The Type II Secreted Lipase/Esterase LesA is a Key Virulence Factor Required for Xylella fastidiosa Pathogenesis in Grapevines

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Pierce’s disease (PD) of grapevines is caused by Xylella fastidiosa (Xf), a xylem-limited gamma-proteobacterium that is responsible for several economically important crop diseases. The occlusion of xylem elements and interference with water transport by Xf and its associated biofilm have been posited as the main cause of PD symptom development; however, Xf virulence mechanisms have not been described. Analysis of the Xf secretome revealed a putative lipase/esterase (LesA) that was abundantly secreted in bacterial culture supernatant and was characterized as a protein ortholog of the cell wall-degrading enzyme LipA of Xanthomonas strains. LesA was secreted by Xf and associated with a biofilm filamentous network. Additional proteomic analysis revealed its abundant presence in outer membrane vesicles (OMVs). Accumulation of LesA in leaf regions associated positively with PD symptoms and inversely with bacterial titer. The lipase/esterase also elicited a hypersensitive response in grapevine. Xf lesA mutants were significantly deficient for virulence when mechanically inoculated into grapevines. We propose that Xf pathogenesis is caused by LesA secretion mediated by OMV cargos and that its release and accumulation in leaf margins leads to early stages of observed PD symptoms.

Xylella fastidiosa (Xf) is a fastidious, xylem-limited gamma-proteobacterium that causes several economically important diseases in crop plants, including grapevine, citrus, periwinkle, almond, oleander, and coffee1,2. In the field, Xf is vector-transmitted by various xylem sap-feeding sharpshooter insects3,4. The Xf subspecies fastidiosa (Xff), exemplified by the California strain Temecula1, causes Pierce’s disease (PD) in grapevine. PD poses a great threat to the winegrowing regions of California5. However, the Xf life cycle and virulence mechanism are not entirely understood2. A characteristic PD symptom is marginal leaf chlorosis progressing to necrosis (leaf scorch). Three general explanations for PD symptom development have been proposed: (i) occlusion of xylem elements and interference with water transport by Xf and its associated biofilm, (ii) plant systemic responses, e.g., growth regulator imbalance, and (iii) Xf-generated phytotoxin5. The occlusion hypothesis extends from early observations of xylem element blockage in PD6. Generally, plant species and regions of the plant body that show the most severe symptoms are those with the greatest proportion of colonized vessels3,7–11, in agreement with the occlusion hypothesis. PD and other Xf diseases are associated with decreased leaf water potential12 and altered carbon isotope incorporation13, also consistent with water stress. Although the occlusion hypothesis widely considered to be supported, other observations are inconsistent with this explanation. The symptoms of wilting and PD are not

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similar and are additive in effect, Xf accumulation in grapevine leaves does not correlate with local PD symptom severity, and in some infections of grapevine and other Xf hosts, severe necrotic symptoms are associated with only minimal vessel occlusion\(^5\).

The secretion of virulence factors by pathogens is an important trigger mechanism for many plant diseases. Unlike closely related pathogens from the genus *Xanthomonas*, *Xff* lacks the type III secretion system (T3SS)\(^14\). However, *Xanthomonas* and *Xf* have in common a similar type II secretion system (T2SS) for a battery of important extracellular enzymes involved in nutrient acquisition and virulence\(^15\). In *Xff*, genes have been identified that code for plant cell wall degrading enzymes (CWDEs) such as polygalacturonase, cellulase, and proteases\(^14,16,17\). These enzymes may aid *Xff* migration inside xylem vessels by degrading the pit membrane and releasing the carbohydrates necessary for bacterial growth and survival\(^17\). Cell wall degradation by CWDEs releases oligosaccharides as a bacterial nutrient source, which can also induce potent plant innate immune responses leading to cell death. Such plant defense responses include production of phytoalexins, fortification of cell walls through callose deposition, oxidative burst, and induction of programmed cell death\(^18–20\).

We report here our analysis of the *Xff* Temecula1 secretome, including comparison with the bacterial surfaceome and outer membrane proteomes. The uncharacterized protein PD1703 was identified as the most abundant secreted protein and its characteristics and likely participation in *Xff*-initiated symptoms of PD are discussed.

**Results**

**Lipases are a highly abundant component of the Xff secretome.** The bacterial proteome was investigated to characterize the subcellular localization of *Xff* proteins as described (Fig. 1A). The identities of soluble supernatant proteins (SSPs) in culture medium were determined after removing bacterial cells and cell fractions through a series of ultracentrifugation and concentration steps. Twenty-four SSPs were identified by LC-MS/MS in the culture supernatant (Table 1). These include many potential plant cell wall-degrading enzymes (CWDEs) such as polygalacturonase, cellulase, and proteases\(^14,16,17\). Mass spectrometry analysis of this particular band revealed two 42 KDa (PD1703, PD1702) proteins and one 46 KDa (PD1211) putative uncharacterized protein.
Table 1. X. fastidiosa Temecula 1 soluble supernatant proteins (SSPs) identified in the secretome. *Protein accession number at UniProt Knowledgebase (UniProtKB; http://www.uniprot.org/). †Protein localization as predicted by PSORTb v. 3.0.2 Subcellular Localization Prediction Tool (http://www.psort.org/psortb/; C: cytoplasmic; OM: outer membrane; P: periplasmic; EC: extracellular; U: unknown). ‡Theoretical protein molecular weight shown in kDa. §Position of signal peptide cleavage site as predicted by SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/). □Prediction of non-classical protein secretion by SecretomeP 2.0 Server (http://www.cbs.dtu.dk/services/SecretomeP/) used for proteins without signal peptide. Score >0.5 indicates non-classical secretion.

The high amino acid sequence homology of these three proteins allowed us to estimate their relative abundance from their numbers of assigned spectra (Fig. S1). PD1703 (64.5%; 783) was more abundant in the secretome than PD1702 (34.2%; 268) and PD1211 (1.3%; 10) (Fig. 1B). In silico analysis predicted that PD1703 is a secreted lipase/esterase protein with high sequence similarity to the cell wall-degrading enzyme LipA from Xanthomonas oryzae pv. oryzae (Xoo) and Xanthomonas campestris pv. vesicatoria (Xcv). To confirm the lipase/esterase functions of PD1703, we evaluated the enzymatic activity of Xff SSPs against short- and long-chain substrates. As reported for Xoo LipA, Xff SSPs degraded p-nitrophenyl butyrate and the short-chain triacylglyceride tributyrin (C4), but not the long-chain triacylglyceride triglycerin (C10) (Fig. S2).

**Xff secretes LesA as cargo in outer membrane vesicles.** Gram-negative bacteria produce outer membrane vesicles (OMVs) that contain biologically active proteins and perform diverse biological functions. Production of OMVs by Xff was recently reported elsewhere. The complete OMV protein cargo has been described for many Gram-negative bacterial species, including Xff’s closely related pathogen Xanthomonas campestris pv. campestris (Xcc) but not for Xff. The OMV fraction was negatively stained and examined using transmission electron microscopy (TEM) (Fig. 2A), revealing the expected vesicles. Our investigation of OMV protein cargo by LC-MS/MS identified 11 proteins (Table 2). Interestingly, the most abundant SSP, LesA (PD1703), was also part of the OMV cargo. Immunoblot analysis confirmed the localization of Xff LesA in the secretome and OMV proteome (Fig. 2C). In addition to LesA, the secreted proteins PD1702, PD0731 and PD1427 were identified in the OMV cargo, indicating that delivery of these proteins in the host could also be mediated by OMVs. Four outer membrane proteins (PD1709, PD1807, PD0313 and PD1283) were identified in the Xff OMV proteome. We previously identified MopB (PD1709) as the major Xff outer membrane protein (OMP) PD1807 and PD1283 were also found in our OM preparation (Table S2). The presence of MopB in both the XffOM and OMV was confirmed by immunoblot (Fig. 2D). The elongation factor Tu (EF-Tu), which is a PAMP in many gram-negative bacteria, was abundant in the Xff outer membrane fraction, but was not identified in Xff secretome or as part of the OMV cargo (Table S2; Fig. 2E).
LesA is localized in the secreted filamentous network. To visualize the distribution pattern of Xff-secreted material, we examined bacterial cells in culture using scanning and transmission electron microscopy (SEM and TEM). Negatively stained cell clusters show that Xff produces cell aggregates embedded in a dense secreted material possibly composed of extracellular polysaccharide (EPS) and proteins that are weakly attached to the bacterial surface (Fig. 3A). The same dense material surrounds planktonic cells (Fig. 3B). SEM analysis of the bacterial culture revealed that Xff secretes a filamentous network of unknown composition similar to the matrix seen surrounding cells aggregates in the xylem vessels of infected grapevines (Fig. 3C,D). TEM analysis of the secreted matrix revealed its close localization to Xff cells in vitro (Fig. 3E). Immunogold labeling and TEM revealed that LesA is embedded in the Xff secreted network (Fig. 3F).
LesA accumulates abundantly in leaf regions with minimal Xff titer and is associated with early stages of PD symptom development. Although LesA accumulated in both OMVs and the secreted matrix of Xff cells, its localization in Xff-infiltrated host tissue was unknown. To search for LesA and other potential secreted virulence factors in the infected host, we compared the total leaf proteome of Xff-infected and non-infected grapevines. Of the 524 proteins found, six were of Xff origin (Table 3). LesA (the most abundant secreted protein) and MopB (the most abundant outer membrane protein) were identified in infected host tissue. The surface protein Hsf (PD0744) was found in infected grapevine leaves, although it was not previously identified in the secretome of cultured Xff. This suggests that both expression and secretion of Hsf may be triggered during infection of grapevine leaves. As expected, no Xff protein was found in non-infected grapevine leaves.

The accumulation of LesA in Xff-infected host tissue confirmed our hypothesis that LesA is highly expressed and secreted not only in vitro but also during the Xff infection/colonization process. We postulated that LesA has an important role in Xff pathogenesis in grapevines similar to that seen in Xoo, where it elicits callose deposition and programmed cell death in rice

LesA elicits a hypersensitive response in grapevine. LipA from Xoo elicits an innate immune response in rice mediated by cell wall degradation, induces callose deposition, and triggers programmed cell death

LesA is required for virulence of X. fastidiosa in grapevines. To test whether LesA has a role in Xff virulence, we generated a lesA mutant using homologous recombination and compared the performance of both proteins after inoculation in grapevines. Our Xff lesA mutant lacked lipase and esterase activities and expresses no LesA protein (Fig. 6C). Interestingly, the Xff lesA mutant was predominantly found in the biofilm mode of growth when cultivated in liquid media, unlike the wild-type strain (Fig. 6D), and formed large cell

Table 2. Proteins identified in X. fastidiosa Temecula 1 outer membrane vesicle (OMV) proteomic analysis. *Protein accession number at UniProt Knowledgebase (UniProtKB; http://www.uniprot.org/). †Protein localization as predicted by PSORTb v. 3.0.2 Subcellular Localization Prediction Tool (http://www.psort.org/psortb/). OM: outer membrane; EC: extracellular; U: unknown. ‡Theoretical protein molecular weight in kDa.

| Accession number | Protein description                      | Gene name | SSP | Surface | OM | Prot. local.† | Theor. Mw‡ | Sequence coverage | Matched peptides |
|------------------|-----------------------------------------|-----------|-----|---------|----|---------------|------------|------------------|-----------------|
| Q87DF4           | Outer membrane protein XadA             | PD0731    | √   | √       | —  | U             | 97.5       | 55.0%            | 61              |
| Q87AW0           | Putative uncharacterized protein        | PD1703    | √   | √       | —  | U             | 46.4       | 67.0%            | 22              |
| Q87AW1           | Putative uncharacterized protein        | PD1702    | √   | √       | —  | U             | 42.7       | 63.0%            | 11              |
| Q87AL6           | Outer membrane protein ompW             | PD1807    | √   | —       | √  | OM            | 31.1       | 19%              | 6               |
| Q87AV4           | Outer membrane protein mopB             | PD1709    | √   | √       | √  | OM            | 42.3       | 15%              | 5               |
| Q87AA4           | Fimbrial protein                        | PD1924    | —   | √       | √  | EC            | 15.3       | 15%              | 2               |
| Q87BM1           | Bacteriocin                             | PD1427    | √   | √       | —  | EC            | 150.3      | 8%               | 7               |
| Q87EJ4           | Serine protease                         | PD0313    | √   | —       | —  | OM            | 101.3      | 4%               | 4               |
| Q87C13           | TonB-dependent receptor                 | PD1283    | √   | —       | √  | OM            | 102.7      | 4%               | 3               |
| Q87C82           | Putative uncharacterized protein        | PD1211    | √   | √       | —  | U             | 46.4       | 6%               | 2               |
| Q87BF0           | Hemolysin-type calcium binding protein  | PD1506    | √   | —       | √  | OM            | 164.2      | 4%               | 2               |
Figure 3. Electron microscopy of Xff cells and the secreted filamentous network. (A) Negative staining of Xff aggregates showing abundant secreted material in which bacterial cells (white arrow) are embedded. (B) Closer view of the secreted material surrounding planktonic Xff cells isolated from the same culture shown in A. (C,D) Scanning electron microscopy of Xff showing bacterial cells surrounded by the secreted filamentous network (white arrows). (E,F) Immunogold detection of LesA in the secreted filamentous network (black arrows) surrounding Xff cells (E). LesA was abundant in the secreted network as shown in F (black arrows).
aggregates (Fig. 6E). The inability of the lesA mutant to elicit typical PD symptoms was confirmed by significantly reduced symptomatic leaves (50%; \( p < 0.05 \)) observed in infected grapevines compared with plants inoculated with the parental strain (Fig. 6F).

Wild-type \( Xff \) and its quorum-sensing mutants have distinct patterns of expression for lesA. Cell–cell signaling plays an important role in the virulence of many plant pathogenic bacteria. \( Xff \) produces the quorum-sensing signaling molecule DSF (diffusible signaling factor) that regulates bacterial pathogenesis. In \( Xanthomonas campestris \) pv. \( campestris \) (\( Xcc \)) and \( Xanthomonas oryzae \) pv. \( oryzae \) (\( Xoo \)), DSF-deficient mutants have reduced virulence.\(^{33,34} \) \( Xff \) \( rpfF \) mutants deficient in DSF production possess a hypervirulent phenotype when inoculated into grapevines.\(^{30} \) The \( rpfF \) gene is required for DSF production in both \( Xff \) and \( Xanthomonas \) species.

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**Table 3.** \( X. fastidiosa \) Temecula 1 proteins found in infected grapevine leaf proteomic analysis. \(^a\)Protein accession number at UniProt Knowledgebase (UniProtKB; http://www.uniprot.org/). \(^b\)Protein localization as predicted by PSORTb v. 3.0.2 Subcellular Localization Prediction Tool (http://www.psort.org/psortb/; C: cytoplasmic; OM: outer membrane; U: unknown). \(^c\)Theoretical protein molecular weight in kDa.

| Accession number\(^a\) | Protein description | Gene name | SSP | Surface | OMV | OM | Prot. local.\(^b\) | Theor. Mw\(^c\) | Sequence coverage | Matched peptides |
|-------------------------|--------------------|-----------|-----|---------|-----|-----|-----------------|----------------|-----------------|-----------------|
| Q87AW0 | Putative uncharacterized protein | PD1703 | √ | √ | — | — | U | 42.4 | 19.6% | 7 |
| Q87AV4 | Outer membrane protein MopB | PD1709 | √ | √ | √ | — | OM | 42.3 | 11.1% | 3 |
| Q87BC0 | 60 kDa chaperonin | PD1538 | — | — | — | — | U | 57.7 | 6.2% | 2 |
| Q87B34 | Glyceraldehyde-3-phosphate dehydrogenase | PD1626 | — | — | — | — | OM | 36.0 | 5.0% | 2 |
| P63774 | 10 kDa chaperonin | PD1537 | — | — | — | — | C | 10.0 | 25.3% | 2 |
| Q87DE1 | Surface protein | PD6744 | — | — | — | — | U | 203.1 | 1.46% | 2 |

**Figure 4.** Detection of LesA movement in grapevine leaves. (A) Grapevine leaf showing the three segments, each 1 to 2 cm in the radial direction, used to detect LesA: I (inside), M (middle) and O (outside). (B) Viable \( Xff \) cell assay of leaf segment \( n = 3 \) extracts revealed ~10-fold reduction in the bacterial population at the leaf edges (outside) than at the center (inside). The bacterial titration was carried out using a standard curve of five dilutions (Fig. S4; \(^*\)One-way ANOVA test; \( R^2 = 0.9817; p < 0.01 \)). (C,D) The amount of LesA detected in grapevine leaves differs slightly in the inside and outside segments (C) (**Mann-Whitney test; \( p = 0.1 \)). However, the relative abundance of LesA per number of \( Xff \) cells (D) is significantly greater in the outside area than in middle and inside segments (**One-way ANOVA test; \( p < 0.05 \)), suggesting that the secreted LesA is moving from the \( Xff \)-crowded inside area to the symptomatic leaf extremities where the bacteria is scarcely present. This experiment was conducted twice with similar results. Picture of leaf was taken by Rafael Nascimento.
Interestingly, Xff rpfC mutants overproduce DSF and have a hyperattachment phenotype, which makes them deficient in virulence and movement in grapevine xylem vessels. For specific virulence-related genes, messenger RNA accumulation for rpfC and rpfF mutants and the wild type was assessed by RT-PCR. The lesA gene was downregulated in the rpfC mutant (Fig. 7).

**Agrobacterium harboring X. fastidiosa LesA is hypervirulent.** To assess whether LesA increases A. tumefaciens virulence, we expressed the Xff lesA gene under the control of its own promoter for an in-planta virulence assay (Fig. 8). When inoculated into walnut plants, which are highly susceptible to this pathogen, the infection is usually localized to a region of tumor formation at the site of infection; however, *Agrobacterium* harboring Xff LesA displayed a systemic necrotic response. The first signs of necrosis appeared in walnut plants inoculated with Agro-A281-LesA at eight weeks post inoculation, but not in plants that were mock-inoculated (PBS) or those that contained an empty vector (Fig. 8A,B). Interestingly, walnut plants inoculated with Agrobacterium expressing Xff LesA were dead at 12 weeks post inoculation (Fig. 8C). When inoculated into grapevines, the same phenotype was not observed (data not shown).

**Discussion**

PD symptom development has long been thought to result from blockage of xylem vessels by *Xf* biofilm and associated gels and tyloses, which leads to water stress in the distal parts of the infected plant and to PD. Our results support an alternative mechanism independent of water stress: a phytotoxic effect resulting from the action of a Xff enzyme, the LesA lipase/esterase encoded by the PD1703 locus. Xff LesA is similar to the type II secreted protein LipA, present in all sequenced xanthomonad genomes, and in many other Gram-negative bacteria. LipA from the rice pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a 42 kDa α/β hydrolase fold protein with lipase/esterase function and short-chain specificity. Recently, LipA from *Xoo* was characterized as a cell wall-degrading enzyme with a carbohydrate-binding domain essential to the protein’s virulence function. In *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*), LipA is expressed from an early stage of tomato leaf infection and is required for wild-type virulence. Additionally, treatment with *Xoo* LipA elicited callose deposition and programmed cell death in rice leaves and roots and an active site serine-to-alanine substitution reduced but did not completely suppress virulence.

Is the Xff lipase/esterase secretion associated with the PD pathogenic process? The preponderance of evidence seems to support this hypothesis. The SSP profile identified LesA as the most abundantly secreted protein. The Xff SSP preparations cleaved p-nitrophenol butyrate and tributyrin, but no long chain triacylglycerides (Fig. S2A,B).
identical to the action of the type II secreted lipase LipA. The short-chain specificity of LesA indicates that this enzyme has both esterase and lipase activities.

Figure 6. *X. fastidiosa lesA* mutants are deficient in virulence. (A) Lipase activity was analyzed around *Xff lesA* mutant (*lesA*) and WT (Tem1) colonies grown in PD3-agar medium supplemented with 1% trybutirin. (B) Esterase activity of proteins from *lesA* and WT by 4-methylumbelliferyl butyrate (4-MUB) assay. Esterase activity is diminished significantly in the *lesA* mutant. (C) Immunoblot detection of LesA in *Xff lesA*− and WT total proteins. The LesA protein was not detected in the mutant, confirming the mutagenesis of the *lesA* gene. (D) Analysis of biofilm formation in liquid culture, showing the predominance of the biofilm growth mode in *lesA*− when compared to WT. (E) Scanning electron micrographs of *Xff* culture showing the aggregation of *lesA*− cells typically found in biofilms. (F) Pierce's disease symptoms in grapevines. Thompson Seedless infected with the wild-type strain Temecula1, the *lesA*− strain, and uninfected (mock inoculated). Data is shown as the difference from uninfected controls (average of 26 plants). Plants inoculated with *Xff lesA*− strain have significantly less PD symptoms (*One-Tailed T-test with Welch's correction and p value of 0.0351) than Temecula1 infected plants. Pictures were taken by Hossein Gouran.

Figure 7. LesA is down regulated in an *Xff* quorum-sensing mutant. Comparison of RNA accumulation in mutants and wild-type by RT-PCR revealed that *lesA* is regulated by *rpfC*. RNA was extracted from *Xff* cells grown in PD3 medium (three flasks/condition; 10⁷–10⁸ cells/mL) at 28 °C and 120 rpm. The 16S rRNA gene was used as an endogenous control. Unpaired t test with Welch's correction was used for statistical analysis (one-tailed; *p* < 0.01).
An association is suggested not only by the presence of homologs of PD1703 in Xoo- and Xcv-infected rice\textsuperscript{21,31,36} and tomato\textsuperscript{22}, respectively, but also by its absence from Xf strain EB92-1, found in elderberry. EB92-1 infects and survives in grapevines for many years but does not cause symptoms and provides effective biocontrol against Xff. A genome draft of the EB92-1 strain revealed that 10 potential pathogenicity effectors were missing, including LesA (PD1703) and another predicted type II secreted enzyme present in our SSP profile, the serine protease PD095637,\textsuperscript{38} On the other hand, lipase/esterase genes of Xff are present in the genome of an Xf virulent strain responsible for citrus variegated chlorosis (CVC). The CVC 9a5c strain genes XF0357, XF0358 and XF2151 are apparent homologs of the Xff Temecula1 genes PD1703 (LesA), PD1702 and PD1211, respectively. In a previous study, the corresponding CVC proteins were not reported in the Xff 9a5c secretome, possibly because the secreted protein fraction was underrepresented due to depletion by the cell washing procedure used\textsuperscript{16,39}. We cannot eliminate the possibility of different secreted virulence factors among various Xf subspecies; however, LesA could also play a role in Xff 9a5c pathogenesis.

Our proteomic analysis (Table 3) revealed that LesA is one of six Xff proteins identified in infected grapevine leaves, clearly indicating that LesA is abundantly secreted by Xff during plant infection/colonization in addition to in liquid culture (Tables 1,2). If LesA is partially responsible for PD symptoms, there should be a spatial association of LesA with the leaf symptom pattern of radial transition from green tissue around the point of petiole attachment to scorching that begins at the leaf margins (Fig. 4A). Previous findings\textsuperscript{32} showed a gradual decrease in Xff titer proceeding from the leaf blade center to the leaf margin, counter to the pattern of symptom development. We have demonstrated that on a per-Xff-cell basis, LesA accumulates most abundantly near the leaf margins in a distribution positively correlated with the development of scorch symptoms (Fig. 4D).

As a direct demonstration of the ability of LesA to induce symptoms, LesA was produced in E. coli and a crude extract was pressure-infiltrated into grapevine leaf blade, inducing local necrosis. The empty-vector control did not induce necrosis and the catalytic triad control, S200A-LesA, induced only minute regions of necrosis (Fig. 5), showing further similarity to Xoo LipA\textsuperscript{21}. The weak reaction of grapevine leaf to the extract from E. coli expressing Xff S200A-LesA may result from residual lipase/esterase activity. Another serine esterase retained a few percent of the wild-type activity in an active site serine-to-alanine replacement mutant\textsuperscript{40}.

The co-localization of LesA and leaf marginal necrosis at a distance from the central leaf region of greatest Xff cell accumulation raised questions about LesA secretion and translocation. Two broad hypotheses were considered:

**Figure 8. Virulence assay in walnut plants.** Xff lesA gene was expressed in A. tumefaciens under the control of its own promoter. Agrobacterium harboring Xff LesA became hypervirulent. Walnut plants inoculated with agrobacterium expressing Xff LesA (Agro-A281-LesA), but not from the mock inoculation (PBS) and empty vector (A,B), presented the first signs of disease at eight wpi and were dead at 12 wpi (C). Pictures were taken by Hossein Gouran.
such as LesA.

Xff gacA mutants expressed less LesA and developed significantly less severe disease symptoms when inoculated with less virulent strains and growth conditions.

Material and Methods

**Xff strains and growth conditions.** The WT Temecula1 strain of Xylella fastidiosa subspecies fastidiosa (Xff, ATCC 700964) and Xff lesA mutant were grown in PD3 medium\(^6\) with aeration (120 rpm) at 28 °C. Plate cultures were prepared in the same medium with the addition of 1.5% agar. PD3 medium was supplemented with kanamycin (75 μg/mL) for selective growth of the lesA mutant. The rpfF and rpfC strains used in this study were kindly provided by Prof. Steven E. Lindow.

Isolation of secreted proteins and outer membrane vesicles from culture supernatants.** Xff cells were harvested by centrifugation at 8000 × g for 15 min at 4 °C. The culture supernatant was transferred to 38.5 mL tubes and centrifuged at 38,000 × g for 1 h at 4 °C in a SW28 rotor (Beckman Coulter, USA). The supernatant was collected (the remaining pellet was discarded), transferred to 12 mL tubes and centrifuged at 150,000 × g for 3 h...
at 4 °C (SW41 Ti rotor, Beckman Coulter). The supernatant, containing SSPs, was concentrated 75 to 100× using Amicon Ultra-15 3 K filters units (Millipore). The pellet, containing OMVs, was resuspended in 300μL PBS (pH 7.4) for subsequent SDS-PAGE analysis. For electron microscopy analysis, the vesicle pellet was resuspended in 50 mM HEPES buffer (pH 6.8).

**Outer membrane and total protein extraction.** Outer membrane protein extraction was performed as described27. To prepare total protein extracts, bacteria from a 2 mL culture (four to six days old) were harvested by centrifugation at 8000 × g for 15 min at 4 °C and washed three times with 1 mL washing buffer containing 10 mM Tris-HCl (pH 8.8), 3 mM KC1, 50 mM NaCl, 5 mM EDTA and 1 mM PMSF and were centrifuged for 2 min at 3000 × g. The pelleted cells were then lysed with 200μL 10 mM Tris (pH 8.8) with 0.5% w/v SDS, 5 mM EDTA, and 1 mM PMSF. After adding DTT to 100 mM, the sample was boiled 3 min and stored at −80 °C.

**Bacterial surface digestion.** *Xff* cells were harvested by centrifugation at 8000 × g for 15 min at 4 °C and washed three times with 1 mL PBS. A total of 4 × 10⁸ cells were resuspended in 100μL filtered and sterilized 50 mM ammonium carbonate buffer (pH 7.5). Digestions were carried out with 20 μg sequencing grade modified trypsin (Promega) in the presence of 5 mM DTT for 15 min at 37 °C. The digestion mixture was centrifuged at 3500 × g for 10 min at 4 °C and the supernatant (containing the peptides) was collected. The trypsin reaction was stopped by adding formic acid to 0.1%. The reaction was filtered using Amicon Ultra Microcon 3 K filters units (Millipore), and the flow-through peptides were held at −20 °C for later analysis.

**Grapevine leaf protein extraction.** Grapevine (*Vitis vinifera* L. cv. ‘Thompson Seedless’) leaves from *Xff*-infected (n = 5) and non-infected (n = 5) plants were collected about one meter above the point of inoculation (12 wpi). Plant tissues were flash frozen in liquid nitrogen, lyophilized and kept at −80°C. Proteins were extracted using a phenol extraction procedure as described61. Each leaf sample (Fig. 4A) was ground in liquid nitrogen using a pestle and mortar containing 1% (w/w) PVPP. One hundred mg plant material was resuspended in 600μL extraction buffer (0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl pH7.5, 0.5 M EDTA, 1 mM PMSF and 2% β-mercaptoethanol). The suspension was homogenized three times (1 min each) using a MM300 TissueLyser (Qiagen). An equal volume of UltraPure buffer-saturated phenol (Life Technologies, USA) was added and the mixture was rehomogenized as described above. After centrifugation at 12,000 × g for 15 min at 4°C, the upper phenol phase was removed and the remaining pellet used for re-extraction in extraction buffer. Proteins were precipitated from the phenol phase using five volumes of 100 mM ammonium acetate in methanol overnight at −20°C followed by centrifugation at 12,000 × g for 15 min at 4°C. Protein pellets were washed four times with 4 mL 100 mM ammonium acetate in methanol and dried 10 min in the hood. Proteins were solubilized with urea buffer (7 M urea, 2 M thiourea, 40 mM Tris, 2% Chaps and 18 mM DTT). The protein concentration was determined according to Bradford’s method using BSA as the standard.

**Protein preparation and mass spectrometry analysis.** *Xff* SSPs and OMV proteins were resolved by SDS-PAGE prior to in-gel digestion to reduce the amount of non-protein contaminants in the samples. Peptides from the cell shaving (surfacome) procedure were desalted using Aspire RP30 desalting tips (Thermo-Fisher Scientific) and subjected directly to LC/MSMS analysis. For the in-gel digestion used for SSPs and OMV proteome analysis, gel pieces were washed twice with 100μL 50 mM ammonium bicarbonate (AmBic; pH 8.0), followed by dehydration with acetonitrile (ACN; three to four times the total volume of gel pieces) for 10 to 15 min. Proteins were reduced for 30 min at 56°C in a solution of 10 mM DTT and 50 mM AmBic. Gel pieces were dehydrated again, followed by replacement of ACN by 55 mM iodoacetamide in 50 mM AmBic. Gel pieces were incubated 20 min in the dark at room temperature, followed by two washes with 150 to 200μL of 50 mM AmBic for 15 min each. Gel pieces were dehydrated with ACN, dried by speed vacuum centrifugation and subjected to trypsin digestion overnight. Peptides were extracted by adding 60% ACN and 0.1% trifluoroacetic acid (TFA) in water, to the gel pieces, followed by sonication for 10 min. The solution containing the peptides was mixed with the supernatant resulting from the tryptic digestion, followed by speed vacuum centrifugation. Digested peptides were then desalted using Aspire RP30 desalting tips and resuspended in loading buffer.

The digested peptides were analyzed using a QExactive mass spectrometer (Thermo Fisher Scientific) coupled with an Easy-LC (Thermo Fisher Scientific) and a nanospray ionization source. The peptides were loaded onto a trap (100 micron, C18 100 Å 5U) and desalted online before separation using a reverse phased column (75 micron, C18 200 Å 3U). The gradient duration for separation of peptides was 60 min using 0.1% formic acid and 100% ACN for solvents A and B respectively. Data was acquired using a data-dependent ms/ms method with a full scan range of 300 to 1600 Da and a resolution of 70,000. The ms/ms method's resolution was 17,500 with an isolation width of 2 m/z with normalized collision energy of 27. The nanospray source was operated using 2.2 KV spray voltage and a heated transfer capillary temperature of 250°C. Raw data was analyzed using X!Tandem and visualized using Scaffold Proteome Software (Version 3.01). Samples were searched against Uniprot databases appended with the cRAP database, which recognizes common laboratory contaminants. Reverse decoy databases were also applied to the database prior to the X!Tandem searches.

For the grapevine proteomic analysis, leaf proteins were precipitated using a ProteoExtract protein precipitation kit (Calbiochem) followed by dehydration overnight in a sterile fume hood. The protein pellet was resuspended in 50 mM AmBic (pH 8.0) and subjected to an in-solution tryptic digestion. Digested peptides were then desalted and subjected to LC/MSMS as described above.

**Electron microscopy analysis of outer membrane vesicles.** Outer membrane vesicles were resuspended in 50 mM HEPES buffer (pH 6.8) and fixed with 4% paraformaldehyde in 1 M Sorenson’s phosphate buffer (pH 7.4). Copper grids (400 mesh) supported with formvar coating were used for electron microscopy. Ten μL fixed
OMVs were placed in the grids and allowed to settle for 10 min. The excess sample was removed with filter paper, followed by quickly staining with 1% ammonium molybdate. Grids were air-dried completely before visualization in a Philips CM120 (FEI/Philips Inc.) electron microscope at 80 KV.

**Western-blot analysis.** To detect LesA, anti-LesA antibody was diluted in PBS-M 1% (PBS plus 1% non-fat dried milk; 1:1000), followed by detection using HRP-conjugated goat anti-rabbit antibody (1:2000 for grapevines and 1:4000 for Xff samples) (Life Technologies, USA). Blocking and washing steps were performed with PBS-M 5% (PBS plus 5% non-fat dried milk) and PBS-T 0.1% (PBS plus 0.1% Tween 20), respectively. Developments were carried out using ECL Plus western blotting detection reagents (GE Life Sciences, USA). To detect MopB and EF-Tu in Xff samples, polyclonal antibodies (dilutions of 1:20,000 for MopB and 1:10,000 for EF-Tu) were used, followed by detection using HRP-conjugated goat anti-rabbit antibody (1:20,000). Blocking and washing steps were carried out as described for LesA. Blots were developed using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, USA). The anti-LesA polyclonal antibody was generated by immunizing rabbits with LesA immunogenic synthetic peptides (GeneScript, USA). The anti-MopB and anti-EF-Tu polyclonal antibodies were kindly provided by George Bruening (Plant Pathology, UC Davis).

**Immunogold electron microscopy.** Immunogold electron microscopy (IEM) was performed using fresh cultures of Xff. The sample containing Xff cells and the filamentous network secreted in the culture supernatant were fixed with 4% paraformaldehyde in 1 M Sorenson's phosphate buffer (pH 7.4). The fixed sample was embedded in LR White resin as described. Ultra-thin sections were cut and placed onto coated grids (200 mesh; treated with glow-discharge), followed by blocking with 1% fish gelatin for 30 min. Grids were blotted with anti-LesA (1:1500) antibody for 1 h at RT and washed with PBS. The primary antibody was detected using anti-rabbit (1:50) antibody coupled to 10 nm gold particles. Unbound conjugate was removed using a sequence of washing steps with PBS. The preparation was negatively stained with 1% ammonium molybdate, air-dried, and visualized in a Philips CM120 (FEI/Philips Inc.) electron microscope at 80 KV.

**Lipase and esterase activity assays.** Tributyrin (C4), tricaprin (C10), and a mixture of tryglycerides (C2 to C10) (Sigma-Aldrich, USA) were used as substrates for LesA activity in a plate assay. The triglyceride substrates (0.5%; v/v) were prepared in a buffer containing 100 mM Tris-HCl (pH 8.0), 25 mM CaCl2, sonicated at 30 W for three min to emulsify the substrates, mixed with an equal volume of 2% agarose solution and solidified in Petri plates. Fifty μL Xff soluble supernatant proteins (700 ng/μL) were added to the wells and assayed for a zone of clearance for 24 to 48 h at RT. The culture medium PD3 was used as a negative control. For the Xff mutant lipase activity assay, tributyryl (3%; v/v) was emulsified in PD2 agar medium using a Polytron PT 3100 prior to autoclaving. Esterase activity was also determined using 4-methylumbelliferyl butyrate (4-MUB) substrate as previously described. For the SSPs esterase activity assay, pNP butyrate (pNP-C4) was used as the substrate in a spectrophotometric assay, which was performed at A405 after a 10 min incubation of pNP-C4 (1 to 5 mM) with Xff soluble supernatant proteins (1 μg/well) in 50 mM Tris-HCl (pH 7.5) at 37°C.

**LesA detection in grapevine leaves by ELISA.** To detect LesA in grapevine (Vitis vinifera L. c.v. ‘Thompson Seedless’) by ELISA, leaves from Xff-infected (12 wpi) and non-infected plants were collected and divided in three sections (inside, middle and outside; ~2 cm radial dimension each). Ninety mg leaf tissue was homogenized in 900 μL coating buffer (0.1 M sodium carbonate buffer, pH 9.6) for three min using a MM300 TissueLyser (Qiagen, USA). The number of Xff cells present in each homogenized sample was determined using a double-antibody sandwich (DAS)-ELISA assay (Agdia Inc., USA) following manufacturer’s instructions. For LesA detection, the homogenized solution was used to coat (100 μL/well) a ninety-six-well Maxisorp microtiter plate (NUNC, USA) for two h at RT. The wells were washed two times with PBS-T 0.1% (PBS plus 0.1% Tween 20) and blocked for one h at RT with PBS-M 5%. The plate was washed three times with PBS-T 0.1%, followed by incubation with anti-LesA (1:1000) antibody in PBS-M 1% for one h at 37°C. The plate was washed three times with PBS-T 0.1% followed by incubation with HRP-conjugated anti-rabbit (1:1000) in PBS-M 1% for one h at 37°C. The plate was washed four times with PBS-T 0.1% and developed with TMB (3,3’,5,5’-tetramethylbenzidin e). One-way ANOVA and Mann-Whitney tests were carried out using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). A value of p < 0.05 was considered statistically significant.

**Hypersensitive response assay in grapevine leaves.** The heterologous expression of wild type LesA and its mutated version, S200A-LesA, in which the amino acid Ser-200 of the active triad was replaced by alanine, was accessed after cloning both genes in the pJexpress 401 (DNA2.0, USA) vector for expression under the T5 promoter. The insertion of cloned genes was verified by PCR using primers for sites flanking LesA genes, followed by transformation of ElectroMAX DH5α competent cells (Life Technologies, USA). For heterologous expression, cells were grown in LB medium supplemented with kanamycin (50 μg/mL) at 37°C and 200 rpm to an A600 of 0.8. IPTG (1 mM) was added to the culture, followed by incubation for three h at 30°C and 120 rpm. Total protein was extracted from E. coli cells using a Celllytic B Plus kit (Sigma-Aldrich, USA). The protein concentration was determined according to Bradford’s method using BSA as the standard. Total proteins from LesA, S200A-LesA, and empty vector-expressing E. coli were used for syringe infiltration in greenhouse-grown grapevine (Vitis vinifera L. c.v. ‘Thompson Seedless’) leaves. One hundred μg/spot of protein (1 μg/μL) was infiltrated into each leaf spot. HR-like lesions in the infiltrated area were photographed 24 h after inoculation.

**Isolation of the lesA mutant strain.** The mutagenesis cassette was chemically synthesized (GenScript, USA) after insertion of a kanamycin resistance gene within Ser-200, the first amino acid of the active triad. The entire open reading frame of PD1703 (494 bp at 5’ and 670 bp at 3’) was chosen as flanking homology regions in this cassette. The synthesized pUC57-PD1703::kan was electroporated into Xff WT Temecula1 as described.
The symptoms were observed at eight and 12 weeks post inoculation.

**Grapevine infection and disease quantification.** Grapevines (‘Thompson Seedless’) were inoculated with 20 μL Xff suspension containing ~2 × 10^7 cells. The plants were inoculated with 10 μL on the first day and reinoculated with 10 μL on the second day, with an independently grown Xff culture used for each inoculation. The bacteria were introduced into each plant at three to four inches above the soil using a number 0 insect pin. Twenty-five plants were inoculated with Xff wild type strain, 26 plants with lesA mutant, and 26 plants with PBS (mock inoculation). Quantification of symptoms was performed using 18 randomly selected plants/treatment at 14 wpi. We show the average percent scouring of the vine with standard error, with nodes without leaves and petioles being excluded from this analysis. Leaf Point System: 0-24% scouring of leaf = 0 points, 25-49% = 1 pt, 50-74% = 2 pts, 75-100% = 3 pts. Values for individual leaves were then summed and divided by the total possible scouring (3 × number of leaves) to give one value per vine. One-Tailed T-test with Welch's correction was carried out using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). A value of p < 0.05 was considered statistically significant.

**RNA extraction and real-time RT-PCR.** Xff 3A2 (WT), rpfC and rpfF cells were grown in 50 mL PD3 liquid medium (three flasks/condition) until the cultures reached 10^7 to 10^8 cells/mL. RNA was extracted using the miRNeasy Mini Kit (Qiagen, USA) following manufacture’s instructions. cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix (Life Technologies, USA). RT-PCR reactions were performed using TaqMan Universal PCR Master Mix (Life Technologies, USA) on the StepOne Real-Time PCR System (PE Applied Biosystems, USA). The level of gene transcription was normalized to 16S rRNA and expressed as a relative difference. The statistical analysis was performed using GraphPad Prism software, version 5 (Graph-Pad Software, San Diego, USA), using the Unpaired t test with Welch’s correction. The data was considered significant when p < 0.05.

**Agrobacterium virulence assay in walnut plants.** BPROM, a bacterial sigma70 promoter recognition program, was used to predict the potential promoter for lesA. This software predicted one promoter region starting at 229 bps 5’ of the lesA start codon. The lesA open reading frame and the 500 bps 5’ of the intergenic space, which was predicted to include the promoter region for lesA, were cloned into the binary vector pDU97.1005. Expression of LesA was tested by detecting protein activity on a tributyrin agar plate. The presence of LesA activity ensured that the predicted 500 bps 5’ of this gene contain the active lesA promoter region, since no other promoter was present in the binary vector. Subsequently, positive colonies with LesA activity were transformed into a disarmed Agrobacterium strain (EHA101-PCH32) by electroporation. The lesA region and empty vector containing Agrobacterium strains were infected into walnut (Chandler) by creating an incision in the bark on the main stem. Ten μL resuspended bacterial culture (10^8 cells in PBS) was placed on the incision and wrapped with parafilm. The symptoms were observed at eight and 12 weeks post inoculation.

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**Author Contributions**
R.N., H.G., H.W.G., G.B., L.R.G. and A.M.D. conceived and designed the experiments; R.N., H.G., H.W.G., H.O.A., A.T. and P.A.F. performed the experiments; S.C. performed the in silico analysis; B.J.R. supervised the work of S.C.; R.N., H.G., H.W.G., G.B., L.R.G. and A.M.D. wrote the paper with contributions from all authors.

**Additional Information**
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