Characterization of the Serralysin-Like Gene of ‘*Candidatus Liberibacter solanacearum*’ Associated with Potato Zebra Chip Disease

Aravind Ravindran, Panatda Saenkham, Julien Levy, Cecilia Tamborindeguy, Hong Lin, Dennis C. Gross, and Elizabeth Pierson†

First and sixth authors: Department of Plant Pathology and Microbiology, second, third, and seventh authors: Department of Horticultural Sciences, and fourth author: Department of Entomology, Texas A&M University, College Station 77843; and fifth author: Department of Agriculture-Agricultural Research Service, CDPG, San Joaquin Valley Agricultural Sciences Center, Parlier, CA 93648.
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ABSTRACT

The nonculturable bacterium ‘*Candidatus Liberibacter solanacearum*’ is the causative agent of zebra chip disease in potato. Computational analysis of the ‘*Ca. L. solanacearum*’ genome revealed a serralysin-like gene based on conserved domains characteristic of genes encoding metalloprotease enzymes similar to serralysin. Serralysin and other serralysin family metalloprotease are typically characterized as virulence factors and are secreted by the type I secretion system (T1SS). The ‘*Ca. L. solanacearum*’ serralysin-like gene is located next to and divergently transcribed from genes encoding a T1SS. Based on its relationship to the T1SS and the role of other serralysin family proteases in circumventing host antimicrobial defenses, it was speculated that a functional ‘*Ca. L. solanacearum*’ serralysin-like protease could be a potent virulence factor. Gene expression analysis showed that, from weeks 2 to 6, the expression of the ‘*Ca. L. solanacearum*’ serralysin-like gene was at least twofold higher than week 1,

indicating that gene expression stays high as the disease progresses. A previously constructed serralysin-deficient mutant of *Serratia liquefaciens* FK01, an endophyte associated with insects, as well as an *Escherichia coli* lacking serralysin production were used as surrogates for expression analysis of the ‘*Ca. L. solanacearum*’ serralysin-like gene. The LsoA and LsoB proteins were expressed as both intact proteins and chimeric *S. liquefaciens*-‘*Ca. L. solanacearum*’ serralysin-like proteins to facilitate secretion in the *S. liquefaciens* surrogate and as intact proteins or as a truncated LsoB protein containing just the putative catalytic domains in the *E. coli* surrogate. None of the ‘*Ca. L. solanacearum*’ protein constructs expressed in either surrogate demonstrated proteolytic activity in skim milk or zymogram assays, or in colorimetric assays using purified protein, suggesting that the ‘*Ca. L. solanacearum*’ serralysin-like gene does not encode a functional protease, or at least not in our surrogate systems.

‘*Candidatus Liberibacter*’ spp. are nonculturable, gram-negative, phloem-limited bacteria associated with economically important diseases of potato, citrus, and many other crops (Gottwald 2010; Haapalainen 2014; Liefting et al. 2009; Munyaneza et al. 2010). ‘*Candidatus Liberibacter solanacearum*’ was confirmed as the causative agent of zebra chip (ZC) disease on potato based on graft transmission (Secor et al. 2009), and evidence indicates that ‘*Ca. Liberibacter*’ spp. cause other diseases (e.g., ‘*Ca. L. asiaticus*’-associated citrus huanglongbing). ZC is an important disease of potato in the United States and many countries throughout the Americas and in New Zealand. ‘*Ca. L. solanacearum*’ is vectored to potato by the tomato or potato psyllid (*Bactericera cockerelli* Sulc) (Liefting et al. 2009; Lin et al. 2011; Munyaneza et al. 2007; Secor et al. 2009; Wen et al. 2009). Characteristic symptoms of ZC include leaf chlorosis and purpling, stunted plants with swollen internodes, proliferation of axillary buds and aerial tubers, and plant decline leading to death (Munyaneza et al. 2007). Depending on the seasonal timing of infection, ZC can dramatically reduce tuber yields and typically causes a brown defect in the tubers, rendering them unmarketable. ZC affects all market classes of potato by reducing tuber yield and quality, and all cultivated potato varieties are susceptible to the disease (Diaz-Montano et al. 2014; Lévy et al.

2015). Factors that contribute to ‘*Ca. L. solanacearum*’ virulence are largely unknown.

Based on the absence of both the type III and type II secretion systems (T3SS and T2SS, respectively) in the ‘*Ca. L. solanacearum*’ genome, Lin et al. (2011) postulated that the type I secretion system (T1SS) is the primary mechanism for the delivery of bacterial products important for ‘*Ca. L. solanacearum*’ interactions with the host plant. In a search for potential virulence factors, computational analysis of the ‘*Ca. L. asiaticus*’ genome resulted in the identification of a gene encoding a serralysin-family protein (CLIBASIA_01345) located next to a T1SS gene cluster (Cong et al. 2012). Moreover, comparative genomics revealed that serralysin-like genes are widely distributed in ‘*Ca. Liberibacter*’ genomes (Cong et al. 2012). Wang and Trivedi (2013) reported that the ‘*Ca. L. asiaticus*’ gene is highly expressed in citrus phloem, speculating that it may be important in ‘*Ca. L. asiaticus*’ virulence.

Serralysin, first described from *Serratia* spp., is an approximately 50-kDa metalloprotease (EC 3.4.24.40) originally isolated from the digestive system of silkworm (Miyata et al. 1971). Proteins in the serralysin family are characterized as bacterial extracellular metalloproteases having a highly conserved zinc-binding motif (HEXXHXUGUXH), a Met-turn (SXXMY) sequence, and multiple calcium-binding glycine-rich repeats (GGXGXG). Kaibara et al. (2012) reported that *Serratia liquefaciens* FK01, an endophytic species associated with insects, produced a serralysin with proteolytic activity that contributed to its ability to parasitize insects. Metalloproteases resembling serralysin are produced by several types of gram-negative bacteria, including many *Serratia* and *Pseudomonas* spp., *Dickeya chrysanthemi*, and *Proteus mirabilis* (Baumann 2011; Maeda 1996). In all cases, translocation occurs through a type I secretion channel and, in most of these reports, the serralysin functions in virulence. For example, a serralysin metalloprotease was

†Corresponding author: E. Pierson; E-mail: eapierson@tamu.edu

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identified as a factor that inhibited adhesion of immune cells and bacterial clearance, serving as a virulence factor in *S. marcescens* infection of silkworm (*Bombyx mori*) (Ishii et al. 2014). In plants, the production of antimicrobial proteins and peptides is a major component of plant defense responses, and metalloproteases have been shown to be important bacterial virulence factors (Castro and Fontes 2005). Thus, hypothetically, a functional serralsin family metalloprotease could be a potent, conserved ‘*Ca. Liberibacter*’ virulence factor in plants.

Analyses of the genomes of both ‘*Ca. L. solanacearum*’ haplotypes LsoA and LsoB that cause ZC (strains HenneA and ZC1, respectively) revealed the presence of a gene encoding a serralsin-like protein located next to a gene cluster encoding a T1SS (Lin et al. 2011). In this study, we report on the similarities between the ‘*Ca. L. solanacearum*’ serralsin-like gene and other genes in the serralsin family in terms of the conserved motifs required for proteolytic activity. In order to examine the functionality of the protein encoded by this gene, a previously constructed serralsin-defective mutant of *S. liquefaciens* FK01 (Kaibara et al. 2012) was used as a surrogate for protein expression studies. The *S. liquefaciens* wild-type strain FK01 produces two serralsin-like metalloproteases, *ser1* and *ser2*, and serralsin-deficient mutants were generated by deletion of one or both of the genes (Kaibara et al. 2012). The mutants demonstrated loss of proteolytic activity in assays using skim milk agar and casein zymography, and genetic complementation restored proteolytic activity (Kaibara et al. 2012). Consequently, the *S. liquefaciens* FK01 double mutant *S. liquefaciens* FK04 was selected as a surrogate for examining whether

the ‘*Ca. L. solanacearum*’ serralsin-like gene encodes a functional protease. An *Escherichia coli* strain (DE3), which does not produce serralsin, also was used as a surrogate.

MATERIALS AND METHODS

Bacterial strains, growth conditions, plasmids, and primers.

Populations of the potato or tomato psyllid vector *Bactericera cockerelli* (Central) infected with either ‘*Ca. L. solanacearum*’ haplotypes LsoA or LsoB from separate colonies described previously (Nachappa et al. 2011) were provided by Dr. Cecilia Tamborindeguy. Total DNA (insect and bacterial) was isolated from infected psyllids using the method of Lévy et al. (2011). Initial screening of ‘*Ca. L. solanacearum*’ in psyllids used the ‘*Ca. L. solanacearum*’ TX 16/23 F/R-specific primers described by Ravindran et al. (2011). In all infected psyllids used in this study to amplify the ‘*Ca. L. solanacearum*’ serralsin-like gene, a single visible band 383 bp in length was obtained, confirming that each psyllid carried ‘*Ca. L. solanacearum*’. The ‘*Ca. L. solanacearum*’ haplotype carried by each insect was confirmed using haplotype-specific primers, as described by Wen et al. (2013).

The bacterial strains and plasmids are listed in Table 1 and primers in Table 2. The *S. liquefaciens* strains were provided by Dr. K. Iiyama (Kaibara et al. 2012). The *S. liquefaciens* and *E. coli* DH5α liquid cultures were grown in a Luria-Bertani (LB) medium at 28°C and supplemented with ampicillin or carbenicillin (100 µg ml⁻¹). The plasmid pBBR1 MCS-4 was provided by Dr. M. Kovach (Kovach et al. 1995).

TABLE 1. Bacterial strains and plasmid details

Plasmids and strains	Description ^a	Source
Plasmids		
pBBR1-MCS4	Broad-host range plasmid cloning vector, Amp ^R (Car ^R or Pip ^R)	Kovach et al. (1995)
pET-14b	<i>Escherichia coli</i> expression vector (His-Tag), Amp ^R (Car ^R or Pip ^R)	Novagen, Madison, WI
Bacterial strains		
<i>Serratia liquefaciens</i> FK01	Wild-type strain	Kaibara et al. (2012)
<i>S. liquefaciens</i> FK04	<i>ser1/ser2</i> -double mutant of FK01	Kaibara et al. (2012)
<i>E. coli</i> DH5α	Competent cells	ThermoFisher Scientific
<i>E. coli</i> BL21 (DE3)	Competent cells	ThermoFisher Scientific
<i>S. liquefaciens</i> FK04		
pBBR1 <i>Sl ser2</i>	1,979 bp, <i>EcoRI/BamHI</i> , Car ^R	This study
pBBR1 LsoA <i>ser</i>	1,983 bp, <i>EcoRI/BamHI</i> , Car ^R	This study
pBBR1 LsoB <i>ser</i>	1,479 bp, <i>EcoRI/BamHI</i> , Car ^R	This study
pBBR1 chimeric <i>ser1</i> -LsoA	3,140 bp, <i>XhoI/PstI</i> , Car ^R	This study
pBBR1 chimeric <i>ser1</i> -LsoB	2,652 bp, <i>XhoI/PstI</i> , Car ^R	This study
pBBR1 chimeric <i>ser2</i> -LsoA	2,754 bp, <i>EcoRI/BamHI</i> , Car ^R	This study
pBBR1 chimeric <i>ser2</i> -LsoB	2,266 bp, <i>EcoRI/BamHI</i> , Car ^R	This study
<i>E. coli</i> BL21 (DE3)		
pET14b LsoB <i>ser</i>	1,535 bp, <i>XhoI/BamHI</i> , Car ^R	This study
pET14b LsoA <i>ser</i>	2,001 bp, <i>XhoI/BamHI</i> , Car ^R	This study
pET14b LsoB <i>ser</i> -300	1,238 bp, <i>XhoI/BamHI</i> , Car ^R	This study

^a Amp^R, Car^R, and Pip^R indicate resistant to ampicillin, carbenicillin, and piperacillin, respectively.

TABLE 2. Primer details^a

Primers	5'–3'	Source
Lso-ser qPCR F	ATCTTGGTCTAGTATTGGTGG	This study
Lso-ser qPCR R	CCATTACATCCCATCCATCAG	This study
Lso-ser F <i>EcoRI</i>	CACCTTgaattcTAGCGGAAGGAGATTCTTA	This study
Lso-ser R <i>BamHI</i>	ATTCGTggatccGCAAACGAGTACAACAATAGCC	This study
<i>ser2</i> F <i>EcoRI</i>	CACCTTgaattcGAGAGCAGCGTCAGAACCC	Kaibara et al. (2012)
<i>ser2</i> R <i>BamHI</i>	ATTTCCggatccGTAATAGAGCGGTGGGCATA	Kaibara et al. (2012)
ProF (-811) <i>XhoI</i>	ACAActcgagGGATCCCCATGATCGTCAG	Kaibara et al. (2012)
<i>ser1</i> -N R	AATTTCCATGTCTTCGCTGGCCGGGTGTTTCACG	This study
Lso <i>ser1</i> -C F	GCGAAGACATGGAAATTAACCTAGATGCGAGAGATG	This study
<i>ser2</i> -N R	AATTTCCATGTTCGCCAGTTCAGGGTGCAA	This study
Lso <i>ser2</i> -C F	TGGGCAACATGGAAATTAACCTAGATGCGAGAGATG	This study
Lso-C ser R <i>PstI</i>	ATTCGTctgcagGCAAACGAGTACAACAATAGCC	This study
Lso-ser F1 <i>XhoI</i>	CACCTTctcgagGTGGATGATATCAAGCCTGTG	This study
Lso-ser R1 <i>BamHI</i>	TGCGggatccTCAATCACTAAAATCAAG	This study
LsoB-ser-300 F <i>XhoI</i>	CACCTTctcgagATGGAAATTAACCTAGATGCGAGAGATG	This study

^a Sequences are underlined with solid lines and dotted lines are complementary.

Analysis of the serralysin-like gene in ‘*Ca. L. solanacearum*’ and ‘*Ca. L. asiaticus*’. The genome sequences for strains representing the two different ‘*Ca. L. solanacearum*’ haplotypes LsoA and LsoB (HenneA and ZC1, respectively) are available at the National Center for Biotechnology Information (NCBI) for analysis. Comparison of serralysin-like genes among different ‘*Ca. Liberibacter*’ spp. was performed using IMG gene content analysis (<https://img.jgi.doe.gov/cgi-bin/w/main.cgi>). To compare the serralysin gene of *Serratia* spp. with the serralysin-like gene of ‘*Ca. Liberibacter*’ spp., the CLUSTAL X Multiple Sequence Alignment program was used (Thompson et al. 1997). Protein sequences used in the analysis are available in the NCBI database and included the following sequences: Ser1 (GenBank accession BAK39731) and Ser2 (GenBank accession BAK39733) from *S. liquefaciens* FK01, serralysin (GenBank accession WP_004934318) from *S. marcescens*, the serralysin-like proteins from ‘*Ca. L. solanacearum*’ ZC1 (haplotype LsoB) (GenBank accession WP_013461860) and HenneA (haplotype LsoA) (GenBank accession KJZ82330.1), and the serralysin-like protein from ‘*Ca. L. asiaticus*’ strains gxpsy (GenBank accession WP_015452346) and psy62 (GenBank accession WP_015452346).

Expression of the serralysin-like gene by quantitative polymerase chain reaction assays. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis was used to determine the level of expression of the ‘*Ca. L. solanacearum*’ serralysin-like gene in tomato plants that had been infested with bacteriiferous psyllids. For this experiment, insects carrying LsoB were used. Total RNA was extracted using an RNeasy Mini Kit (Qiagen) from leaves of plants at week 1 (when insects were removed from plants) and weeks 2, 3, 4, and 6 after insect infestation. As a negative control at each time point, RNA also was extracted from plants treated with psyllids without ‘*Ca. L. solanacearum*’. RNA samples were treated with TURBO DNase (Ambion) according to the manufacturer’s protocol. Total RNA samples were quantified using spectrophotometry (Nano-Drop Technologies, Inc.). Total RNA was reverse-transcribed into cDNA using a Super Script Vilo cDNA Synthesis kit (Invitrogen) following the manufacturer’s protocol. The cDNA was quantified using spectrophotometry and samples were diluted to 10 ng μl^{-1} . qRT-PCR primers were designed for the serralysin-like gene and the *recA* housekeeping genes of ‘*Ca. L. solanacearum*’. The following reaction mix was used for each 20- μl qRT-PCR mixture: 10 μl of SYBR GreenER qPCR SuperMix Universal, 8.16 μl of nuclease-free water, 0.04 μl of ROX reference dye, 0.4 μl of Lso-ser qPCR F primer (200 nM final), 0.4 μl of Lso-ser qPCR R primer (200 nM final), and 1 μl of template DNA (10 ng μl^{-1}). qRT-PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR System. Data were analyzed using the comparative cycle threshold (Ct) method, in which all Ct values were normalized to the *recA* housekeeping gene. The $2^{-\Delta\Delta C_t}$ was calculated as $[2^{-C_t \text{ of each time point} - C_t \text{ recA}}] - [2^{-C_t \text{ week 1} - C_t \text{ recA}}]$ to determine the relative expression of the ‘*Ca. L. solanacearum*’ serralysin-like gene in weeks 2, 3, 4, and 6 compared with week 1.

Cloning of the ‘*Ca. L. solanacearum*’ serralysin-like gene and *S. liquefaciens* serralysin. A single ‘*Ca. L. solanacearum*’ primer set (Lso-ser F/R) was designed based on the conserved regions of the serralysin-like gene sequence (including the native ribosome-binding site) in both haplotypes as determined from analysis of the HenneA and ZC1 genomes. The serralysin-like genes of both haplotypes were amplified from the genomic DNA of ‘*Ca. L. solanacearum*’-infected psyllids having either LsoA or LsoB. A fragment containing the complete sequence of the *ser2* gene was amplified from genomic DNA of *S. liquefaciens* FK01 by PCR using primer pair ser2F/ser2R (Kaibara et al. 2012). All three amplified fragments were cloned separately into the *EcoRI*-*Bam*HI site in the multiple cloning locus of pBBR1 MCS-4, then transformed into *E. coli* DH5 α . The presence of each insert was confirmed using colony PCR and verified by sequencing. The confirmed plasmids (pBBR1 LsoA *ser*, pBBR1 LsoB *ser*, and pBBR1 SI *ser2*)

were transformed separately via electroporation into *S. liquefaciens* FK04 (*ser1/ser2* double mutant) and *E. coli* DE3 (does not make serralysin) to test for protease activity.

Screening for protease activity. Skim milk agar plate assays were used to detect proteolytic activity after incubation at 28°C for 5 days. Skim milk agar contained 10% skim milk powder (Oxoid; ThermoFisher Scientific), 0.5% peptone, and 1.5% agar (autoclaved for 5 min). The formation of clear zones around colonies was diagnostic of proteolytic activity. For the zymography proteolytic assays, 20 μl of cell culture was mixed with a 2 $\times$ zymogram sample buffer (Bio-Rad) incubated at 20°C for 1 h. A 20- μl portion of the denatured samples was then separated by electrophoresis on a 10% polyacrylamide gel containing 0.05% casein with a 10 mA constant current (maximum voltage was 100 V) at room temperature until the blue tracking dye reached the bottom of the gel. The gel was incubated in a renaturing buffer (2.5% Triton X-100, vol/vol) for 30 min, repeated a second time, and then incubated in zymogram developing buffer for 16 h with gentle agitation at 37°C. The gel was stained with Coomassie Brilliant Blue R-250 and then destained (Kaibara et al. 2012). Areas of digestion appearing as clear bands against the stained background indicated protease activity.

Construction of chimeric *Serratia*-‘*Ca. L. solanacearum*’ serralysin-like genes. To circumvent possible problems with protein secretion in our surrogate systems, a two-step PCR method was used to make chimeric serralysin-like genes composed of all combinations of the 5’ sequence from the *S. liquefaciens* *ser1* or *ser2* serralysin genes and the 3’ sequence encoding the putative catalytic regions from the LsoA or LsoB serralysin-like genes. The first step was to PCR amplify both of the 5’ serralysin sequences from genomic DNA of *S. liquefaciens* FK01 (using forward primers ProF [-811] and ser2F and the reverse primers ser1-N R and ser2-N R for *ser1* and *ser2* amplification, respectively), and both of the 3’ ‘*Ca. L. solanacearum*’ sequences from the genomic DNA samples of psyllids carrying either LsoA or LsoB (using forward primers Lso-C ser1F and Lso-C ser2F and the reverse primer Lso ser-C R). The next step used the 5’ and 3’ amplicons as templates to join them by PCR amplification to form the four different, complete chimeric serralysin-like genes. This was made possible because the 5’ sequence reverse primers (ser1-N R and ser2-N R) and 3’ sequence forward primers (Lso-C ser1F and Lso-C ser2F) carry the overlap sequence required to join the PCR products to form a chimeric sequence. The second PCR was performed using either the *ser1* or *ser2* forward primer (ProF [-811] and ser2F) and the ‘*Ca. L. solanacearum*’ reverse primer (Lso ser-C R) (Supplementary Fig. S1). The chimeric amplicons were cloned into pBBR1 MCS-4 (Kovach et al. 1995), resulting in plasmids pBBR1 *ser1*-LsoA, pBBR1 *ser1*-LsoB, pBBR1 *ser2*-LsoA, and pBBR1 *ser2*-LsoB. The plasmids were transferred separately into *E. coli* DH5 α and colony PCR confirmed the presence and expected size of each chimeric *Serratia*-‘*Ca. L. solanacearum*’ serralysin-like gene. From there, sequences were transferred separately into *S. liquefaciens* FK04 and tested as above for proteolytic activity.

Proteolytic activity of the complete LsoA and LsoB serralysin-like proteins and a truncated LsoB protein. The complete LsoA and LsoB serralysin-like genes were amplified using the LsoA *ser* primer set (Lso-ser F1/Lso-ser R1) and LsoB *ser* primer set (Lso-ser F1/Lso-ser R), respectively, and then inserted separately into the *Xho*I-*Bam*HI site within the multiple cloning locus of the high-copy protein expression vector pET14b (Novagen), resulting in pET14b LsoA *ser* and pET14b LsoB *ser*, respectively. Because the 300-bp region at the N-terminal end of the LsoB serralysin-like gene does not contain the domains hypothetically involved in catalytic activity, a truncated construct containing just the putative catalytic domains was constructed (e.g., without other parts of the protein that potentially could negatively affect expression, secretion, or activity in the *E. coli* DE3 surrogate). For the truncated construct, the 300-bp region at the N-terminal end of the LsoB serralysin-like gene was excluded. The remaining 1,238-bp sequence was amplified using forward and reverse primers

LsoB-ser-300 F and Lso-ser R and was cloned into pET14b, resulting in plasmid pET14b LsoB *ser*-300. All plasmids were verified by sequencing and then transformed into *E. coli* DE3.

DE3 cells harboring pET14b LsoA *ser*, pET14b LsoB *ser*, or pET14b LsoB *ser*-300 were grown overnight at 37°C in 3 ml of LB supplemented with ampicillin (100 µg/ml). A 1:100 dilution of an overnight cell culture was added to 100 ml of LB supplemented with ampicillin (10 µg/ml), grown to an optical density at 600 nm of 0.4 to 0.5, then induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 h. After induction with IPTG, cells were harvested, resuspended in cell lysis buffer A (20 mM phosphate buffer [pH 7.8]

containing 0.5 M NaCl and 8 M urea), and lysed via sonication. The protein expression was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and proteolytic activity was evaluated using the zymogram assay (as above). The LsoB and LsoB *ser*-300 proteins were purified using affinity chromatography (Ni-NTA column). The column was washed with buffer B (20 mM phosphate buffer [pH 7.8] containing 10 mM imidazole, 0.5 M NaCl, and 8 M urea) and the protein was eluted with buffer C (20 mM NaH₂PO₄ [pH 4.0], 0.5 M NaCl, and 8 M urea). The purified protein was transferred to a dialysis bag to remove the urea; the buffer used contained a urea gradient (4 M, 2 M, 1 M, 0.5 M, and

Fig. 1. Serralyisin-like genes from ‘*Candidatus Liberibacter solanacearum*’ haplotypes LsoA and LsoB. **A**, Polymerase chain reaction amplification of the ‘*Ca. L. solanacearum*’ serralyisin-like gene from total genomic DNA obtained from psyllids carrying either the LsoA or LsoB haplotype. Lane N, water control; lane 1, LsoA; lane 2, LsoB; lane M, molecular size marker (1-kb DNA ladder, 250 to 10,000 bp). **B**, Comparison of orthologs of the serralyisin-like gene cluster of ‘*Ca. L. solanacearum*’ strain ZC1 (LsoB haplotype), ‘*Ca. L. solanacearum*’ strain HenneA (LsoA haplotype), ‘*Ca. L. solanacearum*’ strain gxpsy, and ‘*Ca. L. asiaticus*’ strain psy62 for similar orthologs. Shading indicates ortholog groups and the size of the bar approximately corresponds to gene size. This comparison was performed using JGI-IMG/ER (<https://img.jgi.doe.gov/cgi-bin/w/main.cgi>). Upstream genes of serralyisin are a type I secretion ATPase (PrtD), a type I secretion membrane fusion protein (HlyD), and a C₄-dicarboxylate transporter (DctA).

0 M). The protein purity was checked by SDS-PAGE analysis, and proteolytic activity was evaluated using the zymogram assay and a colorimetric assay. For the colorimetric assay, azocasein was used as a substrate with the purified proteins, as described by (Secades and Guijarro 1999). Enzyme solutions (120 µl) were mixed with 480 µl of 1% azocasein (Sigma-Aldrich) in Tris buffer containing MgCl₂, and the mixtures were then incubated at 30°C for 30 min. To precipitate nondigested azocasein, 600 µl of 10% trichloroacetic acid (TCA) was added and incubated on ice for 30 min, after which the reactions were centrifuged (15,000 × g at 4°C for 10 min). Supernatants (800 µl) were neutralized by adding 200 µl of 1.8 N NaOH. The absorbance of supernatants was measured using a spectrophotometer (415 nm), where increased absorbance relative to the control indicated the presence of proteolytic activity.

RESULTS

Analysis of the serralysin-like genes in ‘*Ca. L. solanacearum*’ and ‘*Ca. L. asiaticus*’. The ‘*Ca. L. solanacearum*’ serralysin-like gene sequences for haplotypes LsoA and LsoB were

identified from the genomic sequences of ‘*Ca. L. solanacearum*’ strains HenneA and ZC1, respectively, in the NCBI database. The BlastP analysis identified the closest match to these sequences as being ‘*Ca. Liberibacter asiaticus*’ strains gxpsy and psy62. The complete sequences of the serralysin-like genes from LsoB haplotype (1,479 bp, ‘*Ca. L. solanacearum*’ strain ZC1) and ‘*Ca. L. asiaticus*’ strain gxpsy have 493 and 497 amino acids, respectively. In contrast, the serralysin-like genes from the LsoA haplotype (1,983 bp, predicted from ‘*Ca. L. solanacearum*’ strain HenneA) and ‘*Ca. L. asiaticus*’ strain psy62 are predicted to encode much larger proteins, having 661 and 665 amino acids, respectively (Fig. 1A). In both ‘*Ca. Liberibacter*’ spp., the TISS genes are located upstream of the serralysin-like gene (Fig. 1B). Similarly, the TISS genes for all four strains encode homologs for an ATPase (PrtD), a membrane fusion protein (HylD), and a C₄-dicarboxylate transporter (DctA). Because of errors in the genome annotation of LsoA strain HenneA, we observed that the TISS genes (ATPase, PrtD, and HylD) are erroneously broken (Fig. 1B). The serralysin-like proteins of ‘*Ca. Liberibacter*’ spp. were compared with authentic serralysin protein sequences from *S. marcescens* and *S. liquefaciens*

Fig. 2. Alignments of multiple serralysin family protein sequences from *Serratia* and ‘*Candidatus Liberibacter*’ spp. Conserved motifs associated with metalloprotease activity are shown in the dark boxes, including sequences encoding (I) a zinc-binding motif (HEXXHXUGUXH), (II) a Met-turn (SXMXY) motif, (IIIA–D) glycine-rich repeats (GGXGXD), and (IV) an ABC exporter motif (DXXX).

FK01 (Kaibara et al. 2012). Conserved motifs for serralysins, including the zinc-binding domain (HEXXHXUGUXH), the Met-turn sequence (SXXMY), the glycine-rich calcium-binding repeat sequences (GGXGXD), and the ABC exporter (DXXX) sequence (Fig. 2) all were identified in the predicted serralysin-like metalloproteases from both '*Ca. L. solanacearum*' haplotypes and both '*Ca. L. asiaticus*' strains. The authentic serralysin genes from both *Serratia* spp. contained four glycine-rich calcium-binding repeat sequences (designated IIIA, B, C, and D) (Fig. 2). In contrast, both '*Ca. L. solanacearum*' strains had only one repeat (position IIIA) (Fig. 2); the sequence of the binding repeat was (GGAGND) and (GGTGND) for the '*Ca. L. solanacearum*' strains ZC1 and HenneA, respectively.

Expression of a '*Ca. L. solanacearum*' serralysin-like gene in tomato. To assess the expression of the '*Ca. L. solanacearum*' serralysin-like gene in planta, tomato leaves infected with the LsoB haplotype were collected at weeks 1, 2, 3, 4, and 6 following infestation with insects, time points before visible disease symptoms were apparent. qPCR analysis of gene expression with normalization of Ct values to the *recA* housekeeping gene showed that, at weeks 2, 3, 4, and 6, the expression of the '*Ca. L. solanacearum*' serralysin-like gene was 2.1- to 3.4-fold higher than at week 1, indicating that gene expression stays high as the disease progresses ($2^{-\Delta\Delta C_t}$ values were 2.1 ± 0.4 , 2.4 ± 0.6 , 2.9 ± 0.6 , and 3.4 ± 2.7 at weeks 2, 3, 4, and 6, respectively, compared with week 1). In contrast, no amplification of the serralysin gene was observed in tomato plants infested with insects without '*Ca. L. solanacearum*'.

Functional studies of the '*Ca. L. solanacearum*' serralysin-like proteins. The complete sequences of the serralysin-like gene from the LsoA and LsoB haplotypes were cloned separately into a broad-host-range expression vector pBBR1-MCS4. As a control, the complete sequence of the *S. liquefaciens* serralysin *ser2* gene (1,979 bp) was cloned into pBBR1-MCS4. Plasmids were transformed separately into the serralysin-deficient, *ser1/ser2* double mutant *S. liquefaciens* FK04 and *E. coli* DE3 to test for protease activity. Because pBBR1-MCS4 is a low-copy plasmid, we were not able to clearly see the bands on an SDS-PAGE gel, including the positive control. The insertions were moved to pET14b, which is a high-copy expression plasmid; then, the protein sizes of the serralysin-like genes from the LsoA and LsoB haplotypes (approximately 75 and 56 kDa, respectively) were visible on SDS-PAGE gels, confirming the production of proteins at the predicted sizes (Supplementary Fig. S2).

On skim milk agar medium, neither of the '*Ca. L. solanacearum*' serralysin-like proteins expressed in *S. liquefaciens* FK04 exhibited enzymatic activity (Fig. 3A). In contrast, protease activity for authentic serralysin produced by *S. liquefaciens* FK01 or the double mutant *S. liquefaciens* FK04 with the *ser2* gene showed clear zones extending a few millimeters from the edge of the colony. In the casein zymography assay, surrogate cell culture supernatants and pellets were assayed for clear bands of proteolytic activity against a dark background (Fig. 3B). The *S. liquefaciens* serralysin (*ser2*) gene completely restored protease activity to the *S. liquefaciens*

Fig. 3. Proteolytic activity on skim milk agar and casein zymography. **A**, The skim milk agar plate was incubated at 28°C. Section 1, FK01; 2, FK04 double mutant; 3, FK04/pBBR1 LsoB *ser*; 4, FK04/pBBR1 LsoA *ser*; and 5, FK04/pBBR1 SI *ser2*. **B**, Casein zymography for proteolytic activity. **B1**, *Serratia liquefaciens* FK04 supernatant: Lane M, protein marker; lane 1, FK01; lane 2, FK04; lane 3, FK04/pBBR1 LsoB *ser*; lane 4, FK04/pBBR1 LsoA *ser*; and lane 5, FK04/pBBR1 SI *ser2*. **B2**, *Escherichia coli* DE3 supernatant and **B3**, pellet: Lane M, protein marker; lane 1, FK01; lane 2, FK04; lane 3, DE3/pBBR1 LsoB *ser*; lane 4, DE3/pBBR1 LsoA *ser*; and lane 5, DE3/pBBR1 SI *ser2*.

FK04 serralysin-deficient mutant (FK04/pBBR1 *Sl ser2*), whereas neither of the '*Ca. L. solanacearum*' serralysin-like genes restored protease activity in this mutant (FK04/pBBR1 *LsoB ser* or FK04/pBBR1 *LsoA ser*) (Fig. 3B1). Expression of '*Ca. L. solanacearum*' serralysin-like genes in the nonserralysin-producing surrogate *E. coli* DE3 (DE3/pBBR1 *LsoB ser* and DE3/pBBR1 *LsoA ser*) also failed to convey protease activity (Fig. 3B2 and B3). Unexpectedly, there was no protease activity detected in the supernatant of *E. coli* harboring the pBBR *Sl ser2* construct (Fig. 3B2), whereas proteolytic activity was detected in the cell pellet (Fig. 3B3). Apparently, the *S. liquefaciens* serralysin was not secreted by *E. coli* DE3. Incompatibility between the signal peptide and secretion system for the two species also may be responsible for unsuccessful secretion of the '*Ca. L. solanacearum*' serralysin-like proteins expressed in either *E. coli* DE3 or *S. liquefaciens* FK04.

Functional studies of chimeric and truncated '*Ca. L. solanacearum*' serralysin-like proteins. To account for the potential secretion incompatibility, we constructed chimeric sequences. For chimeric sequences, the entire region encoding the serralysin signature sequences encoding the putative catalytic domains from '*Ca. L. solanacearum*' were retained. This included the entire 3' region of the '*Ca. L. solanacearum*' gene encoding the zinc-binding domain, the Met-turn sequence, and the glycine-rich repeat. This '*Ca. L. solanacearum*' sequence was joined with the 5' region of the *S. liquefaciens* FK01 sequence encoding the N terminal end of the serralysin protein thought to be involved in secretion. This process resulted in four different chimeras produced using combinations of the 5' sequences from *S. liquefaciens* *ser1* and *ser2* and the 3' sequences from the serralysin-like genes of the *LsoA* and *LsoB* haplotypes. The chimeric *Serratia*-'*Ca. L. solanacearum*' serralysin-like protein was expressed in *S. liquefaciens* FK04 and the cell culture supernatants and pellets were assayed on skim milk agar and on a casein zymogram. None of the four chimeric serralysin-like gene-constructs (FK04/pBBR1 chimeric *ser1*-*LsoB*, FK04/pBBR1 chimeric *ser1*-*LsoA*, FK04/pBBR1 chimeric *ser2*-*LsoB*, and FK04/pBBR1 chimeric *ser2*-*LsoA*) restored protease activity to the serralysin-deficient mutant FK04 (data not shown). In contrast, serralysin protease activity was restored to the FK04 mutant by complementation with pBBR1-*ser2* carrying the intact serralysin gene from *S. liquefaciens*.

As an alternative approach to chimeric sequences, we also constructed a truncated *LsoB* serralysin-like gene that carried only the three motifs associated with putative proteolytic activity in serralysin (i.e., excluding 300-bp 5' sequences that may not be involved in catalytic activity). As mentioned, the entire *LsoA* serralysin-like gene and *LsoB* serralysin-like gene and the truncated *LsoB* serralysin-like gene construct were cloned separately into the high-copy expression vector pET14b that generates a His-tagged protein for assays of proteolytic activity (zymogram and colorimetric assays). These plasmids were introduced into the *E. coli* DE3 surrogate. We focused on *LsoB* for the high expression vector study because it is typically the more virulent pathogen in our assays with potato (unpublished). As predicted, the complete *LsoB* serralysin-like genes resulted in the expression of proteins in *E. coli* DE3 of 56 kDa (Fig. 4A1), as visualized on SDS-PAGE gels; the truncated construct produced a protein of 44 kDa (Fig. 4A2). Neither of the intact proteins showed proteolytic activity in a zymogram assay (not shown). Neither the purified intact *LsoB* protein nor the truncated *LsoB* protein showed proteolytic activity in the colorimetric assay (Fig. 4B).

DISCUSSION

The importance of T1SS as the primary mechanism for secretion of '*Ca. L. solanacearum*' products involved in pathogen-microbe interactions was suggested by Lin et al. (2011) based on the absence of both T3SS and T2SS in the '*Ca. L. solanacearum*' genome. The finding that '*Ca. L. solanacearum*' encodes a putative serralysin-

like gene (CKC_02265) clustered together with a predicted T1SS locus identified the '*Ca. L. solanacearum*' serralysin-like gene as a highly interesting candidate virulence factor. Serralysin-like proteins are a family of metalloprotease produced by a wide range of microorganisms (Dahler et al. 1990; Maeda and Morihara 1995). Some of these extracellular metalloproteases have been either demonstrated or suggested to play critical roles in bacterial virulence to plants (Castro and Fontes, 2005). For example, *Erwinia chrysanthemi* produces a metalloprotease (PrtA) involved in virulence to modify or degrade host plant cell proteins during soft rot infection (Charkowski 2006; Dahler et al. 1990).

The in silico alignment of the predicted amino acid sequences for the '*Ca. L. solanacearum*' serralysin-like proteins with the predicted amino acid sequences of other serralysin family proteins showed a conserved zinc-binding motif, a Met-turn motif, a single glycine-rich calcium-binding repeat sequence, and an ABC exporter signal motif. These conserved motifs suggested that the '*Ca. L. solanacearum*' serralysin-like protein was a member of the serralysin family of metalloproteases (Bode et al. 1993). The number of glycine-rich calcium-binding sequence repeats varies among bacterial strains along with the size of the protein (Basu and Apte 2008; Baumann 2011; Linhartová et al. 2010). The serralysin protein sequences from *Serratia* spp. have four glycine-rich calcium-binding repeats regions (Fig. 2), whereas both '*Ca. L. solanacearum*' haplotype sequences have only one (IIIA) (Fig. 2).

Fig. 4. Recombinant serralysin-like protein purity assayed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and protease activity assayed by a colorimetric assay. **A1**, Purified *LsoB* serralysin-like proteins from construct pET14b *LsoB ser* and **A2**, purified truncated (5' deletion, ~300 bp) protein from construct pET14b *LsoB ser-300*. Lane M, protein marker (10 to 250 kDa); lane Cru., crude; lane FT, flow through; lane W1, wash 1; lane W2, wash 2; lane 1, elution fraction 1; lane 2, elution fraction 2; and lane 3, elution fraction 3. **B**, Colorimetric assay for protease activity from the culture supernatant of wild-type *Serratia liquefaciens* FK01, purified protein from construct pET14b *LsoB ser*, and purified protein from construct pET14b *LsoB ser-300*.

Similarly, the two '*Ca. L. asiaticus*' sequences also appear to have only one GGXGXD sequence (IIIA) (Fig. 2) but neither sequence matched the consensus sequence perfectly, both having GSXGXD sequences. (Delepelaire 2004) reported that the three amino acids following the consensus repeat sequence also are important, and may form a short β -strand associated with the calcium-binding region (Baumann 2011). The three amino acids following the IIIA repeats in the serralyisin sequences and the '*Ca. L. asiaticus*' sequences are all hydrophobic isoleucine or valine amino acids; two of three amino acids in the '*Ca. L. solanacearum*' sequences are hydrophobic. This pattern is less conserved following the other repeats. A characteristic of serralyisin and its homologs is the binding of multiple calcium ions to the repeats-in-toxin (RTX) domain having these glycine-rich repeats. The binding of calcium ions is believed to facilitate the folding of serralyisin and stabilizes the protein (Baumann 1994; Baumann et al. 1993). Our analysis suggests that, although the '*Ca. L. solanacearum*' serralyisin-like gene encodes a protein with many of the putative catalytic domains, it lacks a prominent RTX domain rich in calcium-binding repeat sequences, which may have functional significance in terms of folding, catalytic activity, or both.

To investigate whether the '*Ca. L. solanacearum*' serralyisin-like protein has functional protease activity, we used the *S. liquefaciens* FK01 mutant system (Kaibara et al. 2012) and *Escherichia coli* DE3 as expression surrogates. Our results reveal the importance of surrogate for demonstrating protease activity. The '*Ca. L. solanacearum*' serralyisin-like genes did not restore protease activity to the serralyisin-deficient *S. liquefaciens* FK04 mutant or convey activity to *E. coli*. In contrast, the *S. liquefaciens* serralyisin (*ser2*) gene completely restored protease activity in the serralyisin-deficient *S. liquefaciens* FK04 mutant. However, proteolytic activity was detected only in the cell pellet (not the supernatant) of *E. coli* harboring the pBBR SI *ser2* construct, suggesting that the *S. liquefaciens* serralyisin was not secreted by *E. coli* DE3. Incompatibility between the signal peptide and secretion system appears to be important for exoprotease activity even for an authentic serralyisin, which indicated that choice of surrogate is important for demonstrating exoprotease activity. We hypothesized that failure to separate the '*Ca. L. solanacearum*' serralyisin-like proteins from the cell pellet when expressed in either *S. liquefaciens* or *E. coli* could have accounted for the lack of proteolytic activity in the supernatant.

To account for the potential secretion incompatibility, we constructed chimeric sequences having the 5' sequence (potentially encoding factors related to secretion) from *Serratia ser1* or *ser2* and the 3' sequence (potentially encoding catalytic activity) from LsoA or LsoB. However, none of the chimeric serralyisin-like proteins demonstrated protease activity in the *S. liquefaciens* cell pellet or supernatant fractions. When the complete LsoB serralyisin-like gene and the truncated gene having just the putative catalytic domain were expressed using a high-expression plasmid and purified from *E. coli* DE3, no proteolytic activity was observed for either purified protein, despite strong expression and yield in the pellet fractions. Although we obtained good separation of the protein from other cellular components, protein folding and stabilization could still have been an issue for activity in this experiment.

Although the '*Ca. L. solanacearum*' serralyisin-like protein was produced at sufficiently high levels in both *E. coli* and *S. liquefaciens* for visualization on gels, the lack of proteolytic activity combined with the lack of a prominent RTX domain suggests several hypotheses: (i) the gene does not encode a functional metalloprotease; (ii) the protein does not fold properly on its own, may require other components to aid in folding, or cannot fold properly in these surrogates; (iii) the protein is missing some other requirement for protease activity (e.g., an additional component, chaperone, or cofactor); or (iv) the expression or activity levels are somehow below our technological capacity for detection. Our finding that the '*Ca. L. solanacearum*' serralyisin-like gene expression increases 2.1- to 3.4-fold from the time insects are

removed to 2, 3, 4, and 6 weeks after infection suggests a possible role for this protein in pathogenicity or survival in the plant host. Interestingly, the expression of the '*Ca. L. asiaticus*' serralyisin-like was observed to be 5.5-fold higher in planta compared with within an Asian citrus psyllid, suggesting a more important role for the protein in plant-microbe compared with insect-microbe interactions (Yan et al. 2013). Although observations using our surrogate system indicate that the '*Ca. L. solanacearum*' serralyisin-like gene does not encode a functional serralyisin-like metalloprotease, we hope our studies will provide useful insight for future work using other surrogates or assays. Nevertheless, the pattern of gene expression in planta suggests further work is needed to define the role of this gene in plant-pathogen interactions.

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