Identification of SlpB, a cytotoxic protease from *Serratia marcescens*

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Abstract

The Gram-negative bacterium and opportunistic pathogen *Serratia marcescens* causes ocular infections in healthy individuals. Secreted protease activity was characterized from 44 ocular clinical isolates and a higher frequency of protease positive strains was observed among keratitis isolates compared to conjunctivitis isolates. A positive correlation was determined between protease activity and cytotoxicity to human corneal epithelial cells in vitro. Deletion of *prtS* in clinical keratitis isolate K904 reduced, but did not eliminate, cytotoxicity and secreted protease production. This indicated that PrtS is necessary for full cytotoxicity to ocular cells and implied the existence of other secreted protease(s) and cytotoxic factors. Bioinformatic analysis of the *S. marcescens* Db11 genome revealed three additional open reading frames predicted to code for serralysin like proteases noted here as *slpB*, *slpC*, and *slpD*. Induced expression of *prtS* and *slpB*, but not *slpC* and *slpD*, in strain PIC3611 rendered the strain cytotoxic to a lung carcinoma cell line; however, only *prtS* induction was sufficient for cytotoxicity to a corneal cell line. Strain K904 with deletion of both *prtS* and *slpB* genes was defective in secreted protease activity and cytotoxicity to human cell lines. PAGE analysis suggests that SlpB is produced at lower levels than PrtS. Purified SlpB demonstrated calcium dependent and AprI-inhibited protease activity and cytotoxicity to airway and ocular cell lines in vitro. Lastly, genetic analysis indicated that the type 1 secretion system gene, *lipD*, is required for SlpB secretion. These genetic data introduce SlpB as a new cytotoxic protease from *S. marcescens*. 
Introduction

Microbial keratitis (MK) is a blinding disease with poor visual outcomes even with effective antibiotics and antifungal agents (1-3). In addition to being a major cause of hospital-acquired infections such as ventilator-associated pneumonia (4, 5), *Serratia marcescens* is a common cause of MK (1, 2, 6-8), yet the virulence factors involved in this process are poorly understood. In general, bacterial secreted factors including hemolysins and proteases contribute to the pathogenesis of bacterial corneal infections (9-12). There are several studies characterizing the importance of proteases from *S. marcescens* isolates in ocular infections, most recently reviewed by Matsumoto (13). Serralysin is a cytotoxic factor capable of killing mammalian cells in vitro (14, 15). Purified serralysin is sufficient to cause keratitis when injected into rabbit eyes, and promotes the spread of bacteria through the corneal stroma (16-18). Additionally, serralysin can degrade components of the human immune system, such as PAR-2, *in vitro*, and this may impact corneal pathogenesis (13, 19-21). Serralysin, and serralysin-family metalloproteases, such as alkaline protease from *Pseudomonas aeruginosa*, can proteolyze mammalian cell-surface proteins thereby modulating cell physiology. A recent example is the protease-mediated activation of the epithelial sodium channel leading to a cell surface that is more amenable for bacterial colonization (22, 23). Several bacteria including *Serratia* species invade eukaryotic airway cells in a protease-dependent manner (24-27).

Few ocular clinical isolates of *S. marcescens* have been characterized for their ability to secrete proteases. These include 3 strains analyzed by Hume, et al., in Australia, (28) and 3 strains from Pinna, et al., in Italy (29). Here we characterized secreted
protease activity from more than 40 ocular isolates of *S. marcescens* collected at a tertiary care hospital in the North Eastern United states. Arbitrarily selected isolates were tested for cytotoxicity, and higher levels of cytotoxicity were associated with high-protease producing strains.

Multiple proteases have been biochemically isolated from a highly virulent keratitis isolate of *S. marcescens*, but the genes responsible for these proteins were not determined (30). Only the gene for the serralysin protease, *prtS*, has been cloned and characterized by sequence analysis (31). Here, we report the identification of three uncharacterized serralysin-like proteases in the genome of *S. marcescens*. To determine which of the metalloproteases mediated cytotoxicity, we cloned and induced expression of each of these genes in trans and in cis in a nearly non-cytotoxic laboratory strain of *S. marcescens*. Only two of the proteases conferred cytotoxicity and elevated secreted protease activity: PrtS (serralysin), and the novel protease named serralysin-like protease B, SlpB. Further genetic analysis using a clinical corneal isolate suggested that SlpB together with PrtS contribute to secreted protease activity by *S. marcescens*. 
Materials and Methods

Bacterial growth and strains. Bacteria were cultured in lysogeny broth (LB) or LB agar (LB broth supplemented with 1.5% w/v agar) (32), and incubated at 30°C. Milk agar plates were prepared using brain heart infusion agar supplemented with 1% (w/v) skimmed milk powder. Liquid cultures were incubated using a New Brunswick TC-7 rotor at speed setting 8 (~62 rpm). S. marcescens strains used for genetic analysis are listed in Table 1. Ocular clinical isolates were obtained from the Charles T. Campbell Laboratory of Ophthalmic Microbiology at the University of Pittsburgh Medical Center.

Escherichia coli strains used in this study were EC100D-pir-116 (Epicentre) and S17-1 \( \lambda \) pir (33); Saccharomyces cerevisiae strain InvSc1 (Invitrogen) was used for cloning.

Tissue culture and cytotoxicity assays. Human lung carcinoma cell line A549 (34) were propagated and maintained in tissue culture media consisting of Dulbecco’s modified Eagle’s medium (MEM) supplemented with 10% fetal bovine serum (Sigma). Human corneal Limbal epithelial (HCLE) cells (35) were maintained in keratinocyte serum free medium with L-Glutamine (Life Technologies), supplemented with bovine pituitary extract (25 µg/ml), embryonic growth factor (0.2 ng/ml), and CaCl\(_2\) (0.3 mM).

Cytotoxicity was measured using Alamar Blue and Presto Blue reagents (Life Technologies) as previously described by Wingard, et al. (36). Briefly, bacterial cultures were grown for 18-20 h in LB medium with antibiotics if a plasmid was used, and L-arabinose (0.2%) was added to induce expression from the \( P_{BAD} \) promoter when desired. Cultures were normalized by adjustment to OD\(_{600}\) = 2.0 by addition of LB medium. Culture aliquots were added to microcentrifuge tubes and spun at 14,000 x g for 2 minutes.
followed by filter sterilization of the supernatants using 0.22 µm filters. Two-hundred
microliters of filtered bacterial cultures were added to epithelial cell line layers in 24 well
dishes with 300 µl of culture medium. After 4 hours of incubation, cell layers were
washed three times with 0.5 ml of phosphate buffered saline (PBS) and suspended in
culture medium with Alamar Blue or Presto Blue viability indicators (Life Technologies)
and measured as previously described (36). Alternatively, cell layers were not washed
following challenge and were analyzed with Presto Blue. In all cases, cytotoxicity values
were determined relative to positive and negative control values, i.e., cytotoxicity of the
mock (negative control) was set to 0% cytotoxicity and detergent treated (triton X-100 at
0.25% v/v) was set to 100% cytotoxicity. The % cytotoxicity was determined using the
following formula: 100 x ((Mock value-experimental value)/(Mock value – detergent
treated value)).

Protease assay
Milk agar plates were used for primary analysis of secreted protease activity. Bacteria
were patched on milk agar plates and incubated for 24 to 48 h at 30˚C, after which zones
of clearance around the colonies were noted. For quantitative analysis, protease activity
was measured from normalized spent culture supernatants (OD_{600} = 2.0) using azocasein
(Sigma) as a colorometric substrate as previously described (37, 38). Normalized
cultures were centrifuged and filtered to remove bacteria. A 150 µl aliquot of the filtered
cell-free supernatant was mixed with 250 µl of azocasein (2% w/v) and incubated for 30
minutes at 37˚C. The reaction was stopped by addition of 1.2 ml of 0.6 N trichloroacetic
acid and the mixture was incubated for 15 minutes on ice. The tubes were centrifuged at
8000 x g for 10 minutes. Cold NaOH (1.4 ml of 1 N solution) was added to wells of a 24
well dish and 1.2 ml of the centrifuged culture mixture was added to the NaOH solution.
Colorometric analysis of liberated azol dye was measured at 440 nm with a plate reader,
and divided by the original culture OD600.

**Molecular biology**

The N-terminal polyhistidine tagged *slpB, slpC and slpD* under control of the *P_{BAD}*
promoter on p15a-based plasmid pMQ125 (39) were generated by replacing *prtS* on
pMQ356 (23) but maintaining the N-terminal His7 tag. This was done by digesting
pMQ356 with Sall and SmaI that cut in the *prtS* gene and then replacing the entire *prtS*
gene using primers that amplify the *slp* genes and have regions of homology to the His7-
tag and vector backbone. The recombination was carried out using *Saccharomyces
cerevisiae* for in vivo recombination (39, 40).

To place the chromosomal candidate protease genes and *prtS* under control of
*P_{BAD}* promoter, the open reading frames (ORF) for *prtS* and the candidate proteases were
cloned into pMQ200 (39), an oriR6K-based suicide plasmid, using *S. cerevisiae*
recombineering. The plasmid was introduced into the PIC3611 strain by conjugation and
the insertions were verified by PCR.

To generate chromosomal deletions of *prtS*, the full length *prtS* gene was cloned
into the pMQ236 (39) allelic replacement vector generating pMQ263. This plasmid was
then digested with AgeI, which cuts in three places in *prtS*, and then recircularized with
T4 ligase. The resulting mutant allele retains 108 base pairs of the *prtS* upstream of the
AgeI site and 618 base pairs downstream of the AgeI site. The *prtS* deletion plasmid was
moved into K904 followed by introduction of pMQ337 which has the I-SceI gene under control of the \( PBAD \) promoter. After addition of I-SceI with L-arabinose, chromosomal \( \text{prtS} \) deletions were screened for kanamycin susceptible isolates that had lost pMQ236, first by a reduction in protease activity on milk agar plates, and then by PCR using primers outside the \( \text{prtS} \) ORF.

To mutate \( \text{slpB} \) by plasmid insertion, a 606 base pair internal fragment of \( \text{slpB} \) was cloned into pMQ118 (39). This plasmid, pMQ218, was introduced into K904 by conjugation and kanamycin resistant isolates were screened by PCR for the plasmid disruption of the \( \text{slpB} \) gene. The plasmid inserts after base pair 747 out of 1419 base pairs, which truncates the \( \text{slpB} \) gene after codon 249 out of 472 amino acids, and adds on 28 codons from the inserting plasmid sequence until reaching a stop codon. The resulting predicted 277 amino acid polypeptide has the protease domain, but lacks the secretion domain. The insertion was verified by PCR analysis.

To generate the \( \text{slpB} \) deletion allele, the \( \text{slpB} \) ORF along with 602 bp of upstream DNA and 523 bp of downstream DNA was cloned into allelic replacement vector pMQ460. A PvuI-PvuI fragment was removed from the \( \text{slpB} \) gene removing 657 base pairs. This in frame deletion mutation is predicted to remove 219 amino acids from the N-terminal half of the 472 amino acid protein. The deleted residues from amino acid 49-187 include the predicted catalytic site.

\textbf{LipD mutation.} Strain C23M13 with a transposon mutation in the \( \text{lipD} \) ORF was identified in a genetic screen for mutations that suppress the elevated protease phenotype of \( \text{crp} \) mutants in \( S. \marcescens \) PIC3611 (37). The \( \text{lipD::tn} \) allele was cloned into allelic
replacement vector pMQ310 (Table 1) to generate pMQ445. The resulting plasmid was moved into PIC3611 by conjugation, and allelic replacement was carried out. The introduced lipD mutation was verified by PCR and loss of lipase and protease activities using plate based assays as previously described (37).

**PAGE analysis of secretomes.** Trichloroacetic acid precipitated secretomes that had been normalized to OD$_{600}$=2 were separated on 8-16% polyacrylamide gels and stained with Coomassie Brilliant Blue. Gels were imaged using an infrared imager (Li-Cor, Odyssey) using the 700 nm channel. Band intensities from the digital images were analyzed using ImageJ Software (NIH). Bands of interest were cut out with a clean razor and proteins were identified using mass spectrometry at the University of Pittsburgh Biomedical Mass Spectrometry Center.

**RT-PCR.** RNA was extracted from cultures grown in LB broth to OD$_{600}$ = 3.0 using a Qiagen RNA Easy Kit and cDNA preparations were performed as previously described (41). Two rounds of DNase treatment were performed to remove chromosomal DNA. 16S rDNA primers were used to normalize signals and to test for chromosomal DNA contamination (Table 2). Control reactions lacking reverse transcriptase were included and verified a lack of chromosomal DNA contamination. The experiment was performed twice with independent RNA samples with similar results.

**SlpB and AprI purification and analysis.** The slpB open reading frame expressed from arabinose-regulated expression plasmid, pMQ436, in *E. coli* EC100D pir-116 cells.
culture was grown overnight in the presence of gentamycin and 10 ml was used as an inoculum for His7-slpB induction in 1 L cultures. The cultures were grown in LB supplemented with gentamycin and 0.2% w/v arabinose was added to the culture when it achieved OD_{600}=0.8. Protein was expressed at 37°C for 4-6 hours. The SlpB protein was purified from the insoluble material under denaturing conditions following protocols previously described for PrtS and AprA (23). In vitro protein refolding and protease activity were assessed using a fluorescent peptide and casein substrates, as previously described (42). The AprI protease inhibitor was expressed and purified as previously described (23).

Statistical analysis

2-tailed Student’s T-test, one way ANOVA with Tukey’s post-test, and Pearson’s correlation were performed with Prism 5, significance was set to p<0.05.

Results

Secreted protease activity from ocular clinical isolates

Protease activity, secreted by ocular clinical isolates of S. marcescens, was analyzed. Isolates were obtained from patients with conjunctivitis (n=7), endophthalmitis (n=2), and keratitis (n=35). Bacteria were patched onto milk agar plates and after 24 h at 30°C, zones of clearance around the bacteria were counted as a positive indication of secreted protease activity, with any zone ≥ 1mm being considered positive. A total of 38 were positive (86%) for clearance at 24 h, although all produced at least a small zone of
clearance by 48-72 hours. Similar results were observed at 37˚C, for example clearing zones on milk plates around patches of strain K904 were 3.6±0.3 mm at 30˚C and 3.6±0.4 mm at 37˚C (n≥7, p=0.96). By 48 hours the zones increased to 4.9±0.8 mm at 30˚C and 5.5±0.7 mm at 37˚C (n=21, p=0.02).

Three of the seven conjunctivitis isolates had low levels of proteolysis on milk agar plates (no zone by 24 hours), whereas only three of the 35 keratitis isolates were protease deficient at 24 h (p=0.0478, Fisher’s Exact Test). As previously reported, we observed that the laboratory strain PIC3611 used in this study did not produce a zone of clearing at 24 h, but did exhibit a small zone of protease activity on milk plates by 48 h (37).

Secreted protease activity was measured from culture supernatants of a subset of the strains analyzed with milk agar plates. Since keratitis is the most frequent ocular infection caused by *S. marcescens*, further analysis primarily used keratitis isolates. Protease activity is shown in Figure 1A and followed a strain dependent pattern. Of the 21 tested keratitis strains, all but two (K1189 and K1496) demonstrated elevated levels of proteolysis relative to laboratory strain PIC3611 (p<0.05, ANOVA with Dunnett’s multiple comparison test).

**Cytotoxicity of ocular clinical isolates to a corneal cell line in vitro is strain dependent**

Cytotoxicity of secreted components produced by ocular clinical isolates and laboratory strain PIC3611 to HCLE cells was investigated. In contrast to the laboratory strain, many of the clinical isolates were highly toxic to the HCLE cell layers (Figure 1B).
Cytotoxicity and protease activity of supernatants from PIC3611 and clinical conjunctivitis and keratitis isolates were found to have a positive correlation (Pearson’s r=0.925, p<0.001; Figure S1).

Mutation of *prtS* in clinical isolate K904 confers loss of secreted protease and cytotoxicity to human epithelial cells. To test whether PrtS contributes to the cytotoxicity of *S. marcescens* keratitis isolates to corneal cells in vitro, which has not previously been reported, a deletion allele of the *prtS* gene was imparted onto the chromosome of strain K904 by allelic replacement. This in frame deletion allele is missing amino acids 37-297 of the predicted protein which includes the zinc-dependent metalloprotease domain. Secreted fractions from the K904 Δ*prtS* mutant strain were defective in protease activity, with a >50% reduction, and this deficit could be complemented by the intact *prtS* gene on a plasmid (Figure 2A). Cytotoxicity to human corneal cells was likewise reduced (Figure 2B). It was of interest that the secreted protease activity and cytotoxicity were not completely eliminated in supernatants of the K904 Δ*prtS* mutant (mock treated cells had 0% cytotoxicity and LB medium had no detectable protease activity, data not shown), suggesting a contribution by another secreted protease(s).

Predicted serralysin-like proteins were identified following *in silico* analysis of *S. marcescens* genomes. In search of additional proteases, we scanned the *S. marcescens* Db11 genome (Wellcome-Trust Sanger Center) using the PrtS amino acid sequence as a query. We identified 3 ORFs with high similarity to serralysin that were also found in
the genomes of more recently sequenced \textit{S. marcescens} strains (43, 44) using an NCBI BLAST search (45). These predicted proteins are here noted as \textit{serralysin-like} proteases B, C and D (Table S2).

The amino acid identities of the predicted proteins to serralysin were 52.6, 50.3, and 45 percent, respectively. Phylogenetic analysis indicates that of the three predicted proteases, SlpB is closest by sequence identity to serralysin, and is also most similar to alkaline protease from \textit{Pseudomonas aeruginosa}, AprA (Figure S2).

Serralysins are peptidases, a subset of the larger M10 metalloprotease group that includes mammalian matrix metallopeptidases (MMPs). Serralysin family proteins have an N-terminal HEXXHXXGXXH sequence involved in Zn2+ coordination in their N-terminus and catalytic activity in addition to repeats in toxin (RTX) motifs (GGXGXD) in the C-terminus involved in binding to calcium and secretion via type I secretion systems (46). The C-terminal domain is noted by the Protein Family Database (47) designation pfam00353. Predicted SlpB, SlpC, and SlpD proteins have the N-terminal HEXXHXXGXXH domain and RTX repeats, as well as a highly conserved M-turn methionine residue proximal to the active site (Table S2). Notably, serralysin and SlpB have four GGXGXD motifs, whereas SlpC and SlpD have two.

\textbf{Native expression of \textit{slpB}, \textit{slpC}, and \textit{slpD} is detectable in a laboratory and a clinical isolate of \textit{S. marcescens}.}

We tested whether the genes noted above from the Db11 genome were present in the genomes of strains PIC3611 and K904 used in this study. PCR analysis revealed that the \textit{prtS}, \textit{slpB}, \textit{slpC}, and \textit{slpD} genes were present in both genomes (data not shown). This
does not, however, prove that these putative metalloprotease genes are transcribed. To test whether \textit{slpB}, \textit{slpC}, and \textit{slpD} were expressed under laboratory conditions, RNA was harvested and RT-PCR was performed. For both PIC3611 and K904 amplicons corresponding to the \textit{slpB}, \textit{slpC}, and \textit{slpD} genes and 16S and \textit{prtS} controls were detectable from RT-treated RNA (Figure 3). Furthermore, expression was relatively higher for each of the protease genes in the clinical isolate, K904, compared to the laboratory strain, PIC3611 (Figure 3).

\textit{Induced expression of serralysin and \textit{slpB}, but not \textit{slpC} and \textit{slpD} increased extracellular protease activity.} The putative chromosomal protease genes for \textit{prtS}, \textit{slpB}, \textit{slpC}, and \textit{slpD} were placed under control of an arabinose-inducible promoter in the laboratory strain PIC3611. This strain was chosen because of a relatively low basal level of extracellular protease activity compared to the clinical isolates (Figure 1A), and this low level would facilitate measuring increases in extracellular protease levels. Arabinose-induction of poly-histidine (His\textsubscript{7}) tagged versions \textit{prtS} or \textit{slpB} from an episomal plasmid resulted in a clear increase in extracellular protease activity, whereas, no increase in protease levels were detected from supernatants of cells expressing the His\textsubscript{7}-tagged versions of \textit{slpC} and \textit{slpD} genes or a negative vector control (Figure 4A). As a second test, the chromosomal \textit{prtS}, \textit{slpB}, \textit{slpC}, and \textit{slpD} genes were expressed under control of the \textit{P\textsubscript{BAD}} promoter integrated in front of each gene in the PIC3611 strain. Similar to episomal induction of these genes, chromosomal expression of \textit{prtS} and \textit{slpB} conferred increased secreted protease activity compared to the empty vector control, whereas \textit{slpC} and \textit{slpD} expression did not (Figure S3A). Induction of a control non-
protease gene, pigA pigment biosynthetic gene, showed little background protease activity.

**Induced expression of serralysin and SlpB increased cytotoxicity to human epithelial cell lines in vitro.** We tested whether induced expression of serralysin and the other putative proteases in PIC3611 could influence the cytotoxicity of bacterial secretomes to human epithelial cells in vitro. Unlike the vector negative control, expression of *prtS* induced bacterial cytotoxicity to A549 airway cells (Figure 4B). Induced expression of *slpB*, but not *slpC* or *slpD*, was similar to *prtS* induction with cytotoxicity to A549 cells (Figure 4B). A similar pattern was observed when *prtS*, *slpB*, *slpC*, and *slpD* were expressed from an episomal plasmid (Figure S3B). Interestingly, unlike cell line A549, the human ocular cell line HCLE was less susceptible to secreted proteases induced in PIC3611, where only *prtS* induction caused significant levels of cytotoxicity (Figure 4C).

**Alkaline protease inhibitor (AprI), from *P. aeruginosa* inhibits PrtS and SlpB mediated proteolysis and cytotoxicity.** AprI was recently shown to be able to inhibit purified PrtS protease activity in vitro (23). This was confirmed in our model system, where induced PrtS was inhibited by AprI in a dose dependent manner (Figure S4A). A similar dose dependent inhibition of SlpB activity by AprI was observed (Figure S4A). Since AprI was able to inhibit both PrtS and SlpB, it was used as another way to assess whether protease activity was responsible for the observed cytotoxicity to
epithelial cells. When AprI was added at 250 nM cytotoxicity to A549 cells was significantly reduced (Figure S4A-B). AprI was also able to inhibit secreted cytotoxicity capability from strain K904 to A549 cells (Figure S4C). Together these data support that protease activity is responsible for the observed cytotoxic effects and increased the scope of proteases known to be inhibited by AprI.

Mutation of slpB in clinical isolate K904 confers loss of secreted protease activity and epithelial cell-type specific cytotoxicity.

To test the role of SlpB in secreted protease activity and cytotoxicity, allelic replacement of the slpB gene on the chromosome of K904 with a deletion allele of slpB was performed. The mutation results in an in frame deletion of 219 amino acids removing the active site and most of the N-terminal protease domain. The ΔslpB mutant was defective in secreted proteolysis (Figure 5A-B) and the defect could be complemented by slpB on a plasmid (Figure 5A-B). Mutation of prtS caused a more severe reduction in protease activity than deletion of slpB (Figure 5B, p<0.05). Consistent phenotypes were recorded with directed insertional mutation of the slpB gene in strain K904 (data not shown). This mutation was achieved by recombination of an internal fragment of slpB on a suicide plasmid into the chromosome. The plasmid inserts after base pair 747 out of 1419 base pairs. Mutation of both prtS and slpB in the same strain conferred a further reduction in secreted protease activity suggesting an independent and additive contribution from PrtS and SlpB (Figure 5A). The ΔslpB mutant was slightly less cytotoxic to HCLE cells than the parental strain, but the reduction was not significant (Figure 5C). Secretomes from the ΔprtS
mutant were significantly less cytotoxic to HCLE cells than from the ∆slpB mutant, but produced equal cytotoxicity to secretomes from the double mutant (Figure 5C).

A clear difference to HCLE cells was observed when A549 cells were challenged with bacterial supernatants, where neither mutation of prtS or slpB reduced cytotoxicity of K904 (Figure 5D). However, the double mutant, ∆prtS ∆slpB, was almost completely defective in cytotoxicity to HCLE cells (Figure 5D). This suggests that either PrtS or SlpB alone is sufficient for cytotoxicity to A549 cells.

The cytotoxicity assays used previously in this study measures both loss of cells due to proteolytic removal of cells (cell detachment) and cell death after 4 hours of challenge with bacterial supernatants. As an alternative approach, the cytotoxicity assays were performed without washing away detached HCLE cells after 4 hours and 24 hours of exposure to K904 and mutant secretomes. After 4 hours, the majority of HCLE cells exposed to K904 supernatants were dead supporting that the K904 secretomes lead to HCLE cell death (Figure 6A). K904 secretomes produced 92% cytotoxicity after 24 hours of exposure (Figure 6B). The prtS mutant was less cytotoxic than the parental strain (77%, p<0.05). As observed at 4 hours, the slpB mutant was as cytotoxic as the parental strain (90%) after 24 hours. Strikingly, the prtS slpB double mutant was the least cytotoxic at 59% (Figure 6B). These experiments further support that the S. marcescens secretomes are cytotoxic to HCLE cells and reveal HCLE cytotoxicity requiring SlpB that was evident after a 24 hour challenge.

**Secretion of SlpB requires type I secretion protein LipD.**
PrtS is secreted through a type I secretion system (T1SS) composed of three proteins LipB, LipC, and LipD (48, 49). Here a genetic approach was used to assess whether SlpB is also secreted through the LipBCD T1SS. To do so, slpB expression was induced in the wild-type PIC3611 strain and an isogenic strain with a transposon mutation in lipD. Secreted protease activity should be highly reduced in the lipD mutant compared to the wild-type strain with a functional LipBCD system if this T1SS is used to secrete SlpB. A prtS expression plasmid (pprtS) was included as a control to verify the approach.

Compared to a vector control, elevated secreted protease activity was measured from the wild-type strain (PIC3611) expressing prtS and slpB, but not the lipD::tn strain expressing prtS and slpB (Figure S5). These data indicate, that like PrtS, SlpB requires LipBCD for secretion.

SlpB encodes an active, Ca\(^{2+}\)-regulated protease

Recombinant SlpB was expressed and purified from E. coli to characterize its biochemical and functional properties \textit{in vitro}. The protein expressed robustly in E. coli and could be purified to homogeneity under denaturing conditions in guanidinium hydrochloride. The protein was then refolded using protocols previously described for other similar serralysin metaloproteases (23).

The protein was refolded by rapid dilution into buffers in the presence and absence of Ca\(^{2+}\), a structural cofactor that has been shown to regulate the folding of RTX proteins and other members of the serralysin protease family (42). The purified protein was first diluted into buffers containing 2 mM Ca\(^{2+}\), which is predicted to saturate the binding sites in the C-terminal RTX domain, and activity was monitored using a
fluorescent substrate. Rapid dilution into buffers containing millimolar Ca$^{2+}$ concentrations resulted in robust protease activity, as monitored by the cleavage of the fluorescent peptide substrate (Figure S6). This activity was not seen in the buffer controls or in refolding reactions that lacked Ca$^{2+}$, consistent with the activation of other serralysin family proteases (23).

To further characterize these Ca$^{2+}$-regulated activities, Ca$^{2+}$ titrations were performed and protease activity was assessed using this fluorescent peptide (Figure S6B). The purified SlpB protease showed no observable protease activity when refolded into buffers containing less than 15 mM Ca$^{2+}$. Between 15-40 mM Ca$^{2+}$, protease activity increased with increasing Ca$^{2+}$. At and above 50 mM Ca$^{2+}$, the protease activity appears saturated and did not increase with further Ca$^{2+}$ addition. The apparent affinity of the Ca$^{2+}$-induced activity was 22 mM and was cooperative with a Hill coefficient of 5.5.

Given the apparent sequence and putative structural similarity between SlpB and other members of the serralysin protease family, the AprI protease inhibitor from *P. aeruginosa* was evaluated for its ability to block SlpB activity. Previous studies have shown that the AprI inhibitor binds with high affinity to *P. aeruginosa* AprA and other serralysin proteases (23, 50). AprI was purified and co-incubated in reactions containing refolded SlpB in the presence and absence of saturating Ca$^{2+}$. When incubated at stoichiometric concentrations, the AprI protease inhibitor effectively blocked the SlpB protease activity (Figure S6C). This binding and protease inhibition suggests that the structure of the folded SlpB protease is likely similar to that of other serralysin family members. Further, the Ca$^{2+}$-regulated activation and AprI inhibition demonstrates that SlpB functions as a bona fide protease.
Purified PrtS and SlpB were cytotoxic to HCLE cells in vitro and SlpB is found at lower concentrations than PrtS in K904 secretomes.

Having purified SlpB, we tested whether recombinant PrtS and SlpB were cytotoxic to HCLE cells. Both PrtS and SlpB were cytotoxic to HCLE cells in a dose dependent manner and could be inhibited by equimolar AprI (Figure 7A-B). This suggests that rather than being less cytotoxic than PrtS to HCLE cells, extracellular SlpB is produced at lower levels or is less stable.

To investigate relative amounts of PrtS and SlpB in K904 secretomes, PAGE analysis was performed. The major band in the K904 secretome was ~50 kDa (Figure 8A-B). The 50 kDa band was highly reduced in the ΔprtS strain (Figure 8A-B). The ΔslpB mutant secretomes had the 50 kDa band at similar levels to the parental K904 strain, and the band was completely absent in the double mutant, suggesting that PrtS and SlpB together constitute this band. Mass spectrometry analysis confirms that the band in the ΔprtS secretomes is SlpB and the band in the ΔslpB secretomes is PrtS. These data suggest that PrtS is made at higher levels or is more stable than SlpB leading to its relative importance in cytotoxicity to corneal cells.

Discussion

Proteolysis of host immune components and eukaryotic surface proteins plays an important role in the pathogenic process of a number of pathogens (51). A variety of model systems have demonstrated that secreted proteases are key virulence factors, facilitating invasion of bacteria into mammalian cells, cleavage of host innate immune
factors such as immunoglobulins and surfactant protein-D, and disruption of tight junctions and epithelial cell integrity (12, 21, 26, 51). In polymicrobial infections, proteases could potentiate virulence by other microbes, not only through enabling bacterial invasion, but through degrading host immune components and activating host proteins such as matrix metalloproteases through proteolysis (52).

Published data implicates *S. marcescens* the secreted metalloproteases PrtS as being a virulence factor (13). Nevertheless, previous studies have assessed protease production by a very limited number of clinical isolates and its role in cytotoxicity to ocular cells has not been tested in vitro. Here, we tested a much larger number of ocular isolates from the three major eye infections caused by *S. marcescens*: conjunctivitis, endophthalmitis, and keratitis. *S. marcescens* is most prevalent as an agent of keratitis, with many studies listing it as the second most common cause of contact-lens associated keratitis behind *P. aeruginosa* (2, 6, 53-56). *S. marcescens* is associated with common contact lens associated inflammation complications (57) and activates inflammation through both the TLR4 / MD-2 / MyD88 and MyD-88 / IL-1R1 pathways (58).

Serralysin itself is sufficient to induce an immune response (21).

In this study, all tested ocular isolates produced some secreted protease activity if allowed to incubate on protease detection agar for 72 hours. However, a range of secreted proteolysis was observed, and interestingly isolates with delayed protease activity were more prevalent among conjunctivitis strains that are associated with a less severe infection. The reason for the different levels of secreted protease activity between strains are not at this point fully understood, but the differences in expression levels of *prtS* and *slpB* were much lower in the low-protease producing PIC3611 compared to the
highly protease producing K904 suggest that differential regulation is behind the different levels of protease production. However, amino acid differences influencing protease activity could be responsible for strain differences in secreted protease activity. Using strain K904 as a representative keratitis isolate that secreted more cytotoxic and proteolytic activity than a wild-type laboratory strain, PIC3611, we obtained evidence suggesting that there may be proteases other than serralysin secreted by the clinical isolate, K904. First, a previous study describes multiple secreted proteases purified from a particularly virulent keratitis isolate of *S. marcescens* (30). Although biochemical data were obtained, the identities of the proteins were not determined. Second, deletion of the *prtS* gene was not sufficient to completely eliminate secreted protease activity by clinical isolate K904. These observations suggested the existence of other protease genes in the genome of *S. marcescens*, a prediction that was validated by searching the published genome of strain Db11 (44). Transcripts from each of the three ORFs could be detected in both a clinical and laboratory strain, and was much higher in the clinical isolate, supporting the conclusion that they were not cryptic ORFs. To obtain insight into the function of these putative proteases, the putative metalloprotease ORFs and positive control *prtS* were placed under transcriptional control of the inducible *PBAD* promoter on the chromosome of a largely non-proteolytic strain. Of *slpB*, *slpC*, and *slpD*, only *slpB* and the positive control *prtS* conferred measurable protease activity. Each of the independently cloned genes was also induced from a low-medium copy plasmid and the identical trend was observed. The potential reasons why *slpC* and *slpD* expression did not generate detectable secreted protease activity include that our assays used only casein as a substrate, and SlpC and SlpD may have specificities
that exclude casein. Similarly, SlpC and SlpD may have pH optima or other requirements not met in our assay conditions. Additionally, these putative proteases may not be efficiently secreted under the tested conditions or are not stable.

One of the outcomes of this study is that PrtS was apparently more important for toxicity to HCLE cells than SlpB, since induced expression of \textit{prtS} from a non-cytotoxic strain conferred much higher levels of cytotoxicity to HCLE cells than expression of \textit{slpB}.

Additionally, mutation of \textit{prtS} had a much more deleterious effect on cytotoxicity from clinical isolate K904 than did mutation of \textit{slpB}. However, given that purified SlpB was similarly cytotoxic to PrtS, it may be that PrtS is more efficiently secreted or more stable than SlpB. PAGE analysis using single and double mutants is consistent with this model, but further biochemical analysis will be required to determine whether the difference is due to reduced secretion, lower stability of SlpB compared to PrtS, and other mechanisms.

Nevertheless, cytotoxicity from purified SlpB and reduced cytotoxicity from a K904 \textit{prtS slpB} double mutant compared to the K904 \textit{prtS} mutant support a potential role for SlpB in cytotoxicity to ocular cells.

The serralysin protease may have differential cytotoxicity by host cell type. A study by Ishii et al demonstrated that \textit{S. marcescens} supernatants enhanced the release of phagocytic hemocytes into the silkworm hemolymph, but did not kill the silkworm (59). Further elegant biochemical analysis revealed that serralysin contributed to the loss of hemocyte adhesion through degradation of adhesion molecules on the hemocyte membrane. The lack of cytotoxicity seen in that study may have to do with differences in bacterial strains, differences in susceptibilities of silkworm hemocytes to human epithelial cell lines, or differences in the timing of the experimental analysis which were
not explicitly stated in the insect study (59). Conversely, other groups found that serralysin was cytotoxic to HeLa cells (14) and human embryonic lung fibroblasts (15), and proteases deficient mutants of *Serratia* Sp. SCBI were defective in cytotoxicity to a Buffalo green monkey kidney cell line. Here, we observed that A549 airway cells were more susceptible than HCLE corneal cells to *S. marcescens* proteases.

This study identifies SlpB as a new cytotoxic factor secreted by *S. marcescens* and supports that under the experimental conditions used in this study, PrtS and SlpB are the major secreted cytotoxic proteins of *S. marcescens*. Lastly, the metal-dependent nature of *S. marcescens* metalloproteases suggests that non-toxic metal chelators could be of use in prevention of protease-associated tissue damage.

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References


monotherapy for the empiric and optimal coverage of bacterial keratitis based on incidence of infection. Cornea 32:830-834.


Figure Legends

Figure 1. Secreted protease activity by ocular clinical isolates of *S. marcescens* correlates with cytotoxicity to human corneal cells. A. Secreted protease activity from strain PIC3611 (WT) and ocular clinical isolates of *S. marcescens* measured using azocasein. B.

Figure 2. Serralysin (PrtS) is required for the majority of secreted protease activity and cytotoxic power of supernatants from a keratitis isolate of *S. marcescens*. A. Secreted protease activity from bacterial supernatants measured by azocasein (n=7). B.

Cytotoxicity of secreted supernatants to HCLE cells measured by Alamar blue (n=6). Vector = pMQ125, ppptS = pMQ356. Mock treated cells had 0% cytotoxicity, and detergent treated
cells had 100% cytotoxicity. Mean and standard deviations are shown. Asterisk indicates a difference from K904 + vector by ANOVA with Tukey’s post-test.

**Figure 3. Elevated metalloprtease gene transcription in keratitis isolate K904.**

Semi-quantitative RT-PCR analysis of gene expression of control and putative metalloprotease genes from *Serratia marcescens* strain PIC3611 (WT) and clinical ocular isolate K904. RNA was collected from cells at OD_{600}=3, purified and found to be free of contaminating chromosomal DNA. The 16S ribosomal gene was used a control for input cDNA. Expression of *prtS* and three putative metalloprotease genes was elevated in the clinical strain K904 relative to the laboratory strain PIC3611. The experiment was performed four times using RNA from three independent experiments. All experiments demonstrated the same trend for each gene. A representative experiment is shown.

**Figure 4. Secreted protease activity from strain PIC3611 with induced expression of putative metalloprotease genes.** A-C. Induction of His7-tagged putative metalloprotease genes from a p15a-based plasmid in strain PIC3611 was followed by measuring protease activity and cytotoxic capacity in the filtered supernatants from cultures grown overnight. The negative control was episomal plasmid pMQ125 (vector). Mean and standard deviations are shown and the asterisk indicate a significant difference from the vector control by ANOVA with Tukey’s post-test, n=6. A. Protease production measured with azocasein. B. Cytotoxicity to A549 airway cell line measured with Presto Blue. C. Cytotoxicity to HCLE cell line measured with Presto Blue.
Figure 5. Deletion of *prtS* and *slpB* confers a protease and cytotoxicity defective phenotype. A. Protease activity of stationary phase bacterial supernatants using azocasein as a substrate and normalized by culture densities. N=5 independent replicates from 3 separate experiments. B. Protease assay of complemented strains as above. N=4 independent replicates. Vector = pMQ125, *pprtS* = pMQ356, *pslpB* = pMQ436. C. Cytotoxicity of normalized bacterial supernatants to HCLE cells. N=6 independent biological replicates from 5 separate experiments. D. Cytotoxicity of normalized bacterial supernatants to A549 cells, n=3. In all graphs the means and standard deviations are shown. Asterisk indicates statistical difference from K904 by ANOVA with Tukey’s post-test. Number signs indicate a significant difference between K904 *prtS* and K904 *prtS slpB* by ANOVA with Tukey’s post-test.

Figure 6. Prolonged exposure of HCLE cells to supernatants of K904 and protease mutants suggest a role for SlpB in cytotoxicity to ocular cells. A-B. Cytotoxicity of normalized bacterial supernatants to HCLE cells measured using Presto Blue, n=3. These experiments were done without a washing step, so that detached HCLE cells are included in the assay. Means and standard deviations are shown. Asterisk indicates statistical difference from K904 by ANOVA with Tukey’s post-test. Number signs indicate a significant difference between K904 *prtS* and K904 *prtS slpB* by ANOVA with Tukey’s post-test. A. 4 hour exposure, B. 24 hour exposure.
Figure 7. Purified PrtS and SlpB are cytotoxic to HCLE cells. A-B. Cytotoxicity of purified proteins to HCLE cells was measured with Presto Blue. Mean values (n=8) and standard deviations are shown. Asterisk equals significant difference from no protease control by ANOVA with Tukey’s post-test. Aprl was added at 1000 nM. A. Experiments performed with PrtS, B. Experiments performed with SlpB.

Figure 8. *S. marcescens* strain K904 produces more PrtS than SlpB. A. PAGE analysis of secretomes of K904 and noted isogenic mutants. Secretomes were normalized by optical density, TCA precipitated, and equal volumes were loaded. Large black arrow indicates a ~50 kDa band. Small black arrow indicates SlpB. Small white arrow indicates PrtS. B. Quantification of the 50 kDa band from 4 gels using independent samples. Values were normalized to the 50 kDa band from the K904 secretomes. Means and standard deviations are shown. Asterisk equals significant difference from K904 by ANOVA with Tukey’s post-test.

Table 1. *S. marcescens* strains and plasmids
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854 Plasmids

856 pMQ118  nptII, rpsL, oriT, R6K, URA3, CEN6/ARSH4 (39)
857 pMQ125  orip15a, P<sub>B</sub>, lacZa, oripRO1600, oriT, URA3, CEN6/ARSH4 (39)
858 pMQ131  pBBR1, aphA-3, oriT, URA3, CEN6/ARSH4 (39)
859 pMQ132  pBBR1, aacC1, oriT, URA3, CEN6/ARSH4 (39)
860 pStvZ3  R6K lacZ nptII, oriT, URA3, CEN6/ARSH4 (60)
861 pMQ200  R6K, nptII, oriT, URA3, CEN6/ARSH4, P<sub>B</sub>-lacZa (39)
862 pMQ218  pMQ118 + slpB internal fragment | This study |
863 pMQ236  R6K, nptII, rpsL, oriT, URA3, CEN6/ARSH4, I-SceI site (39)
864 pMQ240  pSC101<sup>a</sup>, aacC1, oriT, P<sub>lac</sub>-I-SceI, URA3, CEN6/ARSH4 (39)
865 pMQ262  pMQ200 + pigAB<sup>+</sup> for induction of prodigiosin | (61) |
866 pMQ263  pMQ236 + prtS | This study |
867 pMQ310  as pMQ236 with hygromycin resistance marker hph | (62) |
868 pMQ322  pMQ263 with prtS Age1-Age1 deletion allele | This study |
| 869 | pMQ356 pMQ125 + His\textsubscript{7}-prtS | This study |
| 870 | pMQ430 pMQ131 + prtS | This study |
| 871 | pMQ431 pMQ131 + slpB | This study |
| 872 | pMQ436 pMQ125 + His\textsubscript{7}-slpB | This study |
| 873 | pMQ437 pMQ125 + His\textsubscript{7}-slpC | This study |
| 874 | pMQ444 pMQ125 + His\textsubscript{7}-slpD | This study |
| 875 | pMQ445 pMQ310 + lipD::tn (aacC-1 marker) | This study |
| 876 | pMQ460 pMQ236 + sacB, allelic replacement vector | This study |
| 877 | pMQ493 pMQ460 + slpB | This study |
| 878 | pMQ497 pMQ460 + slpB-PvuI-PvuI deletion allele | This study |
| 879 | pMQ510 pMQ200 + prtS | This study |
| 880 | pMQ511 pMQ200 + slpB | This study |
| 881 | pMQ512 pMQ200 + slpC | This study |
| 882 | pMQ513 pMQ200 + slpD | This study |
Figure 2

Protease activity (A440/OD600) A

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% Cytotoxicity B

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* indicates statistical significance.
Figure 4

Protease activity (A$_{440}$/OD$_{600}$)

Episomal expression

- vector
- prtS
- slpB
- slpC
- slpD

% Cytotoxicity

Episomal expression

- vector
- prtS
- slpB
- slpC
- slpD

% Cytotoxicity

Episomal expression

- vector
- prtS
- slpB
- slpC
- slpD

AS49

HCLF

Downloaded from http://iai.asm.org/ on December 18, 2017 by guest
Figure 7

A

B

% Cytotoxicity

0 250 500 1000 1000 PrtS (nM) + AprI

% Cytotoxicity

0 250 500 1000 1000 SlpB (nM) + AprI

* * * *