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SlpE is a calcium-dependent cytotoxic metalloprotease associated with clinical isolates of *Serratia marcescens*

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Abstract

Serralyisin-like proteases are found in a wide variety of bacteria. These metalloproteases are frequently implicated in virulence and are members of the widely conserved RTX-toxin family. We identified a serralyisin-like protease in the genome of a clinical isolate of *S. marcescens* that is highly similar to the canonical serralyisin protein, PrtS. This gene was named serralyisin-like protease E, SlpE, and was found in the majority (67%) of tested clinical isolates, but was absent from most tested non-clinical isolates including the insect pathogen and reference *S. marcescens* strain Db11. Purified recombinant SlpE exhibited calcium-dependent protease activity similar to metalloproteases PrtS and SlpB. Induction of *slpE* in the low-protease-producing *S. marcescens* strain PIC3611 highly elevated extracellular protease activity, and extracellular secretion required the *lipD* type 1 secretion system gene. Transcription of *slpE* was highly reduced in an *eepR* transcription factor mutant. Mutation of the *slpE* gene in a highly proteolytic clinical isolate reduced its protease activity, and evidence suggests that SlpE confers cytotoxicity of *S. marcescens* to the A549 airway carcinoma cell line. Together, these data reveal SlpE to be an EepR-regulated cytotoxic metalloprotease associated with clinical isolates of an important opportunistic pathogen.

Keywords

Metalloprotease; Enterobacteriaceae; Virulence factor; Cytotoxicity; Airway cells

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Conflict of interest

The authors declare no conflicts of interest.

1. Introduction

The serralyisin protein, PrtS, is a cytotoxic virulence factor secreted by *Serratia marcescens*, an opportunistic bacterial pathogen. Serralyisin-like proteases are zinc-dependent metalloproteases found in a wide group of bacteria [29]. These metalloproteases are implicated in virulence and have the widely conserved repeats-in-toxin (RTX) domain at their C-terminus [10], [17], [21], [29]. RTX domains have aspartate- and glycine-rich repeats that coordinate binding of calcium (Ca^{2+}) which is necessary for proper folding of the protein [33]. Serralyisin family proteins, including AprA from *Pseudomonas aeruginosa*, PrtS and SlpB from *S. marcescens*, PrtC from *Erwinia chrysanthemi* and many others, are secreted through type I secretion systems [5], [10]. In the case of *S. marcescens*, the secretion system for PrtS is composed of the LipB, LipC and LipD proteins [1], [19].

A few species of bacteria have several metalloprotease genes in their genomes [12], [22], although the reason for multiple serralyisin family metalloproteases is unclear, given that these proteins generally have broad substrate specificity and maintain functionality over a reasonably broad range of temperature and pH [15], [33]. There are four predicted metalloprotease genes in the *S. marcescens* reference strain Db11 genome [27]. These included serralyisin (*prtS*) and genes predicted to code for serralyisin-like proteins, *slpB*, *slpC*, and *slpD*. Of these, only *prtS* and *slpB* produced extracellular metalloprotease activity when the genes were induced [27].

Previously, a protease was cloned from *S. marcescens* strain SM6 that was cytotoxic to HeLa cells in vitro when expressed from *E. coli* [6], [20]. In this study, we identified a protease with identical amino acid sequence to the SM6 protease in a clinical isolate of *S. marcescens*. We propose to rename the SM6 protease SlpE, because it is found in strains beyond SM6, and to follow the serralyisin-like-protease nomenclature [27]. The purpose of this study was to expand upon previous studies [6] [20] by using defined mutations in clinical isolates, determining whether *slpE* was found in genomes of clinical isolates of *S. marcescens*, assessing SlpE cytotoxicity to a human airway cell line relative to PrtS and SlpB, testing whether the *slpE* was regulated by the EepR transcription factor and determining the secretion system required to secrete SlpE.

2. Materials and methods

2.1. Microorganisms and growth conditions

S. marcescens isolates and *E. coli* were grown in lysogeny broth [3] with aeration or on agar plates. Antibiotics were used for conjugations to remove donor strains (tetracycline at 10 µg/ml) or to select for plasmids (kanamycin at 50 µg/ml and gentamicin at 10 µg/ml). *Saccharomyces cerevisiae* strain InvSc1 (Invitrogen) was grown in yeast extract peptone dextrose broth or on synthetic complete medium without uracil to select for plasmids. Microorganisms were grown at 30°C. Pigmented environmental isolate of *S. marcescens* HQS was obtained from a soil sample on LB agar plates supplemented with tetracycline and confirmed as *S. marcescens* by PCR and phenotypic analysis. *S. marcescens* strain TM was isolated from the gut flora of the subterranean termite *Reticulitermes sp.* using 2.5% yeast extract agar plates. The 16S rDNA of the isolate is 99% identical to *S. marcescens*. The

genome of strain TM was sequenced and assembled by Molecular Research DNA (MR DNA, Shallowater, TX, USA) and the genome was annotated and deposited in NCBI Genbank (accession number KT901292.1). Keratitis isolates were obtained from Regis Kowalski at the Charles T. Campbell Laboratory of Ophthalmic Microbiology. Other *S. marcescens* strains are cited later in the text or listed in Table 1.

2.2. DNA analysis

K904 genomic DNA was prepared using a commercial kit (5-PRIME AchievePure DNA cell and tissue), and was sequenced by ACGT Incorporated using Illumina technology to an average coverage of 113x. The contig containing *slpE* was submitted to GenBank (accession number KT901292), and the remaining genomic DNA will be reported in a separate study.

To test various isolates for the *slpE* gene, total DNA was isolated using Quick Extract DNA extraction solution (Epicentre). Primers for the conserved *oxyR* gene were used as a control to ensure the quality of the DNA preparation, and all samples were positive for the *oxyR* amplicon; these were (5' to 3') GGAGGGAAACAATGAATATTCG and GCTGTCGAGTTGCGCCAGC. Two primer sets were used to amplify *slpE*, and if either amplicon was productive, then the strain was considered positive for *slpE*. A subset of *slpE* amplicons was sequenced, verifying that the amplicon was *slpE*. Two primer sets were used to amplify *slpE*. As a first pass, a primer set that amplified an internal amplicon was used (5' to 3') CAAAGATGCGACGGTGACCTATTC and CTAAACTGCCGGGTATCTTCG. If this set was negative for any given bacterial genome, a second set that amplified the entire *slpE* ORF was used (5' to 3') TTATTAAACGATAAAATCAACAGCGAC and GGTAATACCATGAGTGGAACAAAAC. Amplicons were separated on agarose gels and the presence of a *slpE* band of the correct size with either primer set classified the strain as being positive for *slpE*.

2.3. Cloning and mutagenesis

Plasmid pMQ516 was generated to induce expression of *slpE*. The *slpE* open reading frame (ORF) was cloned with an N-terminal His₇-tag under control of the *P_{BAD}* promoter on vector pMQ125 using in vivo cloning with *S. cerevisiae* [24,25]. Plasmids are listed in Table 1.

The *slpE* gene was mutated in strain K904 and K904 *prtS slpB* by insertion of plasmid pMQ543. To make pMQ543, an internal fragment of *slpE* was amplified and cloned into plasmid pMQ118 that is unable to replicate in *S. marcescens* [25]. Mutants were generated by conjugation with *E. coli* bearing pMQ543 and selecting for kanamycin resistance. Kanamycin-resistant transformants were verified for the *slpE*:pMQ543 mutation using PCR and maintained on kanamycin.

2.4. Cytotoxicity assay

Cytotoxicity was measured as previously described using Presto Blue (Life Technologies) [27]. Briefly, monolayers of human airway carcinoma cell line A549 [13] were challenged with filtered (0.22 µm) bacterial growth medium from cultures grown for 20 h and adjusted to OD₆₀₀=2. The cell layers were incubated with supernatants for 4 h at 37°C in a CO₂ (5%)

incubator. The media above the cells was replaced with fresh medium and incubated with Presto Blue for 1 h and fluorescence was measured using a plate reader (Biotek Synergy 2). A549 cells were grown in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%). The detergent Triton X-100 (0.25%) was used to lyse A549 cells to determine maximum cytotoxicity, and LB medium was used as a control to determine the absence of cytotoxicity.

2.5. SlpE purification and analysis

The pMQ516 plasmid with an N-terminal His7-tagged *slpE* ORF was used to transform *E. coli* EC100D *pir-116* cells by electroporation. The resulting cells were grown overnight in the presence of gentamicin and used as an inoculum for 1 L expression cultures. Expression cultures were grown in LB supplemented with gentamicin. Arabinose (0.2% w/v) was added to the cells at OD₆₀₀ ~0.6 to induce *slpE* expression and incubated at 37°C for 4–6 h. The SlpE protein was purified from inclusion bodies under denaturing conditions following protocols previously described for PrtS and AprA [8], [32]. Proteases were refolded by rapid dilution into PBS or PBS supplemented with 2 mM Ca²⁺ and incubated on ice for 15 min prior to analysis. Protease activity was assessed using a fluorescent casein substrate (EnzChek, Invitrogen) or by colorimetric substrate azocasein (Sigma) [8], [9], [26], [32]. The protease activities were assessed in 96-well plate format on a BioTek Synergy 4 multi-mode plate reader. Cleavage of either substrate induces change in fluorescence, EnzChek, or absorbance, azocasein, that was monitored as kinetic or endpoint measurements. For in vitro cleavage reactions, the activity of the refolded protease was assessed at 37° C after dilution to 1–5 uM final concentration. Mean enzymatic velocity was fit under steady-state conditions and normalized to wild type controls. Reactions lacking the proteases were included as controls for basal, background substrate cleavage [8],[32].

2.6. Serralyisin modeling

The serralyisin proteases (SlpB, C, D, E) were modeled using the Swiss Model server [4]. Protease sequences were first aligned using the T-coffee multiple sequence alignment tools and used to model the proteases using the 1.1Å structure *S. marsezensis* serralyisin, PDB code 5D7W. Homology models were subsequently refined using the Chiron server to minimize steric clashes [23].

2.7. Transcriptional analysis

RNA extraction from OD₆₀₀ = 3.0 cultures and quantitative real-time PCR (qRT-PCR) analysis using the comparative CT method were performed as previously described [28]. Primers to detect *slpE* transcript were (5' to 3') ATTCTGATGGGCAAAGTTGG and TAAACTGCCGGGTATCTTCG. Primers to amplify the 16S rRNA gene as an internal control were previously described [28].

2.8. Statistical analysis

Two-tailed Fisher's Exact test and one-way analysis of variance (ANOVA) with Tukey's posttest were performed using Graphpad Prism software. Statistical significance was set p<0.05.

3. Results

3.1. An open reading frame for a serralysin-like protein identified in the genome of clinical *S. marcescens* isolates

An uncharacterized open reading frame was found during partial sequencing of the genome of *S. marcescens* K904, which is an ocular clinical isolate (GenBank accession KT901292.1). This open reading frame is predicted to code for a protein with an amino acid sequence 61% identical to the well-known virulence factor serralysin (PrtS), a metalloprotease. Interestingly, the candidate serralysin protein is absent from the genome of the sequenced reference strain Db11 that was originally isolated from *Drosophila* [16]. The candidate protease is here noted as serralysin-like protease E (SlpE). The predicted SlpE protein is 479 amino acids in length, compared to 504 for PrtS. It follows the same domain structure with a C-terminal predicted Ca^{2+} binding domain with repeating glycine/aspartate rich repeats and an N-terminal zinc-dependent protease domain (Fig. 1A) suggestive of a serralysin-family protease. BLASTP [2] queries indicated that a protein with the identical amino acid sequence, the metalloprotease gene of *S. marcescens* strain SM6, has been cloned and reported by Braunagel and Benedik [6].

In strain K904, the *slpE* gene is upstream from, and in a predicted operon with, a predicted protease inhibitor gene. The *slpE* gene is 920 base pairs downstream from a divergently expressed gene predicted to code for a diguanylate cyclase as predicted by BLASTP.

Comparative sequence analysis of the recently described SlpB cytotoxic metalloprotease indicated that SlpB was closer in sequence to AprA from *Pseudomonas aeruginosa* than PrtS from *S. marcescens*, suggestive of horizontal gene transfer [27]. By contrast, SlpE, is most closely related to PrtS (Fig. 1B). Interestingly, whereas the multiple serralysin-family genes of *E. chrysanthemi* are grouped together on the chromosome [31], the serralysin genes from *S. marcescens* are not.

The serralysin proteases all share a common two-domain fold with an N-terminal protease domain and a C-terminal RTX domain. Given the high sequence identity and similarity across the serralysin family, homology models of the SlpB, C, D, and E proteases were generated. As expected, the serralysin protease structures are highly similar, with only minor changes in and around the active site (Fig. 1C). The Ca^{2+} and Zn^{2+} cofactor sites are conserved across the family and in all the structures analyzed to date. Small local changes surrounding the active site are seen in the various proteases and may account for changes in enzyme kinetics and substrate preference.

Because the *slpE* gene was absent in the Db11 genome, PCR analysis was used to test the prevalence of the *slpE* gene in clinical, environmental and laboratory strains of unknown origin of *S. marcescens*. However, it is likely that the laboratory strains are not clinical isolates because they are pigmented, and the vast majority of clinical isolates are non-pigmented [14]. In total, 25 out of 42 strains (60%) were positive for *slpE* amplicons. Of the clinical isolates, 24 of 36 were positive for *slpE* (67%), whereas only 1 of the 6 non-clinical isolates (17%) produced the *slpE* amplicon ($p=0.032$, Fisher's exact test). A sample gel is

shown in Fig. 2; gels for other strains are not shown. Five of the *slpE* amplicons were tested by sequencing and all were *slpE* DNA, indicating that the primers are specific.

3.2. Induced expression of *slpE* confers extracellular protease activity upon strain PIC3611 and requires the LipBCD type 1 secretion system

The *slpE* ORF was cloned under control of an L-arabinose-inducible promoter on vector pMQ125. The resulting plasmid, pMQ516 (*pslpE*) was moved into *S. marcescens* strain PIC3611 because it is a very low-protease-producing strain [27] that would easily allow observation of increased protease levels. Induced expression of *slpE* conferred a ~8-fold increase in extracellular protease activity compared to the vector control (Fig. 3A). This was comparable to expression of genes for serralyisin (*pprtS*) and *slpB* (*pslpB*) from the same vector (Fig. 3A). These data support the hypothesis that SlpE from strain K904 is a functional protease.

The PrtS and SlpB proteases require the type 1 secretion system composed of LipB, LipC, and LipD (LipBCD) to become extracellular [1], [27]. As a genetic test to determine whether SlpE uses the same secretion system, the *slpE* gene was induced from *pslpE* in strain PIC3611 with a *lipD* mutation. Induced expression of *slpE* increased extracellular protease activity in growth medium from the wild-type PIC3611 strain, but not in the *lipD* mutant (Fig. 3B). These genetic data indicate that SlpE export requires the LipBCD type 1 secretion system.

3.3. Expression of *slpE* is EepR dependent

The transcription factor EepR was recently shown to regulate the *prtS* and *slpB* proteases in a positive manner [7]. We tested whether *slpE* expression was similarly diminished in a *eepR* strain isogenic to K904 using quantitative RT-PCR. The *slpE* transcript was measured in cultures grown to OD₆₀₀=3 (Fig. 4), because *Serratia* metalloproteases are expressed during stationary phase. There was a significant ~14-fold reduction in the *eepR* mutant compared to the isogenic wild-type strain ($p=0.0076$, Student's *t*-test, $n=6$). This result indicates that *slpE* is regulated by the EepR transcription factor.

3.4. Calcium-dependent folding of SlpE

To assess the Ca²⁺-dependent activity of SlpE, the protease was purified and refolded in vitro in the presence or absence of saturating Ca²⁺ (2 mM). Protease activity was then assessed as a function of digestion of a fluorescently labeled casein substrate. When refolded in the presence of 2 mM Ca²⁺, the purified SlpE protease showed robust protease activity (Fig. 5). The measured activity of the SlpE protease was similar to, but slightly reduced from, that measured for PrtS and SlpB. These enzymatic activities were dependent on the presence of Ca²⁺, as proteases refolded in PBS showed minimal hydrolytic activity using the casein assay. This is consistent with prior studies showing that serralyisin folding is mediated by Ca²⁺-binding within the C-terminal RTX domains [27], [33].

3.5. SlpE has redundant function with PrtS and SlpB in killing human airway epithelial cells in vitro

To test whether *slpE* from strain K904 codes for a cytotoxic protease, *pslpE* was induced in low-protease-producing strain PIC3611, and normalized bacteria-free culture supernatants were tested for cytotoxicity to a human airway cell line (A549). This cell type is relevant, as *S. marcescens* is a frequent cause of nosocomial pneumonia [11]. Controls of triton X-100 detergent and PBS were used to establish minimum and maximum values for cell viability (Fig. 6A). Supernatants from strain PIC3611 with the vector control plasmid were not significantly different from the PBS treated cells, demonstrating that supernatants from strain PIC3611 were not cytotoxic under these conditions. Induction of *slpE* conferred detergent-level cytotoxicity to the PIC3611 supernatants (Fig. 6A), suggesting that SlpE is cytotoxic to human airway cells. Similar results were observed with induction of the positive control *prtS* (Fig. 6A).

A high-protease-producing and cytotoxic bacterial strain, K904, was used to test the importance of SlpE in cytotoxicity to A549 airway epithelial cells. After 2 h incubation with normalized bacteria-free supernatants from K904, ~100% cytotoxicity was measured (Fig. 6B), demonstrating that it is much more cytotoxic than strain PIC3611 (Fig. 6A). Mutation of the *slpE* gene alone did not affect cytotoxicity (Fig. 6B). It was predicted that the cytotoxic impact of an *slpE* mutation was masked by the PrtS and SlpB proteases. A *prtS slpB slpE* triple mutant was generated and found to be significantly less cytotoxic to A549 cells than the *prtS slpB* double mutant (Fig. 6B). Thus, only when redundant protease activity was removed were we able to measure the relative contribution of SlpE to cytotoxicity.

When *pslpE* was introduced into the *prtS slpB slpE* mutant and induced, the resulting supernatants had restored protease activity and cytotoxicity (Fig. 7A–B), whereas the vector control did not (Fig. 7A–B). Together, these genetic data indicated that SlpE in combination with PrtS and SlpB contributes to the cytotoxic capacity of the bacterium.

4. Discussion

This study has characterized a member of the serralyisin family proteases that was strongly associated with clinical isolates rather than non-clinical isolates. In this study, SlpE production did cause cytotoxicity to a human cell line relevant to tissue infected by *S. marcescens*, suggesting a role for SlpE in virulence. A previous report suggested that SlpB was acquired through a horizontal gene transfer event, as it is more similar in amino acid sequence to AprA from *P. aeruginosa* than to PrtS [27]. Conversely, the SlpE sequence was found to be highly similar to PrtS (Fig. 1), suggesting that it results from a duplication event. Consistent with this model, structural superposition of the serralyisins showed minimal changes in overall architecture (Fig. 1C). The C-terminal RTX domains and the core of the protease domain fold superposed with minimal deviation. Local changes were seen surrounding the active site and may tune the proteases for different substrates or activities. Comparative genomic and phenotypic analyses will be necessary to determine when the *slpE* gene was introduced into the *S. marcescens* evolutionary tree, and whether this protease correlates with biotype/genotypes that have acquired a higher capacity for virulence [14].

Mutation of *slpE* alone did not alter the cytotoxicity of strain K904-secreted fractions; however, a mutation of *slpE* provided a clear phenotype when mutated from a *prtS slpB* double mutant, indicating redundant function. The redundancy of SlpE with PrtS and SlpB raises questions regarding whether there is a specific benefit to the bacterium of having multiple similar proteins with redundant function. At present, experiments to compare their specificity, optimal temperature and pH ranges have not been performed and may provide insight into why these multiple genes with apparently similar function are maintained in certain strains of *S. marcescens*. Additionally, the genes may be differentially regulated and expressed in different ecological niches. Interestingly, a protease-deficient SM6 mutant isolate lost almost all cytotoxicity to HeLa cells in vitro [20]. This suggests that SM6 has one cytotoxic protease compared to K904, which has PrtS, SlpB and SlpE, or that the mutation was in a positive regulator of multiple proteases, as the SM6 protease mutation was never mapped and could have been in a transcriptional regulator. Such regulators may exist, as the EepR gene that positively regulates *prtS* and *slpB* expression [7] was postulated to regulate *slpE* expression. Consistent with this prediction, we observed that *slpE* expression was highly reduced in an *eepR* mutant strain. Whereas this data indicates that EepR positively regulates *prtS*, *slpB*, and *slpE*, it is likely that these important secreted proteases are regulated by additional transcription factors.

Previous work by the Benedik group studied a protein with the same sequence from *S. marcescens* strain SM6 [6]; this current study builds upon the previous study and adds new information. When expressed in *Escherichia coli*, the SM6 protease was not secreted [6] unless the *E. coli* hemolysin transporter genes *hlyB* and *hlyD* were expressed in trans, indicating that the protease is secreted through a type I secretion system [30]; however, the native *S. marcescens* type I secretion system used to secrete the SM6 protease was not determined until this current study, which implicates LipBCD as the native secretion system (Fig. 3B). Strain SM6 with an unmapped transposon insertion, presumably in the metalloprotease gene, was less cytotoxic to HeLa cells in vitro, and expression of the SM6 metalloprotease gene from *E. coli* increased the cytotoxicity of bacterial culture filtrates to HeLa cells [20]. Here we add to the previous studies by demonstrating that the SlpE/SM6 metalloprotease contributes to the pathogenesis of *S. marcescens* against human airway cells through directed mutation and induced expression of the *slpE* gene using a modern clinical isolate (Fig. 6B). We demonstrate that SlpE is a calcium-dependent protease, most likely resulting from gene duplication with a *prtS* progenitor gene, and is found in the majority of clinical isolates tested, suggesting that it contributes to virulence.

In summary, this study has further characterized a serralyisin-like protease from the contact-lens-associated keratitis *S. marcescens* isolate K904. The results from this study suggest that the SlpE protein is a cytotoxic, calcium-dependent member of the serralyisin metalloproteases, and is more likely to be found in clinical isolates and may be developed as a diagnostic marker for cytotoxic strains.

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Fig. 1. Analysis of *S. marcescens* serralyisin family proteases

A. Schematic diagram of SlpE with predicted domains, determined by BLASTP. **B.** Phylogenetic comparison of amino acid sequences of *S. marcescens* serralyisin family proteases and AprA from *P. aeruginosa* strain PAO1. Tree made using one-click mode tree rendering program from www.Phylogeny.fr with default settings. The substitution numbers per site are proportional to the branch length. **C.** Homology models of the PrtB, C, D and E proteases are shown superposed onto the solved structure of serralyisin (PrtS) (PDB code 5D7W). The serralyisin structure is shown in blue aligned with SlpB (magenta), SlpC (cyan), SlpD (green) and SlpE (orange). A close-up view of the protease domain is shown as a ribbon trace to highlight the local differences of the proteases near the active site. The dashed line indicates the region enlarged. The zinc co-factor, shown in the active site, is shown as a red sphere.

Fig. 2. PCR analysis of *S. marcescens* genomes for the *slpE* gene

Chromosomal DNA from various *S. marcescens* strains was used in PCR reactions to identify an internal amplicon of the *slpE* gene. Amplification of an internal portion of the *oxyR* gene was used as a control for chromosomal DNA quality. Six of the nine strains shown here exhibited a band consistent with the *slpE* gene. K904, K997, K1172 UC1SER and CMS3335 are clinical isolates; Db11, CHASM, CMS376 and Nima are non-clinical isolates strains.

Fig. 3. Genetic analysis to test whether SlpE requires the LipD T1SS protein for secretion
A. Induced expression of *slpE* or positive controls *pstS* and *slpB* from a non-cytotoxic strain conferred cytotoxicity. Asterisk indicates significant difference from the vector control ($p < 0.01$, One Way ANOVA with Tukey's post-test). **B.** Secreted protease activity was detected when *slpE* was induced in the wild type (PIC3611), but not the *lipD* mutant. Asterisk indicates significant difference from the vector control ($p < 0.001$, One Way ANOVA with Tukey's post-test). Protease activity is expressed as A_{440}/OD_{600} .

Fig. 4. Analysis of *slpE* transcription in an *eepR* mutant

Transcription of *slpE* was measured in K904 and an isogenic *eepR* mutant strain at $OD_{600}=3$. The average and standard deviation of 6 independent samples is shown. The asterisk indicates $p=0.0076$, Student's unpaired *t*-test.

Fig. 5. Calcium-dependent structure and activity of SlpE and serralyisin proteases
The Ca^{2+} -dependent activation of serralyisin and related proteases is shown. Calcium is required for the activity of many proteases; this experiment was performed to determine whether SlpE activity requires calcium. The mean steady state reaction velocities for each protease were normalized to that of PrtS after refolding in the presence or absence of 2 mM Ca^{2+} using a fluorescent substrate .

Fig. 6. Genetic evidence supporting *slpE* codes for a protein cytotoxic to a human carcinoma airway epithelial cell line

A549 cells were challenged by bacterial culture filtrates for 4 h and viability was measured by Presto Blue. Detergent and PBS act as positive and negative controls, respectively. **A.** Induced expression of *slpE* or positive control *prtS* from a non-cytotoxic strain conferred cytotoxicity. Asterisk indicates significant difference from the vector control ($p < 0.001$, One Way ANOVA with Tukey's post-test). **B.** Supernatants from a protease triple mutant (*prtS slpB slpE*), but not a single or double protease mutants in highly cytotoxic strain K904 are defective in cytotoxicity. WT indicates strain K904. Asterisk indicates significant difference from the WT ($p < 0.001$, One Way ANOVA with Tukey's post-test).

Fig. 7. Complementation of the *slpE* mutant defects

A. Protease activity (A_{440}/OD_{600}) was measured from culture filtrates from strain K904 and a triple protease mutant. The *slpE* gene on a plasmid restored protease activity. **B.** A549 cells were challenged by bacterial culture filtrates for 4 h and viability was measured by Presto Blue. Detergent (Deterg.) and PBS act as positive and negative controls, respectively. The *slpE* gene on a plasmid restored cytotoxicity. Mean and one standard deviation are shown for all bars. Asterisk indicates significantly less protease activity or cytotoxicity compared to the K904 strain ($p < 0.001$, One-way ANOVA with Tukey's post-test).

Table 1***S. marcescens***

strains and plasmids

Strain	Description	Reference or source
PIC3611	<i>Serratia marcescens</i> wild type, PIC strain number 3611	Presque Isle Cultures
K904	Contact-lens-associated keratitis isolate	[18]
CMS2853	K904 with <i>priS</i> deletion mutation, <i>priS</i>	[27]
CMS4063	PIC3611 <i>lipD::tn</i>	[27]
CMS4098	K904 <i>priS</i> <i>slpB</i>	[27]
CMS4308	K904 <i>slpE</i> :pMQ543	This study
CMS4309	CMS4098 with <i>slpE</i> :pMQ543 “triple mutant”	This study
pMQ118	<i>nptII</i> , <i>rpsL</i> , <i>oriT</i> , <i>oriR6K</i> , <i>URA3</i> , <i>CEN6/ARSH4</i>	[25]
pMQ125	<i>oriP15a</i> , <i>PBAD-lacZa</i> , <i>oriP1600</i> , <i>oriT</i> , <i>URA3</i> , <i>CEN6/ARSH4</i>	[25]
pMQ516	pMQ125 + <i>His^r-slpE</i>	This study
pMQ543	pMQ118 + <i>slpE</i> internal fragment	This study

Table 2Presence or absence of the *slpE* gene in *S. marcescens* strains

Strain	Classification	<i>slpE</i> internal replicon ^a	<i>slpE</i> ORF replicon ^b	<i>oxyR</i> replicon ^c
CHASM	Environmental	+	ND	+
CMS376	Laboratory	–	–	+
CMS3335	Clinical	+	ND	+
Db11	Insect pathogen	–	–	+
HQ	Environmental	–	–	+
K904	Clinical	+	+	+
K912	Clinical	–	–	+
K927	Clinical	+	ND	+
K952	Clinical	+	ND	+
K977	Clinical	+	ND	+
K981	Clinical	+	ND	+
K997	Clinical	+	ND	+
K1054	Clinical	–	–	+
K1064	Clinical	–	–	+
K1097	Clinical	–	+	+
K1154	Clinical	+	ND	+
K1172	Clinical	+	ND	+
K1178	Clinical	+	ND	+
K1263	Clinical	+	ND	+
K1265	Clinical	+	ND	+
K1301	Clinical	+	ND	+
K1327	Clinical	+	ND	+
K1467	Clinical	+	ND	+
K1489	Clinical	+	ND	+
K1496	Clinical	–	–	+
K1674	Clinical	+	ND	+
K1681	Clinical	+	ND	+
K1728	Clinical	+	+	+
K1739	Clinical	–	–	+
K1776	Clinical	+	+	+
K1780	Clinical	+	+	+
K1788	Clinical	–	–	+
K1800	Clinical	–	–	+
K1876	Clinical	–	–	+
K1890	Clinical	+	+	+
K2089	Clinical	+	+	+
K2119	Clinical	–	ND	+
K2272	Clinical	–	–	+
K3082	Clinical	–	ND	+

Strain	Classification	<i>slpE</i> internal replicon ^a	<i>slpE</i> ORF replicon ^b	<i>oxyR</i> replicon ^c
Nima	Laboratory	–	–	+
TM	Environmental	–	–	+
UCISER	Clinical	+	ND	+

^aPrimers amplify an internal region of the *slpE* as an indication of its presence in a genome.

^bPrimers amplify the *slpE* ORF as an indication of its presence in a genome. These were used with positive controls and if the internal primers failed to amplify *slpE*. ND = not determined.

^cThe highly conserved *oxyR* replicon served as a positive control to validate template DNA.