AIDA-I of *Escherichia coli*, and BrkA of *Bordetella pertussis*, are localized to the bacterial poles [11]. Recently, it was demonstrated that the YadA translocator localized solely to the mitochondrial outer membrane when expressed in yeast and that four β-stands are sufficient for mitochondrial localization [12].

*Candidatus Liberibacter asiaticus* is a Gram-negative, fastidious alpha-Proteobacterium, causing huanglongbing (HLB), a devastating disease of citrus worldwide. HLB causes rapid decline and shortens the life span of infected trees [13]. Having a greatly reduced genome of approximately 1.23 Mb, Las bacteria reside in phloem sieve cells of infected citrus plants and are transmitted by the citrus psyllids, *Diaphorina citri* [14,15]. Intriguingly, even with such a small genome size, the Las psy62 genome contains multiple prophage-related regions, and two were identified as prophages/temperate phages, which occupy ca. one-sixteenth of the entire Las genome [15,16]. Within these prophage regions, two hypothetical hypervariable proteins (Hyv and Hyvω) were identified that contained multiple, nearly-identical, leucine-rich repeats (LRRs). The diversity and plasticity of these two genes may have implications for how these intracellular bacteria adapt to their host ecological niches [17].

Prophages in many bacterial genomes are associated with bacterial pathogenicity and biofilm formation [18]. In the present study, we discovered that these two hypervariable proteins encoded by Las prophages are novel autotransporters (redesignated as LasA and LasAω). We determined that LasA and LasAω are polar and surface localized in bacteria in addition to being targeted to the mitochondria when expressed in plant cells. Previously, we demonstrated that the Las bacterium may act as an “energy parasite” by encoding a functional ATP translocase for direct ATP/ADP importation from their host cells [19]. Together, these findings may lead us to understand how these intracellular bacteria modulate their host energy biosyntheses during their pathogenesis.

**Results**

**Characteristics of unique autotransporters, lasA, and lasAω in Las**

The lasA and lasAω, previously reported as *hyv* and *hyvω*, are located in two prophage regions in the Las Psy62 genome [17]. The 2760 bp lasA encodes a 919 amino-acid hypothetical protein with a predicted molecular mass of 38.9 kDa. LasAω has only one partial LRR. The translocator domains of LasA and LasAω share 80% identity at the amino acid level while the passenger domains share 50% identity at the amino acid level. Using the SignalP signal peptide prediction software, no signal sequence was identified in LasA or LasAω and little information about the function of LasAω was obtained from a BLAST search of the NCBI protein database. The LasA and LasAω passenger domains share low level (about 25%) amino acid sequence similarities with the LRR protein of *Coxella psychrethreae* 34H (GenBank accession number: AAZ26055) and the cell wall associated biofilm protein of *Staphylococcus epidermidis* (ZP_06614153). Surprisingly, the passenger domains of LasA and LasAω share similar LRR repeat structures with the Toll-like receptors (TLRs) that function as sentinels of the innate immune system by binding a variety of ligands, including lipopolysaccharide, flagellin and dsRNA, through a LRR ligand-binding domain [20]. LasA and LasAω translocator domains were predicted to contain ten and twelve β-stranded secondary structures respectively by the YASPIN Secondary Structure Prediction program. However, the 3D structure predicted by the I-TASSER program did not form the typical β-barrel structure, which was reported for the translocator domain of the autotransporter NalP from *Neisseria meningitidis* [3,21]. Despite the absence of typical signal peptides and no significant sequence homology with other autotransporters at the amino acid level, sequence analyses predicted that LasA and LasAω possess architectural features of the autotransporter family, including passenger domains with large repeated sequences that form coiled-coils and translocator domains containing β-stranded structures.

LasA is an outer membrane protein and non-cleaved from cells

The full length gene lasA was cloned into the pET102D-TOPO vector and protein expression was induced in *E. coli* BL21 (DE) cells (Invitrogen, Carlsbad, CA). A protein of the expected size for LasA was shown on SDS-PAGE and confirmed by Western blot (Figure 1). LasA was purified under hybrid conditions, and the elution fractions contained two bands detected by SDS-PAGE. The 120 kDa protein band including the 16 kDa fusion tag was verified by Western blot with an antibody against LasA (N terminus, one full repeat and amino acids from part of the translocator domain). No signal was detected for the 40 kDa protein on the same Western blot (Figure 1B).

It has been shown that autotransporter passenger domains are transported to the cell surface and most of them are processed, thus releasing the passenger domain into the culture’s supernatant. To determine the subcellular localization of LasA and its passenger domain, outer membrane proteins, surface-associated proteins and secreted proteins in culture supernatant were isolated. As shown in Figure 1, a 120 kDa protein was detected in outer membrane fraction by SDS-PAGE gel and confirmed by western blot, which suggests LasA containing both passenger domain and translocator domain in these fractions. In contrast no LasA protein was
detected from the culture supernatant using the anti-LasA I antibody (Figure S1A), which suggests that even though the LasA I protein contains the signal information required for cell pole localization in E. coli, its passenger domain was not cleaved and released into the culture's supernatant in E. coli.

The surface-associated protein was isolated from cell pellets and a Western blot was performed using an anti-LasA I antibody. No specific binding signal was observed (Figure S1A). Taken together, the results indicate that the LasA I is an outer membrane protein and its passenger domain was not cleaved and was still tightly associated with the translocator domain in E. coli.

Polar localization of LasA I and LasA II

Several autotransporters from a variety of rod-shaped pathogenic bacteria are polar-localized in the bacterial [11]. We examined the localization of LasA I and LasA II by constructing GFP fusion proteins. The expression of GFP and GFP fusion proteins was detected by Western blot with an anti-GFP antibody (Figure S1B). When GFP was fused with the translocator domain of lasA I (pET102-gfp-lasA I-TD), or the full length lasA II gene (pET102-gfp-lasA II), the expression of GFP was observed at the cell poles of E. coli by confocal laser scanning microscopy (CLSM) (Figure 2D–I). In the control panel, the expression of GFP (pET102-gfp) without the fusion partner was observed in the whole cell, which indicates GFP itself is not directed to bacterial cell poles (Figure 2A–C).

Exportation of the LasA I and LasA II passenger domains by the translocator domains

Although the typical N-terminal signal sequence found in most autotransporters was not identified in LasA I, our results showed that the passenger domain of the LasA I protein is localized at the E. coli cell surface. Immuno-dot blot results showed strong signals indicating LasA I passenger domain is transported out of the bacterial cells when expressed in E. coli, and no signal was observed in the control strain of E. coli (Figure 3). Proteinase K-treated E. coli containing the LasA I constructs did not bind the LasA I antibody, indicating that the passenger domain of LasA I was degraded on the surface of the bacterial cells. LasA I degradation by proteinase K was confirmed by SDS-PAGE and Western blot. Proteinase K-treated E. coli cells expressing LasA I contained no signal while the untreated control cells contained the full 120 kDa protein (Figure 1). Proteinase K has no ability to cross the bacterial membrane and only digest the surface protein of intact...
bacteria. This confirmed that the passenger domain of LasA,
was digested on the surface of the bacterial cells.

The LasA translocator domain not only exports its native
passenger domain but also the LasA-GFP fusion protein to the
cell surface. When GFP alone was expressed, the GFP protein
stayed inside the cells and no GFP binding signal was detected
with an anti-GFP antibody. However, when GFP was fused
with the translocator domain of LasA, or the full length lasA
gene, the GFP proteins were detected on intact bacterial
surfaces with an anti-GFP antibody (Figure 4). After proteinase
K digestion, immuno-dot blot results showed no GFP signal
(data not shown). However, when the whole-cell lysate was
handled with proteinase K and analyzed by SDS-PAGE, a slight
reduction in the intensity of the fusion proteins was observed
with Coomassie blue staining (data not shown). This indicated
that not all fusion proteins expressed in E. coli are exposed on
the surface of the bacteria and that the translocation of the
GFP fusion protein is not as efficient as with the native LasA
passenger domain.

To further confirm the surface localization of LasA, an
immunofluorescence assay (IFA) was performed. The E. coli
cells expressing LasA were not labeled by anti-LasA antibody
and the E. coli cells expressing the LasA protein can be detected by
Western blot and immuno-dot blot. The absence of surface
labeling indicates that the LasA protein produced in E. coli may
not be secreted and folded properly.

LasA targeting to mitochondria

The autotransporter YadA translocator domain was
expressed in yeast and imported into the mitochondria, which
did not interfere with mitochondrial function [12]. To investigate
the potential function and cellular localization of LasA and
LasAI in plant cells and the role of the LasA and LasAI
translocator domains, full-length LasA, full-length LasAI and
the translocator domain of LasA were cloned into the pGDY
vector and transformed into Agrobacterium tumefaciens strain
GV 2660. Transient expression results showed no detectable

GFP in tobacco plants inoculated with these constructs, except
with the pGDY vector alone. Co-inoculation of tobacco leaves
with a P19 gene silencing suppressor and pGDY-lasA-TD
construct facilitated GFP expression (data not shown). Only a
few cells had detectable GFP in the infiltrated zone when the
full length lasA or lasAI constructs were co-inoculated with the
gene silencing suppressor. CLSM and propidium staining
results showed that the expression of pGDY-lasAI-TD
appeared to localize in the mitochondria. MitoTracker labeling
and CLSM confirmed that GFP-lasAI-TD targeted to the
mitochondria in tobacco leaves. As shown in Figure 5 and
G1, the appearance of yellow mitochondria confirmed the
localization of the pGDY-lasAI-TD fusion protein. The
autofluorescence of chloroplasts was not observed by
MitoTracker detection using a 560nm low-pass filter as shown
by differential interference contrast (DIC) (Figure 5 D and H).
In contrast, GFP alone in whole cells expressed mainly in the
nucleus and did not show yellow mitochondria (Figure 5 C). Our
results demonstrated that the translocator domain of LasA
contains sufficient structural information for targeting
mitochondria. No obvious cell death response was observed in
the infiltrated leaf zone; however, mitochondria aggregation
was observed when infiltrated with full length lasA and lasAI
constructs (Figure 6A2 and A3). In infiltrated leaves, enlarged
mitochondria and morphology change in chloroplast were
observed and both of them are detached from cell wall (Figure
6B2). In addition, aggregation and changes in mitochondrial
morphology were observed in infected periwinkle (Figure 6C2).
Collectively, these results suggest that lasA and lasAI may
affect mitochondria and chloroplast function and manipulate
energy production during Las infection.

Discussion

We previously reported the genetic diversity and
characteristics of two hypervariable proteins (HyvI and HyvII)
from the Psy62 Las genome and global Las isolates that
contain up to 12 nearly identical tandem repeats [17]. In the present study, we discovered that LasA<sub>I</sub> and LasA<sub>II</sub> are two novel autotransporters. Most known autotransporters are virulence proteins in animal and human pathogens [2]. Typically autotransporters contain an N-terminal signal peptide, a passenger domain and a C-terminal translocator domain. However, no typical signal peptide was predicted in LasA<sub>I</sub> or LasA<sub>II</sub>, and while the amino acid sequences of LasA<sub>I</sub> and LasA<sub>II</sub> translocator domains contained predicted β-stranded structures, they shared no homology with translocator domains from other autotransporters. We propose that these proteins are new members of the autotransporter family because the translocator domains of LasA<sub>I</sub> and LasA<sub>II</sub> not only deliver their native passenger domains, but also exported GFP fusion proteins (GFP-LasA<sub>I</sub>-TD and GFP-LasA<sub>II</sub>-TD) onto the bacterial cell surface. These findings reveal that LasA<sub>I</sub> and LasA<sub>II</sub> are unique autotransporters and the T5SS may play an important role in Las pathogenesis. Furthermore, using transient gene expression in tobacco leaves, we demonstrated that LasA<sub>I</sub> contains sufficient structural information for targeting the host mitochondria as do other members of the autotransporter family [12].

Secreted proteins play a central role in the interactions of bacteria and their hosts. Gram-negative bacteria have evolved several specialized secretion systems to deliver effectors into their hosts, such as the Type I secretion system (T1SS) and the Type IV secretion system (T4SS). As an intracellular bacterium, Las does not have a T3SS or a T4SS, but may use the Sec secretion system [15,22]. Among the known bacterial secretion systems, the autotransporter or T5SS is the simplest pathway. Since the first report in the 1980s, the autotransporter family has been continuously expanding. Most of the characterized T5SS secreted proteins contribute to the virulence of animal or human pathogens [2]. The relatively few autotransporters reported from plant pathogens include the adhesins HecA/HecB of *Erwinia chrysanthemi* [23] and the XatA of *Xylella fastidiosa* which is important for virulence and is also associated with bacterial autoaggregation and biofilm formation [24]. In addition, the EstA autotransporter was reported as a member of the esterase family from the rice root colonizing and beneficial bacterium, *Pseudomonas stutzeri* A15 [25].

The amino acid sequences of autotransporters are highly divergent except for the conserved translocator domain. LasA<sub>I</sub> and LasA<sub>II</sub> proteins are unique autotransporters because they share no homology with any other members of the autotransporter family. Only coiled-coil domains were predicted and no signal peptides or anchor domains were identified in the LasA<sub>I</sub> and LasA<sub>II</sub> proteins when using the Trimeric Autotransporter Adhesins (TAAs) domain annotation tool [26]. Since the translocator domains of LasA<sub>I</sub> and LasA<sub>II</sub> have the ability to export their native passenger domains and GFP fusion proteins to the *E. coli* cell surface, the C-terminal translocator domains of LasA<sub>I</sub> and LasA<sub>II</sub> may form β-barrel structures through which the passenger domain can pass [2]. However, using the I-TASSER program to predict the 3D structures of LasA<sub>I</sub> and LasA<sub>II</sub>, the β-barrel structures of these translocator domains are atypical [21]. In other translocator

![Figure 5. Mitochondrial localization of the Las autotransporter LasA<sub>I</sub>. A-G1: confocal laser scanning micrographs. GFP expression and MitoTracker labeling were detected in tobacco leaves infiltrated with pGDY and pGDY-lasA<sub>I</sub>-TD plasmids, respectively. A, E: GFP detection with 505-530 nm BP filter; B, F: MitoTracker detection with 560 nm LP filter; C, G: merged scans; D, H: differential interference contrast (DIC) micrographs of tobacco cells with chloroplasts (red arrows). E1, F1, G1: magnifications of yellow boxes in panels E, F and G. Mitochondria (yellow arrows). doi: 10.1371/journal.pone.0068921.g005](https://www.plosone.org/doi/10.1371/journal.pone.0068921.g005)
Las Encodes Two Novel Autotransporters

Figure 6. Mitochondria aggregation and morphology in plant cells. A1-A3: confocal laser scanning micrographs. MitoTracker labeling was detected in tobacco leaves infiltrated with pGDY (A1), pGDY-lasA (A2) and pGDY-lasAII (A3). Arrows indicate normal and aggregated mitochondria respectively. B1-B4: transmission electron microscopy micrographs. B1: normal mitochondria (red arrow), chloroplast (blue arrow) and cell wall (green arrow) from infiltrated pGDY. B2: enlarged mitochondria (red arrow), abnormal chloroplast (blue arrow) and detached cell wall (green arrow) from infiltrated pGDY-lasA. B3: normal mitochondria (arrow) from healthy periwinkle. B4: aggregated abnormal mitochondria (arrow) from Las-infected periwinkle.

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domains, such as that of NalP from Neisseria meningitidis, the crystal structure contains a 12-stranded β-barrel with a hydrophilic pore filled by an N-terminal α-helix [3]. It is not surprising that the predicted structures of the LasAII and LasAI translocator domains are different from other translocators as there are no conserved amino acids between the Las translocator domains and the known translocator domains. The crystallized structures of LasAII and LasAI will be important for understanding the passenger domain export mechanism.

LasAI and LasAII localize at bacterial poles as do other reported autotransporter members, including IcsA and SepA of Shigella flexneri, AlpA-I of diffusely adherent E. coli and BrkA of Bordetella pertussis [11]. It has been shown that NalP from spherically shaped N. meningitides and BrkA from B. pertussis localize at the pole of E. coli, suggesting that autotransporters contain information required for polar localization [11]. It is interesting to note that in the IcsA protein of S. flexneri two regions within the passenger domain were involved in pole targeting [27]. In contrast, the LasAII translocator domain alone has the ability to target the bacterial poles. Further investigation should identify the region(s) essential for LasAII and LasAI to localize to the bacterial poles.

Typically T5aSS autotransporters exposed at the cell surface are proteolytically cleaved at the junction of the passenger domain and the outer-membrane embedded translocation domain [2]. Although LasAII and LasAI were surface-localized autotransporters, LasAI was present in greater amounts in whole-cell lysates and outer membrane but undetectable in the culture supernatant, indicating either that its inefficient cleavage in E. coli or that the cleaved passenger domain remains tightly associated with the translocator domain. The passenger domains of T5cSS, such as Hia from H. influenzae, are usually not cleaved and stay tightly associated with the cells [4]. In E. coli, BrkA is proteolytically cleaved at the bacterial surface, and the extracellular domain, though cleaved, remains tightly associated with the translocator domain [11]. Further efforts to confirm the LasAII and LasAI surface localization failed by IFA, although production of LasAII and GFP fusion proteins in E. coli was confirmed by immuno-dot blot and Western blot. The exported native passenger domains could not bind the primary antibody, indicating that they may not fold properly on the cell surface of E. coli. This was also observed with the IcsA of S. flexneri, which can be labeled at the surface of wild type S. flexneri but cannot be labeled in the E. coli cells expressing IcsA [28]. Once a pure Las culture is obtained, it will be possible to confirm the LasAI and LasAII surface localization by IFA and determine whether passenger domains are cleaved.

To understand the function of these novel autotransporters, lasAI and lasAII were constructed for Agrobacterium-mediated transient expression. Because no GFP expression was detected with the infiltrations containing our constructs, we used the gene silencing suppressor p19 to enhance the ectopic expression in plant leaves since post-transcriptional gene silencing (PTGS) is reported as a general feature in Agrobacterium-mediated transient expression [29–31]. As expected, strong GFP expression was observed when the lasAI translocator domain (pGDY-lasAI-TD) was co-infiltrated with the p19 construct; while no GFP was detected when infiltrated with the lasAII translocator domain (pGDY-lasAII-TD) alone. However, only a few GFP expressing cells were detected from the leaves co-inoculated with p19 and the full gene constructs of either lasAI or lasAII (pGDY-lasAI and pGDY-lasAII, respectively). This result may be due to GFP-LasAI and GFP-LasAII fusion proteins improperly folded in the plant cells. Surprisingly, we observed the mitochondria aggregation in these infiltrated cells even though there was no detectable GFP expression. We speculate that LasAI and LasAII may have the ability to self-cleave and produce functional subunits targeted to the mitochondria similar to the autotransporter VacA from Helicobacter pylori [32]. Further investigations are underway to confirm this hypothesis. Collectively, these results suggest lasAI and lasAII from a Las prophage/phage (bacterial virus) can act as inducers of PTGS in plant cells. To the best of our knowledge, this is the first evidence of bacterial prophage/phage gene inducing PTGS in plants. Further characterization of the PTGS conferred by LasAI and LasAII may shed light on the evolution and adaptation of the Las bacterium.
With the aid of the gene silencing suppressor p19, we revealed that the LasA\textsubscript{I}, translocator domain-GFP fusion protein targeted to the mitochondria of tobacco leaf cells. Using the YASPIN secondary structure program, LasA\textsubscript{I} and LasA\textsubscript{II} were predicted to contain at least ten β-stranded structures in the translocator domain. It is worth noting that four β-strands in the YadA autotransporter of \textit{Yersinia} are sufficient for its mitochondrial localization in yeast [12]. Furthermore, several bacterial proteins without typical N-terminal signal sequences also target mitochondria [33], indicating that the lack of signal peptides in LasA\textsubscript{I} and LasA\textsubscript{II} is not exceptional.

Proteins containing tandem repeats are associated with diverse functions, and the variable numbers of tandem repeats affect the pathogenicity or antigenicity of several human and animal pathogens [34]. Deletions or insertions of these repeats within the \textit{lasA}\textsubscript{I} and \textit{lasA}\textsubscript{II} genes were reported in samples of distinct geographical origins and a single origin [17]. It is interesting that the tandem repeats of the LasA\textsubscript{I} and LasA\textsubscript{II} passenger domains contain characteristics of the LRR family of proteins. The LRR proteins are important for immune responses, adhesion, invasion, signal transduction, and DNA/RNA processing [35]. The LRR motif of these proteins forms a “horseshoe-shaped” molecule that provides a versatile scaffold for protein–protein interactions [36]. Several LRR proteins have been shown to be located on the cell surface and play a role in surface adherence and aggregation [37]. Compared to the tobacco cells expressing the GFP-LasA\textsubscript{I} translocator domain, which did not affect mitochondrial morphology, we observed mitochondrial aggregation in cells infiltrated with full length \textit{lasA}\textsubscript{I} or \textit{lasA}\textsubscript{II}. This phenomenon could be explained if the translocator domain was integrated into the mitochondrial outer membrane with the LRR passenger domain facing the cytosol, thus causing mitochondrial aggregation. By transmission electron microscopy, mitochondrial aggregation was observed in Las infected periwinkle, which agrees with our observation that mitochondrial aggregation is caused by LasA\textsubscript{I} and LasA\textsubscript{II} expression in tobacco. Most of the reported LRR proteins contain an N-terminal signal peptide for secretion across the bacterial membrane and a C-terminal membrane attachment region followed by a hydrophobic transmembrane [37]. In contrast, LasA\textsubscript{I} and LasA\textsubscript{II} lack the classical signal sequence but the translocator domain can export the LRR passenger domain across bacterial membranes. AdpC, a LRR protein lacking a signal peptide, was also reported to be located on the outer membrane surface when it was expressed in a heterologous \textit{E. coli} host [38]. Further investigation into whether LasA\textsubscript{I} and LasA\textsubscript{II} passenger domains target mitochondria are critical to understanding the functions of these proteins.

In conclusion, ‘Ca. Liberibacter asiaticus’ is an obligate, intracellular bacterium with a significantly reduced genome. We are the first to demonstrate that Las encodes two novel autotransporters (LasA\textsubscript{I} and LasA\textsubscript{II}) that target mitochondria when expressed in plant cells. Although the functions of these effectors remain to be elucidated, we hypothesize that Las encodes these autotransporters to modulate energy biosynthesis since Las may directly import ATP/ADP from the cytosol of host cell for its energy and biosynthesis [19]. On the other hand, these proteins may serve as suppressors for plant immune responses since Las encodes a functional flagellin that induces PAMP-triggered immunity in tobacco leaves [39]. Future work will focus on the functional elucidation of LasA\textsubscript{I} and LasA\textsubscript{II}, including an investigation into whether LasA\textsubscript{I} and LasA\textsubscript{II} passenger domains target mitochondria, identification of the eukaryotic binding partners, and characterization of protein structures. These studies will lead to a better understanding of Las pathogenesis, and thereby yield a better control strategy for HLB.

\section*{Materials and Methods}

\subsection*{Bacterial strains, plants and cultivation}

Strains and plasmids used in this study are listed in Table 1. \textit{Escherichia coli} Top10 (Invitrogen, Carlsbad, CA) was used as a host for plasmid construction and \textit{E. coli} BL21 (DE3) cells (Invitrogen, Carlsbad, CA) for recombinant protein expression. \textit{E. coli} was grown in Luria-Bertani (LB) medium at 37°C. \textit{Agrobacterium tumefaciens} strain GV2260 was cultured at 28°C in LB and used to mediate transient expression in the leaves of \textit{Nicotiana benthamiana}. Antibiotics were used at the following concentrations: carbenicillin, 50 µg/mL; kanamycin, 50 µg/mL.

\textit{N. benthamiana} seeds were stored at 4°C for 2 days prior to germination. Subsequently the seeds were germinated in chambers programmed for cycles of 16 h light and 8 h dark at 26°C. The seedlings were then transferred into FaFard 4P mix soil in plastic containers and grown in controlled greenhouse conditions.

\section*{Table 1. Strains and plasmids used in this study.}

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Western blotting was performed with a primary antibody was dissolved in SDS-PAGE buffer. The outer membrane protein was isolated on the basis of sarkosyl insolubility [43]. The purified protein was separated using one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue. Membrane transfer was performed by iBlot according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Western blotting was performed with a primary antibody against purified partial LasA (N-terminus, one repeat and part of the translocator domain) from an immunized mouse (ProMab Biotechnologies, Richmond, CA). Goat anti-mouse HRP-conjugated antibody was used as the secondary antibody and detected by chemiluminescence following the manufacturer’s instructions (Life Technologies, Carlsbad, CA).

Protein fraction preparation and Western blot

Whole-cell protein lysates, culture supernatant protein (CS), outer membrane protein (OM) and surface associated protein (AD) were prepared from \textit{E. coli} BL21 containing plasmid pET102-lasA, as previously described [11,41–43]. For the CS fraction, culture supernatants were filtered through 0.22 µm-pore size filters and concentrated approximately 100-fold by passage through Amicon centrifuge tubes with a molecular mass limit of 50 KDa (Millipore, Billerica, MA). The final pellet was dissolved in SDS-PAGE buffer. The outer membrane protein was isolated on the basis of sarkosyl insolubility [43]. Briefly the cells were collected and broken down by sonication. Total membrane proteins were separated by ultracentrifugation at 28000 rpm for 1hr at 4°C. To obtain the outer membrane protein, the pellet was suspended in 20mM Tris buffer (pH7.4) containing 0.5% Sarkosyl and centrifuged again at 28000 rpm for 1hr at 4°C. The final pellet was dissolve in SDS-PAGE buffer. To obtain the AD fraction, the protein secreted but remaining bound to the cell surface, and the cell pellets were suspended in PBS and N-hexadecane was added. The suspensions were vortexed and centrifuged, and the liquid phase was filtered. The proteins were precipitated by acetone and dissolved in SDS-PAGE buffer. Proteins were separated on SDS-PAGE and detected by Western blot as described above.

Immuno-dot blot and proteinase K treatment

Immuno-dot blotting was performed as described previously [39]. The protein expression from bacterial cells containing different constructs was induced as described above. Bacterial cultures were centrifuged at 2,000 g for 10 min at 4°C, washed three times with PBS and adjusted to a final concentration of 0.45 as determined by measurements of the optical density at 660 nm. Three microliters of each serial dilution (1:1, 1:5, 1:10 and 1:20) was spotted onto a nitrocellulose membrane in three replicates. The membrane was air dried and the Western blot procedure was performed as described above. The proteinase K treatment of intact cells was performed in PBS with 10 mM MgCl\(_2\) [44] and then detected by immuno-dot blot. The proteins, either untreated or treated with proteinase K, were recovered for SDS-PAGE analysis and Western blotting.

**Table 2. Primers used in this study.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td><strong>Primers for transient expression</strong></td>
<td></td>
</tr>
<tr>
<td>lasA_F</td>
<td>5’-GCCGATACATTTAGAAAAATACATG-3’</td>
</tr>
<tr>
<td>lasA_R</td>
<td>5’-ATTTGCTGAGTTATGCTAAGGAAAATATAC-3’</td>
</tr>
<tr>
<td>lasA_I</td>
<td>5’-ATGGATTCAGTACCTGAGGACATGAGG-3’</td>
</tr>
<tr>
<td>lasA_TD-F</td>
<td>5’-ATGAGAATCTAGAGCTGAGGACATGAGG-3’</td>
</tr>
<tr>
<td><strong>Primers for protein expression</strong></td>
<td></td>
</tr>
<tr>
<td>GFP-pET-F</td>
<td>5’-CACCATGCGGCAGGCGAGGAGCAAG-3’</td>
</tr>
<tr>
<td>GFP-pET-R</td>
<td>5’-TTACTCTAGCTAGCGGATCC-3’</td>
</tr>
<tr>
<td>lasA_F</td>
<td>5’-CACCATGATATATGAGGACACTAGAAATG-3’</td>
</tr>
<tr>
<td>lasA_R</td>
<td>5’-ATGACTGAAATGAGGACACTAGAAATG-3’</td>
</tr>
</tbody>
</table>

*Restriction enzyme sites are in italics and underlined*
the same way [31]. The final cell suspension with an OD_{600} of 1.0 was mixed with the p19 suppressor and infiltrated into 4 week-old N. benthamiana leaves with a 1 mL needleless syringe. The experiments were performed with ten independent replicates.

After two days of infiltration, the infiltrated zone was excised and the epidermal layers were peeled for mitochondrial staining with MitoTracker Red CMXRos according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA) and imaged using a confocal laser scanning microscope (CLSM), Zeiss LSM 510. GFP was detected with a 505-530 nm BP filter and MitoTracker syringe. The experiments were performed with ten independent replicates.

**Transmission electron microscopy (TEM)**

Mitrids were sampled from healthy and Las-infected periwinkle. The infiltrated zone was sliced from tobacco. The samples were fixed and sectioned for TEM micrographs as described previously [44].

**Supporting Information**

Figure S1. LasA1 protein fractions and GFP fusion proteins. A: Western blot of E. coli containing the pET102-

**References**


Las Encodes Two Novel Autotransporters


