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Identification of SlpB, a Cytotoxic Protease from *Serratia marcescens*

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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 than among conjunctivitis isolates. A positive correlation between protease activity and cytotoxicity to human corneal epithelial cells *in vitro* was determined. 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 another 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 I secretion system gene, *lipD*, is required for SlpB secretion. These genetic data introduce SlpB as a new cytotoxic protease from *S. marcescens*.

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 to 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 analyzed by Pinna et al. in Italy (29). In this study, we characterized secreted protease activity from more than 40 ocular isolates of *S. marcescens* collected at a tertiary care hospital in the northeastern 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 noncytotoxic 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 contributes 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% [wt/vol] agar) (32) and incubated at 30°C. Milk agar plates were prepared using brain heart

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TABLE 1 *S. marcescens* strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
CMS376	WT PIC strain no. 3611	Presque Isle Cultures
C23M13	CMS786 with *lipD*::Tn	37
K904	Contact lens-associated keratitis isolate	60
CMS2853	K904 with *prtS* deletion mutation	This study
CMS3809	K904 with *slpB*::pMQ118	This study
CMS3810	K904 Δ*prtS slpB*::pMQ118	This study
CMS4063	CMS376 *lipD*::Tn	This study
CMS4097	K904 with *slpB* deletion mutation	This study
CMS4098	K904 Δ*prtS* Δ*slpB*	This study
Plasmids		
pMQ118	*nptII*, *rpsL*, oriT, $_{ori}$R6K, *URA3*, *CEN6/ARSH4*	39
pMQ125	*ori*p15a, P_{BAD}-*lacZa*, *ori*pRO1600, oriT, *URA3*, *CEN6/ARSH4*	
pMQ131	$_{ori}$pBBR1, *aphA-3*, oriT, *URA3*, *CEN6/ARSH4*	39
pMQ132	$_{ori}$pBBR1, *aacC1*, oriT, *URA3*, *CEN6/ARSH4*	39
pStvZ3	$_{ori}$R6K *lacZ nptII*, oriT, *URA3*, *CEN6/ARSH4*	60
pMQ200	$_{ori}$R6K, *nptII*, oriT, *URA3*, *CEN6/ARSH4*, P_{BAD}-*lacZ*α	39
pMQ218	pMQ118 + *slpB* internal fragment	This study
pMQ236	$_{ori}$R6K, *nptII*, *rpsL*, oriT, URA3, *CEN6/ARSH4*, *I-SceI* site	39
pMQ240	$_{ori}$pSC101^{ts}, *aacC1*, oriT, P_{lac}-*I-SceI*, *URA3*, *CEN6/ARSH4*	39
pMQ262	pMQ200 + *pigAB′* for induction of prodigiosin	61
pMQ263	pMQ236 + *prtS*	This study
pMQ310	Same as pMQ236 with hygromycin resistance marker *hph*	62
pMQ322	pMQ263 with *prtS* AgeI-AgeI deletion allele	This study
pMQ356	pMQ125 + His$_7$-*prtS*	This study
pMQ430	pMQ131 + *prtS*	This study
pMQ431	pMQ131 + *slpB*	This study
pMQ436	pMQ125 + His$_7$-*slpB*	This study
pMQ437	pMQ125 + His$_7$-*slpC*	This study
pMQ444	pMQ125 + His$_7$-*slpD*	This study
pMQ445	pMQ310 + *lipD*::Tn (*aacC*-1 marker)	This study
pMQ460	pMQ236 + *sacB*, allelic replacement vector	This study
pMQ493	pMQ460 + *slpB*	This study
pMQ497	pMQ460 + *slpB*-PvuI-PvuI deletion allele	This study
pMQ510	pMQ200 + *prtS*	This study
pMQ511	pMQ200 + *slpB*	This study
pMQ512	pMQ200 + *slpC*	This study
pMQ513	pMQ200 + *slpD*	This study

infusion agar supplemented with 1% (wt/vol) 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 λ*pir* (33); *Saccharomyces cerevisiae* strain InvSc1 (Invitrogen) was used for cloning.

Tissue culture and cytotoxicity assays. Human lung carcinoma line A549 cells (34) were propagated and maintained in tissue culture medium 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 alamarBlue and Presto Blue reagents (Life Technologies) as previously described by Wingard et al. (36). Briefly, bacterial cultures were grown for 18 to 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 an optical density at 600 nm (OD_{600}) of 2.0 by addition of LB medium. Culture aliquots were added to microcentrifuge tubes and spun at 14,000 × *g* for 2 min, followed by filter sterilization of the supernatants using 0.22-μm filters. Two hundred-microliter quantities of filtered bacterial cultures were added to epithelial cell line layers in 24-well dishes with 300 μl of culture medium. After 4 h of incubation, cell layers were washed three times with 0.5 ml of phosphate-buffered saline (PBS) and suspended in culture medium with alamarBlue 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 treatment (negative control) was set to 0% cytotoxicity, and that of detergent treatment (Triton X-100 at 0.25% [vol/vol]) was set to 100% cytotoxicity. The percent cytotoxicity was determined using the following formula: 100 × [(mock value − experimental value)/(mock value − detergent treatment 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 colorimetric 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% [wt/vol]) and incubated for 30 min 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 min on ice. The tubes were centrifuged at 8,000 × *g* for 10 min. 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. Colorimetric analysis of liberated azol dye was measured at 440 nm with a plate reader and divided by the original culture OD_{600}.

Molecular biology. The N-terminal polyhistidine-tagged *slpB*, *slpC*, and *slpD* genes under the 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 His_7 tag. This was done by digesting pMQ356 with SalI 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 His_7 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 the control of P_{BAD} promoter, the open reading frames (ORFs) 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 bp of the *prtS* upstream of the AgeI site and 618 bp 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 the control of the P_{BAD} promoter. After addition of I-SceI with L-arabinose, chromosomal *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 *prtS* ORF.

To mutate *slpB* by plasmid insertion, a 606-bp internal fragment of *slpB* was cloned into pMQ118 (39). The resulting plasmid, pMQ218, was introduced into K904 by conjugation, and kanamycin-resistant isolates were screened by PCR for the plasmid disruption of the *slpB* gene. The plasmid inserts after bp 747 out of 1,419 bp, which truncates the *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 *slpB* deletion allele, the *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 *slpB* gene, removing 657 bp. 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 to 187 include the predicted catalytic site.

LipD mutation. Strain C23M13 with a transposon mutation in the *lipD* ORF was identified in a genetic screen for mutations that suppress the elevated protease phenotype of *crp* mutants in *S. marcescens* PIC3611 (37). The *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 an OD_{600} of 2 were separated on 8 to 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.

Reverse transcription-PCR (RT-PCR). RNA was extracted from cultures grown in LB broth to an OD_{600} of 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 rRNA gene primers were used to normalize signals and to test for chromosomal DNA contamination (see Table S1 in the supplemental material). 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. A culture was grown overnight in the presence of gentamicin, and 10 ml was used as an inoculum for His_7-*slpB* induction in 1-liter cultures. The cultures were grown in LB medium supplemented with gentamicin, and 0.2% (wt/vol) arabinose was added to the culture when it achieved an OD_{600} of 0.8. Protein was expressed at 37°C for 4 to 6 h. The SlpB protein was purified from the insoluble material under denaturing conditions by 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. Two-tailed Student's *t* test, one-way analysis of variance (ANOVA) with Tukey's posttest, and Pearson's correlation were performed with Prism 5; significance was set to a *P* value of <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 of ≥1 mm 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 to 72 h. 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 ($n \geq 7$; $P = 0.96$). By 48 h 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 h), whereas only 3 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 Fig. 1A and followed a strain-dependent pattern. Of the 21 tested keratitis strains, all but 2 (K1189 and K1496) demonstrated elevated levels of proteolysis relative to that of lab-

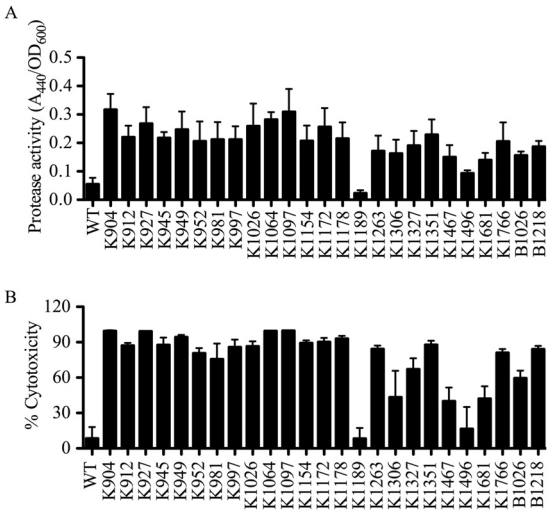

FIG 1 Secreted protease activity by ocular clinical isolates of *S. marcescens* correlates with cytotoxicity to human corneal cells. (A) Secreted protease activity from strain PIC3611 (WT) and ocular clinical isolates of *S. marcescens* measured using azocasein. (B) Cytotoxicity of *S. marcescens* secreted factors to a human ocular epithelial cell line (HCLE).

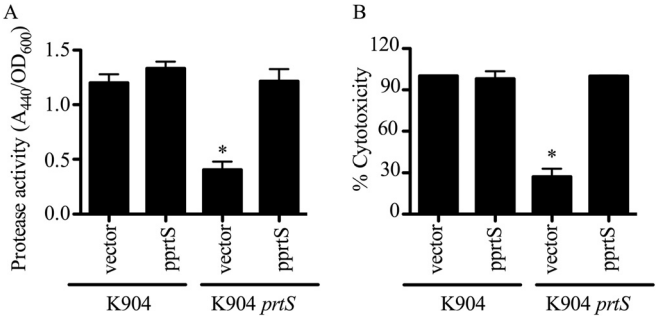

FIG 2 Serralysin (PrtS) is required for the majority of secreted protease activity and cytotoxic power of supernatants from a keratitis isolate of *S. marcescens*. (A) Secreted protease activity from bacterial supernatants measured by azocasein ($n = 7$). (B) Cytotoxicity of secreted supernatants to HCLE cells ($n = 6$). Vector = pMQ125, pprtS = pMQ356. Mock-treated cells had 0% cytotoxicity, and detergent-treated cells had 100% cytotoxicity. Means and standard deviations are shown. The asterisk indicates a difference from K904 plus vector by ANOVA with Tukey's posttest.

oratory 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 (Fig. 1B). Cytotoxicity and protease activity of supernatants from PIC3611 and clinical conjunctivitis and keratitis isolates were found to have a positive correlation (Pearson's $r = 0.925$; $P < 0.001$ (see Fig. S1 in the supplemental material).

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 to the chromosome of strain K904 by allelic replacement. This in-frame deletion allele is missing amino acids 37 to 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 (Fig. 2A). Cytotoxicity to human corneal cells was likewise reduced (Fig. 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 *S. marcescens* strains (43, 44) using an NCBI BLAST search (45). These predicted proteins are here noted as serralysin-like proteases B, C, and D (see Table S2 in the supplemental material).

The amino acid identities of the predicted proteins to serralysin were 52.6, 50.3, and 45%, respectively. Phylogenetic analysis indicates that of the three predicted proteases, SlpB is closest by sequence identity to serralysin, and it is also most similar to alkaline protease from *Pseudomonas aeruginosa*, AprA (see Fig. S2 in the supplemental material).

Serralysins are peptidases, a subset of the larger M10 metalloprotease group that includes mammalian matrix metallopeptidases (MMPs). Serralysin family proteins have an N-terminal HE XXHXXGXXH sequence involved in Zn^{2+} coordination in their N termini 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 (T1SSs) (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 (see Table S2). Notably, serralysin and SlpB have four GGXGXD motifs, whereas SlpC and SlpD have two.

Native expression of *slpB*, *slpC*, and *slpD* is detectable in a laboratory and a clinical isolate of *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 *prtS*, *slpB*, *slpC*, and *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 *slpB*, *slpC*, and *slpD* were expressed under laboratory conditions, RNA was harvested and RT-PCR was performed. For both PIC3611 and K904, amplicons corresponding to the *slpB*, *slpC*, and *slpD* genes and 16S and *prtS* controls were detectable from RT-treated RNA (Fig. 3). Furthermore, expression was relatively higher for each of the protease genes in the clinical isolate K904 than for the laboratory strain PIC3611 (Fig. 3).

Induced expression of serralysin and *slpB*, but not *slpC* and *slpD* increased extracellular protease activity. The putative chromosomal protease genes for *prtS*, *slpB*, *slpC*, and *slpD* were placed

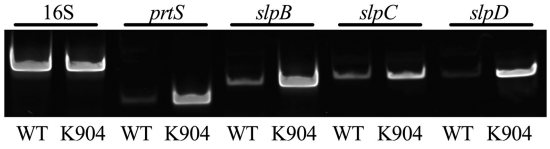

FIG 3 Elevated metalloprotease gene transcription in keratitis isolate K904. Semiquantitative RT-PCR analysis of gene expression of control and putative metalloprotease genes from *Serratia marcescens* strain PIC3611 (WT) and clinical ocular isolate K904 was performed. RNA was collected from cells at an OD_{600} of 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 that in 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. Results from a representative experiment are shown.

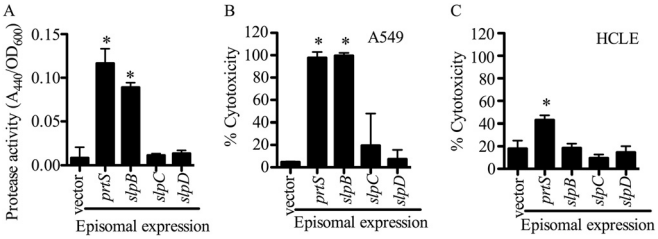

FIG 4 Secreted protease activity from strain PIC3611 with induced expression of putative metalloprotease genes. (A to C) Induction of His_7-tagged putative metalloprotease genes from a p15a-based plasmid in strain PIC3611 was followed by measuring protease activity and cytotoxic capacity in the filtered supernatants from cultures grown overnight. The negative-control was episomal plasmid pMQ125 (vector). Means and standard deviations are shown, and the asterisks indicate a significant difference from the vector control by ANOVA with Tukey's posttest ($n = 6$). (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.

under the 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 those for the clinical isolates (Fig. 1A), and this low level would facilitate measuring increases in extracellular protease levels. Arabinose-induction of polyhistidine (His_7)-tagged versions of *prtS* or *slpB* from an episomal plasmid resulted in a clear increase in extracellular protease activity, whereas no increases in protease levels were detected from supernatants of cells expressing the His_7-tagged versions of *slpC* and *slpD* genes or a negative vector control (Fig. 4A). As a second test, the chromosomal *prtS*, *slpB*, *slpC*, and *slpD* genes were expressed under the control of the P_{BAD} promoter integrated in front of each gene in the PIC3611 strain. Similar to episomal induction of these genes, chromosomal expression of *prtS* and *slpB* conferred increased secreted protease activity compared to the empty vector control, whereas *slpC* and *slpD* expression did not (see Fig. S3A). Induction of a control nonprotease gene, *pigA* pigment biosynthetic gene, showed little background protease activity (see Fig. S3A).

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 (Fig. 4B). Induced expression of *slpB*, but not *slpC* or *slpD*, was similar to *prtS* induction with cytotoxicity to A549 cells (Fig. 4B). A similar pattern was observed when *prtS*, *slpB*, *slpC*, and *slpD* were expressed from an episomal plasmid (Fig. 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 (Fig. 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 (see Fig. S4A in the supplemental material). A similar dose-dependent inhibition of SlpB activity by AprI was observed (see Fig. 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 (see Fig. S4A and B). AprI was also able to inhibit secreted cytotoxicity capability from strain K904 to A549 cells (Fig. S4C). Together, these data support the notion 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 Δ*slpB* mutant was defective in secreted proteolysis (Fig. 5A and B), and the defect could be complemented by *slpB* on a plasmid (Fig. 5B). Mutation of *prtS* caused a more severe reduction in protease activity than deletion of *slpB* (Fig. 5A and B) ($P < 0.05$). 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 bp 747 out of 1,419 bp. 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 (Fig. 5A).

The Δ*slpB* mutant was slightly less cytotoxic to HCLE cells than the parental strain, but the reduction was not significant (Fig. 5C). Secretomes from the Δ*prtS* mutant were significantly less cytotoxic to HCLE cells than from the Δ*slpB* mutant but produced equal cytotoxicity to secretomes from the double mutant (Fig. 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 (Fig. 5D). However, the Δ*prtS* Δ*slpB* double mutant was almost completely defective in cytotoxicity to HCLE cells (Fig. 5D). This suggests that either PrtS or SlpB alone is sufficient for cytotoxicity to A549 cells.

The cytotoxicity assays used previously in this study measure both loss of cells due to proteolytic removal of cells (cell detachment) and cell death after 4 h of challenge with bacterial supernatants. As an alternative approach, the cytotoxicity assays were per-

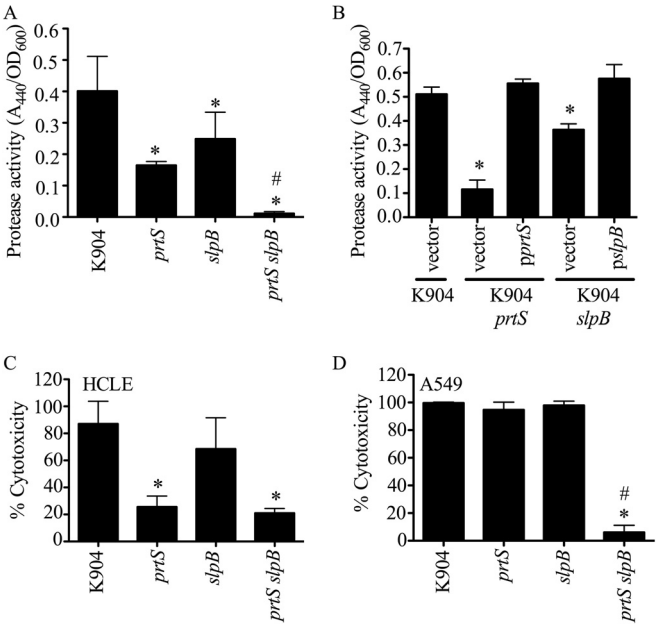

FIG 5 Deletion of *prtS* and *slpB* confers a protease and cytotoxicity defective phenotype. (A) Protease activity of stationary-phase bacterial supernatants using azocasein as a substrate and normalized by culture densities. Data for 5 independent replicates from 3 separate experiments are shown. (B) Protease assay of complemented strains as described above, with 4 independent replicates. Vector = pMQ125, p*prtS* = pMQ356, p*slpB* = pMQ436. (C) Cytotoxicity of normalized bacterial supernatants to HCLE cells, with 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. The asterisk indicates statistical difference from K904 by ANOVA with Tukey's posttest. Pound signs indicate a significant difference between K904 *prtS* and K904 *prtS slpB* by ANOVA with Tukey's posttest.

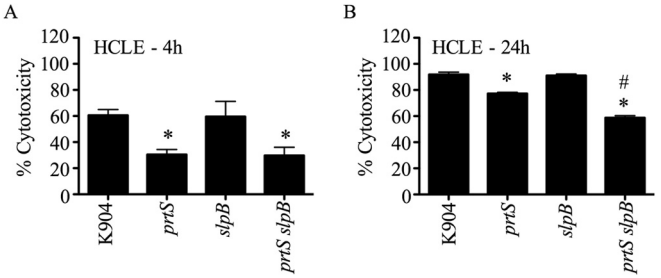

FIG 6 Prolonged exposure of HCLE cells to supernatants of K904 and protease mutants suggest a role for SlpB in cytotoxocity to ocular cells. Cytotoxicity of normalized bacterial supernatants to HCLE cells was measured using Presto blue ($n = 3$). These experiments were done without a washing step so that detached HCLE cells were included in the assay. Means and standard deviations are shown. The asterisk indicates statistical difference from K904 by ANOVA with Tukey's posttest. Pound signs indicate a significant difference between K904 *prtS* and K904 *prtS slpB* by ANOVA with Tukey's posttest. (A) 4-h exposure; (B) 24-h exposure.

formed without washing away detached HCLE cells after 4 h and 24 h of exposure to K904 and mutant secretomes. After 4 h, the majority of HCLE cells exposed to K904 supernatants were dead, supporting the notion that the K904 secretomes led to HCLE cell death (Fig. 6A). K904 secretomes produced 92% cytotoxicity after 24 h of exposure (Fig. 6B). The *prtS* mutant was less cytotoxic than the parental strain (77%) ($P < 0.05$). As observed at 4 h, the *slpB* mutant was as cytotoxic as the parental strain (90%) after 24 h. Strikingly, the *prtS slpB* double mutant was the least cytotoxic, at 59% (Fig. 6B). These experiments further support the notion that the *S. marcescens* secretomes are cytotoxic to HCLE cells and reveal HCLE cytotoxicity requiring SlpB that was evident after a 24-h 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). In this study, 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 (WT) 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 (p*prtS*) 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* (see Fig. S5 in the supplemental material). 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 *in vitro*. The protein was 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 metalloproteases (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 (see Fig. 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 (see Fig. S6B). The purified SlpB protease showed no observable protease activity when refolded into buffers containing less than 15 μM Ca^{2+}. Between 15 and 40 μM Ca^{2+}, protease activity increased with increasing Ca^{2+}. At and above 50 μM Ca^{2+}, the protease activity appeared saturated and did not increase with further Ca^{2+} addition. The apparent affinity of the Ca^{2+}-induced activity was 22 μM 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-

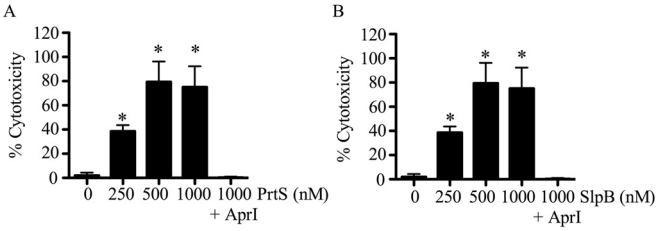

FIG 7 Purified PrtS and SlpB are cytotoxic to HCLE cells. Cytotoxicity of purified proteins to HCLE cells was measured with Presto blue. Mean values ($n = 8$) and standard deviations are shown. The asterisk equals significant difference from no protease control by ANOVA with Tukey's posttest. AprI was added at 1,000 nM. (A) Experiments performed with PrtS; (B) experiments performed with SlpB.

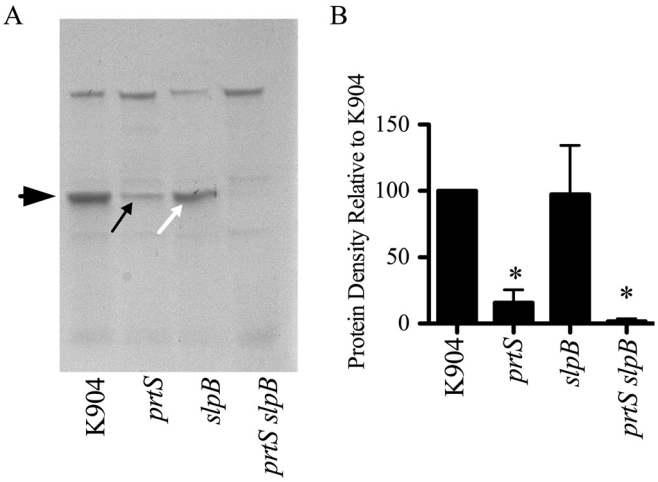

FIG 8 *S. marcescens* strain K904 produces more PrtS than SlpB. (A) PAGE analysis of secretomes of K904 and noted isogenic mutants. Secretomes were normalized by optical density and trichloroacetic acid precipitated, and equal volumes were loaded. The large black arrow indicates an ~50-kDa band. The small black arrow indicates SlpB. The 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. The asterisk equals significant difference from K904 by ANOVA with Tukey's posttest.

incubated in reaction mixtures 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 (see Fig. 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 demonstrate 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 (Fig. 7). 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 (Fig. 8). The 50-kDa band was highly reduced in the Δ*prtS* strain (Fig. 8). The Δ*slpB* mutant secretomes had the 50-kDa band at levels similar to that of 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 Δ*prtS* secretomes is SlpB and the band in the Δ*slpB* 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 also through degrading host immune components and activating host proteins, such as matrix metalloproteases (52).

Published data implicate the *S. marcescens* secreted metalloprotease PrtS as 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*. In this study, 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 h. 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 reasons 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 than in the highly protease-producing K904, suggesting 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 ob-

tained, 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 were 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 P_{BAD} promoter on the chromosome of a largely nonproteolytic 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- to medium-copy-number 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 under 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 *prtS* from a noncytotoxic strain conferred much higher levels of cytotoxicity to HCLE cells than expression of *slpB*. Additionally, mutation of *prtS* had a much more deleterious effect on cytotoxicity from clinical isolate K904 than did mutation of *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 than of PrtS, and other mechanisms. Nevertheless, cytotoxicity from purified SlpB and reduced cytotoxicity from a K904 *prtS slpB* double mutant compared to that from the K904 *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 *S. marcescens* supernatants enhanced the release of phagocytic hemocytes into 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 protease-deficient mutants of *Serratia* sp. strain SCBI were defective in cytotoxicity to a buffalo green monkey kidney cell line. In this study, 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, PrtS and SlpB are the major secreted cytotoxic proteins of *S. marcescens*. Lastly, the metal-dependent nature of *S. marcescens* metalloproteases suggests that nontoxic metal chelators could be of use in prevention of protease-associated tissue damage.

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