Insecticidal Serralysin of *Serratia marcescens* Is Detoxified in M3 Midgut Region of *Riptortus pedestris*

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**Keywords:** insect symbiosis, serralysin, detoxification, *Riptortus pedestris*, *Serratia marcescens* Db11

**INTRODUCTION**

*Serratia* species is frequently found in the midgut bacterial flora of almost all hematopoietic insects, such as mosquitoes and *Rhodnius* species (Demaio et al., 1996; Straif et al., 1998; Gonzalez-Ceron et al., 2003; Azambuja et al., 2004). The gram-negative bacterium, *Serratia marcescens*, acts as an opportunist pathogen in a wide range of animals, including humans and insects (Raymann et al., 2018). *Serratia marcescens* is an entomopathogen that causes bacteremia in the insect hemolymph, resulting in rapid death (Grimont and Grimont, 1978; Chadwick et al., 1990). The gut commensal *S. marcescens* promotes mosquito permissiveness to arboviruses and facilitates arboviral infection via a secreted protein SmEnhancin (Wu et al., 2019). In the gut of a cockroach, *Gromphadorhina portentosa* (Madagascar), *S. marcescens* not only produces active molecule(s) with potent antibacterial properties but also exhibits anti-amoebic effects (Akbar et al., 2018). This entomopathogen produces a virulence factor, called serralysin, which shows insecticidal activity via hemolymph bleeding and inhibition of immune responses.
cell adhesion (Tambong et al., 2014; Ishii et al., 2014a,b). Notably, serralysin degrades the humoral immune factors in insects (Lee et al., 2017a). Serralysin is a conserved metalloprotease among various bacterial species and requires both zinc and a divalent cation for maximal catalysis (Bode et al., 1993; Belas et al., 2004). The entomopathogen Xenorhabdus has a serralysin-type metalloenzyme called PrtA (Massaoud et al., 2011). Proteus and Photorhabdus species also have the serralysin homologs ZapA and AprA, respectively, which inhibit antibacterial activity by destroying antimicrobial peptides, such as defensin and cecropin (Belas et al., 2004; Cabral et al., 2004). The serralysin family is grouped in the metzincin metalloprotease superfamily which is characterized by the zinc-binding motif (HEXXHGXXXHZ; Bode et al., 1993; Salamone and Wodzinski, 1997).

Riptortus pedestris-Burkholderia insecticola symbiosis is a powerful model system for analyzing molecular cross-talks. The bean bug, R. pedestris (Hemiptera: Alydidae), possesses a specialized symbiotic organ in the posterior region of the midgut (referred to as the M4 crypt), where numerous crypts harbor specific gut symbionts of the β- proteobacterial genus, Burkholderia (Kikuchi et al., 2005). In this symbiosis model, Burkholderia-colonized insects (Sym-insects) and insects without gut symbionts (Apo-insects) can be generated in the laboratory depending on oral infection of Burkholderia cells, respectively (Lee et al., 2017b, 2019, 2022). However, the gut immune responses of R. pedestris to exogenous microbes have not yet been clearly elucidated. Using this model, we recently observed that orally infected entomopathogenic S. marcescens cells are resistant to the antimicrobial peptide, trialectin, produced in the salivary glands of R. pedestris (Lee et al., 2017a). However, it is unclear how the pathogenicity of S. marcescens is eventually suppressed in the midgut.

In this study, orally infected S. marcescens were found to colonize the midgut of R. pedestris, but did not exhibit any insecticidal activity against the host. Serralysin was markedly degraded by host-derived factors present in the M1 midgut region. The M3-colonizing S. marcescens cells did not produce serralysin. These results suggest that orally infected S. marcescens lose insecticidal activity through expression of host-derived degrading factors and suppression of serralysin molecules.

**MATERIALS AND METHODS**

**Insect Rearing and Serratia marcescens Inoculation**

*Riptortus pedestris* was maintained in an insectary at 28°C under a long-day cycle of 16 h light and 8 h dark, as described previously (Lee et al., 2020). The nymphs were reared in clean plastic containers with soybean seeds and distilled water containing 0.05% ascorbic acid. The non-pigmented *S. marcescens* strain Db11 was cultured to mid-log phase at 37°C in the Luria-Bertani medium (BD Difco, United States; Lee et al., 2017a). Newly molted second-instar nymphs were inoculated with *S. marcescens* Db11 cells at a concentration of 10⁷ cells/ml.

**Fitness Measurement**

To compare the effect of *S. marcescens* oral infection on the host fitness, nymphs were reared until adulthood and assessed for various fitness parameters, such as emergence rate, survival rate, dry weight, and body length (Lee et al., 2017b). Emergence was monitored daily by inspecting the late-fifth-instar nymphs and counting the number of newly molted adult insects. Survival rates were examined from the second instar to the adult stage when *S. marcescens* was orally infected. For dry weight measurements, adult males were immersed in acetone for 5 min and then completely dried in an oven at 70°C for 10 min.

**Purification of Serralysin**

Serralysin was purified from bacterial cultures according to a previous report (Lee et al., 2017a). Briefly, bacteria were grown at 30°C to an optical density at 600 nm (OD₆₀₀) of 3.0 in the brain heart infusion medium (BD Difco, United States). The supernatant was filtered through a 0.2 μm pore filter (Whatman, United States), ultrafiltered, and replaced with buffer A (50 mM Tris–HCl, pH 8.0) using a Vivaspin 3,000 Da filter (10,000 × g, 30 min, 4°C; Sartorius, United States). The solutions were loaded onto a Mono-Q column equilibrated with buffer A (Metabolomics Research Center for Functional Materials, Kyungsung University; Agilent Technologies 1260 Infinity LC System, United States). The column was eluted with 30 ml of a linear gradient of NaCl (0–1 M) in buffer A at a flow rate of 0.5 ml/min. Active fractions were collected, N-terminally sequenced, and used for further experiments (Lee et al., 2017a).

**Administration of Serralysin and Measurement of Survival Rate**

Purified serralysin was sequentially diluted to a concentration of 1 μg/μl. Each serralysin solution at a different dose (2 μl aliquot) was orally infected or systemically injected into the joint of the hind leg that connects to the 3-day-old adult male, *R. pedestris*. The survival rate was monitored from 1 d after the systemic injection.

**Preparation of Midgut Lysates From Apo- and Sym-Insects**

Midguts from 10 male *R. pedestris* adults were collected in 500 μl of 10 mM phosphate buffer (PB; pH 7.0) containing a protease inhibitor cocktail (Sigma Aldrich, United States). The collected midguts were homogenized using disposable plastic pestles (SciLab Korea, South Korea). After centrifugation at 10,000 × g for 15 min at 4°C, the supernatant was ultrafiltered and replaced with buffer A (50 mM Tris–HCl, pH 8.0) using a Vivaspin 3,000 Da filter (10,000 × g, 30 min, 4°C; Sartorius, Germany). After buffer replacement, the solution was collected and used immediately for further experiments. The supernatant was heat-treated at 90°C for 5 min to inactivate the serralysin-degrading proteins and centrifuged at 20,000 × g for 10 min at 4°C. Bovine serum albumin was used for protein quantification of midgut lysates, and the native protein concentration was determined using the Bradford assay (Kruger, 2009).
**Immunoblot Analyses**

For immunoblot analyses, proteins from M3 lysate or hemolymph were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, United States) using an electro-transfer blottter (Lee et al., 2017a). The membrane was blocked with 10% skim milk in Tris-buffered saline with Tween 20 (TBST) buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.02% Tween 20) for 1 h and washed six times with the TBST buffer. After blocking, the membranes were incubated at room temperature for 1 h with the anti-rabbit serralysin antibody (dilution factor 1:5,000) in TBST containing 5% skimmed milk (Lee et al., 2017a). After washing with TBST six times, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (dilution factor 1:10,000; Santa Cruz, United States) for 30 min. The membranes were washed seven times with TBST and visualized using HRP color development solution in accordance with the manufacturer's instructions.

**Isolation of Midgut-Colonizing Serratia marcescens Cells**

M3 midguts were dissected from 10 fifth-instar nymphs and collected in 100μl of 10 mM PB. M3 midguts were cut several times with fine scissors to break the midgut crypts. One milliliter of 10 mM PB was added to the midgut fragments by gentle pipetting. The solution was then filtered through a 5.0μm pore to remove the midgut isolates. Isolated S. marcescens cells were gently washed with 10 mM PB and collected by centrifugation at 2,000× g. The bacterial cell number was estimated using a hemocytometer.

**Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was isolated from midgut-colonizing or cultured S. marcescens cells (OD_{600nm} = 1.0) using the TRizol reagent (Invitrogen, United States) and RNeasy mini kit (Qiagen, United States), according to the manufacturer’s recommendations. Thereafter, 500 ng of total RNA was converted into cDNA using TOpscript RT DryMix containing oligo-dT primers (Enzymics, South Korea). The synthesized cDNAs were diluted 20-fold, and qRT-PCR was performed on a QuantStudio™3 Real-Time PCR System (Thermo Fisher Scientific Inc., United States). The PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s. The primer sets used for qRT-PCR of serralysin genes are listed in Table 1. The comparative C_{T} (ΔΔC_{T}) method was used to calculate the relative gene expression levels based on the 16S rRNA of S. marcescens cells as an internal control gene (Wilf et al., 2011). All analyses were performed using the QuantStudio™ Design & Analysis Software ver 1.5.2 (Thermo Fisher Scientific Inc.).

**Statistical Analysis**

Statistical significance of the data was determined using an unpaired Student’s t-test, as provided in GraphPad Prism (ver. 8.0; GraphPad Software, United States).

### RESULTS

#### Midgut-Colonizing Serratia marcescens Cells Did Not Affect Host Fitness

Previous studies have shown that S. marcescens Db11 strain is highly pathogenic to Drosophila melanogaster and Apis mellifera when they are systemically injected with this bacterium (Flyg et al., 1980; Kurz et al., 2003; Raymann et al., 2018). However, orally infected S. marcescens Db11 cells can colonize the Drosophila midgut without any virulence (Nehme et al., 2007). Based on these reports, we hypothesized that orally infected S. marcescens cells could colonize the midgut of R. pedestris. The bean bug adults have five different midgut regions (Ohbayashi et al., 2015), M1, M2, M3, M4B, and M4. We examined the colony-forming units (CFUs) of S. marcescens in each midgut region at the indicated times (Figure 1A). Serratia marcescens cells were only detected in M1–M3 regions 2 days post-inoculation (Figures 1C,D). These results suggest that orally infected S. marcescens cells can pass through the digestive tract, such as the foregut region and colonize the midgut. The beneficial symbiont, B. insecticola, colonized M4B and M4 midgut regions (Figures 1C,D). This result is consistent with the previous report that no microorganism or color pigment can pass through M4B region except Burkholderia (Ohbayashi et al., 2015). When we examined the population of orally infected S. marcescens cells in M3 midgut region, the titers of S. marcescens were increased steadily during nymphal development and remained at constant levels of around 10^7 CFUs per individual in adulthood (Figure 1B).

Based on the fact that Burkholderia gut symbionts positively affect the host fitness and immune responses (Kim et al., 2015a; Lee et al., 2017b), we investigated if orally infected S. marcescens is a potent entomopathogen that affects the survival rate and growth of the host. Furthermore, we speculated that Sym-insects with Burkholderia gut symbionts would be more resistant to orally infected S. marcescens than Apo-insects without symbionts. To investigate the effects of S. marcescens cells on either Sym- or Apo-insects, the adult emergence rates of R. pedestris were estimated after oral infection with cultured S. marcescens cells. Orally infected S. marcescens did not affect the growth of R. pedestris (Figure 2A). When the survival rates between Sym- and Apo-insects were compared, all second nymphs infected with S. marcescens were survived until adulthood (Figure 2B). Evaluation of body length and dry weight showed that orally infected S. marcescens did not have any effect on the host, regardless of the presence of symbiotic Burkholderia cells.

#### Table 1 | Primer sets used for qRT-PCR in this study.

| Primer name | Primer sequence (5′→3′) |
|-------------|-------------------------|
| Serralysin 1_F | ATCACCTTCACCGAAGTGG |
| Serralysin 1_R | TGCTTGACGGTTGACTGG |
| Serralysin 2_F | ATGAATACGAGTGTCG |
| Serralysin 2_R | CTTAAAGTTCCTCGCCCGT |
| 16S rRNA _F | GGCCCGGTTGTGAAAGTC |
| 16S rRNA _R | GCTTTACGGCCAGTCTACG |

**Figure 1A**

Midgut-Colonizing Serratia marcescens cells in R. pedestris midgut regions. M1, M2, M3, M4B, and M4 midgut regions were stained with the anti-rabbit serralysin antibody (dilution factor 1:5,000; Santa Cruz, United States) for 30 min. The membranes were washed seven times with TBST and visualized using HRP color development solution in accordance with the manufacturer's instructions.

**Figure 1B**

Survival rates of R. pedestris adults after oral infection with cultured Serratia marcescens cells. Oral infection with S. marcescens cells did not affect the growth of R. pedestris (Figure 2A). When the survival rates between Sym- and Apo-insects were compared, all second nymphs infected with S. marcescens were survived until adulthood (Figure 2B). Evaluation of body length and dry weight showed that orally infected S. marcescens did not have any effect on the host, regardless of the presence of symbiotic Burkholderia cells.
Serralysin metalloprotease is the major toxic substance of *S. marcescens* in host insects (Ishii et al., 2014a). However, our results showed that oral infection with *S. marcescens* did not have any adverse effects on development of *R. pedestris* (Figure 2). These results indicate that orally infected *S. marcescens* does not show any insecticidal activity during the colonization of *S. marcescens* cells in insect gut. Therefore, to compare the insecticidal activity of serralysin based on the infection routes, the survival rate was investigated by oral infection or systemic injection of serralysin purified from the bacterial culture medium. Only systemic injection of serralysin showed insecticidal activity in a dose-dependent manner whereas all tested insects were survived after oral infection (Figure 3). The amino acid sequence of purified serralysin was identified as A-A-T-T-G-Y-D-A-V-D, corresponding to the 17th to 26th amino acids of serralysin 1 (Supplementary Figure 1).

It is questionable how host insects are alive when they are orally infected with insecticidal serralysin. Our previous study provided a biochemical evidence that the antimicrobial peptide trialysin in *R. pedestris* salivary fluid was specifically hydrolyzed by serralysin, leading to the loss of its antimicrobial activity (Lee et al., 2017a). Based on these results, we hypothesized that the insecticidal activity of serralysin would be diminished during digestive tract passage and colonization by unknown factors, thereby facilitating host survival. To prove this hypothesis, purified serralysin was co-incubated with three midgut extracts of Apo-insects, and the activation of serralysin was investigated with immunoblot analyses. Purified serralysin was gradually degraded by the midgut extract in a dose-dependent manner (Figure 4). This result strongly suggests that a host-derived factor capable of degrading serralysin exists in the M1 midgut region (Figure 4A). To determine if the serralysin-degrading activity is derived from the hosts, Apo- or Sym-insects, extracts from the M1 midgut of both hosts were co-incubated with serralysin. Purified serralysin was completely removed by the M1 lysate of Sym-insects, but remained in the M1 lysate of Apo-insects (Figure 4B-i). Moreover, when the supernatant of heat-treated Sym- and Apo-M1 lysates was added to purified serralysin, the host-derived serralysin-degrading factor lost its biological activity (Figure 4B-ii). This result revealed that the
serralysin-degrading factor in the M1 lysate is related to proteinous molecules.

M3-Colonizing Serratia marcescens Cells Do Not Produce Serralysin

Although a serralysin-degrading factor from *R. pedestris* existed in M1 region (Figure 4), orally infected *S. marcescens* cells still colonized the M3 midgut region (Figure 1). To understand the host-gut microbe interactions in M3 region, we investigated if serralysin was detected in M3 tissue. Serralysin was not detected in the M3 tissue (Figure 5). This result provides the possibility that *S. marcescens* cells in M3 region do not produce serralysin during colonization of the host gut. Based on this result, we investigated that serralysin expression in M3 region can be maintained at a low level or completely suppressed. Genome analysis based on the National Center for Biotechnology Information (NCBI) public database showed that two *serralysin* genes were present in *S. marcescens* Db11. The nucleotide and putative amino acid sequences of *serralysin* 1 and *serralysin* 2 showed a similarity of 66.5 and 59.0%, respectively (Supplementary Figure 1). In addition, serralysin 1 and 2 had a catalytic domain related to metzincin at the N-terminus region (data not shown). The phylogenetic analysis revealed that serralysin 1 and serralysin 2 formed independent clades, respectively (Supplementary Figures 2, 3).

When comparing mRNA expression levels between serralysin genes in cultured *S. marcescens*, no difference in the expression level was found (Supplementary Figure 4). The transcriptional levels of *serralysin* genes were compared between in vitro-cultured *S. marcescens* and M3-colonizing *S. marcescens* cells. The expression levels of *serralysin* genes in M3-colonizing *S. marcescens* cells were significantly lower than those of in vitro-cultured *S. marcescens* (Figure 6A). Under systemic injection, M3-colonizing *S. marcescens* cells did not produce serralysin whereas in vitro-cultured *S. marcescens* cells secreted serralysin (Figure 6B).

DISCUSSION

In a previous study, we demonstrated that serralysin, a toxin produced by *S. marcescens* cells, neutralized the
antimicrobial activity of trialysin in the salivary of R. pedestris, resulting in suppressing the host immune response (Lee et al., 2017a). When systemically inoculated into the host, serralsin exhibited insecticidal activity (Figure 3). Based on this result, we hypothesized that the serralsin toxin of orally infected S. marcescens could kill the host insect.
However, orally infected *S. marcescens* cells were found only to colonize the midgut without insecticidal activity (Figure 1). These results led to the idea that orally infected *S. marcescens* cells may have been converted into non-pathogenic microorganisms and then lost their insecticidal activity.

Insect detoxification systems inactivate toxic substances, greatly reducing biological effects by entomopathogenic microorganisms (Hillyer, 2016). In the insect midgut, the mechanism of resistance against toxic substances is composed of complex responses of the defense system, including alteration of toxin-binding proteins (Bravo et al., 2011), sequestration of the toxin by lipophorin (Ma et al., 2012), increased production of primary enzymes (Tartar et al., 2009; Grizanova et al., 2019; Wu et al., 2020), and antioxidant activity (Dubovskiy et al., 2008). These biochemical reactions require various enzymes, such as esterases, uridine diphosphate-glycosyltransferases, cytochrome P450s, glutathione-S-transferases, and monoxygenases (Ramsey et al., 2010; Feng et al., 2021). These enzymes play important roles in the detoxification of toxic substances, such as insecticides and plant secondary metabolites (Li et al., 2007). In this study, we revealed that a host-derived factor that can degrade serralysin is present in the M1 midgut region of *R. pedestris* (Figure 4A). The serralysin-degrading activity was completely lost after heat treatment (Figure 4B). Taken together, the host-derived factors for degrading serralysin are proteinaceous molecules, such as detoxifying enzymes or their complexes. As serine proteases, such as trypsin and chymotrypsin, have a serine hydroxyl residue at the active site and are involved in regulation of enzyme activity, they were candidates for host-derived serralysin-degrading factors (Aiyappa and Harris, 1976). The proteolytic activity of metalloproteases including serralysin was completely inhibited by EDTA, a chelating agent for divalent metal cations (Kida et al., 2007; Ishii et al., 2014a,b). The inhibitor screening for serralysin metalloprotease can be a good strategy to identify host-derived factors.

Orally infected *S. marcescens* cells did not produce serralysin during colonization of the midgut. Transcriptome analysis by RNA sequencing of both gut-colonizing bacteria and cultured bacteria showed that the expression patterns of several genes were completely different (Ohbayashi et al., 2019). When differentially expressed genes were classified into clusters of orthologous groups of the NCBI public database, the expression levels of most genes involved in cell motility, secretion, vesicular transport, and extracellular structures were downregulated in the gut-colonized bacteria (Baumann, 2005; McCutcheon and Moran, 2012; Ohbayashi et al., 2019). Among them, Type II and type IV secretion systems are involved in the extracellular transport of cell wall-degrading enzymes and endotoxins (Sandkvist, 2001; Murdoch et al., 2011; Gallique et al., 2017; Ohbayashi et al., 2019). There are changes in gut-colonizing bacteria, such as small size, increased stress sensitivity, loss of motility, and alteration of cell surfaces (Kim et al., 2013, 2015b; Ohbayashi et al., 2019). Based on these results, we investigated that the gene expression pattern of M3-colonizing *S. marcescens* would be different from that of free-living *S. marcescens*. As expected, the transcriptional and translational expression levels of *serralin* genes dramatically decreased in M3-colonizing *S. marcescens* (Figure 6). This explained the reason the insecticidal serralysin was not found in M3 tissue.

The previous report that lipopolysaccharide (LPS) components are associated with the insecticidal activity of *S. marcescens* may provide a molecular evidence that M3-colonizing *S. marcescens* cannot produce serralysins. In general, LPS is a requisite component for maintaining the physiological properties, such as permeability and biofilm formation (Herlax et al., 2005; Ishii et al., 2014b; Hathroubi et al., 2016). The LPS layer comprises three regions: lipid A moieties, core oligosaccharides, and O-antigen polysaccharides. Among them, the O-antigen residue is highly associated with bacterial pathogenicity. The *waaE* mutant of *Aeromonas hydrophila* which has an O-antigen-deficient LPS is avirulent and loses the ability to stimulate the pro-phenoloxidase system in *Tenebrio molitor* larvae (Noonin et al., 2010). Enterohemorrhagic *Escherichia coli* O157 requires an LPS O-antigen for effective insecticidal activity against...
Bombyx mori (Miyashita et al., 2012). Similarly, the wecA gene of S. marcescens which is involved in the biosynthesis of LPS O-antigen is an important factor for the apoptotic death of host immune cells in B. mori (Ishii et al., 2012). The culture supernatant of the S. marcescens wecA mutant exhibits reduced hemolymph bleeding activity compared to that of the wild-type strain (Ishii et al., 2014b). In S. marcescens, rough-type LPS lacking O-antigen components induced significantly low hemolytic activity (Poole and Braun, 1988; Kurz et al., 2003). Symbiotic Burkholderia colonizing the M4 crypt lacks an O-antigen residue in R. pedestris (Kim et al., 2015b). These molecular evidences suggest the possibility that M3-colonizing S. marcescens cells may lack the LPS O-antigen.

In summary, we revealed that serralysin produced by orally infected S. marcescens cells was degraded by the host-derived factor present in the M1 tissue of R. pedestris. In addition, S. marcescens cells colonized in the M3 region did not produce the entomopathogenic serralysin. This study will provide insight into how orally infected entomopathogenic bacteria can colonize the host midgut without insecticidal activity and the molecular interactions between them in the gut.

**AUTHOR CONTRIBUTIONS**

JL and D-WL conceived and designed the study and wrote the manuscript. JL performed the experiments and analyzed the data. All authors contributed to the article and approved the submitted version.

**FUNDING**

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) and funded by the Ministry of Education (NRF-2020R1I1A1A0106704312). This research was supported by a grant from the Korea Basic Science Institute (National Research Facilities and Equipment Center) and funded by the Ministry of Education (2019R1A6C1010044).

**ACKNOWLEDGMENTS**

We would like to thank Bok Luel Lee and Jong Uk Kim (Pusan National University) for providing serralysin antibodies for this study.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.913113/full#supplementary-material
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